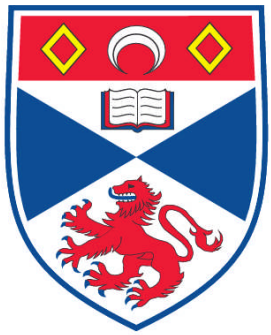
AN INVESTIGATION INTO THE EFFECTS OF A SIMULATED HUMAN GASTRO-INTESTINAL TRACT HAS ON BACILLUS CEREUS AND BACILLUS WEIHENSTEPHANENSIS VIABILITY AND PATHOGENICITY

Elizabeth Ann Hillhouse

A Thesis Submitted for the Degree of PhD
at the
University of St. Andrews



2012

An Investigation into the Effects a Simulated Human Gastro-Intestinal Tract has on *Bacillus cereus* and *Bacillus weihenstephanensis* Viability and Pathogenesis

Elizabeth Ann Hillhouse

This thesis is submitted in partial fulfilment for the degree of
MPhil/PhD
at the
University of St Andrews

Date of Submission
9th September 2011

Figures and Tables

Abbreviations

BHI	Brain Heart Infusion Broth
bp	Base pair
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CFU	Colony forming unit
CT values	Crossing point values
CTG	Cell tracker green
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
EDTA	Ethylenediamine tetracetic acid
Kb	Kilobase
L	Litre
PI	Propidium iodide
MALDI-TOF	Matrix associated laser absorption ionization time of flight
NA	Nutrient Agar
OD_{600}	Optical density at 600 nm
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RNA	Ribonucleic Acid
RT	Room temperature
RT PCR	Reverse transcription PCR
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TAE	Tris-acetic-EDTA buffer
TBE	Tris-borate-EDTA buffer
Tfi	DNA polymerase derived from *Thermus filiformis*
T_m	Melting temperature
V/V	Volume to volume ratio
W/V	Weight to volume ratio
WT	Wild type

1. Candidate's declarations:

I, Elizabeth Hillhouse hereby certify that this thesis, which is approximately 40,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

I was admitted as a research student in October, 2006 and as a candidate for the degree of PhD in October, 2007; the higher study for which this is a record was carried out in the University of St Andrews between 2006 and 2011.

Date …… signature of candidate ……

2. Supervisor's declaration:

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of …… in the University of St Andrews and that the candidate is qualified to submit this thesis in application for that degree.

Date …… signature of supervisor ……

3. Permission for electronic publication: (*to be signed by both candidate and supervisor*)

In submitting this thesis to the University of St Andrews I understand that I am giving permission for it to be made available for use in accordance with the regulations of the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. I also understand that the title and the abstract will be published, and that a copy of the work may be made and supplied to any bona fide library or research worker, that my thesis will be electronically accessible for personal or research use unless exempt by award of an embargo as requested below, and that the library has the right to migrate my thesis into new electronic forms as required to ensure continued access to the thesis. I have obtained any third-party copyright permissions that may be required in order to allow such access and migration, or have requested the appropriate embargo below.

The following is an agreed request by candidate and supervisor regarding the electronic publication of this thesis:

Access to printed copy and electronic publication of thesis through the University of St Andrews.

Date …… signature of candidate …… signature of supervisor ……

A supporting statement for a request for an embargo must be included with the submission of the draft copy of the thesis. Where part of a thesis is to be embargoed, please specify the part and the reasons.

Abstract

Bacillus cereus is one of the known causes of diarrhoeal food poisoning. In their natural environment of soil surviving as spores facilitates their colonisation of raw food ingredients enabling their access to the food chain. Recently psychrotrophic strains of *B. cereus* have been reclassified based on divergent cold shock gene (*cspA*) sequences and renamed *B. weihenstephanensis*. It is the modified *cspA* gene that is thought to confer the psychrotolerant phenotype witnessed by these strains. Aside from *cspA*, *B. cereus* and *B. weihenstephanensis* are closely related, leading to questions about its pathogenicity and ability to mediate diarrhoeal food poisoning outbreaks.

Food producers use a variety of processes to limit microbial contamination within food products. Although effective against vegetative cells, spores are often resistant and as such can persist within this environment. Chilled temperatures (4^0C) are often used to limit the growth of any contaminating microbes. Under such conditions *B. cereus* spores would remain dormant however *B. weihenstephanensis* spores have been shown to germinate and outgrow under refrigerated conditions. This could result in the consumption of both *B. cereus* and *B. weihenstephanensis* spores and vegetative cells. The effect that the human gastro-intestinal tract (GI) has on *B. cereus* and *B. weihenstephanensis* vegetative cells and spores is unclear. This study showed no difference in the viability of *B. cereus* or *B. weihenstephanensis* strains to survive and grow within a simulated human GI tract. Vegetative cells were revealed to die quickly in the stomach. Spore viability was shown to reduce in the stomach environment by approximately 10^4-fold. With a larger initial inoculum, 10^7spore/ml, viable spores were still recorded after 4 hours. These spores subsequently germinated within the small intestinal simulation and the resulting vegetative cells rapidly proliferated.

Mass spectrometry illustrated the ability of vegetative cells from both *B. cereus* and *B. weihenstephanensis* to produce an array of secreted proteins whose function were predominately related to virulence and pathogenesis. *B.*

weihenstephanensis strain 10202 was shown to produce the potent cytotoxin, CytK-1, while other *B. weihenstephanensis* and *B. cereus* tested strains possessed either or both Nhe and Hbl toxins. The primary diarrhoeal virulence factor/haemolysin BL was shown to be present in the supernatant of each strain through western blotting. Significantly smaller concentrations of each protein were detected, however, under simulated human GI tract conditions when compared to optimal conditions. The effects of the simulated human GI tract on virulence gene expression were monitored through real time PCR. No pattern between *B. cereus* and *B. weihenstephanensis* strains was found confirming that virulence gene expression is strain specific. Some genes were shown to be significantly upregulated such as *fur,* (the ferric iron uptake regulator and *groEL*, encoding a molecular chaperone. The expression of others however was reduced such as haemolysin BL components, *hblA* and *hblC*. Overall there were no significant differences detected between *B. cereus* and *B. weihenstephanensis* strains in their ability to survive the human GI tract and express virulence factors associated with diarrhoeal food poisoning.

It has been a long road to complete my thesis and there are many people who deserve my thanks. Firstly my supervisor Peter who has been very patient and understanding when editing my manuscript. Thank you to Dr Catherine Botting of the mass spectrometry dept for her help and advice. Dr Andrew Desbois for critically reading my work and offering me help, advice, encouragement and tough love when required. Dr Shirley Graham for helping to provide a great and fun working environment.

My Unilever team were a great support system and I am very grateful to Dr Peter McClure, Jan McClure and Dr Clive Blackburn.

Lorraine and Craig Black who opened their home to me many times and Marian and Dave who blew my mind with figs. Lorraine also endured hours of Betty ‘what if’ situation or could I experiments and for that I am incredibly grateful. Charlie Carton Smith helped without knowing some times (the odd stolen tip) but was also my sounding board and awesome tennis partner.

Stephanie and Simon Yapp and Louise Mckale, have been my rocks over the last two years and without their unfailing support, encouragement and belief I would never have finished. My Aunty Frances who provided me with an awesome office and much appreciated listening ear. Finally my incredibly supportive parents are just awesome, they have helped in countless ways, too numerous to mention. I know that the best thank you I could give them is to finally submit.

Chapter One:
Introduction

1.1 *B. cereus*; it is a family affair

B. cereus, an opportunistic human pathogen, can cause two types of food poisoning, emetic and diarrhoeal, along with more serious systemic infections (Kotirantaa et al, 2000; Stenfors Arnsen *et al*, 2007; Granum *et al*, 2007). The primary cause of *B. cereus* mediated diarrhoeal food poisoning is the presence of spores and vegetative cells within foodstuff, which is subsequently ingested. Its primary habitat of soil often facilitates its ability to colonise raw food ingredients. Within this environment *B. cereus* strains survive as spores. Sporulation (covered in section 1.2) is a survival strategy that permits vegetative cells to transform into spores allowing them to persist within an environment where conditions can no longer support vegetative growth (Setlow & Kornberg, 1970; Moir & Smith, 1990; Nichoson *et al*, 2001; Setlow, 2003). Although the consequence of ingesting this bacterium is well known the fate of the ingested cells and spores within the human gut remains unclear.

B. cereus strains can be mesophilic or psychrotrophic, illustrating a wide growth temperature spectrum possibly evolving from their presence in so many varied environments (Johnson, 1984; Claus & Berkeley, 1986). Psychrotrophic strains of *B. cereus* have been reclassified into a separate species called *B. weihenstephanensis* (Lechner *et al*, 1998). This categorization was based on modifications in the cold shock protein gene, *cspA* (Kotiranta *et al*, 2000). Mutations in this area have resulted in the loss of psychrotolerance in strains. Aside from alterations within the *cspA* gene, the species are very closely related. Conventional PCR studies have shown that *B. weihenstephanensis* strains possess many of the virulence genes associated with diarrhoeal food poisoning making their presence a concern in processed foods that are often stored at low temperatures (Bartoszewic *et al*, 2008). The *B. cereus* family also include two further members, *B. thuringiensis* and *B. anthracis*.

During sporulation *B. thuringiensis* manufactures parasporal crystalline toxins (Schnepf *et al*, 1998; Bartoszewicz *et al*, 2008; Swiecicka, 2008). This characteristic is used to identify *B. thuringiensis* strains from other members

of the *B. cereus* group. Like *B. cereus*, *B. thuringiensis* is widely distributed in the environment and is often isolated from soil, water, and the digestive tract of both invertebrates and vertebrates (Ischimatsue *et al*, 2000; Swiecicka & De Vos, 2003; Swiecicka & Mahilon, 2005; Bizzarri & Bishop. 2006; Hendriksen *et al*, 2006, Swiecicka & Mahillon, 2006; Bartoszewic *et al*, 2008). Due to the pathogenic nature of the toxins it produces towards insects, *B. thuringiensis* is sprayed on an array of crops as an insecticide. Genetic comparison between *B. thuringiensis* and *B. cereus* has revealed that discriminating between these two species is difficult (Priest *et al*, 2004). The primary distinction is the presence of the plasmid encoding the crystalline toxin, which in their natural habitat or under laboratory conditions can be lost making *B. cereus* and *B. thuringiensis* strains virtually identical. *B. cereus* strains containing the *B. thuringiensis* toxin plasmid have also been reported, highlighting the exchange of genetic material within these two species. (Carlson *et al*, 1994).

Sequencing of the *B. thuringiensis* serovar konkukian str. 97-27 genome revealed the presence of the toxin genes, known to be involved in diarrhoeal food poisoning (Han et al, 2006). Very few cases of diarrhoeal food poisoning mediated by *B. thuringiensis* have been reported (Samples and Buetter, 1983; Warren *et al*, 1984; Green *et al*, 1990; Jackson *et al,* 1995; Jackson *et al* 1995). It is however possible that *B. thuringiensis* has a greater role in gastroenteritis cases than previously thought but the difficulty in differentiating between these species impacts our ability to know absolutely.

B. anthracis is derived from an ancestral *B. cereus* strain that suffered distinct selective pressure and has gained specific pathogenic properties (Hill *et al* 2004). As the causative agent of anthrax, *B. anthracis* is possibly the most widely known and feared member of the *B. cereus* family. The genes involved in causing anthrax are distributed over two extra-chromosomal plasmids known as pXO1 and pXO2, both of which are required for full virulence (Anderson *et al*, 2005). Some of the virulence genes associated with diarrhoeal food poisoning were found when the genome of *B. anthracis* Ames strain was sequenced. However, a mutation within the *plcR* gene, the

transcriptional regulator of virulence genes within *B. cereus*, renders it non-functional resulting in no expression of diarrhoeal food poisoning (Agaisse *et al*, 1999; Mignot *et al*, 2001; Jenson *et al*, 2003; Daffonchio *et al*, 2006; Stenfor Arnesen *et al*, 2007). *B anthracis* is the most monomorphic species within the family with strains clustering together tightly in phylogenic trees. Its possible, because of the rapid death caused by anthrax, that *B. anthracis* does not exchange the same amount of genetic material as the less pathogenic *B. cereus* and *B. thuringiensis*, which would account for the high level of diversity witnessed in these spp. (Hill *et al*, 2004; Han *et al* 2006).

Based on genetic evidence many believe that *B. cereus*, *B. thuringiensis* and *B. anthracis* should be considered as one species (Ash *et al*, 1991; Helgason *et al*, 2004). Studies have shown that the chromosomal diversity within the 16S and 23S rRNA genes is virtually identical (Priest *et al*, 2004). Extensive testing via genomic mapping, pulse field gel electrophoresis of chromosomal DNA, multilocus sequence typing (MLST), Box PCR fingerprinting and amplified fragment length polymorphism (AFLP) have also highlighted very few genomic differences, strengthening the call for one species (Helgason, *et al*, 2004; Priest *et al*, 2004). Based on their varied pathology however these strains have remained individual species (Priest *et al*, 2004; Daffonchio *et al*, 2006)

Although the genome similarity between *B. cereus*, *B. weihenstephanensis*, *B. thuringiensis* and *B. anthracis* is significant, only *B. cereus* and *B. weihenstephanensis* pose a concern food for producers. In order to prevent contamination of finished products, sterilization processes are used. Although less severe conditions result in the rapid death of vegetative cells, spores are resilient to the mild heat and chemical treatments that are routinely used to decontaminate and preserve food and dairy products. The ability of these species to sporulate allows them to persist in environments that no longer support microbial growth and impacts greatly on their potential pathogenesis.

1.2 Sporulation and Germination; A survival strategy

The ability for some bacterial species to produce endospores was discovered in the 19th century by Tyndall, Cohn and Koch (Nicholson *et al*, 2000; Nicholson, 2002). The ubiquitous nature of *B. cereus* results in its presence on a host food material, which could poses a health risk to consumers if ingested. *B. cereus* vegetative cells become spores as a part of a survival strategy which allows the bacterium to remain viable under a host of conditions that would otherwise result in rapid vegetative cell death. The conditions surrounding sporulating cells can greatly influence the resulting spores properties (Nicholson *et al,* 2000; Nicholson, 2002; Setlow, 2003).

The mechanism of sporulation within the *Bacillus* family has been studied in detail (Figure 1.2). Once begun the process cannot be reversed. Initially the cell divides asymmetrically creating a mother cell and a pre-spore cell. A copy of the genome is present in each cell at this stage (Nicholson, 2002; Errington, 2003; Piggot & Hilbert, 2004; Henriques & Moran, 2007). The smaller pre-spore eventually becomes the spore centre after a series of developmental stages. The mother cell engulfs, like a phagocyte, the pre-spore cell in an action that results in the creation of the spore's characteristic double membrane (Errington, 2003; Piggot & Hilbert, 2004; Henriques & Moran, 2007). The spore structure is further enhanced by the addition of peptidogylcan between the inner and outer membranes that surround the pre-spore (Moir. 1990; Setlow, 2003; Foulger & Errington, 2006; Partridge & Errington, 2006). To the inner membrane a thin layer of peptidoglycan is added while a distinct, denser layer of peptidolgycan is then added to the outer membrane and forms the spore cortex (Errington, 2003; Setlow *et al*, 2003; Henriques & Moran, 2007).

The spore cortex plays an essential role in keeping dormancy by employing physical and osmotic pressure and maintaining the core's dehydrated status (Errington, 2003; Piggot & Hilbert, 2004; Henriques & Moran, 2007). Active transport and diffusion are used to place Ca^{2+} ions and dipicolinic acid (DPA) in the inner cell compartment (Moir, 1990; Setlow, 2000; Vepachedu & Setlow, 2004). It is believed that these molecules help in heat resistance by

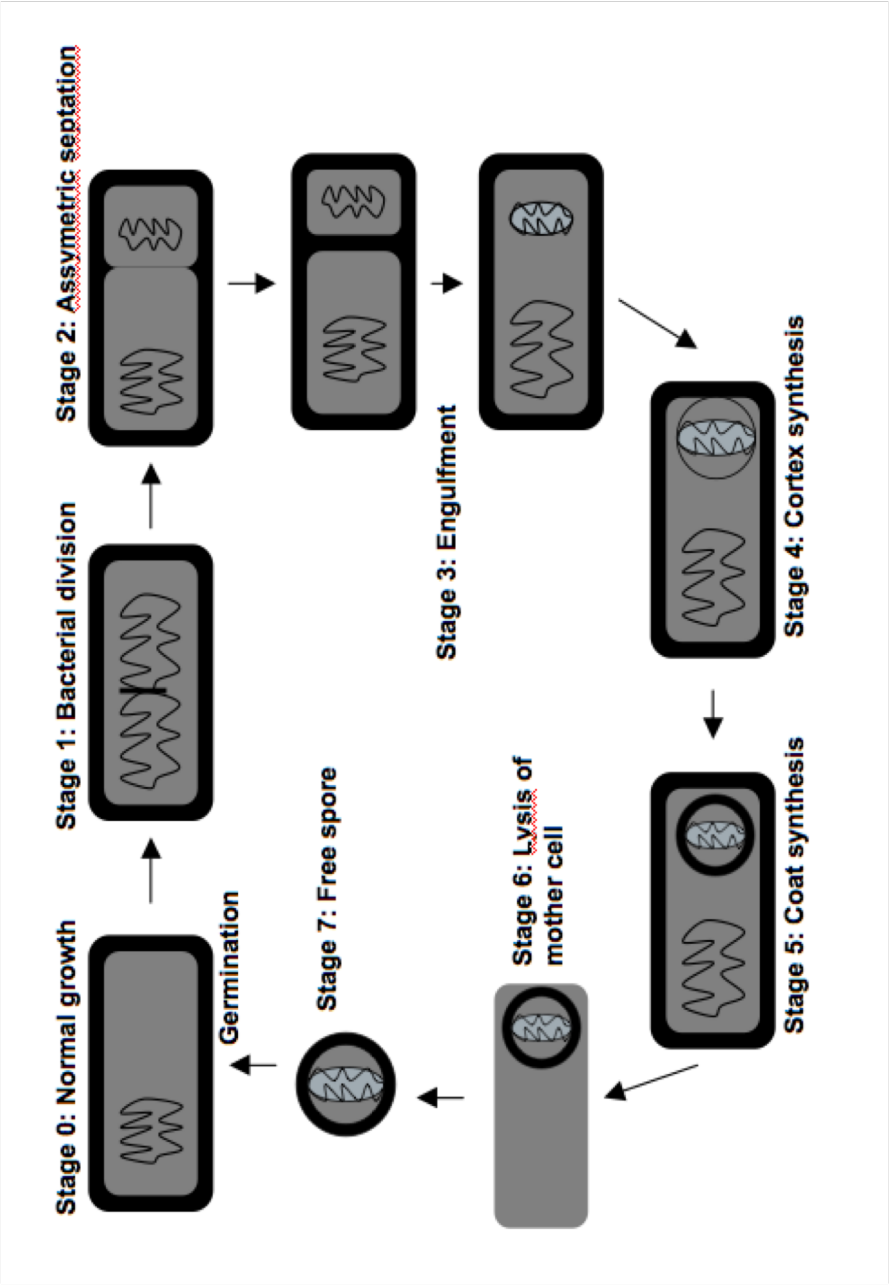


Figure 1.2 Sporulation Cycle. One of the main virulence mechanisms *B. cereus* possesses is the ability to, under hostile growth conditions, turn from vegetative cells to spores via a process called sporulation.

Stages in Sporulation

1) Conditions surrounding vegetative cells change initiating sporulation
2) Cell divides creating one large and one small compartment.
3) The larger compartment subsequently engulfs the smaller creating the characteristic double membrane spore contain
4) The spore structure is enhanced by the addition of peptidoglycan between the inner and outer membranes
5) Active transport and diffusion results in Ca2+ ions accumulating in the inner cell while diplicolinate acid (DPA) gathers in the pre-spore
6) The mother cell is the site for the production of coat-associated proteins, which eventually surround the outer membrane.
7) Maturation of the pre-spore into a spore coinciding with increased resistance to stresses and the development of the characteristic phase-bright look.
8) The mother cell ruptures leaving the fully formed spore

Image adapted from Timothy Paustian diagram at http://lecturer.ukdw.ac.id/dhira/BacterialStructure/Inclusions.html

possibly becoming stabilising agents (Moir, 1990). Large concentrations of α and β small acid soluble proteins (SASPs) present in the pre-spore help confer the mature spore's resistance (Moir, 1990 ; Mohr *et al*, 1991; Fairhead Setlow & Setlow, 1993; Callahan Fox & Fox, 2009; Vyas *et al*, 2011). SASPs protect spore DNA by binding and inducing a conformational change in its structure from B to A type (Murray *et al,* 1998; Callahan Fox & Fox, 2009). In conjunction with the construction of the spore cortex the outer cell wall protein coat is manufactured (Errington, 2003; Piggot & Hilbert, 2004; Henriques & Moran, 2007). Cell wall proteins are produced in the mother cell and transported to the outer cell membrane where they form the hardened exterior. The spore coat plays a role in maintaining spore resistance. It acts as a barrier to any substrates trying to enter the spore cortex including peptidoglycan-hydrolysing enzymes (Nicholson *et al,* 2000; Henriques & Moran, 2007). Essential to continued spore viability is the maintenance of the cellular functions required for germination (Nicholson *et al*, 2000). Damage to any part of the germination pathway would result in spores remaining dormant permanently. During sporulation spores prepare to be dormant for an unknown period of time as it is impossible to predict how long they will require to remain in this state (Nicholson *et al*, 2000).

The response regulator *SpoOA* controls the initial stages of sporulation (Burbulys *et al*, 1991; Phillips & Strauch, 2002; Steil *et al*, 2003). Genes for cell division and the sigma regulators needed for early spore development are controlled by phosphorylated *SpoOA* (Errington, 2003; Hilbert & Piggot, 2004, Steil *et al*, 2005) From septation onwards gene expression in the mother and pre-spore is under the control of the sigma factors, σ^E, σ^F, σ^G, σ^K with additional regulation supplied by *spoIIID, GerR and GerE*. (Moir, 1990; Errington, 2003; Hilbert & Piggot, 2004; Steil *et al*, 2005; Henriques & Moran, 2007; Camp Wang & Losick, 2011). These factors monitor the expression of specific early and late developmental genes needed to ensure sporulation occurs correctly. Early in the sporulation process σ^E is found within the mother cell while σ^F is present in the pre-spore body. Later these sigma factors are replaced with σ^K, σ^G respectively (Eichenberger *et al*, 2003; Steil *et al*, 2005;

Wang *et al*, 2006; Camp Wang & Losick, 2011). In order for σ^K and σ^G controlled late developmental genes to be expressed, σ^E and σ^F need to be deactivated (Camp Wang & Losick, 2011). Negative feedback loops created by σ^K and σ^G have been reported to directly affect the presence of σ^E and σ^F, removing them and thus allowing the continued expression of late developmental genes resulting in full spore formation (Zhang & Kroos, 1997; Zhang Struffi & Kroos, 1999; Camp Wang & Losick, 2011). The ability for σ^E, σ^F, σ^G, σ^K to work together, quickly, ensures that gene expression is in sync with the evolving morphological structure of the spore (Errington, 2003; Hilbert & Piggot, 2004; Stragier & Losick, 1996; Steil *et al*, 2005).

Despite being dormant and metabolically inert, spores monitor any changes in their surroundings. The flexible spore surface layers respond to hydration levels by expanding and contracting (Driks, 2003; Westphal *et al*, 2003; Henriques *et al*, 2007). Molecules, known as germinants, interact with the spore and can initiate a change in lifestyle from dormant to active. This process, known as germination, can be triggered by a host of factors; nutrients play a major role and can take the form of sugars, amino acids or purine nucleosides (Nicholson *et al*, 2000; Setlow Cowan & Setlow, 2003; Moir, 2006). Once initiated germination cannot be halted despite any loss of the germinant molecule (Moir & Smith, 1990). Following the commencement of germination, changes within the spore body can be detected; DPA and Ca^{2+} are released from the spore core and replaced with water. Their release prompts the full hydrolysis of the spore cortex and breakdown of the peptidoglycan layer by core lytic enzymes (CLE), CwlJ and SleB (Foster & Johnstone, 1989; Popham *et al*, 1999; Boland *et al*, 2000; Chirakkal *et al*, 2002; Moir, 2006) The initial release of small amounts of DPA by SleB during the first stages of germination is essential for the release of greater amounts of Ca-DPA and the activation of the second CLE, CwlJ (Bagyan & Setlow, 2002; Moir, 2006; Magge *et al*, 2008, Setlow *et al*, 2009). SASPs are broken down and used as the building blocks of protein synthesis (Moir & Smith, 1990). Vegetative outgrowth is finally cemented by the renewal of RNA and

DNA replication leading to cell division (Setlow, 1983; Moir & Smith, 1990, Moir, 2006).

A tough barrier to most chemicals, the spore coat does retain a permeable structure that allows germinants to communicate with the spore (Behravan *et al*, 2000; Setlow, 2003; Henriques & Moran, 2007). The binding of the germinant to a receptor located within the inner membrane starts a cascade of molecular events culminating in vegetative cell growth (Paidhungat & Setlow, 2000; Setlow, 2003). Different germinants require corresponding receptors resulting in the need for more than one receptor (McCann *et al*, 1996; Barlass *et al*, 2002). Receptors in *B. subtilis* are encoded by the homologous operons, *gerA*, *gerB* and *gerK* (Paidhungat & Setlow, 2001; Setlow Cowan & Setlow, 2003). *GerA*, *gerB* and *gerK* each encode three proteins; two integral membrane proteins and one pre-lipoprotein (Paidhungat & Setlow, 2001; Setlow Cowan & Setlow, 2003; Cooper & Moir, 2011). Functioning germination receptors are essential to enable *B. cereus* to recover from the self-imposed dormant state.

The ubiquitous nature of *B. cereus* and its ability to form spores, which could possibly survive conditions employed by food producers to limit bacterial contamination, causes widespread concern.

1.3 Routes to the food chain and beyond

The ability of *B. cereus/B. weihenstephanensis* vegetative cells to become spores when surrounded by conditions that do not support cellular growth offers them a huge advantage in surviving food production. Processes such as heat treatments and chemical exposure, which result in the cell death of many contaminating bacteria, have little effect on spores. The removal of all bacteria except *B. cereus/B. weihenstephanensis* spores creates a non-competitive environment within food products allowing *B. cereus/B. weihenstephanensis* to grow uninhibited (Stenfors Arensen *et al*, 2008). This could have an impact on both food spoilage and poisoning as once spores germinate, new vegetative cells could grow freely without competition. Spores are known to have adhesive properties, linked to the appendages that project

from the spore body (Stalheim & Granum, 2001). Food processing equipment can become a reservoir for *B. cereus/B. weihenstephanensis* spores growing as biofilms, which can continue the cycle of contamination (Wiencek *et al*, 1990; Tauveron *et al*., 2006; Faille *et al*., 2007; Stenfors Arnsen *et al*, 2008).

Refrigeration is used as a mechanism to reduce/contain microbial growth. Within this environment psychrotrophic *B. weihenstephanensis/B. cereus* cells would thrive as shown by both species over the last 20 years being regularly isolated from diary products and machinery (Te Giffel *et al*, 1997; Larsen & Jørgensen, 1999; Stenfors *et al*, 200). REPFEDs (refrigerated processed food of extended durability) are also at risk from contamination. These foods have been created in order to meet new demands from consumers who are seeking meals that are minimally processed with strong organoleptic characteristics (Nauta *et al*, 2003). Unlike previous processed foods that are treated to numerous preservation processes including challenge testing, REPFED's are only subjected to a mild heat treatment (Nauta *et al*, 2003). The presence of *B. cereus* or *B. weihenstephanensis* spores within REPFED foods is of great concern as mild heat treatments and refrigeration has been shown to be insufficient to inhibit these bacteria from germinating and growing. In the UK REPFED meals had a market value of £500 million in 2000 (Nauta *et al*, 2003). As consumers grow more aware of health and food issues, the desire for these products over the last 12 years has not diminished. The minimal processing these foods undergo, the potential for *B. cereus/B. weihenstephanensis* contamination on raw food ingredients and improper storage/reheating in the home environment all contribute to *B. cereus* and *B. weihenstephanensis* mediated food poisoning outbreaks. In order to understand the fate of any ingested spores or cells further work is needed as little is known about the effect of the human GI tract (Gilbert, 1983; Gilbert & Kramer, 1986).

1.4 Barrier to Disease; Human Gastro-Intestinal Tract

The gastro-intestinal (GI) tract ensures all energy and nutrients from foods are extracted while processing the rest as waste. It comprises mouth, pharynx, oesophagus, stomach, small intestine, large intestine and anus. The GI tract

contains millions of bacteria, which live a commensal lifestyle with their host (Ganzle *et al*, 1999). These microbes offer some protection against invading bacterial like *B. cereus* however the physiology of the GI tract itself provides a strong barrier to infection (Huang & Adams, 2004; Kristiffersen *et al*, 2007). It is only when *B. cereus* cells/spores can overcome this environment and diarrhoeal associated virulence factors can be produced that a diarrhoeal food poisoning infection is possible.

The stomach provides a strong barrier to *B. cereus* cells and spores invasion by the maintained low pH and the presence of pepsin. Stomach pH, normally acidic, is dependent on food consumed with maximum pH levels recorded after a larger meal (Conway *et al*, 1987; McLauchlan *et al*, 1989; Dressman *et al*, 1990; Ruas-Madiedo *et al*, 2002; Huang, & Adams, 2004). The less acidic the pH the more likely a higher proportion of ingested cells and spores will survive this environment. The presence of food also offers a buffering effect to the decreasing pH, sheltering contaminating cells/spores (Conway *et al*, 1987; McLauchlan *et al*, 1989; Dressman *et al*, 1990; Ruas-Madiedo *et al*, 2002). The period of time spores and cells spend in the stomach environment influences to their viability (Ruas-Madiedo *et al*, 2002). Liquids pass relatively quickly but food items can take between 2-4 hours to empty into the small intestine. The longer cells and spores spend in the stomach the greater their exposure to the increasing acidic pH and pepsin (Dressman *et al*, 1990; Huang, & Adams, 2004).

Once food reaches the small intestine, bile salts and digestive enzymes are produced to continue the degradation process, releasing nutrients from the food (Jackson & McLaughlin, 2006). Bile acids break down fats contained with food. However as a by-product, bile acids also show significant bactericidal effects against *B. cereus* (Kristiffersen *et al*, 2007). The presence of bile and lipases within this environment could have a negative effect on both cell growth and spore germination while also neutralising possible secreted toxins by denaturing foreign secreted proteins (Kristiffersen *et al*, 2007; Tam *et al*, 2006; Fang *et al*, 2009). Transit time is also between 2-4 hours however pH at 8 is more alkaline than that of the stomach (Huang, & Adams, 2004). Onset of

symptoms relating to diarrhoeal food poisoning are 12-17 hours post ingestion indicating that spores can germinate in these surroundings and vegetative cells can somehow stick to the wall of the small intestine and remain there after transit.

1.5 *B. cereus* adhesion

The ability of pathogenic bacteria to adhere to any surface can result in increased virulence. Hydrophobic interactions mediate the attachment of bacteria to surfaces like oral cavities, contact lens, surgical and dental biomaterials, food, food production equipment and human epithelium (Wiencek *et al*, 1990; Tauveron *et al*, 2006). Hydrophobic *B. cereus* spores are commonly isolated from raw food ingredients leading to concern from food producers about contaminated surfaces within food production lines (Faille, Fontaine & Benezech 2001; Stalheim & Granum, 2001). In order to limit the number of attached spores and vegetative cells on food production machinery, cleaning in place (CIP) systems have been designed (Rouillard, 1998). CIP methods vary but the standard CIP regime involves water rinse, 1% sodium hydroxide at 70^0C for 10 min, water rinse, 0.8% nitric acid at 70^0C for 10 min, water rinse (Rouillard, 1998; Hornstal *et al*, 2007). This process is effective against vegetative cells, however, spore viability has been reported to only drop by 40% (Faille, Fontaine & Benezech 2001; Hornstal *et al*, 2007). Unaffected hydrophobic spores could colonise equipment leading to contaminated work surfaces and eventually contaminated food products. The ability of *B. cereus* spores to adhere to surfaces is not only important during food processing but is an additional virulence factor if spores are consumed and encounter the human GI tract.

B. cereus has been shown to be significantly more adhesive to surfaces than other members of the *Bacillus spp.* due to its ability to produce highly hydrophobic spores that preferentially attach to hydrophobic surfaces (Ronner *et al*, 1990; Husmark, 1993; Andersson *et al*, 1995; Stalheim & Granum, 2001). A greater level of hydrophobicity is found in *B. cereus* spores because of abundant levels of protein held within a balloon like structure that surrounds the spore, known as the exosporium (DesRoiser & Lara, 1984; Weincek *et al*,

1990, Sylvestre Couture-Tosi & Mock, 2002; Tauveron *et al*, 2006). Several studies performed during the 1960s showed that the exosporium is composed of a paracrystalline basal layer with a hair-like outer region (Roth & Williams, 1963; Gerhad & Ribi, 1964; Hachisuka *et al*, 1964, Moberty *et al*, 1966, Kramer & Roth, 1968). Further microscopic investigations revealed that individual appendages are tubular in shape with an average length of 2.5μm (Kozuka & Tochikubo, 1985; Husmark & Ronner, 1992; Tauveron *et al*, 2006). It has been postulated that appendages make the first contact with a surface and as such promote spore adhesion (Stalheim & Granum, 2001; Tauveron *et al*, 2006).

Predominately consisting of protein, appendages can suffer proteolytic degradation by proteases such as pepsin (Stalheim & Granum, 2001). The human stomach contains significant levels of pepsin while the small intestine comprises a host of degradative enzymes. Removal of spore appendages has lead to a reduction in spore attachment to hydrophobic glass however there was no difference in the adherence of spores with and without appendages to stainless steel (Husmark & Ronner, 1992). The role appendages play in spore adhesion and attachment is unknown however it is possible that in static cultures conditions they do not offer any additional advantage. However under continuous flow conditions, like those found in the small intestine, appendages could enhance spore 'stickiness' allowing greater numbers of spores to attach to the epithelium (Stelhiem & Granum, 2001).

Epithelial attachment is a feature of many Gram-negative pathogenic bacteria such as *Escherichia coli*, *Shigella spp*, *Vibro cholera* and *Pseudomonas aeruginosa*. The ability of *B. cereus* to adhere to epithelial cells is thought to greatly increase their pathogenesis. In 1995 *B. cereus* strain NVH100 caused a persistent diarrhoeal food poisoning infection resulting three people requiring treatment for seven days and one person receiving intravenous care for five weeks (Granum *et al*, 1995). The ability of NVH100 spores to attach to the epithelial cell wall within the small intestine and germinate *in situ* allowed secreted toxins direct access to target cells resulting in a chronic infection (Andersson Granum & Ronner, 1998; Granum *et al*, 1995). *B. cereus* spore

hydrophobicity and the ability to attach to epithelial cells is known to be strain dependent. Ingesting strains that are only slightly hydrophobic may result in the mild symptoms normally associated with diarrhoeal food poisoning. However, consuming strains whose spores that are more hydrophobic could result in a more severe, continuing infection. The ability of hydrophobic *B. cereus* spores to adhere to epithelial cells is an important start to ultimately causing diarrhoeal food poisoning. *B. cereus* mediates diarrhoeal food poisoning through the expression and secretion of a host of virulence factors including specific toxins. To date there have been numerous outbreaks of *B. cereus* mediated diarrhoeal food poisoning, some resulting in death (Lund *et al*, 2000).

1.6 Prevalence of *B. cereus* mediated food poisoning outbreaks

Hauge (1950) was the first to show a correlation between the presence of *B. cereus* cells and spores and an attack of food poisoning (Hauge, 1950). Since then a greater awareness as to the pathogenic effects of *B. cereus* has led to diarrhoeal food poisoning cases being recorded all over the world.

Between 1989-1993 outbreaks of *B. cereus* food poisoning were recorded in five European countries (Figure 1.6). In England and Wales between 1992-2005 45 outbreaks were recorded by the health protection agency (HPA), affecting around 399 people (http://www.hpa.org). As previously described, *B. cereus* elicits two types of food poisoning (emetic and diarrhoeal) that although have greatly varying symptoms are often counted together under *B. cereus* food poisoning. This can lead to uncertainty as to which cases are genuinely attributed to diarrhoeal food poisoning (Stenfors *et al*, 2007). Determining the number of diarrhoeal food poisoning outbreaks mediated by *B. cereus* is hampered by the possession of similar clinical symptoms to that of other food poisoning bacteria such as *S. aureus* (emetic) and *C. perfringens* (Diarrhoeal). Furthermore due to symptoms being short-lived there is a tendency for cases to go unreported additionally impacting on the awareness of *B. cereus* diarrhoeal infections (Granum *et al*, 2007; Stenfors *et al*, 2008)

B. cereus emetic food poisoning is more prevalent in Britain and Japan possibly due to the diet consumed (Kramer & Gilbert 1989; Granum, 1997). Cereulide (emetic toxin) is produced within foodstuff like rice or pasta that are starch rich (Kramer & Gilbert, 1989; Granum, 1997; Carlin *et al*, 2006; Ehling-Schulz *et al*, 2006). This toxin is exceptionally heat stable, remaining viable after cooking or re heating of the food product (Carlin *et al*, 2006).

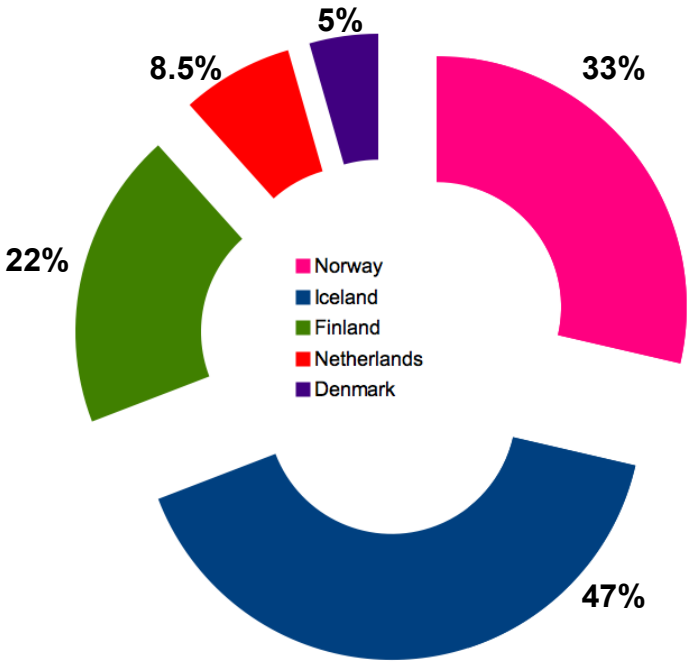


Figure 1.6 Food poisoning cases. Between 1989 and 1993 food poisoning outbreaks in some European countries were recorded. The percentage of cases attributed to *B. cereus* is displayed above based on overall food poisoning figures. Taken from Granum *et a*l, (2007)

As a consequence of people ingesting the toxin within food, not cells or spores, symptoms occur more rapidly than in diarrhoeal cases, between 30 minutes - 6 hours post ingestion (Carlin *et al*, 2006; Ehling-Schulz *et al*, 2006). *B. cereus* mediated diarrhoeal food poisoning is more common in Northern Europe and America and contracted by eating contaminated protein rich foods such as beef, chicken, fish, cheese, milk, sauces and dairy desserts (Krammer & Gilbert, 1989; Granum, 1997; Anderson-Borge *et al*, 2000; Chroma *et al*, 2002; Fagerlund *et al*, 2004; Montville & Matthews, 2005). Diarrhoeal food poisoning is caused by the presence of cells and/or spores within foods that are ingested. Spores can germinate and cells proliferate

resulting in the production of a host of virulence factors culminating in symptoms associated with diarrhoeal disease (Turnbull, 1979; Anderson *et al*, 1998; Kotiranta *et al*, 2000; Guamer & Malagelada, 2003). There has been a considerable effort to determine the toxins involved in *B. cereus* mediated diarrhoeal food poisoning and over the last 10 years several have been identified and well characterised.

1.7 Toxins associated with diarrhoeal food poisoning

1.7.1 Three component cytotoxins; HaemolysinBL and Non-hemolytic Enterotoxin

Extensive investigations into the diarrhoeal toxins produced by *B. cereus* led to the discovery of two/three/component toxins that are unique to this bacterium, haemolysin BL (hbl) and non-haemolysin enterotoxin (nhe) (Beecher *et al*, 1990; Lund *et al*, 2000). Sequencing revealed that genes within each toxin are arranged sequentially and expressed as a single operon. Hbl genes *hblA*, *hblC* and *hblD* produce proteins, B, L1 and L2 respectively (Figure 1.7.1) (Beecher & Wong, 2000). Nhe genes, *nheA*, *nheB* and nheC each express a protein of the same name (Figure 1.7.1) (Lund *et al*, 2000).

There are similarities between the roles each protein plays within these toxins. Both nhe and hbl produce one protein with a binding function and two lytic proteins (Beecher & Wong, 2000; Lund *et al*, 2000). Due to this it is possibly not surprising that homology between *hbl* and *nhe* gene sequences has been identified (Heinrich *et al*, 1993; Ryan *et al*, 1997; Gunebretiere *et al*, 2002; Anderson *et al*, 2005; Dietrich *et al*, 2005; Martinez-Blanch *et al*, 2011). The *nheB* and *nheC* gene share 44% gene sequence homology and they are also genetically similar to the *hblC* gene, which produces L1 (Stenfors Fagerlund & Granum, 2008). In cell culture supernatant it has been shown that nheA and

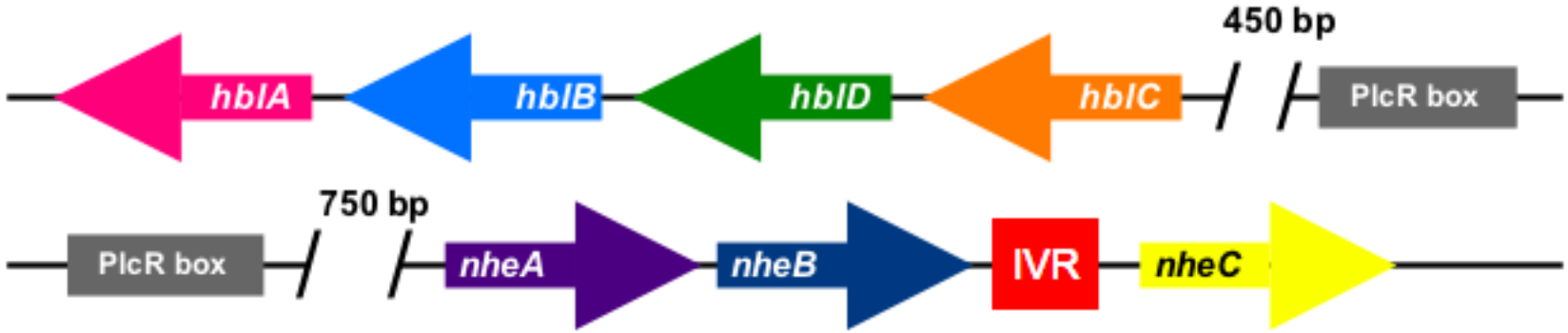


Figure 1.7.1 Haemolysin BL and Non-Haemolysin Enterotoxin gene organisation.
Both *hbl* and *nhe* are organised as operons under the control of the plcR regulator. PlcR boxes can be located upstream of the start codons. Transcription of hbl terminates within *hblB*, resulting in a truncated protein. An inverted repeat has been found within the region spanning nheB/C genes. Diagram based on previous knowledge gained about haemolysin BL and the non-haemolysin enterotoxin.

nheB are present in two forms, the second being a processed form of the larger toxin. Both show indistinguishable activity under *in vitro* conditions (Fagerlund *et al*, 2008; Stenfors Fagerlund & Granum, 2008). There are also multiple homologues of the *hbl* components. They deviate in charge, size and chromatographic behaviour however there is no difference noted in the activity of the L components (Fermanian *et al*, 2000). Sequence variance was witnessed in *hblA*, however it remains functional as a binding protein (Schoeni *et al*, 1999; Hansen *et al*, 2001; Thaenthanee *et al*, 2005). Variant forms of *nhe* have been found in some *B. cereus* strains, however most *nhe* sequences are between 90-100% homologous (Linback *et al*, 2004; Fagerlund *et al*, 2008). Minor differences in *nhe* are centred on the region spanning *nheB-nheC*, which can vary in length in all strains (Arnesen Fagerlund & Granum, 2008).

The presence of two lytic subunits in toxins that interact with only one binding protein does imply that there could be a degree of overlapping function. Although *hbl* and *nhe* express proteins that function in similar ways they are not interchangeable (Lund & Granum, 1997; Fagerlund *et al*, 2008; Stenfors Fagerlund & Granum, 2008). The degree of similarity however witnessed between these toxins poses questions about their evolution. It is highly likely that hbl and nhe toxins arose from a single gene, which through selective and environmental pressures has evolved to produce multiple toxins to elicit a maximal effect (Beecher & Wong, 2000). The greater level of diversity noted within sequenced hbl proteins could be the result of simultaneous duplication or horizontal gene transfer events (Beecher & Wong, 2000). These actions are more likely to occur within hbl as it maps to an area on the *B. cereus* chromosome that is unstable (Beecher & Wong, 2000). The genetic variance found within this toxin can make it difficult to determine the pathogenic potential of *B. cereus* strains.

The fourth member of the hbl operon, *hblB,* is not involved in toxin formation. Sequence analysis showed that *hblB* is 72% identical to *hblA* gene (Schoeni *et al*, 1999; Fagerlund *et al*, 2008). A function for *hblB* has as yet not been found. Hbl is present in around 50% of strains tested to date and is believed

to be the primary virulence factor in *B. cereus* mediated diarrhoeal food poisoning.

1.7.2 The mechanism of action utilized by Haemolysin BL

Hbl components were isolated using isoelectric focusing (IEF) and SDS-Page and designated B (binding) and L (lytic) based on their properties determined via haemolysis assays (Beecher, & MacMillan, 1990). Erythrocytes incubated with the B protein first then L proteins are quickly lysed however incubating with L proteins first then B does not result in any cell lysis (Beecher & Wong, 1997; Beecher & Wong, 2000). Although the HBL components act together they bind independently of each other then subsequently come together to form pores in the lipid bilayers of target cells (Beecher & McMillan, 1991; Beecher & Wong, 1994; Beecher & Wong, 1997). It was thought HBL elicited its effect via the enzymatic breakdown of the target cell membrane however via osmotic protection experiments it was determined that the L proteins along with B form a membrane attack complex resulting in transmembrane pores being created (figure 1.7.2) (Beecher & Wong, 1997; Madegowda *et al*, 2008; Senesi *et al*, 2010). There is a change in the state in the protein B between binding to the cell membrane and when it becomes 'primed' and ready to interact with L1+2. This difference could be a by-product of a conformational change to B once bound to the membrane or due possibly to the oligomerization of B (Beecher & Wong, 2000). Molecules smaller than the pore flood in to and out of the cell creating a negative osmotic gradient that results in the rupture of the cell (figure 1.7.2) (Beecher & Wong, 1997; Madegowda *et al*, 2008; Senesi *et al*, 2010).

Hbl expression causes several characteristic effects; fluid accumulates in rabbit ileal loops, vascular permeability increases and dermonecrotic activity is witnessed, Vero cells are rapidly lysed, and erythrocytes from various animals are lysed (Thompson, 1984; Beecher & MacMillan, 1990; Beecher & Wong, 1994b; Beecher *et al*, 1995a; Beecher *et al*, 2005b; Beecher & Wong, 2000). A unique pattern of discontinuous haemolysis can be witnessed on

blood agar plates. The inhibiting effects of excess HBL components, B and L_1, cause this. Close to the colony the concentration of B and L is at its highest, resulting in the association of B and L proteins before B has bound to cells (Beecher & Wong, 2001). Lysis only occurs at a distance where the rate of B binding to the cell membrane is greater than the concentration of L and B (Beecher & Wong, 1997). The binding of B to cells is the rate-limiting step in cell lysis (Beecher & Wong, 1997).

1.7.3 Non-haemolysin Enterotoxin Mechanism of Action

Although first believed to be non-hemolytic, Fagerlund *et al* (2008) reported that nhe can lyse some mammalian erythrocytes (Fagerlund *et al*, 2008). Its main action however is cytotoxic, effecting epithelial cells by forming pores in their lipid bilayers resulting in cell leakage and ultimately death (Lund *et al*, 2004; Fagerlund *et al*, 2008). Even at low toxin levels nhe can rapidly form pores indicating that its action is not dependent on any surface protein receptors. The exact method of cell lysis via nhe is unknown however the oligomer state of NheB molecules is considered to be important (Linback *et al*, 2004; Arnesen Fagerlund & Granum, 2008). Unlike hbl where all components bind to the target cell membrane only NheB is capable of this. Similar to the A-B type toxins it is possible that receptor and catalytic functions are held within different domains on separate polypeptides allowing NheB to both bind to target cells and the lytic components NheA and NheC (Lindback *et al*, 2004). When tested nheC was not found in culture supernatant. Further binding studies revealed that a ratio of 10:10:1 between nheABC proteins resulted in optimal cell lysis. Over-expression of NheC was shown to affect the binding of NheB to target cells resulting in the inhibition of target cell lysis (Linback *et al*, 2004). In order to maintain the optimal ratios of each component a repression mechanism must be in place. Within the area spanning *nheB* and nhe*C* a DNA inverted repeat has been located (figure 1.6.1). This could result in some translational repression of the NheABC operon thereby reducing the amount of NheC expressed ensuring maximal toxicity (Granum *et al*, 1999; Linback *et al*, 2004).

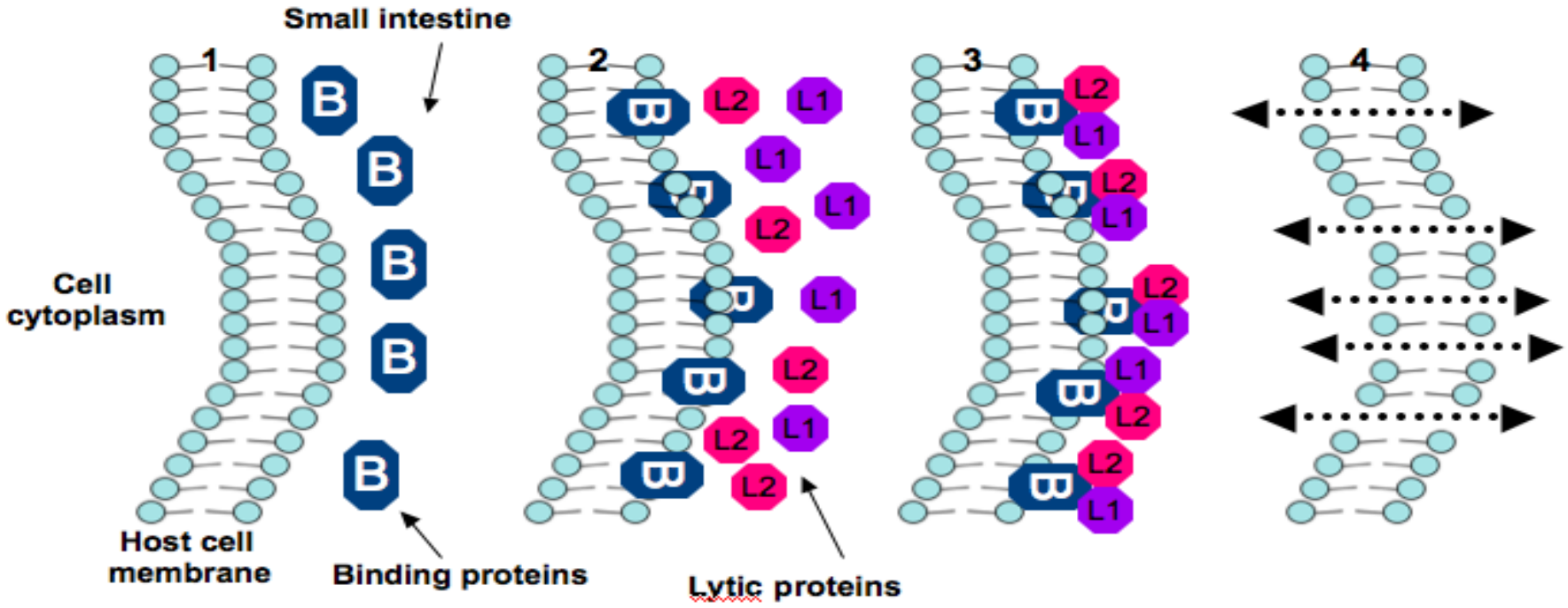


Figure 1.7.2 Haemolysin BL Mode of Action
1. The first step is the production of the haemolysin BL components via the plcR regulator. Once the operon is transcribed B, L_1 and L_2 are exported through the sec pathway to the surrounding environment, normally epithelial cells. **2.** B molecules associate with the cell membrane and bind at roughly equal distances across the host cell membrane. **3.** L_1 and L_2 bind to the membrane and by associating with B form a membrane-attacking complex against the host cell membrane. **4.** This results in deep pores forming allowing small molecules to flood in creating a negative gradient leading to cell lysis. Diagram based on information known about hbl mechanism of action.

Attempts have been made to mutate the individual *nhe* genes with limited success. The introduction of mutations in *nheA* has not been possible as cells deficient in this gene are not viable which raises interesting questions as to a possible secondary function of nheA (Mendelson *et al*, 2004). The occurrence of *nhe* in 100% of *B. cereus* strains tested to date supports this theory (Fagerlund *et al*, 2008; Arnesen Fagerlund & Granum, 2008). The presence of multiple homologs of both *hbl* and *nhe* and a non-functional gene in *hbl* raises interesting questions about the evolution of *B. cereus* strains and the selection pressures they have faced. Both toxins when expressed cause widespread destruction of host cell tissue leading to symptoms associated with diarrhoeal food poisoning. These toxins however are not the only ones in the *B. cereus* arsenal; several single component toxins have been identified and in recent years characterised.

1.7.4 Single component toxins; Cytotoxin K

Some *B. cereus* strains have been shown to produce β-barrel toxins like those generated by *Clostridium* and *Staphylococcus* spp. Sequence identity shows that cytotoxic K (cytK) produced by *B. cereus*, the α-toxin produced by *S. aureus* and the β-toxin expressed by *C. perfringens* share 30% amino acid identity (Lund Buyser & Granum, 2000; Fagerlund *et al*, 2004)**.** The solved crystal structure of α-toxin highlights its mushroom like shape, encompassing stem, rim and cap domains. It has a height of 10nm with the diameter within the pore varying from 1.4 at the narrowest point to 4.6nm (Song *et al*, 1996; Prevost, 1999). The sequence homology between cytK and the α-toxin suggest that cytK could produce pores in lipid bilayers that resemble the α-toxin with a β-barrel structure. Indeed, conductivity assays proved the ability of cytK to produce similar pores with single channel conductance (Hardy *et al*, 2001).

CytK is known to be secreted through the sec pathway as water-soluble monomers that link together to form a pre-pore state on the surface of target cells. The pore forming regions are subsequently pushed into the cell membrane, creating a β-barrel transmembrane pore (Bhakdi, & Tranum-Jensen, 1991). The insertion of a pore creates a pressure gradient resulting in

the movement of ions and small molecules in and out of the cell. Although there is definite sequence similarity between the β-barrel forming toxins their biological purposes are different (Hardy *et al*, 2001). CytK is acutely cytotoxic toward epithelial cells like those located within the small intestine in contrast to the α-toxin, which shows weak activity (Bhakdi, & Tranum-Jensen, 1991). The protease trypsin is a key constituent of the small intestine. Trypsin displays bactericidal properties by catalyzing the hydrolysis of proteins containing leucine and arginine residues. This can affect some of the toxins expressed by *B. cereus* (Lund, Byser, & Granum, 2000). However as *cytK* contains no leucine it remains unaffected (Lund, Byser, and Granum, 2000). Its ability to survive in such inhibitory conditions, coupled with its cytotoxicity on epithelial cells, makes it a very potent toxin.

First discovered after a food poisoning outbreak that resulted in the death of three elderly people in Norway, cytK displayed necrotic and haemolytic properties (Lund Buyser & Granum, 2000). The implicated strain, NVH391/98, was demonstrated through phylogenetic analysis to be distant from the main *B. cereus* group members. Subsequently NVH391/98 was shown to produce a variant form of cytK, denoted cytK-1, which is highly toxic due to its hyper expression levels. CytK distribution is restricted within the *B. cereus* family and only present in a few strains with the highly cytotoxic cytK-1 only present and functional in two strains to date. Strains previously found to contain cytK produce a less cytotoxic protein, denoted cytK-2 (Fagerlund *et al*, 2004; Fagerlund *et al*, 2007). CytK-1/2 display 89% amino acid identity, however differences were detected phenotypically in the pores created by each protein. CytK-2 did not produce the characteristic large pores that were witnessed with cytK-1 (Fagerlund *et al*, 2004; Fagerlund *et al*, 2007). Differences in the expression levels were also detected with less cytK-2 being produced under similar conditions. These factors contribute to cytK-2 being five times less toxic (Fagerlund *et al*, 2004; Fagerlund *et al*, 2007)

Virulence gene expression in *B. cereus* is under the control of the pleiotropic regulator *plcR* (see section 1.8) (Gohar *et al*, 2002). *plcR* regulated genes

contain a plcR recognition box upstream from the start codon. At -86bp and -89bp away from the cytK-1 and cytK-2 genes respectively sits the *plcR* recognition sequence (Agaisse *et al*, 1999; Gohar *et al*, 2002; Brillard & Lereclus, 2004). In the highly toxic NVH391/98 strain the plcR-box ('5-TATGCAATTTCGCATA-3') deviates in one base pair resulting in a mismatch when compared with the normal consensus sequence ($TATGNAN_4TNCATA$) (Agaisse *et al*, 1999; Okstad *et al*, 1999; Gohar *et al*, 2002; Brillard & Lereclus, 2004). This diverged box sequence has only been found in one further gene, InhA-2, a zinc requiring metalloproteinase (Fedhila *et al*, 2003). When the alternative plcR sequence from NVH391/98 was introduced into ATCC14579 there was a slight reduction in ctyK-2 expression, implying that the standard plcR-box sequence is more efficient than the one held in NVH391/98 (Brillard & Lereclus, 2004). This indicates that not only does plcR recognise divergent plcR-box sequences; the deviation in the plcR box is not the reason behind ctyK-1 hyper expression (Ivanova *et al*, 2003; Brillard & Lereclus, 2004). It is possible differences recorded in the σ^A boxes directly preceding the plcR-box at -35 could account for the increased cytK-1 expression in NVH391/98 (Brillard & Lereclus, 2004). Along with the plcR box, a fur (ferric uptake regulator) recognition sequence was also located preceding both cytK genes. It is possible that this iron regulator also has an indirect role in the expression of cytK1/2, however its exact role is unclear.

Although it was first thought that the three component toxins, *nhe* and *hbl* were not produced in NVH391/98 due to a negative result using commercial detection kits, it was later discovered that *nhe* is present, but in a variant form (Fagerlund *et al*, 2007). Thus the highly cytotoxic effect of NVH391/98 cannot be solely due to expression of cytK-1. CytK-2 producing strains contain both *nhe* and *hbl* operons however it is unknown whether the production of all three toxins results in a greater cumulative effect on host cells. CytK1/2 is not the only pore forming toxin that *B. cereus* can express although it is the only one implicated in any diarrhoeal food poisoning outbreak.

1.7.5 Haemolysin II and III

Haemolysin II (*hlyII*) is a 42kDa protein, first described in 1973 and is a member of the ß-PFT (pore forming toxin) family like cytK1/2 (Baida *et al*, 1999; Andreeva *et al*, 2007). Homology studies have shown that that hlyII is 30% similar to the *Staphylococcal* haemolysin, αHL. Through modelling, based on αHL, HlyII has been shown to be a mushroom shape, comprising a cap, stem domain and seven-rim domains (Andreeva *et al*, 2007). The diameter of the pore is at it largest (4nm) inside the cap, however it is believed that the functional diameter of the pore is around 1.2nm. Sequence similarity between these toxins is held within the cap domain (Miles, Bayley, & Cheley, 2002).

Like cytK, upon secretion hlyII proteins assemble to form transmembrane pores in vulnerable cells, leading to cell death. Rapid pore formation in host cell membranes contribute to hlyII being 15 times more potent than its relatives, cytK and αHL (Miles, Bayley, & Cheley, 2002). Once pores have been formed they can not undergo further processing. Pore properties and activity can be influenced by external environmental conditions (Andreeva *et al*, 2007). Oligomerization of hlyII proteins is required for the formation of pores in cell membranes and is temperature dependent with maximal lysis occurring at 25^0C. Out with the optimal temperatures, hlyII molecules do not bind in close enough proximity to each other and subsequently can not oligomerize to form larger pores (Miles *et al*, 2002; Andreeve *et al*, 2007). HlyII also contains leucine and arginine residues that could render the toxin non-functional within the small intestine due to the presence of trypsin (Fagerlund *et al*, 2004). The presence of trypsin and human body temperature being 37^0C could be enough to render this potent toxin, non-functional.

Like other virulence factors, including *cytK*, *nhe* and *hbl* it was believed that plcR regulated hlyII expression however extensive searches resulted in no plcR box being located (Kovalevskiy *et al*, 2007; Rodikova *et al*, 2007). However a gene was found showing homology to the tet family of transcriptional regulators. It was determined through knock out experiments

that this gene was directly involved in the regulation of hlyII and was named, hlyIIR (Kovalevskiy *et al*, 2007; Rodikova *et al*, 2007).

1.7.6 Haemolysin III

A second haemolysin, denoted haemolysin III has been characterised. Initially believed to be a variant form of hlyII or a truncated or proteolytic portion of another haemolysin, it was demonstrated to be encoded for by a separate gene and homology studies revealed it differed from hlyII (Baida & Kuzmin, 1995). HlyII is small, potent toxin (Baida & Kuzmin, 1995). It produces pores within the membrane of susceptible cells. Its action is temperature dependent with optimal lysis witnessed at 25^0C (Baida & Kuzmin, 1996). There are key steps in the action of hlyII and hlyIII; expression of toxin, binding to responsive cells, formation of lesion and finally lysis. All stages in the process are temperature dependent; at low temperatures neither binding nor lysis occurs (Baida & Kuzmin, 1996). Binding to cells appears to happen in a monomeric fashion with multiple monomers binding resulting in the disruption to the human cell wall and membrane just before lysis. It is likely as with *HlyII*, that human body temperature is too high for these haemolysins to be effective toxins

1.8 Additional Virulence Factors

1.8.1 Adhesins

For *B. cereus* to cause diarrhoeal food poisoning it must remain in the small intestine for a significant period of time to allow virulence factors to be expressed. *B. cereus* produce proteins known as adhesins that promote *B. cereus* attachment to the epithelial cell wall and thus greatly enhance virulence. These proteins are classified as virulence factors. The protein EntFM, first believed to be a toxin as it was isolated from every diarrhoeal food poisoning outbreak was recently shown to be a cell wall peptidase (Cwp) and was renamed CwpFM (Tran *et al*, 2010). As a cwp, cwpFM has a role in bacterial adhesion, invasiveness and biofilm formation (Tran *et al*, 2010). Mutating the *cwpFM* gene led to a reduction in the virulence recorded in an insect model due to a dramatic loss in the cells ability to adhere (Tran *et al*, 2010). The ability of *B. cereus* spores and vegetative cells to adhere to the

epithelial layer in the small intestine could dramatically increase their virulence and in turn their pathogenicity.

1.8.2 Phospholipase C

Enhancing the effect of toxins is a host of further non-toxic virulence factors like phospholipase C. Preferentially it hydrolyses phosphatidylcholine (PC-PLC). A plasmid encoded tri-zinc protein, PC-PLC has a molecular mass of 28.5kDa and a wide range of activity, preferring mildly acidic conditions. It quickly lyses membranes that contain high levels of phosphatidylcholine, however also shows activity against sphingomyelin and phostphatidylinositol (PI) (Durham et al, 2007). PI-PLC belongs to a family of intracellular enzymes that play important roles in signal transduction. PIP_2, a component of cell membranes, is cleaved by PI-PLCR and two messenger proteins are produced, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) (Katan, 1998, Martin & Pitzer, 2000). DAG activates a series of signal cascades when it binds to the protein kinase K domain within the membrane (Hofmann & Dixit, 1998). IP3 interacts with the smooth endoplasmic reticulum of eukaryotic cells, which results in the release of calcium ions (Ca2+), which regulate cell proliferation (Hofmann & Dixit, 1998). Phospholipase C is not essential to the growth or survival of *B. cereus* cells but its ability to act against human epithelial cells increases virulence.

1.8.3 Sphingomyelinase

Sphingolipids are present in eukaryotic cell membranes. Prokaryotes can utilize them within hosts to increase their virulence (Heung Luberto & Del Poeta 2006). Several sphingomyelin specific phospholipase C (PLC) activities, termed sphingomyelinase (SMase) exist within *B. cereus* to break down sphingolipids. *B. cereus* mediated SMase have been shown to be $Mg2^+$ dependent neutral nSMase and unlike their mammalian counterparts are not attached to the cell membrane (Obama *et al*, 2003; Hofmann & Dixit, 1998). SMase hydrolysis products are always phosphorylcholine and ceramide. Ceramide molecules are released and have been shown to have a role in cell differentiation and proliferation (Goñi & Alonso, 2002; Hoffmann & Dixit, 1998; Tomiuk *et al*, 1998). In human erythrocytes cooperation between PC-PLC and

sphingomyelinase is required to lyse cells as their membranes are constructed of both sphingomyelin and phosphatidylcholine (Pomerantsev *et al*, 2003). Recently a cytolysin has been identified which has both phospholipase and sphingomyelinase activity.

1.8.4 Cereolysin AB

Cereolysin AB toxin is a 23kDa, zinc metalloenzyme, which encodes two genes, *cerB* and *cerA*. (Gilmore *et al*, 1989). *CerB* has been shown to encode sphingomyelinase while *cerA* is involved in phospholipase C production. Sphingomyelinase rearranges the lipid composition in human erythrocytes in order for phospholipase C to then lyse the cells (Gilmore *et al,* 1989). *cerA* and *cerB* are arranged tandemly and control of their expression is thought to be under *plcR*. This toxin alone has not mediated any food poisoning outbreaks but its expression could impact on virulence.

1.9 Virulence Gene Control

1.9.1 PlcR Quorum Sensing

Almost all known virulence factors in *B. cereus* are controlled by plcR, a pleiotropic regulator that was first discovered in *B. thuringiensis* (Lereclus *et al*, 1996; Goher *et al*, 2008). PlcR regulated genes can be determined by the presence of a characteristic plcR box (Agaisse *et al*, 1999). The box is composed of a palindromic sequence, which is highly conserved ($TATGNAN_4TNCATA$) (Agaisse *et al*, 1999; Slamti & Lereclus, 2002). The area around the active boxes is very AT rich with evidence of σ^A binding sites, suggesting the plcR-controlled genes could be expressed by σ^A RNA polymerase. This box can be found at various locations upstream from the gene it controls. In some cases it can overlap the -35 region or binds closer to -60 region upstream from the gene (Agaisse *et al*, 1999). The variance of the plcR-box position could indicate that transcription can be initiated by varying mechanisms dependent on way the plcR promoter is structurally organised (Agaisse *et al*, 1999). A signal sequence, typical of those found in prokaryotes is present in genes under plcR control. This suggests that proteins expressed could be secreted, possibly through the sec secretion machinery (Agaisse *et al*, 1999).

PlcR is involved in the expression of genes with various functions including haemolysin, enterotoxins, peptidoglycan synthesis, antimicrobial peptides and drug efflux pumps (figure 1.9.1). When Gohar *et al* (2002) created a plcR null mutant in ATCC14579 50% less secreted protein was present in the supernatant when compared to wild type (WT) ATCC14579 (Gohar *et al,* 2002). 2-D gels indicated that 96 protein spots were under the control of plcR and 36 were partially controlled by plcR. Most of the spots corresponded to flagellins and a zinc metalloprotease, inhA2. Flagellins are associated with adherence to host tissue and have been shown to be essential in bacterial virulence. InhA2 works against host defences, breaking down antibacterial peptides (Gohar *et al*, 2002; Gohar *et al*, 2008). Sequence studies showed that there are no plcR boxes upstream from inhA2 or flagellin suggesting that plcR exerts its control in an indirect manner. As well as increasing virulence genes under the control of plcR offer a selective advantage for *B. cereus* strains trying to survive within hostile conditions, like those present in the human GI tract.

In order to express virulence genes, plcR has to be activated. PlcR is activated by the 48aa signal oligopeptide papR. *PapR* can be expressed under various conditions and secreted via the sec pathway (Slamti & Lereclus, 2002; Gohar *et al*, 2008). The active fragment is created during its export resulting in the inducer molecule containing five C-terminal residues (Slamti & Lereclus, 2002; Gohar *et al*, 2008). It is then re-imported into the cell through the opp permease system, which has been shown to be critical to the functioning of plcR (Figure 1.9.1) (Agaisse *et al*, 1999; Gohar *et al*, 2002; Slamti & Lereclus, 2002; Declerck *et al*, 2007; Gohar *et al*, 2008)

Within the 34kDa plcR molecule, helix-turn-helix (HTH) domains and five tetratricopeptide repeats (TPR) and a capping domain can be found (Slamti & Lereclus, 2002; Declerck *et al*, 2007). TPR are present in both prokaryotes and eukaryotes and consist of conserved helical areas that are involved in protein and peptide interactions **(**Bouillaut *et al,* 2007; Declerck *et al*, 2007; Gohar *et al*, 2008). It has been shown that papR binds to the centre of the

TPR domains and somehow triggers a conformational change in the structure of plcR (Agaisse *et al*, 1999; Gohar *et al*, 2002; Slamti & Lereclus, 2002; Bouillaut *et al,* 2007; Declerck *et al*, 2007; Gohar *et al*, 2008). The HTH domains are altered allowing DNA to bind and influence gene expression (Bouillaut *et al,* 2007; Declerck *et al*, 2007). PlcR will only bind DNA in the presence of papR (Agaisse *et al*, 1999; Gohar *et al*, 2002; Slamti & Lereclus, 2002; Bouillaut *et al,* 2007; Declerck *et al*, 2007; Gohar *et al*, 2008). PlcR box regions throughout the chromosome show the highest rate of mutation; however these changes occur out with the conserved core TPR region (Bouillaut *et al*, 2007). PapR and plcR are separated by approximately 70bp and evidence suggests they were once one gene, which has evolved into two separate genes that still require each other to function (Slamti & Lereclus, 2002; Slamti *et al*, 2004; Brillard & Lereclus, 2007; Bouillaut *et al*, 2007; Declerck *et al,* 2007).

PlcR controlled genes are not confined to a pathogenicity island like the toxin genes on plasmids in *B. thuringiensis* and *B. anthracis*. They are spread throughout the chromosome with plcR homologs found in *B. anthracis* and *B. thuringiensis* pointing towards these species diverging from one common ancestor (Agaisse *et al*, 1999; Slamti *et al*, 2004). The quorum like sensing of plcR/papR allows for an effective change in the gene expression profile enabling the continued adaptation of *B. cereus* to their surroundings. Deletion of plcR in *B. cereus* significantly reduced the pathogenicity of the organism but does not abolish it completely indicating that other regulators have a role in *B. cereus* virulence (Gohar *et al*, 2002)

1.9.2 HlyII Regulation

Searches through the regions surrounding the hlyII gene revealed no plcR box, however a ferric iron uptake regulator (fur) sequence was found along with another gene subsequently discovered to be hlyII regulator, hlyIIR (Kovalevskiy *et al*, 2007; Rodikova *et al*, 2007). The presence of both regulatory sequences indicates that hlyII is strictly controlled by both regulator and *fur* (Kovalevskiy *et al*, 2007). *HlyIIR* negatively regulates transcription by binding to a region 48bp from the *hlyII* start site and

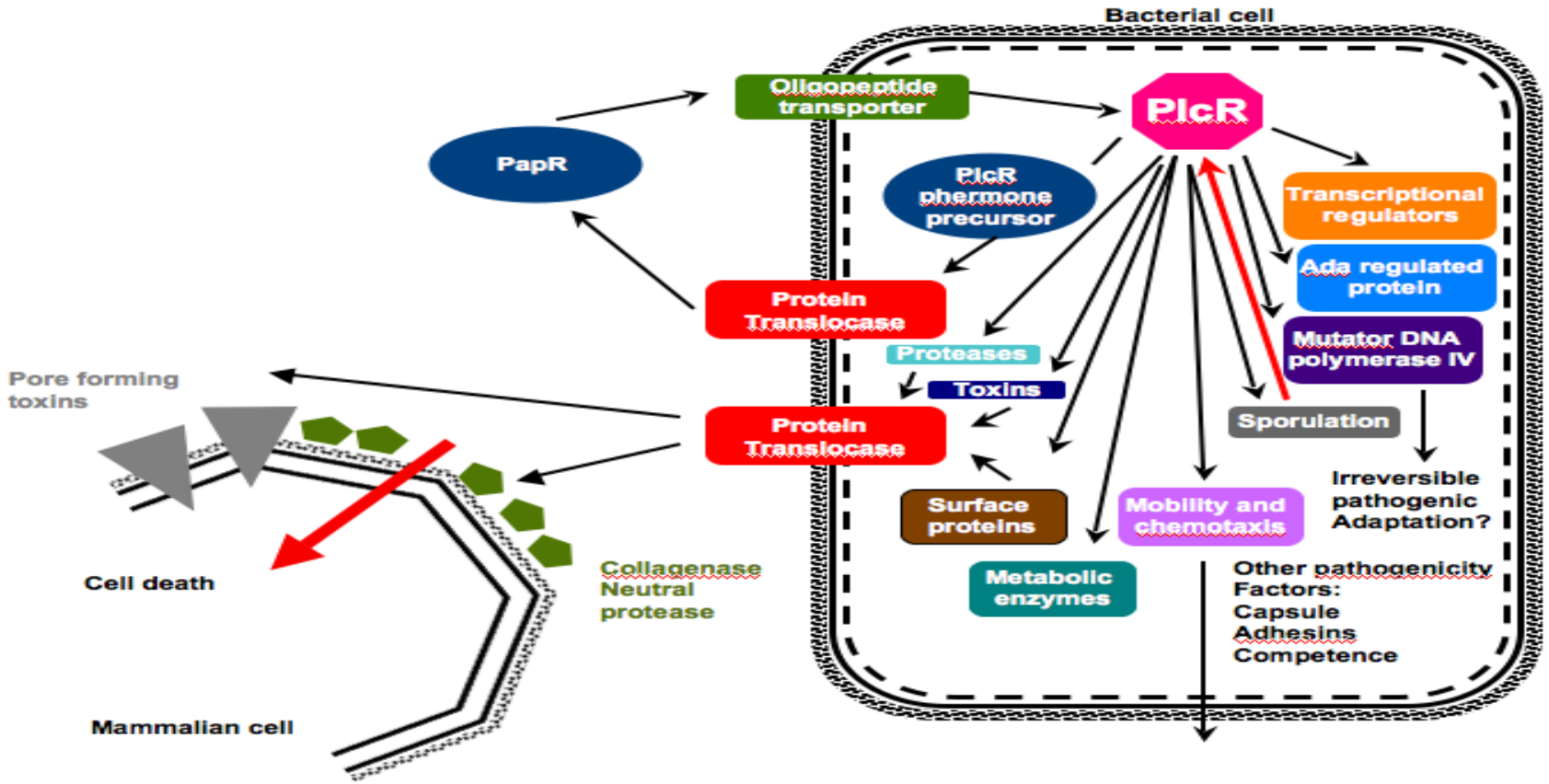


Figure 1.9.1 PlcR Regulon. PlcR transcription regulator controls the expression of many genes involved in virulence and cell survival. Gene expression is controlled by the presence of a plcR-box located upstream from the start codon. PlcR however must be activated before it can mediate any effect. The oligopeptide papR has been shown to interact with plcR, modifying it, resulting in its ability to bind to plcR boxes and initiate gene transcription. Activation of the plcR regulon results in the secretion of many proteins including toxins that can have a negative effect on host cells. Diagram modified from Ivanova *et al*, (2003).

inhibiting the ability of RNA polymerase to change from closed to an open conformation, resulting in less or no hlyII being produced (Andreeva *et al*, 2007; Rodikova *et al*, 2007).

A 44bp, inverted repeat, region located within the hlyII operator has been revealed to interact with HlyIIR (Rodikova *et al*, 2007). HlyIIR produces a 23.5kDa protein that shows homology in its N terminus to the TetR family of transcription factors (Kovalevskiy *et al*, 2007; Rodikova *et al*, 2007). Its unclear as to why such a long length of DNA interacts with hlyIIR, however has been shown to be evolutionary stable and present in all strains of *B. cereus* that contain *hlyII* (Kovalevskiy *et al*, 2007; Rodikova *et al*, 2007). Adaptation to changing environments, pathogenicity, virulence and drug resistance are all partly controlled by TetR repressors via helix-turn-helix domains that bind DNA. Mapping the N terminal region of HlyIIR revealed the presence of helix-turn-helix domains found in around 95% of prokaryotic transcription factors (Kovalevskiy *et al*, 2007).

Toxin production within *B. cereus* has been shown to commence at the beginning of stationary phase however *hlyII* is expressed during log phase suggesting it could be one of the first toxins produced. A small ligand, possibly a cholesterol derivative has been shown to moderate the repressor activity of HlyIIR (Kovalevskiy *et al*, 2007). A possible negative feedback loop could be created where once *hlyII* has been expressed the degraded host cellular cholesterol, released from the cell membrane, binds to the cell surface of *B. cereus* and signals for the termination of *hlyII* expression. *HlyII* and *hlyIIR* are present in all *B. cereus* sequenced genomes except ATCC10897. *HlyIIR* has been implicated in the regulation of 4 further genes, however only hlyII has been shown to be toxic (Kovalevskiy *et al*, 2007).

1.9.3 Ferric Iron Uptake Regulator Repressor Gene *fur*

The ferric iron uptake regulator repressor gene (*fur*) also has a role in the expression of *B. cereus* virulence factors (Harvie *et al*, 2005). The uptake of iron is controlled by *fur* in many bacteria and has been linked to the regulation of over 40 genes across many metabolic pathways. It also plays a role in

oxidative and acid stress and the production of virulence factors. Iron within hosts can be in short supply with it being bound to transferrins, ferritins, haemoglobin and myoglobin (Harvie *et al*, 1995). Within *B. cereus* Fur plays an essential role ensuring intra-cellular iron concentrations are maintained within limits that allow bacterial growth, survival and pathogenesis (Harvie *et al*, 2005). Genetic analysis has revealed that it is involved in the regulation of haemolysin II (hlyII) expression, HlyIIR expression and the expression of a number of predicted cell surface proteins with putative roles in cellular adhesion and invasion (Harvie *et al*, 2005). Genes controlled by *fur*, like *plcR*, have Fur-boxes upstream from their start sites that bind in a metal dependent fashion. A null mutation of *fur* in a *B. cereus* strain resulted in a decrease in virulence when tested in an insect model. Genes encoding toxins CytK and HlyII have Fur boxes within their promoter area and in the case of *hlyII* it overlaps the transcriptional start site (Kovalevskiy *et al*, 2007; Rodikova *et al*, 2007). The location of these Fur-binding site indicates that in the presence of fur, hlyII could be repressed. However the true extent of the impact that Fur has on virulence remains to be determined.

1.10 Cytotoxin Detection

In order to detect the threat of diarrhoeal food poisoning toxins first the presence of *B. cereus* within food or raw materials or in laboratory culture must be ascertained. Previously food or food ingredients were heated to remove the presence of contaminating vegetative bacteria before the enumeration of *B. cereus* spores. This method however is flawed due to the likely presence of *B. cereus* vegetative cells within the sample (Mossel Koopman, & Jongerius, 1967). Mossel *et al* (1967) manufactured a selective medium to culture *B. cereus* known as MY-agar (Mossel Koopman, & Jongerius, 1967). This media differentiates between *B. cereus* group members by accounting for lecithinase production and mannitol fermentation. Polymyxin B can also be incorporated into medium to allow for the specific selection of *B. cereus* (Mossel Koopman & Jongerius, 1967). Once the presence of a *B. cereus* strain is confirmed, the identification of its toxin producing capabilities needs to be determined.

Spira and Goepfert (1972) discovered that *B. cereus* produces a toxin capable of increasing the vascular permeability in rabbit ileal loops. Vascular permeability could also be detected in rabbits and mice when a preparation of crude cell filtrate was injected into their skin. A blueing reaction could be discerned within ten minutes, with this area being directly proportional to toxicity of the prepared strain (Spira & Goepfert, 1972). Both the rabbit ileal loop and vascular permeability assays became the gold standard tests for the presence of *B. cereus* enterotoxins and only recently have molecular methods surpassed those traditional ones and allowed for quicker, cheaper more accurate detection of *B. cereus*.

Conventional PCR allows specific virulence genes to be detected. Although this gives no quantitative information on gene expression it can be useful in determining the potential of an organism to produce specific proteins. PCR has been used in a number of studies involving *B. cereus* to establish the presence of genes involved with food intoxication such as *hbl*, *nhe* components along with *cytK* and other known virulence factors (Mäntynen & Lindström, 1998). Muliplex PCR has also been described, allowing for the assay of multiple virulence genes at once under defined conditions (Minaard *et al,* 2004). Real time PCR allows for specific genes to be monitored and using control genes, data on gene expression levels to be discerned (Flicker *et al*, 2007). These approaches allow for rapid confirmation and quantification of virulence genes within food isolates, raw materials and under laboratory conditions. PCR results are however only as accurate as the primer design and conditions such as stringency and thus care must be taken to ensure the correct product has been amplified.

Chinese hamster ovary cells along with other cell lines have been used to measure the cytotoxicity of *B. cereus* strains. Pathogenic stains destroy the cell monolayer within a short period and this can be directly proportional to the amount of toxin being expressed and secreted. Although this assay can inform as to the cytotoxic nature of the isolated strain it does not distinguish between individual toxins as several are known to elicit the same destruction of cell monolayers.

Commercial immunoassy kits have been designed to help confirm the presence of individual components of the *hbl* and *nhe* toxins (Day *et al*, 1994; (Hsieh *et al*, 1999; Beecher & Wong, 1994; Buchanan & Schultz, 1994). The reverse passive latex agglutination assay (RPLA) produced by Oxoid, detects the L_2 protein from *hbl*. The *Bacillus* diarrhoeal enterotoxin visual immunoassay by 3M™ (Tecra™) detects the *nheA* component of *nhe* along with a secondary non-toxic protein. These kits offer a quick method of determining the presence of each toxin however lack quantitative assessments about the level of toxic components within food, raw materials or media. Polyclonal and monoclonal antibodies have been produced for each member of the three component toxins (Beecher & Wong 1994A; Moravek *et al*, 2004). These have allowed for a greater sensitivity in assaying expression levels of Hbl and Nhe proteins however the process is time consuming and can require lengthy enrichment and protein precipitation steps. Sequence homology seen between *nheB/C* proteins and across toxins, such as L_2 and *nheB* could also affect the specificity of polyclonal raised antibodies.

Recently Moravek *et al* (2004) have utilized monoclonal antibodies directed at the binding component of *hbl* and *nheA* to determine the presence of these toxins via colony immunoblots. *B. cereus* was grown on blood agar plates and secreted toxins transferred to a nitrocellulose membrane where they were probed with specific monoclonal antibodies (Moravek *et al,* 2004). This allowed quick and reliable detection of hbl and nhe on culture plates. As described previously both *Hbl* and *Nhe* are transcribed as operons, indicating that if one protein from these toxins can be detected it is likely that all components have been expressed (Moravek *et al,* 2004).

The rapid identification of *B. cereus* isolates from food and raw materials is of great importance to food producers. By utilizing new techniques along with more traditional approaches the presence of contaminating pathogenic *B. cereus* can be determined within 24 hours, giving the food producer time to take steps to limit consumer exposure.

1.11 Conclusions

The ability of *B. cereus* and *B. weihenstephanensis* to survive, as endospores when the environment around them no longer supports microbial growth is crucial. This strategy allows them to persist where they would otherwise perish, like soil and on raw food ingredients. The ability of spores and vegetative cells to contaminate foodstuffs is of great concern to food producers. Over the last 10 years food preservation/sterilization techniques have changed. Consumers have demanded foods that are less processed resulting in new practices, which may result in the death of all vegetative cells, but spores are often resistant. Chill is frequently used as a mechanism to reduce microbial growth but in such environments it has been shown *B. weihenstephanensis* can grow. Although there is a significant amount of information available about *B. cereus* mediated diarrhoeal food poisoning including toxin expression, whether it is mediated by contaminating spores or vegetative cells is unclear. The effect of the human gastro-intestinal tract on spores and vegetative cell viability within food items is not known. It is however possible for pathogenic *B. cereus* to survive as demonstrated by the diarrhoeal food poisoning outbreaks recorded throughout Europe. *B. weihenstephanensis* is an emerging member of the *B. cereus* group. There is uncertainty as to its ability to be pathogenic and capable of mediating diarrhoeal food poisoning outbreaks.

1.12 Aims and Objectives

- To compare and contrast *B. cereus* and *B. weihenstephanensis* vegetative cells and spores to determine any differences in their ability to grow, their survival and their ability to cause disease under simulated human GI tract conditions.
- To investigate the effect the human gastro-intestinal tract has on the expression of virulence factors associated with diarrhoeal food poisoning.
- To gain more information about *B. weihenstephanensis* and ascertain its potential as a human diarrhoeal food poisoning pathogen.

Chapter Two
Materials and Methods

2.1 Chemical suppliers

General laboratory chemicals of analytical grade were purchased from Sigma-Aldrich Company Ltd, Dorset, UK, and Fisher Scientific Ltd, Leicestershire, UK. Polymerase chain reaction (PCR) DNA oligonucleotide primers were sythesized by Sigma-Aldrich Company Ltd, Dorset, UK. TFI polymerase and PageRuler™ plus prestained protein ladder were purchased from Fermentas Ltd, York, UK. 1Kb DNA ladders were from Bioline Ltd, London, UK. Real time PCR reagents were obtained from Bio Rad Ltd, Hemel Hempstead, UK. dNTPs at a stock concentration of 100mM were purchased from Promega Ltd, Southampton, UK and $MgCl_2$ was from Invitrogen Ltd, Paisley, UK.

DNA and RNA extraction kits (DNeasy/RNeasy Mini Kits) were from Qiagen Ltd, West Sussex, UK. Trypsin was purchased from Promega Ltd, Southampton, UK. Pepsin was from Fisher Scientific Ltd, Leicestershire, UK. Pancreatin and bile acid were purchased from Sigma-Aldrich Company Ltd, Dorset, UK.

Nupage® 10% Bis-Tris 2-12% precast gels, Nupage MES SDS (20x) electrophoresis buffer, Iblot® nitrocellulose transfer stack, and Nupage LDS sample buffer (4x) were all purchased from Invitrogen, Ltd, Paisley, UK.

All disposable plasticware was obtained from Greiner Ltd, Gloucestershire, UK or Nunc, Thermo Fisher Scientific, Northumberland, UK. PCR tubes were obtained from Abgene Ltd, Epson, UK. PCR plates were from Biorad Ltd, Hemel Hempstead, UK. Brain heart infusion broth medium (BHI) was purchased from Merck Ltd, Nottingham, UK. Nutrient agar medium was from Oxoid Ltd, Basingstoke, UK. Agar was bought from Biogene Ltd, Cambridgeshire, UK. Instant blue Coomassie stain was from Triple Red Ltd, Buckinghamshire, UK. Non-interfering protein assay kit was purchased from Calbiochem, Merck Ltd, Nottingham, UK. Western blotting detection agents were purchased GE healthcare Ltd, Bucks, UK.

2.2 Strains, media and growth conditions

All growth media were sterillised by autoclaving at 121^{0}C, 15 psi for 20 minutes. Media, buffer and solutions were prepared using ultra pure deionised water (Elga system, Millipore, Herts, UK)

2.3 *Bacillus* Strains and Growth Media

Four *Bacillus cereus* strains and three *Bacillus weihenstephanensis* strains were used in this study (Table 2.3.1). Strains were selected from recent publications based on their growth characteristics and pathogenesis. Three *B. cereus* strains were isolated from diarrhoeal food poisoning outbreaks allowing direct comparisons to be drawn between these strains and *B. weihenstephanensis*.

All strains were grown in liquid culture (brain heart infusion broth (pH 7.5)) and on solid agar (nutrient agar, pH 8). pH was adjusted using a Hanna pH201 microprocessor pH meter and 10M NaOH or 10M HCL. Growth was measured by both cell enumeration, on nutrient agar plates after twelve-sixteen hours and spectrophometrically in liquid culture (OD_{600}).

2.4 Growth Conditions

Single colonies were picked using sterile disposable loops and inoculated into 5mls of BHI. Overnight culture at 30^{0}C in an orbital incubator at 200rpm provided starter cultures. These cultures were used for subsequent inoculations and then used as experimentally required. Short term storage of cells was obtained through bacteria streaked to single colonies on nutrient agar plates and stored at 4^{0}C for up to four weeks. Gycerol stocks were used for long term storage. To log phase liquid cultures sterile glycerol was added to final concentration of 25% (v/v), vortexed and stored at -80^{0}C.

2.5 Spore crops

Nutrient agar plates were inoculated with 100μl of a log phase culture as determined by OD_{600}. Plates were incubated at 30^{0}C for four days until sporulation was complete. 10ml of sterile PBS was added to each plate and a

Species	Name	Information	Source/Publication
B. cereus	27	Possibly psychrotrophic; Isolated from diarrhoeal food poisoning outbreak.	Wijnands *et al*, 2006
	883-00	Mesophilic; Contains cytotoxin, *cytK-2* gene.	Fagerlund *et al*, 2004
	F2081B/98	Mesophilic; Contains *hbl* and *nhe* genes. Isolated from diarrhoeal food poisoning outbreak.	Guinebretiere *et al*, 2002
	98HMPL63	Mesophilic; Contains *hbl* and *nhe* genes. Isolated from diarrhoeal food poisoning outbreak.	Guinebretiere *et al*, 2002
B. weihenstephanensis	10390	Psychrotrophic; Contains *hblD* and *nheB* genes and showed 90% toxicity towards vero cells	Stenfors *et al,* 2002
	10396	Psychrotrophic; Contains *hblD* and *nheB* genes and showed 80% toxicity towards vero cells	Stenfors *et al*, 2002
	10202	Psychrotrophic; Contains *hblD* gene and showed 90% toxicity towards vero cells	Stenfors *et al*, 2002

Table 2.3.1 *B. cereus* and *B. weihenstephanensis* Strains. Strains were selected based on their growth parameters, known pathogenic nature and their potential pathogenesis.

sterile inoculating loop was used to remove the spores and cells from the surface of the agar. This mixture was then transferred to a sterile 15 ml Falcon tube and centrifuged at 4000xg, 4^0C for 10 minutes The supernatant was removed and the pellet washed 3 times in 10ml of sterile PBS. The final washed pellets were then heat treated at 80^0C for 10 minutes to kill any vegetative cells. Pellets were then resuspended in 1ml PBS, aliquoted and stored at -20^0C.

2.5.1 Spore counts

Viable spore concentrations were determined by colony counts on nutrient agar. Serial dilutions, typically 10^{-1} to 10^{-12} were made using BHI as the diluent. Colonies were counted after 3 days of incubation at 30^0C

2.6 Effects on growth in simulated gastro-intestinal environments of *B. cereus* and *B. weihenstephanensis*

2.6.1 Stomach Simulations

B. cereus and *B. weihenstephanensis* strains were inoculated into BHI and incubated at 30^0C, 200rpm, to mid-log phase as determined by OD_{600} and diluted to a final cell concentrations of 10^3, 10^5 or 10^7cfu/ml within media. Spore suspensions were diluted in sterile BHI to a concentration of 10^3, 10^5 or 10^7 spores/ml. Pepsin (3g/L w/v) was added to BHI broth and the pH reduced using 10M HCL to 1, 2 or 3. Media was sterilised through a 0.2μm filter (Millipore Ltd, Watford, UK). 10 mls of filtered BHI + pepsin was aseptically transferred to a 100ml conical flask. Flasks were then inoculated tp 10^3, 10^5, or 10^7 spores/ml or CFU cells/ml. Cultures were incubated at 37^0C, 200rpm. Cells were enumerated through viable count. Samples were taken hourly for 4 hours, serially diluted, plated on nutrient agar and placed at 37^0C for 12-16 hours.

Growth was also recorded under stomach conditions by monitoring optical density (OD_{600}). 48 well plates were used and 300μl BHI + pepsin at pH 1, 2 or 3 was dispensed into each well. 50μl of cells to give a final concentration 10^3, 10^5 or 10^7 of CFU/ml or spores/ml were inoculated into each well. Random wells were not inoculated, providing negative controls. Plates were

sealed and placed in a, pre-heated (37^0C) plate reader (BioTek Ltd, Bedfordshire, UK). OD_{600} were measured every 6 minutes for 4 hours, shaking for 272 seconds before each reading to disperse clumped cells.

2.6.2 Small Intestinal Simulations

B. cereus and *B. weihenstephanensis* strains were inoculated into BHI and incubated at 30^0C, 200rpm, until cell density reached 10^3, 10^5, or 10^7 CFU/ml as determined by OD_{600}. Spore suspensions were diluted in sterile BHI to a concentration of 10^3, 10^5 or 10^7 spores/ml. Bile acids were added to fresh, sterile BHI at two concentrations, 0.3% (3g/L w/v) and 0.5% (5g/L w/v). pH was adjusted to pH8 with the addition of 10M $NaCl_2$. Media was sterilized through 0.2μm filter. 10ml of BHI + bile acid was transferred to 100ml conical flask. Pancretin (3g/L w/v) was added aseptically, and flasks inoculated with 1ml of 10^3, 10^5 or 10^7 CFU/ml or spores/ml. Cultures were incubated at 37^0C, 200rpm for four hours. Cell number was monitored through viable counts. Samples were taken hourly, diluted and plated on nutrient agar. Plates were incubated at 37^0C for 12-16 hours.

2.6.3 Dual GI tract simulation

B. cereus and *B. weihenstephanensis* strains were inoculated into BHI and incubated at 30^0C, 200rpm, until cell density reached 10^3, 10^5, or 10^7 CFU/ml as determined by OD_{600}. Spore suspensions were diluted to the required concentration (10^3, 10^5 or 10^7 spores/ml). Cultures were initially incubated in simulated stomach media at 37^0C, 200rpm. After 4 hours 1ml of simulated stomach media was removed and inoculated into 10mls of simulated small intestinal media and incubated at 37^0C, 200rpm for a further 4 hours. Cell number was determined through viable counts. Samples taken were diluted and plated on nutrient agar. Plates were incubated at 37^0C for 12-16 hours.

2.7 Cell viability

B. cereus and *B. weihenstephanensis* strains were inoculated into BHI and incubated at 30^0C, 200rpm, until cell density reached 10^3, 10^5, or 10^7 CFU/ml as determined by OD_{600}. Cells were inoculated into simulated stomach media at pH 1, 2 or 3 and incubated for four hours at 37^0C, 200rpm. 4 hours post

inoculation 500μl of culture from each strain was removed and added to a sterile eppendorf containing 500μl of sterile BHI. Cell tracker green (stock 10mM in DSMO) and propdium iodide (stock 3.75mM in ethanol) were added to give final concentrations of 10μM and 1.8μM respectively. The reaction mixture was vortexed and incubated in the dark at 37^0C for 30 minutes. The sample were then centrifuged for 1 minute at 10,000rpm to remove any unbound dye. The resulting pellet was washed three times in 500μl sterile distilled water (dH_20) and resuspended in 20μl of sterile BHI.

2.7.1 Slide Preparation

Slides were cleaned with ethanol. 2μl of the resuspended pellet was placed in the centre of the slide. 1% agarose in TAE was prepared and 2μl placed on the slide quickly follwed by a 13mm coverslip. Clear nail polish was used to seal the coverslip to the slide. Agarose helps to fix the cells in one position. Fluorescently labelled cells taken from the gastric simulations were visualized through a FITC (excitation λ= 490/520: emission λ=528/538nm) and RD-TRPE (excitation λ=490/520: emission λ=528/538nm) fluorescent filters Images were taken using an Olympus IX70 deltavision microscope, Applied Precision Inc, Washington, USA. Captured images were processed and analysed using SoftRox explorer 1.3, Applied Precision Inc, Washington, USA.

2.8 Extraction of DNA from *B. cereus* and *B. weihenstephanensis* cultures

Qiagen DNeasy blood and tissue kit was used to extract genomic DNA from all strains grown under optimal conditions (30^0C, 200rpm, 6 hours) following the protocol for Gram-positive bacteria.

2.8.1 DNA visualisation; preparation, staining and running of agarose gels

DNA was visualised by size fractionation on horizontal, neutral gels at an agarose concentration, typically, of 1% (however, this varied from 0.7% to 2%). Agarose was dissolved in 1 x TAE buffer and poured into a cast (size

dependent on number of samples being ran). Immediately prior to pouring into the case, ethidium bromide was added to give a final concentration of 0.5μg/mL and mixed gently. Small gels were run in 1 x TAE at 70 volts for 1.5 hours and larger gels at 100 volts for 3 hours.

2.8.2 Photography of gels

A VUP GelDoc-It® TS310 transilluminator was used to visualise DNA in the agarose gels with the aid of GeneSnap (Syngene version 6.08).

2.8.3 DNA band sizing and quantification

DNA fragment size was determined by comparison to known band size via a DNA ladder (100ng/μl of λ Hind III DNA, Promega). Yield and purity were determined by nanodrop.

2.9 Conventional and RT real time PCR primer design

Primers for gene amplification were designed using sequence data taken from NCBI gene bank. RT PCR primers were designed using the following guidelines; Amplicon length 80-250bp, 18-24 nucleotides in length, no secondary structures, repeating motifs or primer dimers, melting points for primer pairs were +/- 5^0C, high GC content >50%, 3' should be GC rich to form a GC clamp to enhance annealing and Blast searches were conducted to ensure specific binding. Appendix C shows all primer sequences used in this study. Primer stock concentration of 100mM were diluted to making working concentrations of 10nM. A DNA concentration of 50ng was used throughout all conventional PCR experiments in all strains.

2.10 Conventional PCR

Determiming the presence of selected virulence genes was performed by PCR using extracted genomic DNA. Reactions consisted of Invitrogen 5x Tfi buffer (5μl), 2mM $MgCl_2$ (0.75μl), 0.25μM dNTPsce (0.5μl), Primers (0.5μl each), DNA (1μl), Tfi DNA polymerase (0.5μl) and water (16.25μl). Optimum annealing temperatures were ascertained through gradient PCR and samples were processed under the following condition; Initial denaturation 2 minutes at

95^0C, 35x (1 minute 94^0C, 1 minute (T_m), and 2 minutes at 72^0C), final extension step 5 minutes at 72^0C. Reactions were performed on a Techne TC-512 PCR machine. In order to assess the PCR 10% of the final reaction was electrophoresed on an agarose gel.

2.11 Extraction of RNA from *B. cereus* and *B. weihenstephanensis* cultures

B. cereus and *B. weihenstephanensis* strains were grown under optimal conditions (30^0C, 6 hours, 200rpm) and simulated human GI tract conditions.

2.11.1 Removal of Pancretin from simulated human GI tract cultures

Spore crops were diluted to a concentration of 10^7 spores/ml and inoculated into simulated stomach media at pH 3 and incubated at 37^0C for 4 hours. 10ml of simulated stomach media was then transferred to 100ml of simulated small intestinal media and grown for 4 further hours at 37^0C, 200rpm. Cultures were then spilt into sterile 50ml falcon tubes and centrifuged at 510gs for 10 minutes to pellet pancretin but not any cell biomass. Supernatant was removed and placed in fresh falcon tubes and centrifuged at 4000gs for 20 minutes to pellet cell biomass. The supernatant was then discarded and the pellet resuspended in 1ml of fresh BHI and centrifuged at 2000rpm for 3 minutes. Supernatant was removed and placed in a sterile eppendorf and centrifuged at 12000rpm for 5 minutes to pellet pancretin free biomass. Pellets were resuspended in 200μl in BHI.

2.11.2 RNAprotect®

RNAprotect® from Qiagen was added to the resuspended pellet to stabialize the RNA present within the sample to ensure a high yield and reliable gene expression data. Two volumes of RNAprotect® reagent was added to each strain and incubated at RT for 5 minutes before centrifugation at 8000rpm for 10 minutes.

2.11.3 Qiagen RNeasy blood and tissues extraction kit

After pancretin removal and RNAprotect® step, RNA was extracted using the RNeasy Tissue extraction kit from Qiagen following the protocol for Gram-

positive bacteria. Proteinase K (20mg/ml) was used in conjunction with DNases on-column DNA digestion (contained within Qiagen kit) to limit any DNA and protein contamination within the samples. RNA was eluted in RNA free water. After RNA extraction yield and purity were determined by nanodrop.

2.12 Synthesis of cDNA for RT real time PCR

Using extracted RNA, cDNA was synthesized using Bio-Rad iScript cDNA synthesis kit following the manufacturers protocol.

2.13 Reverse transcription real time PCR with strains 27, F2081B/98, 10390, 10396 and 10202

Reverse transcription real time PCR was performed using BioRad Iscript one-step RT-PCR kit with SYBR green. 100ng of genomic RNA was used in each reaction. This was determined to be the best amount through serial dilutions. The reaction set up; 2X SYBR® Green RT-PCR Reaction Mix (12.5µl), forward primer (10 µM) 0.75µl, Reverse primer (10 µM) 0.75µl, Nuclease-free H_2O x 9µl, RNA (100ng = 1.01 µl), and iScript Reverse Transcriptase (1µl). The reaction protocol; cDNA synthesis: 10 min at 50°C, iScript Reverse transcriptase inactivation: 5 min at 95°C, PCR cycling and detection (35 cycles): 10 sec at 95°C 30 sec at 55°C to 60°C (data collection step) All reactions were performed in triplicate using a Biorad Icycler PCR machine**.** Melt curve analysis was performed at the end of each PCR reaction to ensure no contamination or primer dimers had been formed. The reaction protocol was 1 min at 95°C, 1 min at 55°C, 10 sec at 55°C (80 cycles, increasing each by 0.5°C each cycle).

2.13.1 Reaction Efficiency

Reaction efficiencies for each primer set were determined by using serial dilutions of genomic DNA and Bio-Rad IQ SYBR green supermix. Reaction mixture: 10µl of SYBR supermix, 1µl of each primer, 1µl of genomic DNA and 7µl of ddH_20. PCR conditions were 95^0C (3 mins), (x40) 60^0C (30 sec) - 72^0C

(30 sec), 72^0C (5 mins). The slope value obtained from the standard curve was used to determine changes in gene expression.

2.13.2 Gene expression determining using the Pfaffl equation

An equation taken from the article by Pfaffl *et al* (2001) was used to determine the changes in gene expression when strains were grown under simulated human GI tract conditions. It uses amplification efficiencies determined from serial dilution of genomic DNA and the crossing point value for both the control and treated samples to give a relative expression ratio for each gene investigated (Figure 5.4.2).

2.14 *In vitro* haemolytic assay of *B. cereus* and *B. weihenstephanensis*

Defibrinated horse and sheep blood (2%) (Oxoid Ltd, HPH, UK) was diluted in phosphate buffer saline (PBS). Horse and sheep blood (2%) in dH20 was used as a positive lysis control. To each well of a 96 well plate, 100μl of blood was added. All strains were cultured under optimal or simulated stomach/intestinal/dual conditions. After 4 or 8 hours cultures were centrifuged at 13,000rpm. 10μl of supernatant was removed and added to sheep or horse blood and, where appropriate, serial dilutions made. Blood cell turbidity was measured spectrophotometrically (OD_{570nm}) every 5 minutes over a 4 hours period. To avoid cell clumping the plate was shaken for 220 seconds before every reading. A decline in OD_{570} indicates haemolysis of red blood cells.

2.15 Reversed Passive Latex Agglutination Toxin Detection

BCET-RPLA toxin detection kit was purchased from Oxoid and used following the manufacturers guidelines. Once the test had been started plates were sealed and stored at room temperature on black card. The level of agglutination was judged by eye and given a score (-), (±), (+), (++) or (+++). All strains were tested under both optimal and simulated human GI tract conditions.

2.16 Secreted protein production and concentration from *B. cereus* and *B. weihenstephanensis*

Strains were grown under simulated conditions (8 hours at 37^0C, 200rpm) and under optimal toxin producing condition (6 hours, 32^0C, 200rpm). Cultures were then centrifuged to pellet biomass at 4000xg for 20 minutes. 20mls of supernatant was removed and combined with acetone, in a 1:4 ratio and placed at -20^0C overnight to concentrate any protein present. Samples were then centrifuged at 4000gs for 20 minutes to pellet protein. The supernatant was removed and the remaining pellet allowed to air dry. Once dry pellets were then resuspended in 500μl of 0.5M triethylammonium bicarbonate, TEAB (Sigma-Aldrich), aliquots were taken and stored at -80^0C to prevent loss of protein stability.

Proteins in the supernatant of simulated human GI tract cultures were also concentrated using vivaspin2 5000mw (Vivascience, Thermo Scientific Ldt). Supernatant was loaded on the column and centrifuged at 4000xg for 20 minutes. This process was repeated until all the supernatant had passed through the column and only 300-500μl remained. The flow through was collected and stored. Concentrated protein samples were then eluted by turning the spin column upside down and centrifuging at 4000xg for 3 minutes. Protein yield for both methods was determined using Non-interfering protein assay kit (Calbiochem)

2.17 Isobaric tag for relative and absolute quantitation (iTRAQ) experiment

To quantify proteins secreted under normal and dual simulated human GI tract conditions, iTRAQ was used. iTRAQ labels peptide fragments with specific weighted markers. In one experiment up to 8 conditions/strains can be investigated and compared with results quantifying protein abundance along with identifying proteins present. This would allow comparisons to be drawn between optimal and simulated protein profiles in *B. cereus* and *B. weihenstephanensis* strains. *B. cereus* strain F2081B/98 was initially selected to be tested.

2.17.1 Reducing the proteins and blocking cysteines

F2081B/98 secreted protein samples, 100ug, were to in 0.5M TEAB and SDS (2%w/v) was added to act as a denaturant and the samples were vortexed. **Reducing agents (2-carboxyethyl) were then added to each sample, mixed and incubated at 60^0C for 1 hour. Samples were the centrifuged and cysteine blocking agents (MMTS) were added and incubated for 10 minutes at RT.**

2.17.2 Trypsin Digest

10μl of 100μg reconstituted typsin was added to each F2081B/98 sample tube and vortexed. Tubes were then sealed and incubated at 37^0C overnight (12-16 hours).

2.17.3 Labelling F2081B/98 digest with iTRAQ reagents

Individual ITRAQ tags were reconstituted with 70μl of 70% ethanol and mixed. All vial contents were added to one sample e.g. vial 114 was added to F2081B/98^{optimal}, 115 were added to F2081B/98^{simulated} etc. Samples were then mixed, by inversion, and incubated at room temperature for 1 hour. Samples were then combined into one eppendorf tube and mixed.

2.17.4 Preparing F2081B/98 samples for analysis by MALDI-TOF

Using a speed vacuum the combined F2081B98 tagged sample volume was reduced to 20μl and reconstituted with cation exchange buffer-load to 1ml. The pH of the sample was checked and reduced to between 2.5-3.3 by the addition of further cation exchange buffer-load. The cartridge was conditioned by the injection of cation exchange buffer-clean and the flow through put to waste. 2ml of cation exchange buffer-load was passed through the column and directed to waste (Figure 2.17.3). The sample was then slowly injected (1drop/second) and the flow through collected. The cartridge was washed through with cation exchange buffer-load to remove **TEEP**, SDS, calcium chloride and excess iTRAQ reagents and the flow through was collected. The combined F2081B/98 tagged sample was eluted by the addition of 500μl cation exchange buffer-elute at 1 drop/second and collected in a fresh tube as

a single fraction. Additional trypsin was washed through the cartridge by the passing through 1ml of cation exchange buffer-clean

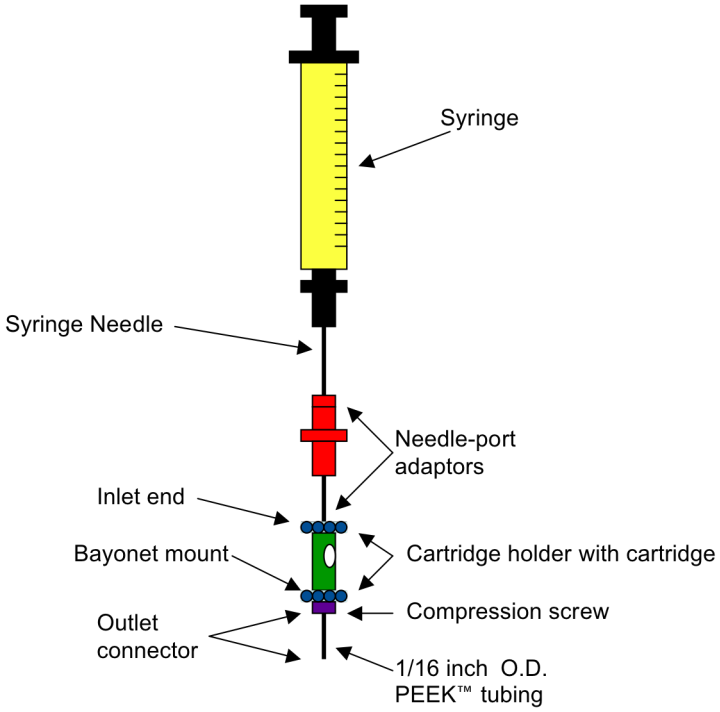


Figure 2.17.4 Cation exchange column

2.17.5 F2081B/98 Sample clean up before MALDI-TOF analysis

F2081B/98 sample was cleaned up using PepClean™ C-18 spin columns (Thermo scientific) 200μl of a 50% methanol solution was washed through the spin columns by centrifugation at 5000xg for 1 minute to wet the resin. This step was repeated twice. 200μl of 0.5% trifluoroacetic acid (TFA) in 5% Acetonitrile (ACN) was added to the spin column to equilibrate it and centrifuged at 1500gs for 1 minute The flow through was discarded and this step was repeated. The F2081B/98 fraction was then loaded on the spin column and centrifuged at 1500xg for 1 minute. This was repeated with the recovered flow-through to ensure complete binding to the column. The spin column was then placed in a new receiver tube and washed with 200μl of 0.5% TFA in 5% ACN and centrifuged for 1 minute at 1500xg. This step was repeated and the flow though discarded. Proteins were eluted by the addition of elution buffer (70% ACN) and centrifuged at 1500xg for 1 minute. Elution

was repeated with further elution buffer and the sample dried in a speed vacuum before suspending in 1-2μl matrix solution prepared just before MALDI-MS analysis.

2.17.6 Q-Star MALDI-TOF mass spectrometer identification of secreted proteins

To identify any secreted proteins present in optimal and simulated human GI tract culture conditions precipated protein samples were trypsinised ovenight at 37^0C. The resulting peptide fragments in each strain sample were analysed on a Q-Star MALDI Mass spectrometer. The resulting peptide data was used to identify proteins present through Mascot (Matrix science).

2.18 Western blotting analysis of the production of haemolysin BL under optimal and simulated human GI tract conditions

Aliquots of secreted protein were placed at 70^0C for 10 minutes to denature proteins**.** Samples of 10ug (optimal secreted protein) and 150ng (simulated secreted protein) were mixed with Loading dye (50 mM Tris-HCl; pH 6.8, 2% SDS, 10% Glycerol,1% β-Mercaptoethanol, 12.5 mM EDTA, 0.02 % Bromophenol Blue) analysed by SDS-PAGE. Duplicate gels were used, one to stain with Coomassie blue to visualise proteins and the second to be used for western blotting. Loading controls taken from the Coomassie stained gels were used to determine changes in haemolysin BL protein amounts between strains under various conditions, time-points and temperatures.

For western blotting one gel was incubated in water and then proteins transferred to a nitrocellulose membrane using the iblot dry blotting system (Invitrogen, Paisley, UK). Protein transfer was confirmed by using a pre-stained protein ladder (Fermentas). The nitrocellulose membrane was incubated in PBS for 5 minutes before blocking (5% (w/v) skimmed milk powered, 0.1% (v/v) Tween-20 in PBS) for 1 hour at room temperature on a rocking platform. *hblA*, *hblC* and *hblD* were detected using polyclonal antibodies (Table 2.18.1). Polyclonal antibodies (1/500 dilution) were added to blocking buffer and membranes incubated at 4^0c overnight on a rocking

platform. The membrane was then washed (3 x 5 minutes in PBS + 0.1% (v/v) Tween-20) and then incubated with a horseradish peroxidase linked secondary antibody (1/30000 dilution) for 1 hour. The membrane was then washed as described previously and proteins were visualised by enhanced chemiluminea scence (GE Healthcare, Amersham). Images were taken on a

Name	Type	Target	Source
hblA	Polyclonal	Binding protein B in Haemolysin BL	Amy Wong
hblC	Polyclonal	Lytic component 1 in Haemolysin BL	Amy Wong
hblD	Polyclonal	Lytic component 2 in Haemolysin BL	Amy Wong
Immuno Pure	Secondary	Host: Goat Antigen: Rabbit IgG	Pierce

Table 2.18.1 Antibodies used in this study. Polyclonal antibodies raised in rabbits were a kind gift from Amy Wong. A 1/500 dilution was used for polyclonal antibodies. Immuno pure antibody was diluted 1/30000.

luminescent image analyser LAS-1000plus at various exposure lengths. (Fujifilm co Ltd, Japan).

2.19 Computer Analysis

Netprimers: http://www.premierbiosoft.com/netprimer/

Allows analysis of primers, including melting temperatures and predicting T_M, and possible secondary structures e.g. hairpins, self-dimers and cross-dimers. Optimizes primer design.

Olig Calculator: hppt://www.pitt.edu/~rsup/oligocalc.html

Quick and easy tool for calculating bp length, melting temperatures and molecular weight of primers and %GC content.

Chapter Three

B. cereus and *B. weihenstephanensis* Ability to Survive Within Conditions Simulating a Human Gastro-Intestinal Tract

3.1 Introduction

With the threat of food contamination, food producers aim to remove all bacteria from final food products. Despite this effort, *B. cereus* can be isolated from various raw food materials and also within end foodstuff (Krammer & Gilbert, 1989; Montville & Matthews, 2005). In The Netherlands, America, Wales and England *B. cereus* is thought to be responsible for 2% of poisoning outbreaks while in France 4-5% of reported cases are attributed to this bacterium (Granum & Baird-Parker, 2000; Who, 2000). Ubiquitous in nature, the ability of *B. cereus* to produce endspores results in their survival during stress, throughout food production, such as high pressure, drying, pasteurization, high/cold temperatures, and irradiation (Jaquette & Beuchat, 1998; Kamat *et al*, 1989; Kotiranta *et al*, 2000; Lechner *et al*, 1999; Raso *et al* 1998; Setlow, 2006; Stuy, 1956). Rosenquist *et al* tested various Danish food items for the presence of *B. cereus* like spp and found high counts in heat treated, starchy products where growth might have occurred from improper cooling after heat treatment (Rosenquist *et al*, 2005). Recently psychrotolerant strains of *B. cereus* have been classified into a separate group, *B. weihenstephanensis* (Lechner *et al*, 1999). Strains from this family can survive and grow at refrigerated temperatures (<7^{0}C) (Lechner *et al*, 1999). The presence of *B. cereus/B. weihenstehanensis* spores within a food product is of concern to food producers but there is little data on spore survival within a human host.

Changing social culture has resulted in minimally processed, ready to eat foods being demanded by consumers. Foods, which once were pasteurised and do not require any further cooking, are often stored at 4^{0}C to limit the germination and growth of any contaminating *B. cereus* or *B. weihenstephanensis* strains (Thorsen *et al*, 2005; Lund *et al*, 2008). It is unclear what effect long-term storage at 4^{0}C would have on *B. cereus* spores but it is unlikely that a chilled environment would result in the loss of spore viability in *B. cereus* or *B. weihenstephanensis* strains (Lechner *et al*, 1999). Under these conditions spores would persist within food items and subsequently be consumed. It is well known that the presence of *B. cereus* spores within foodstuff gives rise to vegetative cells in the small intestine that

produce diarrhoeal food poisoning toxins (Stenfors *et al*, 2008). However what is unclear is the pathogenic potential of *B. weihenstephanensis* spores and cells. Although yet to be implicated in any outbreaks strains do possess the genes of all known *B. cereus* diarrhoeal food poisoning toxins.

A limiting factor in the ability of *B. cereus* or *B. weihenstephanensis* to cause diarrhoeal food poisoning is the human GI tract. Designed to maximise the breakdown of food it also provides a barrier to invading microbes (Jackson & McLaughlin, 2006; Fang *et al*, 2009). The low acidic pH and proteases found in the stomach may have a significant impact on both *B. cereus* and *B. weihenstephanensis* cell and spore viability (Jackson & McLaughlin, 2006). Conditions found within the small intestine could also affect any contaminating bacteria (Kristiffersen *et al*, 2007; Hong *et al*, 2009). Digestive enzymes produced in the pancreas and secreted into the small intestine work in synergy with bile salts to remove nutrients from the passing food (Jackson & McLaughlin, 2006). The presence of bile and lipases could have a negative effect on both cell growth and spore germination while also neutralising any secreted toxins (Kristiffersen *et al*, 2007; Tam *et al*, 2006; Fang *et al*, 2009). The effect this environment has on cell and spore viability remains unknown. Therefore it is important to determine the effect the human GI tract has on *B. cereus*/*B. weihenstephanensis* cells or spores as well as establishing a possible infectious dose.

3.2 Aims

- To investigate the ability of *B. cereus* and *B. weihenstephanensis* cells and spores to survive and grow under simulated human GI tract conditions
- To establish an infective dose of *B. cereus* or *B. weihenstephanensis* cells/spores.

A

Stomach conditions e.g. 37^0c, 0.3% pepsin, pH 1-3

10^7, 10^5, and 10^3 vegetative cells/ml or spores/ml;
4 strains: pathogenic, mesophilic *B. cereus*
3 strains: psychrotolerant *B. weihenstephanensis*

Brain heart infusion broth enriched with pepsin and acidified to pH 1, 2 or 3.

Optical density was monitored and survivors enumerated over the 4-hour incubation period.

B

Small intestinal conditions e.g. 37^0c, 0.3/0.5% bile acids, 0.3% pancreatin

10^7, 10^5, and 10^3 vegetative cells/ml or spores/ml;
4 strains: pathogenic, mesophilic *B. cereus*
3 strains: psychrotolerant *B. weihenstephanensis*

Brain heart infusion broth enriched with bile acids, pancreatin at pH 8

Survivors enumerated over the 4-hour incubation period.

Figure 3.2.1 Experimental Rationale
B. cereus ubiquitous presence in soil results in both cells and spores being found on raw food material. Food contaminated with *B. cereus* or potentially *B. weihenstephanensis* could cause a diarrhoeal food poisoning outbreak, although an infectious dose of spores has not yet been established. In order to determine the effect the GI tract has on *B. cereus* and *B. weihenstephanensis* strains conditions simulating the stomach and small intestine were defined and a spore infectious dose investigated. **A)** Stomach conditions **B)** Small intestinal conditions.

3.3 *B. cereus* and *B.weihenstephanensis* calibration curves

Optical density readings that corresponded to 10^3, 10^5 and 10^7 CFU/ml were determined. Calibration curves for each strain (Figure 3.3.1) were produced and the average results for *B. cereus* and *B. weihenstephanensis* strains produced. The determined OD readings corresponding to various CFU/ml values were subsequently used throughout all survival assays. Spore crops were diluted to 10^3, 10^5 and 10^7spore/ml.

3.4 *B. cereus and B. weihenstephanensis* vegetative cell viability when challenged with a simulated human stomach

The ability of all strains to survive within a simulated human stomach (pH 3, 10^3 CFU/ml) was monitored over a 4-hour period (Figure 3.4.1 A+B). There was no significant difference in the recorded OD_{600} after 4 hours compared to the initial reading. The minimal increase in OD_{600} confirmed that these conditions did not support cell growth. Results were similar when higher inoculum levels were used and at lower pH (10^5 CFU/ml; see Appendix A. 10^7 CFU/ml; data not shown). Similar trends were witnessed in both *B. cereus* and *B. weihenstephanensis* strains leading to the conclusion that under these conditions there was no difference between species.

3.5 Stomach viability assay

A fluorescence microscopy study was utilized to observe cell viability in each strain under simulated stomach conditions. For all strains the stomach conditions induced cell death at pH 3 (Figure 3.5.1). Total loss of viability in each strain was shown to occur within 60 minutes, as cell populations displayed widespread uptake of PI. No difference in viability was found at pH 3 between *B. cereus* and *B. weihenstephanensis* species. No viable cells were found when cells were incubated at pH 1 and pH 2 (data not shown).

Viability counts were performed simultaneously to verify the microscope data. Results confirmed that all strains were sensitive to the simulated human stomach showing essentially no survival, 1 in 10^7 cells after 60 minutes. The data collected showed that any contaminating *B. cereus* or *B. weihenstephanensis* vegetative cells are neutralised in the simulated stomach.

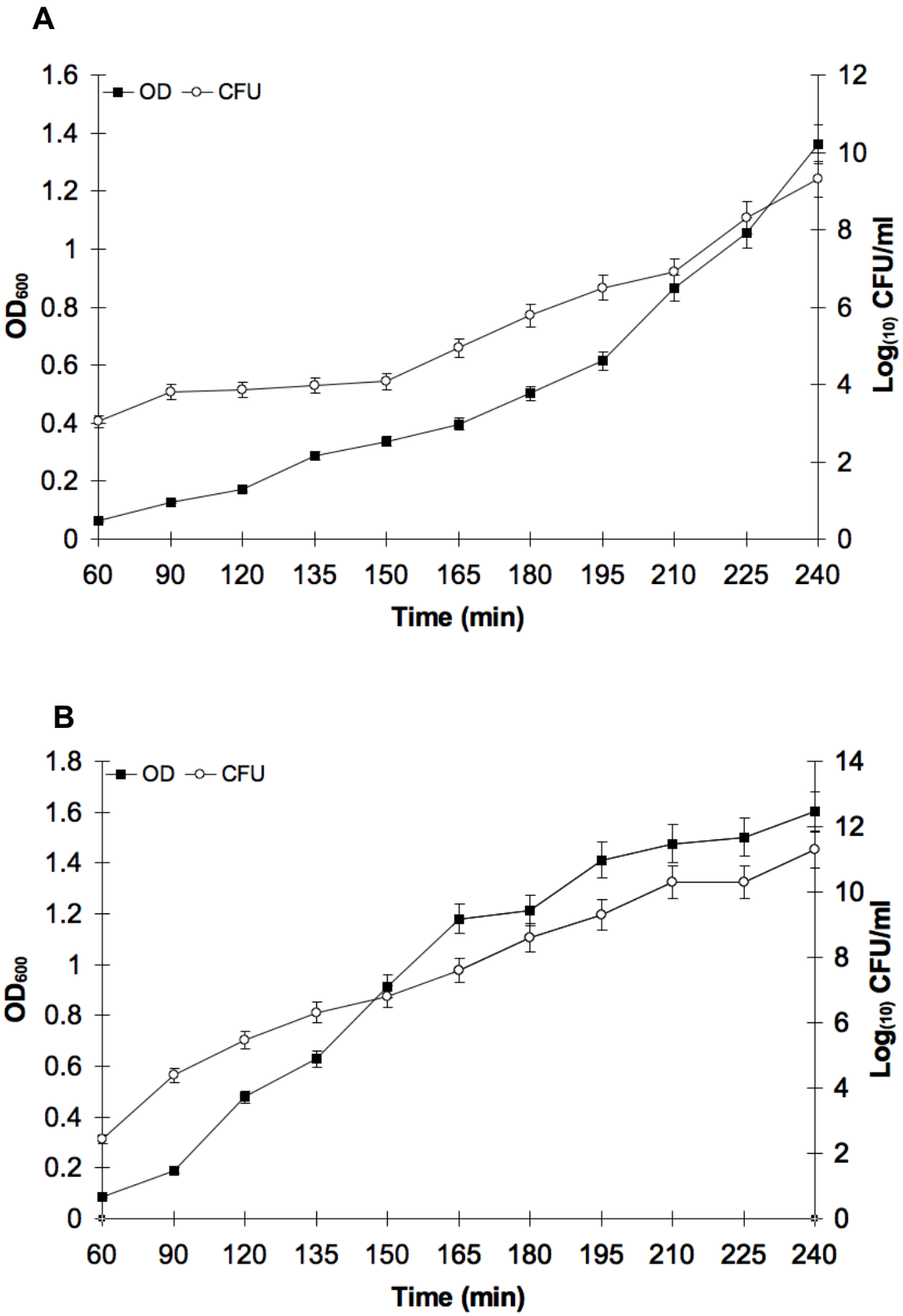


Figure 3.3.1 Calibration curves A. *B. cereus*. **B.** *B. weihenstephanensis*. In BHI medium cell density was measured in each strain and the average shown. Optical density and cell enumeration every 30 minutes at 30^0C. Not all error bars are visible.

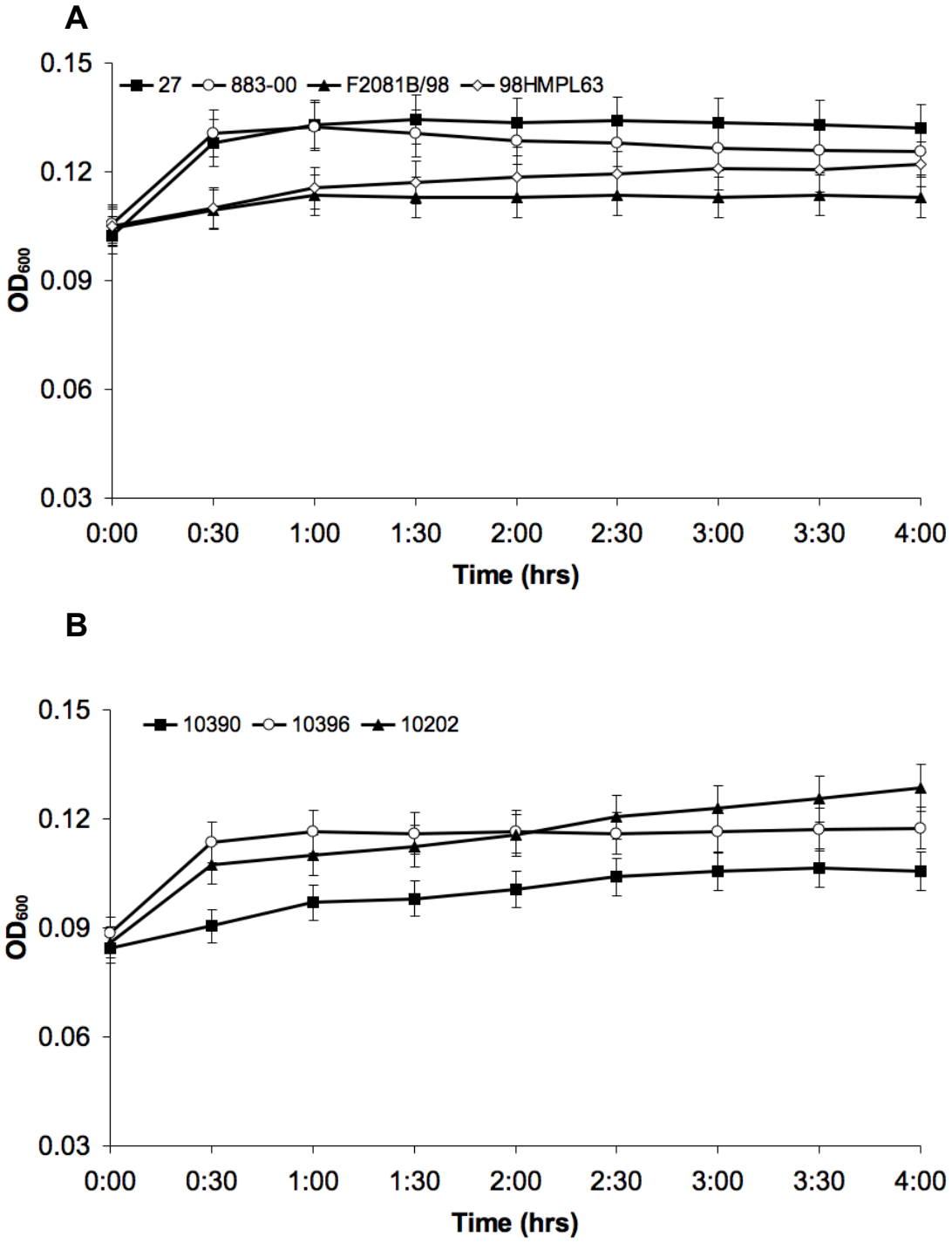


Figure 3.4.1 Vegetative cell OD during stomach simulation. Duplicate cultures of strains were grown until cell density reached 10^3 cfu/ml and subsequently inoculated into simulated stomach media, pH 3 for a period of 4 hours at 37^0c. Optical density readings were taken every 30 minutes. **A.** *B. cereus* strains 27(■), 883-00 (○), F2081B/98 (▲), and 98HMPL63 (◇) **B.** *B. weihenstephanensis* strains 10390 (■), 10396 (○), and 10202 (▲).

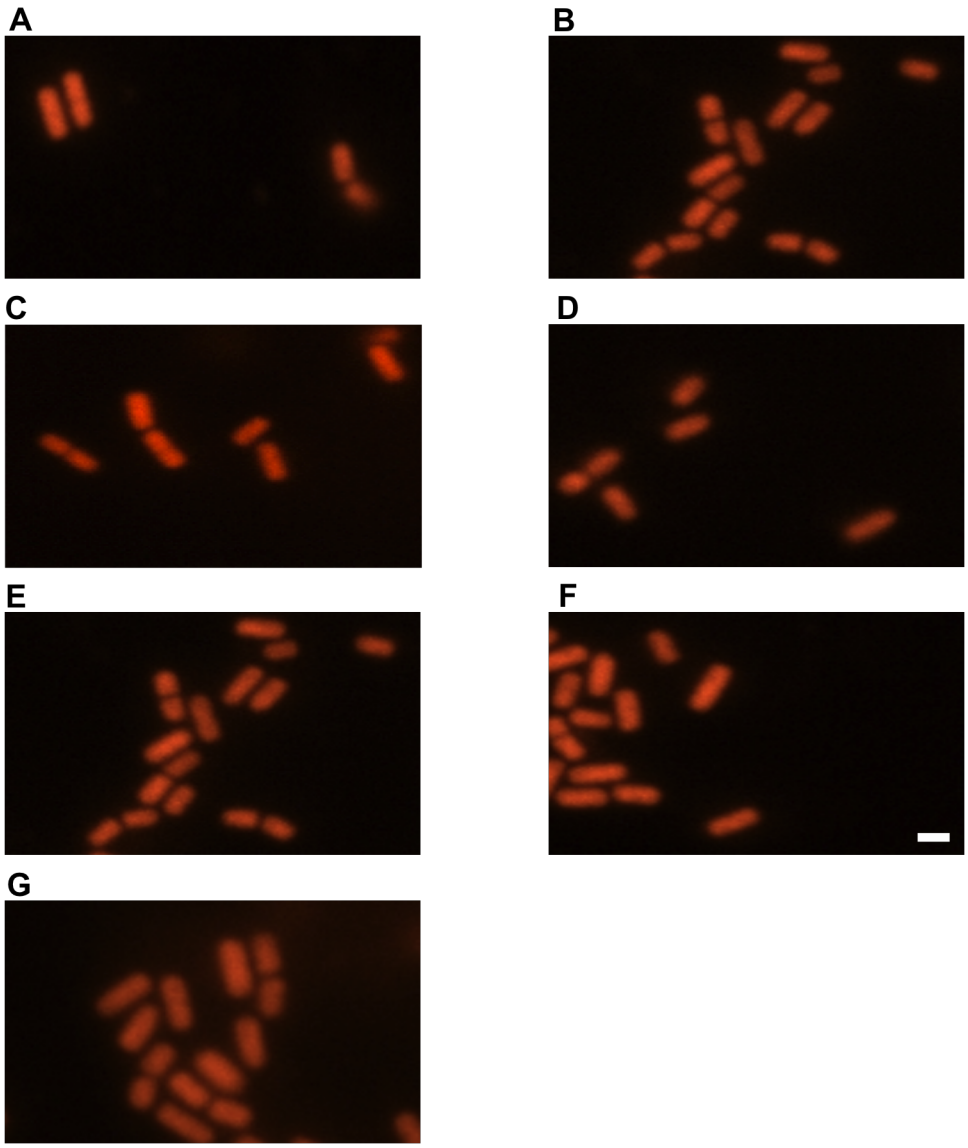


Figure 3.5.1 *B. cereus* and *B. weihenstephanensis* vegetative cell survival with a simulated human stomach. Simulated stomach media were inoculated with 10^7 vegetative cells and grown at 37^0c for 4 hours. Samples were taken hourly and stained with pI and cell tracker green and visualized with a delta vision microscope. **A-D** shows *B. cereus* stains 27, 883-00, F2081B/98 and 98HMPL63 vegetative cells respectively after 60 minutes incubation. **E-G** show *B. weihenstephanensis* strains 10390, 10396 and 10202 vegetative cells after 60-minute incubation. All strains irrespective of origin are stained red indicating that there membrane has been compromised and they are dead. Scale bar = 2μM

3.6 *B. cereus and B. weihenstephanensis* spore outgrowth when challenged with a simulated human stomach

The ability of spores from all strains to germinate and grow in simulated stomach media was investigated. OD_{600} was monitored for each strain over a 4-hour period OD_{600} readings decreased in all *B. cereus* strains (Figure 3.6.1A). Similarly OD_{600} decreased in all *B. weihenstephanensis* strains (Figure 3.6.1B). The increases and decreases in OD_{600} readings were not significantly different between any strain. Similar trends in both *B. cereus* and *B. weihenstephanensis* were witnessed at lower pH and higher initial inoculum levels (10^5 spore/ml; see Appendix A. 10^7 spore/ml; data not shown).

To show that the small decrease in OD_{600} was not caused by germination, spore state was observed via phase-contrast microscopy as differences in the refractive index between the specimen and background are created when an illuminating light is passed through (Kong *et al*, 2010). The refractive index can then be converted into contrast changes. Dormant spores, with a high refractive index appear phase bright while germinated spores appear dark (Kong *et al*, 2010). Spore appearance in all strains was monitored throughout. Under conditions simulating the stomach spores remained phase bright, indicating this environment does not support germination (data not shown).

Spore viability was monitored in addition to OD_{600} and phase state. Almost identical drops in spore numbers were witnessed between *B. cereus* and *B. weihenstephanensis* strains indicating no strain displayed a greater degree of resistance to the acidic conditions. A greater than 4-log reduction in spore number was recorded in all strains with an initial inoculum of 10^5 (Figure 3.6.2 A+B) and a 3-log reduction with an initial inoculum of 10^7 samples (Figure 3.6.3 A+B). No viable spores were detected in any 10^3 samples (data not shown). The reason for the loss in viability is unclear as spores remained phase bright during the simulation. It is possible, however, that the germination receptors located on the spore coat were damaged in the strong acidic environment. This work demonstrates that strains 10202, 10390 and 10396 have the same ability to survive as the diarrhoeal food poisoning causing clinically isolated *B. cereus* strains.

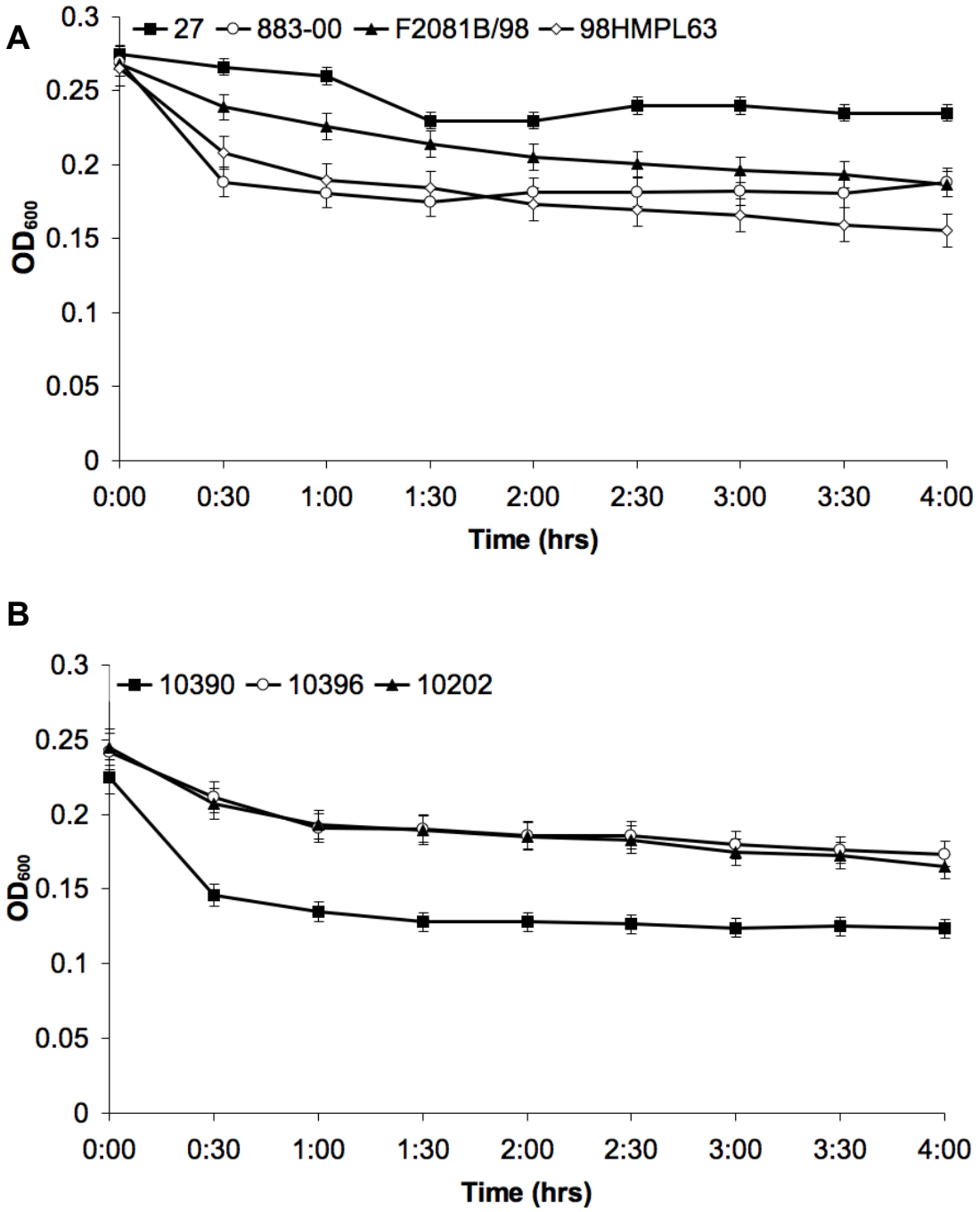


Figure 3.6.1 Spore stomach growth simulations. Fresh spore crops were diluted to 10^3 spore/ml and inoculated into simulated stomach media, pH 3 for 4 hours at 37^0c. Optical density readings were taken every 30 minutes. **A.** 27(■), 883-00 (○), F2081B/98 (▲), and 98HMPL63 (◇) **B.** 10390 (■), 10396 (○), and 10202 (▲).

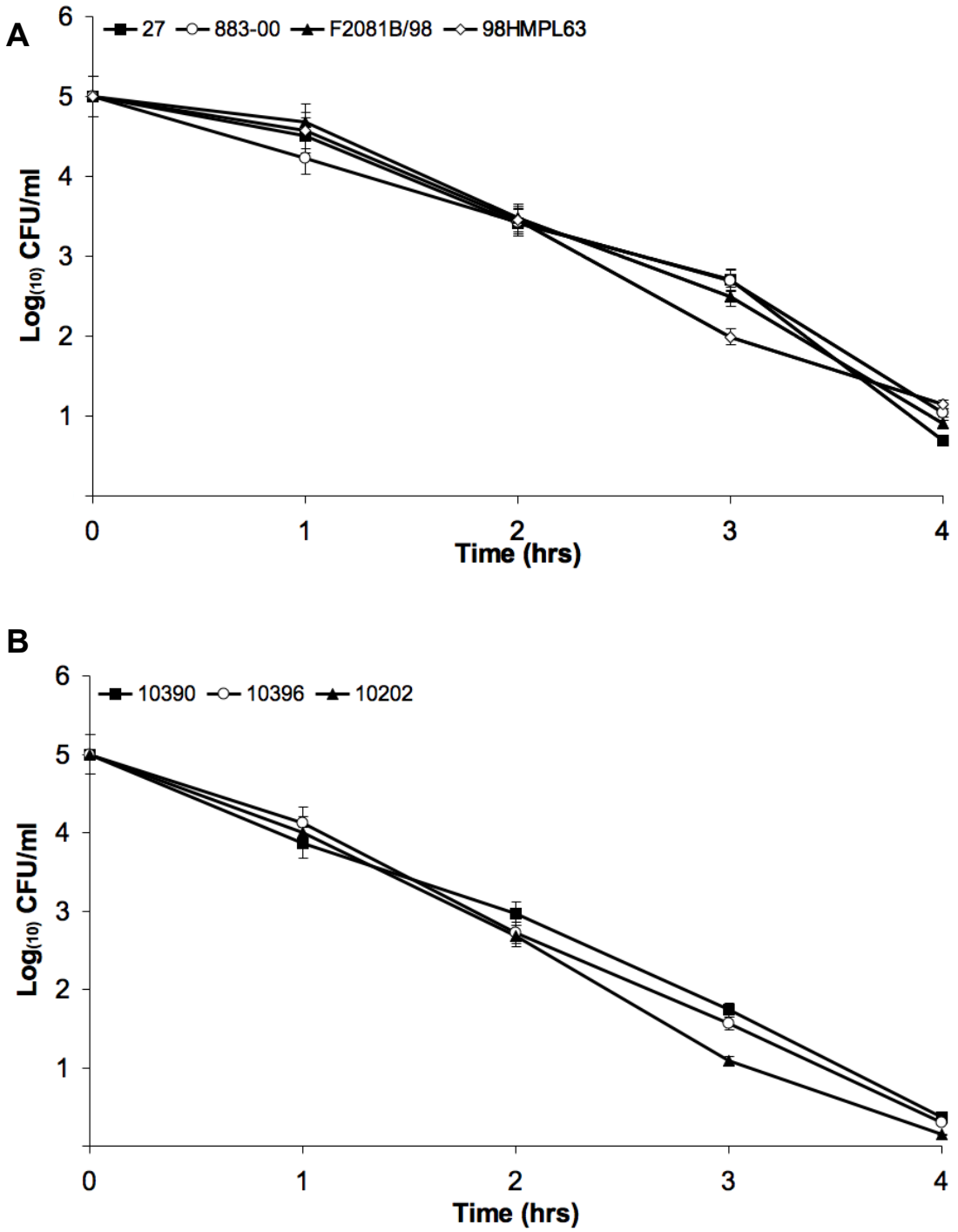


Figure 3.6.2 10^5 spore viability count under simulated stomach conditions. Spore suspensions were diluted to 10^5 spores/ml and inoculated into simulated stomach media at pH 3, 37^0c for 4 hours. Viability was monitored through cell enumeration. **A.** *B. cereus* strains 27(■), 883-00 (○), F2081B/98 (▲), and 98HMPL63 (◇) **B.** *B. weihenstephanensis* strains10390 (■), 10396 (○), and 10202 (▲). Not all error bars are visible.

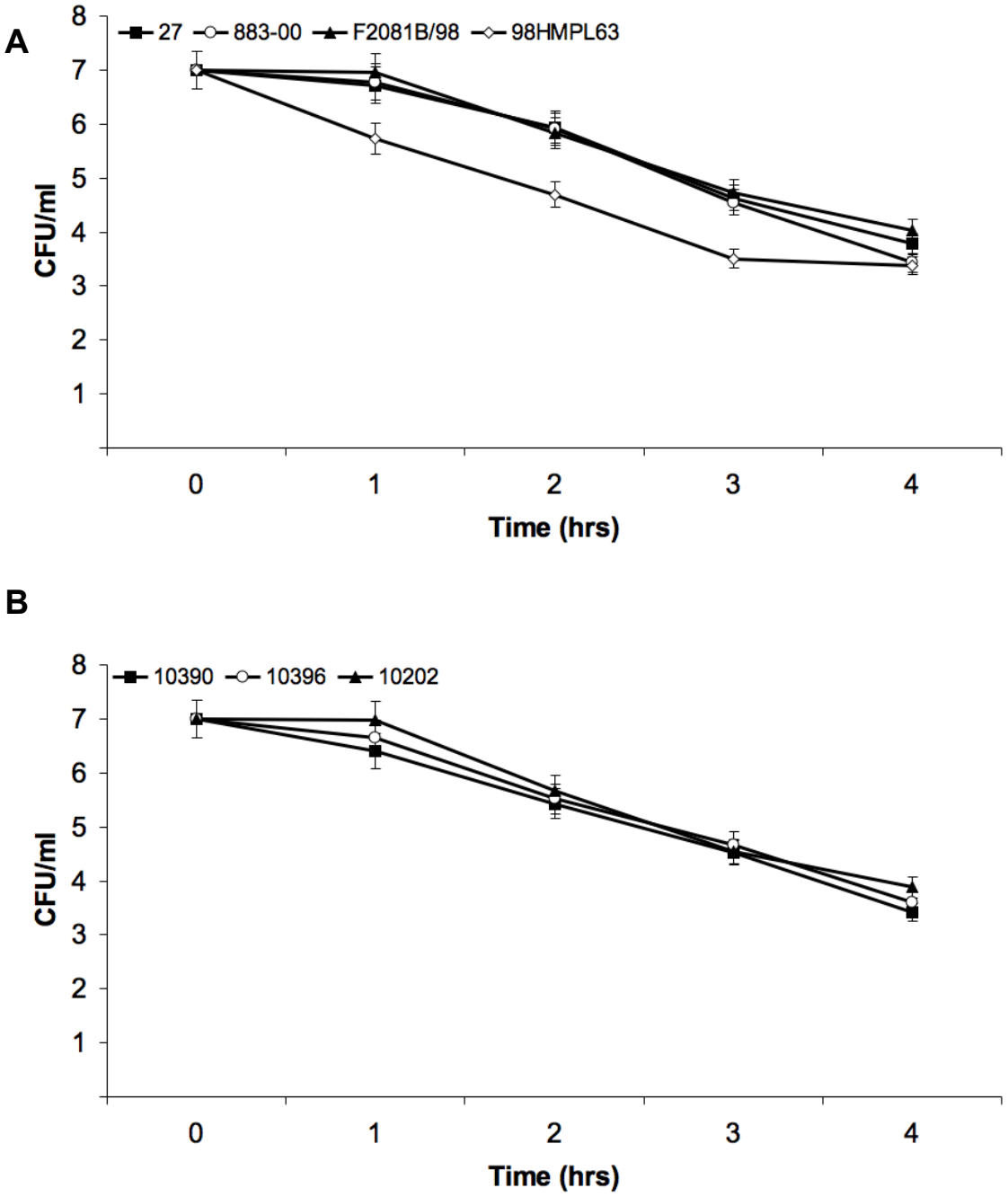


Figure 3.6.3 10^7 spore viability count under simulated stomach conditions. Spore suspensions were diluted to 10^7 spores/ml and inoculated into simulated stomach media at pH 3, 37^0c for 4 hours. Viability was monitored through cell enumeration. **A.** *B. cereus* strains 27(■), 883-00 (○), F2081B/98 (▲), and 98HMPL63 (◇) **B.** *B. weihenstephanensis* strains10390 (■), 10396 (○), and 10202 (▲). Not all error bars are visable.

The potential for both *B. cereus* and *B. weihenstephanensis* spores to survive the stomach conditions mimicked here indicates that *in vivo*, with the buffering effect of food, spores would also remain, however, only after a high inoculum.

3.7 Vegetative cell viability under conditions simulating the small intestine

Food passes from the stomach in to the small intestine. In this environment digestive enzymes and bile work to remove any nutrients and proteins from passing food (Jackson & McLaughlin, 2006). Bile has previously been shown to have a negative effect *B. cereus* cell growth. The ability of *B. cereus* and *B. weihenstephanensis* strains to survive in this environment was investigated.

Viability was monitored through cell enumeration. Results showed that in the presence of 0.5% bile and pancreatin the bacterial cells grew rapidly. Two hours post initial inoculation (10^3cfu/ml) cell numbers had increased by 3-logs in all *B. cereus* strains (Figure 3.7.1A). Cell numbers increased over the next two hours with final figures reaching between 10^7-10^9cfu/ml, however, there was no significant difference between individual strains growth rates. Similar results were witnessed for *B. weihenstephanensis* strains 10202, 10390, 10396 as they were also inhibited by the presence of bile salts and pancreatin (Figure 3.7.1B). Strain 10202 reached the highest cell density (10^7cfu/ml), however, there was no significant difference between the growth rates recorded for the three strains. Simular results were shown when the initial CFU/ml inculation was increased and at the reduced bile concentration of 0.3% (data not shown).

Neither *B. cereus* nor *B. weihenstephanensis* strains were inhibited by the conditions used to simulate the small intestine. There was no significant difference in individual growth, showing that *B. weihenstephanensis* strains can survive and grow as well as *B. cereus* strains.

OD_{600} values were recorded for all strains in the presence of bile and pancreatin. As with the viable cell counts very similar trends were witnessed with varying initial inoculua (Appendix A 10^3 CFU/ml; 10^5 and 10^7 results

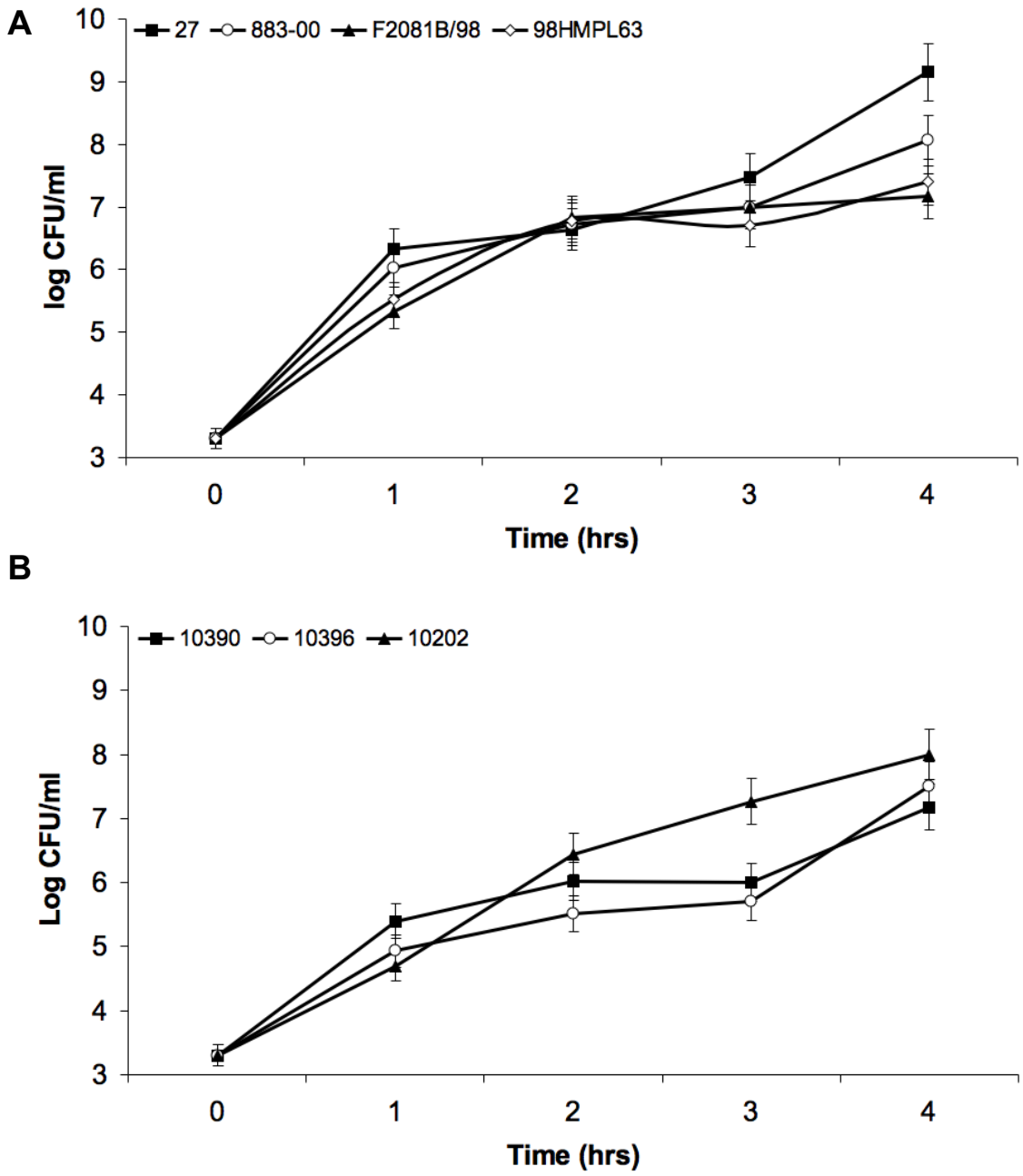


Figure 3.7.1 Vegetative cell viability under simulated small intestinal conditions. Strains were grown under optimal conditions until mid-log phase and then diluted in simulated small intestinal medium to give a cell density of 10^3 cfu/ml. Cells were enumerated through viable counts taken hourly. **A**. B*. cereus* strains 27(■), 883-00 (○), F2081B/98 (▲), and 98HMPL63 (◇). **B.** *B. weihenstephanensis* strains 10390 (■), 10396 (○), and 10202 (▲). Not all error bars are visable

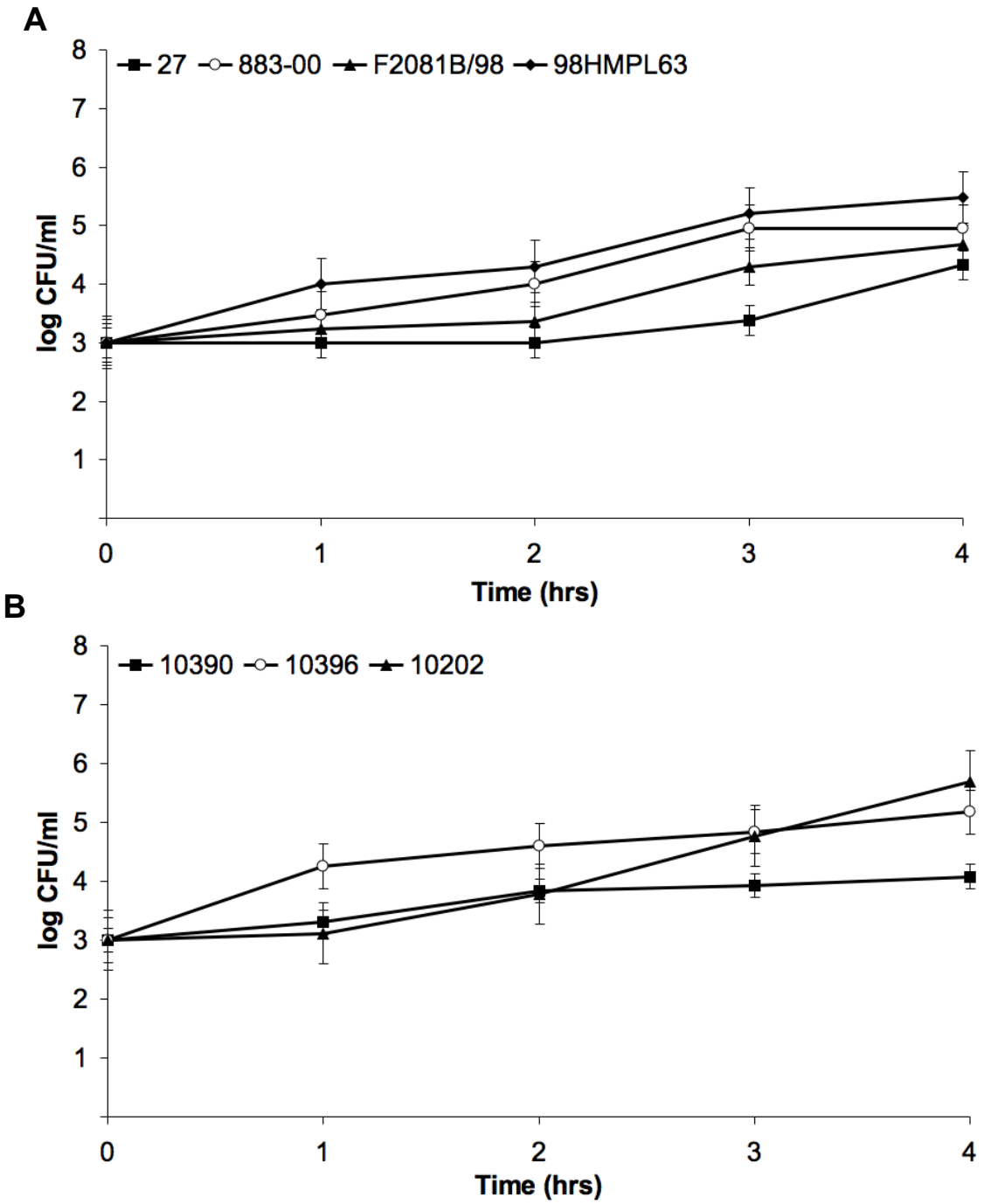


Figure 3.8.1 Spore intestinal viability assay; 10^3spores/ml. Spore suspensions were diluted to 10^3 spores/ml and inoculated into simulated small intestinal media, grown at 37^0c, pH 8, 0.3% bile for a period of 4 hours. Cell number was determined every hour. **A**. B*. cereus* strains 27(■), 883-00 (○), F2081B/98 (▲), and 98HMPL63 (◇). **B.** *B. weihenstephanensis* strains 10390 (■), 10396 (○), and 10202 (▲). Not all error bars are visible.

data not shown)

3.8. Spore viability under conditions simulating the small intestine.

Spores were quantified by enumeration. Under the simulated small intestinal conditions germination and outgrowth was slow in all strains. Independent of the initial inoculums (10^3/10^5/10^7) or bile acid concentrations (0.3/0.5%) recorded growth in *B. cereus* strains 27, 883-00 and F2081B/98 (Figure 3.8.1A (10^3), 3.8.2A (10^5), 0.5% bile acid) was slow. Strain 98HMPL63 germinated faster than the other strains but cell density increased only by 2-logs. Similar trends were found when a larger initial inoculum, 10^7, was used (data not shown). Similar results were obtained in the presence of 0.3% bile acids (data not shown).

Long lag periods were also witnessed in *B. weihenstephanensis* strains (Figure 3.8.1B (10^3), 3.8.2B (10^5)). 10390 and 10396 showed little increase in cell density over 4 hours. Strain 10202 was slow to germinate however cell density reached 10^5cfu/ml within 4 hours. There was no significant difference between *B. cereus* and *B. weihenstephanensis* strains ability to germinate under simulated intestinal conditions.

OD_{600} values were recorded for all strains in the presence of bile and pancreatin. As with the viable cell counts very similar trends were witnessed with varying initial inoculums (Appendix A 10^3 CFU/ml; 10^5 and 10^7 results data not shown)

3.9 Human GI tract simulation using spores

Previously in this study it has been shown that neither *B. cereus* nor *B. weihenstephanensis* vegetative cells can survive the conditions within the simulated stomach. This suggests that food poisoning cases come from the presence of spores within food.

Combining stomach and small intestinal simulations allowed the modelling of spore survival through a complete GI tract. The viability of both 10^5 and 10^7spores/ml was investigated. 10^3 spores/ml was not included as there were

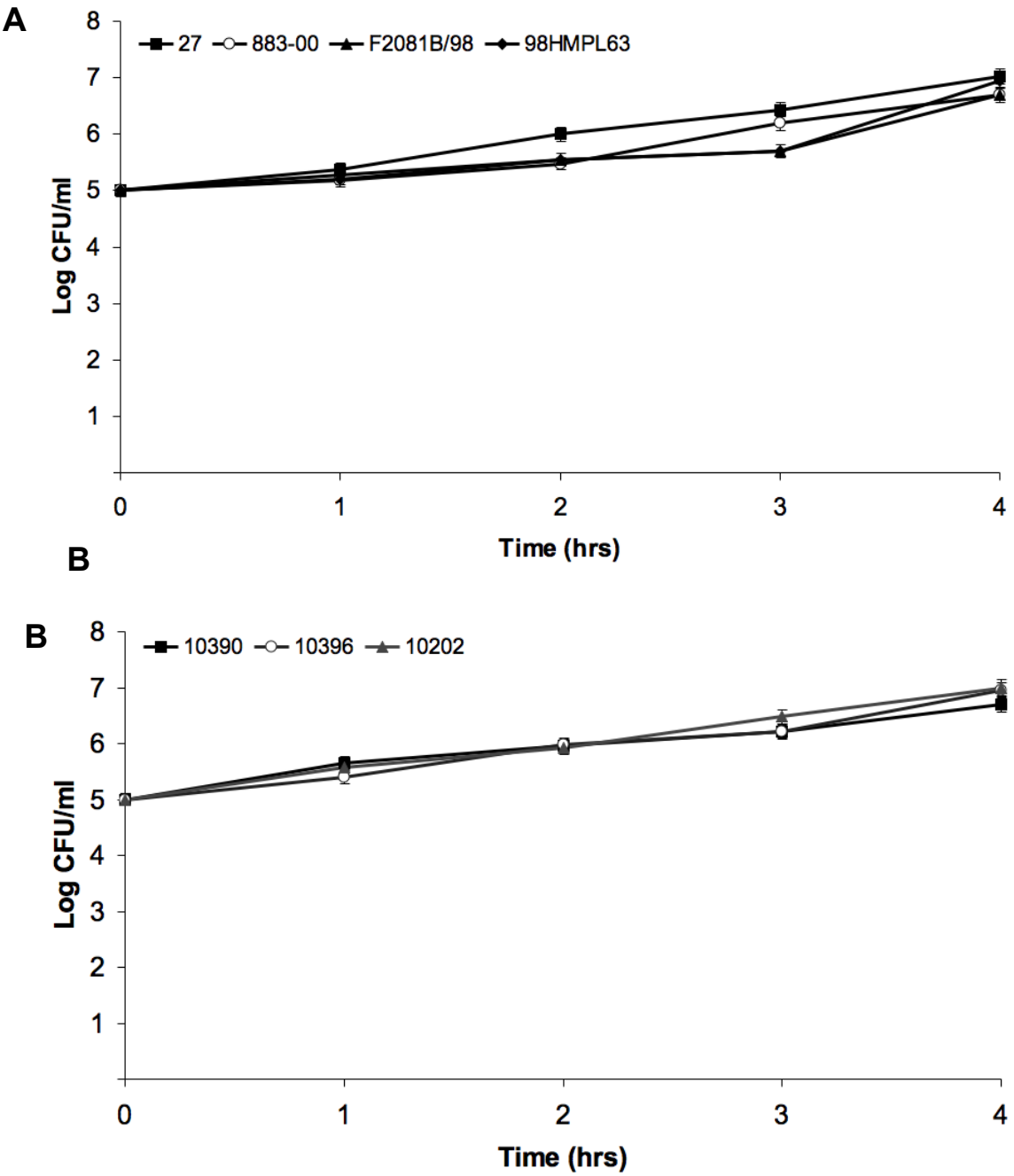


Figure 3.8.2 Spore intestinal viability assay; 10^5spores/ml. Spore suspensions were diluted to 10^5 spores/ml and inoculated into simulated small intestinal media, grown at 37^0c, pH 8, 0.5% bile for a period of 4 hours. Cell number was determined every hour. **A**. B*. cereus* strains 27(■), 883-00 (○), F2081B/98 (▲), and 98HMPL63 (◇). **B.** *B. weihenstephanensis* strains 10390 (■), 10396 (○), and 10202 (▲). Not all error bars are visable.

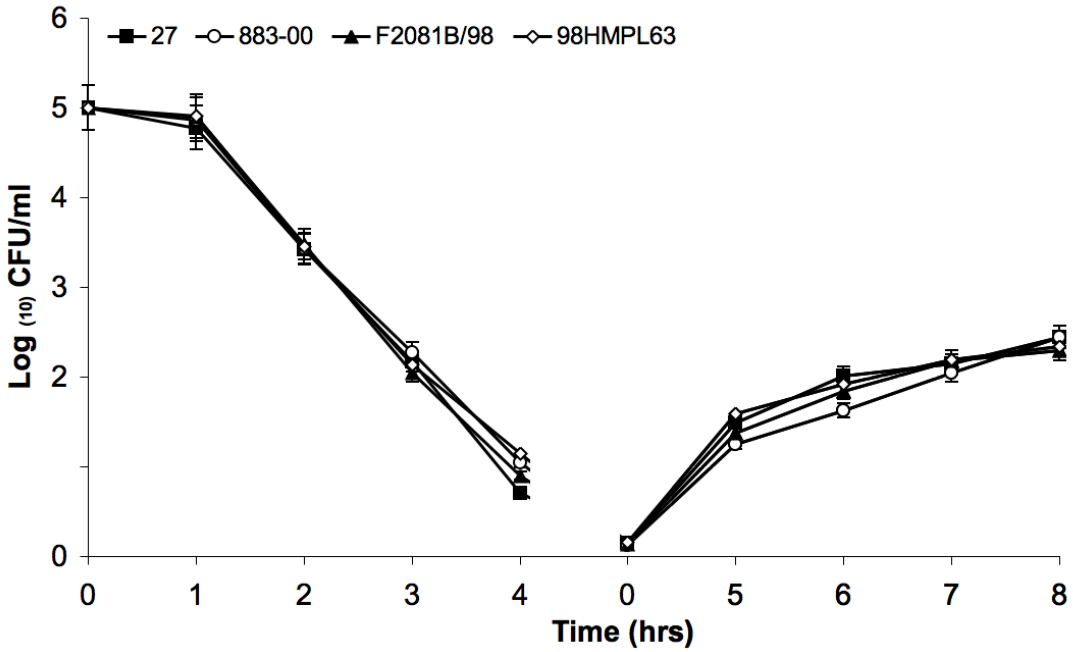
few survivors after the stomach incubation. The ability of all strains to survive simulated conditions was monitored via viable counts. During the first 4 hours within the stomach simulation (pH3) *B. cereus* and *B. weihenstephanensis* 10^5spore/ml samples showed a steady decline in viability (Figure 3.9.1A + B). Within the small intestinal simulation (0.5% bile and pancretin) cell number did recover slightly, by a factor of 10 in all strains. This result was in agreement with previous data collected **(**Figure 3.8.2A+B**)**.

Similar trends in viability were witnessed when the initial inoculum was increased to 10^7spores/ml, however all strains reached higher cell density after 8 hours (Figure 3.9.2). The significant increase in spores surviving the stomach conditions resulted in a greater concentration of spores able to germinate and outgrow in the small intestine. This resulted in, after 4 hours, cell density almost reaching that of the initial inoculum in 10^7 samples. Similar results were found when *B.weihenstephanensis* strains were investigated. After 4 hours in simulated stomach media few spores (<20) were viable from the initial 10^5spore/ml in any strain (Figure 3.9.1B). Over the course of the small intestinal simulation cell density increased in all strains, resulting 10^2 cfu/ml being returned after 8 hours. As with *B. cereus* strains a greater number of cells were returned from the GI tract simulation when the initial inoculum was increased to 10^7spores/ml (Figure 3.9.2B). Similar trends were found when the stomach situlation was pH2 and the bile acid concentration reduced to 0.3% (data not shown). The similarity in the results for *B. cereus* and *B. weihenstephanensis* strains show that under conditions simulating the human GI tract spores from this psychrotolerant species can survive and grow.

3.10 Discussion

The principal function of the gastro-intestinal tract is digestion, accomplished through a series of motility, secretion and absorption events (Liao *et al*, 2009). Pathogenic bacteria such as *B. cereus* are inhibited from colonising the GI tract due to the host suppressing their survival through a variety of methods,

A



B

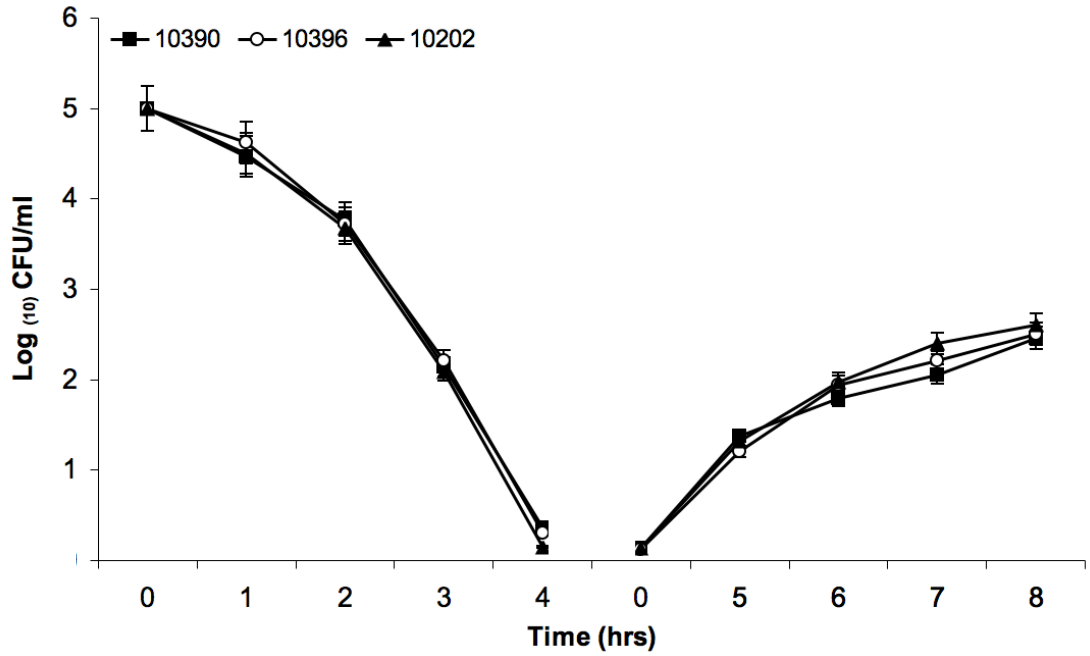


Figure 3.9.1 Spore viability during GI tract simulation; 10^5spores/ml. Spore suspensions were diluted to 10^5 spores/ml and inoculated into simulated stomach media and grown for 4 hours. Spores were then transferred to small intestinal media for 4 hours. Cell density was monitored hourly. **A.** *B. cereus* strains 27(■), 883-00 (○), F2081B/98 (▲), and 98HMPL63 (◇). **B.** *B. weihenstephanensis strains* 10390 (■), 10396 (○), and 10202 (▲). Not all error bars are visable.

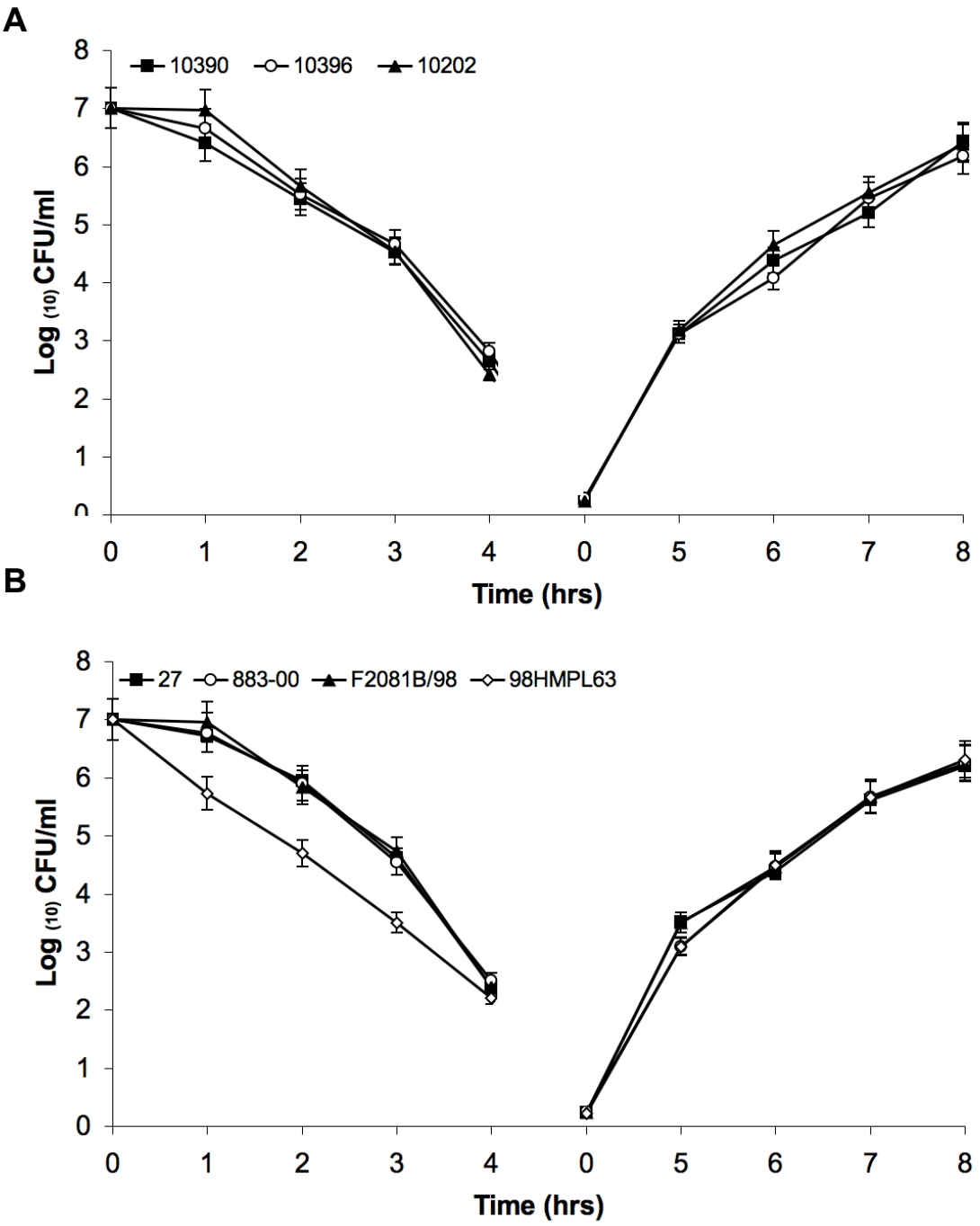


Figure 3.9.2. Spore viability during GI tract simulation; 10^7 pores/ml. Spore suspensions were diluted to 10^7 spores/ml and inoculated into simulated stomach media and grown for 4 hours. Spores were then transferred to small intestinal media for 4 hours. Cell density was monitored hourly. **A.** *B. cereus* strains 27(■), 883-00 (○), F2081B/98 (▲), and 98HMPL63 (◇). **B.** *B. weihenstephanensis strains* 10390 (■), 10396 (○), and 10202 (▲). Not all errors bars a visable.

including rapid food transit times, production of antimicrobial peptides, low pH and the presence of bile salts (Fang *et al*, 2009). This environment does however allow for the establishment of normal gut flora. The density of bacteria along the GI tract can vary, from 10^3 cfu/ml at the stomach/small intestine inlet increasing to 10^{10} cfu/ml in the small intestine itself and peaking at 10^{14}/ml at the colon (Kaper & Sperandio, 2005). In order to establish an infection *B. cereus* needs to penetrate the dense population of gut flora within the small intestine. It has been shown that *Bacillus* species including *B. cereus* are able to exploit the GI tract as a habitat. However, they also survive elevated osmolarity, oxygen starvation, competition for nutrients, the immune response, and exposure to bile and degradative enzymes (Kristiffersen *et al*, 2007; Hong *et al*, 2009). *B. cereus* food poisoning occurs when the balance between the numbers of pathogenic microbes, the production of specific virulence factors and the condition of the host defence mechanism alters (Ganzle *et al*, 1999).

In vivo conditions within the human GI tract are markedly different from those tested *in vitro* models. The innate immune response, presence and competition between intrinsic intestinal flora is not only difficult to simulate *in vitro* but costly (Ganzle *et al*, 1999). Models have been created to incorporate some of the conditions contaminating bacteria encounter when traversing the GI tract. These approaches are primarily used to investigate the interactions between probiotic bacteria and micro-flora. Molly *et al* (1993) constructed a system that represented the human GI tract, named the human intestinal microbial ecosystem (SHIME). Originally composed of five reactors representing the duodenum/jejunum, ileum, caecum, ascending colon, and descending colon. Faecal matter was used to build the microbial communities found within these environments (Molly *et al*, 1993; Mainville *et al*, 2005). In 1995 Minekus developed a model similar to SHIME, called TNO. It incorporated four separate compartments to mimic the stomach, duodenum, jejunum and ileum. Peristaltic contractions were also simulated in each compartment (Marteau *et al*, 1997). This dynamic model was computer controlled and offered the possibility of simulating the changes in pH along with the presence of degradative enzymes and bile acid (Minekus *et al*, 1995; Maathuis *et al* 2010). The SHIME and TNO models could be used to gain

valuable insights into the behaviour of *B. cereus* and *B. weihenstephanensis* cells and spores in the presence of natural flora. These models however are cost prohibitive and numerous systems would be required to test all the strains used in this study. A further complication of using these simulators could be determining viability of a particular strain within the large bacterial background created, as *B. cereus* can be isolated from the faecal matter of healthy individuals. This drawback could lead to false data regarding *B. cereus* spore and cell viability. Traditional methods of assessing potential probiotics involve growing cells and transferring culture to an acidic milieu, using oxgall or pancreatin to simulate the small intestine (Mainville *et al*, 2005). It was this simple and established approach of simulating the human GI tract that was modified in this study to determine the viability of both cells and spores.

It is generally believed that psychrotrophic *B. weihenstephanensis* strains grow poorly at 37^0C while mesophilic *B. cereus* can grow comfortably (Wijnands *et al*, 2006; Kristiffensen *et al*, 2007). Within the experiments conducted during this research there was no significant difference in the growth patterns or viability *of B. weihenstephanensis* spores or cells when compared to their *B. cereus* counterparts.

A major factor in determining *B. cereus* and *B. weihenstephanensis* survival through the human GI tract is the acidification of the stomach (Clavel *et al*, 2004). Resting stomach pH is between 4-5, however, when food enters and digestion commences the pH drops significantly to between 1-2. Once food has been packaged and transited to the small intestine the pH returns to that of resting. The acidic environment of the stomach and the presence of proteases are believed to be potent barriers to the survival of invading bacteria. Clavel *et al* in 2004 investigated the ability of *B. cereus* cells and spores to survive high pH (empty stomach) and low pH (full stomach) environments. Chicken, pea or milk were used as food products within the growth medium. Initially they added between 10^{6-7} CFU or spores/ml and incubated at 37^0c for 6 hours (Clavel *et al*, 2004). Viable counts were used to determine the survivor numbers. Spore viability was shown to be unaffected at all pH values (5.0. 4.5. 2.5 and 1.0) and in some samples germination and

outgrowth was witnessed after two hours (Clavel *et al*, 2004). Results gained during this work were not consistent with those of Clavel *et al*. Spore viability was shown to drop significantly at pH 3 and lower. Varying experimental conditions are the likely cause in the discrepancies within each set of results. The presence of food material within Clavel *et al* simulation could have offered spores an advantage when facing the acidic stomach.

In 2006 Wijnands *et al* investigated the ability of psychrotolerant and mesophilic spores to survive in a simulated stomach. Experimental conditions were based on those of Rotard *et al* (1995) and strains were incubated for 60 minutes. Under those conditions (pH 2.5) they showed that spore viability was reduced by 1-log after 60 minutes (Wijnands *et al*, 2006). These results mimicked those in this study as both *B. weihenstephanensis* and *B. cereus* spore viability fell 1-log after an hour. If Wijnands *et al* had continued their simulation it is possible that they would have witnessed the significant loss of viability to the spore sample that was recorded in this study.

Clavel *et al* (2004) showed that vegetative cells were vulnerable in an acidic stomach environment even when food is present. 10^3 *B. cereus* cells were killed after two hours at pH 3.5 and lower (Clavel *et al*, 2004). Tam *et al* further exhibited *B. cereus* sensitivity to low pH (2-3), with only 0.0008% of cells surviving after 30 minutes when incubated in stomach simulating media without any food material (Tam *et al*, 2006). Vegetative cell vulnerability was verified in this study when no cells were returned from the gastric phase at low pH irrespective of the initial inoculation size. After a generous meal, when stomach pH reaches its maximum, any contaminating *B. cereus* or *B. weihenstephanensis* spores or vegetative cells will only be weakly affected possibly allowing a greater number to travel on the small intestine (Conway *et al*, 1987; McLauchlan *et al*, 1989; Dressman *et al*, 1990; Ruas-Madiedo *et al*, 2002).

Within the small intestine a range of bile concentrations can be found, between 0.2-2%, dependent on food consumed (Kristiffersen *et al*, 2007). Bile emulsifies and degrades fats contained within food while also showing bactericidal effects (Kristiffersen *et al*, 2007). It has been shown to induce

membrane damage in bacterial cells and cause the degradation of DNA and RNA. Tam *et al* recorded the survival rate of an undomesticated *B. subtilis* strain under intestinal conditions. 10^9 spores/ml were inoculated into intestinal fluid containing 0.2% bile, and after a short incubation time between 10^3-10^5 spores were returned (Tam *et al*, 2006). Kristiffersen *et al* showed that domesticated, non pathogenic *B. cereus* strain ATCC14579 could grow on plates containing a bile concentration of 0.005%, however, at concentrations of 0.01% no colonies were recovered (Kristiffersen *et al*, 2007). Wijnands *et al* showed, when testing psychrotrophic *B. cereus* spores that after 4 hours under intestinal conditions CFU only increased by 1-log (Wijnands *et al*, 2006). These results were consistent with data recorded in this study, which also indicated that in both *B. cereus* and *B. weihenstephanensis* strains CFU only increased by 1 log after being incubated in simulated intestinal media for 4 hours, however, it is difficult to make comparison between the work shown here and other studies as components used to simulate the GI tract environments were not consistent.

It has previously been noted that pre-exposure to a simulated human stomach had a positive effect on spore germination and outgrowth in simulated intestinal environment (Wijnands *et al*, 2006). When *B. cereus* strains were pre-incubated at pH 2.5 they grew faster in the small intestine. During the GI tract simulations in this study although spore viability fell during the gastric phase, surviving spores within the small intestinal simulations were able to germinate quickly and outgrow, increasing CFU by 3-logs in 4 hours. This trend was seen in both *B. cereus* and *B. weihenstephanensis* strains calling into question the claim that mesophilic strains germinate better and grow faster in simulated gastro intestinal conditions than psychrotrophic strains (Wijnands *et al*, 2006; Kristiffersen *et al*, 2007).

Pathogenic enteric bacteria such as *Listeria* and *Enterococcus* have been shown to endure higher bile concentrations, 0.3% (Kristiffersen *et al*, 2007). Results gained during this work showed that the vegetative cells from *B. cereus* and *B. weihenstephanensis* strains were unaffected in the presence of 0.3 and 0.5% bile. Pathogenic strains like those used in this study show a greater degree of resistance to the effects of bile. This immunity could be

linked to the production of a bile salt hydrolase (Bsh) (Paramithiotis *et al*, 2006, Fang *et al*, 2009). This enzyme limits the negative impact bile can have on vegetative cells however it is not yet known whether the strains used in this study produce the Bsh protein.

Although *B. cereus* vegetative cells grow well in the small intestine, it is believed the main cause of *Bacillus* mediated diarrhoeal food poisoning stems from the ingestion of spores within foodstuff that then transit the GI tract and germinate in the small intestine. Early work using dogs, rabbits, pigs and mice have shown that bacterial spores are able to germinate in the GI tract (Cartman *et al*, 2008). However the survival of spores in these animals may not translate to a human host (Cartman *et al*, 2008). Looking specifically at chickens, Cartman *et al* showed that spore germination was initiated in the small intestine and that over the course of the study, vegetative cells surpassed the number of inoculated spores (Cartman *et al*, 2008). Mouse experiments have also shown that greater numbers of spores were returned in the faeces than were initially inoculated indicating the spores have undergone a cycle of germination, outgrowth and subsequently sporulation (Tam *et al*, 2006). Rats containing human flora within their GI tract showed that vegetative cells do not survive however spores traverse the environment unaffected (Kristiffersen *et al*, 2007).

Surrounded by the right nutrients spores initiate germination, quickly absorbing water, degradating cortex and spore coat, allowing vegetative growth to resume (Moir, 2006). Kristiffersen *et al* showed that spores remained viable after 15 minutes within media containing 1% bile salts, highlighting the strength of their resistant structures (Kristiffersen *et al*, 2007). The work performed in this study shows that under conditions simulating the small intestine, with bile concentrations of 0.3/0.5%, spore viability was not affected. Over the 4-hour simulation spores germinated increasing CFU by between 10^3-10^5 depending on the initial inoculum level. It has been reported in some enteric bacteria that the presence of bile salts can induce a virulence response. In the case of *B. cereus* it is possible that this change in “lifestyle” from survival to pathogenic growth could result in the production of toxins in

areas of the small intestine that harbour a lower salt concentration (Kristiffersen *et al*, 2007).

The peristaltic actions of the small intestine result in un-germinated spores being shed in human faeces (Fakhry *et al*, 2008; Hong *et al*, 2009a). Appendages projecting from *B. cereus* spores could lead to their attachment to the hydrophobic wall of the small intestine possibly allowing spores to subsequently germinate (Kotiranta *et al*, 2000). The *B. cereus* virulence response can be activated in the small intestine through its interaction with host epithelial cells (Minnard *et al*, 2004). Specific areas have been highlighted within *B. cereus* cells that facilitate this interaction, indicating the presence of specific receptors for adhesion (Minnard *et al*, 2004). *B. cereus* alters the cytoskeletal arrangement of cells resulting in its ability to invade the host epithelium. It has been shown that fully differentiated CaCo-2 cells are lysed in the presence of *B. cereus* (Wijnands *et al*, 2007). The level of destruction caused by the presence of *B. cereus* cells was greater than the numbers of internalized cells, indicating that *B. cereus* does not need to invade cells (Minnard *et al*, 2004). However, colonization of this area results in a strong inflammatory response designed to limit the effect of *B. cereus* (Kristiffersen *et al*, 2007; Hong *et al*, 2009). The effect of the human immune response on spores is currently unknown. It is possible that when faced with a host attack the germinated spores form vegetative cells that are immediately able to sporulate giving *B. cereus* a selective advantage within this environment (Tam *et al*, 2006).

The gastro-intestinal tract is designed to maximise the breakdown of foods, releasing any nutrients presents. Within this environment normal, non-pathogenic gut flora flourish while invading bacterial pathogens, such as *B. cereus* are inhibited (Ganzle *et al*, 1999). Occasionally however the mechanisms the body has in place to suppress the actions of *B. cereus* fail and diarrhoeal food poisoning cases are reported. In order for this to occur cells and spores need to survive the acidic stomach environment before facing bile acids and digestive enzymes in the small intestine. The work carried out here shows that *B. cereus* and *B. weihenstephanensis* cells are rapidly killed in the stomach while spore viability is reduced by a maximum of 10^4-fold over a four hour period. In the small intestine cell viability was

purportedly reduced due to the presence of bile (Kimoto-Nira et al, 2009). Within the human GI tract simulated in this study the addition of bile did not affect cell growth or spore germination in either *B. cereus* or *B. weihenstephanensis* strains.

The results from this work show that there was no difference between the ability of mesophilic *B. cereus* and psychrotrophic *B. weihenstephanensis* spores to survive and germinate within the simulated GI tract. 10^5 spores within a food item that does not go under any further cooking could result in food poisoning. A small number of spores were recovered when 10^5 spores/ml were added to the stomach simulation. With the protective casing of food it is likely that an even greater number of spores would survive than was recorded in this study. The few recovered spores went on to germinate and outgrow, increasing CFU by over 4-logs. Although no difference in survival was recorded between *B. cereus* and *B. weihenstephanensis* strains the ability of *B. weihenstephanensis* to produce similar levels of diarrhoeal toxins remains to be determined.

Chapter Four
Mass Spectrometry Investigation of Secreted Proteins Produced Under Simulated Human Gastro-Intestinal Tract Conditions

4.1 Introduction

Results presented previously revealed that under conditions mimicking the human GI tract both *B. cereus* and *B. weihenstephanensis* spores can survive gastric transit and germinate and grow in the small intestine. The ability to germinate and grow however does not automatically result in the production of virulence factors related to diarrhoeal food poisoning. There is little work reporting the effects that the human GI tract has on the expression of secreted virulence factors however one could hypothesize that within these surroundings, bile acids, proteases and host defences could have a negative effect on any proteins secreted.

Diarrhoeal food poisoning outbreaks have been attributed to *B. cereus* since Hauges experiments in the 1950s confirmed it as an infective agent (Hauge, 1955). *B. weihenstephanensis* has not been implicated as the cause of any food poisoning outbreaks though it has been shown to possess all the toxin genes present in *B. cereus* (Lechner *et al*, 1999; Stenfors *et al*, 2002; Stenfors *et al*, 2008). In order for either *B. cereus* or *B. weihenstephanensis* to cause diarrhoeal food poisoning cells must be able to produce toxins at concentrations high enough to be toxic. High cell density triggers the production of a host of virulence factors through a quorum sensing system using papR and plcR (Figure 1.5 Introduction) (Lechner *et al* 1999; Agaisse *et al*, 1999; Salamitou *et al*, 2000; Slamti *et al*, 2002). The majority of these virulence proteins are secreted from the cell via the sec pathway into the extracelluar millieu (Tjalsma *et al*, 2000). Three toxins are known to cause diarrhoeal food poisoning; haemolysin BL (hbl), non-haemolysin enterotoxins (nhe) and cytotoxic K (cytK). A host of proteases, chaperones and flagellar related proteins are also produced and secreted. These factor impact greatly on the pathogenesis of invading strains by counteracting host defences and enhancing host tissue degradation (Bouillaut *et al*, 2005; Gohar *et al*, 2005; Gilois *et al*, 2007; Cadot *et al*, 2010; Fagerlund, Lindback, & Granum, 2010)
The primary diarrhoeal virulence factor of *B. cereus* is haemolysin BL. It shows haemolytic, cytotoxic, dermonecrotic, and vascular permeability activities against a range of cell types (Beecher *et al*, 1995). The proteins that

constitute haemolysin BL can be detected in culture supernatant using antibodies. In 1994 Beecher & Wong constructed polyclonal antibodies directed against the binding (hblA), and both lytic proteins (hblC and hblD) (Beecher & Wong, 1994). Polyclonal antibodies recognize multiple epitopes on any one antigen (Beecher & Wong, 1994). Sequence variability within hbl genes has been noted previously and as a consequence polyclonal antibodies will bind all hbl derivatives (Beecher, 1995). There are however some inherent drawbacks of using a less specific antibody, firstly large groups of non-specific antibodies can be produced which can result in a high background signal and secondly multiple epitopes may result in cross reactivity giving false positives.

In order to determine secreted proteins produced *B. cereus* and *B. weihenstephanensis*, techniques can be utilised. Precision, sensitivity and high throughput are all advantages of mass spectrometry (MS) based peptide quantification (Boehm *et al*, 2007). Isobaric tag for relative and absolute quantification (iTRAQ™) is a non-gel based method that allows simultaneous conditions to be tested within one experiment resulting in the identification and quantification of proteins present (Boehm *et al*, 2007; Wiese *et al,* 2007). Proteins are initially broken down in to smaller peptide fragments by proteolytic cleavage. Peptide samples and iTRAQ™ reagents are mixed together resulting in the formation of amide bonds between the iTRAQ tags and the proteolytic peptides (Wiese *et al,* 2007). Labelled peptides are pooled creating one sample that is analysed by LC MS/MS (Figure 4.1A) (Aggarwal, Choe & Lee, 2006).

iTRAQ™ Isobaric tags possess 3 regions; mass reporter group (R), balance group (B) and a protein reactive group (N-hydroxysuccinimide) (PRG) (Figure 4.1B) (Thompson *et al*, 2003; Boehm *et al*, 2007; Tweedie-Cullen & Livingston-Zatde, 2009). The weight of the reporter ion (114-121, depending on sample number) is offset by the weight of the balance group resulting in samples being indistinguishable by mass (Thompson *et al*, 2003; Boehm *et al*, 2007). Collision induced dissociation (CID) applied during MS/MS results in the reporter ions liberation from the tag (Aggarwal, Chloe & Lee, 2006; Boja *et al*, 2009; Tweedie-Cullen & Livingston-Zatde, 2009). Measuring the intensity

generated by the release of these low molecular weight reporter ions during CID enables the relative quantification of peptides from each sample (Tweedie-Cullen & Livingston-Zatde, 2009). Moreover the creation of γ- and β-ions at the N and C terminus of peptides during the CID fragmentation allows for the identification of proteins present within each sample through the mascot algorithm (Boehm *et al*, 2007; Wiese *et al,* 2007). iTRAQ™ allows for the investigation of multiple proteomes by enabling the simultaneous quantification and identification of peptides from various conditions (Thompson *et al*, 2003; Aggarwal, Chloe & Lee, 2006).

These techniques can be applied to determine the proteins *B. cereus* and *B. weihenstephanensis* secrete under simulated GI tract conditions, helping to determine not only protein identity but also abundance and could yield novel information about both species secretome.

4.1.1 Aims

- To determine whether under simulated GI tract conditions *B. cereus* and *B. weihenstephanensis* can produce haemolysin BL.
- To map the scretome of both *B. cereus* and *B. weihenstephanensis* strains under optimal and simulated human GI tract conditions

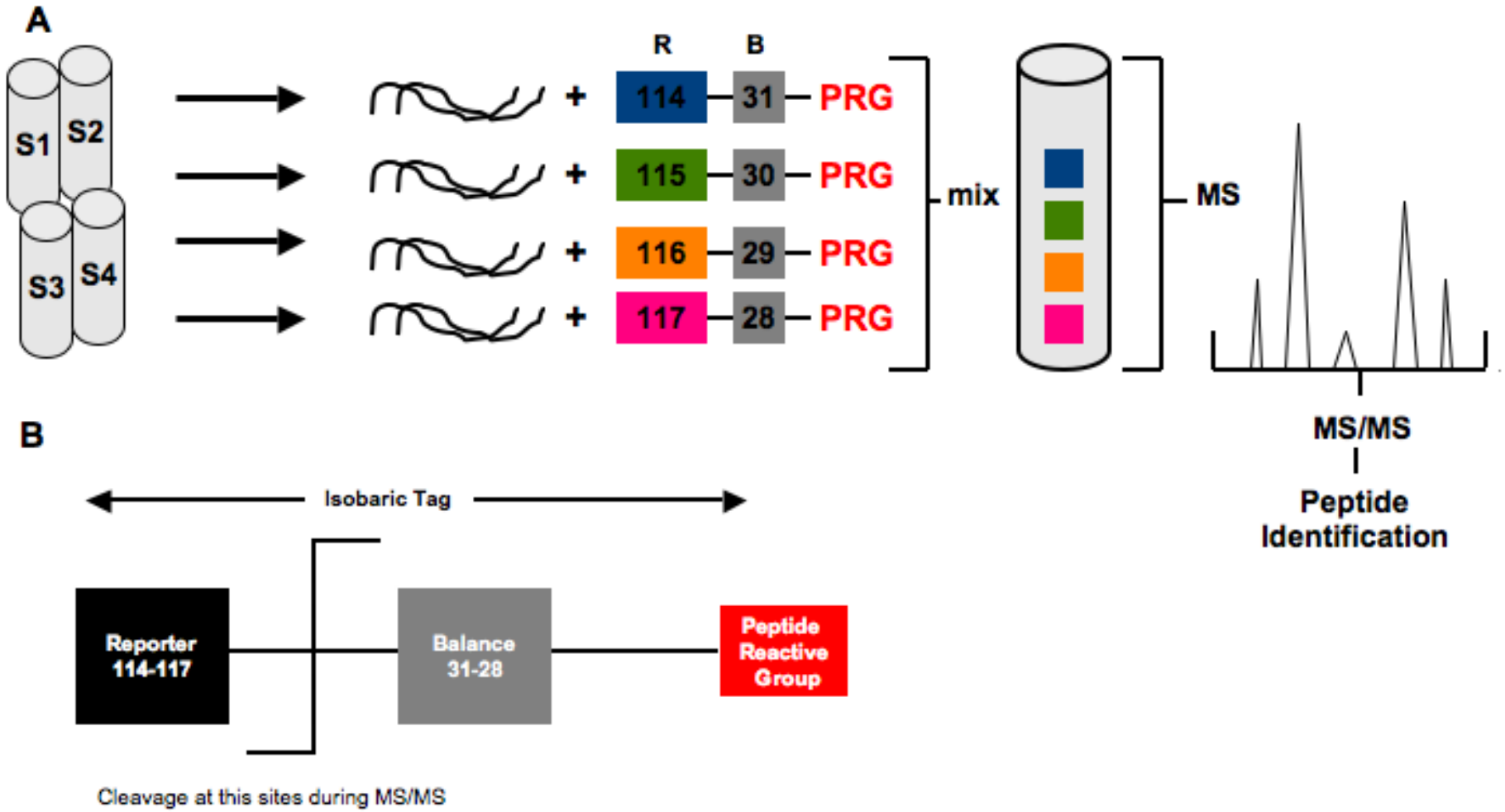
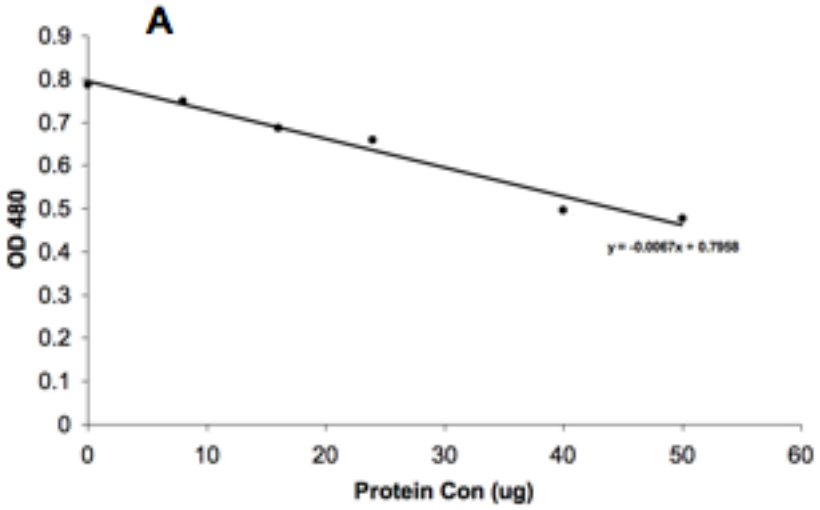


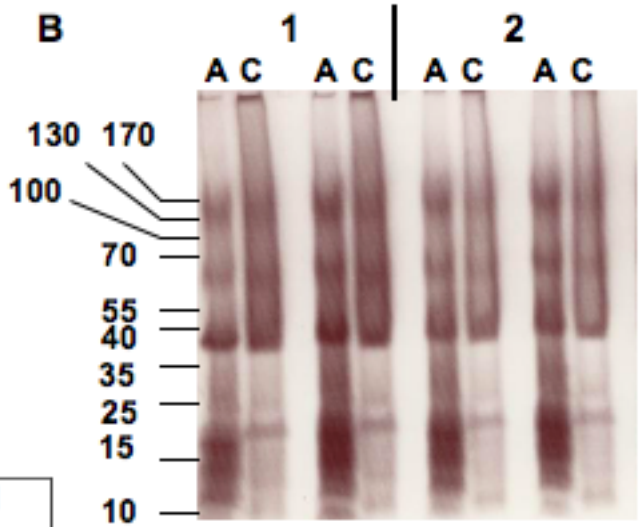
Figure 4.1 iTRAQ™ reagent and workflow. A. Displays stages during an iTRAQ™ experiment. Samples are firstly trypsinized and then labeled with iTRAQ reagents. Samples are then pooled in to one sample and subjected to MS to obtain quantitative peptide data and then tandem MS/MS to allow peptide identification. PRG= protein reactive group **B.** iTRAQ™ reagents contain three regions, reporter, balance and peptide reactive group which when bound to peptides and subjected to MS analysis results in quantitative data and identification of peptides present within each sample. Diagram modified from the applied biosystems technical note.

4.2 Protein extraction, precipitation and quantification

In order to identify proteins secreted by *B. cereus* and *B. weihenstephanensis,* supernatants were harvested from cultures grown under optimal (BHI, pH 7, 30^0C) and simulated human GI tract conditions (Stomach; BHI, pepsin, pH3. Small intestine; pancreatin, bile acids, BHI, pH8, 37^0C) (Glatz & Goepfert, 1976; Christiansson *et al*, 1989) In order to determine any effect the human GI tract has on virulence protein secretions, cultures were compared to those grown under optimal conditions. Protein yield was determined using an non-interfering protein assay kit that uses bovine serum albumin (BSA) as the standard. Optical density readings for BSA samples containing known protein concentrations were plotted and a line of best fit added (Figure 4.2.2A). The equation of the straight line was used to determine secreted protein yields of strains grown under various conditions.

Secreted protein samples were concentrated to ensure that all proteins present could be detected. Using acetone to concentrate supernatant proteins resulted in precipitation and the failure of some proteins to resolubilise Several attempts were made to resolubilise proteins with the addition of sodium dodecyl sulfate (SDS) to TEAB buffer but all were unsuccessful. The precipation witnessed could be in part due to the presence of pancreatin, bile acids and pepsin. A secondary concentration method using vivaspin concentrators was used out to overcome this precipitation. Although all samples were soluble, a comparison of column and acetone concentrated proteins highlighted differences in protein yields with less small MW proteins in the samples (Figure 4.2.2B). These differences could have resulted from protein being lost on the column. Although some precipitation was present when acetone was used to concentrate secreted proteins it was determined that this would be the best method to concentrate proteins for all further experiments.





C

	Optimal (µg/µl)	Simulated (µg/µl)
***B. cereus* Strain**		
27	5.5	5.2
883-00	5.3	5.3
F2081B/98	5.2	5.1
98HMPL63	5.5	4.9
***B. weihenstephanensis* Strains**		
10390	5.4	5.4
10396	5.5	5.4
10202	5.4	5.2

Figure 4.2.2 Protein concentrations and acetone vs column precipitation. A Secreted proteins were concentrated and yield determined through the non-interfering protein assay kit from Calbiochem. Optical density readings were taken and yields determined using BSA as a protein standard. Once concentrations were determined protein samples were frozen and stored at -80^{0}c **B.** Secreted proteins contained within the supernatant of strains grown under a host of conditions were precipitated with ice-cold acetone and stored at -20^{0}c or were passed through a vivaspin concentrating column. A denotes acetone while C refers to column. Strains tested were *B. cereus* 27 and F2081B/98 (**1**) and *B. weihenstephanensis* 10396 and 10202 (**2**). **C.** Table showing protein yields for all strains harvested under optimal and simulated human GI tract conditions.

4.3 Quantification and identification of proteins within the supernatant of *B. cereus* strain F2081B/98 via iTRAQ™

B. cereus strain F2081B/98 was selected for iTRAQ experiments. Previously it had tested positive for hbl and nhe complexes, and both Ctyk and bceT genes indicating its potential pathogenesis. Duplicate supernatant samples from both GI tract simulated and optimal culture conditions were concentrated and the protein yield determined. 100μg of normal and simulated samples were digested overnight with trypsin and then labelled with iTRAQ reagents.

No mascot or quantitative results were returned from the supernatant harvested under simulated human GI tract conditions. Only one protein was identified from the supernatant harvested under optimal conditions, phospholipase C (A0R9W0). The failure under both conditions suggests that the fragmented peptides within each sample did not bind with the iTRAQ reagents successfully, resulting in no proteins being identified. It is possible that increasing the amount of protein within the samples could result in better binding however it has been shown that high protein concentrations can also inhibit the binding of tags to peptide (Thompson *et al*, 2003; Boehm *et al*, 2007). ITRAQ reagents are costly and with seven strains to investigate, MALDI-TOF mass spectrometry was employed. Although this method gives no quantitative information relating to protein abundance it does accurately identify secreted proteins present within the supernatant.

4.4 Detecting proteins present within the supernatant of *B. cereus* and *B. weihenstephanensis* strains

B. cereus is a well known mesophilic bacteria that causes diarrhoeal food poisoning outbreaks when spores/vegetative cells are ingested. These contaminants are transported to the small intestine where they produce a host of virulence factors including toxins. *B. weihenstephanensis* is a newly described species within the *B. cereus* group that comprises the psychrotolerant strains which can survive at refrigerated temperatures (Lechner *et al*, 1999). Although it is unclear if *B. weihenstephanensis* has mediated any diarrhoeal food poisoning outbreaks it has been shown to

possess both three-component toxin, HBL and NHE along with a host of other virulence factors though PCR analysis (Stenfors *et al*, 2002; Wijnands *et al*, 2006; Sensi & Ghelandi, 2010).

By mapping the secretome of both *B. cereus* and *B. weihenstephanensis* strains comparisons can be made and more information gained about the proteins produced under these defined conditions. Initially the secretome of strains grown under simulated human GI tract conditions were investigated. No proteins were identified from any strain during MS analysis under those conditions. Experiments were repeated with increased amounts of protein however no results were returned. It is possible that the addition of large amounts of pancreatin, bile acids and pepsin masked the presence of any secreted proteins or degraded them before MALDI-TOF mass spectrometry analysis. In order to simulate one condition faced by invading *B. cereus* or *B. weihenstephanensis* cells, secreted proteins were harvested after 8 hours at 37^0C in BHI. Proteins were detected, identified and separated into the following categories; toxins, degradative enzymes, metabolism, protein folding, stress response, and flagellar related. Each strain possessed similar protein profiles.

A comprehensive list of all proteins found in each strain along with mowse scores and accession number details can be found in appendix 4.3B. A more detailed study of virulence factors is shown in table 4.4.1.

4.4.1 Toxins

Individual proteins from both three-component toxins were identified in the supernatant of each strain however only F2081B/98 and 10396 contained all proteins. The highly toxic cytK-1 (37kDa) protein was only detected within 10202 supernatant. Fagerlund *et al* (2004) reported a genetic variant of cytK-1, named cytK-2 in *B. cereus* strain 883-00 however it was not detected under the conditions used in this work (Table 4.4.1). The lack of pattern in these results confirmed the previously held belief that expression and production of toxins within the *B. cereus* and *B. weihenstephanensis* groups is strain specific. Previous PCR studies have shown that *B. weihenstephanensis*

strains do possess the highly toxic cytK-1 protein. This is, to our knowledge, the first time the functional protein has been detected (Stenfors *et al*, 2008; Bartoszewicz *et al*, 2008; this study).

4.4.2 Other Virulence Factors

Degradative enzymes are virulence factors that help to break down host cell walls and host produced antimicrobial peptides. The most common enzymes found were phospolipase C (PI-PLC), sphingomyelinase and collagenase. Strains also produced proteins that could impact on their individual pathogenesis. Strain F2081B/98 contained 5-nucleotidase along with neutral proteases (NrpB), and the cell wall peptidase nlpC/p60. NrpB is believed to have a role in the modification of papR in the extracelluar space before it is re-imported through the opp channel to initiate plcR related gene expression. papR is the oligopeptide that acts as the inducer molecule for plcR regulated genes (Bouillaut *et al,* 2007; Declerck *et al*, 2007). nlpC was also present in strain 27, 883-00 and 98HMPL63. The immune inhibitor metalloprotease, InhA2, was found in the supernatant of F2081B/98. In *B. thuringiensis* InhA2 has been shown to hydrolyse insect produced antimicrobial peptides cecropins and attacins, increasing *B. thuringiensis* pathogenicity (Fedhila *et al*, 2003). It is possible that this enzyme has a similar role in *B. cereus* and degrades human antimicrobial peptides produced in response to *B. cereus* invasion.

4.4.3 Other proteins

GroEL, the molecular chaperone was found in strain 98HMPL63. Chaperones have an essential role in protein folding. Under conditions of stress chaperones also work to reduce the build up of protein aggregates that could become lethal to the cell. Its presence in only *B. cereus* strain 98HMPL63 was surprising as 37^0C is above their optimal growth temperature and thus could induce temperature stress. The metalloenzyme peptidase M4 thermolysin was found in 10396. It utilizes metal ions to perform catalytic reactions. A cell wall peptidase, nlpC and putative chitinases were also present. Significantly more proteases were recorded in strain 10396 than 10390 and 10202.

Enolase was also present in F2081B/98, 10390 and 10396. This enzyme is responsible for the conversion of 2-phosphoglycerate (2-PG) to phosphoenolypyruvate (PEP) in the penultimate stage of glycolysis, and thus helps provides energy to growing cells. Thioredoxin, a small ubiquitous protein that primarily acts to protect cells against oxidative stress, however is also induced by other stresses such as increased salt, acid or temperature, was found in 10390.

By far the greatest quantity of protein recorded in the supernatant however was flagellin or flagellar related. The flagellum is responsible for locomotion within *B. cereus/B. weihenstephanensis* and it consists of three structures, base, hook, and filament (Kotiranta *et al*, 2002; Ramarao & Lereclus, 2006; Salvetti *et al*, 2007). The filament is composed of around 20,000 flagellin proteins, arranged in a cylindrical shape. The primary function of the flagellum is movement and under certain conditions cells can become hyper-flagellated resulting in a swarming morphology (Ghelardi *et al*, 2002; Keans & Losick, 2003; Ghelardi *et al*, 2007). The ability to colonise surfaces by swarming has been linked with virulence and pathogenesis and the expression of hbl. Their abundance in the proteins returned from the supernatant is not surprising and confirms the findings of Gohar *et al* (2002) that stated flagellar related proteins made up a significant percentage of protein found in the supernatant of *B. cereus* (Gore *et al*, 2003). Recently there have been links made between the presence of flagella and virulence with strains shown to be significantly less virulent when mutations are made in the genes involved in flagellar construction (Ghelardi *et al*, 2002).

MALDI-TOF mass spectrometry analysis allowed for a non-quantitative assessment of the proteins secreted by *B. cereus* and *B. weihenstephanensis*. In order to gain quantitative data concerning the expression of hbl under simulated human GI tract conditions, polyclonal antibodies were used.

		Hemolysin BL			**Non-hemolytic Enterotoxin**			**Cytotoxin K**	**Other Virulence factors**				
		HblA	HblC	HblD	NheA	NheB	NheC	CytK	PI-PLC	Sph	NrpB	InhA2	Enolase
B. cereus	27	**X**	**X**	**X**	**X**				**X**	**X**	**X**		
	883-00				**X**	**X**							
	F2081B/98	**X**	**X**	**X**	**X**	**X**			**X**	**X**	**X**	**X**	**X**
	98HMPL63	**X**	**X**		**X**	**X**	**X**		**X**	**X**	**X**		
B. weihenstephanensis	10390			**X**	**X**					**X**			**X**
	10396	**X**	**X**	**X**	**X**				**X**	**X**			**X**
	10202				**X**	**X**		**X**	**X**	**X**	**X**	**X**	

Table 4.4.1 Virulence factors identified within the secretome. Crucial virulence factors involved in diarrhoeal food poisoning were found in the secretome of both *B. cereus* and *B. weihenstephanensis* strains and the results are shown above. Only two strains F2081B/98 and 10396 contained all hbl proteins and both the binding and one lytic protein nhe. There was no pattern in the presence of toxin related proteins confirming the idea that pathogenesis is strain specific. All strains produced sphingomyelinase (sph) and all but two contained phosphatidylinositol-specific phospholipase C (PI-PLC), which along with sph is involved in the break down of host cell walls. The highly toxin cytotoxic K protein was identified in strain 10202 although previously this strain was shown to lack the cytk gene. This is the first example of cytK expression within *B. weihenstephanensis*.

4.5 Expression of haemolysin BL under optimal culture conditions

The presence of each individual protein comprising haemolysin BL was confirmed using western blots. Spores were inoculated into BHI and grown under previously defined optimal conditions (30^0C, 150rpm, BHI, pH7, 5 hours) (Glatz & Goepfert, 1976; Christiansson *et al*, 1989). 10μg of concentrated protein samples from each supernatant was transferred to a nitrocellulose membrane and probed with antisera raised to hblA, hblC and hblD. To visualize protein present a secondary antibody (anti rabbit HRP) was applied to the membrane. Due to the absence of a antibody control, a reference band (36kDa) that was consistently expressed was selected as a loading control.

HblA was detected in the supernatant of all *B. cereus* and *B. weihenstephanensis* strains (Figure 4.5.1 A+B). Significant concentrations of the B protein were detected in each strain, resulting in a white halo like appearance on the blot. Although PCR has previously shown the *hblA* gene to be present in *B. weihenstephanensis* strains this is the first study where its expression has been recorded. HblC was detected in *B. cereus* strains 27, F2081B/98 and 98HMPL63 but not non-pathogenic 883-00 (Figure 4.5.2A) Previous experiments have shown however, the presence of hblC in 883-00 supernatant (Fagerlund *et al*, 2004). The presence of hblC within *B. weihenstephanensis* strains was also confirmed (Figure 4.5.2B). Loading controls possibly show that significantly less hblC is detected in all strains when compared to hblA. The reason for this difference is unclear however the age and specificity of the antibody could be a factor. HblD was detected in all strains (Figure 4.5.3A+B). There was significantly more hblD protein than the secondary lytic protein hblC. Under optimal conditions pathogenic *B. cereus* strains were confirmed as haemolysin BL producers, able to express each individual protein. It was also found that *B. weihenstephanensis* strains could produce hblA, hblC and hblD proteins, all of which are required to constitute a functional toxin. Variance within the expression level of each protein was discovered, although the reasons for these discrepancies were undetermined.

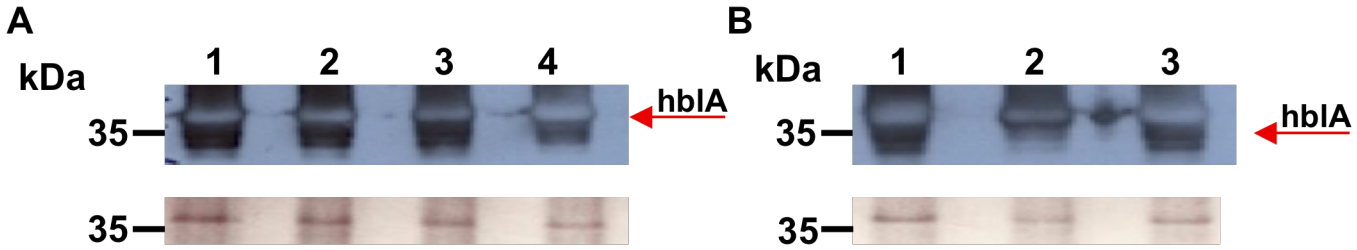


Figure 4.5.1 HblA under optimal conditions. **A**. Numbers 1-4 denote *B. cereus* strains, 27 883-00, F2081B/98 and 98HMPL63 respectively. **B**. 1-3 denotes *B. weihenstephanensis* strains 10390, 10396, and 10202**.**

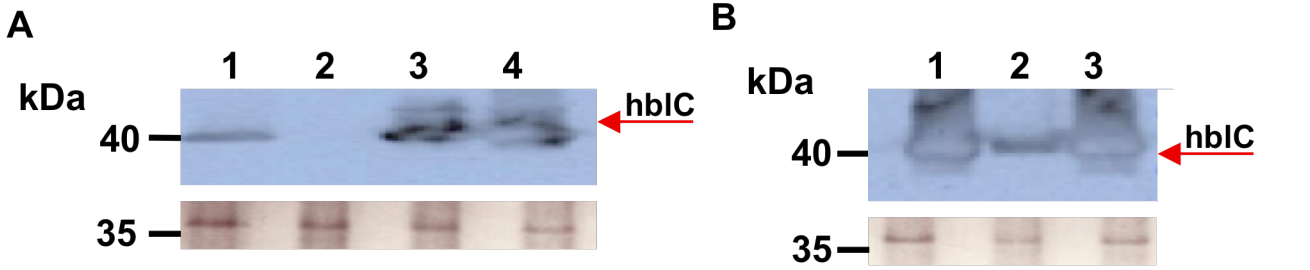


Figure 4.5.2 HblC under optimal conditions. **A**. Numbers 1-4 denote *B. cereus* strains, 27, 883-00, F2081B/98 and 98HMPL63 respectively. **B**. 1-3 denotes *B. weihenstephanensis* strains 10390, 10396, and 10202**.**

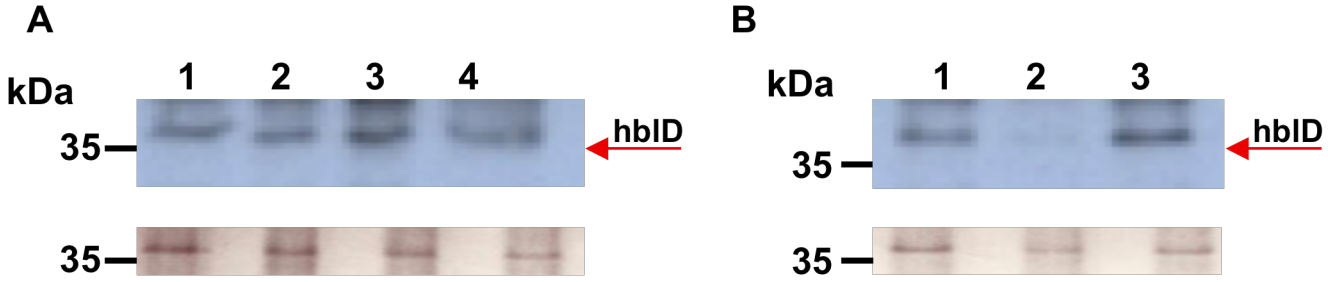
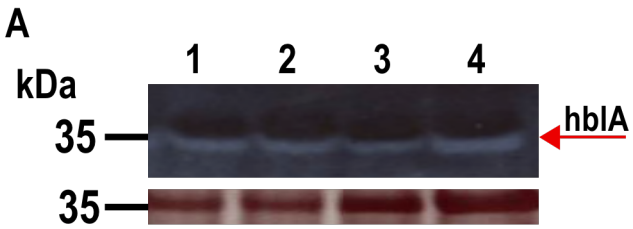


Figure 4.5.3 HblD under optimal conditions A. Numbers 1-4 denote *B. cereus* strains, 27, 883-00, F2081B/98 and 98HMPL63 respectively. **B**. 1-3 denotes *B. weihenstephanensis* strains 10390, 10396, and 10202**.**



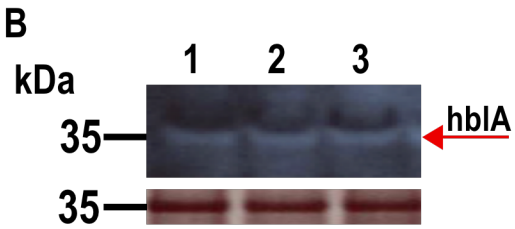
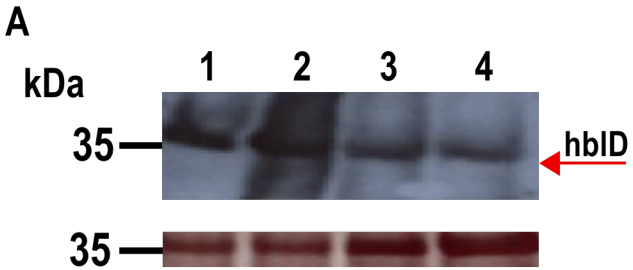


Figure 4.6.1 HblA under simulated conditions. **A** Numbers 1-4 denote *B. cereus* strains, 27, 883-00, F2081B/98 and 98HMPL63 respectively. **B.** Numbers 1-3 denotes *B. weihenstephanensis* strains 10390, 10396, 10202.



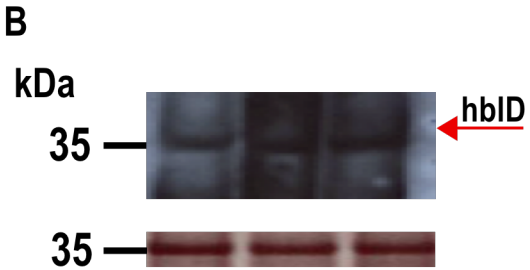
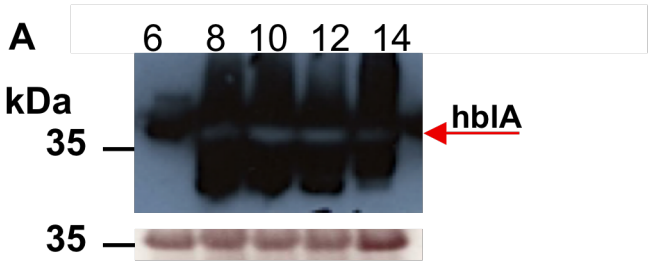


Figure 4.6.2 HblD under simulated conditions. **A** Numbers 1-4 denote *B. cereus* strains, 27, 883-00, F2081B/98 and 98HMPL63 respectively. **B.** Numbers 1-3 denotes *B. weihenstephanensis* strains 10390, 10396, 10202.



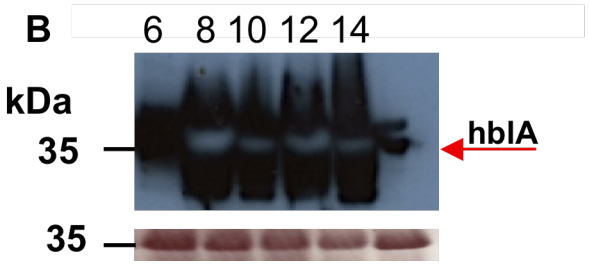
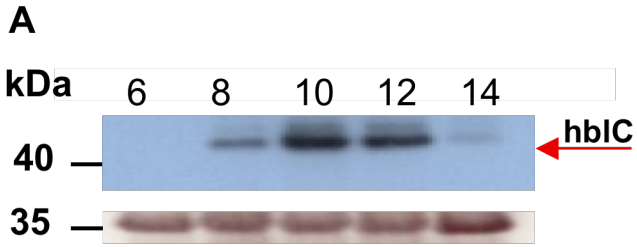


Figure 4.7.1 Time point western blot analysis of hblA. Samples were taken after 6, 8, 10, 12 and 14 hours post inoculation under optimal conditions. **A**. F2081B/98 **B**. 10396



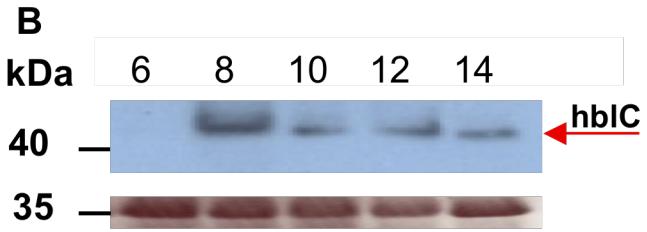
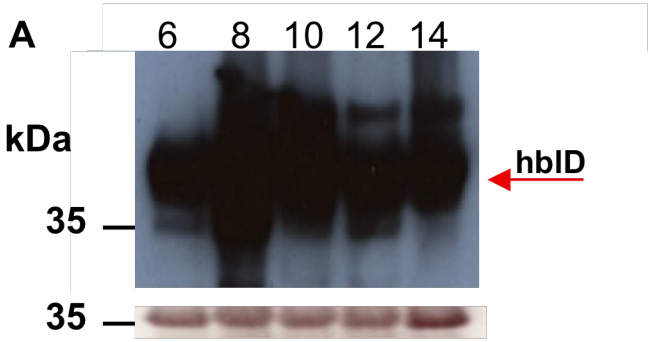


Figure 4.7.2 Time point western blot analysis of hblC. Samples were taken after 6, 8, 10, 12 and 14 hours post inoculation under optimal conditions. **A**. F2081B/98 **B**. 10396



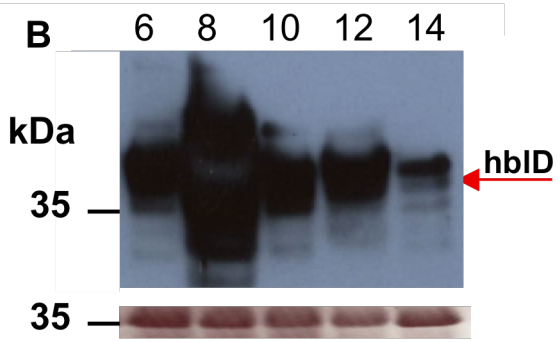


Figure 4.7.1 Time point western blot analysis of hblD. Samples were taken after 6, 8, 10, 12 and 14 hours post inoculation under optimal conditions. **A**. F2081B/98 **B**. 10396

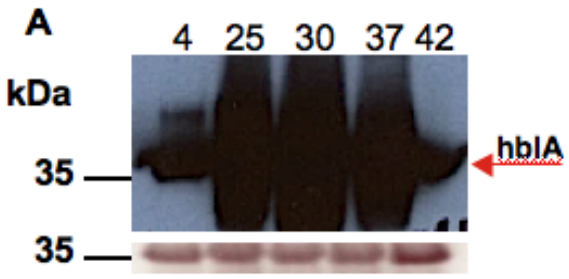
It could be postulated the both the antibody specificity and strain specific expression ratios could be responsible.

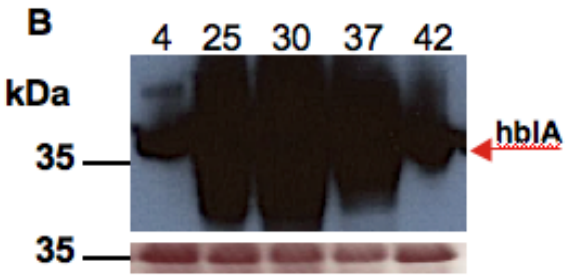
4.6 Expression of haemolysin BL under simulated human GI tract conditions

Supernatants were harvested from cultures grown under simulated human GI tract conditions that were initially inoculated with 10^7 *B. cereus or B. weihenstephanensis* spores. Previous work has shown that the greatest number of spore survivors from the stomach simulation was gained when the initial inoculum was 10^7spores/ml. Therefore in order to ensure the maximal amount of protein was present in the supernatant an initial inoculum 10^7 spores/ml was used. Increasing the protein concentration within the membrane from 20μg to 150μg resulted in the appearance of faint bands

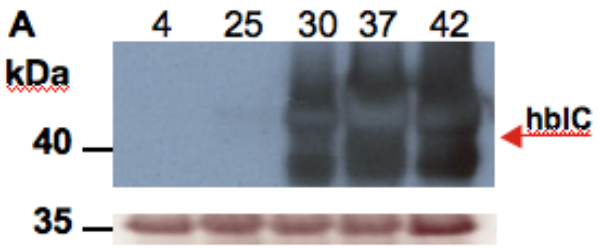
hblA was detected in all *B. cereus* and *B.weihenstephanensis* strains (Figure 4.6.1A+B). No hblC was detected in any strain when membranes were probed with antisera (data not shown). The reasons for the absences of this lytic protein were unclear. Under optimal conditions hblC expression was reduced when compared to that of hblA and hblD. It is possible that within the simulation high levels of proteases result in the small amount of hblC produced being quickly degraded. HblD was present in both *B. cereus* and *B. weihenstephanensis* strains when tested (Figure 4.6.2A+B). Significantly more hblD was detected than hblA although comparable protein amounts were tested. The simulated media contained a host of proteases that are also present within a human GI tract (Kristiffersen *et al*, 2007; Tam *et al*, 2006; Fang *et al*, 2009). These enzymes attack and degrade any proteins secreted by *B. cereus* or *B. weihenstephanensis* cells. This could possibly account for the differences detected in hbl protein levels under optimal and simulated conditions. In comparison to optimal condition blots, 15 times more protein, isolated from simulated cultures, was added to each membrane however detected protein bands were still weak. The reason for the variance witnessed in the hbl protein levels is unclear. As an operon the genes corresponding to

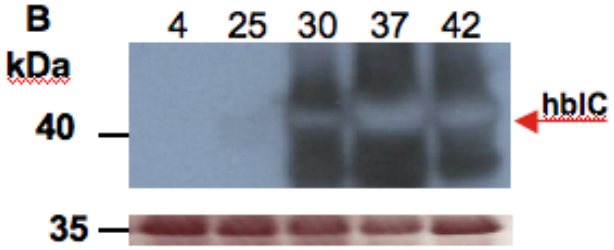
each protein are expressed sequentially and as such a similar level of expression is expected if translationary coupled.



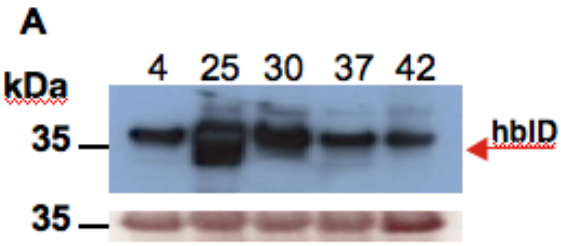


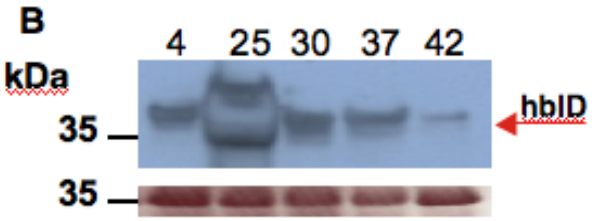
F
a
S
c
c
2
d
B





F
a
S
c
c
2
d
B





F
a
S
c
c
2
d
B

Under conditions simulating the GI tract both hblA (binding protein) and hblD (lytic protein) were detected in *B. cereus* and *B. weihenstephanensis* strains. It is believed that this is first case of individual hbl proteins being detected under conditions simulating the human GI tract.

4.7 Time course experiment of haemolysin BL expression under optimal conditions

The onset of diarrhoeal symptoms is between twelve and sixteen hours post ingestion of contaminated food. Dependent on the meal consumed the maximum GI transit time is believed to be eight hours. Therefore cells and spores spend a further four to eight hours in the small intestine before the host feels any negative effects.

Using a time course experiment, expression of individual hbl proteins can be monitored over this period. Strain F2081B/98 and 10396 were selected as *B. cereus* and *B. weihenstephanensis* representatives as little difference between the individual strains had been witnessed in earlier experiments.

Secreted proteins were harvested from BHI inoculated with 10^7 F2081B/98 and 10396 spores after 6, 8, 10, 12, and 14 hours. The initial start time of 6 hours was selected as at that point spores would have been in the simulated intestinal media for 2 hours. In that time some spores could have germinated and resulting vegetative cells produce secreted proteins. hblA, hblC and hblD were detected after 6 hours. HblA was present in abundance, with a significant level of protein recovered in both strains between hours 8-12 (Figure 4.7.1A+B). HblC was recorded at time points 8, 10 and 12 in strain F2081B/98 with samples 10 and 12 having the greatest protein concentration (Figure 4.7.2A). Similar levels of hblC were recorded in 10396 from 8 to 14 hours (Figure 4.7.2B). Both strains appeared to produce significantly less hblC than hblA. Substantial levels of hblD protein were recorded at each time point in strain F2081B/98 (Figure 4.7.3A). In 10396 time point 8 had the highest concentration of hblD. As the time course continues less protein was detected (Figure 4.7.3B).

4.8 Investigation of the effect varying temperatures on Haemolysin BL production

The optimal growth temperature for *B. cereus* is 30^0C however as a mesophilic it can grow at temperatures between 25^0C-37^0C (Johnson, 1984; Claus & Berkeley, 1986). *B. weihenstephanensis* has been reported to grow at 4^0C while being uncultivable at 40^0C and above (Lechner *et al*, 1999). In order to determine if temperature has an effect on the production of hbl proteins, strains F2081B/98 and 10396 were grown at 4^0C, 25^0C, 30^0C, 37^0C and 42^0C and the presence of each protein determined through western blot.

HblA was found at all temperatures with the greatest concentrations at 25^0C, 30^0C, and 37^0C in both strains (Figure 4.8.1A+B). Detecting hblA at 42^0C after 8 hours indicated that both *B. cereus* and *B. weihenstephanensis* spores were able to germinate and produce virulence factors at what was thought to be an inhibitory temperature. HblC was only present at 30^0C, 37^0C and 42^0C samples in both strains (Figure 4.8.2A+B). The reason for its absence at 25^0C was unknown as spores had germinated and outgrowth was witnessed. HblD was present at all temperatures in both strains with the greater concentration recorded at 25^0C (Figure 4.9.3A+B). In contrast to previous work, which indicated 30^0C was optimal for toxin production it was witnessed that the greater concentrations of some proteins were produced at 37^0C.

Overall this work has illustrated the ability of both *B. cereus* and *B. weihenstephanensis* strains to produce a host of virulence factors associated with diarrhoeal food poisoning. It detailed the expression of the primary diarrhoeal virulence factor, hbl in conditions mimicking the human GI tract. It was shown that under these conditions hblA and hblD were present in all strains supernatant. The potent cytK-1 was identified in the supernatant of *B. weihenstephanensis* strain 10202, indicating that this psychrotroph could be pathogenic to humans. This study also revealed that *B. weihenstephanensis* can grow and produce the hbl proteins at 42^0C, contradicting previous work that said *B. weihenstephanensis* could not be cultured at 42^0C. It is possible that their growth spectrum is wider than previously thought or that strains have individual growth ranges. Taking these results in conjunction with those

under simulated conditions it can be postulated that temperature is not the limiting factor in hbl production under simulated GI tract conditions but the presence of bile and proteases, which affect the concentration of each component.

4.9 Discussion

The ability of bacteria to secrete virulence factors including toxins greatly increases their virulence. In order for bacterial toxins to access their site of action in target host cells they first need to cross the bacterial membrane (Fagerlun Linback & Granum, 2010). One quarter or proteins secreted by the non-pathogenic bacterium *B. subtilis* during stationary phase are associated with metabolising carbohydrates, in contrast *B. cereus* predominately secrets toxins, proteases or phospholipids (Gohar *et al*, 2005). These changes in the function of secreted proteins could have occurred as *B. cereus* was evolving to live in a human host thereby the primary source of nutrients changing from carbohydrates to proteins (Gohar *et al*, 2005). The by-product of *B. cereus* adapting, allowing them to inhabit humans is their ability to harm the host and cause diarrhoeal food poisoning.

iTRAQ is a technique developed by Applied Biosystems which both quantifies and identifies proteins within a complex mix. Through the use of isobaric reagents four or eight experimental states can be compared at once. Within this study, this method was used to investigate the secretome of *B. cereus* strain F2081B/98. The bacterium was grown under optimal and simulated human GI tract conditions allowing information to be gained relating to the effect that the simulated GI tract could have on secreted proteins and overall what the impact could be on strain virulence. Upon MALDI-TOF mass spectrometry analysis no peptides were identified as tagged in any of the simulated GI tract or optimal samples. Removal of iTRAQ filter resulted in one hit corresponding to phospholipase C in the optimal sample. It is highly likely that under optimal conditions more than one protein is present within the supernatant. Insufficient binding of the proteolysed peptides and iTRAQ reagents could explain the lack of tagged peptides when samples were analysis. The reasons however as to why this may have happened are

unclear. It is also possible that protein could have been lost through the cation exchange chromatography. During cation exchange the sample was eluted as one fraction as only small amounts of protein were thought to be contained within each sample. Subsequently experiments have shown that the secretrome of F2081B/98 contains a considerable number of proteins including virulence factors like haemolysin BL. It is possible that eluting the sample in more than one fraction during cation exchange may have resulted in the detection of more proteins.

Haemolysin BL is the primary virulence factor in diarrhoeal food poisoning however initially Beecher and MacMillian isolated the components from *B. cere*us strain F837/76 from a surgical wound (Beecher & MacMillian, 1990). Properties of these components were determined through haemolysis assays and were identified as B (binding) and L (lytic). Subsequently the purified L component was shown to comprise of two proteins of 36kDa and 45kDa and thus were named L_1 and L_2 respectively (Beecher & MacMillian, 1991). Immuno-flourescence highlighted that the 35kDa B protein binds to the surface of target cells after which the lytic proteins associate with B. All three proteins are required for maximal toxicity however only trace amounts of L1 and L2 are needed to lyse erythrocytes when B is bound (Beecher & MacMillian, 1991). Beecher, Schoeni and Lee Wong (1994) constructed polyclonal antibodies against the individual hbl proteins allowing for the rapid identification of toxin producing *B. cereus* strains (Beecher, Schoeni & Lee Wong, 1994). Monoclonal antibodies also directed at B, L_1 and L_2 have been manufactured from strain B4ac (Dietrich *et al*, 1999). These antibodies have only been used to determine the presence of hbl proteins within the culture medium, therefore enabling the identification of hbl producing strains. In this study polyclonal antibodies were used to monitor hbl expression under simulated human GI tract conditions. This is the first time that the presence of each individual hbl protein has been assessed under conditions mimicking those found in humans using antibodies.

Under optimal culture conditions all strains were shown to produce hbl proteins. Significantly less hblC was detected in comparison with hblA/D. The

genes encoding *hblA/C/D* have been shown to be transcribed as an operon therefore the reason for less hblC within the culture medium is unknown at this time (Dietrich *et al*, 1999; Granum *et al*, 1999; Linback *et al*, 2004). No difference was recorded in the expression of hbl components between *B. cereus* and *B. weihenstephanensis* strains under any conditions tested. Although the presence of the *hblACD* operon has been previously identified, this is the first time that hbl proteins have been shown to be produced by *B. weihenstephanensis* (Lechner *et al*, 1999; Puβ *et al*, 1999; Pacova *et al*, 2003; Stenfors *et al*, 2002; Stenfors *et al*, 2008). Under the simulated human GI tract conditions less hblA and hblD were recorded and no hblC was detected in comparison to optimal culture conditions. To determine if temperature was a factor in hbl expression temperature shift assays were performed. No one temperature consistently produced the highest concentration of each protein, although hblA concentration in 10390 and hblC in F2081B/98 was greatest at 37^0C. It has previously been suggested that *B. cereus* and *B. weihenstephanensis* strains have adapted to a human environment based on the array of proteases they produce (Rasko *et al*, 2004). It is possible that these strains have partly adapted to the conditions found within human and thus express certain proteins maximally at 37^0C. Further work is required to determine if other virulence factors are maximally produced at this temperature.

Hemolysin BL is not the only toxin or virulence factor *B. cereus* manufacture. In order to gain a greater understanding of the proteins produced, each strains secretome was mapped using tandem mass spectrometry. Gohar *et al* (2005) used 2D gel electrophoresis to probe the secretome of *B. cereus* strain ATCC14579. 23 spots on the gel corresponded to toxins or degradative enzymes (Gohar *et al*, 2005). Toxin components hblC, hblD, nheA and nheB were identified along with the cytotoxin, cytK. Additionally, enolase, peptidase T, groEL and flagellin were also found within the supernatant (Luo *et al*, 2006). Gilois *et a*l (2007) also investigated secreted proteins produced by *B. cereus* strain ATCC14579. Two hours post stationary phase, 80% of the secreted protein compared contained flagellins, toxins, proteases and phospholipases which were also found within the *B. cereus* and *B.*

wiehestephanensis strain used in this study using MALDI-TOF mass spectrometry (Gilois *et al*, 2007). During this work no hemloysin BL proteins were detected in strains 883-00 and 10202 via MS analysis however subsequent western blots did show all components present apart from hblC in 883-00. Only strain 98HMPL63 produced all nhe proteins under these experimental conditions. The expression profile of particular toxins was explored further by using time course experiments running from late exponential (T_{-1}) to late stationary phase (T_5) growth. The cytK toxin was shown to increase until T_2 in stationary phase after which it declined. The hemolysin BL lytic protein hblC presence was recorded until T_3/T_4 hours. The second lytic hbl protein, hblD and the non-haemolytic enterotoxin proteins, nheA and nheB were all present during the changing growth phases from exponential (T_{-1}) to stationary (T_2) (Gilois *et al*, 2007). Secreted protein profiles can change based on the demands placed on cells from the external environment. Giliois *et al* (2007) suggested that there are specific timeframes for the expression of virulence factors, implying that some proteins that were not detected in this study could still be produced by each strain.

Further to the hbl and nhe toxins, cytK-1 was detected in *B. weihenstephanensis* strain 10202. Showing 30% homology to the β barrel toxin found in *Staphylococcus aureus* and *Clostridium perfringens*, cytK-1 is a cytotoxin with significant cytotoxic ability. Secreted in a soluble form cytK molecules come together to form transmembrane pores (Bhakdi, & Tranum-Jensen, 1991; Song *et al*, 1996; Lund, Byser, and Granum, 2000; Hardy *et al*, 2001). Two variant forms have been identified and denoted cytK-1 and cytK-2. The second form, believed to be a truncated version of cytK-1, is dramatically less toxic (Fagerlund *et al*, 2004). Blast analysis showed that the cytK protein isolated from strain 10202 showed significant homology to that of the ctyK-1 protein group. Previous PCR analysis has shown cytK to be both present in few *B. weihenstephanensis* strains, indicating that like *B. cereus* it has a very narrow distribution (Guinebretiere *et al*, 2006; Hendriksen Hansen, & Johanson, 2006; Shadrin *et al*, 2007; Bartoszewicz *et al*, 2008; Stenfors *et al*, 2008). Confirmation of gene presence does not automatically result in expression. This is believed to be the first reported occasion where ctyK-1 has

been detected within the culture supernatant of a *B. weihenstephanensis* strain. The implication of this result could be serious for food producers concerned about psychrotrophic *B. weihenstephanensis*. Historically concerns about the presence of psychrotrophic *Bacillus* have focused on food spoilage however recent work, including this study, has shown that *B. weihenstephanensis* can not only survive within a human host but also produce toxins associated with food poisoning. When investigated further both *B. cereus* and *B. weihenstephanensis* also express more proteins deemed virulence factors.

A significant amount of protein identified from the supernatant of each strain was flagellin or flagellar related in both this study and also previous work (Bouillaut *et al*, 2005; Gilois *et al*, 2007). Flagella have been shown to play an important role in the virulence of many bacterial pathogens including *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Listeria monocytogenes*. Flagella have numerous functions including roles in adhesion, biofilm formation and host invasion. However primarily they are responsible for locomotion (Liv & Ochaman, 2007). Specialised forms of flagella driven motility are associated with increased secretion of virulence proteins (Ghelardi *et al*, 2002; Senesi *et al*, 2002; Fedhila *et al*, 2004; Chen *et al*, 2009). Changes in environmental conditions can trigger cell differentiation from normal swimmers to elongated, hyperflagellated swarming cells (Ghelardi *et al*, 2002; Kearns & Losick, 2003). Swarm cells move together like multi-cellular rafts enabling quick movement over solid areas enabling them, like biofilms to colonise surfaces quickly. This could be greatly important when cells are faced with the small intestine where in order to cause disease they need to colonise the epithelium. One transcriptional regulator (plcR) is believed to control the expression of many virulence factors shown to be produced by the strains in this study.

Around 80% of extracellular proteins manufactured through stationary phase are controlled by plcR, the pleoitrophic gene regulator (Agaisse et al, 1998; Bouillaut *et al*, 2005). PlcR is activated through the binding of the peptide papR, which itself is plcR regulated. Inactivate papR molecules are exported

from the cell via the sec secretion system. Once in the supernatant it is processed by nprB becoming active and then re-imported through the opp channel. NprB along with InhA2/1 and collagenase were all found in the supernatant of ATCC14579 (Bouillaut *et al*, 2005; Gilois *et al*, 2007; Cadot *et al*, 2010). Cadot *et al* (2010) suggested that all strains tested to date produced nprB and InhA1. In this work only F2081B/98 and 10202 were positive for both nrpB and InhA2, strains 27 and 98HMPL63 contained nprB alone.

The ability of a particular substance or protein to cause an immune reaction within the human body is referred to as immunogenicity. The immunogenicity of intestinal bacteria depends on the degree of contact with the lymphoid tissue (Hohmann Schmidt & Rowley, 1979; Wold *et al*, 1989). Such an abundance of flagellar related proteins in the supernatant of both spp. suggest that they could interact significantly with human epithelial and lymphoid tissue. In *B. anthracis*, enolase and nlpC have been shown to be immunogenic (Chitlaru *et al*, 2006; Delvecchio *et al*, 2006; Walz *et al*, 2007). It is possible that these proteins also serve a similar function in *B. cereus* and *B. weihenstephanensis*. Enolase was found in strains F2081B/98, 10390 and 10396 and nlpC in 10396. It is possible that further proteins that are secreted by *B. cereus* or *B. weihenstephanensis* also act as immunogens. The presence of these proteins within the small intestine could alert the human body to the presence of *B. cereus* or *B. weihenstephanensis* cells enabling a host immune response. Further work is required to investigate this.

Investigating the secretome of both *B. cereus* and *B. weihenstephanensis*, resulted in no significant difference being found between these species. All strains produced a host of proteins involved in virulence, with many overlapping. *B. weihenstephanensis* strain 10202 was the only strain to produce cytK-1, the potent cytotoxin. This result along with the evidence of further toxins being secreted by *B. weihenstephanensis* strains illustrates this species potential pathogenicity. The overlapping expression profiles of different toxins and toxin components could allow both *B. cereus* and *B. weihenstephanensis* strains, when within the small intestine, to have a multi

strike function allowing them to attack through several different mechanisms. The ability to co-ordinately express many different toxins and virulence factors could increase their pathogenicity. This could have wide ranging implications when coupled with the knowledge that *B. weihenstephanensis* strains could also survive within a simulated human GI tract as well as *B. cereus*.

Chapter Five
Real Time PCR Detection of Changes in Gene Expression After Exposure to Conditions Simulating A Human Gastro-Intestinal Tract

5.1 Introduction

Previous work illustrated that *B. cereus* and *B. weihenstephanensis* spores can survive and grow within simulated human GI tract conditions as well as under optimal growth conditions and could produce a variety of virulence factors, many related to diarrhoeal food poisoning. However little is known about how the expression of virulence genes is affected by these same conditions.

B. cereus express genes with both major and minor roles in virulence. The production of these virulence factors is controlled by the transcription factor, plcR. First identified in *B. thuringiensis* as the regulator of phosphoinositide phospholipase C (PI-PLC) production, *plcR* was subsequently shown to govern the expression of over 100 genes (Lereclus *et al*, 1996; Goher *et al*, 2008; Agaisse *et al*, 1999). Thereafter it was identified as a pleiotropic regulator in all *B. cereus* group members. Interestingly a mutation within *plcR* in *B. anthracis* inhibits the production of any toxins associated with diarrhoeal food poisoning (Mingot *et al*, 2001; Pomersantsev, Pomerantseva, & Leppla, 2004; Satalla *et al*, 2010).

Through a two-component system using the oligopeptide papR, *plcR* controls the expression of the main diarrhoeal toxins, HBL, NHE and cytK (see Figure 1.5) (Declerck *et al*, 2007). HBL and NHE are each composed of three proteins encoded by separate genes each as one operon (Beecher & Wong, 1994; Lund & Granum, 1995). HBL is haemolytic and when culture supernatant containing this toxin is incubated with red blood cells they are quickly lysed (Beecher & Wong, 1994a; Beecher & Wong, 1994b) In contrast NHE is cytotoxic, forming pores in epithelial lipid bilayers (Lund *et al*, 2000). Both are widely accepted as the primary virulence factors in *B. cereus* and mediate the majority of diarrhoeal food poisoning cases reported. CytK was first discovered during a food poisoning outbreak in France, which resulted in the deaths of three people (Fagerlund *et al*, 2004). Two forms of this toxin have been described, cytK-1 and cytK-2. CytK-2 is a truncated version of cytK-1 and is significantly less harmful (Fagerlund *et al*, 2007). The distribution of cytK-1 within the *B. cereus* family is narrow, around 30% of

strains, in contrast with HBL and NHE, which are found in around 75% and 100% of strains tested to date respectively (Dietrich *et al*, 1995; Lund & Granum 1997).

Other regulators have been shown to also play a role in *B. cereus* virulence. The ferric ion uptake regulator (*fur*) plays essential roles within *B. cereus*, ensuring intracellular levels of iron remain constant allowing growth, survival and pathogenesis (Harvie *et al*, 2005; Ollinger *et al*, 2006). *Fur* has been linked to the expression of hemolysin II, a pore-forming toxin like cytK, along with surface proteins that have links to adhesion and invasion. Like *plcR*, *fur* boxes, where fur binds to affect transcription of controlled genes, can be found upstream of genes (Harvie *et al*, 2005). Mutations in *fur* resulted in *B. cereus* displaying reduced virulence in an insect model, proving its role in pathogenesis (Harvie *et al*, 2005).

Under stressful conditions numerous proteins are produced to ensure cells remain viable. The hostile environment *B. cereus* and *B. weihenstephanensis* are presented with when ingested could result in the production of proteins that although are not toxic to humans, contribute to virulence. These proteins work to help *B. cereus* and *B. weihenstephanensis* survive within stressful environments. *GroEL* and *SigB* are two such proteins. *GroEL* is a molecular chaperone involved in ensuring proteins fold correctly (Chang *et al*, 2003; Yushan *et al*, 2010). *SigB* is an alternative sigma factor involved in the ability of *B. cereus* to adapt to stressful conditions (Kalman *et al*, 1990; Antelmann *et al*, 1997). It is involved in the expression of the general stress protein regulon (gsr), which is expressed under various stresses to ensure cells remain viable.

Investigating the effects a simulated human GI tract has on the expression of these virulence factors could expand the knowledge of how *B. cereus* and *B. weihenstephanensis* can or could cause diarrhoeal food poisoning. New techniques such as qPCR can be employed to monitor the expression of selected genes in real time, under defined conditions.

Traditional PCR products are quantified by running samples on an agarose gel at the end of the reaction. Real time PCR (qPCR) differs from conventional PCR as the rate of amplicon production can be monitored in real time, instead of at the end point, producing sensitive, simple and easily reproducible results (Bustin, 2000; Dussault & Pouliot, 2006; Postollec *et al,* 2011). Since the original description of real time PCR using ethidium bromide the process has evolved and now various detection mechanisms exist to identify DNA within a real time reaction (Monis Giglio, & Saint, 2005).

Numerous specific intracalculating dyes, which bind dsDNA have been developed. In both commercial and in-house master mixes the most frequently used is SYBR® green as not only is its fluorescence 13 x greater when bound to dsDNA, it is more cost effective than other systems (Vitzthum *et al*, 1999; Monis Gilglio, & Saint, 2005). The SYBR green/DNA complex absorbs blue light (λ_{max}=488nm) and emits green light (λ_{max}=522nm) (Fricker *et al*, 2007). The recorded fluorescence is plotted against the cycle number and an amplification curve generated. It is from this curve that CT (cycle threshold) values are taken. SYBR green will however bind to any dsDNA resulting in contaminated samples producing a false positive signal. Using single-step protocols, where RNA is converted to cDNA within the reaction instead of part of a two-step system, minimises the risk of DNA contamination and experimental variation (Wong and Medrano, 2005). Amplicons produce a characteristic melting temperature (T_m), which correspond to the specific sizes of a sequence detected during gel electrophoresis (Monis Giglio & Saint, 2005). Melt curve analysis can be performed after RT-PCR to determine the presence of any contaminants as different T_m within a sample would be detected.

RT-PCR (reverse transcriptase) turns sample mRNA in to cDNA allowing the relative analysis of gene transcripts. By comparing varying culture conditions information on the effects various environments have on gene expression can be gained. RT-PCR was used to determine any changes in gene expression of selected virulence factors under simulated human GI tract conditions.

5.1.1 Aims

- To determine through conventional PCR experiments the virulence genes present in the *B. cereus* and *B. weihenstephanensis* strains employed in this work
- To determine the effects a simulated human GI tract has on the expression of selected virulence genes through RT-PCR.
- To investigate *B. cereus* and *B. weihenstephanensis* strains ability to lyse sheep and horse blood.

5.2 The confirmation of selected virulence factors within *B. cereus* and *B. weihenstephanensis* strains using conventional PCR

Results presented in chapter 4 showed the ability of both *B. cereus* and *B. weihenstephanensis* strains to produce a host of virulence factors under defined conditions. The work presented was both qualitative and quantitative but further investigation about the expression of specific virulence factors was needed. By using RT-PCR gene expression can be monitored in real time under simulated human GI tract conditions, which were previously described. Stages in the RT PCR experiments are shown in figure 5.2.

Strains were grown overnight at 30^0c, (BHI, pH 7.5, 200rpm) and DNA extracted using Qaigen DNeasy extraction kit. DNA quality and yield was measured using a nano drop machine and aliquots of 50μg were stored at -20^0c. The toxins hemolysin BL (*hbl*) and non-hemolysin enterotoxin (*nhe*), virulence regulators plcR and Fur, and stress proteins *groEL* and *sigB* were selected as markers of virulence. The presence of the actin homolog and housekeeping genes *MreB* and *rpoB* were also determined. These genes were used as controls as throughout the experiment as their expression remained constant. This allowed changes in the genes of interest to be determined. Primers were designed based on the ATCC14579 published sequence held within the National Centre for Biotechnology Information database. The presence of each virulence gene was determined for all *B. cereus* and *B. weihenstephanensis strains* (table 5.2). With few exceptions (27; *nheC, cytK*. 883-00; *hblD* and *nheC*. F2081B/98; *cytK*. 98HMPL63; *nheC*, *cytK*, and *sigB*. 10390; *nheC*, and *cytK*. 10202; *hblD* and *sigB*) each gene was detected in every strain. It is possible that hbl and nhe genes were not found because of genetic diversity within hbl/nhe sequences. *Hbl* maps to a region of the *B. cereus* chromosome, which is unstable resulting in insertions or deletions within gene coding regions (Beecher & Wong, 2000). *CytK* is potentially a lethal toxin however within *B. cereus* and B. *weihenstephanensis* strains it has a narrow distribution.

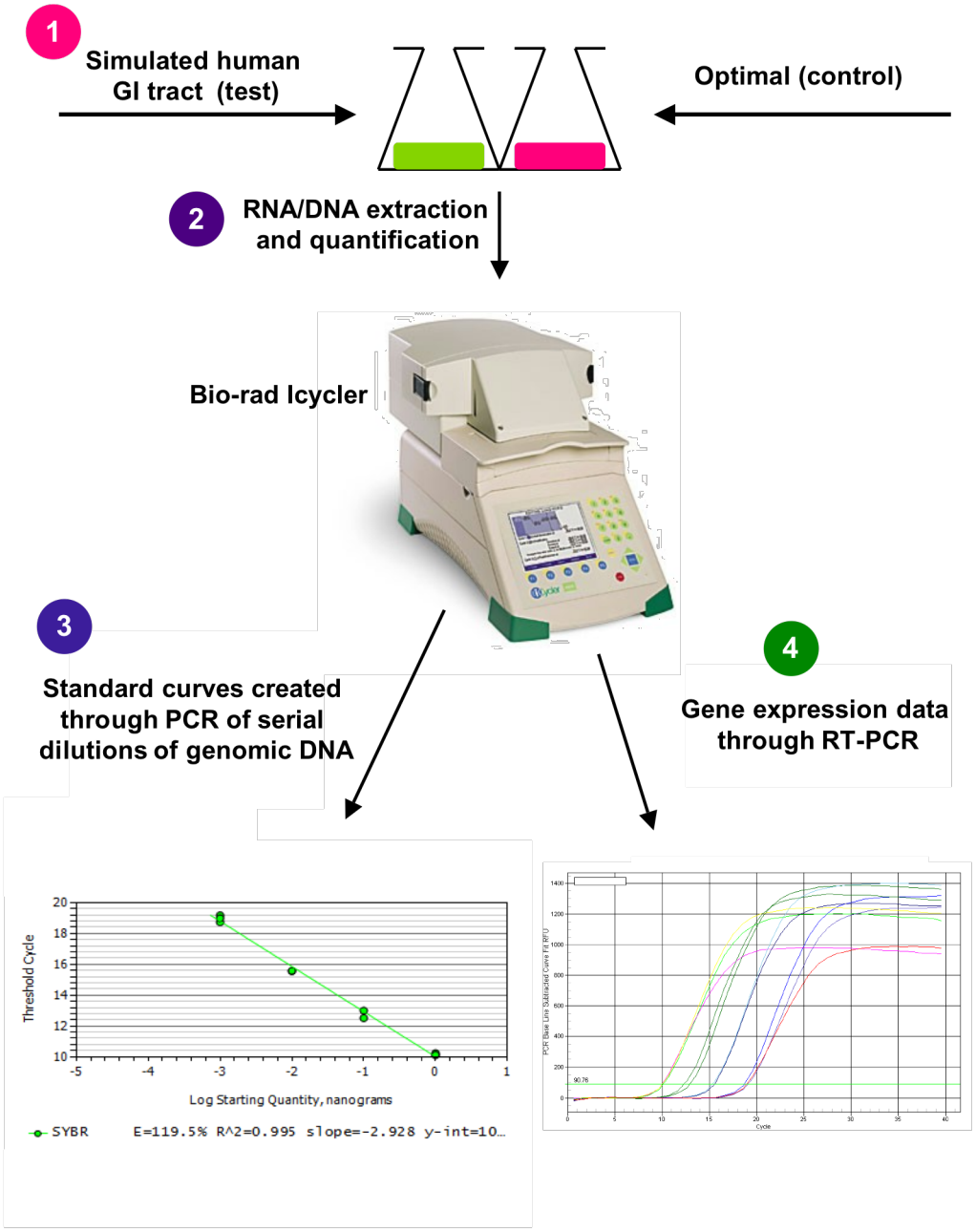


Figure 5.2 Stages in real time PCR experiment. 1) Strains were incubated under simulated GI tact and optimal conditions. 2) DNA and RNA was extracted from each strain using qiagen DNA/RNAeasy kits. 3) Extracted DNA was used to determine primer sets efficiency and slope values used in the subsequent RT-PCR equations. 4) Extracted RNA used in RT-PCR reactions to determine difference in gene expression under varying conditions.

		Hemolysin BL			**Non-Hemolysin Enterotoxin**			**Cytotoxic K**	**Virulence Regulators**		**Stress Proteins**		**Housekeeping Genes**	
		hblA	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*cytK*	*plcR*	*Fur*	*groEL*	*sigB*	*mreB*	*rpoB*
B. cereus	27	X	X	X	X	X			X	X	X	X	X	X
	883-00	X	X		X	X		X	X	X	X	X	X	X
	F2081B/98	X	X	X	X	X	X		X	X	X	X	X	X
	98HMPL63	X	X	X	X	X			X	X	X		X	X
B. weihenstephanensis	10390	X	X	X	X	X			X	X	X	X	X	X
	10396	X	X	X	X	X	X	X	X	X	X	X	X	X
	10202	X	X		X	X	X	X	X	X	X		X	X

Table 5.2 PCR virulence gene confirmation. A selection of virulence factors thought to be important in diarrhoeal food poisoning, bacterial survival and persistence were selected and there presence within the genomes of each strain determined via PCR. The (X) denotes the genes detection. In some strains e.g. 883-00 only one or two genes from hbl or nhe were found. It is possible that the gene sequence within these strains have diverged from those of published sequences that were used to design these primers.

5.3 *B. cereus* and *B. weihenstephanensis* strains RNA extraction and quantification for RT-PCR experiments

B. cereus strains 27 and F2082B/98 and *B. weihenstephanensis* strains 10390, 10396 and 10202 were grown under both simulated GI tract and optimal conditions after 8 hours duration similar to western blot conditions. The rational behind comparing optimal and simulated conditions comes from the knowledge that virulence gene expression is at its highest under optimal conditions. By comparing these two different growth environments the effects that a human GI tract has on virulence gene expression can be determined. Qiagens's RNA protect was used to stabilize any RNA or DNA present within the samples. Once extracted RNA purity and yield were determined via nano drop. Aliquots of 100μg of RNA were made and stored at -80^{0}c until required.

5.4 Determining virulence gene expression under simulated GI tract controls by RT real time PCR

Primers were designed based on key characteristics required for successful RT-PCR reactions for each of the genes of interest (see appendix C). As previously described extracted DNA was used to generate standard curves for each gene, in each strain which provided information on the PCR efficiency (E) (see appendix 5 and materials and methods 3.6). Two reference genes were also selected and primers designed accordingly. The expression of these genes is believed to remain constant throughout and can account for difference in quality or amount of RNA through normalisation (Radonic, Thulke et al. 2004).

RT-PCR reactions were performed in triplicate with treated and control RNA samples for each primer set. Gene expression ratios were determined using the average crossing point (CP) values for treated and control reactions (Figure 5.4.2). The CP value is generated when SYBR green fluorescence increases above a pre-determined threshold created by the RT-PCR software. Reading the levels of fluorescein within each sample sets the threshold. Fluorescein is an internal control contained within the PCR master mix, which helps account for the difference in background SYBR or loading difference

A

$$E = 10^{[-1/slope]}$$

B

$$\text{Expression Ratio} = \frac{(E_{target})^{\Delta CT_{target}\,(control - sample)}}{(E_{ref})^{\Delta CT_{ref}\,(control - sample)}}$$

Figure 5.4.2 Pfaffl RT-PCR equation for the determination of reaction and expression ratios. A. Equation used to determine the efficiency of the reaction taken from the data produced by performing serial dilutions of *B. cereus* and *B. weihenstephanensis* DNA. **B.** The equation used to determine the expression ratio using the PCR efficiency of the target and reference genes (E target, E ref) and the difference in crossing point (Ct) value between the control and treated samples.

		B. cereus		*B. weihenstephanensis*		
	Genes	**27**	**F2081B/98**	**10390**	**10396**	**10202**
Control (optimal)	*nheA*	13.97 +/-0.0243	17.68 +/-0.1393	13.39 +/-0.3408	13.52 +/-0.0008	18.90 +/-0.2269
	nheB	14.36 +/-0.1205	20.37 +/-0.1627	14.93 +/-0.5067	13.82 +/-0.0644	20.66 +/-0.4560
	nheC	15.25 +/-0.2218	19.70 +/-0.0136	16.70 +/-1.3757	16.70 +/-0.0554	15.92 +/-0.0554
	hblA	14.48 +/-0.0639	15.55 +/-0.1817	14.36 +/-0.2171	12.71 +/-0.8049	14.62 +/-0.5364
	hblC	13.99 +/-0.3248	17.16 +/-0.0106	12.55 +/-0.4542	13.41 +/-0.1276	17.71 +/-0.2412
	hblD	20.78 +/-0.7810	19.27 +/-0.0392	14.34 +/-0.0461	17.16 +/-0.0362	19.02 +/-0.0367
	plcR	23.09 +/-0.0802	18.48 +/-0.0924	25.22 +/-0.1379	23.99 +/-0.3996	19.87 +/-0.5967
	fur	22.06 +/-0.1262	20.37 +/-0.3909	18.02 +/-0.2750	14.96 +/-0.1678	17.39 +/-0.3350
	groEL	16.36 +/-0.0005	16.79 +/-0.1552	16.40 +/-0.5000	15.92 +/-0.0252	17.97 +/-0.2014
	SigB	14.58 +/-0.3921	19.36 +/-0.2312	17.15 +/-0.3497	15.20 +/-0.8897	16.50 +/-0.5174
Treated (simulated)	*nheA*	14.18 +/-0.0569	18.96 +/-0.0058	13.61 +/-0.1109	16.28 +/-0.1060	17.64 +/-0.0130
	nheB	15.81 +/-0.4207	20.38 +/-0.6018	14.61 +/-0.1311	20.36 +/-0.2715	20.77 +/-0.3013
	nheC	16.29 +/-0.5374	17.54 +/-0.1042	17.08 +/-0.9955	17.08 +/-0.0753	16.42 +/-0.4560
	hblA	14.94 +/-0.1840	15.87 +/-0.2634	19.11 +/-0.4725	14.21 +/-0.0436	14.95 +/-0.2812
	hblC	14.52 +/-0.2451	17.59 +/-0.0012	13.23 +/-0.4844	18.58 +/-0.0419	17.34 +/-0.1797
	hblD	15.99 +/-0.4923	21.39 +/-0.0285	15.12 +/-0.1893	16.16 +/-0.8594	19.10 +/-0.1209
	plcR	24.17 +/-0.3579	18.20 +/-0.0513	23.99 +/-0.6283	24.64 +/-0.3205	17.43 +/-0.1928
	fur	21.82 +/-0.4418	17.71 +/-0.0674	16.40 +/-0.0135	23.87 +/-0.2414	17.30 +/-0.0189
	groEL	18.88 +/-0.3524	16.79 +/-0.1630	13.73 +/-0.2339	15.57 +/-0.0944	16.71 +/-0.0944
	SigB	14.65 +/-0.0482	19.46 +/-0.4375	14.61 +/-0.0881	15.17 +/-0.3887	16.90 +/-0.0977

Table 5.4 Real-time PCR CT values. The CT values were obtained when the level of fluorescence increased to that of higher than the background signifying the point at which gene expression is increased. The earlier the CT value the more abundant the transcripts. These CT values were used to the determine gene expression ratios. Standard deviation (SD) was determined for all samples.

within the first 10 cycles of the PCR. The Pfaffl (2001) equation allows the gene expression ratio to be determined by using the PCR efficiency values gained during the creation of standard curves, the delta CP values for the gene of interest along with the reference gene sample.

Average CT values (Table 5.4) were used to determine changes in gene expression under simulated GI tract conditions in comparison to that of optimal conditions. Lower CP values indicate a higher level of cDNA within the sample. The ratio obtained for the gene of interest and the reference genes were plotted and fold difference changes in the rates of expression shown.

5.5 Investigating the effects of a simulated human GI tract on the expression of the three component toxin, *nhe*

NheA and *nheB* presence was confirmed in each strain however *nheC* was only recorded in *B. cereus* strain F2081B/98 and *B. weihenstephanensis* strains 10396 and 10202. In *B. cereus* strain F2081B/98 *nheA* expression was reduced by 2 fold however there was no change detected in strain 27 (Figure 5.5.1A). A 2-fold reduction in *nheA* expression was also witnessed in *B. weihenstephanensis* strain 10396. In contrast however nheA in both 10390 and 10202 appeared to be up regulated by 3.3 and 1.8 fold respectively (Figure 5.5.1B). A similar pattern was detected for *nheB* within *B. weihenstephanensis* strains. 10390 showed a 4.5 fold increase in expression while 10396 showed a 2-fold reduction in the level of *nheB* (Figure 5.5.2B). A decrease in expression was also recorded in *B. cereus* strain 27 however strain F2081B/98 remained the same (5.5.2A). *B. cereus* strains showed increased levels of *nheC* (Figure 5.5.3A). Strain 27 displayed a 1.3 fold difference while F2081B/98 increased by 3.6 fold. A reduction in *nheC* expression was recorded in both *B. weihenstephanensis* strains 10396 and 10202, 2 and 3 fold respectively (Figure 5.5.3B). The reason for these differences in gene expression are unclear but the random nature of the results supports the previously held ideas about toxin expression varying significantly from strain to strain.

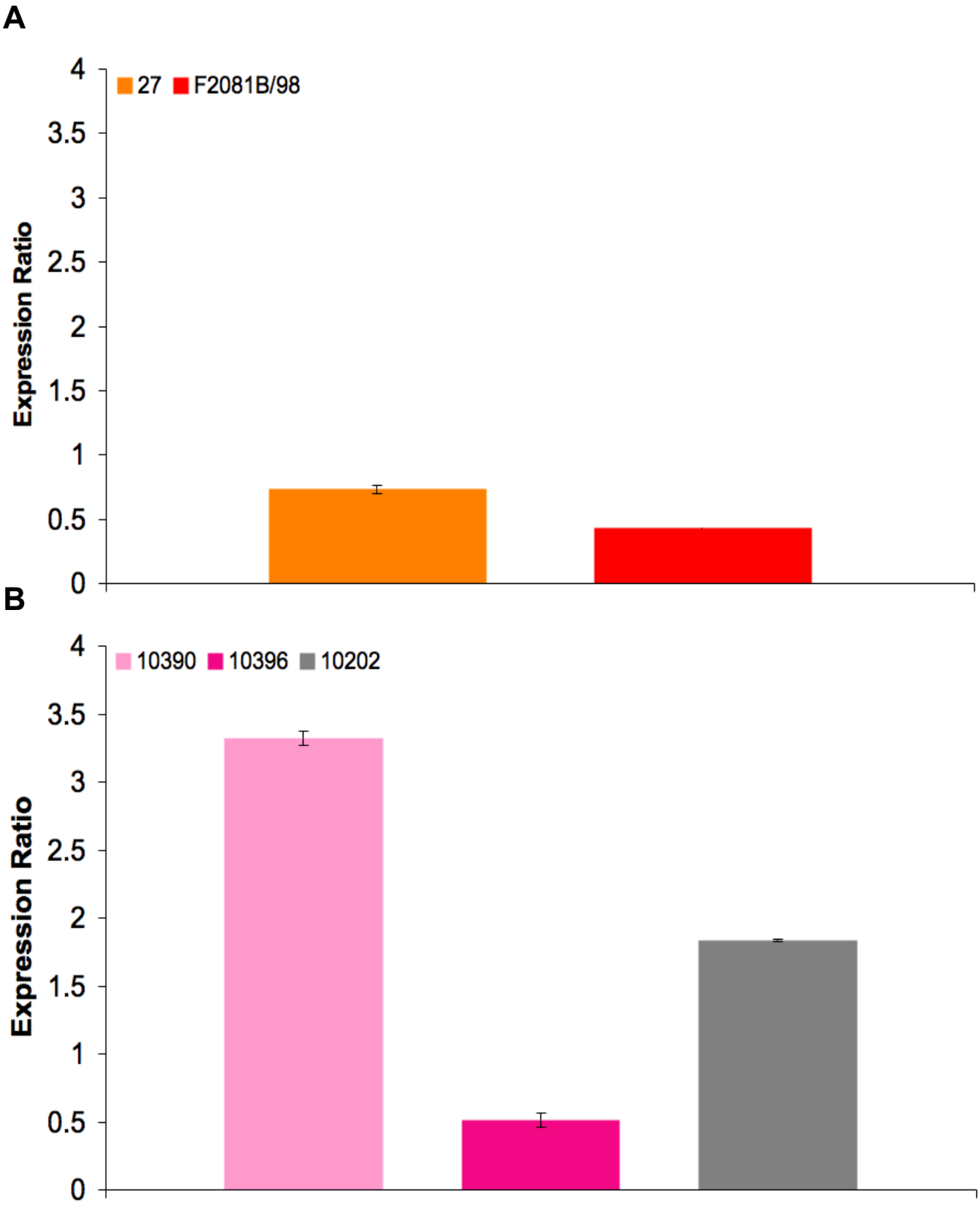


Figure 5.5.1 Expression ratios of *nheA* under simulated human GI tract conditions. **A**. *B. cereus* strains. **B.** *B. weihenstephanensis* strains

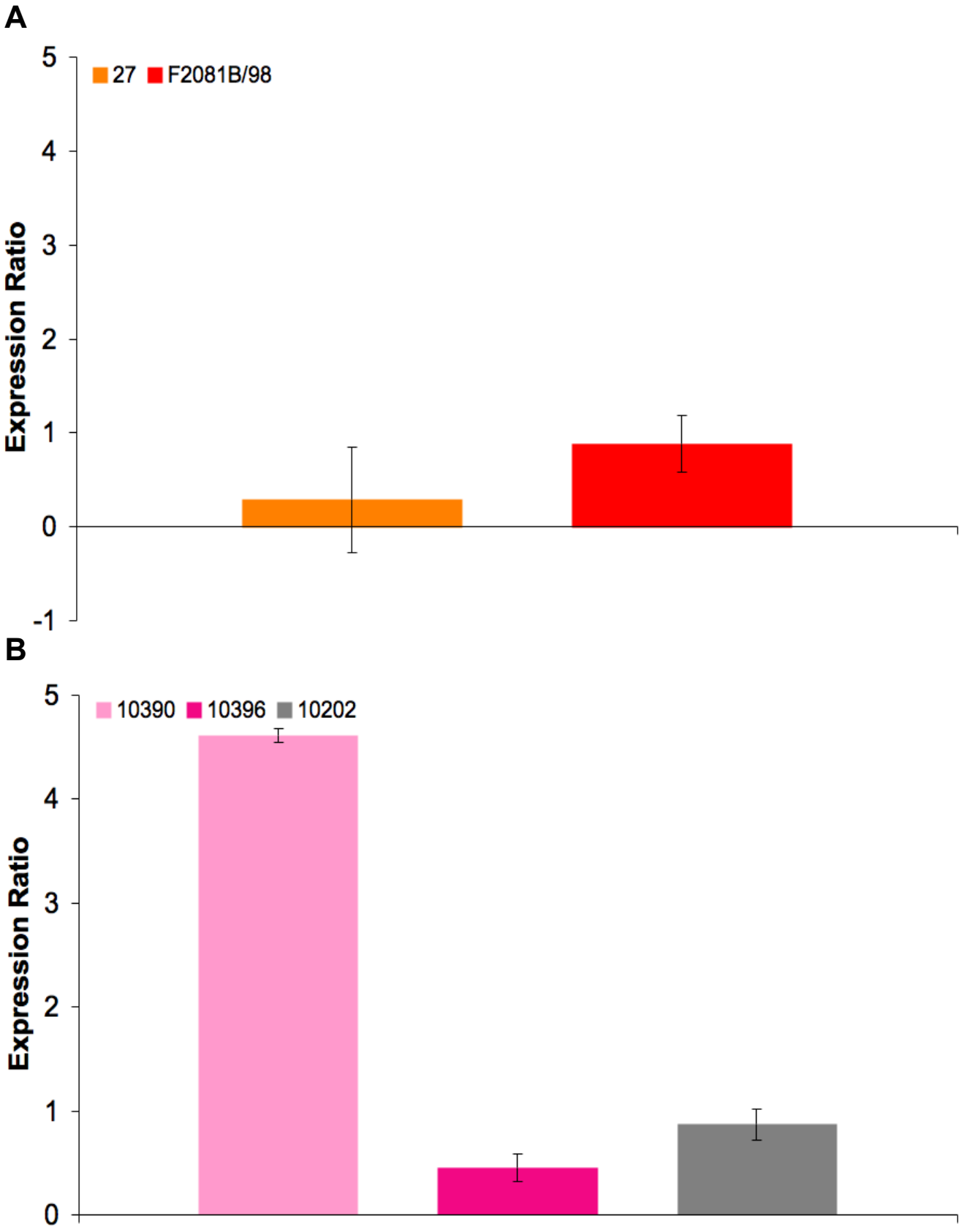


Figure 5.5.2 Expression ratios of *nheB* under simulated human GI tract conditions. **A**. *B. cereus* strains. **B.** *B. weihenstephanensis* strains

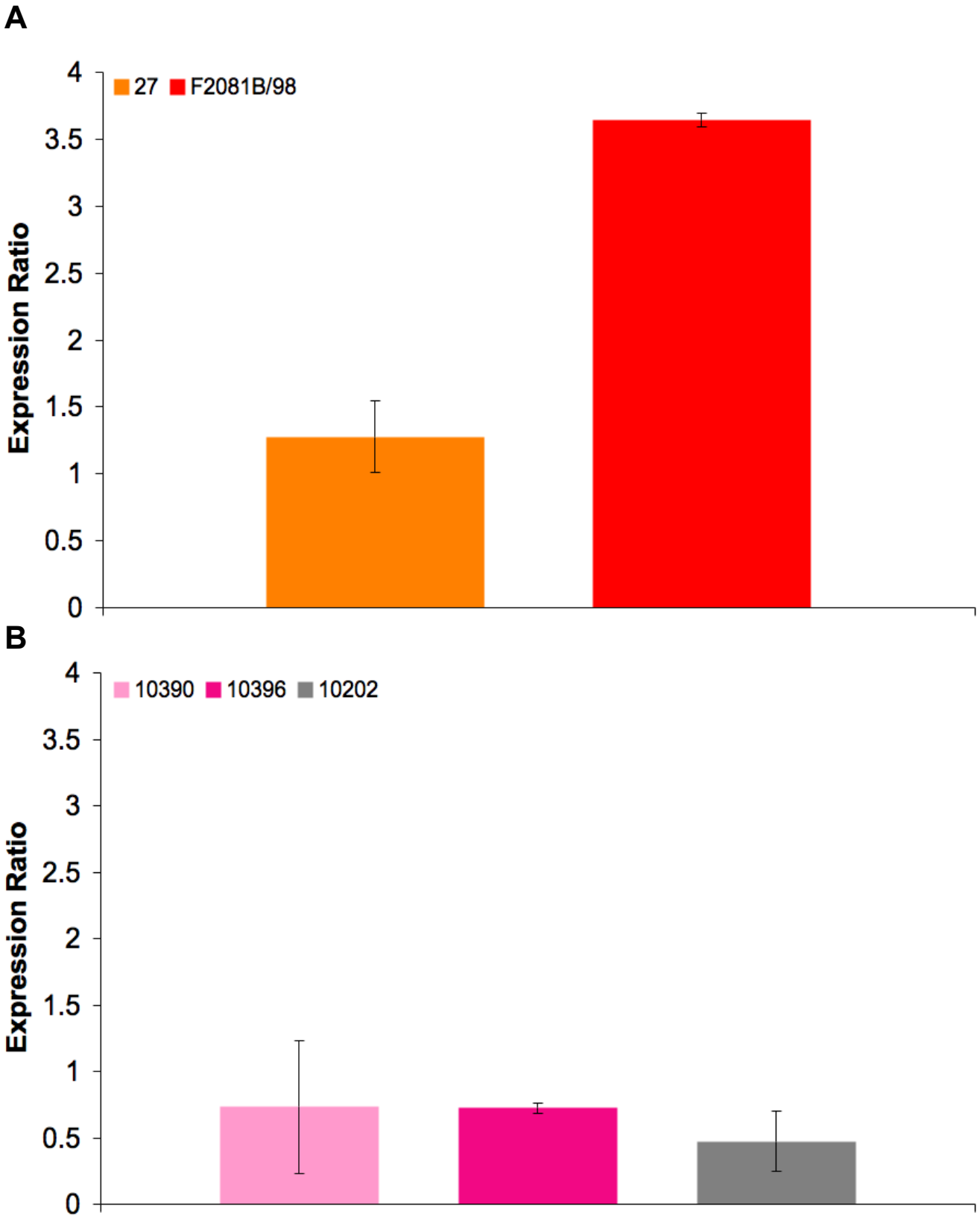


Figure 5.5.3 Expression ratios of *nheC* under simulated human GI tract conditions. **A**. *B. cereus* strains. **B.** *B. weihenstephanensis* strains

5.6 Investigating the effects of a simulated human GI tract on the expression of the three component toxin, *hbl*

Hemolysin BL genes *hblA* and *hblC* were detected in each strain using conventional PCR. *HblD* however was not amplified in *B. cereus* strain 883-00 and *B. weihenstephanensis* strain 10202. A 2-fold decrease was recorded in *hblA* expression in strain 27 while strain F2081B/98 was unaffected by the simulated GI tract conditions (Figure 5.6.1A). *B. weihenstephanensis* strains 10396 and 10202 both recorded a 2.3 fold increase in *hblA* while 10390 *hblA* expression remained constant (Figure 5.6.1B). In *B. cereus hblC* expression was down regulated in strains 27 and showed no change in F2081B/98 (Figure 5.6.2A). In *B. weihenstephanensis* strain 10202 no change in *hblC* expression was detected however in strain 10390 its expression was significantly down regulated by greater than 2-fold. Strain 10396 recorded significantly elevated levels of *hblC* expression, 2.3 fold greater under simulated conditions (Figure 5.6.2B). *HblD* expression was significantly up-regulated by 3 fold in strain 27 while it remained the same in strain F2081B/98 (Figure 5.6.3A). The expression level of *hblD* was also increased *in B. weihenstephanensis* strains 10390 and 10396 by 2.1 and 2.2 fold respectively (Figure 5.6.3B). The reason for the increased expression of some genes within this operon is unclear, as it would be expected if one gene was up-regulated they all would be as they are co-expressed. Like the results found for *nhe* these finding confirm that toxin expression is strain dependent.

These results show that the genes this make up the primary virulence factors (toxins) in diarrhoeal food poisoning are expressed under simulated GI tract conditions, similar to that of optimal conditions. In some cases specific gene expression was shown to be up-regulated.

5.7 Investigating the effects of a simulated human GI tract has on the expression of the transcription activator, *plcR*

The *PlcR* gene was present in each strain as confirmed by conventional PCR. By monitoring *plcR* observations about potential pathogenesis of strains can be made as this protein regulates the expression of all known virulence factors in *B. cereus* and *B. weihenstephanensis* (Agaisse *et al*, 1999; Gohar *et al*, 2002; Slamti & Lereclus, 2002; Gohar *et al*, 2008). In *B. cereus* strain 27

there was minimal increase of 1.4 fold however F2081B/98 remained at the same level as under optimal conditions (Figure 5.7.1A). 10202 was the only *B. weihenstephanensis* strain to show a higher level of *plcR* expression, 3.5 fold (Figure 5.7.1B). A 3-fold reduction in plcR was recorded in strain 10396. The increase in expression levels in strains 27 and 10202 could account for the high transcription levels of *hblD* and *nheC* and *nheA* and *hblA* genes respectively. No change in expression was however recorded in F2081B/98 and 10390, and with strain 10396 a decrease was recorded, possibly indicating that another transcriptional regulator was involved in the control of toxin genes.

5.8 Investigating the effects of a simulated human GI tract has on the expression of the ferric uptake regulator, *fur*

The presence of the gene *fur* was confirmed in each strain by conventional PCR. *Fur* the ferric iron regulator has been linked to virulence within *B. cereus*. Strain 27, 10396, and 10202 all showed greater than a 2-fold decrease in fur expression under simulated GI tract conditions (Figure 5.8.1A +B). In stark contrast fur expression was significantly increased in strains F2081B/98 and 10396 by 4 and 10.4 fold respectively. The reason for these vastly different results is currently unclear.

5.9 Investigating the effects of a simulated human GI tract has on the expression of the transcription factor, *sigB*

The presence of the transcriptional regulator *sigB* was confirmed in each strain apart from F2081B/98 and 10202. *SigB* is a general sigma factor involved in the regulation of starvation/survival stress response. There was no change in the expression rate within the *B. cereus* strains (Figure 5.9.1A). *B. weihenstephanensis* strains 10390 and 10396 showed elevated expression levels, 1.4 and 1.3 fold difference respectively. No difference was recorded between optimal and simulated conditions in strain 10202 (Figure 5.9.1B**).**

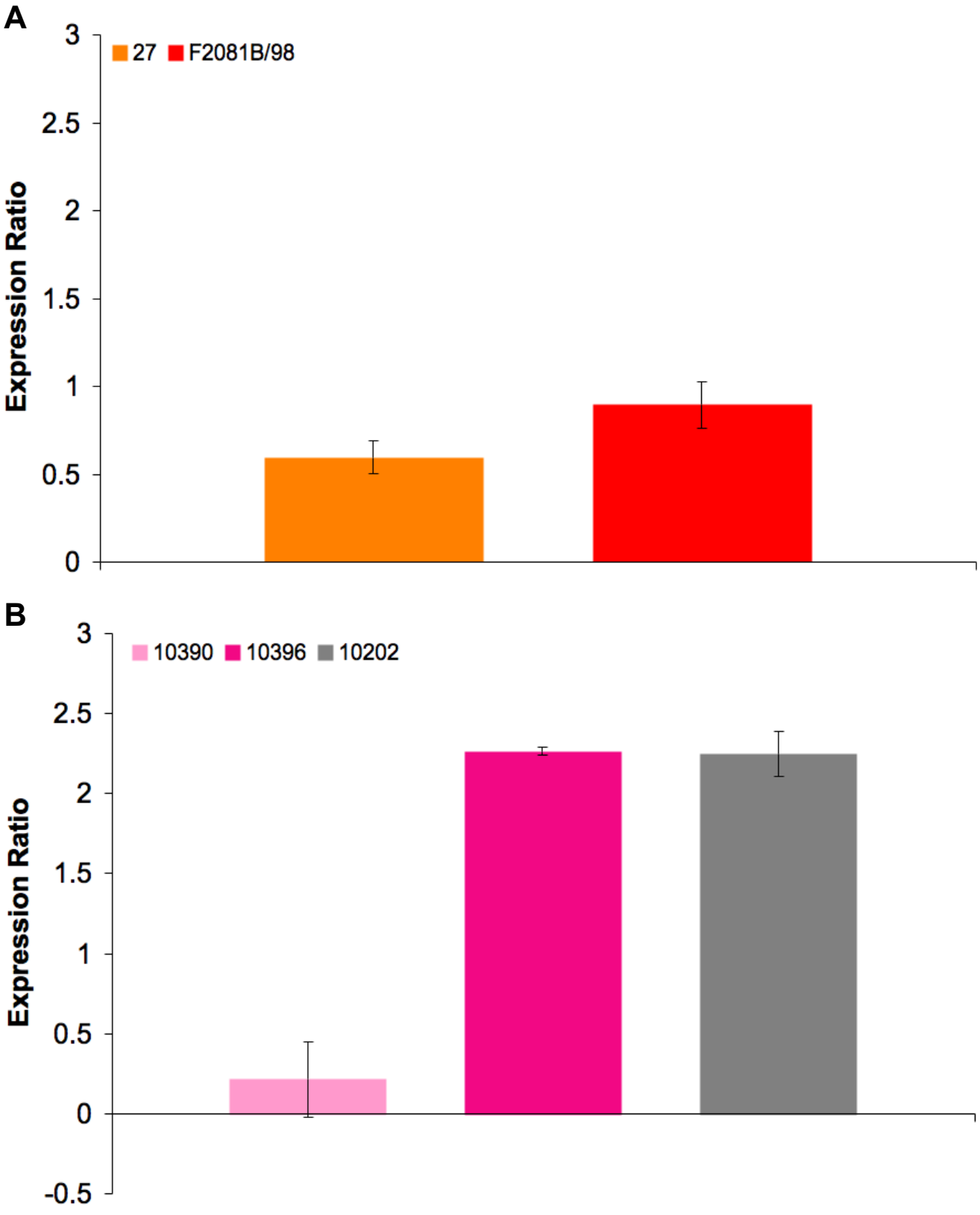


Figure 5.6.1 Expression ratios of *hblA* under simulated human GI tract conditions. **A**. *B. cereus* strains. **B.** *B. weihenstephanensis* strains

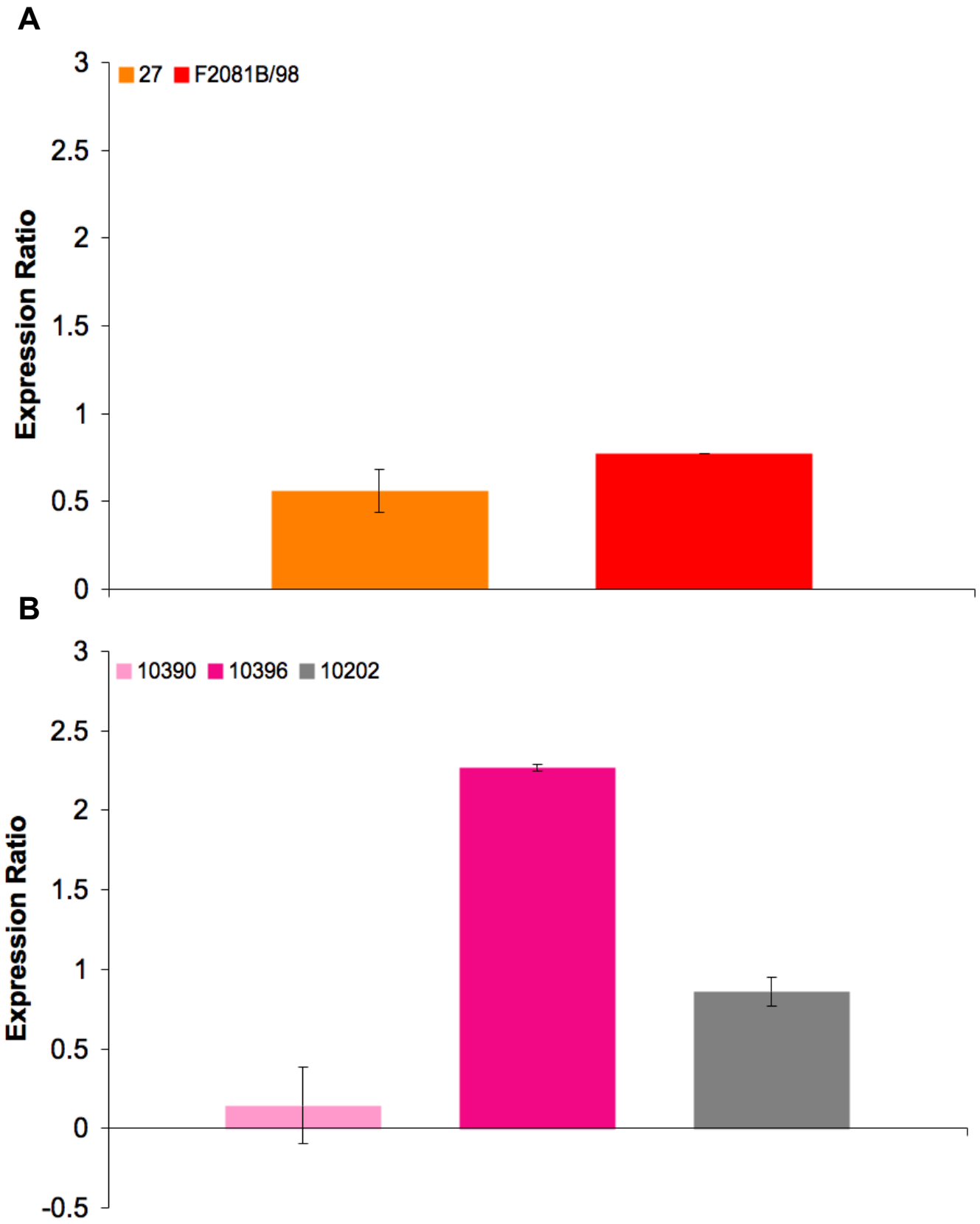


Figure 5.6.2 Expression ratios of *hblC* under simulated human GI tract conditions. **A**. *B. cereus* strains. **B.** *B. weihenstephanensis* strain

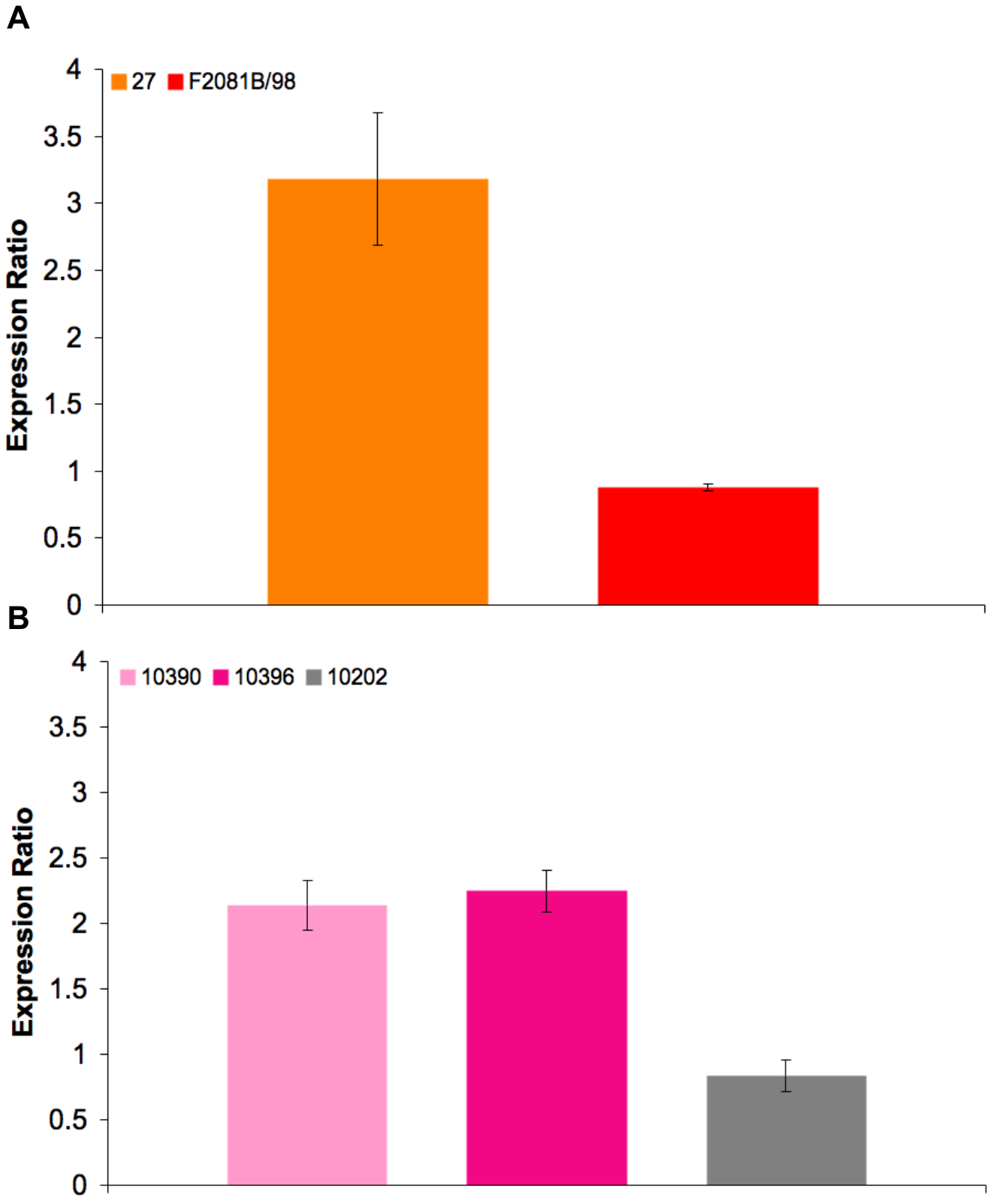


Figure 5.6.3 Expression ratios of *hblD* under simulated human GI tract conditions. **A**. *B. cereus* strains. **B.** *B. weihenstephanensis* strains

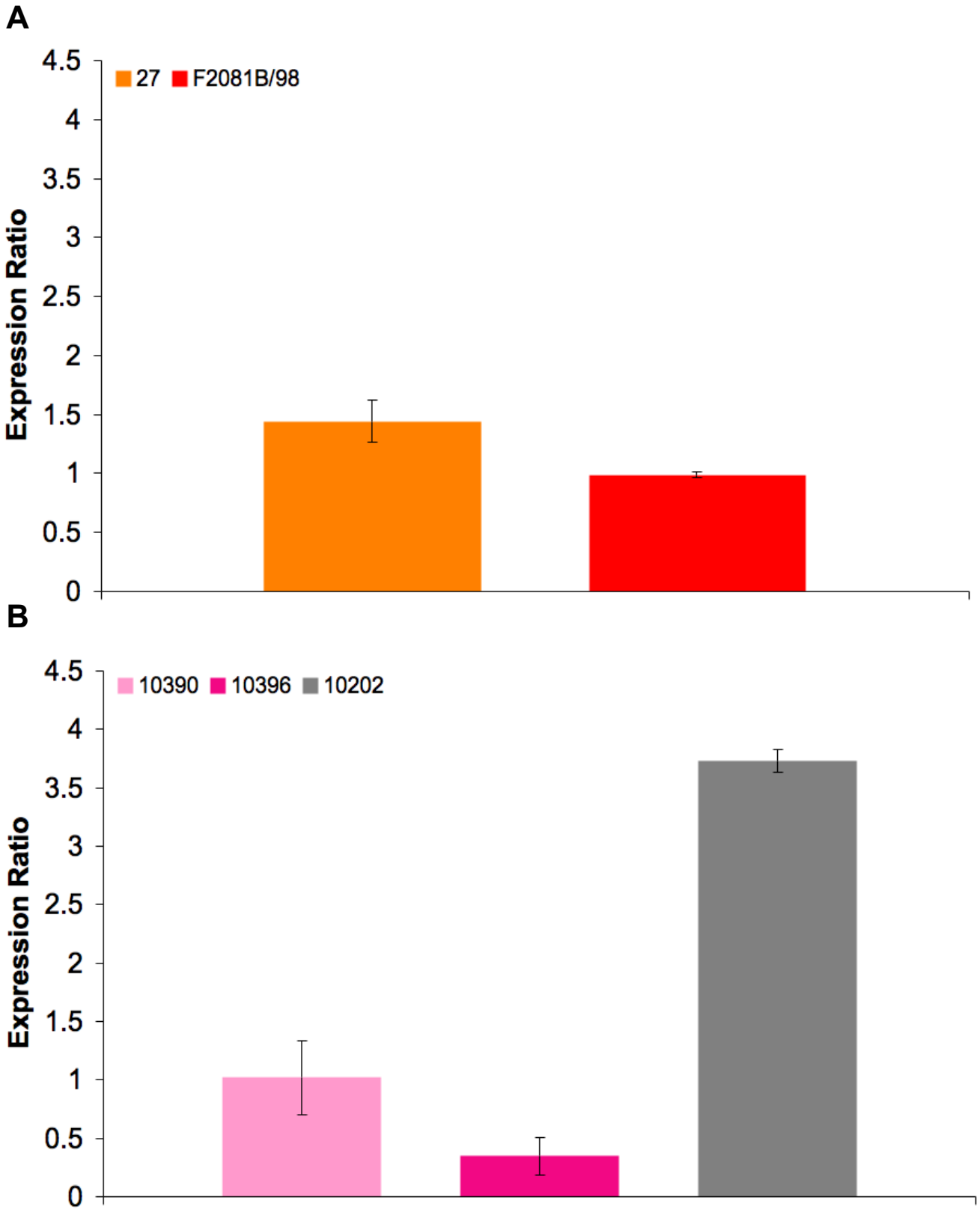


Figure 5.7.1 Expression ratios of *plcR* under simulated human GI tract conditions. **A**. *B. cereus* strains. **B.** *B. weihenstephanensis* strains

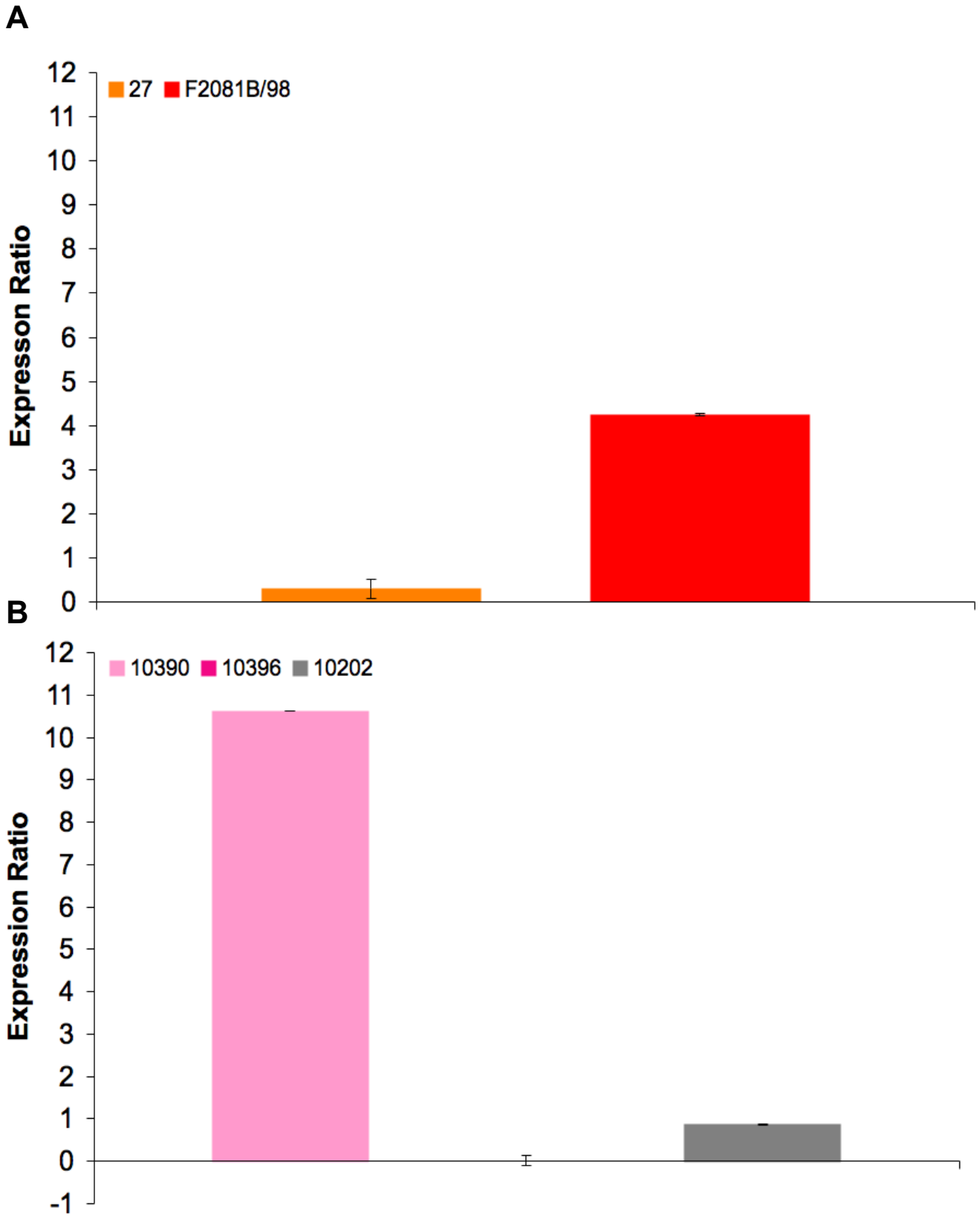


Figure 5.8.1 Expression ratios of *fur* under simulated human GI tract conditions. **A**. *B. cereus* strains. **B.** *B. weihenstephanensis* strains

5.10 Investigating the effects of a simulated human GI tract has on the expression of the molecular chaperone, *groEL*

GroEL is part of a two-protein chaperone complex, which works to ensure that under stressful conditions proteins are folded correctly increasing both the potential for survival and pathogenesis. Strain 27 showed a reduction in groEL expression when optimal and simulated conditions were compared (Figure 5.10.1A). In contrast F2081B/98 displayed a 2.7 fold increase in expression. Both *B. weihenstephanensis* strains 10390 and 10202 showed a slight increase in expression, 1.5 and 1.7 respectively (Figure 5.10.1B). Strain 10396 showed the same rate in expression as recorded under simulated conditions.

5.11 Verification of RT-PCR gene expression results using haemolysis assay and the Oxoid BCET-RPLA kit

Supernatant taken from each strain grown both under simulated and optimal conditions was incubated with sheep and horse red blood cells (RBC) to determine if strains were haemolytic. Sheep RBC contain greater amounts of sphingomyelin than horse RBC resulting in faster haemolysis as *B. cereus* can produce sphingomyelinase (Oda *et al*, 2010). Rapid (<3 minutes) haemolysis was witnessed when supernatant from simulated cultures was incubated with sheep RBC (data not shown). The same result was witnessed when supernatant from cultures grown under optimal conditions were incubated with sheep RBC (data not shown). As was described in the literature horse RBC took longer to lyse but all strains showed haemolytic activity under both conditions (data not shown). Although this assay does not inform to the actual cause of the rapid RBC haemolysis it does verify that all strains produce toxins possibly capable of lysing human RBC.

The Oxoid BCET-RPLA kit allows for the detection of nheA, one of the lytic proteins compromising the enterotoxin nhe, using a specific antibody. The degree of reaction is measured by monitoring the agglutination within the sample. No reaction is determined as – while the degree of agglutination is ranked from + to +++, with +++ showing the greatest level.

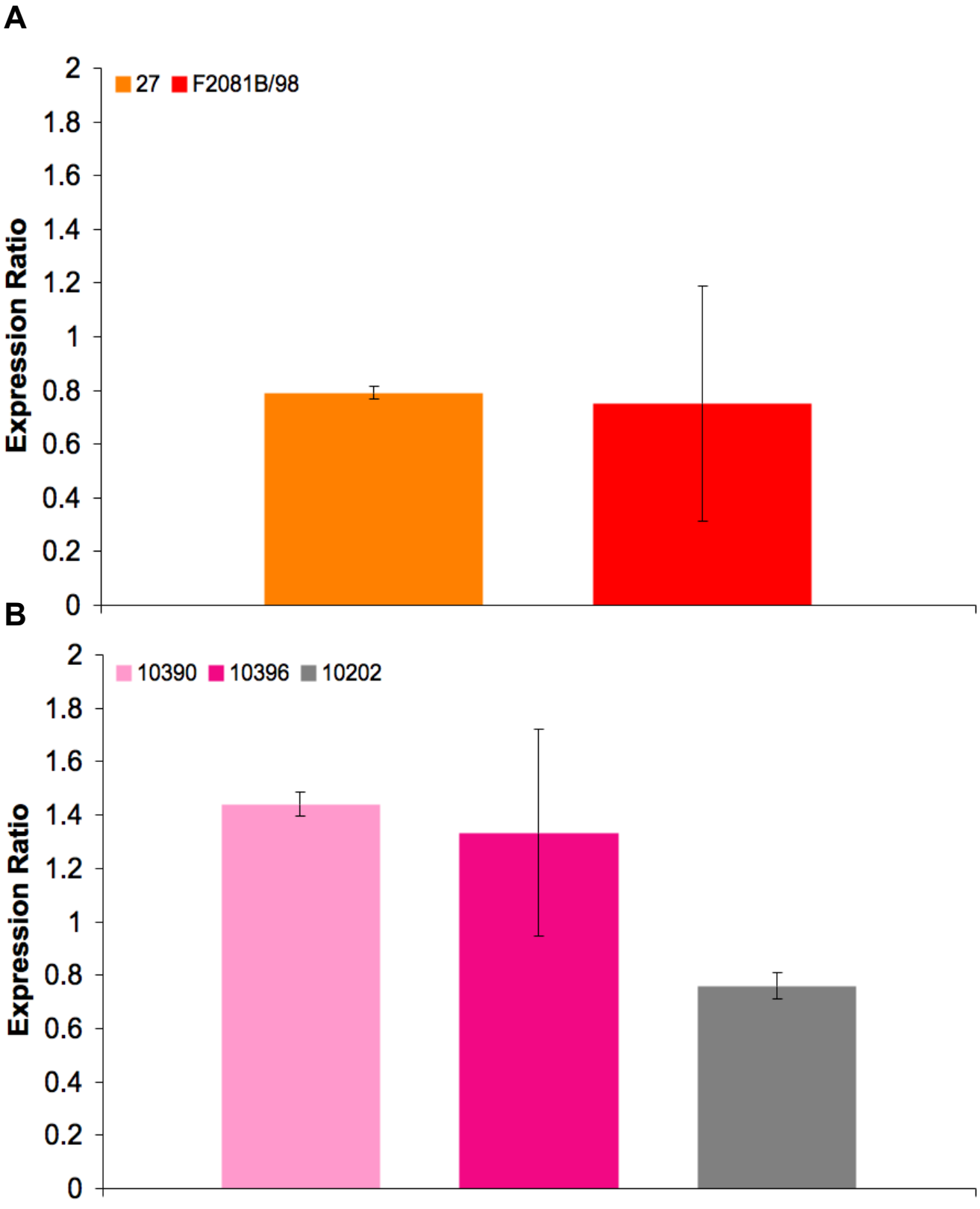


Figure 5.9.1 Expression ratios of *sigB* under simulated human GI tract conditions. **A**. *B. cereus* strains. **B.** *B. weihenstephanensis* strains

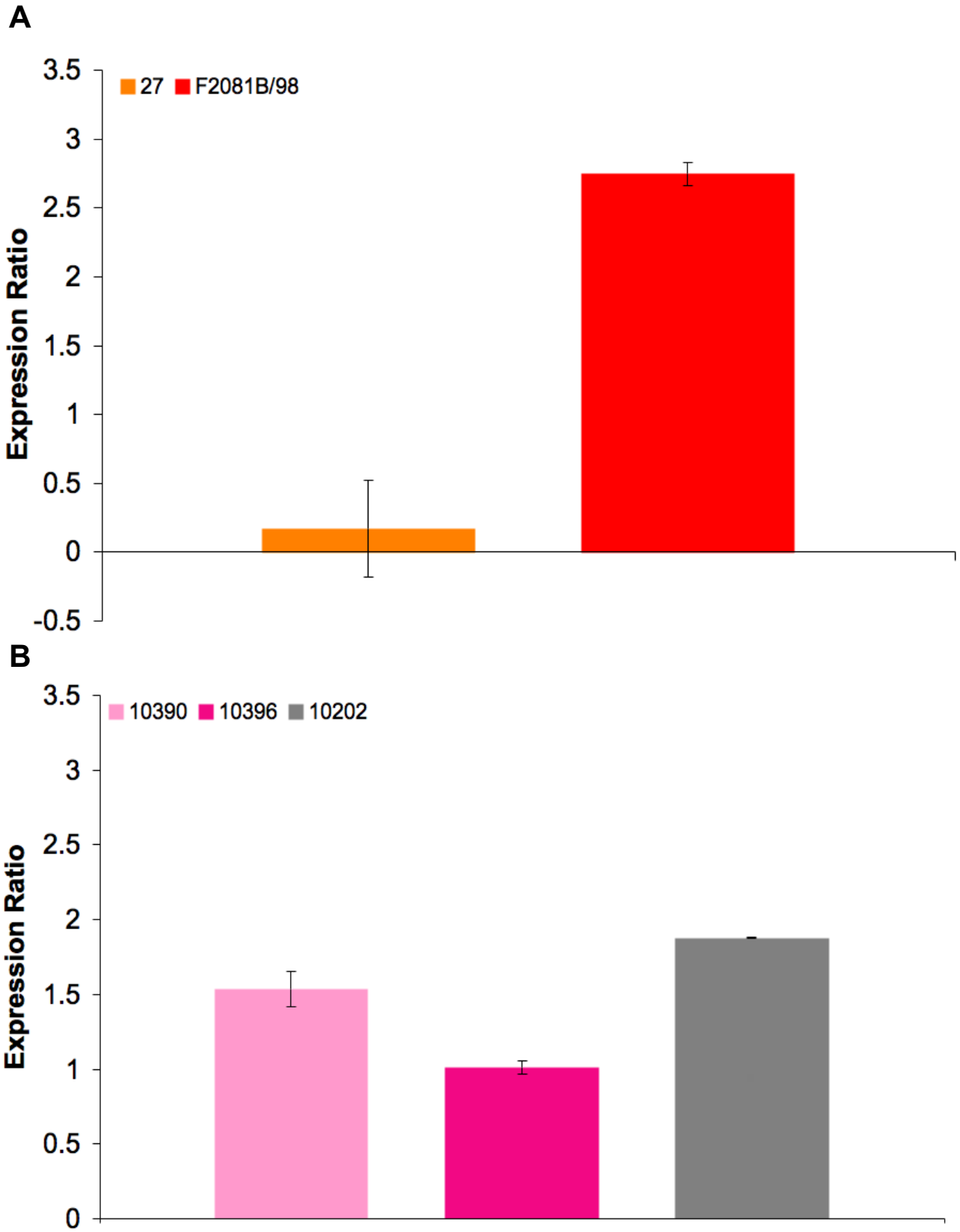


Figure 5.10.1 Expression ratios of *groEL* under simulated human GI tract conditions. **A**. *B. cereus* strains. **B.** *B. weihenstephanensis* strains

All strains tested, grown under simulated GI tract conditions displayed the maximum agglutination, +++ (data not shown). These results corroborated previous findings that both *B. cereus* and *B. weihenstephanensis* strains can produce nheA under simulated conditions although in some cases gene expression is reduced. Similar results were found when supernatant was taken from strains grown under optimal conditions and tested. Taken at face value these results have shown that under simulated conditions both *B. cereus* and *B. weihenstephanensis* strains can produce components of toxins involved in diarrhoeal food poisoning.

5.12 Discussion

The results in this chapter show that both *B. cereus* and *B. weihenstephanensis* strains contain some or all of the virulence factors associated with diarrhoeal food poisoning. *B. weihenstephanensis* strains all expressed toxin genes and further virulence genes, indicating that it could be as pathogenic as *B. cereus*. Under conditions mimicking the human GI tract specific virulence genes were shown to be expressed and in some strains, significantly up regulated. No pattern in the expression of *hbl* or *nhe* specific genes was witnessed between the individual *B. cereus* and B. *weihenstephanensis* strains.

The removal of residual genomic/plasmid DNA is required for a successful RT-PCR experiment as this allows for the accurate recognition of specific RNA sequences. This was tackled in this study by performing on column digestion of any DNA present along with melt curve analysis after every RT-PCR experiment to ensure no contamination. Being able to monitor gene expression under a host of different environmental conditions is one of the key uses of RT-PCR along with allowing for rapid and sensitive method of assaying strains virulence potential (Gore *et al*, 2003).

Prior to RT-PCR various culture assays and animal tests have been employed to enable the detection and characterisation of diarrhoeal enterotoxins. Fluid accumulation in rabbit ileal loops along with vascular permeability assays, skin reactions, cytotoxicity assays using Vero cell and $CaCo_2$ cells are all used to determine strain toxicity (Kramer & Gilbert, 1984; Kramer & Gilbert,

1989; Jackson, 1993; Lund & Granum, 1996; Pruβ *et al*, 1999; Hardy *et al*, 2001; From *et al*, 2005). More recently two companies have developed kits, which allow for the rapid identification of hbl (Tecra) and nhe (Oxoid) toxins. These kits both contain antibodies designed to react with one protein produced from each toxin. Results are displayed as either positive or negative however these tests have however been shown to be inaccurate under certain conditions and thus better methods of toxin detection are required (Beecher & Wong, 1994; Day *et al*, 1994; Buchanan & Schultz 1994; Fletcher & Logan, 1999). Conventional PCR has been used to determine the presence of toxin genes within isolated strains. The detection of a toxin gene however does not automatically define in the pathogenicity of a particular strain (Stenfors *et al*, 2008). RT-PCR techniques are used to determine the number of contaminating cells within a food source (Martinez-Blanch *et al*, 2009). Investigating the effect the human GI tract has on the expression of virulence genes could help determine how *B. cereus* and potentially *B. weihenstephanensis* become pathogenic within this environment.

The ability of *B. cereus* and *B. weihenstephanensis* to adapt to their surroundings is crucial to their survival and growth. The expression of key genes can confer resistance to a wide variety of stress factors, some of which could be encountered in the human GI tract (Hecker & Volker 1998). As one of the first alternative sigma factors, *sigB* was found in *B. subtilis* in 1979 (Haldenwang & Losick, 1979; Cotter & Hill, 2003). Subsequently in 1993 it was found to be the regulator of a stress/starvation regulon, which includes a significant number of general stress response genes (GSR) (Haldenwang, 1995; Hecker *et al*, 1996; Price *et al*, 2001). *SigB* has been shown to mediate a role in the stress response in the pathogens, *L. montocytogenes, S. aureus and B. cereus*. There has even been a putative link between *sigB* and the psychrotolerance witnessed in some *B. cereus/B. weihenstephanensis* strains (de Vries *et al*, 2004; van Schaik *et al*, 2004). This could be of considerable interest to food producers as chilled temperatures are used in the preservation of minimally processed foods (van Schaik & Abee, 2005). In this study *B. weihenstephanensis* strains 10390 and 10396 showed a slight increase in the expression of *sigB* compared to optimal conditions. It is possible that these strains are using the GSR mediated by *sigB* to help their

survival and growth within the GI tract simulation. Strains 10202, 27 and F2081B/98 however showed no change possibly indicating that these strains were not as affected by the conditions, or that another mechanism was employed to minimise the stress damage. In order to determine whether these hypotheses could be true, further experimentation is required.

Hbl and nhe enterotoxins are each composed of three genes, encoding three proteins. *HblCDA* and *nheABC* genes are grouped together in two-polycistronic operons that are believed to be under the control of *plcR* (Clair *et al*, 2010). The presence of an inverted repeat between *nheB/C* possibly acts as a translational repressor, which results in the production of less *nheC* in comparison to the other two proteins (Granum O'Sullivan, & Lund, 1999; Linback *et al*, 2004; Stenfors *et al* 2008; Sensi & Ghelardi, 2010). Although the mode of action that these toxins use to exert their effects are different around 40% amino acid identity between the individual *hbl* and *nhe* proteins has been shown. Maximal toxicity results when all components of each toxin are present. Moravek *et al* (2006) noted that there was an extremely broad variation in the level of enterotoxins produced by individual *B. cereus* strains. This difference between strains was also recorded in this study, with varying expression ratios found in each strain, resulting in no discernable pattern.

Gore *et al* (2003) used nucleic acid sequence based amplification (NASBA) to monitor the expression of *hblC* in a *B. cereus* strain grown within a contaminated model food system. They discovered that maximal *hblC* expression appeared after 15 hours, which reflected a cell load of $6.0x10^7$ CFU/ml (Gore *et al*, 2003). Similar cell concentrations were found after 8 hours in the GI tract simulation within this study and strain 10390 showed a 2.3-fold increase in *hblC* expression relative to growth in BHI at 30^0C. Gore *et al* (2003) also showed that *hblC* expression remained constant for a period of 35 hours before declining; indicating once *B. cereus* or *B. weihenstephanensis* begin to produce the *hbl* toxin its expression is continued for a significant amount of time (Gore *et al*, 2003). Also investigated was a food isolated strain *B. circulans;* it was shown that maximal *hblC* expression occurred when the cell density reached $9.8x10^4$ CFU/ml. This figure is only marginally lower than the generally considered infectious range of 10^5-

10^8xCFU/ml. It is possible that food poisoning strains, being more virulent than food-associated strains can survive within the human GI tract and thus grow quicker and produce large amounts of toxins (Gore *et al*, 2003). Controlling cellular iron levels along with various other substances is imperative to the survival of any bacterial cell. *Fur*, the ferric iron uptake regulator has been linked to the virulence of *B. cereus* strains (Harvie *et al*, 2005). In both strains F2081B/98 and *B. weihenstephanensis* strain 10390 fur expression was significantly increased. In strain 10390 a 10.5 fold difference in comparison to optimal conditions was recorded. It is possible in the simulated GI tract that there is a build up of intracellular iron within these two strains accounting for the increased production of fur. It is also possible as Harvie *et al* concluded that *fur* is involved in the regulation of key virulence factors including *hbl* and *nhe* (Harvie *et al*, 2005). Both F2081B/98 and 10390 showed increased transcription of the genes making up *nhe* and *hbl* and these changes could potentially be attributed to *fur*. In order to determine the reason behind such a vast up-regulation of *fur*, further work is needed.

The primary regulator of *B. cereus* virulence factors however is *plcR*. It has been shown to mediate the transcriptional control of over 100 genes (Lereclus *et al*, 1996; Goher *et al*, 2008; Aggaise et al, 1999).

Lechner *et al* (1996) showed that phosphatidylinositol-specific-phospholipase C (*plcA*) in *B. thuringiensis* was under the control of a transcriptional regulator named *plcR*. Further characterisation of *plcR* showed that this gene is a global regulator of virulence genes within *B. thuringiensis* (Aggaise *et al,* 1999). Previous work has illustrated the high genetic similarity between *B. thuringiensis* and *B. cereus*, making it very difficult to discriminate between them. In order to determine whether this regulator was present in *B. cereus* southern blots using the sequenced *B. thuringiensis plcR* were created and used to probe various *B. cereus* strains (Agaisse *et al*, 1999). *B. thuringiensis* and *B. anthracis* toxin genes are maintained on plasmids, separate from genomic DNA. *PlcR* however was shown to map to several areas within one section of the *B. cereus* chromosome, dismissing the idea of a pathogenicity island where all virulence genes are located (Agaisse *et al*, 1999). A highly conserved palindromic sequence ($TATGNAN_4TNCATA$) acts as the

recognition sequence within the *plcR* promoter (Aggaise *et al*, 1999). This area, known as the *plcR*-box, was used as a marker to determine the presence of other *plcR* controlled genes and it was discovered that over 100 genes contained this sequence (Aggaise et al, 1999).

Subsequent proteomic and gene knockout experiments showed that around 80% of extracellular proteins manufactured by *B. cereus* during stationary phase are controlled by *plcR* (Bouilluant *et al,* 2003). These proteins included phospholipases, hemolysins (hbl/nhe), cytotoxins (cytK), and proteases, which are all known virulence factors (Gohar *et al*, 2003). There has however been little work done on determining *plcR* expression under conditions mimicking the human GI tract. In *B. weihenstephanensis* strain 10202, plcR expression was up-regulated. The activity of *plcR* is conditional on the actions of a secreted signalling peptide known as *papR* (Delerck *et al*, 2002). When the intracellular concentration of *papR* increases reaching a certain threshold, it interacts with *plcR* forming a complex that binds to the *plcR* box increasing the expression of *plcR*, *papR* and various virulence factors (Delerck *et al*, 2002). Along with autoregulation, the sporulation gene *spo0A* also plays a role in the expression of *plcR*. During exponential growth *spo0A* binds to either side of *plcR* and represses its expression however once cells enter stationary phase the repression is lifted as *spo0A* is required (Gohar *et al*, 2002). It is possible that the increase in *plcR* expression recorded could be due to *B. weihenstephanensis* cells just entering stationary phase thus requiring the upregulation of *plcR* and *plcR* regulated genes. In order to determine if this hypothesis is possible a time course of *plcR* expression through the dual GI tract simulation and beyond needs to be investigated. This study shows the first evidence of *plcR* expression under GI tract simulated conditions and highlighted that that these surroundings had little effect on *plcR* expression. This is also the first work to demonstrate the actual expression of *plcR* within *B. weihenstephanensis* strains and not just confirm the presence of the gene through conventional PCR. A ΔplcR strain showed a significant decrease in pathogenicity, however it was not abolished (Gohar *et al*, 2002). Clair *et al* (2010) showed evidence suggesting that neither the *plcR* quorum sensing system nor *plcR* itself was enough to account for the virulence seen within the *B. cereus* family.

Very recently, expression of *plcR*, and *hbl* has been shown to be controlled by *ResD Fnr* and *CcpA* (Senesi & Ghelardi, 2010). *ResD* is one part of the two-component response regulator *ResDE*, which modifies *hbl* expression by binding to the promoter region of *hbl's* operon (Senesi & Ghelardi, 2010). *Fnr* also bind to the promoter region while at specific cis regions *ccpA* binds to DNA subsequently modifying transcription (Senesi & Ghelardi, 2010). These same transcriptional regulators have also been shown to influence *nhe* expression. Therefore the production of *hbl* and *nhe* is very tightly regulated within *B. cereus* and potentially *B. weihenstephanensis*. Duport *et al* (2006) reported that under conditions of anaerobiosis, and full aerobiosis, mutations in *resDE* resulted in the upregulation of both *nhe* and *hbl* (Duport *et al,* 2006). Through resDE a link is made between the cell's redox condition and the expression of enterotoxins (Duport *et al*, 2006). Due to the ability of *resDE* to control the expression of toxins in oxygen limited conditions it is possible that within a human small intestine *resDE* could help *B. cereus* to adapt and become toxigenic. Laouami *et al* (2011) linked L-lactate dehydrogenase A (*LdhA*) with fermentative growth and toxinogenesis showing that a mutation in *ldhA* resulted in the down regulation of putative virulence factors in *B. cereus* including *nhe* (Laouami *et al*, 2011). Further investigations revealed that together *resDE*, *fnr* and *ldhA* regulate *nhe* expression, not *plcR* as previously thought. This complex of transcriptional regulators and *ldhA* could work together to control further virulence factors because when *ldhA* was mutated pathogenesis decreased (Laouami *et al*, 2011). Within the work conducted here the expression of *resDE/Fnr/CcpA/ldhA* were not investigated however their ability to control the expression of *hbl* and *nhe* could explain the increased expression of nheA, nheB, *nheC, hblA*, *hblC and hblD* in both *B. cereus* and *B. weihenstephanensis* strains.

Previously it is was understood that *plcR* was the only regulator of virulence genes within the *B. cereus* family (apart from *B. anthracis*) now the management of these genes has become significantly more complicated as various levels of control and controllers are discovered. A considerable amount of work remains to be completed investigating gene expression under conditions simulating the human GI tract. This work has however shown that the conditions used here to simulate the GI tract did not negatively effect the

expression of any virulence gene tested, although some expression ratios were low. It is possible that this is also the case in vivo where a small number of contaminating cells can trigger an infection. It was also shown that not only can *B. weihenstephanensis* express the same toxins, virulence factor and transcriptional regulators as *B. cereus* in some cases their expression were greatly increased. This work continues to show that *B. weihenstephanensis* strains contaminating foodstuffs are of great concern as they could prove to be just as pathogenic as *B. cereus*.

Chapter Six
Final Conclusions and Future Work

6.0 Final Conclusions and Future Work

The objectives of this thesis were to determine and compare the effect that passage through conditions simulating the human gastro intestinal tract has on the growth and survival of vegetative cells and spores of *B. cereus* and psychrotrophic *B. weihenstephanensis.* Furthermore the potential pathogenicity of *B. weihenstephanensis* and *B. cereus* under the same conditions was also compared. The knowledge obtained could then be employed to inform on risk assessment of relevant manufactured chilled food product safety.

6.1 The route to diarrhoeal food poisoning is caused by the presence of spores within foodstuff

The primary niche of *B. cereus* and *B. weihenstephanensis* vegetative cells and spores is soil. They are regularly isolated from raw food ingredients alongside contaminated food processing machinery. The presence of spore and vegetative cells within food products poses a risk to human health if consumed in high enough numbers. However, the effect that passage through the human GI tract has on cell and spore viability is unclear. Four *B. cereus* and three *B. weihenstephanensis* strains were used in this work to model the growth and survival of cells and spores under conditions simulating those present in the human GI tract. *B. cereus* and *B. weihenstephanensis* cells were shown to lose viability quickly in the simulated stomach environment at all pH values tested (1, 2, and 3). Spore viability was also negatively affected with a 4-log reduction in spore numbers recorded after four hours for strains of both species. These results indicate that in order for spores to pass through the stomach to the small intestine a large concentration is required.

Conditions found within the small intestine limit the effect of invading bacterial pathogens (Tam *et al*, 2006; Kristiffersen *et al*, 2007). The small intestinal simulation in this study focused purely on the effects of bile acid and digestive enzymes. This environment had no detrimental effect on the viability of spores or vegetative cells. However, in each strain tested spore germination proceeded slowly with only small increases in CFU recorded after 4 hours. In contrast vegetative cells from each strain were able to proliferate quickly increasing by 10^4 CFU/ml after 4 hours. Pathogenic strains like those used in

this study show a greater level of resistance to the effects of bile. This immunity could be linked to the production of a bile salt hydrolase (Bsh) (Paramithiotis *et al*, 2006, Fang *et al*, 2009). This enzyme limits the negative impact bile can have on vegetative cells. At this time it is unclear whether the strains used in this study produce the protein required to convey this protection.

The results gained and depicted in figure 6.1 suggest that any *B. cereus* or *B. weihenstephanensis* vegetative cells ingested would die in the stomach. Spores, if ingested in large enough numbers, do survive and are able to germinate and once in vegetative form, rapidly proliferate in the small intestine. Based on these results it could be postulated that the vegetative cells of *B. cereus* and *B. weihenstephanensis* pose little threat if consumed, however, spores if present in high numbers can survive passage through the stomach and grow in the small intestine.

6.2 There is no difference in the survival characteristics of cells and spores between *B. cereus* and *B. weihenstephanensis* strains

B. weihenstephanensis is a newly described member of the *B. cereus* family (Lechner *et al*, 1999). Identified as psychrotrophic it can grow at refrigerated temperatures (<7^{0}C). There is little data on their ability to survive under conditions that simulate the human GI tract. The work conducted here showed that there was no difference in recorded spore survival between *B. cereus* isolates and *B. weihenstephanensis* strains. *B. weihenstephanensis* vegetative cells suffer a similar fate to *B. cereus* and therefore are killed quickly in the acidic stomach. Spores however were shown to survive this environment and go on to germinate in the small intestine.

6.3 All strains were shown to produce a variety of virulence factors under both laboratory and simulated GI tract culture conditions

The ability of *B. cereus* and *B. weihenstephanensis* cells to express virulence factors under lab culture conditions and conditions simulating those present in

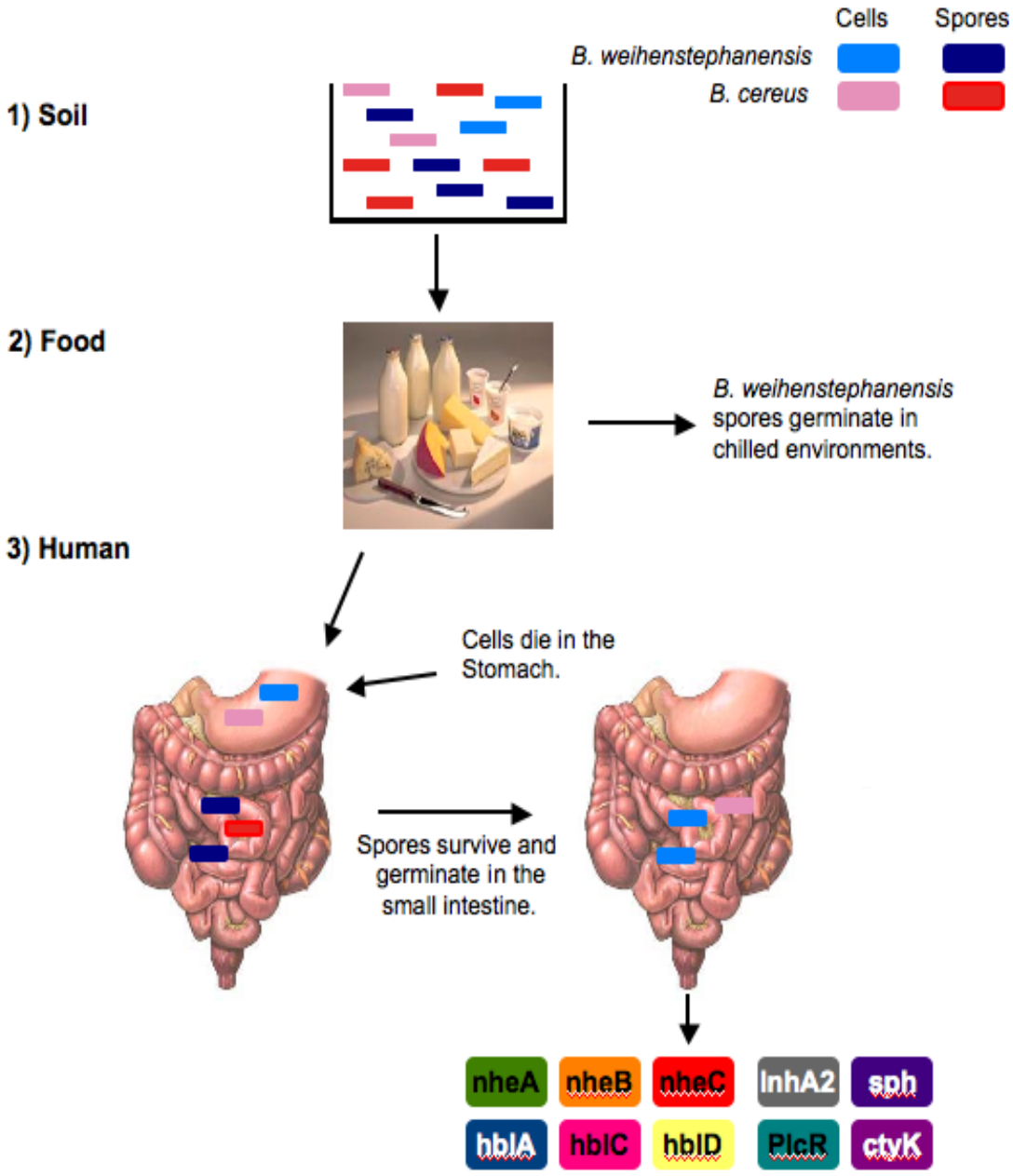


Figure 6.1: Routes to Diarrhoeal Food Poisoning. This model, based on the results gained during this thesis, illustrates the fate of ingested *B. cereus* or *B. weihenstephanensis* cells and spores when they enter the human GI tract. 1) Spores and cells from both species can be found in soil which in turn results in their isolation from many food products. 2) *B. weihenstephanensis* spores can germinate in chilled conditions and the resulting vegetative cells could lead to spoilage and potentially emetic food poisoning. 3) Cells die quickly when they encounter the acidic stomach suggesting *B. weihenstephanensis* and *B. cereus* cells are not a threat to consumers. Spores however can survive and pass to the small intestine where they germinate slowly, eventually producing virulence factors associated with dirarrhoeal food poisoning.

the human GI tract were investigated. Virulence factors including diarrhoeal food poisoning toxins were expressed and secreted under both conditions as shown by tandem mass spectrometry and western blotting. Tandem mass spectrometry highlighted that strain supernatant predominately consisted of virulence factors including proteases, haemolysins, cytotoxins, flagellin, phospholipids and sphingomyelinase. Within the supernatant of *B. weihenstephanensis* strain 10202 the potent cytotoxin cytK was detected. Although the presence of the *cytK* gene had been confirmed in *B. weihenstephanensis* strains previously this was to our knowledge the first time the cytotoxin had been found in *B. weihenstephanensis* supernatant.

Polyclonal antibodies raised against the haemolysin BL proteins were used to show that under both laboratory and simulated GI tract conditions these proteins were present. However, significantly less protein was detected when supernatants of strains grown under simulated GI tract conditions were tested in comparison to laboratory conditions. It is possible that gastric proteases added to the media to simulate the human GI tract could have degraded the secreted hbl proteins, possibly explaining the reduced amount of protein detected.

Conventional PCR was used to confirm the presence of genes known to confer virulence within *B. cereus* and *B. weihenstephanensis*. Each strain was shown to possess the genes required to produce both *nhe* and *hbl* diarrhoeal toxins along with other known virulence factors. In order to determine if the simulated GI tract environment was negatively effecting gene expression, RT-PCR was employed. It was shown that under simulated conditions genes were both up and down regulated when compared to expression levels under laboratory conditions depending on the strain. Notably *B. weihenstephanensis* strains were shown to up-regulate the expression of *nhe* and *hbl* along with transcriptional regulators *plcR* and *fur*.

6.4 There was no discernible difference in the type or degree of virulence factor expression between *B. cereus* and *B. weihenstephanensis* strains under conditions simulating the human GI tract

PCR studies have shown that *B. weihenstephanensis* strains possess all the virulence factors associated with diarrhoeal food poisoning. The presence of a gene, however, does not guarantee its expression. The work shown here highlights the ability of three *B. weihenstephanensis* strains to secrete the virulence factors associated with diarrhoeal poisoning. These results along with those from the previous chapter illustrate the pathogenic potential of *B. weihenstephanensis*

The work carried out within this thesis is novel showing the ability of *B. weihenstephanensis* to survive a simulated human GI tract and express virulence factors in this environment. This work has also highlighted that vegetative cells die quickly in the stomach environment and thus if psychrotrophic *B. weihenstephanensis* spore germinate in a chilled environment it is likely that they would be killed once ingested. This suggests that although *B. weihenstephanensis* could cause diarrhoeal food poisoning it is more likely that if present within food as vegetative cells, it could contribute to spoilage or possibly even emetic food poisoning. This work has expanded our knowledge about *B. weihenstephanensis* and confirmed its ability to become a serious human pathogen. The results gained here will help the food industry design appropriate risk assessments for *B. weihenstephanensis*.

6.5 Future Work

The simulation constructed in this study was crude and only determined the effect of acidic pH, pepsin, bile acids and pancreatin on selected cells and spore concentrations. Developing a better method to mimic the surroundings of a human GI tract would be advantageous in determining the true effect that this environment has on growth and viability. One of the biggest barriers to invading pathogens like *B. cereus/B. weihenstephanensis* is the presence of natural gut flora. These were not involved in this simulation because of the inherent difficultly in representing every species and difficulty in discriminating species microbiologically to determine growth and survival. The gut flora also varies from person to person based on age, ethnicity, diet and geographical location. Model GI tracts including bacteria have been constructed; however, they are primarily used to determine the effectiveness of probiotics (Molly *et al*, 1993). The SHIME (simulated human intestinal microbial ecosystem) and

TNO intestinal model are computer-controlled systems, which incorporate the majority of conditions found within a human stomach and small intestine (Molly *et al*, 1993; Minekus *et al*, 1995; Mainville *et al*, 2005; Maathuis *et al*, 2010). There are however some inherent difficulties; numerous systems would be required to test a cross section of society as well as a different reaction for each species tested. Despite these drawbacks developing a better test system to model the human GI tract would provide a greater insight into how *B. cereus/B. wiehenstephanensis* cells and spore remain viable and cause disease.

Mammalian cell models that allow valuable information on *B. cereus* and *B. weihenstephanensis* virulence are not easily available (Arnsen *et al*, 2011). Insect larvae from *Galleria mellonella* can be used to analyse *B. cereus* (Salamitou *et al*, 2000; Fedhila *et al*, 2002; Bouillant *et al* 2005). Arnsen *et al* (2011) used this insect model to determine the pathogenesis of *B. cereus* and *B. weihenstephanensis* strains at 15^0c and 37^0c. Overall they found that *B. weihenstephanensis* strains were significantly less virulent at 37^0c than 15^0c indicating that their presence within REPFED (refrigerated processed food of extended durability) foods would not constitute a danger to the public in term of diarrhoeal food poisoning (Arnsen *et al*, 2011). Although strain cytotoxicity in a mammalian model was never determined during the work presented in this thesis, *B. weihenstephanensis* strains were shown to produce virulence factors including toxins at 37^0c through mass spectrometry. The expression of some toxin genes within *B. weihenstephanensis* strains were also shown to be up regulated in conditions simulating the human GI tract at 37^0c. It maybe useful, due to the disparity of the results, to perform this assay to determine if the strains used in this study are cytotoxic in the *G. mellonella* insect model.

It is possible that fluorescent proteins GFP (green fluorescent protein)/CFP (cyan fluorescent protein)/DsRed (red fluorescent protein)/YFP (yellow fluorescent protein) could be used to tag each gene constituting the *hbl/nhe* toxins. The supernatant from cultured strains could then be incubated with Vero or Caco-2 cells. Using fluorescent microscopy the action of all diarrhoeal food poisoning toxins could be monitored under conditions simulating those found in the host. This would not only allow for a greater understanding of

how these toxins act and bind together but could also give quantitative details on *hbl/nhe* expression levels. Lewis & Marston (1999) developed plasmid vectors containing GFP, which were shown to integrate through either a single or double crossover event into the chromosome of *B. subtilis*. Using these vectors to tag individual hbl/nhe proteins would allow their actions to be monitored in real time providing greater information on how these proteins interact to form potent toxins.

Further molecular experiments could be performed to monitor gene expression by utilising microarrays. Biochips and microarray technology have been developed to quickly determine the pathogenic nature of food isolate strains (Lucchini Thompson & Hinton, 2001; Gabig-Ciminska *et al*, 2005; Lui *et al*, 2007). Developing the real time PCR experiments further and using DNA micro-arrays could result in a wealth of information about the expression of all genes within *B. cereus/B. weihenstephanensis* under varying conditions. Sergeev *et al* (2006) constructed an array, which used 18 virulence genes as markers to identify *B. cereus* group isolates. This multiplex allowed detection of 96 traits in 7 different samples, in triplicate to be monitored on one slide (Sergeev *et al*, 2006). Using microarrays to determine pathogenic *B. cereus/B. weihenstephanensis* strains offers a rapid detection method to food producers. Greater information could be gained by individually investigating the transcriptome of *B. cereus* and *B. weihenstephanensis* strains. Cases of diarrhoeal food poisoning occur via the expression of a host of virulence factors that together constitute a complex gene expression network. Therefore enterotoxin expression and subsequent pathogenesis is strain dependent. Understanding fully the mechanism by which *B. cereus* and potentially *B. weihenstephanensis* cause food poisoning would be greatly beneficial (Wijnands *et al*, 2002).

Agaisse, H., Gominet, M., Okstad, O.A., Kolsto, A.B. and Lereclus, D. (1999) PlcR is a Pleiotropic Regulator of Extracellular Virulence Factor Gene Expression in *Bacillus thuringiensis*. *Molecular Microbiology* **32**, 1043-1053.

Aggarwal, K., Choe, H.L., and Lee, K.H. (2006) Shotgun Proteomics using the iTRAQ Isobaric Tags. *Briefings in Functional Genomics and Proteomics* **5**: 112-120

Andersen Borge, G.I., Skeie, M., Sorhaug, T., Langsrud, T. and Granum, P.E. (2001) Growth and Toxin Profiles of *Bacillus cereus* Isolated from Different Food Sources. *International Journal of Food Microbiology* **69**, 237-246.

Anderson, I., Sorokin, A., Kapatral, V., Reznik G., Bhattacharya, A., Mikhaliova, N., Burd, H., Joukov, V., Kaznadzey, D., Walunas, T., Larsen, N., Pusch, G., Liolios, K., Grechkin, Y., Lapidus, A., Goltsman, E., Chu, L., Fonstein, M., Ehrlich, S.D., Overbeek, R., Kyrpides, N., and Ivanova, N. (2005) Comparative Genome Analysis of Bacillus cereus Group Genomes with *Bacillus subtilis*. *FEMS Microbiology Letters* **250**, 175-184.

Andersson, A., Granum, P.E., and Ronner (1998) The Adhesion of *Bacillus cereus* Spores to Epithelial Cells Might be an Additional Virulence Mechanism. *International Journal of Food Microbiology* **39**, 93-99.

Andersson, A., Ronner, U. and Granum, P. E. (1995) What Problems Does the Food Industry have with the Spore-Forming Pathogens *Bacillus cereus* and *Clostridium perfringens*? *International Journal of Food Microbiology* **28**, 145-55.

Andreeva, Z.I., Nesterenko, V.F., Fomkin, M.G., Ternovsky, V.I., Suzina, N.E., Bakulina, A.Y., Solonin, A.S. and Sineva, E.V. (2007) The Properties of *Bacillus cereus* Hemolysin II Pore Depends on Environmental Conditions. *Biochimica et Biophysica Acta* **1768**, 253-263.

Antelmann, H., Schmid, R., and Hecker, M. (1997) The NAD Synthetast NadE (OutB) of *Bacillus subtilis* is a σ^B –Dependent General Stress Protein. *FEMS Microbiology Letters* **153**, 405-409

Arnsen, L.S., Granum, P.E., Buisson, C., Bohlin, J., and Nielsen-LeRoux, C. (2011) Using an Insect Model to Access Correlation Between Temperature and Virulence in *Bacillus weihenstephanensis* and *Bacillus cereus*. *FEMS Microbiology Letters* **317**, 196-202

Ash, C., Farrow, J.A.E., Dorsch, M., Stackebrandt, E., Collins, M. (1991) Comparative Analysis of *Bacillus anthracis*, *Bacillus cereus*, and Related Species on the Basis of Reverse Transcriptase Sequencing of 16S rRNA. *International Journal of Systematic Bacteriology* **41**, 342-346.

Bagyan,I. and Setlow, P. (2002) Localization of the Cortex Lytic Enzymer CwlJ in Spores of *Bacillus subtillis*. *Journal of Bacteriology* **184**, 1219-1224.

Baida, G.E. and Kuzmin, N.P. (1996) Mechanism of Action of Hemolysin III from *Bacillus cereus*. *Biochimica et Biophysica Acta* **1284**, 122-124.

Baida, G.E., Budarina, Z.I., Kuzmin, N.P. and Solonin, A.S. (1999) Complete Nucleotide Sequence and Molecular Characterisation of Hemolysin II Gene from *Bacillus cereus*. *FEMS Microbiology Letters* **180**, 7-14.

Barlass, P.J., Houston, C.W., M.O., C. and Moir, A. (2002) Germination of *Bacillus cereus* Spores in Response to L-Alanine and to Inosine: The Roles of gerL and gerQ Operons. *Microbiology* **148**, 2089-2095.

Bartoszewicz, M., Bideshi, D.K., Kraszewska, A., Modezelewska, E., and Swiecicka, I. (2008) Natural Isolates of *Bacillus thuringiensis* Display Genetic an Psychrotrophic Properties Characteristic of *Bacillus weihenstephanensis*. *Journal of Applied Microbiology* **10**, 1967-1975

Beecher, D.J., and MacMillan, J.D. (1990) A Novel Bicomponent Hemolysin from *Bacillus cereus*. *Infection and Immunity* **58**, 2220-2227.

Beecher, D.J. and Macmillan J.D. (1991) Characterization of the Components of hemolysin BL from *Bacillus cereus*. *Infection and Immunity* **59**, 1778-1784.

Beecher, D.J., Olsen, T.W., Somers, E.B., and Wong, A.C.L. (2000) Evidence for Contribution of Triparitite Hemolysin BL, Phosphatidylcholine-Preferring Phospholipase C, and Collagenase to Virulence of *Bacillus cereus* Endophthalmitis. *Infection and Immunity* **68**, 5269-5276

Beecher, D.J., Schoeni, J.L., and Wong, A.C. (1995) Enterotoxic Activity of Hemolysin BL from *Bacillus cereus*. *Infection and Immunity* **63**, 4423-4428

Beecher, D.J., and Wong, A.C.L. (1994) Improved Purification and Characterisation of Hemolysin BL, a Hemolytic Dermonecrotic Vasculat Permeability Factor from *Bacillus cereus*. *Infection and Immunity* 62, 980-986

Beecher, D.J., and Wong, A.C.L. (1994a) Identification and Analysis of the Antigens Detected by Two Commercial *Bacillus cereus* Diarrheal Enterotoxin Immunoassay Kits. *Applied and Environmental Microbiology* **60**, 4614-4616.

Beecher DJ & Wong AC (1994b) Identification of Hemolysin BL-Producing *Bacillus cereus* Isolates by a Discontinuous Hemolytic Pattern in Blood Agar. *Applied and Environmental Microbiology* **60**, 1646–1651.

Beecher, D.J., and Wong, A.C.L. (1994c) Identification of Hemolysin BL-Producing *Bacillus cereus* Isolates by a Discontinuous Hemolytic Pattern in Blood Agar. *Applied and Environmental Microbiology* **60**, 1646-1651.

Beecher DJ & Wong AC (1997) Tripartite Hemolysin BL from *Bacillus cereus*. Hemolytic Analysis of Component Interactions and a Model for its

Characteristic Paradoxical Zone Phenomenon. *Journal of Biological Chemistry* **272**, 233–239.

Beecher, D.J., and Wong, A.C.L. (2000) Tripartite Haemolysin BL: Isolation and Characterization of Two Distinct Homologous Sets of Components from a Single *Bacillus cereus* Isolate. *Microbiology* **146**, 1371-1380.

Behravan J., Chirakkal, H., Masson, A. and Moir, A. (2000) Mutations in the gerP Locus of *Bacillus subtilis* and *Bacillus cereus* Affect Access of Germinants to their Target in Spores. *Journal of Bacteriology* **182**, 1987-1984

Besten, H.M.W., Mataragas, M., Moezelaar, R., Abee, T. and zwietering, M.H. (2006) Quantification of the Effects of Salt Stress and Physiological State on Thermotolerance of *Bacillus cereus* ATCC10987 and ATCC 14579. *Applied and Environmental Microbiology* **72**, 5884-5894.

Bhakdi, S., and Tranum-Jensen, J. (1991) Alpha-toxin of *Staphylococcus aureus*. *Microbiological Reviews* **55**, 733-751.

Bizzarri, M.F., and Bishop, A.H. (2006) Recovery of *Bacillus thuringiensis* in Vegetative Form from the Phylloplane of Clover (*Trifolium hybridum)* During a Growing Season. Journal of Invertebrate Pathology **94**, 38-47

Boehm, A.M., Putz, S., Altenhofer, D., Sickmann, A., and Falk, M. (2007) Precise Protein Quantification Based on Peptide Quantification Using iTRAQ. *BMC Bioinformatics* **21**, 214-232.

Boja, E.S., Phillips, D., French, S.A., Harris, R.A., and Balaban, R.S. (2009) Quantitative Mitochondrial Phosphoproteomics Using iTRAQ on an LTQ-Orbitrap with High Energy Collision Dissociation. *Journal of Proteome Research* **9**, 4665-4675

Boland, F.M., Atrih, A., Chirakkal, H., Foster, S.J. and Moir, A. (2000) Complete Spore-cortex Hydrolysis During Germination of *Bacillus subtilis* 168 requires SleB and YpeB. *Microbiology* **146**, 57–64.

Bouillaut, L., Perchat, S., Arold, S., Zorrilla, S., Slamti, L., Henry, C., Gohar, M., Declerck, N. and Lereclus, D. (2008) Molecular Basis for Group-specific Activation of the Virulence Regulator PlcR by PapR heptapeptides. *Nucleic Acids Res* **36**, 3791-3801.

Bouillaut, L., Ramarao, N., Buisson, C., Gilois, N., Gohar, M., Lereclus, D. and Nielsen-Leroux, C. (2005) FlhA Influences *Bacillus thuringiensis* PlcR-regulated Gene Transcription, Protein Production, and Virulence. *Appl Environ Microbiol* **71**, 8903-8910.

Brillard, J., and Lereclus, D. (2004) Characterization of a Small PlcR-Regulated Gene Co-Expressed with Cereolysin O. *BMC Microbiology* **7**, 52

Buchanan RL & Schultz FJ (1994) Comparison of the Tecra VIA kit, Oxoid BCET-RPLA Kit and CHO Cell Culture Assay for the Detection of *Bacillus cereus* Diarrhoeal Enterotoxin. *Letters in Applied Microbiology* **19**, 353–356.

Burbulys, D., Trach, K. A. and Hoch, J. A. (1991). Initiation of Sporulation in *Bacillus subtilis* is Controlled by a Multicomponent Phosphorelay. *Cell* **64**, 545–552.

Bustin, S.A. (2000) Absolute Quantification of mRNA Using Real-Time Reverse Transcription Polymerase Chain Reaction Assay. *Journal of Molecular Endocrinology* **25**, 169-193

Cadot, C., Tran, S-L., Vignaud, M-L., De Buyser, M-L., Kolstø, A-B., Brisabois, A., Nguyen-The, C., Lereclus, D., Guinebretiere, M-H., and Ramarao, N. (2010). InhA1, NprA, and HlyII as Candidates for Markers to Differentiate Pathogen from Nonpathogenic *Bacillus cereus* Strains. *Journal of Clinical Microbiology* **48**, 1358-1365

Callahan, C., Fox, K., and Fox, A., (2009) The Small Acid Soluble Proteins (SASP α and SASP β) of *Bacillus weihenstephanensis* and *Bacillus mycoides* Group 2 are the most Distinct Among the *Bacillus cereus* Group. *Molecular Cellular Probes* **23**, 291-297

Camp, A.H., Wang, A.F., and Losick, R. (2011) A Small Protein Required for the Switch from σF to σ^G During Sporulation in *Bacillus Subtilis*. *Journal of Bacteriology* **193**, 116-124.

Carlin, F., Fricker, M., Pielaat, A., Heisterkamp, S., Shaheen, R., Salonen, M.S., svensson, B., Nguyen-the, C. and Ehling-Schulz, M. (2006) Emetic Toxin Producing Strains of *Bacillus cereus* Show Distinct Characteristics within the *Bacillus cereus* Group. *International Journal of Food Microbiology* **109**, 132-138.

Cartman, S. T., La Ragione, R.M., and Woodward, M.J. (2008) *Bacillus subtilis* Spores Germinate in the Chicken Gastrointestinal Tract. *Applied and Environmental Microbiology* **74**, 5254-5258

Chang, Y-H., Shangkuan, Y-H, Lin, H-C., and Liu, H-W. (2003) PCR Assay of the *groEL* Gene for Detection and Differentiation of *Bacillus cereus* Group Cells. *Applied and Environmental Microbiology* **69**, 4502-4510.

Chen, J.L., Chiang, M.L. and Chou, C.C. (2009) Survival of the Acid-Adapted *Bacillus cereus* in Acidic Environments. *International Journal of Food Microbiology* **128**, 424-428.

Chirakkal, H., O'Rourke, M., Atrih, A., Foster, S.J., Moir, A. (2002) Analysis of Spore Cortex Lytic Enzymes and Related Proteins in *Bacillus subtilis* Endospore Germination. *Microbiology* **148**, 2383-2392

Chitlaru, T.M., Gat, O., Gozlan, Y., Ariel, N., and Shafferman, A. (2006) Differential Proteomic Analysis of the *Bacillus anthracis* Secretome: Distinct

Plasmid and Chromosome CO2-Dependent Cross Talk Mechanisms Modulate Extracellular Proteolytic Activities. *Journal of Bacteriology* **188**, 3551-3571.

Choma, C. and Granum, P.E. (2002) The Entertotoxin T (BcET) from Bacillus cereus can Probably not Contribute to Food Poisoning. *FEMS Microbiology Letters* **217**, 115-119.

Christiansson, A., Satyanarayan Naidu, A., Nilsson, I., Wadstrom, T. and Pettersson, H.-E. (1989) Toxin Production by *Bacillus cereus* Dairy Isolates in Milk at Low Temperatures. *Applied and Environmental Microbiology* **55**, 2595-2600.

Clair, G., Roussi, S., Armengaud, J., and Duport, C. (2010) Expanding the Known Repertoire of Virulence Factors Produced by *Bacillus cereus* Through Early Secretome Profiling in Three Redox Conditions. *Molecular and Cellular Proteomics* **9**, 1486–1498.

Claus D and Berkeley CW. (1986) The Genus *Bacillus*. *In: Bergey's Manual of Systematic Bacteriology*. **2**. Sneath PHA (Ed). Williams, Wilkins, Baltimore. **34**: 1105-1139,

Clavel, T., Carlin, F., Lairon, D., Nguyen-The, C. and Schmitt, P. (2004) Survival of *Bacillus cereus* Spores and Vegetative Cells in Acid Media Simulating Human Stomach. *Journal of Applied Microbiology* **97**, 214-219.

Conway, P.L., Gorbach, S.L., and Goldin B.R. (1987) Survival of Lactic Acid Bacteria in the Human Stomach and Adhesion to Intestinal Cells. *Journal of Dairy Science* **70**, 1-12/

Cooper, G.R., and Moir, A. (2011) Amino Acid Residues in the GerAB Protein Important in the Function and Assembly of the Alanine Spore Germination Receptor of *Bacillus subtilis* 168. *Journal of Bacteriology* **193**, 2261-2267.

Cotter, P.D., and Hill, C. (2003) Surviving the Acid Test: Responses of Gram-Positive Bacteria to Low pH. Microbiology and Molecular Biology Reviews **67**, 429-453

Daffonchio, D., Raddadi, N., Merabishvili, M., Cherif, A., Carmagnola, L., Brusetti, L., Rizzi, A., Chanishvili, N., Visca, P., Sharp, R. and Borin, S. (2006) Strategy for Identification of *Bacillus cereus* and *Bacillus* Strains Closely Related to *Bacillus anthracis*. *Applied and Environmental Microbiology* **72**, 1295-1301.

Day, T.L, Tatani, S.R, Notermans, S., and Bennett, R.W. (1994) A Comparison of ELISA and RPLA for Detection of *Bacillus cereus* Diarrhoeal Enterotoxin. *Journal of Applied Bacteriology* **77**, 9–13.

Declerck, N., Bouillaut, L., Chaix, D., Rugani, N., Slamti, L., Hoh, F., Lereclus, D. and Arold, S.T. (2007) Structure of PlcR: Insights into Virulence

Regulation and Evolution of Quorum Sensing in Gram-Positive Bacteria. *Proceedings of the National Academy of Sciences* **104**, 18490-18495.

Desrosier, J.P., and Cano Lara, J. (1983) Synthesis of the Exosporium During Sporuation of *Bacillus cereus*. Microbiology **130**, 935-940.

Dietrich, R., Fella, C., Strich, S. and Martlbauer, E. (1999) Production and Characterisation of Monoclonal Antibodies Against the Hemolysin BL Enterotoxin Complex Produced by *Bacillus cereus*. *Applied and Environmental Microbiology* **65**, 4470-4474.

Dietrich, R., Moravek, M., Burk, C., Granum, P.E. and Martlbauer, E. (2005) Production and Characterisation of Antibodies against Each of the Three Subunits of the *Bacillus cereus* Nonhemolytic Enterotoxin Complex. *Applied and Environmental Microbiology* **71**, 8214-8220.

Dressman J.B., Berardi, R.R., Dermentzoglou, L.C., Russell, T.L., Schmaltz, S.P., Barnett, J.L., and Jarvenpaa, K.M. (1990) Upper Gastrointestinal (GI) pH in Young, Healthy Men and Women. *Pharmacological Research* **7**, 756-761

Duport, C., Zigha, A., Rosenfeld, E., and Schmitt, P. (2006) Control of Enterotoxin Gene Expression in *Bacillus cereus* F4430/73 Involves the Redox-Sensitive ResDE Signal Transduction System. *Journal of Bacteriology* **188**, 6640-6651

Durban, M.A., Silbersack, J., Schweder, T., Schauer, F. and Bornscheuer, U.T. (2007) High Level Expression of a Recombinant Phospholipase C from *Bacillus cereus* in *Bacillus subtilis*. *Applied Genetics and Molecular Biotechnology* **74**, 634-639.

Dussault, A. A. and Pouliot. M. (2006). "Rapid and Simple Comparison of Messenger RNA Levels Using Real-time PCR." *Biological Procedures Online* **8**, 1-10.

Ehling-Schulz, M., Fricker, M., Grallert, H., Rieck, P., Wagner, M. and Scherer, S. (2006) Cereulide Synthetase Gene Cluster from Emetic *Bacillus cereus*: Structure and Location on a Mega Virulence Plasmid Related to *Bacillus anthracis* Toxin Plasmid pXO1. *BMC Microbiology* **6**, 1-11.

Eichenberger, P., Jensen, S. T., Conlon, E. M., van Ooij, C., Silvaggi, J., Gonzalez-Pastor, J-E., Fujita, M., Ben-Yehuda, S., Strangler, P., Liu, J.S., and Losick, R. (2003). The σ^E Regulon and the Identification of Additional Sporulation Genes in *Bacillus subtilis*. *Journal of Molecular Biology* **327**, 945–972.

Errington, J. (2003). Regulation of Endospore Formation in *Bacillus subtilis*. *Nature Reviews Microbiology* **1**, 117–126.

Fagerlund, A., Brillard, J., Furst, R., Guinebretiere, M-H., and Granum, P.E. (2007) Toxin Production in a Rare and Genetically Remote Cluster of Strains of the *Bacillus cereus* Group. *BMC Microbiology* **7**, 43-51.

Fagerlund, A., Linback, T., and Granum, P.E. (2010) *Bacillus cereus* Cytotoxins Hbl, Nhe and CytK are Secreted via the Sec Translocation Pathway. *BMC Microbiology* **30**, 304

Fagerlund A, Lindback T, Storset AK, Granum PE & Hardy SP (2008) *Bacillus cereus* Nhe is a Pore-forming Toxin with Structural and Functional Properties Similar to the ClyA (HlyE, SheA) Family of Haemolysins, able to Induce Osmotic lysis in Epithelia. *Microbiology* **154**: 693–704.

Fagerlund A, Ween O, Lund T, Hardy S.P & Granum P.E (2004) Genetic and Functional Analysis of the cytK Family of Genes in *Bacillus cereus*. *Microbiology* **150**: 2689–2697.

Faille, C., Fontaine, F., and Benezech, T. (2001) Potential Occurrence of Adhering Living *Bacillus* Spores in Milk Product Processing Lines. *Journal of Applied Microbiology* **90**, 892-900.

Faille, C., Tauveron, G., Le Gentil-Lelievre, C., and Slomianny, C. (2007) Occurrence of *Bacillus cereus* Spores with a Damaged Exosporium: Consequences on the Spore Adhesion on Surfaces of Food Processing Lines. *Journal of Food Protection* **70**: 2346–2353.

Fairhead, H., Setlow, B. and Setlow, P. (1993) Prevention of DNA Damage in Spores and in Vitro by Small, Acid-soluble Proteins from *Bacillus* Species. *Journal of Bacteriology* **175**, 1367–1374.

Fakhry, S., Sorrentini, I., Ricca, E., De Felice, M., and Baccigalupi, L. (2008) Characterization of Spore Forming *Bacilli* Isolated from the Human Gastrointestinal Tract. *Journal of Applied Microbiology* **105**, 2178-2186

Fang, F., Li, Y., Bumann, M., Raftis, E.J., Casey, P.G., Cooney, J.C., Walsh, M.A., O'Toole, P.W. (2009) Allelic Variation of Bile Salt Hydrolase Genes in *Lactobacillus salicarius* Does Not Determine Bile Resistance Levels. *Journal of Bacteriology* **191**, 5743-5757

Fedhila, S., Gohar, M., Slamti, L., Nel, P. and Lereclus, D. (2003) The *Bacillus thuringiensis* PlcR-Regulated Gene *inhA2* is Necessary but Not Sufficient for Virulence. *Journal of Bacteriology* **185**, 2820-2825.

Fedhila, S., Nel, P and Lereclus, D. (2002). The InhA2 Metalloprotease of *Bacillus thuringiensis* Strain 407 is Required for Pathogenicity in Insects Infected Via the Oral Route. *Journal of Bacteriology* **184**, 3296-3304.

Fermanian, C. and Wong, A.C.L. (2000) Improved In Vitro Detection of Hemolysin BL from *Bacillus cereus*. *International Journal of Food Microbiology* **57**, 1-8.

Fletcher, P., and Logan, N.A. (1999) Improved Cytotoxicity Assay for *Bacillus cereus* Diarrhoeal Enterotoxin. *Letters in Applied Microbiology* **28**, 394-400

Foster, S.J., and Johnstone, K. (1989) Pulling the Trigger: The Mechanism of Bacterial Spore Germination. *Molecular Microbiology* **4**, 137-141.

Foulger, D. & Errington, J. (1989). The Role of the Sporulation Gene *spoIIIE* in the Regulation of Prespore-specific Gene Expression in *Bacillus subtilis*. *Molecular Microbiology* **3**, 1247–1255.

Fricker, M., Messelhausser, U., Busch, U., Scherer, S., Ehling-Schulz, M. (2007) Diagnostic Real Time PCR Assays for the Detection of Emetic *Bacillus cereus* Strains in Food and Recent Food-Borne Outbreaks. *Applied and Environmental Microbiology* **73**, 1892-1898

From, C., Pukall, R., Schumann, P., Hormazabal, V. and Granum, P.E. (2005) Toxin Producing Ability Among *Bacillus* spp. Outside the *Bacillus cereus* Group. *Applied and Environmental Microbiology* **71**, 1178-1183.

Gohar, M., Faegri, K., Perchat, S., Ravnum, S., Okstad, O.A., Gominet, M., Kolsto, A.B. and Lereclus, D. (2008) The PlcR Virulence Regulon of *Bacillus cereus*. *PLoS one* **3**, e2793.

Gabig-Ciminska, M., Liu, Y., and Enfors, S-O. (2005) Gene-based Identification of Bacterial Colonies with an Electric Chip. *Analytical Biochemistry 345*, 27--276

Gänzle, M. G., S. Weber, and W. P. Hammes. (1999) Effect of Ecological Factors on the Inhibitory Spectrum and Activity of Bacteriocins. International *Journal of Food Microbiology* **46**, 207–217.

Gerhardt, P., and Ribi, E. (1964) Ultrastructure of the Exosporium Enveloping Spores of *Bacillus cereus*. Journal of Bacteriology. 88, 1774–1789.

Ghelardi, E., Celandroni, F., Salvetti, S., Barsotti, C., Baggiani, A. and Senesi, S. (2002) Identification and Characterization of Toxigenic *Bacillus cereus* Isolates Responsible for Two Food-Poisoning Outbreaks. *FEMS Microbiology Letters* **208**, 129-134

Gherlardi, E., Celandroni, F., Salvetti, S., Ceragioli, M., Beecher, D.J., Senesi, S. and Wong, A.C.L. (2005) Swarming Behaviour and Hemolysin BL in *Bacillus cereus*. *Applied Environmental Microbiology* **73**, 4089-4093.

Ghelardi, E., Celandroni, F., Salvetti, S., Ceragioli, M., Beecher, D.J., Senesi, S., and Wong, A.C.L. (2007) Swarming Behaviour and Hemolysin BL Secretion in *Bacillus cereus*. *Applied and Environmental Microbiology* **73**, 4089-4093

Gilbert, R.J., and Kramer, J.M. (1984) *Bacillus cereus* Enterotoxins: Present Status. *Biochemistry Society Transactions* **12**, 198–200.

Gilbert RJ & Kramer JM (1986) *Bacillus cereus* Food Poisoning. Progress in Food Safety (Proceedings of Symposium) (Cliver DC & Cochrane BA, eds), pp. 85–93. Food Research Institute, University of Wisconsin-Madison, Madison, WI.

Gilmore, M.S., Cruz-Rodz, A.L., Leimeister-Wachter, M., Kreft, J. and Goebel, W. (1989) A *Bacillus cereus* Cytolytic Determinant, Cereolysin AB, which Comprises the Phospholipase C and Sphingomyelinase Genes: Nucleotide Sequence and Genetic Linkage. *Journal of Bacteriology* **171**, 744-753.

Gilois, N., Ramarao, N., Bouillaut, L., Perchat, S., Aymerich, S., Nielson-LeRoux, C., Lereclus, D. and Gohar, M. (2007) Growth Related Variations in the *Bacillus cereus* Secretome. *Proteomics* **7**, 1719-1728.

Glatz, B.A., and Goepfert, J.M. (1976) Defined Conditions for Synthesis of *Bacillus cereus* Enterotoxin by Fermenter-Grown Cultures. *Applied and Environmental Microbiology* **32**, 400-404.

Goher, M., Faegri, K., Perchat, S. Ravnum, S., Okstad, O.A., Gominet, M., Kolsto, A-B., Lereclus, D. (2008) The plcR Virulence Regulon of *Bacillus cereus*. *PLos one* **3**, e2793

Gohar, M., Gilois, N., Graveline, R., Garreau, C., Sanchis, V. and Lereclus, D. (2005) A Comparative Study of *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus anthracis* Extracellular Proteomes. *Proteomics* **5**, 3696-3711.

Gohar, M., Okstad, O.A., Gilois, N., Sanchis, V., Kolsto, A.-B. and Lereclus, D. (2002) Two Dimensional Electrophoresis Analysis of the Extrecellular Proteome of *Bacillus cereus* Reveals the Importance of the PlcR Regulon. *Proteomics* **2**, 784-791.

Goni, F.M. and Alonso, A. (2002) Sphingomyelinases: Enymology and Membrane Activity. *FEBS Letters* **531**, 38-46.

Gore, H.M., Wakeman, C.A., Hull, R.M., and McKilip, J.L. (2003) Real-time Molecular Beacon NASBA Reveals *hblC* Expression from *Bacillus* spp. in Milk. *Biochemical and Biophysical Research Communication* **311**, 386-390

Granum, P.E. and Baird-Parker, T.C. (2000) *Bacillus* species. In The Microbiological Quality and Safety of Food, vol. II. ed. Lund, B.M.,Baird-Parker, A.C. and Gould, G.W. pp. 1029–1039. Gaithersburg: Aspen Publishers

Granum PE, O'Sullivan K, Lund T. (1999) The Sequence of the Nonhaemolytic Enterotoxin Operon from *Bacillus cereus*. *FEMS Microbiology Letters* **177**, 225-229.

Granum, P.E., Tomas, J.M., Alouf, J.E. (1995) A Survey of Bacterial Toxins Involved in Food Poisoning; A Suggestion for Bacterial Food Poisoning Toxin Nomenclature. *International Journal of Food Microbiology* **28**, 1290-144

Green, M., Heumann, M., Sokolow, R. Foster, L.R., Bryant, R., Skeels, M. (1990) Public Health Implications of the Microbial Pesticide *Bacillus thuringiensis*: An Epidemiological Study, Oregon, 1985-86. *American Journal of Public Health* **80,** 848-852

Guamer, F., and Maagelada, J-R. (2003) Gut Flora in Health and Disease. *Lancet* **361**, 512-519

Guinebretiere, M.-H., Broussolle, V. and Nguyen-The, C. (2002) Enterotoxigenic Profiles of Food-Poisoning and Food-Borne *Bacillus cereus* Strains. *Journal of Clinical Microbiology* **40**, 3053-3056.

Guinebretiere, M., Thompson, F.L., Sorokin, A., Normand, P., Dawyndt, P., Ehling-Schulz, M., Svensson, B., Sanchis, V., Nguyen-The, C., Heyndrickx, M. and De Vos, D. (2008) Ecological Diversification in the *Bacillus cereus* Group. *Environmental Microbiology* **10**, 851-865.

Guinebretiere, M.-H., Velge, P., Couvert, O., Carline, F., Debuyser, M.-L. and Nguyen-The, C. (2010) Ability of *Bacillus cereus* Group Strains to Cause Food Poisoning Varies According to Phylogenetic Affiliation (Groups I to VII) Rather than Species Affiliation. *Journal of Clinical Microbiology* **48**, 3388-3391.

Hachisuka, Y., Kozuka, S., and Tsukikawa, M. (1964) Exosporia and Appendages of *Bacillus* Species. *Microbiology and Immunology* **28**. 619- 624

Haldenwang, W. G. (1995). The Sigma Factors of *Bacillus subtilis*. *Microbiological Reviews* **59**, 1–30.

Haldenwang, W.G., and Losick, R. (1979) A Modified RNA Polymerase Transcribes a Cloned Gene Under Sporulation Control in *Bacillus subtilis*. *Nature* **282**, 256-260

Han, C.S., et al (2006) Pathogenomic Sequence Analysis of *Bacillus cereus* and *Bacillus thuringiensis* Isolates Closely Related to *Bacillus anthracis*. *Journal of Bacteriology* **188**, 3382-3390.

Hansen, B.M. and Hendriksen, N.B. (2001) Detection of Enterotoxin *Bacillus cereus* and *Bacillus thuringiensis* Strains by PCR Analysis. *Applied and Environmental Microbiology* **67**, 185-189.

Hardy, S.P., Lund, T. and Granum, P.E. (2001) CytK Toxin of *Bacillus cereus* Forms Pores in Planar Lipid Bilayers and is Cytotoxic to Intestinal Epithelia. *FEMS Microbiology Letters* **197**, 47-51.

Harvie, D.R., Vilchez, S., Steggles, J.R. and Ellar, D.J. (2005) *Bacillus*

cereus Fur Regulates Iron Metabolism and is Required for Full Virulence. *Microbiology* **151**, 569-577.

Hauge S (1950) Food Poisoning Caused by *Bacillus cereus* (in Norwegian, English abstract). *Nord Hyg Tidsskr* **31**, 189–206.

Hauge S (1955) Food Poisoning Caused by Aerobic Spore Forming *Bacilli*. *Journal of Applied Bacteriology* **18**, 591–595.

Hecker, M., Schumann, W., and Volker, U. (1996) Heat Shock and General Stress Response in *Bacillus subtilis*. *Molecular Microbiology* **19**, 417-428

Hecker, M., and Volker, U. (1998) Non-Specific, General and Multiple Stress Resistance of Growth-Restricted *Bacilus subtilis* Cells by the Expression of the σ^B Regulon. *Molecular Microbiology* **29**, 1129-1136

Heinrichs, J.H., Beecher, D.J., MacMillan, J.D. and Zilinskas, B.A. (1993) Molecular Cloning and Characterization of the *HblA* Gene Encoding the B Component of Hemolysin BL from *Bacillus cereus*. *Journal of Bacteriology* **175**, 6760-6766.

Helgason, E., Tourasse, N. J., Meisal, R., Caugant, D. A. and Kolsto, A. B. (2004). Multilocus Sequence Typing Scheme for Bacteria of the *Bacillus cereus* Group. *Applied and Environmental Microbiology* **70**, 191-201

Hendriksen, N. B. and Hansen, B. M. (2002). Long-term Survival and Germination of *Bacillus thuringiensis var*. *Kurstaki* in a Field Trial. *Canadian Journal of Microbiology* **48**, 256-261

Henriques, A.O. and Moran Jr, C.P. (2007) Structure, Assembly and Function of the Spore Surface Layers. *Annual Reviews in Microbiology* **61**, 555-588.

Heung, L.J., Luberto, C., Del Poeta, M. (2006) Role of Sphingolipids in Microbial Pathogenesis. *Infection and Immunity* **74**, 28–39

Hilbert, D. W., and Piggot, P. J. (2004) Compartmentalization of Gene Expression During *Bacillus subtilis* Spore Formation. *Microbiology and Molecular Biology Reviews* **68**, 234-262

Hill KK, Ticknor LO, Okinaka RT et al. (2004) Fluorescent Amplified Fragment Length Polymorphism Analysis of *Bacillus anthracis*, *Bacillus cereus,* and *Bacillus thuringiensis* Isolates. *Applied Environmental Microbiology* **70**, 1068–1080.

Hofmann, K., and Dixit, V.M. (1998) Ceramide in Apoptosis-Does it Really Matter. *Trends in Biochemical Sciences* **23**, 374-377

Hohmann, A., G. Schmidt, and D. Rowley. (1979) Intestinal and Serum Antibody Responses in Mice after Oral Immunization with *Salmonella*,

Escherichia coli and *Salmonella-Escherichia coli* hybrid strains. Infection and Immunity 25, 27-33.

Hong, H.A., To, E., Fakhry, S., Baccigalupi, L., Ricca, E., and Cutting, S. (2009) Defining the Natural Habitat of *Bacillus* Spore-Formers. *Research in Microbiology* **160**, 375-379

Hornstra, L.M., de Leeuw, P.L.A., Moezelaar, R., Wolbert, E.J., de Vries, Y.P., de Vos, W.M. and Abee, T. (2007) Germination of *Bacillus cereus* Spores Adhered to Stainless Steel. *International Journal of Food Microbiology* **116**, 367-371.

Hsieh, Y.M., Sheu, S.J., Chen, Y.L., and Tsen, H.Y. (1999) Enterotoxigenic Profile and Polymerase Chain Reaction Detection of *Bacillus cereus* Group Cells and *Bacillus cereus* Strains from Food and Food-borne Outbreaks. *Journal of Applied Microbiology* **87**, 481-490

Huang, Y. and Adams, M.C. (2004) In Vitro Assessment of the Upper Gastrointestinal Tolerance of Potential Probiotic Dairy Propionibacteria. *Internation Journal of Food Microbiology* **91**, 253-260.

Husmark, U. (1993). Adhesion Mechanisms of Bacterial Spores to Solid Surfaces. Chalmers University of Technology. PhD Thesis.

Husmark, U., and Ronner, U. (1992) The Influence of Hydrophobic, Electrostatic and Morphologic Properties on the Adhesion of *Bacillus* Spores. *Biofouling* **5**, 1-6.

Ichimatsu, T., Mizuki, E., Nishimura, K., Akao, T., Saltoh, H., Higuvhi, K, and Ohba, M. (2000) Occurrence of *Bacillus thuringiensis* in Fresh Waters of Japan. Current Microbiology **40**, 217-220.

Ivanova, N., *et al* (2003) Genome Sequence of *Bacillus cereus* and Comparative Analysis with *Bacillus anthracis*. *Nature* **423**, 87-91.

Jackson, S.G. (1993) Rapid Screening Test for Enterotoxin-Producing *Bacillus cereus*. *Journal of Clinical Microbiology* **31**, 972-974

Jackson, S.G., Goodbrand, R.B., Ahmed, R and Kasatiya, S. (1995) *Bacillus cereus* and *Bacillus thuringiensis* Isolated in a Gastroenteritis Outbreak Investigation. *Letters in Applied Microbiology* **21**, 103-105

Jackson, A.D., and McLaughlin, J. (2006) Digestion and Absorption. Surgery **24**, 250-254

Jaquette, C. B., and Beuchat, L. R, (1998) Combined Effects of pH, Nisin, and Temperature on Growth and Survival of Psychrotrophic *Bacillus cereus*. *Journal of Food Protection* **61**, 563-570.

Jenson, G.B., Hansen, B.M., J, E. and Mahillon, J. (2003) The Hidden Lifestyle of *Bacillus cereus* and Relatives. *Environmental Microbiology* **5**, 631-

640.

Johnson, K.M. (1984) *Bacillus cereus* Foodborne Illness: An Update. *Journal of Food Protection* **47**, 145-153

Kalman S., Duncan, M.L., Thomas, S.M., and Price, C.W. (1990) Similar Organization of the *sigB* and *spoIIA* Operons Encoding Alternate Sigma Factors of *Bacillus subtilis* RNA Polymerase. *Journal of Bacteriology* **172**, 5575-5585

Kamat A.S., Nerkar D.P., and Nair P.M. (1989) *Bacillus cereus* in Some Indian Foods, Incidence and Antibiotic, Heat and Radiation Resistance. *Journal of Food Safety* **10**, 31–41.

Kaper, J.B., and Sperandio, V. (2005) Bacterial Cell-to-Cell Signalling in the Gastrointestinal Tract. *Infection and Immunity* **73**, 3197-3209

Katan, M. (1998) Families of Phosphoinositide-Specific Phospholipase C: Structure and Function. *Biochimica et Biophysica Acta* **1436**, 5-17

Kearns, D.B. and Losick R. (2005) Cell Populations Heterogeneity During Growth of *Bacillus subtilis. Genes & Development* **19**, 3083-3094

Keynan, A. (1978) Spores VII (Chambliss, C. & Vary, J. C., eds.), pp. 43-53, *American Society for Microbiology*, Washington

Kimoto-Nira, H., Kobayashi, M., Nomura, M., Sasaki, K., Suzuki, C. (2009) Bile Resistance in *Lactotococcus lactis* Strains Varies with Cellular Fatty Acid Composition: Analysis by Using Different Growth Media. *Journal of Food Microbiology* **131**, 183-188

Kong, L., Zhang, P., Setlow, P., and Li, Y-Q. (2010) Characterisation of Bacterial Spore Germination Using Integrated Phase Contrast Microscopy, Raman Spectroscopy and Optical Tweezers. *Analytical Chemistry* **82**, 3840-3847

Kort, R.A., O'Brien, A.C., van Stokkum, I.M., Oomes, S.J.C.M., Crielaard, W., Hellingwerf, K.J. and Brul, S. (2005) Assesment of Heat Resistance of Bacterial Spores from Food Product Isolates by Fluorescence Monitoring of Dipicolinic Acid Release. *Applied and Environmental Microbiology* **71**, 3556-3564.

Kotiranta, A., Haapasalo, M., Kair, K., Kerosuo, E., Olsen, I., Sorsa, T., Meurman, J.H., and Lounatmaa, K. (1998) Surface Structure, Hydrophobicity, Phagocytosis, and Adherence to Matrix Proteins of *Bacillus cereus* Cells with and without the Crystalline Surface Protein Layers. *Infection and Immunity* **66**, 4895-4902.

Kotiranta, A., Lounatmaa, K. and Haapasalo, M. (2000) Epidemiology and Pathogenesis of *Bacillus cereus* Infections. *Microbes and Infection* **2**, 189-198.

Kovlevskiy, O.V., Lebedev, A.A., Surin, A.K., Solonin, A.S., Antson, A.A. (2007) Crystal Structure of *Bacillus cereus* HlyIIR, a Transcriptional Regulator of the Gene for Pore-forming Toxin Hemolysin II. Journal of Molecular Biology **365**, 825-834

Kozuka, S., and Tochikubo, K. (1985) Properties and Origin of Filamentous Appendages on Spores of *Bacillus cereus*. *Microbiology and Immunology* **29**, 21-37**.**

Kramer, J.M., and Gilbert, R.J. (1989) *Bacillus cereus* and other *Bacillus* species. Foodborne Bacterial Pathogens (Doyle MP, ed), pp. 21–70. Marcel Dekker, New York.

Kramer, M. J., and Roth, I. L. (1968). Ultrastructural Differences in the Exosporium of the Sterne and Vollum Strains of *Bacillus anthracis*. *Canadian Journal of Microbiology* **14**, 1297–1299.

Kristiffersen, S.M., Ravnum, S., Tourasse, N.J., Okstad, O.A., Kolsto, A-B., and Davies, W. (2007) Low Concentrations of Bile Salts Induce Stress Responses and Reduce Motility in *Bacillus cereus* ATCC14579. *Journal of Bacteriology* **189**, 5302-5313

Laouami, S., Messaoudi, K., Alberto, F., Clavel, T., and Duport, C. (2011) Lactate Dehydrogenase A Promotes Communication Between Carbohydrate Catabolism and Virulence in *Bacillus cereus*. *Journal of Bacteriology* **193**, 1757-1766

Larsen, H.D., and Jørgensen, K. (1999) Growth of *Bacillus cereus* in Pasteurized Milk Products. *International Journal of Food Microbiology* **46**, 173–176.

Lechner, S., Mayr, R., Francis, K.P., Purss, B.M., Kaplan, T., Wiessner Gunkel, E., Stewart, G.A.B., and Scherer, S. (1998) *Bacillus weihenstephanensis* sp. Nov. is a New Psychrotolerant Species of the *Bacillus cereus* Group. *International Journal of Systematic Bacteriology* 48, 1373-1382.

Lereclus, D., Agaisse. H., Gominet, M., Salamitou, S., and Sanchis, V. (1996) Identification of a *Bacillus thuringiensis* Gene that Positively Regulates Transcription of the Phosphatidylinositolspecific Phospholipase C Gene at the Onset of the Stationary Phase. *Journal of Bacteriology* **178**, 2749–2756.

Lereclus, D., Agaisse, H., Grandvalet, C., Salamitou, S., and Gominet, M. (2000) Regulation of Toxin and Virulence Gene Transcription in *Bacillus thuringiensis*. *International Journal of Medical Microbiology* **290**, 295–299.

Lewis, P.J., and Marston, A.L. (1999) GFP Vectors for Controlled Expression and Dual Labelling of Protein Fusions in *Bacillus Subtilis*. *Gene* **227**, 101-109

Liao, R., Duan, L., Lei, C., Pan, H., Ding, Y., Zhange, Q., Chen, D., Shen, B, Yu, Y., and Liu, W. (2009) Thiopeptide Biosynthesis Featuring Ribosomally Synthesized Precursor Peptides and Conserved Posttranslational Modifications. *Chemistry and Biology* **16**, 141-147

Lindback, T., Fagerlund, A. and Rodland, M.S. (2004) Characterization of the *Bacillus cereus* Nhe Enterotoxin. *Microbiology* **150**, 3959-3967.

Lindback, T., Økstad, O.A., Rishovd, A.L., and Kolstø, A.B. (1999) Insertional Inactivation of *hblC* Ecoding the L2 Component of *Bacillus cereus* ATCC 14579 Hemolysin BL Strongly Reduces Enterotoxigenic Activity, but not the Haemolytic Activity Against Human Erythrocytes. *Microbiology* 145: 3139–3146.

Liu, R., and Ochman, H. (2007) Origins of Flagellar Gene Operons and Secondary Flagellar Systems. Journal Bacteriology 189, 7098-7104

Lucchini, S., Thompson, A., and Hinton, J.C.D. (2001) Microarrays for Microbiologists. *Microbiology* **147**, 1403-1414

Lund, T., De Buyser, M. and Granum, P.E. (2000) A New Cytotoxin from *Bacillus cereus* that May Cause Necrotic Enteritis. *Molecular Microbiology* **38**, 254-261.

Lund, T., and Granum, P.E. (1996) Characterisation of a Nonhaemolytic Enterotoxin Complex from *Bacillus cereus* Isolated after a Foodborne Outbreak. *FEMS Microbiology Letters* **141**, 151–156.

Lund, T. and Granum, P.E. (1997) Comparison of Biological Effect of the Two Different Enterotoxin Complexes Isolated from Three Different Strains of *Bacillus cereus*. *Microbiology* **143**, 3329-3336.

Maathuis, A.J.H., Keller, D., Farmer, S. et al (2010) Survival and Metabolic Activity of the GanedenBC^{30} Strain of *Bacillus coagulans* in a Dynamic *In Vitro* Model of the Stomach and Small Intestine. *Beneficial Microbes* **1**, 31-36

Madegowda, M., Eswaramoorthy, S., Burley, S.K., and Swaminathan, S. (2008) X-ray Crystal Structure of the B Component of Hemolysin BL from *Bacillus cereus*. Proteins 71, 534–540.

Magge, A., Granger, A.C., Wahome, P.G., Setlow, B., Vepachedu, V.R., Loshon, C.A., Peng, L., Chen, D., Li, Y.Q., Setlow, P. (2008) Role of Dipicolinic Acid in the Germination, Stability, and Viability of Spores of *Bacillus subtilis*. *Journal Bacteriology* **190**,4798-4807.

Mainville, I., Arcand, Y. and Farnworth, E.R. (2005) A Dynamic Model that Simulates the Human Upper Gastrointestinal Tract for the Study of Probiotics. *International Journal of Food Microbiology* **99**, 287-296.

Mäntynen, V., and Lindström, K (1998). A Rapid PCR Based DNA Test for Enterotoxic *Bacillus cereus*. *Applied and Environmental Microbiology* **64**, 1634-1630

Marteau, P., Minekus, M., Havenaar, R. and Huis In't Veld, J.H.J. (1996) Survival of Lactic Acid Bacteria in a Dynamic Model of the Stomach and Small Intestine; Validation and the Effect of Bile. *Journal of Dairy Sciences* **80**, 1031-1037.

Martin, S.F., and Pitzer, G.E. (2000) Solution Conformations of Short-Chain Phospoatidylcholine Substrates of the Phosphatidylcholine-Preferring PLC of *Bacillus cereus*. *Biochimica at Biophysica Acta* **1464**, 104-112

Martinez-Blanch, J.F., Sanchez, G., Garay, E., and Aznar, R. (2009) Development of a Real-time PCR Assay for Detection and Quantification of Enterotoxigenic Members of *Bacillus cereus* Group in Food Samples. *International Journal Food Microbiology* **135**,15–21

Martinez-Blanch, J.E., Sanchez, G., Garay, E., and Aznar, R. (2011) Evaluation of Phenotypic and PCR-based Approaches for Routine Analysis of *Bacillus cereus* group. *Antonie van Leeuwenhoek* **99**, 697-709

McCann, K.P., Robinson, C., Sammons, R.L., Smith, D.A. and Corfe, B.M. (1996) Alanine Germination Receptors of *Bacillus subtilis. Letters in Applied Microbiology* **23**, 290–294.

McLauchlan, G., Fullarton, G.M., Crean, G.P., McColl, K.E.L. (1989) Comparison of the Gastric Body and Antral pH: a 24 hour Ambulatory Study in Healthy Volunteers. *Gut* **30**, 573– 578

Mendelson, I., Tobery, S., Scorpio, A., Bozue, J., Shafferman, A. and Friedlander, A.M. (2004) The NheA Component of the Non-Hemolytic Enterotoxin of *Baciilus cereus* is Produced by *Bacillus anthracis* but is not Required for Virulence. *Microbial Pathogenesis* **37**, 149-154

Mignot, T., Mock, M., Robichon, D., Landier, A., Lereclus, D., and Fouet, A. (2001) The Incompatibility between the *PlcR*- and *AtxA* Controlled Regulons may have Selected a Nonsense Mutation in *Bacillus anthracis*. *Molecular Microbiology* **42**, 1189–1198.

Miles, G., Bayley, H. and Cheley, S. (2002) Properties of *Bacillus cereus* Hemolysin II: A Heptameric Transmembrane Pore. *Protein Science* **11**, 1813-1824.

Minekus, M., Marteau, P., Havenaar, R., Huis inTt Veld, J.H.J. (1995) A Multicompartmental Dynamic Computer-controlled Model Simulating the Stomach and the Small Intestine. *Alternatives to Laboratory Animals* **23**, 197–209.

Minnaard, J., Delfederico, L., Vasseur, V., Hollmann, A., Rolny, I., Semorile, L. and Perez, P.F. (2006) Virulence of *Bacillus cereus*: A

Multivariate Analysis. *International Journal of Food Microbiology* **116,** 197-206.

Minnaard, J., Lievin-Le Moal, V., Coconnier, M., Servin, A.L. and Perez, P.F. (2004) Disassembly of F-Actin Cytoskeleton after Interaction of *Bacillus cereus* with Fully Differentiated Human Intestinal Caco-2 Cells. *Infection and Immunity* **72**, 3106-3112.

Mitchell, C., Skomurski, J. F., Vary, J.C. (1986) Effect of Ion Channel Blockers on Germination of *Bacillus megaterium* Spores. *FEMS Microbiology Letters* **34**, 211-214

Moberty, B.J., Shafa, F., and Gerhardt, P. (1966) Structural Details of Anthrax Spores During Stages of Transformation into Vegetative Cells. *Journal of Bacteriology* **92**, 220-228

Mohr, S. C., N. V. H. A., Sokolov, C., He, and P. Setlow. (1991) Binding of Small Acid-soluble Spore Proteins from *Bacillus subtilis* Changes the Conformation of DNA from B to A. *Proceedings of the National Academy of Science* **88**, 77-81.

Moir, A. (2006) How Do Spores Germinate? *Journal of Applied Microbiology* **101**, 526-530.

Moir, A. and Smith, D.A. (1990) The Genetics of Bacterial Spore Germination. *Annual Reviews of Microbiology* **44**, 531-553.

Molly, K., De Smet, I., Nollet, L., Vande Woestyne, M., Verstraete, W. (1996) Effect of Lactobacilli on the Ecology of the Gastrointestinal Microbiota Cultured in the SHIME Reactor. *Microbial Ecology in Health and Disease* **9**, 79–89.

Monis, P.T., Giglio, S., and Saint, C. P. (2005) Comparison of SYT09 and SYBR Green I for Real Time Polymerase Chain Reaction and Investigation of the Effect of Dye Concentration on Amplification and DNA Melting Curve Analysis. *Analytical Biochemistry* **340**, 24-34.

Moravek, M., Dietrich, R., Buerk, C., Broussolle, V., Guinebretiere, M.H., Granum, P.E., Nguyen-The, C., and Martlbauer, E. (2006) Determination of the Toxic Potential of *Bacillus cereus* Isolates by Quantitative Enterotoxin Analyses. *FEMS Microbiology Letters* **257**, 293–298.

Moravek, M., Wegscheider, M., Schulz, A., Dietrich, R., Burk, C., and Martlbauer, E. (2004) Colony Immunoblot Assay for the Detection of Hemolysin BL Enterotoxin Producing *Bacillus cereus*. *FEMS Microbiology Letters* **238**, 107-113

Mossel, D.A.A., Koopman, M.J. and Jongerius, E. (1967) Enumeration of *Bacillus cereus* in Foods. *Applied Microbiology* **15**, 650-653

Murray, T., Popham, D. L., Pearson, C.B., Hand. A., and Setlow, P. (1998) Analysis of Outgrowth of *Bacillus subtilis* Spores Lacking Penicillin-Binding Protein 2a. *Journal of Bacteriology* 180, 6493-6501

Nauta, M.J., Litman, S., Barker, G.C., and Carlin, F. (2003) A Retail and Consumer Phase Model for Exposure Assessment of *Bacillus cereus*. *International Journal of Food Microbiology* **83**, 205-218

Nicholson, W.L. (2002) Roles of *Bacillus* Endospores in the Environment. *Cellular and Molecular Life Science* **59**, 410-416

Nicholson, W.L,. Munakata, N., Horneck, G., Melosh, H.J., Setlow, P. (2000) Resistance of *Bacillus* Endospores to Extreme Terrestrial and Extraterrestrial Environments. *Microbiology and Molecular biology Reviews* **64**, 548–72

Obama, T., Fujii, S., Ikezawa, H., Ikeda, K., Imagawa, M., and Tsukamoto, K. (2003) His151 and His296 Are the Acid-Base Catalytic Residues of *Bacillus cereus* Sphingomyelinase in Sphingomyelin Hydrolysis. *Biological Pharmacological Bulletin* **26**, 920-926

Oda, M., Takahashi, M., Matsuno, T., Uoo, K., Nagahama, M., Sakurai, J. (2010) Haemolysis Induced by *Bacillus cereus* Sphingomyelinase. *Biochimica et Biophysica Acta* **1798**,1073–1080.

Okstad, O.A., Hegna, I., Lindback, T., Rishovd, A. and Kolsto, A. (1999) Genome Organization is not Conserved between *Bacillus cereus* and *Bacillus subtilis*. *Microbiology* **145**, 621-631.

Ollinger, J., Song, K-B., Antelmann, H., Hecker, M., and Helmann, J.D. (2006) Role of the Fur Regulon in Iron Transport in *Bacillus subtilis*. *Journal of Bacteriology* **188**, 3664-3673

Pacova, Z., Svec, P., Stenfors, L. P., Vyletelova, M., and Sedlacek, I. (2003) Isolation of the Psychrotolerant Species *Bacillus weihenstephanensis* from Raw Cow's Milk. *Czech Journal of Animal Science* **48**, 93-96.

Paidhungat, M., and Setlow, P. (1999) Isolation and Characterization of Mutations in *Bacillus subtilis* that allow Spore Germination in the Novel Germinant d-alanine. *Journal of Bacteriology* **181**, 3341–3350.

Paidhungat, M. and Setlow, P. (2000) Role of Ger Proteins in Nutrient and Non-nutrient Triggering of Spore Germination in *Bacillus subtilis*. *Journal of Bacteriology* **182**, 2513–2519.

Paidhungat, M., and Setlow, P. (2001) Localization of a Germinant Receptor Protein (GerBA) to the Inner Membrane of *Bacillus subtilis* Spores. *Journal of Bacteriology* **183**, 3982–3990.

Paramithiotis, S., Melissari, I. and Drosinos, E.H. (2006) *In vitro* Assessment of Properties Associated with the Survival through the

GastroIntestinal Tract of *Staphylococci* Isolated from Traditional Sausage Fermentation. *Food Microbiology* **23**, 663-671.

Partridge SR, Errington J. (1993) The Importance of Morphological Events and Intercellular Interactions in the Regulation of Prespore-specific Gene Expression During Sporulation in *Bacillus subtilis*. *Molecular Microbiology* **8**, 945-955.

Periago, P.M., Abee, T. and Wouters, J.A. (2002) Analysis of the Heat-Adaptive Response of Psychrotropic *Bacillus weihenstephanensis*. *International Journal of Food Microbiology* **79**, 17-26.

Pfaffl, M. W. (2001). A New Mathematical Model for Relative Quantification in Realtime RT-PCR. *Nucleic Acids Res* **29**(9): e45.

Phillips, Z. E., and Strauch, M. A. (2002). *Bacillus subtilis* Sporulation and Stationary Phase Gene Expression. *Cellular and Molecular Life Sciences* **59**, 392–402.

Pomerantsev, A.P., Kalnin, K.V., Osorio, M. and Leppla, S.H. (2003) Phosphatidylcholine-Specific Phospholipase C and Sphingomyelinase Activities in Bacteria of the *Bacillus cereus* Group. *Infection and Immunity* **71**, 6591-6606.

Pomerantsev, A.P., Pomerantseva, O.M. and Leppla, S.H. (2004) A Spontaneous Translational Fusion of *Bacillus cereus* PlcR and PapR Activates Transcription of PlcR-dependent Genes in *Bacillus anthracis* via Binding with a Specific Palindromic Sequence. *Infection and Immunity* **72**, 5814–5823.

Popham, D.L., Helin, J., Costello, C.E. and Setlow, P. (1996) Muramic Lactam in Peptidoglycan of *Bacillus subtilis* Spores is Required for Spore Outgrowth but not for Spore Dehydration or Heat Resistance. *Proceedings of the National Academy of Sciences* **93**, 15405–15410.

Postollec, F., Falentin, H., Pavan, S., Combrisson, J., and Sohier, D. (2011) Recent Advances in Quantitative PCR (qPCR) Applications in Food Microbiology. *Food Microbiology* **28**, **1-14**

Prevost, G. (1999). The Bi-component *staphylococcal* Leukocidins and C-haemolysins (toxins). In The Comprehensive Sourcebook of Bacterial Protein Toxins, pp. 402–418. Edited by J. H. Freer & J. E. Alouf. London, UK: Academic Press.

Price, C.W., Fawcett, P., Ceremonie, H., Su, N., Murphy, C.K., and Youngman, P. (2001) Genome-Wide Analysis of the General Stress Response in *Bacillus subtilis*. *Molecular Microbiology* **41**, 757-774

Priest, F.G., Barker, M., Baillie, L.W.J., Homes, E. C., and Maiden, M.C.J. (2004) Population Structure and Evolution of the *Bacillus cereus* Group. *Journal of Bacteriology* **186**, 7959-7970

Pruβ, B.M., Dietrich, R., Nibler, B., Martlbauer, E. and Scherer, S. (1999) The Hemolytic Enterotoxin HBL is Broadly Distributed Among Species of the *Bacillus cereus* Group. *Applied and Environmental Microbiology* **65**, 5436-5442.

Radonic, A., Thulke, S., MacKay, I.M., Landt, O., Siegert, W., and Nitsche, A. (2004) Guideline to Reference Gene Selection for Quantitative Real-time PCR. *Biochemical and Biophysical Research Communications* **313**, 856-62.

Ramarao, N., and Lereclus, D. (2006) Adhesion and Cytotoxicity of *Bacillus cereus* and *Bacillus thuringiensis* to Epithelial Cells are FlhA and PlcR Dependent, Respectively. *Microbes Infection* **8**, 1483–1491.

Rasko, D.A., Altherr, M.R., Han, C.S. and Ravel, J. (2005) Genomics of the *Bacillus cereus* Group of Organisms. *FEMS Microbiology Reviews* **29**, 303-329.

Raso, J., M. M., Gongora-Nieto, G. V., Barbosa-Canovas, and B. G. Swanson. (1998) Influence of Several Environmental Factors on the Initiation of Germination and Inactivation of *Bacillus cereus* by High Hydrostatic Pressure. *International Journal of Food Microbiology* **44**,125–132.

Rodikova, E.A., Kovalevskiy, O.V., Mayorov, S.G., Budarina, Z.I., Marchenkov, V.V., Melnik, B.S., Leech, A.P., Nikitin, D.V., Shlyapnikov, M.G. and Solonin, A.S. (2007) Two HlyIIR Dimers Bind to a Long Perfect Inverted Repeat in the Operator of the Hemolysin II Gene from *Bacillus cereus*. *FEBS Letters* **581**, 1190-1196.

Ronner, U., Husmark, U., and Henriksson, A. (1990) Adhesion of *Bacillus* Spores in Relation to Hydrophobicity. *Journal of Applied Microbiology* **69**, 550-556

Rosenquist, H., Smidt, L., Andersen, S.R., Jensen, G.B., Wilcks, A. (2005) Occurrence and Significance of *Bacillus cereus* and *Bacillus thuringiensis* in Ready-to-eat Food. *FEMS Microbiology Letters* **250**, 129–136.

Roth, I.L., and Williams, R.P. (1963) Comparison of the Fine Structure of Virulent and Avirulent Spores of *Bacillus anthracis*. Texas Reports on Biology and Medicine **21**, 394–399.

Ruas-Madiedo,P., Hugenholtz, J., and Zoon, P. (2002). An Overview of the Functionality of Exopolysaccharides Produced by Lactic Acid Bacteria. *International Dairy Journal* **12**, 163-171.

Ryan, P.A., MacMillan, J.D. and Zikinskas, B.A. (1997) Molecular Cloning and Characterization of the Genes Encoding the L1 and L2 Components of Hemolysin BL from *Bacillus cereus*. *Journal of Bacteriology* **179**, 2551-2556.

Salamitou, S., Ramisse, F., Brehelin, M., Bourguet, D., Gilois, N., Gominet, M., Hernandez, E. and Lereclus, D. (2000) The PlcR Regulon is Involved in

the Opportunistic Properties of *Bacillus thuringiensis* and *Bacillus cereus* in Mice and Insects. *Microbiology* **146**, 2825-2832.

Salvetti, S., Ghelardi, E., Celandroni, F., Ceragioli, M., Giannessi, F. and Senesi, S. (2007) *FlhF*, a Signal Recognition Particle like GTPase is Involved in the Regulation of Flagellar Arrangement Motility Behaviour and Protein Secretion in *Bacillus cereus*. *Microbiology* **153**, 2541-2552.

Samples, J.R., and Buettner, H. (1983) Corneal Ulcer Caused by a Biologic Insecticide (*Bacillus thuringiensis*). *American Journal of Ophthalmology* **95**, 258–260.

Sastalla, I., Matese, L.M., Pomersantseva, O.M., Pomersantsev, A.P., Keane-Myers, A., and Leppla, S.H. (2010) Activation of the Latent plcR Regulon in *Bacillus anthracis*. *Microbiology* **156**, 2982-2993

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelsom, J., Zeigler, D.R., and Dean, D.H. (1998) *Bacillus thuringiensis* and its Pesticidal Crystal Protein. Microbiology and Molecular Biology Reviews **62**, 775-806

Schoeni, J.L. and Wong, A.C.L. (1999) Heterogeneity Observed in the Components of Hemolysin BL, an Enterotoxin Produced by *Bacillus cereus*. *International Journal of Food Microbiology* **53**, 159-167.

Schoeni, J.L. and Wong, A.C.L. (2005) *Bacillus cereus* Food Poisoning and Its Toxins. *Journal of Food Protection* **68**, 636-648.

Senesi, S., Celandroni, F., Salvetti, S., Beecher, D. J., Wong, A. C. L. and Ghelardi, E. (2002) Swarming Motility in *Bacillus cereus* and Characterization of a *fliY* Mutant Impaired in Swarm Cell Differentiation. *Microbiology* **148**, 1785–1794.

Senesi, S., and Ghelardi, E. (2010) Production, Secretion and Biological Activity of *Bacillus cereus* Enterotoxins. *Toxins* **2**, 1690-1703

Senesi, S., Salvetti, S., Celandroni, F., and Ghelardi, E. (2010) Features of *Bacillus cereus* Swarm Cells. *Research in Microbiology*. **161**, 743-749

Sergeev, N., Distler, M., Vargas, M., Chizhikov, V., Herold, K.E., Rasooly, A. (2005) Microarray Analysis of *Bacillus cereus* Group Virulence Factors. *Journal of Microbiological Methods* **65**, 488-502

Setlow, B., Cowan, A.E. and Setlow, P. (2003) Germination of Spores of *Bacillus subtilis* with Dodecylamine. *Journal of Applied Microbiology* **95**, 637–648.

Setlow, B., Loshon, C.A., Genest, P.C., Cowan, A.E., Setlow, C. and Setlow, P. (2002) Mechanisms of Killing Spores of *Bacillus subtilis* by Acid, Alkaii and Ethanol. *Journal of Applied Microbiology* **92**, 362-375.

Setlow, B., McGinnis, K.A., Ragkousi, K. and Setlow, P. (2000) Effects of Major Spore-specific DNA Binding Proteins on *Bacillus subtilis* Sporulation and Spore Properties. *Journal of Bacteriology* **182**, 6906–6912.

Setlow, B., Peng, L., Loshon, C.A., Li, Y.Q., Christie, G., and Setlow, P. (2009) Characterization of the Germination of *Bacillus megaterium* Spores Lacking Enzymes that Degrade the Spore Cortex. *Journal of Applied. Microbiology* **107**, 318-328.

Setlow, P. (1983) Germination and outgrowth. In The Bacterial Spore, Vol. II ed. Hurst, A. and Gould, G.W. pp. 211–254. London: Academic Press.

Setlow, P. (2003) Spore Germination. *Current Opinion in Microbiology* **6**, 550-556

Setlow, P. (2006) Spores of *Bacillus subtilis*: Their Resistance to and Killing by Radiation, Heat and Chemicals. *Journal of Applied Microbiology* **101**, 514-525

Setlow, P., and Kornberg, A. (1970) Biochemical Studies of Bacterial Sporulation and Germination. *Journal of Biological Chemistry* **245**, 3637-3644.

Shadrin, A.M., Shapyrina, E.V., Siunov, A.V., Severinov, K.V., and Solonin, A.S. (2007) *Bacillus cereus* Pore-forming Toxins Hemolysin II and Cytotoxin K: Polymorphism and Distribution of Genes Among Representatives of the Cereus Group. Mikrobiologiia 76, 462–470.

Slamti, L. and Lereclus, D. (2002) A Cell-Cell Signaling Peptide Activates the PlcR Virulence Regulon in Bacteria of the *Bacillus cereus* Group. *The EMBO Journal* **21**, 4550-4559.

Slamti, L., Perchat, S., Gominet, M., Vilas-Boas, G., Fouet, A., Mock, M., Sanchis, V., Chaufaux, J., Gohar, M. and Lereclus, D. (2004) Distant Mutations in *PlcR* Explain Why Some Strains of the *Bacillus cereus* Group are Nonhemolytic. *Journal of Bacteriology* **186**, 3531-3538.

Song, L., Hobaugh, M.R., Shustak, C., Cheley, S., Bayley, H., and Gouaux, J.E. (1996) Structure of *staphylococcal* A-hemolysin, a Heptameric Transmembrane Pore. *Science* 274, 1859–1866.

Spira, W.M., and Goepfert, J.M. (1972) *Bacillus cereus*-induced Fluid Accumulation in Rabbit Ileal Loops. Applied Microbiology 24, 341–348.

Stalheim, T., and Granum, P.E. (2001) Characterisation of Spore Appendages from *Bacillus cereus* Strains. *Journal of Applied Microbiology* **91**, 839-845

Steil, L., Hoffmann, T., Budde, I., Völker, U. and Bremer, E. (2003). Genome-wide Transcriptional Profiling Analysis of Adaptation of *Bacillus subtilis* to High Salinity. *Journal of Bacteriology* **185**, 6358–6370

Steil, L., Serrano, M., Henriques, A.O., Volker, U. (2005) Genome-wide Analysis of Temporally Regulated and Compartment-specific Gene Expression During Spore Development in *Bacillus subtilis*. *Microbiology* **15**, 399–420

Stenfors Arnsen, L.P., Fagerlund, A., and Granum, P.E (2008) From Soil to Gut: *Bacillus cereus* and its Food Poisoning Toxins. *FEMS Microbiology Reviews* **32**, 579-606.

Stenfors Arnesen, L., Granum, P.E., Buisson, C., Bohlin, J., and Nielsen-LeRoux, C. (2011) Using an Insect Model to Assess Correlation Between Temperature and Virulence in *Bacillus weihenstephanensis* and *Bacillus cereus*. *FEMS Microbiology Letters* **317**, 196-202

Stenfors, L.P., Mayr, R., Scherer, S. and Granum, P.E. (2002) Pathogenic Potential of Fifty *Bacillus weihenstephanensis* Strains. *FEMS Microbiology Letters* **215**, 47-51.

Stenfors Arnesen, L.P., O'Sullivan, K. and Granum, P.E. (2007) Food Poisoning Potential of *Bacillus cereus* Strains from Norwegian Dairies. *International Journal of Food Microbiology* **116**, 292-296.

Stuy, J. H. 1956. Studies on the Mechanism of Radiation Inactivation on Micro-organisms. Inactivation of Germinating Spores of *Bacillus cereus*. *Biochimica et Biophysica Acta* **22**, 241–246.

Swiecicka, I. (2008) Natural Occurrence of *Bacillus thuringiensis* and *Bacillus cereus* in Eukaryotic Organisms: A Case for Symbiosis. Biocontrol Science and Technology **18**, 221-239

Swiecicka, I., and De Vos, P. (2002) Properties of *Bacillus thuringiensis* Isolated from Bank Voles. Journal of Applied Microbiology **94**, 60-64

Swiecicka, I., and Mahillon, J. (2006) Diversity of Commensal *Bacillus cereus Sensu Lato* Isolated from the Common Sow Bug (*Porcellio scaber*, Isopoda). FEMS Microbiology Ecology **56**, 132-140

Sylvestre, P., Couture-Tosi, E., and Mock, M. (2002) A Collagen-like Surface Glycoprotein is a Structural Component of the *Bacillus anthracis* Exosporium. *Molecular Microbiology* 45, 169–78

Tam, N.K.M., Uyen, N.Q., Hong, H.A., Duc, L.H., Hoa, T.T., Serra, C.R., Henriques, A.O., and Cutting, S.M. (2006) The Intestinal Life Cycle of *Bacillus subtillis* and Close Relatives. *Journal of Bacteriology* **188**, 2692-2700

Tauveron, G., Slomianny, C., Henry, C., and Faille, C. (2006) Variability Among *Bacillus cereus* Strains in Spore Surface Properties and Influence on their Ability to Contaminate Food Surface Equipment. *International Journal of Food Microbiology* **110**, 254–262.

Te Giffel MC, Beumer RR, Granum PE & Rombouts FM (1997) Isolation and Characterisation of *Bacillus cereus* from Pasteurised Milk in Household Refrigerators in The Netherlands. *International Journal Food Microbiology* **34**, 307–318

Thaenthanee, S., Wong, A.C., and Panbangred, W. (2005) Phenotypic and Genotypic Comparisons Reveal a Broad Distribution and Heterogeneity of Hemolysin BL Genes Among *Bacillus cereus* Isolates. *International Journal of Food Microbiology* **105**, 203–212.

Thompson, N.E., Ketterhagen, M.J., Bergdoll, M.S., and Schantz EJ (1984) Isolation and Some Properties of an Enterotoxins Produced by *Bacillus cereus*. *Infection and Immunity* **43**, 887–894.

Thompson, A., Schafer, J., Kuhn, K., Kienle, S., Schmidt, G., Neumann, T., Johnstone, R., Mohammed, A.K., Hamon, C. (2003) Tandem Mass Tags: A Novel Quantification Strategy for Comparative Analysis of Complex Protein Mixtures by MS/MS. *Analytical Chemistry* **75**, 1895-1904

Thorsen, L., Hansen, B.M., Nielsen, K.F., Hendriksen, N.B., Phipps, R.K., and Budde, B.B. (2006) Characterization of Emetic *Bacillus weihenstephanensis*, a New Cereulide-producing Bacterium. *Applied Environment Microbiology* **72**, 5118–5121.

Tjalsma, H., Bolhuis, A., Jongbloed, J.D.H., Bron, S., and van Dijl, J.M. (2000) Signal-peptide-dependent Protein Transport in *Bacillus subtilis*: a Genome-based Survey of the Secretome. *Microbiology Molecular Biology Review* **64**, 515–547.

Tomiuk, S., Hofmann, K., Nix, M., Zumbansen, M., and Stoffel, W. (1998) Cloned Mammalian Neutral Sphingomyelinase: Functions in Sphingolipid Signalling. *Proceedings of the National Academy of Sciences* **95**, 3638-3643

Tran, S-L., Guillemet, E., Ngo-Camus, M., Clybouw, C., Puhar, A., Moris, A., Gohar, M., Lereclus, D., Ramarao, N. (2010) Haemolysin II is a *Bacillus cereus* Virulence Factor that Induces Apoptosis of Macrophages. *Cellular Microbiology* **13**, 92-108

Turnbull, P.C., Jorgensen, K., Kramer, J.M., Gilbert, R.J., and Parry, J.M. (1979) Severe Clinical Conditions Associated with *Bacillus cereus* and the Apparent Involvement of Exotoxins. *Journal Clinical Pathology* **32**, 289–293.

Tweedie-Cullen, R. Y., and Livingstone-Zatchej, M. (2009). Quantitative Analysis of Protein Expression using iTRAQ and Mass Spectrometry. *Nature Protocols*. 89

van Schaik, W., and Abee, T. (2005) The Role of σ^B in the Stress Response of Gram-Positive Bacteria-Targets for Food Preservation and Safety. *Current Opinions in Biotechnology* **16**, 218–224.

van Schaik, W., Tempelaars, M.H., Wouters, J.A., de Vos, W.M., and Abee, T. (2004) The Alternative Sigma Factor σ^B of *Bacillus cereus*:

Response to Stress and Role in Heat Adaptation. *Journal of Bacteriology* **186**, 316-325

Vepachedu, V.R. and Setlow, P. (2004) Analysis of the Germination of Spores of *Bacillus subtilis* with Temperature Sensitive *spo* Mutations in the *spoVA* Operon. *FEMS Microbiology Letters* **239**, 71–77

Vitzthum, F., Geiger, G., Bisswanger, H., Brunner. H., and Bernhagen, J. (1999) A Quantitative Fluorescence-based Microplate Assay for the Determination of Double-stranded DNA using SYBR Green I and a Standard Ultraviolet Transilluminator Gel Imaging System. *Analytical Biochemistry* **276**, 59-64.

de Vries, Y.P., Hornstra, L.M., de Vos, W.M., and Abee, T. (2004) Growth and Sporulation of *Bacillus cereus* ATCC14579 Under Defined Conditions: Temporal Expression of Genes for Key Sigma Factors. *Applied and Environmental Microbiology* **70**, 2514-2519

Vyas, J., Cox, J., Setlow, B., Coleman, W.H., and Setlow, P. (2011) Extremely Variable Conservation of γ-Type Small, Acid-Soluble Proteins from Spores of Some Species in the Bacterial Order *Bacillales*. *Journal of Bacteriology* **193**, 1884-1892

Walz, A., Mujer, C.V., Connolly, J.P., Alefantis, T., Chafin, R., Dake, C., Whittington, J., Kumar, S.P., Khan, A.S., and DelVecchio, V.G. (2007) *Bacillus anthracis* Secretome Time Course Under Host-Simulated Conditions and Identification of Immunogenic Proteins. *Proteome Science* **5**, 5-11

Wang, S.T., Setlow, B., Conion, E.M., Lyin, J.L., Imamura, D., Sato, T., Setlow, P., Losick, P., and Eichenberger, P. (2006) The Forespore Line of Gene Expression in *Bacillus subtiliis*. *Journal of Molecular Biology* **357**, 16-37

Westphal, A.J., Price, B.P., Leighton, T.J. and Wheeler, K.E. (2003) Kinetics of Size Changes of Individual *Bacillus thuringiensis* spores in Response to Changes in Relative Humidity. *Proceedings of the National Academy of Sciences* **100**, 3461–3466.

Wiencek, K.M., Klapes, N.A., and Foegeding, P.M. (1990) Hydrophobicity of *Bacillus* and *Clostridium* spores. *Applied and Environmental Microbiology* **56**, 2600–2605.

Wiese, S., Reidegeld, K.A., Meyer, H.E., and Warscheid, B. (2007) Protein Labeling by iTRAQ: A New Tool for Quantitative Mass Spectrometry in Proteome Research. *Proteomics* **7**, 340-350

Wijnands, L.M., Dufrenne, J.B., van Leusden, F.M. (2002) Vol. 250912001 RIVM report, pp. 983-8.

Wijnands, L.M., Dufrenne, J.B., van Leusden, F.M., and Abee, T. (2007) Germination of *Bacillus cereus* Spores is Induced by Germinants from Differentiated Caco-2 Cells, a Human Cell Line Mimicking the Epithelial Cells of the Small Intestine. *Applied and Environmental Microbiology* **73**, 5052-5054

Wijnands, L.M., Dufrenne, J.B., Zwietering, M.H., and van Leusden, F.M. (2006) Spores from Mesophilic *Bacillus cereus* Strains Germinate Better and Grow Faster in Simulated Gastro-Intestinal Conditions than Spores from Psychrotrophic Strains. *International Journal of Food Microbiology* **112**, 120-128

Wold, A.E., Dahlgren, U.I.H., Hanson, L.A., Mattsby-Baltzer, I., and Midvetdt, T. (1989) Difference Between Bacterial and Food Antigens in Mucosal Immunogenicity. *Infection and Immunity* **57**, 2666-2673

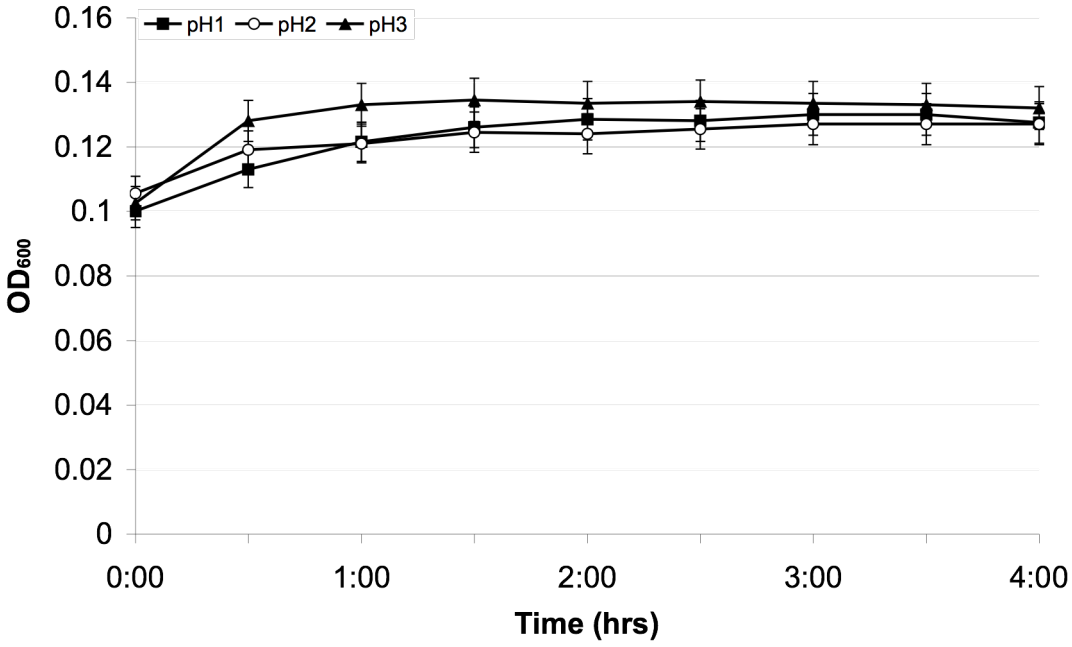
Wong, M.L., Medrano, J.F. (2005) Real-time PCR for mRNA Quantitation. *Biotechniques* **39**, 75-85.

Yushan, H., Lei, L., Weijia, L., and Xiaoguang, C. (2010) Sequence Analysis of the *groEL* Gene and Its Potential Application in Identification of Pathogenic Bacteria. *African Journal of Microbiology Research* **4**, 1733-1741

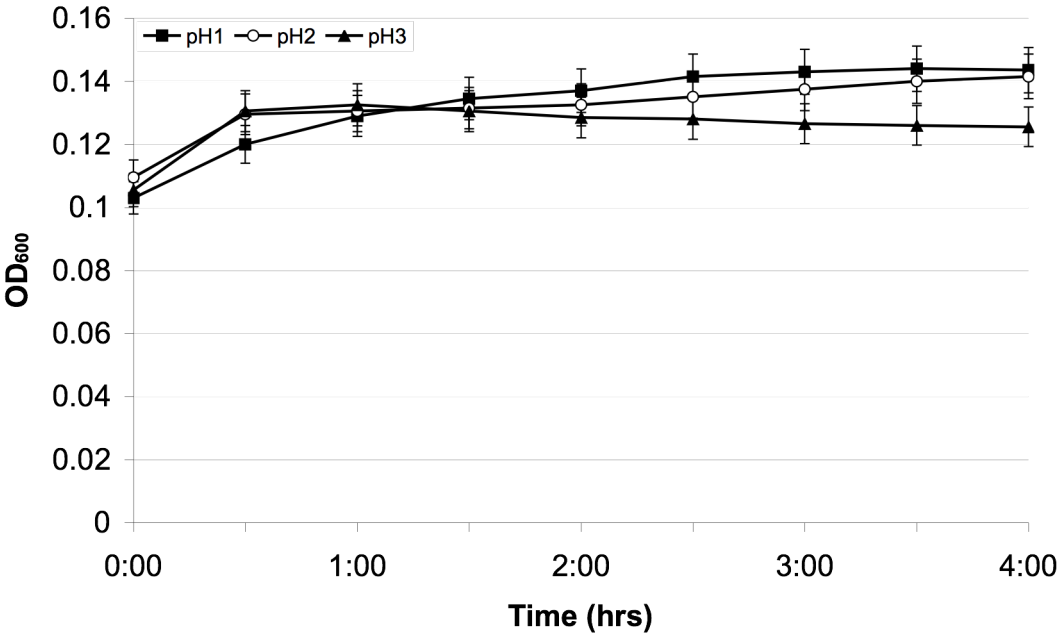
Zhang, B., and Kroos, L. (1997) A Feedback Loop Regulates the Switch From Once Sigma Factor to the Next in the Cascade Controlling *Bacillus subtilis* Mother Cell Gene Expression. *Journal of Bacteriology* **179**, 6138-6144

Zhang, B., Struffi, P., and Kroos, L. (1999) σ^K Can Negatively Regulate *sigE* Expression by Two Different Mechanisms During Sporulation of *Bacillus subtilis*. *Journal of Bacteriology* **181**, 4081-4088

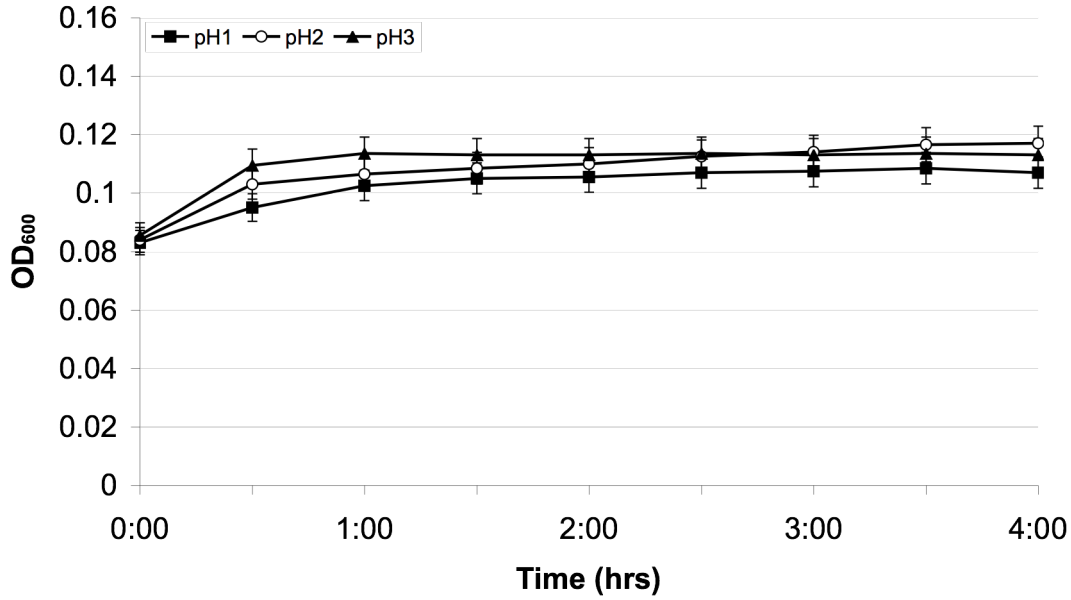
Appendix A
Vegetative Cell Stomach Simulation (10^5CFU/ml)
B. cereus
27



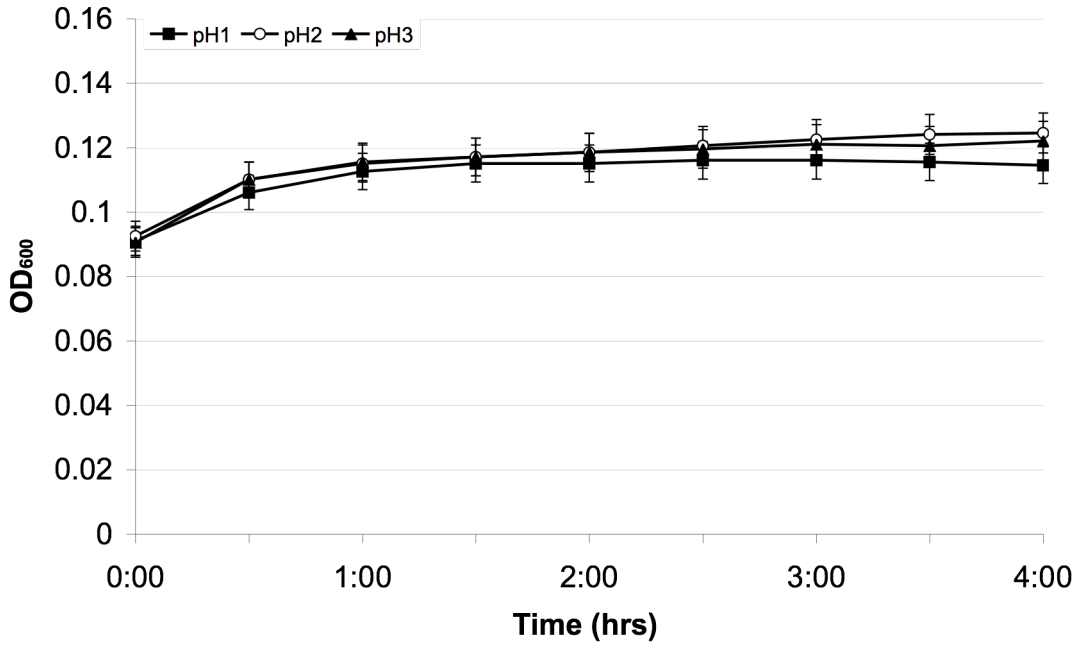
883-00



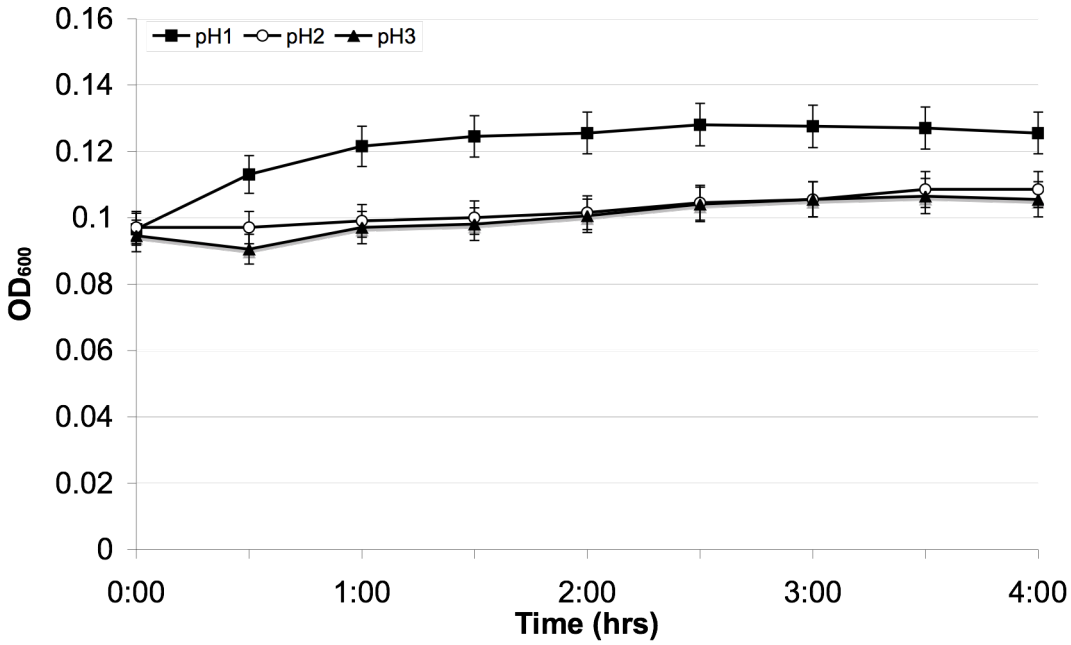
F2081B98



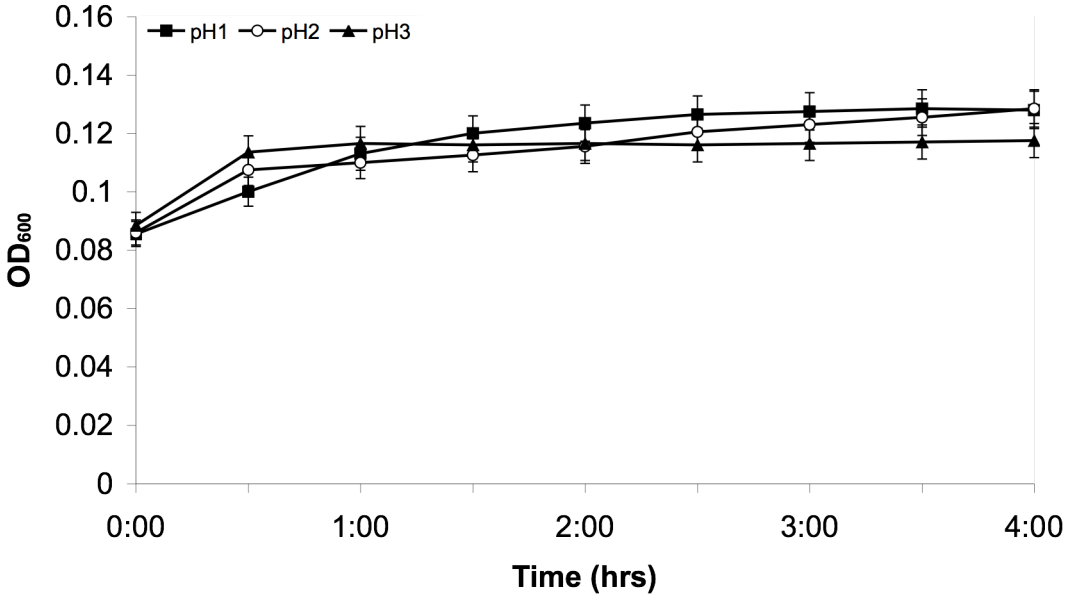
98HMPL63



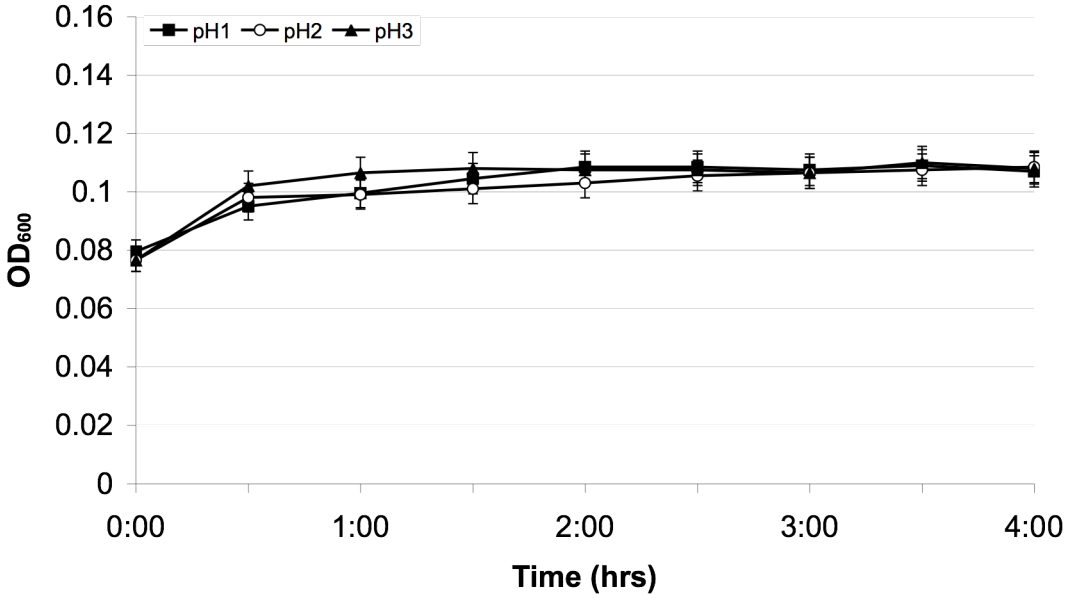
B. weihenstephanensis
10390



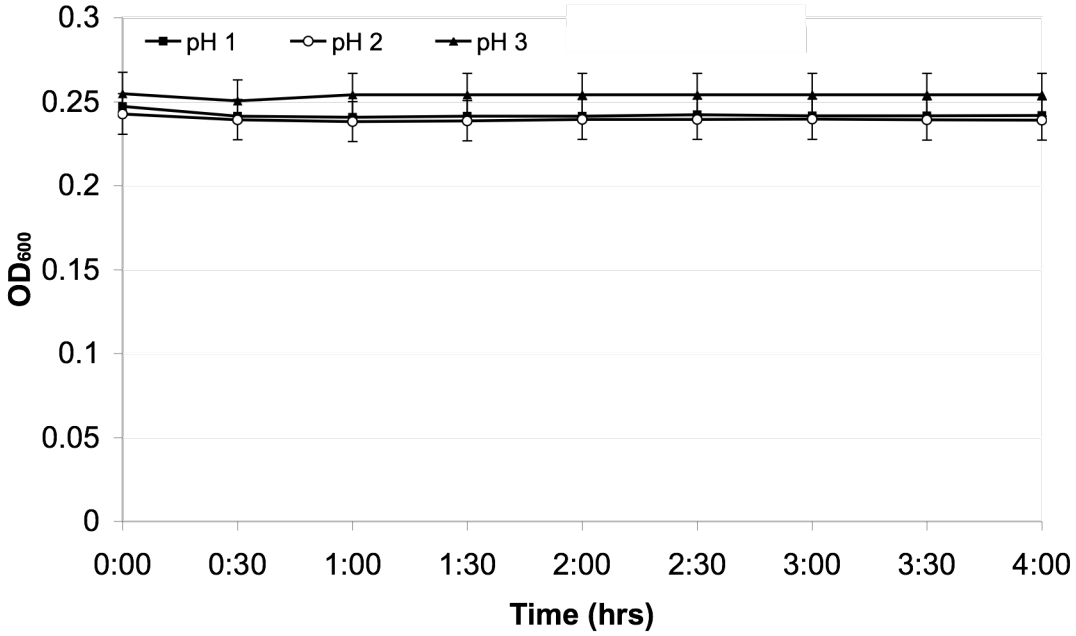
10396



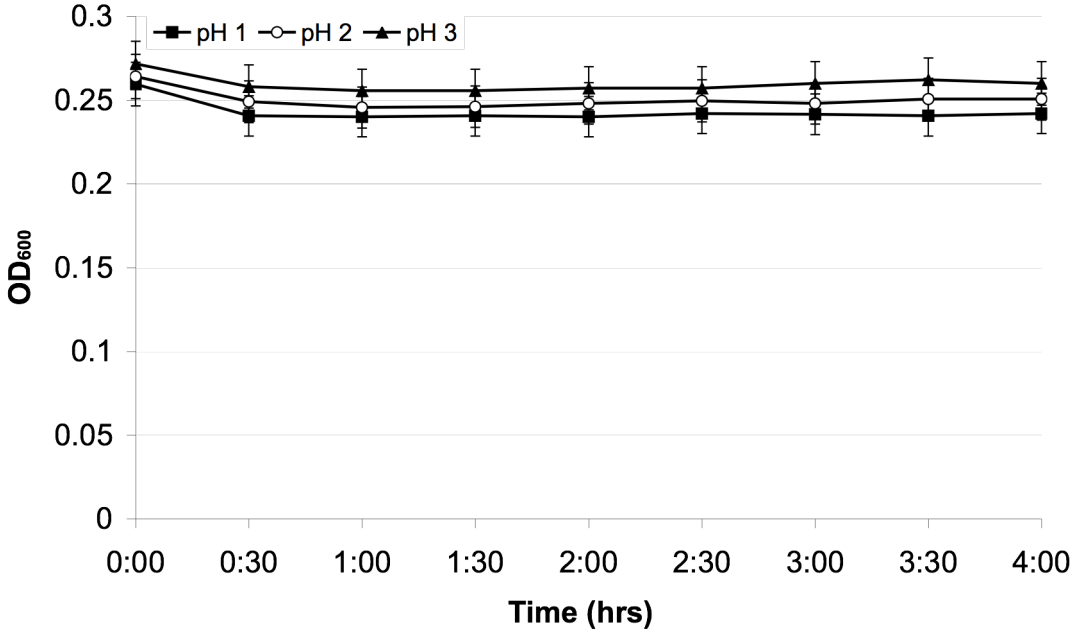
10202


pH1
pH2
pH3
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
OD600
0:00
1:00
2:00
3:00
4:00
Time (hrs)

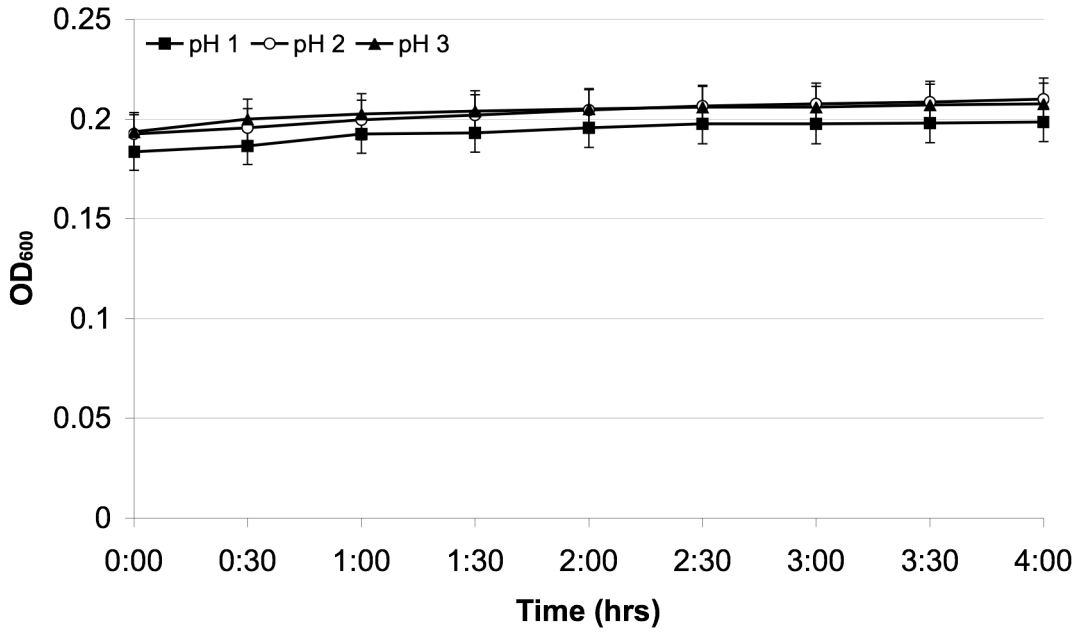
Individual Spore Stomach Simulation Data (10^5spores/ml)
B. cereus
27



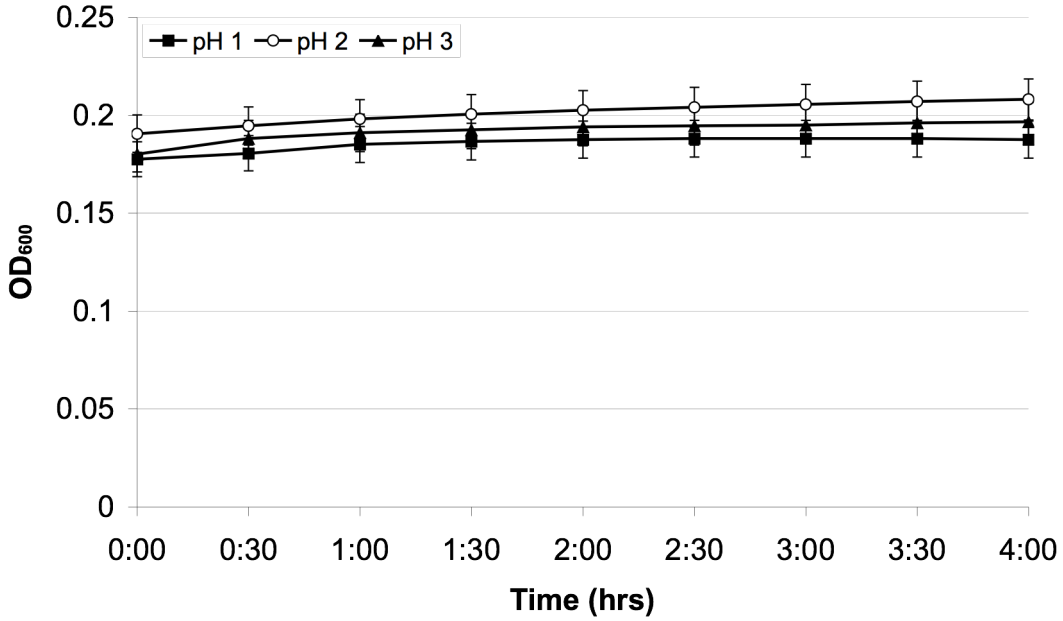
883-00



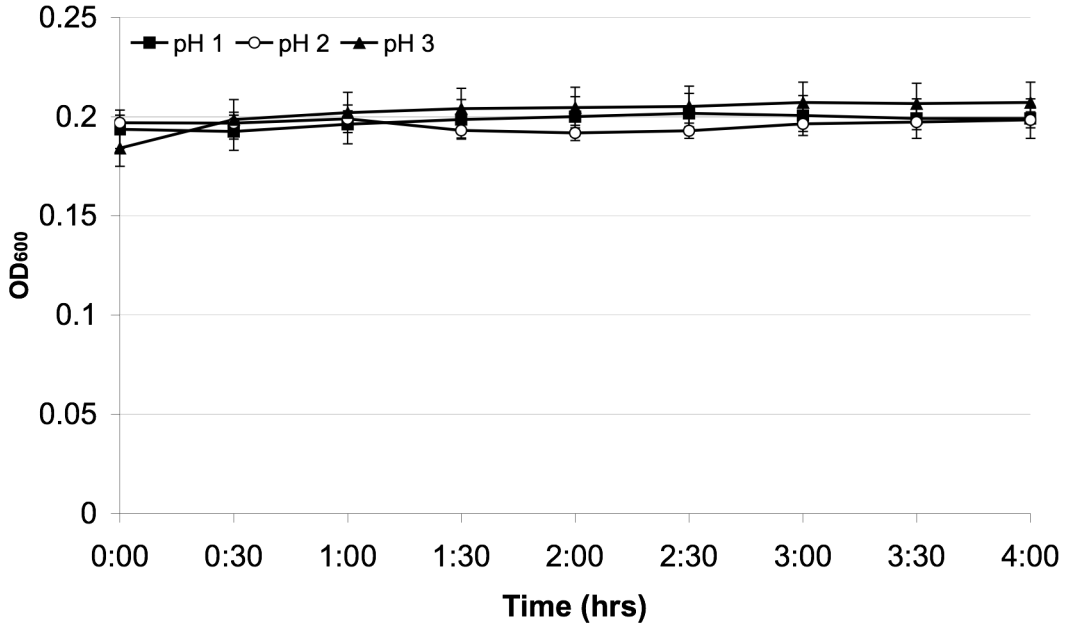
F2081B/98



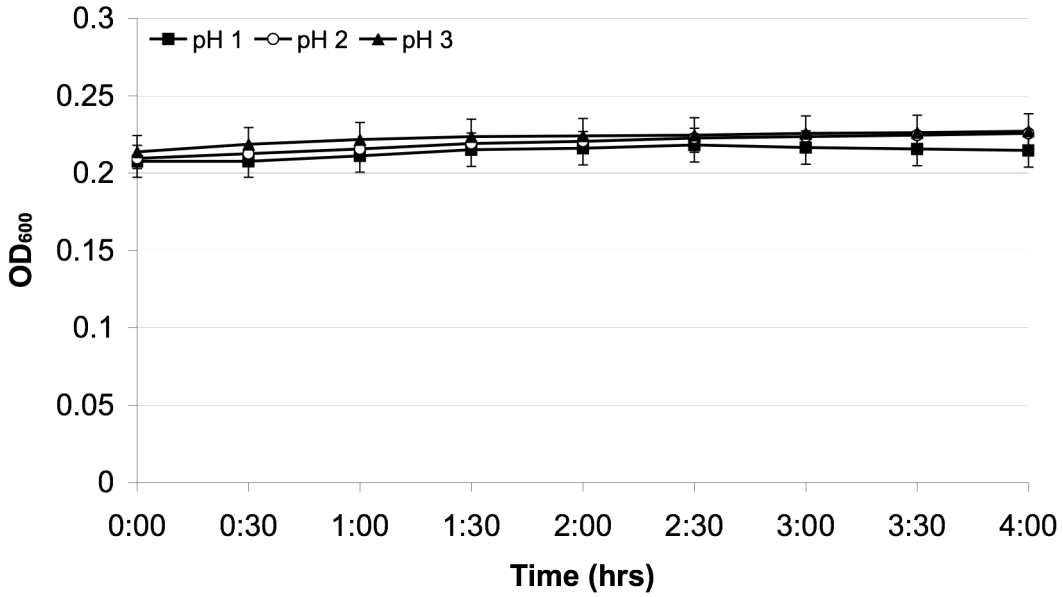
98HMLP63



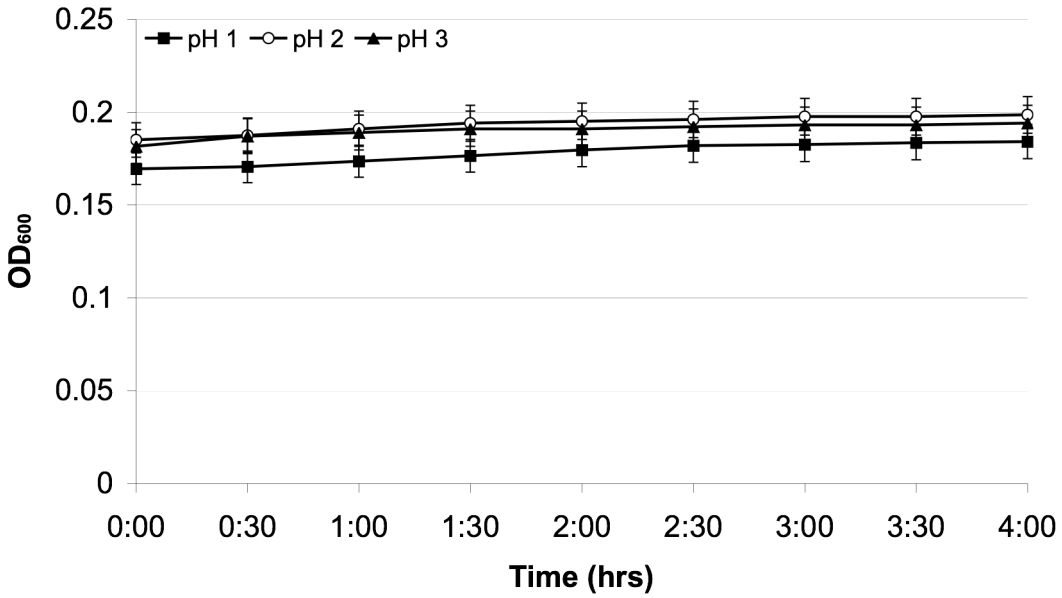
***B. weihenstephanensis*;**
10390



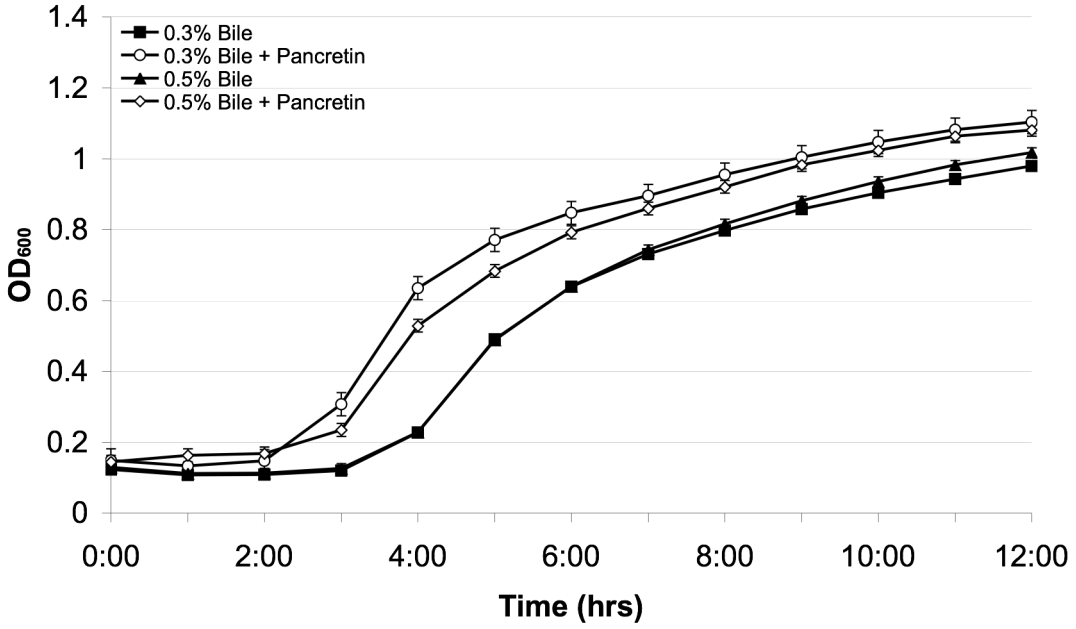
10396



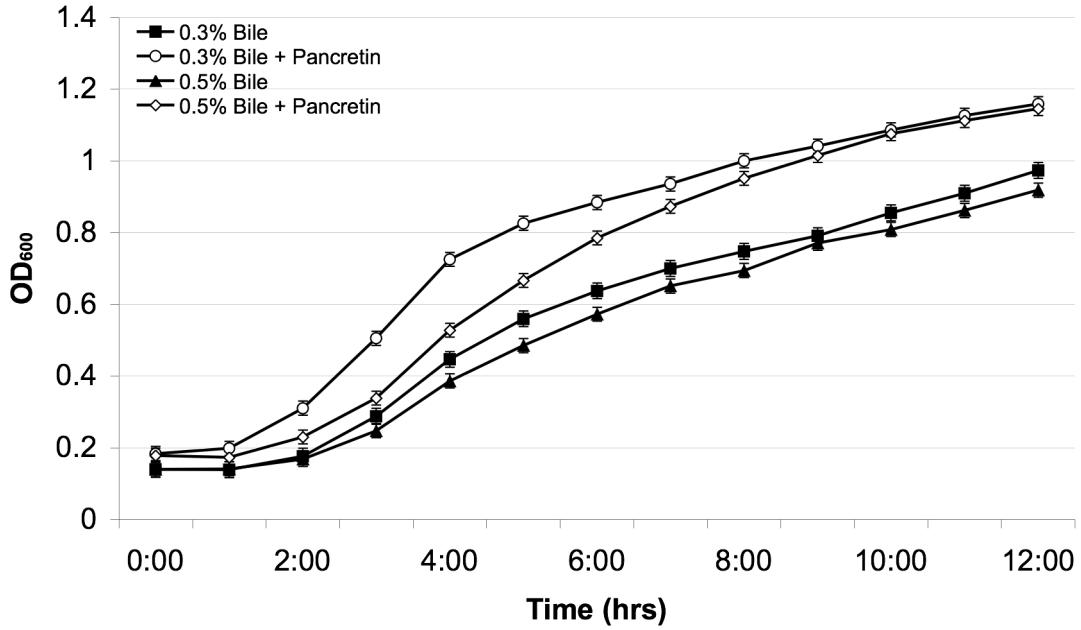
10202


pH 1
pH 2
pH 3
0.25
0.2
0.15
0.1
0.05
0
OD600
0:00
0:30
1:00
1:30
2:00
2:30
3:00
3:30
4:00
Time (hrs)

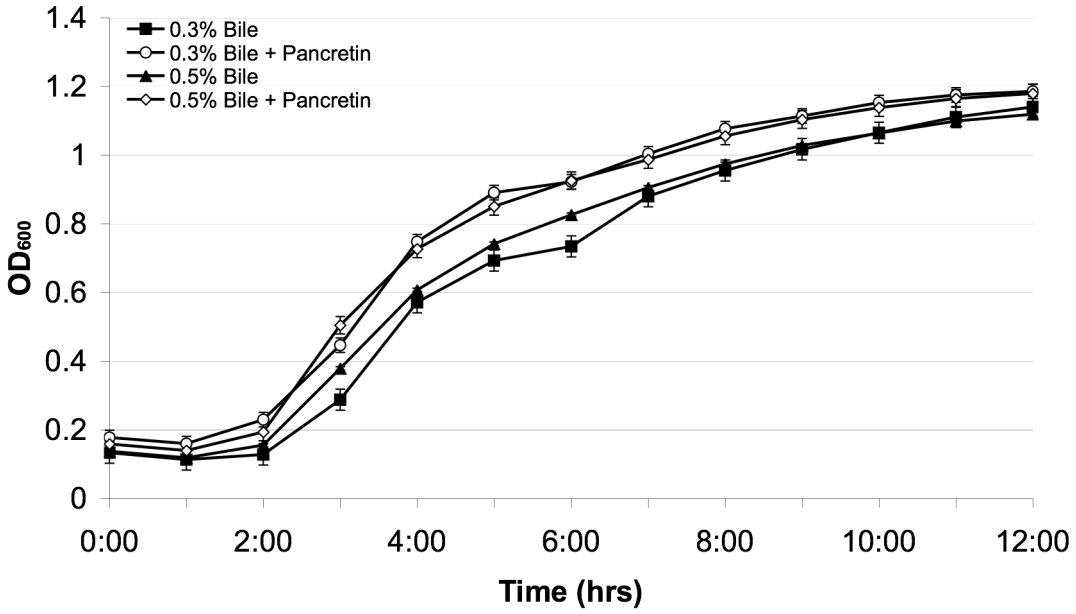
Small Intestinal Simulation; 10^3CFU/ml Vegetative cell
B. cereus
27



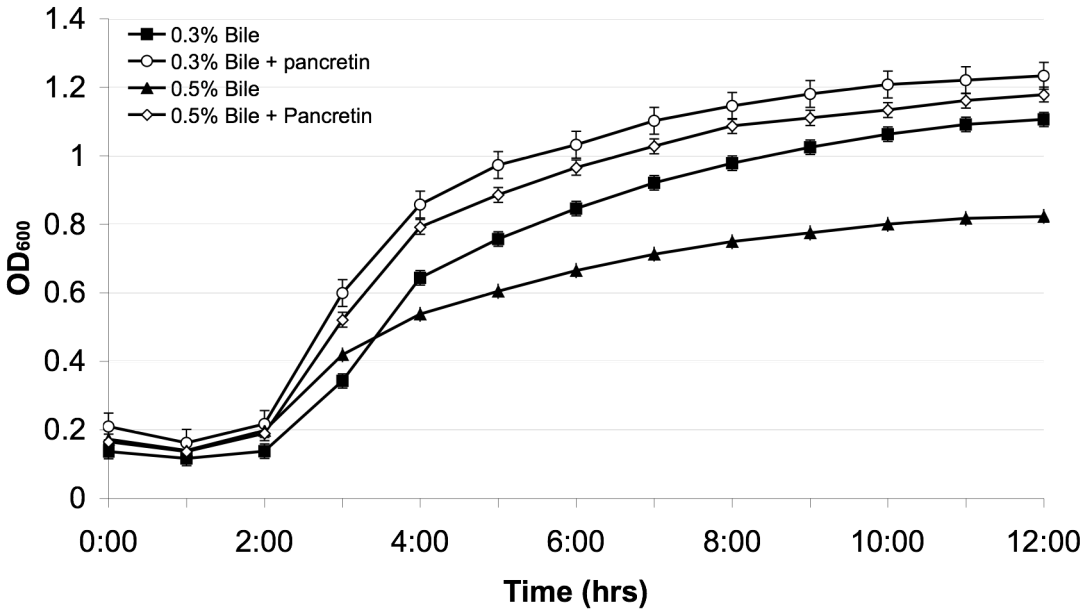
883-00



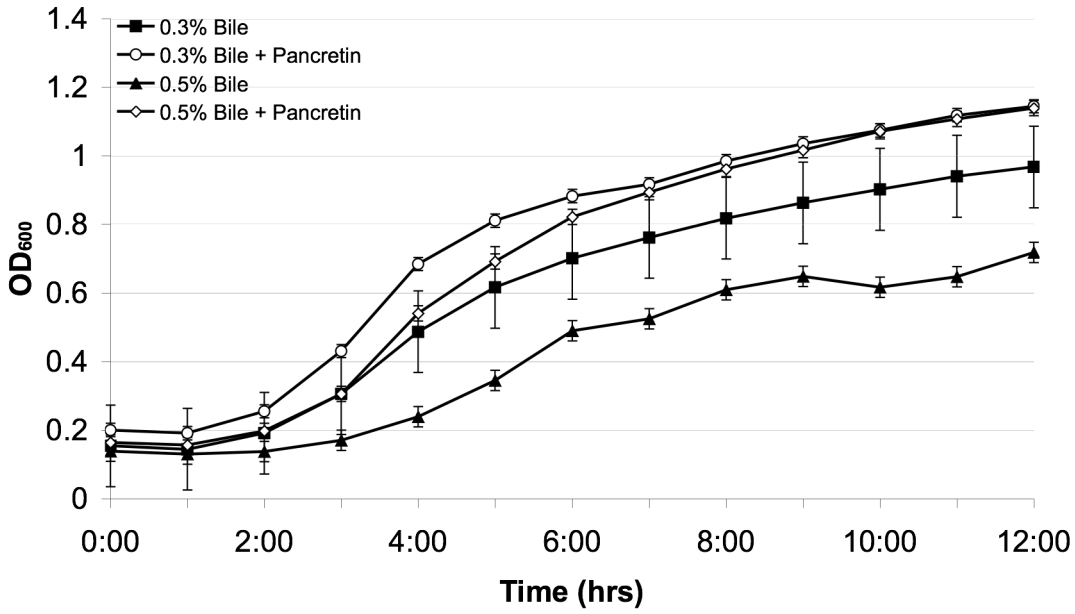
F2081B/98



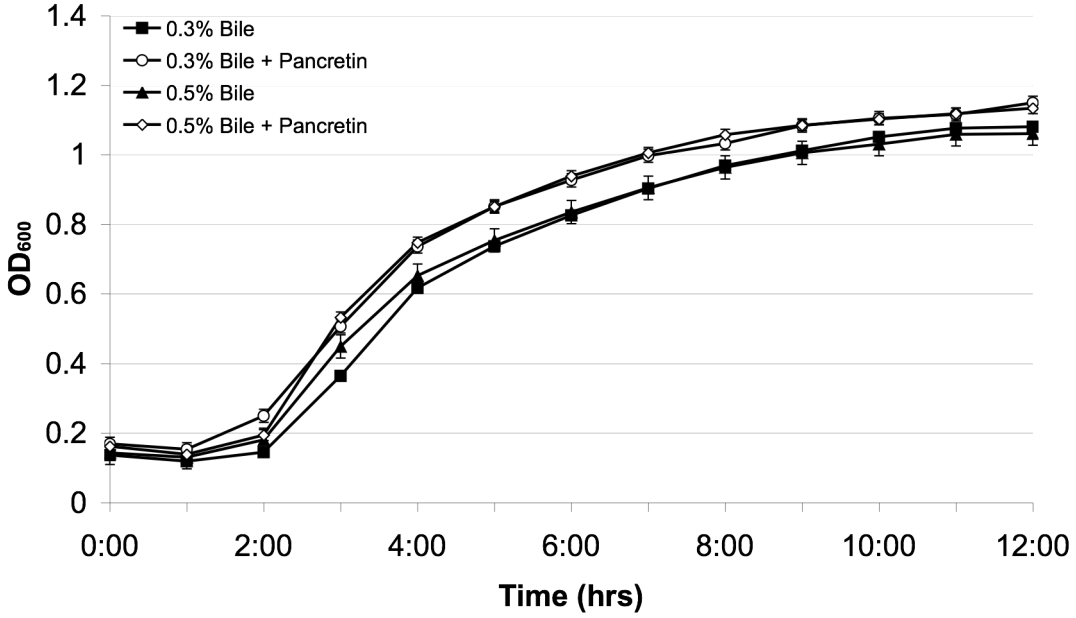
98HMPL63



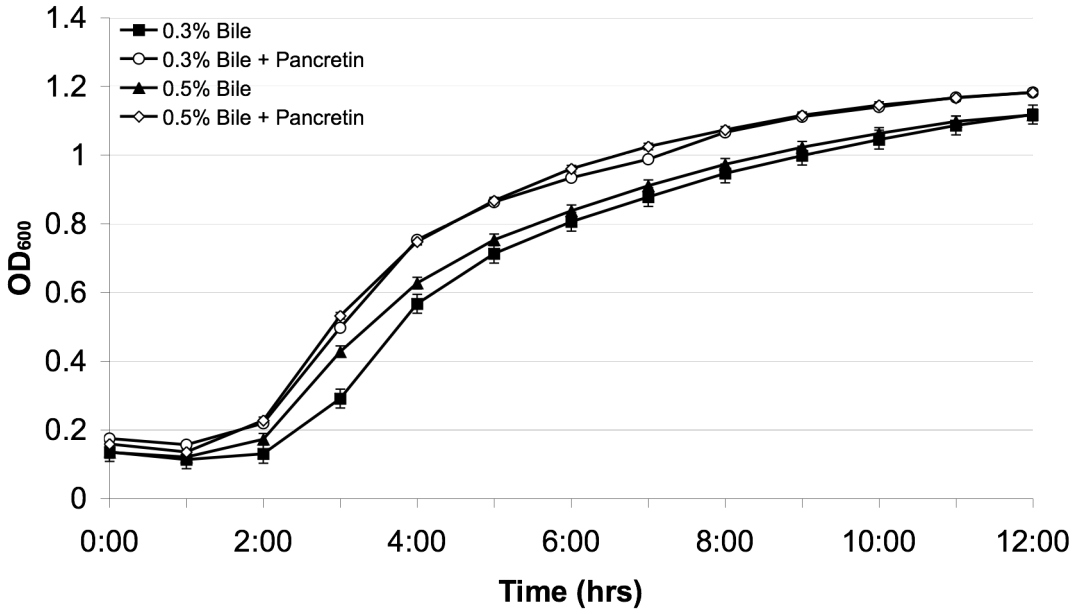
B. weihenstephanensis;
10390



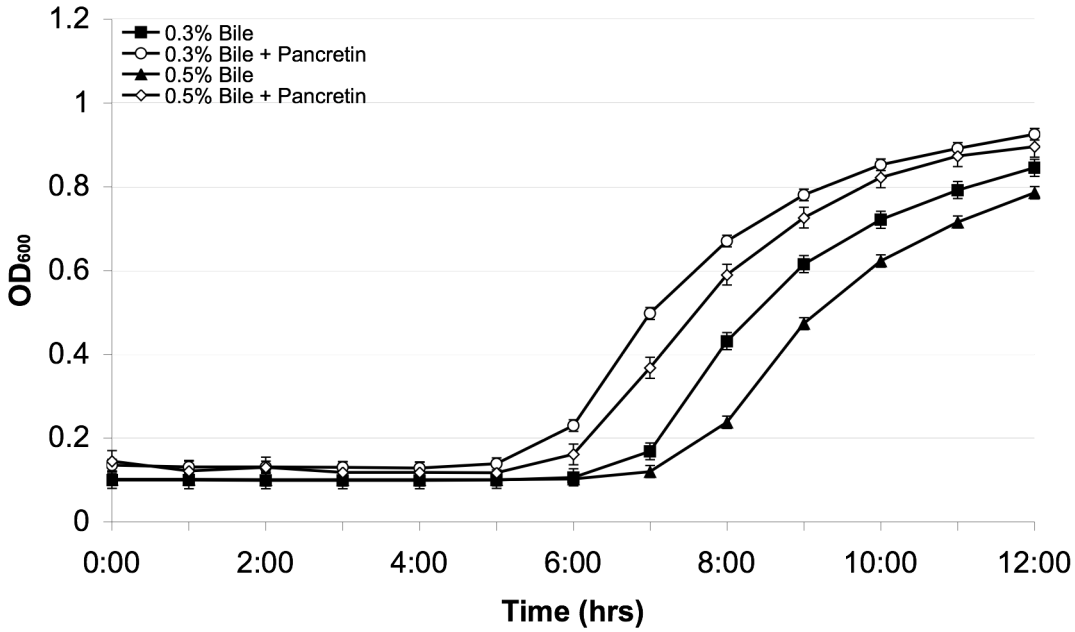
10396



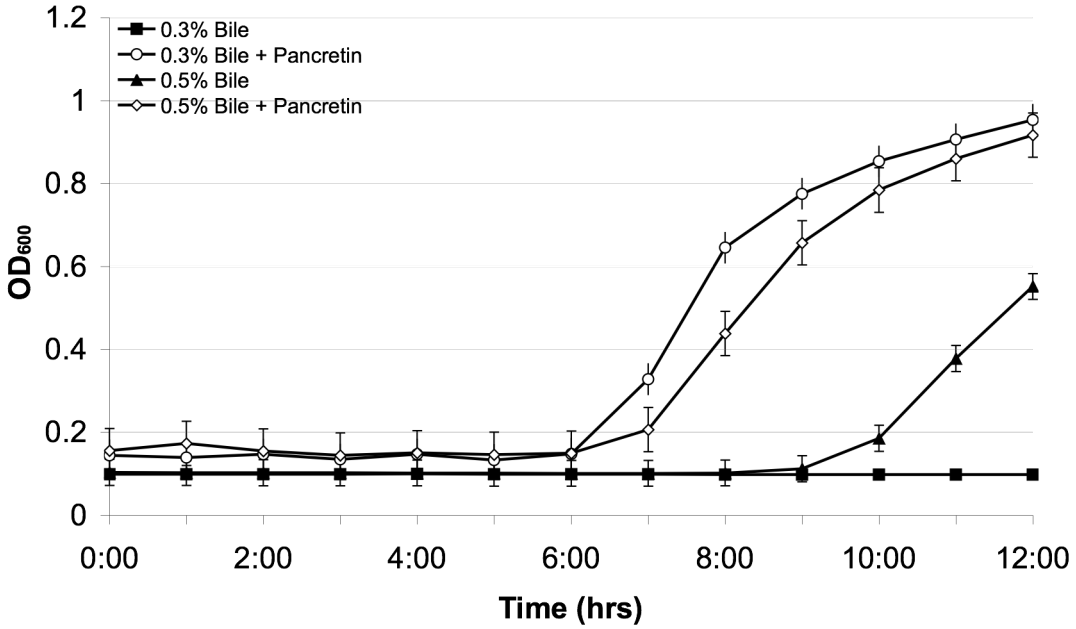
10202



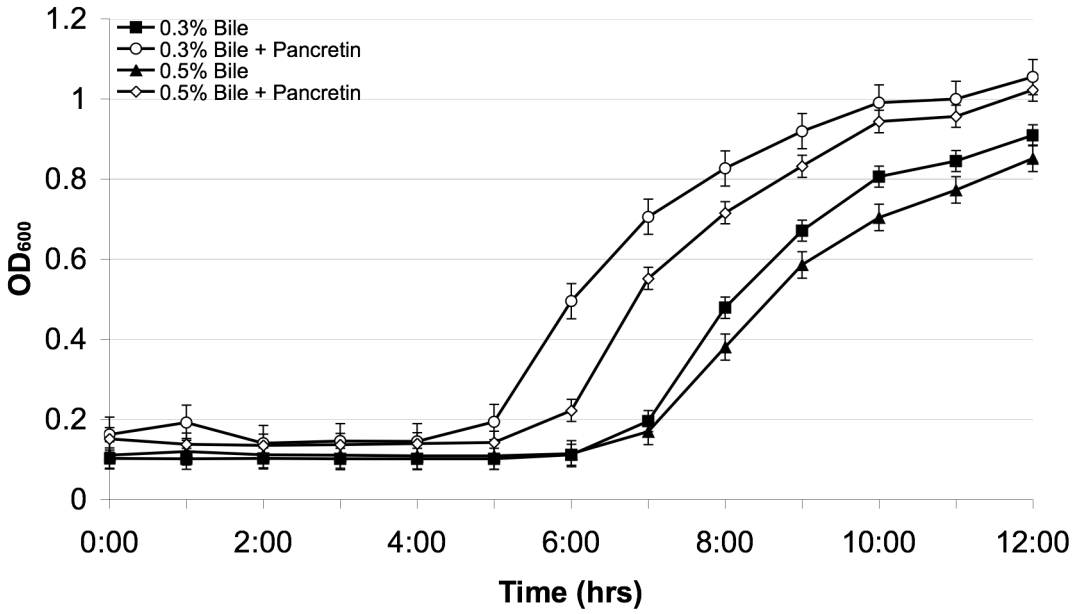
Small Intestinal Simulation; 10^3spores/ml
B. cereus
27



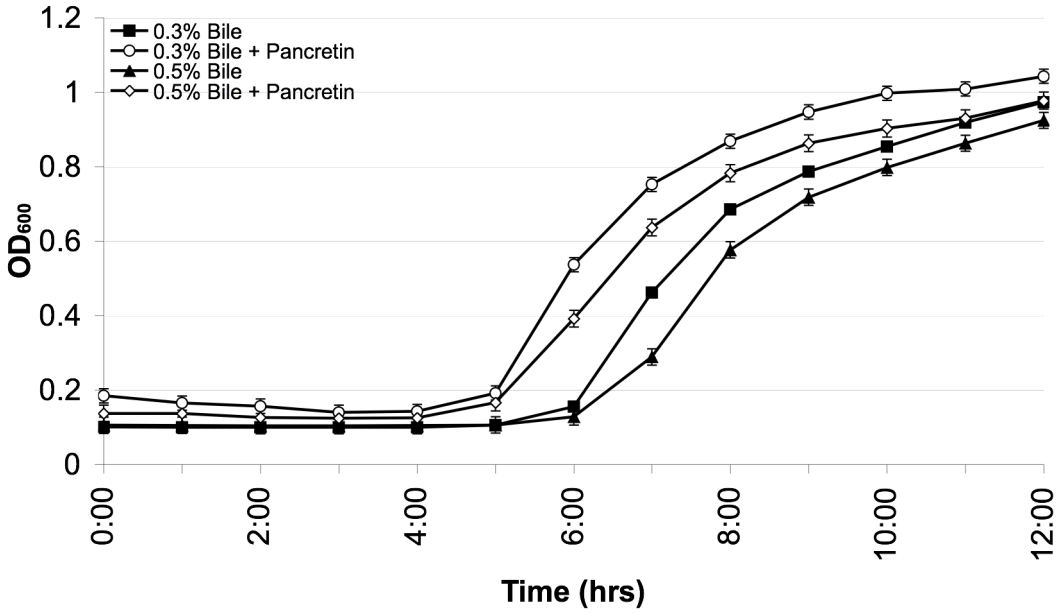
883-00



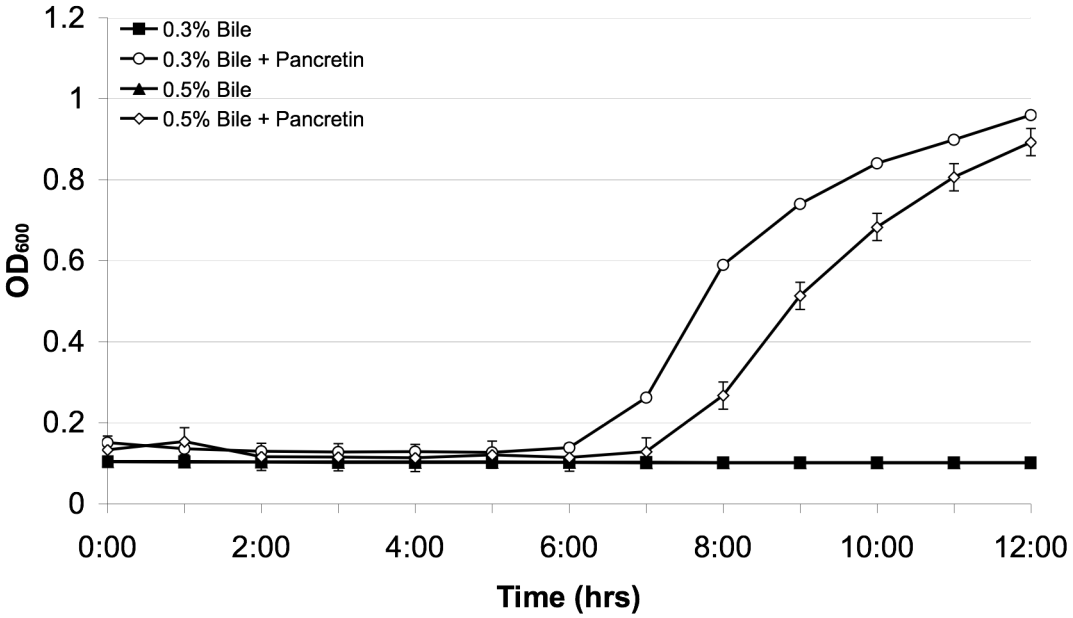
F2081B/98



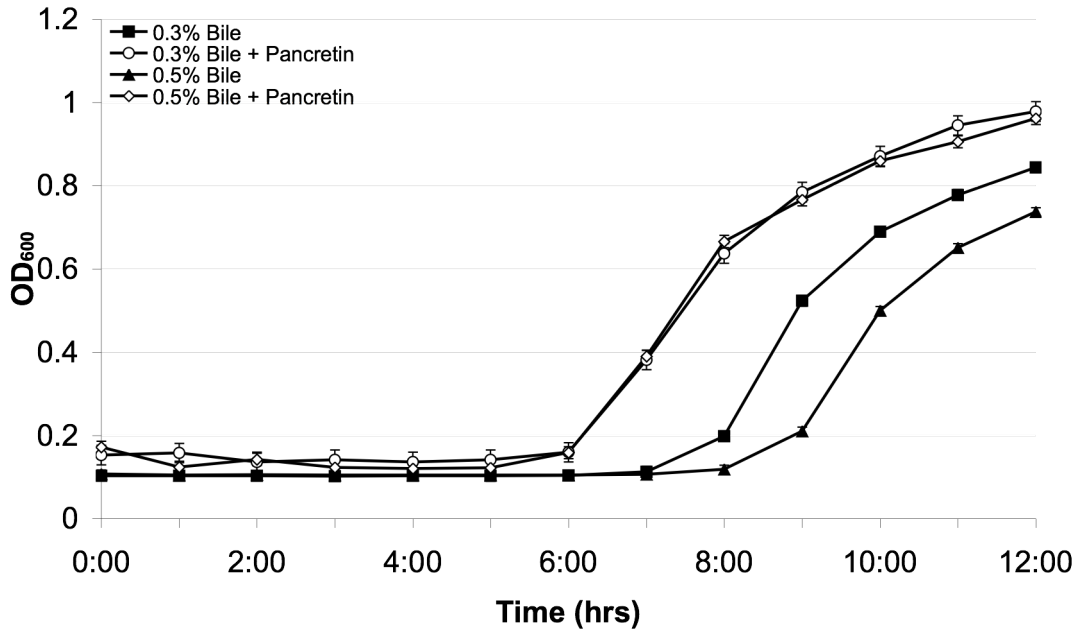
98HMPL63



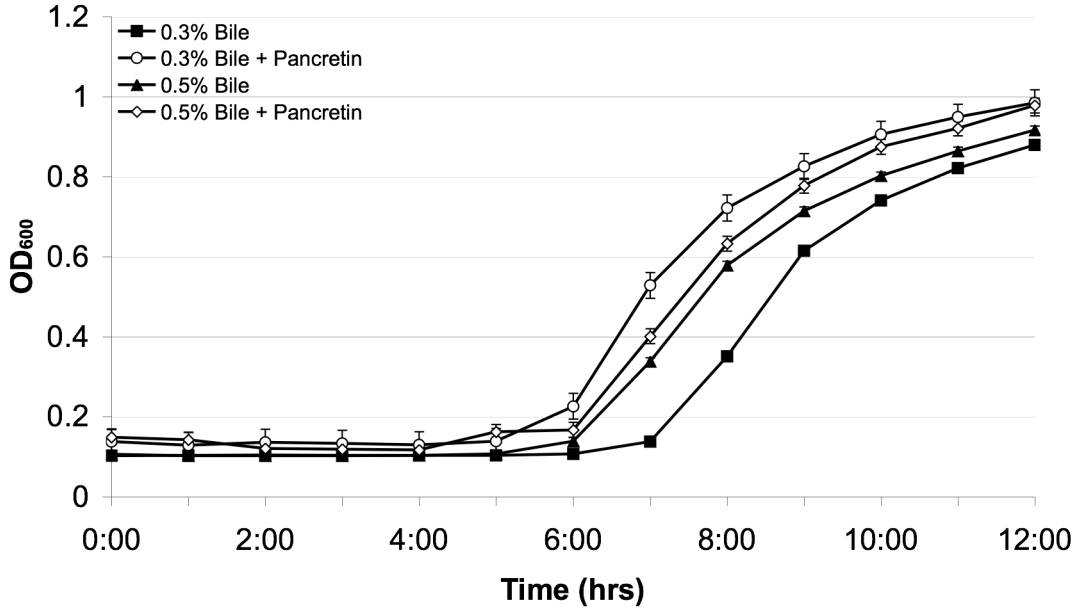
***B. weihenstephanensis*;**
10390



10396


0.3% Bile
0.3% Bile + Pancretin
0.5% Bile
0.5% Bile + Pancretin
OD600
1.2
1
0.8
0.6
0.4
0.2
0
0:00
2:00
4:00
6:00
8:00
10:00
12:00
Time (hrs)

10202


0.3% Bile
0.3% Bile + Pancretin
0.5% Bile
0.5% Bile + Pancretin
OD600
1.2
1
0.8
0.6
0.4
0.2
0
0:00
2:00
4:00
6:00
8:00
10:00
12:00
Time (hrs)

Appendix B
***B. cereus* Mascot Results**
27

Gene	Accession Number	MW	pI	% Coverage	Mowse	Matched	Homology	Identity
Putative microbial collagenase	B5UIY5	109.9	5.42	10	364	28	0	28
Putative microbial collagenase	B7IWT0	110	5.32	8	329	27	0	27
nheA	Q2TM57	43.7	5.21	13	269	22	0	22
Putative microbial collagenase	B3ZE05	110.6	5.22	7	265	17	0	17
Putative microbial collagenase	Q4MQT1	110	5.26	8	258	22	0	22
Flagellin	Q73AJ3	49.8	5.44	9	206	11	0	11
Flagellin	A0RC95	52.3	5.2	8	206	11	0	11
Flagellin	Q2WI55	43.6	5.1	10	155	11		11
Sphingomyelin phosphodiesterase C	B7HBV7	37.6	6.1	18	145	11	0	11
Phospholipase C	Q3EXN7	32.2	6.45	14	144	19	0	19
Flagellin	Q2EI47	43.1	5.43	5	130	8	0	8
hblD	A0RCP7	40.1	6.85	6	79	6	0	6
flgL	B5UR12	32.0	5.09	9	79	10	0	10

Flagelar hook protein	B5UR13	48.1	5.22	5	60	3	0	3
nprB	Q2PUL5	65.5	5.92	8	59	5	0	5
hblA	AORFT7	42.2	5.28	5	57	5	0	5
hblC	AORFT9	50.6	5.28	2	57	2	0	2
Flagellin	Q2EI31	37.0	4.96	9	55	4	0	4
Cell wall hydrolase	A0RL24	61.1	9.56	4	53	3	0	3

883-00

Gene	Accession Number	MW	pI	Mowse	% Coverage	Matched	Homology	Identity
Collagenase	B7IWT0	110.0	5.32	304	6	23	8	9
nheA	Q2TM57	43.7	5.21	296	21	28	3	15
Collagenase	B5UIY5	109.9	5.42	252	7	22	6	7
Flagellin	Q73AJ3	53.3	5.44	237	17	14	6	3
Flagellin	A0RC95	110.6	5.2	229	12	13	6	3
Collagenase	B3ZE05	43.6	5.22	197	4	14	3	7
Flagellin	Q2EI55	110.0	5.1	195	14	16	5	3
Collagenase	Q4MQT1	32.1	5.26	175	4	20	8	0
Phospholipase C	Q3EXMN7	36.9	6.45	150	14	12	4	7
Sphingomyelin phosphodiesterase C	B7HBU7	43.1	6.1	125	23	14	3	0
nheB	A0RCP7	43.1	6.85	107	11	14	2	1
Flagella hook protein	B5URI5	32.0	6.85	96	9	7	2	3

nrpB	Q2PUI5	65.5	5.92	87	8	6	0	1
Flagellar capping protein	B5URI1	50.3	5.86	66	6	2	1	0

F2081B/98

Gene	Accession Number	MW	pI	Mowse	% Coverage	Matched	Homology	Identity
nheB	Q3EL00	43.7	5.2	706	52	120	0	15
Flagellin	A0RC95	52.3	5.2	614	24	43	0	9
Collagenase	Q81I63	109.0	5.2	613	18	51	0	17
Flagellin	Q2EI54	43.7	5.4	606	27	38	0	9
Flagellin	Q2EI47	43.1	5.4	597	27	34	0	9
Flagellin (fragement)	Q2EI46	38.8	5.1	589	30	38	0	8
Flagellin (fragment)	Q2EI45	37.8	4.9	559	27	39	0	8
hblA	A0RCP7	36.2	6,9	516	31	30	0	11
Flagellin (fragment)	Q2EIA2	43.1	5.2	481	27	34	0	6
nheB	Q3Y6N2	43.7	6.9	476	36	20	0	10
Flagellin (fragement)	Q2EI66	44.7	5.4	475	24	54	0	11
Flagellin (fragment)	Q2EI57	34.4	5	470	25	37	0	7
Flagellin	Q73AJ3	36.7	5.1	469	25	35	0	7
Flagellin (fragment)	Q2EIB0	49.2	5.2	464	18	34	0	6
Phospholipase C	B5UJA3	32.3	5.4	464	36	63	0	6
Flagellin (fragment)	Q2EI71	34.6	7.1	440	26	29	0	10
Sphingomyelin phosphodiesterae	Q3EXN6	36.5	5	428	27	71	0	7

nheA	Q3Y6M4	43.6	6.1	381	30	33	0	7
Protease	B7HJB8	43.8	5.2	355	23	62	0	9
Flagellin (fragment)	Q2EI68	35.2	8.4	308	19	23	0	9
Phosphatidylinositol diacylglycerol-lyase	B7HDN6	38.1	5.2	305	25	31	0	5
Flagellin (fragment)	Q2EI30	40.1	6.3	291	14	22	0	7
Protease	B5UVH5	43.8	5.8	282	19	46	0	4
hblD	Q1XFJ8	16.4	8.6	279	47	33	0	7
1-phosphatidylinositol phosphodiesterase	Q3EUR0	37.8	4.9	236	21	26	0	6
GroEL	A0R8W4	57.4	6.7	235	11	8	0	4
Phosphatidylinositol diacylglycerol-lyase	A9US05	37.8	4.5	201	14	19	0	4
Protease	B7HFP9	64.6	6.3	179	8	5	0	3
hblC	Q9L4L8	50.1	6	161	9	8	0	5
Enterotoxin FM	Q2TMX3	44.5	5.7	146	10	9	0	5
NlpC/P60	B7IT71	45.2	9.6	133	10	9	0	4
hblA (fragment)	A7GNS4	44.8	9.6	133	7	17	0	3
Haemolytic enterotoxins	A7GNS4	44.8	5.2	116	7	17	0	4
Surface layer domain protein	A1BYN6	49.5	5.2	116	5	9	0	2
flgK	B7IQA0	48.0	5.9	114	9	3	0	3
Peptidase M4 thermolysin	A7GP51	61.9	5.3	114	4	16	0	3
Acyl carrier protein	A0RHM3	8.8	5.7	112	10	3	0	1
InhA	B5UJ97	87.9	3.9	109	3	5	0	3
Hypothetical exported protein	Q3EQX7	18.6	5.6	105	10	2	0	1

Enolase	A0RKS3	46.4	9	103	11	12	0	3
Flagelar hook associated protein	B7HI46	48.2	4.7	100	9	3	0	3
5’-nucleotidase	A0RFV3	57.9	5.2	95	6	3	0	2
Uncharacterised protein	B5UK42	14.7	6	90	21	7	0	3
Flagellar hook-associated protein	Q63DA1	50.8	9.8	87	7	4	0	3
Alkyl hydroperoxide reductase,	A0R944	20.7	5.9	72	22	3	0	3
50S Ribosomal protein	A0R8H0	12.5	4.7	64	25	8	0	3
NIpC/P60	B7HFV4	56.1	7.6	64	4	6	0	2
flgL	B5URI2	32.0	5.1	60	5	1	0	1
Fibronectin type III domain protein	B7HFV3	47.3	7.6	54	7	6	0	3
FlgK	A9VNE6	47.7	5.2	54	7	2	0	2
DNA binding protein HU	A0RBW4	12.9	8.9	53	11	2	0	1
Bacillolysin	Q3EJ76	21.1	4.7	51	6	1	0	1
S-layer protein	A0RAK5	64.9	8.5	51	2	3	0	2

98HMPL63

Gene	Accession Number	MW	pI	Mowse	% Coverage	Matched	Homology	Identity
Flagellin	Q2E187	45.3	5.48	580	32	29	6	15
Collagenase	B7IWT0	110.0	5.32	508	12	32	8	13
Collagenase	B5UIY5	109.9	5.42	502	35	35	8	11
Collagenase	B32E05	109.9	5.22	385	30	30	0	8
Collagenase	Q4MQT1	109.8	5.26	364	35	26	0	6
Collagenase	B7JPE4	110.6	5.2	320	26	17	4	4
Flagellin	Q2EI65	36.7	5.16	306	17	12	3	3
Flagellin	Q2EI30	40.1	5.83	271	12	19	5	2
nheA	B32DK7	43.7	5.22	259	19	17	3	11
Sphingomyelin phosphodiesterase C	B7HBU7	37.0	6.1	254	17	9	9	0
Flagellin	Q2EI46	38.7	5.08	251	9	9	2	3
Flagellin	B32P89	52.3	5.2	241	9	5	2	3
Phosphollipase C	Q3EXM7	48.5	5.31	218	5	7	0	1
Neutral Protease	B7HFP9	64.6	5.97	148	7	10	0	1
hblD	A0RCP7	43.1	6.85	109	10	10	3	2
nheB	Q1XFH2	23.4	9.18	109	10	9	3	2
Glycosylphosphatidylinositol diacylglycerol lyse	A0RHD2	38.1	6.27	95	9	9	2	0
1-phosphatidylinositol	Q3EUR0	37.8	6.67	95	9	7	2	0

phosphodiesterase								
Flagellar hook associated protein	Q630AI	50.8	5.9	85	7	9	0	1
Flagellar hook protein flgL	B5URI2	32.0	5.09	73	9	7	4	0
hblA	A0RFT7	42.2	5.28	72	5	7	3	0
Murein endopeptidase	A0RC26	43.7	8.89	69	5	5	2	0
Acyl carrier protein	A0RHM3	8.8	3.37	65	18	5	2	1
60K chaperonin	A9LHQ7	19.7	4.59	55	10	6	1	2

B. weihenstephanensis
10390

Gene	Accession Number	MW	pI	Mowse	% Coverage	Matched	Homology	Identity
Collagenase	B7IWT0	110.0	5.3	269	6	11	1	5
Collagenase	B32E05	110.6	5.2	237	5	13	1	5
Flagelin	Q73AJ3	49.8	5.4	157	13	7	2	0
Fagelin	Q2EI65	34.3	5.1	153	19	7	3	0
Sphingomyelinase phosphodiesterase	B7HBU7	37.6	6.1	146	23	11	1	1
Flagelin	A0RC95	52.3	5.2	144	12	7	3	0
Phospholipase C	Q3EXN7	32.1	6.5	141	11	14	6	3
Flagelin	Q2EIB0	36.1	5.1	138	18	7	3	0
nheA	Q2TM57	43.7	5.2	129	11	8	3	4
Acyl carrier protein	A0RHM3	8.8	3.9	99	18	6	0	3
hblD	A0RCP7	43.1	6.9	93	6	8	0	4
nheB (fragment)	QIXFH2	23.4	9.2	93	11	8	0	4
Glycosylphosphatidlinositol diaglgycerol-lyase	A0RHD2	38.1	6.3	86	10	10	0	0
1-phospatidylinositol phosphodiesterase	Q3EUR0	37.8	6.7	86	10	10	0	0
Enolase	A0RKS3	46.4	4.7	82	8	9	2	3
Thioredoxin	A0RJC4	11.5	4.5	66	15	3	1	2

10390

Gene	Accession Number	MW	pI	% Coverage	Mowse	Matched	Homology	Identity
nheA	Q2TM57	43.7	5.2	60	794	57	0	17
Flagellin	A0RC95	52.3	5.2	33	699	39	0	11
Flagelin	B3Z764	39.2	5.2	41	682	34	0	13
Flagellin	Q2EI54	43.0	5.4	38	663	33	0	12
Collagenase	B7H9W3	110.1	5.2	16	658	31	0	18
Flagellin	Q2EI47	43.2	5.4	37	646	27	0	2
Flagellin	Q2EI45	37.8	4.9	41	640	36	0	12
nheB	A0RCP7	43.1	6.9	39	637	28	0	14
hblD	B7JJJ9	43.7	5.4	41	606	41	0	13
Flagellin	Q73AJ3	49.8	5.4	26	542	31	0	7
Flagellin (Fragment)	Q2EIA2	36.2	5.2	28	522	28	0	8
Flagellin (Fragment)	Q2EI57	34.4	5.1	29	506	28	0	8
Flagellin	Q2EI65	36.7	5.2	27	506	28	0	8
Flagellin	B7JIM6	47.3	5.5	25	504	27	0	10
Flagellin	Q2EI95	39.7	5.8	29	495	29	0	9
Flagellin	Q2EIBO	36.1	5.1	28	491	28	0	8
Flagellin	Q2EI66	44.0	5.0	26	487	31	0	8
Flagellin	B3ZP89	48.5	5.3	24	479	29	0	9
Phospholipase C	B5UJA3	32.4	7.1	31	442	15	0	10
Flagellin (Fragment)	Q2EI55	43.6	5.1	23	430	29	0	7
Flagellin	Q2EI93	39.9	5.4	28	423	23	0	8
Flagellin	Q2EI87	45.3	5.5	22	422	22	0	8
Flagellin	Q2EI86	46.5	5.9	24	408	23	0	8
nheB	Q3Y6N2	43.1	6.9	26	397	20	0	8
Flagellin	Q2EI30	40.1	5.8	23	352	21	0	7

Protease	B7HJB8	43.8	8.4	28	324	63	0	9
Flagellin (fragment)	Q2EI32	33.7	4.9	22	304	19	0	6
Phosphatdylcholine-hydrolyzing phospholipase C	Q5J8N8	22.1	6.8	32	296	10	0	6
nheA (Fragment)	Q1XFJ8	16.4	4.9	44	250	17	0	7
hblC	Q9L4L8	50.1	5.7	16	234	8	0	8
hblA	A0RFT7	42.2	5.3	17	221	7	0	6
InhA	B7HW94	87.9	5.5	7	218	8	0	7
nrpB	B7HFP9	64.6	6.0	12	218	5	0	4
Phosphatidylinositol diacylglycerol-lyase	B7HDN6	38.1	6.3	14	213	14	0	4
nheA	A7GNS4	44.0	5.2	17	209	13	0	6
hblA (Fragment)	Q1XFF0	32.8	5.2	18	203	6	0	5
GroEL	A0R8W4	57.4	4.8	9	189	6	0	4
Phospholipase C	A7GLA8	32.4	6.5	14	178	8	0	4
5’-Nucleotidase	A0RFV3	57.9	6.0	9	177	6	0	5
Surface layer domain protein	A1BYN6	49.5	5.9	9	173	17	0	3
Uncharacterised protein	B5UK42	14.7	9.8	40	166	11	0	4
Enolase	A0RKS3	46.4	4.7	9	152	11	0	5
Flagellin (Fragment)	Q2EI27	35.0	5.2	11	147	5	0	3
Peptidase M4 thermolysin	A7GP51	61.8	5.7	6	143	5	0	3
Enlongation factor TU	A0R8H8	42.9	4.9	11	134	5	0	4
hblD	Q32TH7	41.6	5.4	13	129	5	0	3
Flagellar capping	B5V357	51.5	5.4	2	120	2	0	2

protein								
DNA binding protein	A0RBW4	12.9	8.9	13	116	3	0	2
Chitinase	B7HA12	49.7	6.6	11	112	4	0	4
Uncharacterised protein	A0RC88	43.4	5.3	3	111	6	0	3
NIplC/p60	B7HFV4	50.1	7.6	9	107	7	0	3
Microbial collagenase metalloprotease	A316EI	12.3	4.6	4	105	11	0	4
Flagellar hook protein	B9IWF2	42.6	5.4	7	103	4	0	3
Fibronectin type III domain protein	B7HFV3	47.3	7.6	7	93	4	0	3
FIgL	B5URI1	32.0	5.1	5	89	1	0	1
Uncharacterised protein	B5UV19	23.5	8.9	6	83	1	0	1
NlpC	A0RCW0	43.9	9.7	8	80	4	0	3
NlpC	B7IT71	45.2	9.6	8	80	4	0	3
Thioredoxin	A0RJC4	11.5	4.5	15	79	3	0	1
Anthrolysin O	B3YP38	56.6	7.6	3	77	1	0	1
S-layer protein	Q3EXA6	63.6	7.0	4	77	2	0	2
Alkyl hydroperoxide reductase, subunit C	A0R944	20.1	4.8	12	70	1	0	1
Sphingomyelin phosphodiesterase	Q3EN6	36.5	6.1	12	69	7	0	3

10202

Gene	Accession Number	MW	pI	% Coverage	Mowse	Matched	Homology	Identity
Collagenase	B7IWT0	110.0	5.2	11	521	35	3	32
Collagenase	B5UIY5	109.9	5.2	11	481	39	33	6
nheA	Q2TM57	43.7	5.4	21	332	32	8	24
Flagellin	Q2EIA2	36.2	5.1	25	315	12	1	11
Flagellin	Q73AJ3	49.8	5.2	18	313	12	1	1
Flagellin	Q2EI57	34.4	5.2	27	313	12	5	7
Flagellin	Q2EI65	36.7	5.3	25	313	12	5	7
Collagenase	B3ZE05	110.0	5.2	7	310	25	14	9
Collagenase	Q4MQT1	109.9	5.1	7	309	25	18	7
Flagellin	A0RC95	52.3	6.1	18	304	12	7	5
Flagellin	Q2IB0	36.1	5.0	25	298	12	7	5
Sphingomyelin phosphodiesterase C	B7HBV7	37.5	5.3	29	274	18	0	18
Flagellin	Q2EI66	44.6	4.9	21	268	12	8	4
Collagnase	B7JPE4	109.8	5.4	7	267	10	16	2
Flagellin	Q2EI45	37.8	6.0	21	266	13	8	4
Flagellin	Q2EI47	43.1	6.9	17	242	14	6	4
nheA (fragment)	Q1XFL0	25.1	5.1	32	225	6	9	4
nheB	A0RCP7	43.1	6.9	11	203	12	10	4
Flagellin	Q2EI56	43.1	5.1	16	180	21	4	2
Neutral Protease B	B7HFP9	64.6	6.0	10	180	5	10	2
Phospholipase C	Q3EXN7	32.2	6.5	14	172	18	4	5
Flagellin	Q2EI30	40.1	5.8	14	167	13	0	1
Phosphatidylinositol	B7HDN6	38.1	6.3	13	158	10	10	18

diacylglycerol lyase								
nheB	Q3Y6N2	43.1	6.9	11	145	14	9	3
FlgL	B5URI2	32.0	5.1	9	133	4	10	1
Phosphatidylinositol diacylglycerol lyase	Q3EVR0	37.8	6.8	10	123	9	0	1
flgL	B7HI46	48.2	5.2	7	91	9	0	3
nheA (fragment)	QIXFJ8	16.4	5.0	20	82	7	3	1
Flagellar hook protein	Q63DA1	50.8	5.9	5	82	5	2	1
Murein endopeptidase	A0RCZ6	43.7	9.0	5	78	7	0	1
Anthrolysin O	B3YP38	56.6	7.6	6	74	9	0	1
hblB	A0RFT7	42.2	5.3	5	73	2	1	0
Phosphatidylinositol diacylglycerol lyase	A9VS05	37.8	6.3	6	73	3	1	0
cytK	B3Z0A1	37.0	8.6	7	62	3	0	0
Acyl carrier protein	A0RHM3	8.8	3.9	18	60	4	0	1

Appendix C
Conventional PCR Primers

Gene	Sequence
nheA Forward	CGA GTT GCT TCA TTC CTG TAA GCG
nheA Reverse	AAG AGC TGC TTC TCT CGT TTG GCT
nheB Forward	AGT GAA GCA AGC TCC AGT TCA TGC
nheB Reverse	AGC GGG ATT CCT GCA TCT TGA CTA
nheC Forward	GGC GCA AAC GAC ATC GAG CAT ATT
nheC Reverse	GTC CGC CAG CAA GGC ATG TTA TTA
hblA Forward	ATT CCA ACC TTG ATT GCT GGT GGC
hblA Reverse	GCA TTT GGC GCA TTT GTT CCA GTC
hblC Forward	TCT CGC AAC ACC AAT CGT TCA AGC
hblC Reverse	CTT GCG CAG TTG CCA CAT CAG TAT
hblD Forward	GCA TTG GCT GAA ACA GGG TCT CAT
hblD Reverse	TTG TGG AAT CGT TGC CTG TTG ACC
cytK Forward	CAA CAC CTG CTG CTT ACG CTC AAA
cytK Reverse	TTT CCA ACC CAG TTT GCA GTT CCG
plcR Forward	TAT GTC ACC AAT CGG AAG TGA GCC
plcR Reverse	CGT AAG CAG CCA CAT GAT ATT GCC
fur Forward	AACGTGAAGCAACAGTTCGTGTGC
fur Reverse	CGC ACC TTC TTG GCG TAA GTC ATA
groEL Forward	GCA CAG AAC AAA TCG AAG CTC GCA
groEL Reverse	GGT GCA TTT GGT TCT GGC TTG TCA
sigB Forward	TGG TGA AGC GCA GGA AAG GTT AGT
sigB Reverse	CGG TGA ACG CTG CAA ATG ATC TGT
rpoB Forward	ATC GTA GCT CGT TTC CGT GGT GAA
rpoB Reverse	TTT ACT TCC GCA CCA ACG CGA ATG
mreB Forward	TGG TTG TTG ATA TCG GTG GCG GTA
mreB Reverse	ACG GTC CAT AAT GTC TGC CGC TAA

Real Time PCR Primers

Gene	Sequence
nheA Forward	AGA GAA GCA GCT CTT CGC ATT CAG
nheA Reverse	CAC AAA CTG AAG CAG AGG CAA CAC
nheB Forward	AGG GCC AAT TGC CAT TAT TGG TGG
nheB Reverse	GCA CAG CTG GTA TCG TAT TAG CGA
nheC Forward	CTG GTG GTG TAC TTT GTG TAG CGT
nheC Reverse	GGA TAG AAT TTC TGG CGC ACA AGC
hblA Forward	TCA TTA CAC CAC CAG CAA TCC ACG
hblA Reverse	ATG TCC GGG CAT TTG GAG GTC ATA
hblC Forward	CAA CGC CAA TCG TTC AGG CAG AAA
hblC Reverse	TGG AGC CTT AGC AAG TCC GAA TGT
hblD Forward	TAG CCT GGT CAA TTG GTG GTG GAT
hblD Reverse	ATC GGT TTA GGA ACA GCA GCT GGT
plcR Forward	TCA CCA ATC GGA AGT GAG CCG AAT
plcR Reverse	CCA TAA CGT CGT TTC AAT GTT CAA GGG
fur Forward	GCA ACG TGA AGC AAC AGT TCG TGT
fur Reverse	TTC GGA GAC GGT GTT TCA CGC TAT
groEL Forward	AAG GTA TCG TTG CAG GTG GTG GTA
groEL Reverse	TCG TCA AAT CGC AAT CAA CGC TGG
sigB Forward	ACA GAT CAT TTG CAG CGT TCA CCG
sigB Reverse	TGG CAG TTC GGT AGC GAG AAT TGA
rpoB Forward	ACA CGG TGG TGG CGG TAT TAT CTT
rpoB Reverse	TTG ATT CAC GCC TGG TGG CAA TTC
mreB Forward	TGG TTG TTG ATA TCG GTG GCG GTA
mreB Reverse	CGC TGA AAG AAC AGC TGA GGC ATT

Real time PCR Efficiency and R2 values;
B. cereus
27

	% PCR Efficiency	R^2 Value	Slope	E Value
PlcR	96.3	0.998	-3.414	1.9630
Fur	84.7	0.997	-3.753	1.8469
MreB	90.3	0.997	-3.578	1.9032
SigB	93.3	0.990	-3.495	1.9325
GroEL	100.3	0.998	-3.316	2.0025
NheA	83.2	1.000	-3.804	1.8318
NheB	104.2	0.997	-3.223	2.0430
NheC	186.3	1.000	-2.189	2.8630
HblA	116.8	1.000	-2.976	2.1678
HblC	80.1	0.999	-3.912	2.0572
HblD	104.9	0.997	-3.210	2.0489
rpoB	109.9	0.998	-3.106	2.0987

F2081B/98

	% PCR Efficiency	R^2 Values	Slope	E Value
PlcR	93.5	1.000	-3.489	1.9347
Fur	94.9	0.996	-3.450	1.9492
MreB	118.1	1.000	-2.953	2.1809
SigB	139.6	1.000	-2.636	2.3953
GroEL	93.8	0.999	-3.481	1.9376
NheA	107.9	0.990	-3.147	2.7856
NheB	99.8	0.999	-3.327	1.9979
NheC	92.4	1.000	-3.518	1.9242
HblA	95.6	0.995	-3.431	1.9564
HblC	94.9	0.997	-3.451	1.9488
HblD	109.2	0.999	-2.168	2.9024
rpoB	98.2	0.998	-2.254	1.9488

B. weihenstephanensis
10390

	% PCR Efficiency	R^2 Values	Slope	E Value
PlcR	98.6	0.997	-3.356	1.9860
Fur	92.1	1.000	-3.527	1.9210
MreB	111.3	0.999	-3.077	2.1134
SigB	91.1	1.000	-3.557	1.9105
GroEL	90.2	1.000	-3.582	1.9018
NheA	92.0	0.999	-3.529	1.9202
NheB	81.7	1.000	-3.858	1.8164
NheC	98.2	0.999	-3.685	1.9142
HblA	89.5	0.994	-3.601	1.8954
HblC	103.6	0.999	-3.239	2.0358
HblD	97.8	0.992	-3.377	1.9775
rpoB	103.9	0.997	-3.232	2.0389

10396

	% PCR Efficiency	R^2 Values	Slope	E Value
PlcR	129.5	1.000	-2.771	2.2955
Fur	88.5	0.997	-3.634	1.8844
MreB	107.8	1.000	-3.147	2.0785
SigB	98.3	0.997	-3.364	1.9827
GroEL	104.7	0.994	-3.214	2.0470
NheA	109.9	1.000	-3.106	2.0987
NheB	100.9	0.996	-3.301	2.0088
NheC	108.6	0.997	-3.132	2.0859
HblA	101.0	0.998	-3.299	2.0097
HblC	104.7	0.999	-3.213	2.0476
HblD	105.8	0.999	-3.199	2.0577
rpoB	103.9	0.998	-3.832	2.3809

10202

	% PCR Efficiency	R^2 Values	Slope	E Value
PlcR	117.2	0.999	-2.968	2.1723
Fur	84.6	0.989	-3.757	1.8457
MreB	97.6	0.998	-3.383	1.9763
SigB	92.1	0.982	-3.528	1.9206
GroEL	93.7	1.000	-3.483	1.9369
NheA	93.3	0.993	-3.493	1.9332
NheB	113.2	0.999	-3.042	2.1317
NheC	100.8	1.000	-3.304	2.0075
HblA	98.6	1.000	-3.357	1.9856
HblC	99.0	0.999	-3.327	1.9979
HblD	92.0	0.998	-3.531	1.9196
rpoB	98.5	1.000	-3.289	1.9347