Detection and Further Characterisation of the Toxins and Associated Genes of *Bacillus cereus*

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Declaration

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from published or unpublished work of others has been acknowledged in the text and a list of references is given.

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Statement of the Contribution of Others

This project was supervised by Dr. Graham Burgess, Microbiology and Immunology, James Cook University, Townsville Australia and Dr. Jane Oakey, Oonoonba Veterinary Laboratory, Queensland Department of Primary Industries and Fisheries, Townsville Australia. All editorial and proofreading assistance was obtained from Dr. Graham Burgess and Dr. Jane Oakey.

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Abstract

The food poisoning bacterium *Bacillus cereus* produces a large array of potentially pathogenic substances including four haemolysins, three different types of phospholipase C, the emetic toxin (cereulide) and at least five enterotoxins. The relative importance of these metabolites to the pathogenicity of *B. cereus* strains has not been fully elucidated. The major goals of this project were to evaluate existing toxin detection methods, develop improved methods of detecting pathogenic strains of *B. cereus* and to characterise the genes associated with the production of cereulide.

A large number of foodborne and clinical strains of *B. cereus* were tested for diarrhoeal toxin production using previously reported methods, including polymerase chain reaction (PCR), gel diffusion haemolysis, Vero cell cytotoxicity and two commercially available diarrhoeal toxin detection kits. The genes for all five of the diarrhoeal toxins (haemolysin BL, enterotoxin T, non-haemolytic enterotoxin, enterotoxin FM and cytotoxin K) have been characterised and subsequently PCR primers have been designed to detect these genes. The PCR methods for three of these toxins (HBL, enterotoxin T and enterotoxin FM) were utilised to determine the prevalence of toxin genes in *B. cereus*. The gene for enterotoxin FM was the most commonly detected with 86.8% of the isolates containing this gene, followed by haemolysin BL (50%) and enterotoxin T (42.6%). The Vero cell cytotoxicity assay was deemed to be the most useful detection method due to its ability to detect actual toxicity, regardless of which of the five diarrhoeal toxins the strain in question was able to produce.

Currently there are no simple and reliable methods available for detection of emetic strains of *B. cereus*. The most commonly used method of detecting emetic strains of *B. cereus* is the HEp-2 cell cytotoxicity assay. Cereulide causes vacuolation of the HEp-2 cell mitochondria. This effect is transitory and often difficult to identify. Finlay *et al*. (1999) improved this method by utilising the tetrazolium salt MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide). Although this method was sensitive and removed the subjectivity inherent in the original method, the MTT assay produces an insoluble, crystalline formazan end product that requires an additional step to solubilise the product before the absorbance readings can be taken. This method was improved by replacing MTT with the next generation tetrazolium salt, MTS (3-(4,5-

dimethythiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium). The advantages of MTS over MTT include the rapidity of colour development, the storage stability of MTS and the ability to return the sample to the incubator to await further colour development. A large number of *B. cereus* strains were tested using the HEp-2/MTS assay. The results correlated exactly with the HEp-2 mitochondrial assay. However, the sensitivity of the assay was increased and the visualisation of results relied upon observing a colour change reaction that negated the subjectivity of the original method.

Cereulide bears a close resemblance to metabolites produced by non-ribosomal peptide synthetases (NRPS) from the genera *Bacillus* and *Streptomyces*. Turgay and Marahiel (1994) developed universal primers to detect a 500 base pair (bp) region that has been highly conserved in all of the NRPS genes sequenced thus far. These primers were utilised to determine if production of cereulide is linked to peptide synthetases. Two previously reported emetic strains of *B. cereus* were tested using the NRPS primers, resulting in 497 bp products, which were subsequently cloned and the nucleotide sequence determined. The nucleotide and translated amino acid sequences showed a high degree of homology with other peptide synthetases, such as surfactin, gramicidin, bacitracin, tyrocidine and lichenysin. Primers were designed from variable regions of the NRPS consensus sequence to be specific for the *B. cereus* NRPS gene sequence. A PCR-ELISA detection system was also developed to increase the specificity of the assay. Analysis of a large number of emetic and non-emetic strains of *B. cereus* showed that the PCR primers distinguished between emetic and non-emetic strains. This PCR method will greatly improve food laboratories' abilities to detect strains of *B. cereus* capable of causing emesis and also enable a preventative approach to be applied to the control of emetic food poisoning.

A wide variety of other *Bacillus* species were tested for toxin genes using previously published enterotoxin PCRs and the novel cereulide PCR developed in this study. One strain of *B. thuringiensis* (BT1) contained all three of the enterotoxin genes that were targeted. One strain of *B. circulans* (2715) contained the gene for enterotoxin FM. A strain of *B. licheniformis* (BL1) and a strain of *B. subtilis* (BS1) contained the gene for cereulide.

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List of Abbreviations

<	lesser than
>	greater than
%	percentage
α	alpha
aa	amino acid
approx	approximately
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
β	beta
BCET-RPLA	*Bacillus cereus* enterotoxin – reverse passive latex agglutination
BDE-VIA	*Bacillus* diarrhoeal enterotoxin – visual immunoassy
BHI	brain heart infusion
BHIG	brain heart infusion with glucose
BLASTn	basic local alignment sequencing tool (nucleotide)
BLASTp	basic local alignment sequencing tool (protein)
bp	base pairs
°C	degrees Celsius
Ca	calcium
CADM	complete amino acid defined medium
CAMP	Christie, Atkins & Munch-Petersen
cfu	colony forming units
cpe	cytopathic effect
dig	digoxigenin
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP's	generic term for single deoxyribonucleotide dATP = deoxyadenine triphosphate dCTP = deoxycytosine triphosphate dGTP = deoxyguanine triphosphate dTTP = deoxythymine triphosphate dUTP = deoxyuracil triphosphate
e-value	Expect value
EDTA	ethylene diamine tetra-acetic acid
e.g.	for example (Latin: *exemplum gratii*)
ELISA	enzyme linked immunosorbent assay
F	*Bacillus* strains originally from the Public Health Service, London, UK
FBS	foetal bovine serum
g	grams
g	centrifugal acceleration relative to Earth's gravity
H	hydrogen
H_2O	water
HBL	haemolysin BL
HCl	hydrogen chloride
hrs	hours
i.e.	that is (Latin: *id est*)
IH	*Bacillus* strains originally from the University of Helsinki, Department of Applied Chemistry and Microbiology, Finland

K	potassium
Kb	kilobases
KCl	potassium chloride
KDa	kilodaltons
kg	kilograms
L	litres
LB	Luria-Bertani medium
M	molar
MADM	minimum amino acid defined medium
Mb	megabases
mg	milligrams
Mg	magnesium
min	minutes
ml	millilitres
mm	millimetres
mM	millimolar
MTS	3-(4,5-dimethythiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium
MTT	3-(4,5- dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na	sodium
NaCl	sodium chloride
NC	*Bacillus* strains originally from the Nagoya City Public Health Institute, Nagoya City, Japan
ng	nanograms
NHE	non-haemolytic enterotoxin
nm	nanometres
NRPS	non-ribosomal peptide synthetase
NVH	*Bacillus* strains originally from the Norwegian School of Veterinary Science, Olso, Norway
PC2	physical containment level 2
PC3	physical containment level 3
PCH	phosphatidylcholine hydrolase
PCR	polymerase chain reaction
PEMBA	polmyxin pyruvate egg-yolk bromothymol blue agar
pers. comm.	personal communication
pH	potential of hydrogen
PI	phosphatidylinositol
PIH	phosphatidylinositol hydrase
p-pant	4’-phosphopantetheine
rev	revolutions
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
sec	seconds
sp.	species
spp.	species (plural)
TBST	tris buffered saline with Tween 20
U	units
UK	United Kingdom
µg	micrograms
µl	microlitres
µm	micrometres

USA	United States of America
UV	ultraviolet
V	volts
w/v	weight/volume
XTT	sodium (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl])-2*H*-tetrazolium-5-carboxanilide

USA	United States of America
UV	ultraviolet
V	volts
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Chapter 1

General Introduction

and Literature Review

1.1 General Introduction

Despite improving levels of hygiene and sanitation in Australia and around the world, the incidence of foodborne disease is believed to be increasing. A recent estimate suggests that half a million cases of acute gastroenteritis occur in Australia each year (Arnold and Munce, 1997). The reason for the increase in reported cases of gastroenteritis is probably due to a number of factors, including: better reporting and diagnostic techniques, changes in eating habits and the identification of new human pathogens. The relative importances of these factors are unknown. However, the increase in reports of foodborne disease is believed to reflect a true increase in incidence despite factors such as increased awareness and ability to detect foodborne microorganisms (Crerar *et al.*, 1996).

The Gram-positive bacterium *Bacillus cereus* is a common cause of food poisoning in Australia and around the world. Reports indicate that this organism is responsible for between 8-39% of food poisoning outbreaks (Crerar *et al.*, 1996; Jenson and Moir, 1997). However, the causative agent for the majority of food poisoning outbreaks is not determined, indeed the majority of food poisoning cases are not reported due to the short duration and mildness of symptoms. Therefore, it is difficult to accurately quantify the true incidence of any one food poisoning agent. This is particularly the case with *B. cereus* food poisoning, where two distinct syndromes have been documented, and symptoms can greatly vary in their characteristics, duration and severity.

Bacillus cereus is a heat resistant bacterium that is capable of surviving most cooking procedures due to the production of highly resistant spores. It is a common environmental bacterium that contaminates food through the soil. This organism is capable of prolific growth in a variety of foods and is capable of producing a variety of toxins that can induce two distinct food poisoning syndromes. Methods for identifying toxigenic strains of *B. cereus* are extremely lacking due to the unclear pathogenicity of food poisoning syndromes caused by this organism. It is important that diagnostics are improved in this area because these bacteria are a significant problem in the food industry. In particular, *B. cereus* is a problem in the dairy industry where species of *Bacillus* commonly form biofilms, and the rice industry, which is most commonly

implicated in emetic cases of *B. cereus* food poisoning. Improved diagnostics will enable the food industry to adopt more effective quality control procedures to ensure that products are not contaminated with toxigenic strains of *B. cereus*.

1.2 The Taxonomy of Genus *Bacillus*

1.2.1 *Bacillus*

The genus *Bacillus* is a heterogenous collection of Gram positive, spore-bearing rods. The genus comprises over 100 validly described species (DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, 2004 http://www.dsmz.de/bactnom/nam0379.htm) and many additional species of uncertain taxonomic standing. Sporangial morphology is commonly used for division of the genus into three distinct groups: those containing oval spores, those with oval spores that distinctly swell the sporangium, and those with spherical spores (Harwood, 1989).

1.2.2 *Bacillus* subgroup 1

Bacillus anthracis, Bacillus cereus, Bacillus mycoides and *Bacillus thuringiensis* have all been placed in *Bacillus* subgroup 1 based on their large cell width and their spores, which do not distend the sporangium (Harwood, 1989). More recently *Bacillus* subgroup 1 has been expanded to include *B. pseudomycoides* (Nakamura, 1998) and *B. weihenstephanensis* (Lechner *et al*., 1998).

Two distinct syndromes of food poisoning can be induced by strains of *B. cereus*. The two syndromes, the diarrhoeal and emetic, are mediated by the action of specific toxins. The diarrhoeal syndrome is a mild illness characterised by diarrhoea, whereas, the emetic illness is more severe and characterised by vomiting. This bacterium has also been implicated in bovine mastitis, severe systematic and pyrogenic infection, gangrene, septic meningitis, cellulitis, panophthalmitis, lung abscesses, endocarditis and endophthalmitis (Johnson, 1984; Callegan *et al*., 1999).

Anthrax, the highly infectious disease of animals and humans, is caused by the bacterium *B. anthracis*. Anthrax begins with the introduction of spores into the body, usually via minor abrasions, insect bites or inhalation. The spores then germinate and the bacteria multiply. The infection may spread to the regional lymph nodes and then progress to a high titre bacteraemia. The pathogenesis of *B. anthracis* infections depends on the production of three specific virulence factors: an anti-phagocytic capsule and two toxins - the oedema and lethal toxins (Agaisse *et al.*, 1999).

The third important member *Bacillus* subgroup 1 is *B. thuringiensis*, which is used extensively as an insecticide. More than 500 tonnes of product (equivalent to $>5 \times 10^8$ bacteria) are sprayed annually in the US alone (Anon., 1999). The insecticidal activities of *B. thuringiensis* are due to the production of proteinaceous crystals consisting of δ-endotoxins (cry toxins) during sporulation. The crystals are ingested by insect larvae and are dissolved in the midgut. The released toxins then bind to and are inserted into the membrane of the midgut epithelial cells via insect-specific receptors, creating trans-membrane leakage pores that cause cell lysis and the death of the insect (Agaisse *et al*., 1999).

1.2.3 Homology in *Bacillus* subgroup 1

Following an extensive biochemical, physiological and morphological study of the genus Priest *et al*. (1988) failed to find characters that would consistently differentiate the four species of *Bacillus* subgroup 1. The 16S rRNA sequences of the four taxa are also highly homologous (>99%) and differ only within the range expected for a single species (Ash *et al.*, 1991). Indeed, recent research suggests that the four reported species are actually the same species. The only discernable genetic difference between them was found to be the presence/absence of particular transposable elements, such as the *B. anthracis* virulence plasmids: pX01 and pX02 (Vilas-Boas *et al*., 2002). Currently the division of these bacteria into different species is based mainly on their differences in pathogenicity e.g. *B. anthracis* is a pathogen of mammals and *B. thuringiensis* is a pathogen of insects. Many researchers now believe that *B. anthracis, B. mycoides* and *B. thuringiensis* should be grouped as subspecies of *B. cereus* (Ash *et al.*, 1991; Agaisse *et al.*, 1999).

1.3 *Bacillus cereus*: The Organism and its Characteristics

1.3.1 The history of *Bacillus cereus* food poisoning

Outbreaks of food poisoning due to *Bacillus* spp. have been described since the beginning of the century (Lund, 1990). The first confirmed outbreak of *B. cereus* food poisoning occurred in Norway in 1950. The food vehicle was vanilla sauce, which had been prepared a day in advance and stored at room temperature before serving. Consumption of contaminated vanilla sauce resulted in a diarrhoeal illness. The sauce was later found to contain 2.5×10^7 to 1.1×10^8 *B. cereus* per millilitre. Four related outbreaks were described involving more than 600 people. To provide further evidence that *B. cereus* was the causative agent Steinar Hauge inoculated sterile sauce with *B. cereus*, incubated it for 24 hours and then consumed the sauce. The onset of symptoms occurred 12 hours later. Subsequently, *B. cereus* was recognised as an important cause of food poisoning worldwide (Johnson, 1984).

1.3.2 The characteristics of *Bacillus cereus*

This facultatively anaerobic bacterium is characterised by large vegetative cells, typically 1.0 μm by 3.0-5.0 μm in chains. The normal temperature growth-range of *B. cereus* is 8 to 55°C, with optimum growth around 28-35°C. Psychrotrophic strains are not uncommon and can grow at temperatures as low as 4-5°C, however *B. cereus* is generally classed as a mesophile. Growth and enterotoxin production have been observed in rice meal after 24 days at 4˚C. However, it is believed that growth of psychrotrophic strains to high numbers in refrigerators and the subsequent production of enterotoxin after ingestion is more significant than toxin production in foods at low temperatures. (Adams and Moss, 1995; Jenson and Moir, 1997).

The reported pH growth range for *B. cereus* is 4.3 to 9.3. Raevuori and Genigeorgis (1975) obtained limited growth of the bacterium when grown in meat at pH 4.35. The minimum range of water activity for vegetative growth of this organism is 0.912-0.950 (Jenson and Moir, 1997).

1.3.3 The isolation and identification of *Bacillus cereus*

In an outbreak investigation of *B. cereus* food poisoning, implicated foods, faecal and vomitus specimens all contain large numbers of organisms. Therefore, enrichment techniques are usually not required. The most commonly used selective medium is polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA). When grown on PEMBA, *B. cereus* produces typical crenated colonies that retain the turquoise blue of the pH indicator (bromothymol blue) due to their inability to ferment mannitol and hence produce acid. A zone of egg-yolk precipitation is produced through lecithinase activity, which involves the cleaving of lecithin (phosphatidylcholine) into phosphorylcholine and diglyceride (Figure 1.1). Polymyxin is used in the medium as a selective agent to suppress Gram negative bacteria (Adams and Moss, 1995; Johnson, 1984).The bacterium can also be grown on blood agar where it produces lavender colonies with beta haemolysis. However, blood agar is unsuitable for the detection of low levels of *B. cereus* in the presence of other organisms (Baron *et al*., 1994).

Following presumptive identification of *B. cereus,* confirmatory identification can be made using the Holbrook and Anderson spore stain. This test involves staining spores with malachite green, lipid globules with sudan black and counter staining the vegetative cell with safranin. No other *Bacillus* sp. with the typical *B. cereus* colony, cell and spore morphology possesses lipid globules in the cytoplasm (Jenson and Moir, 1997). Biochemical confirmation can be based on an isolates' ability to produce acid from glucose but not from mannitol, xylose or arabinose (Adams and Moss, 1995).

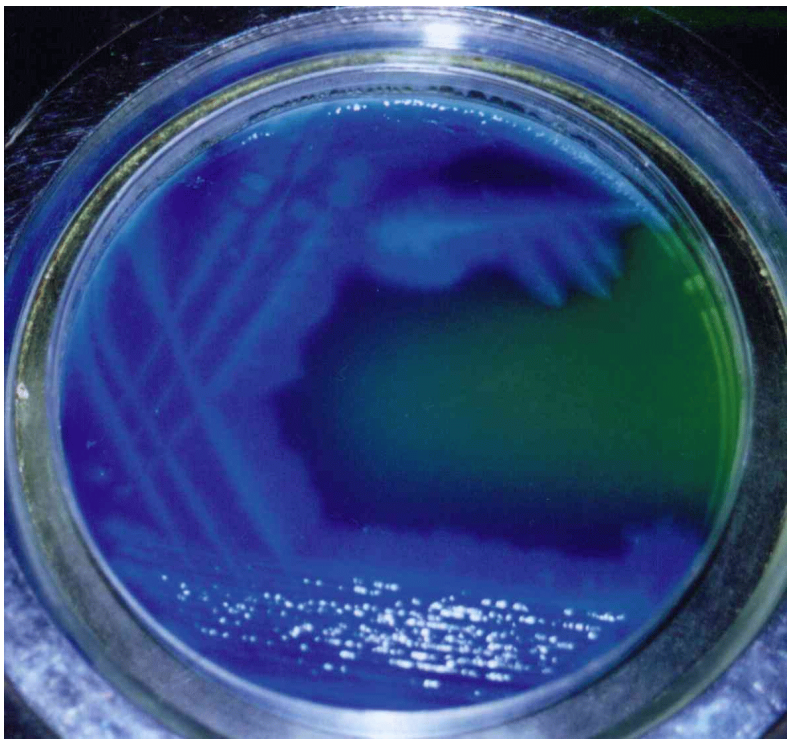


Figure 1.1 *Bacillus cereus* grown on PEMBA. Characterised by turquoise blue, crenated colonies surrounded by a zone of egg-yolk precipitation

Molecular techniques can also be used to confirm *B. cereus* isolates. Te Giffel *et al*. (1997) developed a polymerase chain reaction (PCR) method with DNA probes based on the variable region V1 of the 16S rRNA of *B. cereus* and *B. thuringiensis*. The researchers stated that they had developed a rapid and sensitive method to distinguish these two very closely related organisms.

The confirmation of *B. cereus* as the causative agent responsible for foodborne disease is dependant upon a combination of food consumption history, symptoms and detection of the bacterium in the implicated food and/or a patients vomitus or faeces. It is also necessary to demonstrate either that the same serotype isolated from the vomitus/faeces is also present in the implicated food, or that the isolate is enterotoxigenic (Jenson and Moir, 1997).

1.3.4 Serotyping of *Bacillus cereus*

Bacillus cereus may be differentiated into 18 serotypes based upon flagella (H) antigens, of which eight have been implicated in foodborne illness. Serotypes 1, 3, 4, 5, 8 and 12 have been associated with emetic illness while serotypes 1, 6, 8, 9, 10 and 12 have been associated with diarrhoea (serotypes 1, 8, and 12 have been associated with both syndromes) (Jay, 1997). Gilbert and Parry (1977) used this serotyping scheme to type cultures from 84 outbreaks of *B. cereus* food poisoning. Type 1 was present in 66% of emetic outbreaks and only 22% of diarrhoeal outbreaks. Other serotypes implicated in emetic outbreaks by Gilbert and Parry were types 3, 4, 5, 8 and untypable, while serotypes 2, 6, 8, 9 and 12 were associated with the diarrhoeal form of disease. Shinagawa *et al*. (1992) also discovered serotype 1 to be the most commonly isolated serotype from emetic outbreaks of *B. cereus* (serotype 1: 23%; serotype 8: 2%; untypable: 2%).

1.3.5 The genome of *Bacillus cereus*

Genetic maps of *B. cereus* strains have indicated that the chromosomal size may vary from 2.4 to more than 5.5 Mb. It is believed that *B. cereus* may exist either as one large chromosome with smaller extrachromosomal elements, or as a small chromosome with large extrachromosomal elements. The genome reportedly has a constant region of 2.4 Mb and a less stable region which is more easily mobilised into other genetic elements. The less stable part of the genome is localised to one region of the chromosome and is believed to be subject to frequent relocations between the chromosome and episomal elements (Carlson and Kolstø, 1994).

Beverley (1988) isolated plasmids from five *B. cereus* strains with apparent sizes of 730 kb (ATCC 6464), 600 kb (F2038/78), 450 kb (strain 41), 400 kb (ATCC 33018) and 290 kb (F4810/72). These large plasmids were separated in a pulse-time-dependent manner, indicating they were linear (large circular plasmids do not enter the gel). Linear plasmids have previously been reported in other bacteria e.g. *Borrelia* sp. and *Streptomyces* sp. (Carlson and Kolstø, 1994).

The complete genome sequence of the *B. cereus* type strain ATCC 14579 has recently been determined (Ivanova *et al*., 2003). Analysis of the sequence has confirmed the close relationship between *B. cereus*, *B. anthracis* and *B. thuringiensis* (Ivanova *et al*., 2003; Read *et al*., 2003).

1.4 The Ecology of *Bacillus cereus*

1.4.1 *Bacillus cereus* in the environment

This organism has been found in soil, dust and water that has run off soil, in particular, soils containing low levels of organic matter. The presence of this organism in plant foods is most likely due to soil contamination rather than a specific association between the microorganism and plants (Jenson and Moir, 1997).

1.4.2 *Bacillus cereus* in food

This bacterium is widespread in the environment and enters the food chain through contaminated food and water. The organism is present in most raw foods of plant origin with numbers especially high in some samples of spices and cereals (Jenson and Moir, 1997). The high resistance of the spores to desiccation allows *B. cereus* to survive on most dried food products. A survey of garlic, cinnamon, pepper, chilli, oregano and thyme found *B. cereus* in all samples at levels of 1×10^2 to 1×10^6 cfu/g (te Giffel *et al*., 1996). Eyles *et al*. (1989) found *B. cereus* in 100% (25/25) of flour samples tested. However, levels of the organism were low.

Rice is the most commonly implicated food in emetic cases of *B. cereus* gastroenteritis. Most samples of rice have low levels of *B. cereus* present. Fried or cooked rice has been implicated in approximately 95% of cases of *B. cereus* food poisoning with emetic symptoms (Jenson and Moir, 1997), indicating that there may be a relationship between substrate and emetic toxin (or cereulide) production. Many food poisoning cases have occurred where bulk rice is prepared in advance. Resistant spores (particularly serotype H1) may survive cooking to germinate, grow and produce cereulide during storage. This scenario commonly occurs in Chinese restaurants and take-away

establishments. Leftover portions of boiled rice from bulk cooking are allowed to "dry off" at room temperature and, when required, are either reheated by boiling or, more usually, flash fried before service. Vegetative cell growth is rapid in cooked rice at room temperature and is enhanced by the addition of beef, chicken or egg (Riemann and Bryan, 1979). Gilbert and Parry (1977) found *B. cereus* in 25 of 252 samples of boiled rice and 49 of 204 samples of fried rice. Levels of the bacterium ranged from 100 - 1 × 10^5 cfu per gram in boiled and fried rice. In the United Kingdom *B. cereus* was isolated from 98 of 108 (91%) of rice samples tested (Jenson and Moir, 1997).

Bacillus cereus is of particular concern in the dairy industry for several reasons. Firstly, the spores are very hydrophobic and attach to the surfaces of the pipelines of the dairy processing plant, where they might germinate, multiply and resporulate. Secondly, pasteurisation is insufficient to kill the spores, while competition from vegetative bacteria is eliminated. Thirdly, several strains of *B. cereus* are psychrotrophic and are capable of growth in milk at temperatures as low as 4-6˚C (Andersson *et al.*, 1995). Ahmed *et al*. (1983) sampled 400 milk and milk products in USA over a five month period for the presence of *B. cereus*. The organism was isolated from 9, 35, 14 and 48% of raw milk, pasteurised milk, Cheddar cheese and ice cream samples, respectively. The levels of *B. cereus* found did not exceed 100/mL in raw milk, 1,000/mL in pasteurised milk, 200/g in Cheddar cheese and 3,800/mL in ice cream. Despite the frequency at which dairy products are found to be contaminated with *B. cereus*, no outbreaks have been reported from consumption of milk and milk products, except for a few cases involving cream and certain desserts. Presumably the high numbers of the organism required to elicit symptoms causes visible spoilage of milk and other dairy products and this deters consumption of the product (Ahmed *et al.*, 1983). Granum (1997) suggested that milk drinkers may be partially protected against *B. cereus* food poisoning through immunity acquired by continuous consumption of this organism.

Bacillus cereus has been isolated from a variety of other foods including beans, cocoa, fish, dried potatoes, lentils, oil and meat. An outbreak of diarrhoeal food poisoning occurred after a university field day in South Carolina, USA. People at the field day who ate barbecued pork were five times more likely to develop symptoms than those who did not eat pork. The pork was unrefrigerated for 18 hours after cooking.

Subsequent tests showed that the leftover pork contained >10^5 cfu/g of enterotoxigenic *B. cereus* (Luby *et al*., 1993).

1.5 Symptoms of *Bacillus cereus* Food Poisoning

1.5.1 The diarrhoeal syndrome

The diarrhoeal illness caused by *B. cereus* is characterised by abdominal pain, profuse watery diarrhoea and rectal tenesmus. The illness is usually quite mild, however, cases that require hospitalisation have occurred. The incubation period for this syndrome is 8-16 hours after the consumption of the contaminated food and symptoms last for 12-24 hours. Nausea and vomiting are not commonly associated with the diarrhoeal illness. Levels of *B. cereus* found in the implicated foods responsible for this syndrome range from 5×10^5 to 9.5×10^8 cfu/g (Adams and Moss, 1995).

The diarrhoeal syndrome of *B. cereus* food poisoning can be mediated by at least five distinct enterotoxins. The pH and proteolytic enzymes of the upper gastrointestinal tract digest these toxins if they are preformed in foods (Granum *et al*., 1993). Therefore, the diarrhoeal illness is proposed to be due to bacteria growing in the intestine. Spores, which have survived digestion, are believed to germinate in the intestine and grow to produce viable cells, which subsequently produce toxin. The action of these toxins at the molecular level is not well established. However, they are known to reverse the absorption of fluid, sodium, and calcium and to cause malabsorption of glucose and amino acids (Mantynen and Lindstrom, 1998).

Two recent outbreaks of *B. cereus* food poisoning in Norway were associated with eating stew. The infective dose in these cases was estimated to be 10^4 to 10^5 cells. Of the 17 people affected in the outbreaks, three were hospitalised, one of which was for three weeks. Researchers suggested that in extended cases such as this, strains of *B. cereus* colonise the small intestine and cause more severe symptoms by producing enterotoxin at the site of colonisation (Granum, 1997).

1.5.2 The emetic syndrome

The emetic illness is more acute than the diarrhoeal illness and symptoms occur only 1-5 hours after the ingestion of the contaminated food. The illness is characterised by nausea and vomiting, which lasts for 6-24 hours. The similarity of this illness to that of *Staphylococcus aureus* food poisoning has been noted (Jay, 1996). Levels of *B. cereus* in foods associated with the emetic illness range from 1.0×10^3 to 5.0×10^{10} cfu/g (Adams and Moss, 1995).

Cereulide is regarded as the most dangerous of the toxins produced by *B. cereus.* This is highlighted by a rare case where a 17-year old boy and his father developed acute gastroenteritis after eating spaghetti and pesto that had been prepared four days earlier. The boy died within two days due to fulminant liver failure and rhabdomyolysis. The father developed hyperbilirubinemia and rhabdomyolysis, and recovered. High concentrations of cereulide were found in both the residue from the pan used to reheat the food and the boy's liver and bile (Mahler *et al*., 1997). In most cases of emetic food poisoning illness occurs following ingestion of precooked food held for too long at unsatisfactory storage temperatures (Jenson and Moir, 1997).

Emetic food poisoning induced by *B. cereus* is regarded as an intoxication due to the fact that viable cells are not necessary for illness to occur. In contrast, the diarrhoeal syndrome of food poisoning is mediated by the production of toxins within the gastrointestinal tract of the host and is therefore regarded as an infection.

1.6 Epidemiology of *Bacillus cereus*

1.6.1 The incidence of *Bacillus cereus* food poisoning

The reported incidence of *B. cereus* food poisoning varies widely between different countries. It is apparent that the major factors in this variation are diet and the differing reporting procedures between countries. In most cases the true prevalence of food poisoning is grossly under-reported. Moreover, the reporting rate of illness caused by *B. cereus* may be underestimated due to the relatively short duration of both disease

syndromes (<24 hours). In addition the frequency at which individual people are affected is usually not monitored. Consequently the full extent of *B. cereus* food poisoning is unknown.

In the USA, from 1988 to 1992, a total of 2,423 foodborne disease outbreaks affecting 77,375 people were reported. In approximately 40% of these cases the aetiology was confirmed, and *B. cereus* was responsible for 21 outbreaks, affecting 433 people. Chinese food was the most commonly implicated vehicle of transmission (Notermans and Batt, 1998).

In the Netherlands, from 1992 to 1994, a total of 1,543 outbreaks and 1,087 other cases of foodborne disease were reported, involving a total of 7,567 people. The causative agent was identified in only 8.3% of the cases. The most frequently isolated pathogen was *B. cereus*, which was implicated in 40 of the outbreaks. The most commonly implicated food vehicle was Chinese foods (Notermans and Batt, 1998). The Netherlands and Norway experience the highest reported incidence of outbreaks from this organism. However, *B. cereus* has been the focus of much research in these countries and this may explain the high isolation rate from food poisoning outbreaks (Granum, 1997).

From 1980 to 1995 *B. cereus* accounted for 7.4% of all bacterial foodborne outbreaks of known aetiology in Australia (Crerar *et al.*, 1996). However, this number is probably a minor representation of the true incidence of food poisoning caused by this organism due to under reporting and the mildness of the majority of cases. In New South Wales, Australia, from 1977-1984, *B. cereus* was associated with 39% of all incidences of foodborne disease investigated. It was most frequently associated with rice (Jenson and Moir, 1997). It is apparent that the incidence of *B. cereus* food poisoning varies greatly between reports.

The type of illness most commonly encountered in countries also varies. In Japan, the emetic illness is reported about 10 times more frequently than the diarrhoeal form of the disease. In Europe and North America the diarrhoeal illness is reported more frequently.

This variation is presumably due to the differences in diet and nutrition that exist between these countries (Granum, 1997).

1.6.2 Transmission of *Bacillus cereus*

Asymptomatic carriage of *B. cereus* has been reported in 14 to 43% of people. In addition, during the acute phase of the illness, faeces may contain up to 10^9 organisms/gram and bacteria can also be isolated from the vomitus. This suggests that human contacts such as food handlers may be important sources of *B. cereus* in foods (Jenson and Moir, 1997). The belief however, is that the organism most commonly enters the food chain through contaminated soil or water.

Raw foods of plant origin are the major source of *B. cereus*. The majority of outbreaks associated with this organism occur when food has been held for too long at unsatisfactory storage temperatures. For instance, Johnson (1984) found that the numbers of *B. cereus* can double in 25-60 minutes in boiled rice held at 30°C. This rate is reported to be higher if protein sources such as chicken, beef or egg are present (Jenson and Moir, 1997).

Crielly *et al.* (1994) isolated *B. cereus* and *B. licheniformis* more commonly than other species of *Bacillus* in milk at all stages of processing. The researchers found that *B. cereus* was associated with cattle feed throughout the year. However, the bacteria were more common in raw milk during the summer months. Although *B. licheniformis* was more frequently found in higher numbers, *B. cereus* grew to dominate the bacterial population when grown at ambient temperatures. Crielly *et al*. (1994) suggested that post-pasteurisation contamination may not necessarily be the most important source of *B. cereus* in milk and milk products.

Food implicated in *B. cereus* food poisoning illnesses usually contain at least 10^5 cfu/g. However 10% of outbreaks have been associated with food containing less than this (Jenson and Moir, 1997). Kramer and Gilbert (1989) surveyed the data from a large number of *B. cereus* disease outbreaks. Levels of *B. cereus* involved in the diarrhoeal syndromes studied varied from 1.2×10^3 to 1.0×10^8 organisms/gram with a median

value of approximately 1 x 10^7 organisms/gram. The data from the emetic outbreaks indicated that the numbers of *B. cereus* in implicated foods ranged from 1.0×10^3 to 5.0×10^{10} organisms/gram with a median value of 1×10^7 organisms/gram. Granum (1997) suggested that food with more than 10^4 organisms/gram of *B. cereus* may not be safe for consumption.

1.7 The Virulence Factors of *Bacillus cereus*

The virulence factors of *B. cereus* remain uncertain, partly because it produces a large number of proteins that potentially possess toxigenic activity and partly because these proteins are difficult to isolate. Illness associated with this organism may be mediated by the synergistic effect of a number of products. The major factors which are believed to influence the virulence of *B. cereus* are listed and explained below. The factors that are believed to induce food poisoning are outlined in Table 1.1.

1.7.1 The diarrhoeal toxins

1.7.1.1 Haemolysin BL

A three component complex system, designated haemolysin BL (HBL) is believed to be the major diarrhoeal toxin of *B. cereus*. Beecher and MacMillan (1990) identified a three component toxin that was only active when all three components were present. The researchers found that the individual components of HBL were not haemolytic, however when the components were recombined haemolytic activity was restored. The rabbit ileal-loop and vascular permeability assays provided further evidence of this, as they also required all components to be present for a positive reaction (Thompson *et al.*, 1984; Beecher and MacMillan, 1991; Beecher *et al.*, 1995).

The B component of HBL has a molecular mass of 35 kDa and is encoded by the gene *hblA*. The component's role as a binding protein was verified by an immunofluorescent staining procedure that detected the attachment of purified B component to sheep erythrocytes (Beecher and MacMillan, 1991). The researchers showed that when the components were added separately, in the reverse order, to sheep erythrocytes,

haemolysis did not occur. This suggests that the B component is required to bind to the erythrocytes first before the lytic (L) component can act. Heinrichs *et al*. (1993) provided further evidence of the role of the B component by cloning and expressing the gene *hblA* in *Escherichia coli*. The protein, expressed in *E. coli*, produced the characteristic ring shaped haemolysis (see section 1.8.1.5) when combined with purified L component from *B. cereus*. Analysis of the components using gel filtration chromatography revealed that cell lysis is mediated by the L components, designated L_1 (36 kDa) and L_2 (45 kDa). The extent of haemolysis, however, is determined primarily by the concentration of the B component, and apparently only trace amounts of L are required to cause haemolysis (Beecher and Wong, 1997).

The gene for the B component of HBL was cloned and sequenced by Heinrichs *et al*. (1993). The genes encoding for the L_1 and L_2 components were subsequently cloned and sequenced in the same laboratory (Ryan *et al.,* 1997). The genes *hblC* (L_1) and *hblD* (L_2) were found arranged in tandem and separated by only 37 bases. The gene *hblA* was located immediately downstream from the gene encoding the L_1 protein. Ryan *et al*. (1997) suggested that the four genes, including a gene *hblB* of unknown function, are cotranscribed and constitute an operon.

Various studies have found that approximately half of *B. cereus* strains produce diarrhoeal enterotoxin (Pirttijarvi *et al.*, 1996). Mantynen and Lindstrom (1998), using PCR, found that 41.3% of the amplification products (n = 80) of *B. cereus* strains tested hybridised with a *hblA* probe in a Southern blot. Granum *et al*. (1996) analysed 321 *B. cereus* isolates and found that 239 (74%) displayed cytotoxicity in the Vero cell assay. However, PCR and hybridisation assays detected the B-component of HBL in only 127 of these strains (53% of the cytotoxic strains). It is evident from these results that HBL is not the only product of *B. cereus* which is capable of inducing cytotoxicity and most likely enterotoxicity.

1.7.1.2 Enterotoxin T

A single component protein (41 kDa) with enterotoxic activity was identified by Agata *et al*. (1995) and subsequently designated enterotoxin T. The protein exhibited Vero cell cytotoxicity, positive vascular permeability reaction and fluid accumulation in the ligated mouse ileal loop. Enterotoxin T was also lethal to mice upon injection. The enterotoxin T gene (*bceT*) was subsequently cloned and expressed in *E. coli*, resulting in the production of the 41 kDa protein (Agata *et al*., 1995). Recent studies have indicated that enterotoxin T may not be able to induce food poisoning (Hansen *et al*., 2003; Choma and Granum, 2002).

Studies utilising PCR to determine the prevalence of enterotoxin T gene in strains of *B. cereus* vary in their results. Agata *et al*. (1995) found the gene *bceT* in 100% (10/10) of the isolates tested. The bacteria included four strains obtained from diarrhoeal syndrome foodborne illnesses, three strains from emetic syndrome foodborne illness and three environmental strains. Granum *et al*. (1996) detected the *bceT* gene in only 40% (37/71) of the isolates tested. Hsieh *et al*. (1999) reported that the gene was present in 50% (14/28) of *B. cereus* food isolates and 57% (17/30) outbreak-associated strains. Mantynen and Lindstrom (1998) only found the gene in the model strain (B-4ac) of 58 strains of *B. cereus.* These discrepancies may be due to the use of different PCR primers or to varying populations in different regions.

1.7.1.3 Non-haemolytic enterotoxin

Evidence for the existence of a third enterotoxin of *B. cereus* was first discovered by Granum *et al*. (1996). The researchers found that 2 of 7 food poisoning strains tested did not produce HBL or enterotoxin T, therefore, another toxin must have been responsible for the illnesses noted. Another three-component enterotoxin complex, which is now known as non-haemolytic enterotoxin (NHE), was implicated as being responsible (Lund and Granum, 1996). The complex is composed of three proteins with molecular masses of approximately 39, 45 and 105 kDa. Similarly to HBL, all components are required for maximum cytotoxic activity. The complex is highly cytotoxic to Vero cells but is non-haemolytic. The 45 kDa component is the main target

antigen detected in the commercial *Bacillus* Diarrhoeal Enterotoxin Visual Immunoassay (Tecra). The sequence of the 39 kDa protein overlaps with five of the six amino acids in the N-terminal of the L_1 protein of HBL published by Beecher and Wong (1994). Furthermore, the three toxic components were all recognised by a polyclonal antiserum reported to detect enterotoxin from *B. cereus* (Lund and Granum, 1996). The NHE genes are commonly found in strains of *B. cereus*. Andersen-Borge *et al*. (2001) found NHE genes in 100% (n=11) of *B. cereus* isolates tested.

1.7.1.4 Enterotoxin FM

A novel enterotoxin gene was identified from the *B. cereus* strain FM1. The gene for enterotoxin FM was subsequently cloned and sequenced (Asano *et al.*, 1997). The cloned gene was designated *entFM*. To determine the location of the gene, total DNA from *B. cereus* FM1 was separated into plasmid and chromosome fractions by $CsCl_2$ density gradient centrifugation and blotted onto nylon membrane. DNA on the membrane was subjected to hybridisation with a radioactive probe specific to the *entFM* gene. The results indicated that the *entFM* gene was on the chromosome (Asano *et al.*, 1997). Subsequently Asano *et al*. (1997) designed PCR primers to amplify the full protein-coding region of the enterotoxin. The primers were used to show that the gene is present in a large variety of *B. thuringiensis* strains. The enterotoxin gene did however have minor differences in amino acid sequences. Hsieh *et al*. (1999) used the same primers to survey a large number of *Bacillus* spp. The *entFM* gene was found in 78 of the 84 (93%) *B. cereus* strains, one of the three (33%) *B. mycoides* strains and seven of the nine (78%) *B. thuringiensis* strains. In addition, PCR results showed that 27 of the 28 (96%) *B. cereus* food isolates and all 30 (100%) of the outbreak-associated strains contained the gene *entFM*. In this extensive study by Hsieh *et al*. (1999) the *entFM* gene was found to be the most prevalent enterotoxin gene for *B. cereus* group.

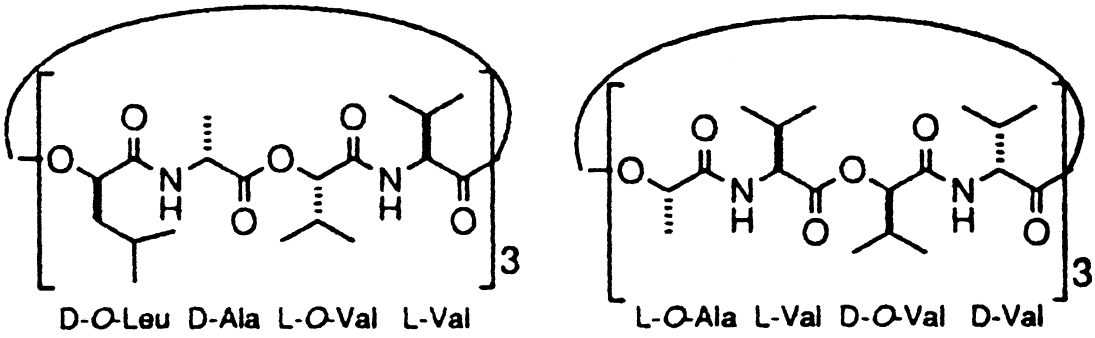
1.7.1.5 Cytotoxin K

A new toxin named cytotoxin K was recently isolated from a *B. cereus* strain that caused a serious food poisoning outbreak in which three people died. Cytotoxin K is a 34 kDa protein that is believed to cause necrotic enteritis. Sequence and structural analysis revealed that the toxin is similar to the α-haemolysin of *Staphylococcus aureus* and the β-toxin of *Clostridium perfringens* (Lund *et al*., 2000). The distribution of cytotoxin K seems to be limited. Ghelardi *et al*. (2002) did not find the toxin gene (*cytK*) in 20 strains of *B. cereus* tested.

1.7.2 The emetic toxin (cereulide)

Due to difficulties in purifying cereulide, very little is known about this peptide. Cereulide is an extremely stable compound, it can survive trypsin and pepsin treatments, pH 2-11 and heating of 121°C for 90 min (Jenson and Moir, 1997).

A peptide produced by *Bacillus cereus*, which was termed the vacuolation factor, was shown to cause vacuolation of mitochondria when exposed to HEp-2 (human carcinoma of the larynx) cells (Hughes *et al*., 1988). The HEp-2 cell vacuolation factor was extracted and purified by Agata *et al*. (1994). The toxin responsible was a 1.2 kDa dodecadepsipeptide that was subsequently named cereulide. Agata *et al*. also determined the structure of the toxin: (D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$. Cereulide was shown to have a very similar structure to the potassium ionophore valinomycin (Figure 1.2). Like cereulide, valinomycin also inflicts mitochondrial damage when added to HEp-2 cells and induces emesis in *Suncus murinus* (Agata *et al.*, 1995). Mikkola *et al*. (1999) found that the action of cereulide was similar to that of valinomycin over a wide range of tests. They concluded that the toxic effects were due to cereulide being a potassium ionophore.



Cereulide Valinomycin

Figure 1.2 The structures of cereulide and valinomycin (Agata *et al*., 1994).

The process whereby cereulide is formed is currently unknown. The toxin may be the translation product of a *B. cereus* gene or the result of enzymatic conversion of a certain substrate in the growth medium (Notermans and Batt, 1992). The production of cereulide has been suggested to be related to sporulation (Shinagawa *et al.*, 1991). Cereulide only seems to be produced when *B. cereus* is grown on particular substrates, particularly rice and other farinaceous materials. Fried or cooked rice has been implicated in nearly 95% of all emetic food poisoning outbreaks (Jenson and Moir, 1997).

Various artificial growth media have been tested to ascertain which medium is optimal for *B. cereus* growth and cereulide production. Shinagawa *et al*. (1992) found that growth of *B. cereus* in cooked rice suspension resulted in much higher titres of cereulide than growth in brain heart infusion (BHI) broth, trypto-soya broth, trypto-soya agar and cooked rice agar. Agata *et al*. (1999) found cereulide titres were highest when *B. cereus* was grown in skim milk compared to growth in BHI, trypto-soya broth, and nutrient broth. Agata *et al*. also developed a minimum amino acid-defined medium (MADM) by subtracting amino acids from complete amino acid-defined medium (CADM) to leave only the amino acids that were essential for growth and cereulide production. The resulting medium included only three essential amino acids, valine,

leucine and threonine. Detectable titres of cereulide were produced when *B. cereus* was grown on this medium. The development of this synthetic medium may facilitate the purification of cereulide as purification from other culture media such as skim milk requires many steps and is frequently not practical.

Cereulide's capacity to induce vomiting in humans is not completely understood. Oral and intraperitoneal application of cereulide to *Suncus murinus* (musk shrew) has been shown to provoke vomiting (Agata *et al.*, 1995). The researchers found that vagotomy or a 5-hydroxytryptamine ($5\text{-}HT_3$) receptor antagonist completely inhibited the emetic effect. Therefore it was concluded that cereulide stimulates the vagus afferent through binding to the $5\text{-}HT_3$ receptor which results in vomiting.

The prevalence of emetic strains of *B. cereus* in the environment and in particular foods is unknown. Mikami *et al*. (1994) found 16 of 310 (5.2%) isolates from various foods and environmental sources were cereulide producing strains (as shown by the HEp-2 vacuolation assay). All of the cereulide producing strains isolated were of the H1 serotype. Shinagawa *et al*. (1991) found that 23 of 27 (85.2%) isolates from *B. cereus* vomiting-type food poisoning were serotype H1.

Table 1.1 The toxins of *Bacillus cereus* and their properties

Toxins	Associated Illness	Associated Genes	Protein/Peptide Size
Haemolysin BL	Diarrhoeal	B - *hblA*	B - 35 kDa
		L_1 - *hblC*	L_1 - 36 kDa
		L_2 - *hblD*	L_2 - 45 kDa
Enterotoxin T	Diarrhoeal	*bceT*	41 kDa
Enterotoxin FM	Diarrhoeal	*entFM*	45 kDa
Non-haemolytic enterotoxin	Diarrhoeal	*nheA*	NheA - 45 kDa
		nheB	NheB - 39 kDa
		nheC	NheC - 105 kDa
Cytotoxin K	Diarrhoeal	*cytK*	34 kDa
Cereulide	Emetic	unknown	1.2 kDa

Based on data from: Beecher and Wong, 2000; Agata *et al*., 1995; Asano *et al*., 1997; Lund *et al*., 1999; Lund *et al*., 2000; Agata *et al*., 1994.

1.7.3 Haemolysins

1.7.3.1 Haemolysin 1

Haemolysin 1 (or cereolysin) is a thiol activated protein that cross-reacts with streptolysin-O and has a molecular weight of about 55 kDa. This protein is responsible for the main haemolysis observed in *B. cereus* and is lethal when injected into mice. Haemolysin 1 is heat labile but is not susceptible to proteolysis. It is inhibited by cholesterol and serum (Granum, 1994; Jenson and Moir, 1997).

1.7.3.2 Haemolysin 2

Considerably less is known about haemolysin 2. It is heat labile and susceptible to proteolytic enzymes. The molecular weight of the protein is approximately 30 kDa. It is not susceptible to cholesterol. The *in vivo* toxicity of this protein has not yet been established (Granum, 1994; Jenson and Moir, 1997).

1.7.4 Phospholipases C

Phospholipases C specific for various phospholipids have been isolated from several bacteria, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *B. cereus* (Yamada *et al*., 1988). Three different phospholipases C have been reported from *B. cereus*, they have all been cloned and are well characterised.

1.7.4.1 Phosphatidylinositol hydrase

Phosphatidylinositol hydrase (PIH) is a 34 kDa enzyme which hydrolyses phosphatidylinositol (PI) and PI-glycan-containing membrane anchors, which are important structural components of one class of membrane proteins. PIH is non-haemolytic and does not require any ions for biological activity. This enzyme is not genetically linked to the two other phospholipases C (Granum, 1994).

1.7.4.2 Phosphatidylcholine hydrolase

Hydrolysis of lecithinin (egg yolk reaction) is a major criterion for the identification of *Bacillus* spp. Most strains of the *B. cereus* group, such as *B. cereus*, *B. thuringiensis* and *B. mycoides*, possess lecithinase activity. The reaction is catalysed by the phospholipase C, phosphatidylcholine hydrolase (PCH) which hydrolyses lecithin (Schraft and Griffiths, 1995). PCH also hydrolyses phosphatidylethanolamine and phosphatidylserine. PCH requires the presence of either zinc or calcium ions for activity (Granum, 1994).

1.7.4.3 Sphingomyelinase

Sphingomyelinase is responsible for the hydrolysis of sphingomyelin. The enzyme requires magnesium for activity and is inhibited by zinc and calcium. Hsieh *et al*. (1999) designed PCR primers based on the reported sequence of four different strains of *B. cereus*. Positive PCR results were obtained for all *Bacillus* subgroup 1 strains tested. This suggests that sphingomyelinase alone cannot be responsible for the symptoms elicited by this organism as not all strains are capable of causing disease. However, the researchers showed that this PCR method was accurate for the identification of *B. cereus* group cells as 100% of these strains were positive and no positives occurred when other species of *Bacillus* and a large variety of foodborne organisms (including some that produced sphingomyelinase) were tested.

PCH (*cerA*) and sphingomyelinase (*cerB)* are genetically linked. The two genes are separated by only 79 bases, between which is situated a promoter sequence and a ribosomal binding site (Gilmore *et al*., 1989). The synergistic effect of these two enzymes results in haemolysis of mammalian erythrocytes (Schraft and Griffiths, 1995). It has also been observed that the combination of *Staphylococcus aureus* sphingomyelinase and *B. cereus* PCH resulted in a total lysis of an erythrocyte preparation in 60 minutes. Neither enzyme alone affected greater than 2% lysis of the erythrocyte population over 180 minutes. The observations of synergism and the close genetic linkage of the sphingomyelinase and PCH genes suggests that the two enzymes function naturally together as an effective cytolysin (cereolysin AB). This deduction is supported by the α-toxin of *Clostridium perfringens* which displays both sphingomyelinase and PCH activities (Gilmore *et al.*, 1989).

1.7.5 *PlcR*: A regulator of extracellular virulence factor gene expression

PlcR is a gene that controls the expression of several non-specific extracellular virulence factors. This gene is common to all the members of the *Bacillus* subgroup 1. The *plcR* gene from *B. anthracis* was shown to be non-functional (Agaisse *et al.*, 1999). However, insertion of a functional gene from *B. cereus* lead to the expression of a number of factors that are usually not observed in *B. anthracis* (e.g. haemolysins,

proteases and phospholipases). The inactivated *plcR* gene is believed to be a major influencing factor in the differences that are observed between *B. anthracis* and *B. cereus* (Mignot *et al*., 2001).

PlcR was originally identified as a transcriptional activator of *plcA*, the gene that encodes phosphatidylinositol-specific phospholipase C (Lereclus *et al*., 1996). However, it is now known that *plcR* controls the expression of a large regulon comprising at least 14 genes. Polypeptides produced by *plcR*-regulated genes include degradative enzymes, cell-surface proteins and toxins, all of which can be considered as potential virulence factors (Agaisse *et al.*, 1999).

PlcR controls the expression of several toxin genes including HBL, NHE, cytotoxin K, phosphatidylinositol hydrase, phosphatidylcholine hydrolase and sphingomyelinase (Agaisse *et al.*, 1999; Lund *et al*., 2000). Therefore, this transcriptional activator can be considered an important virulence regulator for members of the *B. cereus* subgroup 1.

Three of the *plcR*-regulated genes, *plcA*, *plcB* and *hblA* have been mapped in *B. cereus* ATCC 14579, *B. thuringiensis* ssp. *canadensis* HD224, *B. thuringiensis* spp. *berliner* 1715 and *B. thuringiensis* spp. *gelichiae*. The chromosome maps of these four strains were very similar and the three genes in question were not clustered, suggesting that *plcR*-regulated genes are dispersed on the chromosome. Subsequently additional *plcR*-regulated genes were mapped in *B. cereus* ATCC14579. The genes were found to be spread out over one-half of the genome, clearly demonstrating that *plcR*-regulated genes do not form a cluster (Agaisse *et al.*, 1999).

Sequence analysis of the promoter region of *plcR*-regulated genes revealed a highly conserved palindromic region (TATGNAN$_4$TNCATA). This region is believed to be the specific recognition target for PlcR activation. Subsequent deletion and nucleotide substitution analysis demonstrated the importance of the conserved region in the activation process (Agaisse *et al.*, 1999).

1.7.6 The spore

The vegetative cells of *B. cereus* are not particularly resistant to environmental stresses such as heat, radiation or chemicals. However, the spores produced by this bacterium are highly resistant due to their metabolic dormancy and tough physical nature (Jenson and Moir, 1997).

All members of the genus *Bacillus* can initiate the process of sporulation when one or more nutrients becomes limiting for growth. The first noticeable event in sporulation is an unequal division of the cytoplasm, resulting in a small and a large progeny, each with a complete genome. The small compartment (forespore) is destined to become the mature spore, while the large compartment (mother cell) engulfs the forespore. Eventually after a series of morphological changes the mother cell lyses and releases the mature spore into the environment (Setlow, 1994).

Mature spores have no detectable metabolism (i.e. are dormant) and can survive for extremely long periods in the absence of nutrients. Long term studies on spore survival indicate that >80% of spores in a population of *Bacillus* spp. can survive for up to a year. Similar studies suggest that spores can survive for up to 100-2000 years in the environment (Setlow, 1994). Parry and Gilbert (1980) conducted a study to determine the heat resistance of *B. cereus* spores at 95°C. The researchers found that isolates of serotype H1 were more resistant. They suggested that the preparation of rice might select for serotype 1 and therefore explain why this serotype is most commonly implicated in foodborne outbreaks.

Probably the most apparent physical difference between vegetative cells and the more resistant spore is the amount of water present in the cell. Growing cells are comprised of 75-80% water (3-4 g water/g dry weight), whereas, the spore core has a water content of 0.5-1 g water/g dry weight (Setlow, 1994). Reduced water content is the major factor in determining spore heat resistance. The temperature at which sporulation takes place has a significant impact on the heat resistance of the resultant spores, with spores produced at a higher temperature being generally more heat resistant than spores of the same strain produced at lower temperatures. The reason for this is believed to be a decrease in

water content of the spores when they are produced at higher temperatures (Setlow, 1994).

Spores of *B. cereus* are relatively moderately resistant to heat when compared to other mesophilic *Bacillus* spp. such as *B. subtilis*, *B. licheniformis* and *B. coagulans* (Jenson and Moir, 1997). However, the spores are still extremely resistant to heat. In addition, spores are even more resistant to cold: spore *D* values increase about 10-fold for each 10°C fall in temperature (Setlow, 1994).

1.8 Toxin Detection Methods

1.8.1 Diarrhoeal toxin detection methods

1.8.1.1 Commercial diarrhoeal enterotoxin immunoassay kits

Two immunological assays are available for the detection of diarrhoeal toxins of *B. cereus*. The TECRA *Bacillus* Diarrhoeal Enterotoxin Visual Immunoassay (BDE-VIA) is manufactured by Bioenterprises Pty Ltd (Roseville, Australia) and supplied by TECRA diagnostics (Batley, UK). The kit is a microtitre plate-based immunoassay which can be read visually or with an automated plate reader. OXOID (Basingstoke, UK) market the *Bacillus cereus* Enterotoxin Reverse Passive Latex Agglutination (BCET-RPLA) kit which is manufactured by Denka, Japan.

The validity of these assays has been brought into question due to conflicting results with each other and with cell culture assays. Day *et al*. (1994) detected enterotoxin in the supernatants of 13 strains of *B. cereus* using the BDE-VIA but only six with the BCET-RPLA. One of the seven strains which failed to react in the BCET-RPLA had previously been shown to induce illness in a monkey feeding test and four of the other six had been implicated in food poisoning outbreaks. Day *et al*. subsequently stated that the TECRA BDE-VIA is more reliable than the OXOID BCET-RPLA. This statement is supported by a number of researchers (Buchanan and Shultz, 1994; Rusul and Yaacob, 1995).

Buchanan and Schultz (1994) compared the two kits with a CHO cell cytotoxicity assay. The researchers found that while the results from the three assays correlated for a number of strains, it was apparent that the two kits detected different antigens. Further evidence was provided by the observation that no cross-reactivity occurred from the positive controls of each kit.

Beecher and Wong (1994) tested the three purified components of HBL with the BCET-RPLA. The results indicated that the kit is specific for the L_2 component of HBL. To further validate these results the haemolytic activity of the positive control provided by the BCET-RPLA was tested. The control showed no activity when alone or when combined with the B component or B plus L_2. However, the control did induce lysis when combined with the B and L_1 components, therefore supporting the inference that the BCET-RPLA detects the L_2 component of HBL.

Beecher and Wong (1994) also determined the antigens detected by the TECRA BDE-VIA. The researchers used the detecting antibody/enzyme conjugate to probe Western blots of culture supernatant from *B. cereus* F837/76 (a highly enterotoxigenic strain). The darkest bands on the Western blot were at 40 and 41 kDa. These proteins were purified and then shown to react strongly with the BDE-VIA. The two proteins were apparently non-toxigenic as they failed to react in the vascular permeability reaction. Recently however, Lund and Granum (1996) showed that the BDE-VIA detects the 45 kDa protein of NHE.

It is apparent that neither commercial enterotoxin detection kit can be used as the definitive test for detection of diarrhoeal toxin production. The BCET-RPLA may be useful for the detection of HBL. The kit only detects the L_2 component of the complex and therefore may produce some false positives due to one or both of the other components being absent. This eventuality may be rare however as Ryan *et al*. (1997) reported that the three genes for the toxin were very close on the genome and formed an operon. The BDE-VIA may be useful for the detection of NHE. Once again however only one of the three components are detected and a reaction may not always reflect a toxigenic isolate.

1.8.1.2 Cell cytotoxicity assays

A number of cell lines are susceptible to the diarrhoeal toxins. The most commonly used are Vero (monkey kidney) and CHO (Chinese hamster ovary) cell lines. Buchanan and Schultz (1994) found that the CHO cell cytotoxicity assay was more sensitive than both the BDE-VIA and the BCET-RPLA. Christiansson *et al*. (1989) compared the HeLa S3, Vero and HEL (human embryonic lung) cell lines to determine their comparative sensitivity to diarrhoeal toxins. HEL was discovered to be more susceptible than the other two cell lines. Jackson (1993) found the McCoy cell line also to be a sensitive cell culture system.

The majority of cell cytotoxicity assay protocols involve adding filtered bacterial supernatant to a cell line and observing if the filtrate affects the cells. The results are interpreted in a number of ways. Although cell cytotoxicity assays are an inexpensive and convenient method of diarrhoeal toxin detection they present many drawbacks. Many non-specific extracellular virulence factors are produced by strains of *B. cereus*, which may be cytotoxic to the cell line. Moreover this detection method is time consuming (>3 days for results) and requires the constant maintenance of cell lines.

1.8.1.3 Conventional detection methods

The vascular permeability reaction was traditionally used for the detection of diarrhoeal toxins. Culture filtrates or supernatants are injected intradermally into the backs of depiliated rabbits. After three hours the rabbit is injected intravenously with 4 mL of 2% Evans blue dye. Zones of blueing and necrosis are measured at the site of intradermal injection after an additional hour. The blueing is produced as a result of the alteration of the permeability of the blood vessels by the toxin (Jenson and Moir, 1997).

The ligated rabbit ileal loop assay (young New Zealand white rabbits) involves intraluminally injecting 1 or 2 mL of test material into 5 or 10 cm ileal loops, respectively. The reaction is considered positive if the ratio of the volume of fluid accumulation to loop length is >0.5 (Jenson and Moir, 1997). The growth medium used greatly influences a given strain to produce a response. Spira and Goepfert (1972) found

Brain Heart Infusion broth (BHI) to be the best for this assay. The ligated ileal loop assay correlates well with the vascular permeability reaction. However, both methods are non-specific and require the handling and maintenance of rabbits which is expensive.

1.8.1.4 Polymerase chain reaction

The polymerase chain reaction is a fast and extremely sensitive method of detecting whether a bacterium possesses the toxin gene in question. This method has been used extensively for all of the diarrhoeal toxin-producing genes (see section 1.7.1). However, the presence of a toxin gene does not necessarily indicate that the bacterium is capable of producing the protein in concentrations sufficient to produce disease and does not indicate the presence of any co-factors that may be required for toxin synthesis. Various studies have stated that >50% of *B. cereus* isolates are capable of producing diarrhoeal toxins (Agata *et al.*, 1995; Granum *et al.*, 1996; Hsieh *et al.*, 1999). However, a much smaller proportion is believed to be capable of inducing diarrhoeal disease. PCR may be used as a powerful tool in conjunction with other assays which indicate how much enterotoxin is produced i.e. cell cytotoxicity assays, vascular permeability reaction and ligated ileal loop assay.

1.8.1.5 Gel diffusion assay for haemolysin BL

Beecher and MacMillan (1990) found that HBL exhibits a unique ring-shaped pattern of haemolysis in a gel diffusion assay. Lysis does not begin at the well edge but rather several millimetres away. With time the cells closer to the well are lysed but the haemolytic zone does not increase much beyond the initial diameter (Beecher and Wong, 1994). The unusual haemolytic pattern is believed to be reliant upon the concentration of the B component. Near the well, concentrations of B are believed to saturate the membrane and inhibit association with the L component (which causes lysis). Further from the well concentrations of the B component are lower, allowing association with the L component, and therefore lysis, to occur. With time, continued diffusion allows the concentration of the B component nearer the well to decrease and

haemolysis can occur (Beecher and MacMillan, 1994). This method could be a very efficient and inexpensive method of identifying isolates that produce HBL.

1.8.2. Cereulide detection methods

Currently simple, convenient methods are not available for the detection of cereulide. The most commonly used methods are cell culture assays. Szabo *et al*. (1991) found that of seven cell lines tested Int 407, CHO and HEp-2 were all equally sensitive with the former being preferred for ease of interpreting results. Cereulide causes vacuolation of the mitochondria in these cell lines, which can be visualised under a light microscope (Hughes *et al.*, 1988). Finlay *et al*. (1999) reported a more sensitive HEp-2 cell based assay. The method utilised 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which is regarded as an indicator of cell viability. This assay detects cereulide production due to the toxin's effect on HEp-2 cell mitochondria. Beattie and Williams (1999) reported that the MTT method was also highly sensitive with CHO cells.

Starch hydrolysis can be used as a screening assay for emetic strains. Isolates of *B. cereus* that produce cereulide are unable to hydrolyse amylose (Shinigawa *et al*., 1979; Nishikawa *et al*., 1996; Agata *et al*., 1996). This can be observed on nutrient agar supplemented with starch. Following bacterial growth, the agar plate is flooded with iodine and colonies are examined for surrounding clear zones that indicate starch hydrolysis.

Another assay recently reported was based upon loss of motility and mitochondrial swelling in boar spermatozoa (Andersson *et al.*, 1998). The paralysed spermatozoa exhibited swollen mitochondria, but no depletion of cellular ATP or damage to the plasma membrane integrity. The 50% effective concentration of purified cereulide to boar spermatozoa was 0.5 ng of toxin/ml of boar semen. Similar effects were also induced by 2 ng/ml of valinomycin. The researchers surmised that the symptoms provoked by cereulide on the boar spermatozoa were due to the toxin acting as a membrane channel-forming ionophore, damaging mitochondria and blocking the phosphorylation required for the motility of boar spermatozoa. The sensitivity of the

boar spermatozoa assay was recently improved by a new method that assesses the dissipation of the mitochondrial inner membrane transmembrane potential ($\Delta\psi m$) (Hoornstra *et al*., 2003). A sensitive chemical assay using high performance liquid chromatography (HPLC) with ion trap mass spectrometry has also been developed in the same laboratory (Haggblom *et al*., 2002).

An appropriate animal model for the study of cereulide has not been found to date. Cereulide has been shown to induce emesis in rhesus monkeys after intragastric administration (Shinagawa *et al.*, 1995). Emesis can also be induced in *Suncus murinus* (musk shrew) both by oral and intraperitoneal application (Agata *et al.*, 1995). However, these animals are difficult to obtain and are unsuitable for routine testing.

1.9 Control of *Bacillus cereus* Food Poisoning

It is virtually impossible to ensure that plant foods are totally free from spore forming soil bacteria such as *B. cereus*. However, if food is cooked and stored correctly *B. cereus* should not cause serious problems unless growth is allowed to occur. Problems may arise if food is processed in a way that only vegetative cells are destroyed leaving spores to germinate without competitors. To combat this situation cooked foods must be stored below 5°C or above 60°C. The majority of *B. cereus* food poisonings occur because cooked food is allowed to cool at unsatisfactory storage temperatures.

1.10 Major Objectives of the Project

The first major objective of this project was to evaluate existing diarrhoeal toxin-detection methods to determine their relative efficacies in detecting toxigenic strains of *B. cereus*. An additional objective in this phase of research was to establish the incidence of specific toxin genes in clinical and food isolates of *B. cereus*, and to gain some insight into the relative significance of the four documented diarrhoeal toxins.

The second major objective was to determine the genes that are associated with the production of the emetic toxin (cereulide). The genes that are responsible for the

synthesis of this toxin have not been discovered. The characterisation of these genes would augment the research into the production of cereulide and thereby facilitate the development of improved diagnostic methods.

The third major objective of this thesis was to develop an improved method of detecting emetic strains of *B. cereus*. Due to the unavailability of a rapid, easy-to-use detection method food products currently cannot be screened for the presence of toxigenic strains. In addition, it is difficult for health authorities to implicate emetic isolates of *B. cereus* following food poisoning outbreaks. The aim is to produce a rapid, sensitive method that can easily be adopted by quality control laboratories and health authorities.

Chapter 2

General Materials and Methods

2.1 Bacterial strains used in the study

Bacillus isolates were kindly donated from a variety of sources, which are listed below:

- Lars Andrup, Nat. Inst. Occup. Health, Copenhagen, Denmark.
- Maria Andersson, Department of Applied Chemistry and Microbiology, University of Helsinki, Finland.
- Per Einar Granum, Department of Pharmacology, Microbiology and Food Hygiene, Norwegian School of Veterinary Science, Oslo, Norway.
- Melissa Toh, Department of Food Science and Technology, University of New South Wales, Sydney, Australia.
- John Bates, Queensland Health Scientific Services, Brisbane, Queensland.
- In addition, isolates were sourced from a survey of supermarket salad bars in Townsville, Australia (Horwood, 1998).

Bacteria were classified into groups based upon descriptions of the isolate from the source (Table 2.1, 2.2, 2.3, 2.4).

Table 2.1 *Bacillus cereus* diarrhoeal and non-emetic isolates

Strain Designation[a]	Description[b]	Case History	Source
F4094/73	non-emetic	unknown	Lars Andrup
ATCC14579	non-emetic	*B. cereus* type strain	Maria Andersson
F528	diarrhoeal	diarrhoeal outbreak	Maria Andersson
F3453	non-emetic	unknown	Maria Andersson
IH41064	diarrhoeal	diarrhoeal outbreak	Maria Andersson
IH41385	non-emetic	unknown	Maria Andersson
NVH0075/95	produces NHE	diarrhoeal outbreak	Per Einar Granum
NVH1230/88	produces HBL and NHE	diarrhoeal outbreak	Per Einar Granum
CK1[c]	produces cytotoxin K	unknown	Per Einar Granum
CK2[c]	cytotoxin K -ve	unknown	Per Einar Granum
3153, 3154, 3155, 3156, 3259, 3261, 3262, 3261, 3262, 3263, 3264, 1994, 1996, 1997, 2174, 2175 2884, 3164, 3166	diarrhoeal	unknown	Melissa Toh

[a] F, Public Health Service, London, UK; ATCC, American Type Culture Collection; IH, University of Helsinki, Department of Applied Chemistry and Microbiology, Helsinki, Finland; NVH, Norwegian School of Veterinary Science, Oslo, Norway;

[b] Description of isolate provided by supplying researcher

[c] Isolate designation was not provided

This group encompasses all of the *B. cereus* isolates that the supplying researchers described as non-emetic (i.e. does not produce cereulide) or associated with outbreaks

of diarrhoeal food poisoning. All isolates were tested for purity by culturing on PEMBA and confirmed as *B. cereus* by the Holbrook Anderson spore stain.

Table 2.2 *Bacillus cereus* emetic isolates

Strain Designation[a]	**Description**[b]	**Case History**	**Source**
F47, F4426, F5881, NC7401, F4810/72	emetic	emetic outbreak	Maria Andersson
NCF, NCG, NCY, NC954, NC1044, NC1078	emetic	food poisoning incident	Melissa Toh
NC7401, NC90T, NC88F, NC1310, NC1287, NC1219, NC1204	emetic	faeces	Melissa Toh
NC1184, NC1149, NC1154	emetic	vomitus	Melissa Toh
NC1315, NC1291, NC1260, NC1245, NC1240, NC1237	emetic	foods	Melissa Toh
NC1246, NC1128	emetic	soil	Melissa Toh

[a] NC, Nagoya City Public Health Institute, Nagoya, Japan.

[b] Description of isolate provided by supplying researcher

This group encompasses all of the isolates that the supplying researchers described as emetic (i.e. produces cereulide). All isolates were tested for purity by culturing on PEMBA and confirmed as *B. cereus* by the Holbrook Anderson spore stain.

Table 2.3 *Bacillus cereus* food isolates

Strain Designation	Description	Case History	Source
Bc1 to Bc30	food isolates	isolated from supermarket salad bars	Paul Horwood

All isolates were tested for purity by culturing on PEMBA and confirmed as *B. cereus* by the Holbrook Anderson spore stain.

Table 2.4 *Bacillus* spp. isolates

Strain Designation	***Bacillus* Species**	Source
T376, T377	*Bacillus amyloliquefaciens*	Melissa Toh
BA1[a]	*Bacillus anthracis*	John Bates
T2715	*Bacillus circulans*	Melissa Toh
T3269	*Brevibacillus laterosporus*	Melissa Toh
T18, T368, T369, T370, T371, T372, T373, T374, T375, BL1[a]	*Bacillus licheniformis*	Melissa Toh
T16	*Bacillus megaterium*	Melissa Toh
T17	*Bacillus pumilus*	Melissa Toh
T378, T379	*Bacillus sphaericus*	Melissa Toh
T11, T12, T13, T14, T15, T745, BS1[a]	*Bacillus subtilis*	Melissa Toh
BT4100, BT1[a]	*Bacillus thuringiensis*	Melissa Toh

[a] Isolate designation was not provided

This group of bacteria encompasses all of the *Bacillus* isolates obtained that were not classified as *B. cereus*. All isolates were named as received from supplying institution. All isolates were tested for purity by culturing on PEMBA.

2.2 Maintenance of *Bacillus* spp. Culture Collection

2.2.1 Cryopreservation of *Bacillus* isolates

All cells were stored in tryptone soya broth (Oxoid) with 10% glycerol (Appendix 1) at -70°C. Frequently used isolates were also stored on nutrient agar slopes (Appendix 1) at 4°C. All isolates on slopes were refreshed from -70°C stocks monthly.

2.2.2 Examination of strains for purity

All imported strains were examined for purity by plating onto PEMBA. Single colonies were selected and used for all subsequent cryopreservation and re-inoculation procedures.

2.2.3 Holbrook and Anderson spore stain

Following examination for purity *B. cereus* species confirmation was conducted using the Holbrook and Anderson spore stain. Microscopic examination for the presence of lipid globules in the vegetative cells replaces the need for a large number of biochemical tests. Holbrook and Anderson (1980) established that *B. cereus* was the only *Bacillus* species capable of possessing lipid globules in their vegetative cells following growth on PEMBA. This test is particularly useful for differentiating *B. cereus* from *B. thuringiensis* , which exhibits similar growth characteristics on PEMBA yet does not contain lipid globules.

Approximately 1-2mm in diameter of bacterial colony, from overnight growth on PEMBA, was used to prepare a film on a standard microscope slide. Following the fixation of bacteria to the slide by air-drying, the slide was flooded with 5% w/v malachite green. The slide was heated with a flaming alcohol swab until steam production was evident. However, care was taken to ensure that the liquid did not boil. The slide was left on the rack for two minutes and then washed with running water and blotted dry with paper towelling. Sudan black (0.3% w/v in 70% ethyl alcohol) was flooded over the slide and left for 15 minutes. The slide was washed for 5 seconds with

xylene and then blotted dry using paper towelling. The slide was flooded with 0.5% w/v safranin for 20 seconds and washed under running water. The slide was blotted dry and examined under a microscope using the oil immersion lense.

The *Bacillus* spores stain pale green, are central in position and do not swell the sporangium. Lipid globules are stained black and the vegetative cytoplasm strains red. This appearance following the Holbrook and Anderson spore stain, coupled with the typical colony formation on PEMBA, confirms the identification of *B. cereus*.

2.3 Maintenance of Cell Lines (HEp-2 and Vero cells)

2.3.1 Culture and maintenance of cell lines

Vero cells were kindly donated by Jan Smith (TropBio Pty Ltd, Townsville Australia) and the HEp-2 cells were kindly donated by Annalese Semmler (University of Tasmania, Hobart Australia). Cells were grown in DMEM (Gibco) with 10% foetal bovine serum. Cell lines were passaged every 3-4 days using ATV. All cell lines were incubated at 37°C.

2.3.2 Cryopreservation of cells

Healthy and rapidly dividing cells (log phase) were grown to form a confluent monolayer before freezing. Cells were lifted from the surface using ATV. The cell suspension was centrifuged at 1000 *g* for 5 minutes. The pellet (cells) was resuspended in DMEM with 10% foetal bovine serum and 10% dimethyl sulfoxide (DMSO), to give a final concentration of approximately 1 x 10^6 cells/ml (cell count - Section 2.3.4). Aliquots of 1ml were transferred to cryopreservation tubes and frozen at -70°C overnight before being placed in liquid nitrogen. One tube was thawed and checked for viability through culture after 4 days (Fraser, 1999; Mather and Roberts, 1998).

2.3.3 Thawing of cells

Cells were thawed by immersion in a 37°C waterbath. Following surface sterilisation of the tube with 70% ethanol, the cells were added to 10 ml of DMEM with 10% foetal bovine serum.

2.3.4 Cell count

Cell counts were conducted using a haemocytometer (Counting Chamber, Weber Scientific International), using the trypan blue dye exclusion test. Cell suspension and trypan blue (0.2%) were thoroughly mixed in equal volumes, added to the haemocytometer and examined under a light microscope. The concentration of viable cells/ml of medium was determined by counting the unstained cells in a number of the 1x1 mm squares and then applying the following formula:

$$\text{concentration of viable cells (cells /ml)} = \frac{\text{no. of cells counted}}{\text{no. of squares counted}} \times 10^4 \times 2$$

(Fraser, 1999)

2.4 Bacterial Culturing for Enterotoxin Assays

Bacillus spp. cultures were inoculated into 10 ml of brain heart infusion (Oxoid) with 0.1% glucose (BHIG) (Appendix 1). The broths were incubated in a rotary shaker at 30°C, 150 rev/min for 24 hours. Following incubation, 100 µl of this broth was used to inoculate a fresh tube of BHIG. The culture was further incubated in a rotary shaker at 30°C, 150 rev/min for 24 hours.

The resulting culture was centrifuged at 5000 *g* for 5 minutes. The supernatant was filtered through a 0.22 µm syringe filter (Millipore) into a 1.5 ml sterile tube (Eppendorf).

2.5 PCR General Methods

2.5.1 Boiling extraction method

Bacteria were plated for single colony formation on nutrient agar (OXOID) and incubated at 30°C overnight. Approximately 1 to 2 mm in diameter of colony was transferred to 200 µl of TE buffer (Appendix 1) in a 1.5 ml tube (Eppendorf) and placed in a boiling water bath for 15 minutes to lyse the cells. Cell debris was removed by centrifugation at 15,000 *g* for 5 minutes. The supernatant was transferred to a clean 1.5 ml tube and stored at 4°C (Hansen and Hendriksen, 2001).

2.5.2 Gel electrophoresis

High purity agarose (Sigma) was added to 100 ml of 1x TAE (Appendix 1) to the required final concentration (1% w/v and 2% w/v were used in this thesis). The solution was mixed and microwaved for approximately 3 minutes until boiling. The solution was allowed to cool (warm to touch) and 0.5 µg of ethidium bromide/ml of gel solution was added. The warm gel was poured into a gel cast (Pharmacia) and well combs were fitted. Following setting of the gel the plate was overlayed with 1x TAE.

Gel loading solutions were prepared as follows:

PCR samples:

- 10 µl of PCR product
- 2 µl of 6x loading dye

DNA marker:

- 1 µl of DNA marker (1 Kb Plus DNA Ladder - Invitrogen)
- 9 µl of 1x TAE
- 2 µl of 6x loading dye

Solutions (12 µl) were added to the wells. The gel was run at 90V for 1 hour. The gel was removed from the casting and examined for specific-sized DNA fragments under UV light by comparison with the DNA marker.

Chapter 3

Evaluation of Diarrhoeal Toxin

Detection Methods

3.1 Introduction

The pathogenicity of *Bacillus cereus* in gastrointestinal infections has been associated with the ability to produce toxins. A large array of potentially pathogenic substances have been reported from this organism, including four haemolysins, three different types of phospholipase C, cereulide and at least five enterotoxins (Kotiranta *et al*., 2000). The toxins produced by *B. cereus* are reviewed in Section 1.7.1.

B. cereus has been reported to produce five distinct toxins that are capable of inducing the diarrhoeal illness: haemolysin BL (HBL), enterotoxin T, enterotoxin FM, non-haemolytic enterotoxin and cytotoxin K. The relative importance of these enterotoxins is yet to be determined. Haemolysin BL was the first characterised toxin of *B. cereus* that was implicated in the pathogenesis of diarrhoeal food poisoning (Beecher and Macmillan, 1990). However, numerous strains have been implicated in diarrhoeal food poisoning that were unable to produce HBL (Agata *et al*., 1995; Granum *et al*., 1996; Lund and Granum, 1996; Lund *et al*., 2000). It is apparent that none of the enterotoxins described to date are solely responsible for the diarrhoeal illness.

Numerous methods have been developed to differentiate between diarrhoeal and non-diarrhoeal strains of *B. cereus*. Toxin detection methods previously utilised include animal toxicity (Spira and Goepfert, 1972), cell culture cytotoxicity (Jackson, 1993; Buchanan and Schultz, 1994), ELISA (Beecher and Wong, 1994) and PCR (Agata *et al*., 1995; Asano *et al*., 1997; Mantynen and Lindstrom, 1998; Lund *et al*., 2000). Section 1.8.1 of this thesis has a comprehensive review of the commonly used diarrhoeal toxin detection methods. None of these methods can be used as the definitive assay for detection of diarrhoeal strains of *B. cereus*.

This phase of the study was conducted to compare the relative effectiveness of the various diarrhoeal toxin detection methods that are commonly used in laboratories. Further elucidation of the comparative importance and prevalence of the five diarrhoeal toxins of *B. cereus* was also an objective of this research.

3.2 Materials and Methods

3.2.1 Bacterial strains

Sixty-eight *B. cereus* isolates were tested using previously established enterotoxin detection methods. The assays selected for evaluation in this survey were the Vero cell cytotoxicity assay, the gel diffusion assay, two commercial ELISA kits and PCR. The *B. cereus* strains surveyed included emetic, enterotoxic and food isolates (Table 3.1). The isolates were divided into three groups: the emetic strains - comprised 11 emetic food poisoning isolates; the food strains - comprised 30 *B. cereus* strains isolated from supermarket salad bars; diarrhoeal strains - a heterogenous group of isolates consisting of diarrhoeal food poisoning isolates, positive and negative control imports, and Tecra positive control isolates. The emetic and diarrhoeal isolates were tested using all of the methods mentioned above. However, the food isolates were only tested using PCR and Vero cell cytotoxicity.

Table 3.1 *Bacillus cereus* strains used in the enterotoxin survey

Strain Groupings[a]	Strain Designations
Diarrhoeal isolates	F4094/73, ATCC14579, F528, F3453, IH41064, IH41385, 3153, 3154, 3155, 3156, 3259, 3261, 3262, 3263, 3264, 1994, 1996, 1997, 2174, 2175, 2884, 3164, 3166, NVH0075/95, NVH1230/88, CK1, CK2
Emetic isolates	F47, F4426, F5881, NC7401, F4810/72, NCF, NCG, NCY, NC954, NC1044, NC1078
Food isolates	Bc1 to Bc30

[a] See Tables 2.1, 2.2 and 2.3 for complete descriptions of *Bacillus* strains

3.2.2 Vero cell cytotoxicity assay

Vero cells were maintained in DMEM with 10% foetal bovine serum (FBS) at 37°C. Cells were trypsinised and diluted to 10^6 cells/ml in normal growth medium. Falcon 96-well flat bottom cell-culture plates (Becton Dickinson) were seeded with 100 µl of this dilution. Plates were incubated at 37°C for approximately 24 hours or until the cells formed a confluent monolayer (Buchanan and Schultz, 1994).

B. cereus was cultured as described in Section 2.4. Cell-free supernatant was added (100 µl/well) to the left-hand wells and then serially diluted (2-fold) across the plate. The plates were incubated at 37°C for 18 hours. Following incubation, plates were examined under a light microscope for damage to the Vero cells. Reactions were considered positive if >50% of Vero cells had detached from the plate.

3.2.3 Gel diffusion assay for HBL

Haemolysin BL agar (Beecher and Lee Wong, 1994) was made containing 50 mM Tris-HCL, 150 mM NaCl, and 1% purified agar. Sheep blood (Oxoid) (2.5%) was added following autoclaving of the agar solution at 121°C for 30 min. Wells (6 mm diameter) were made in the agar by punching a hole with sterile pipette tips that had been widened by having their ends cut off with a sterile pair of scissors.

B. cereus strains were cultured as outlined in Section 2.4. Supernatant (10 µl) was added to each well and plates were incubated at 25°C. Wells were examined for discontinuous haemolysis every hour for 12 hours.

3.2.4 Tecra *Bacillus* Diarrhoeal Enterotoxin - Visual Immunoassay (BDE-VIA)

The Tecra *Bacillus* Diarrhoeal Enterotoxin - Visual Immunoassay (BDE-VIA) was conducted according to the manufacturer's instructions.

3.2.5 Oxoid *Bacillus* Enterotoxin - Reverse Passive Latex Agglutination (BCET-RPLA)

The Oxoid *Bacillus* Enterotoxin - Reverse Passive Latex Agglutination (BCET-RPLA) was conducted according to the manufacturer's instructions.

3.2.6 PCR methods

Previously published PCR methods were used for detection of the enterotoxin genes. The target genes to be detected and the primers used are listed below in Table 3.2. All primers were synthesised by GeneWorks, Australia.

Table 3.2 Previously published PCR primers for the detection of enterotoxin genes

Target Gene	Primers	Primer Sequences	Product Size	Reference
hblA	hblA1 hblA2	5'-GCTAATGTAGTTTCACCTGTAGCAAC-3' 5'-AATCATGCCACTGCGTGGACATATAA-3'	874 bp	Mantynen and Lindstrom, 1998
bceT	bceT1 bceT2	5'-TTACATTACCAGGACGTGCTT-3' 5'-TGTTTGTGATTGTAATTCAGG-3'	428 bp	Agata *et al*, 1995
entFM	entFM1 entFM2	5'-ATGAAAAAAGTAATTTGCAGG-3' 5'-TTAGTATGCTTTTGTGTAACC-3'	1269 bp	Asano *et al*, 1997

3.2.6.1 Haemolysin BL PCR protocol

The PCR reaction mixture (50 µl) contained sterile distilled H_2O, 1x PCR buffer (MBI Fermentas), 1.5 mM $MgCl_2$, 200 µM (each) of dNTP's (Promega), 1 U of Taq DNA polymerase (MBI Fermentas), 1 µM of each primer (hblA1 and hblA2 - Table 3.2), and 4 µl of template (as prepared in Section 2.5.1).

Amplification was performed in an Eppendorf Mastercycler. The PCR reaction conditions were as follows: 1 cycle at 94°C for 10 min, followed by 35 cycles at 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, and finally 1 cycle at 72°C for 7 min. The amplification results were resolved by 1% agarose gel electrophoresis (as outlined in Section 2.5.2).

3.2.6.2 Enterotoxin T PCR protocol

The PCR reaction mixture (50 µl) contained sterile distilled H_2O, 1x PCR buffer (MBI Fermentas), 1.5 mM $MgCl_2$, 200 µM (each) of dNTP's (Promega), 1 U of Taq DNA polymerase (MBI Fermentas), 1 µM of each primer (bceT1 and bceT2 - Table 3.2), and 4 µl of template (as prepared in Section 2.5.1).

Amplification was performed in an Eppendorf Mastercycler. The PCR reaction conditions were as follows: 35 cycles at 94°C for 20 sec, 57°C for 20 sec, and 72°C for 20 sec. The amplification results were resolved by 1% agarose gel electrophoresis (as outlined in Section 2.5.2).

3.2.6.3 Enterotoxin FM PCR protocol

The PCR reaction mixture (50 µl) contained sterile distilled H_2O, 1x PCR buffer (MBI Fermentas), 1.5 mM $MgCl_2$, 200 µM (each) of dNTP's (Promega), 1 U of Taq DNA polymerase (MBI Fermentas), 1 µM of each primer (entFM1 and entFM2 - Table 3.2), and 4 µl of template (as prepared in Section 2.5.1).

Amplification was performed in an Eppendorf Mastercycler. The PCR reaction conditions were as follows: 35 cycles at 94°C for 20 sec, 52°C for 20 sec, and 72°C for 20 sec. The amplification results were resolved by 1% agarose gel electrophoresis (as outlined in Section 2.5.2).

3.3 Results

3.3.1 Vero cell cytotoxicity

The majority (82.4%) of the *B. cereus* isolates induced CPE in the Vero cell cytotoxicity assay (Table 3.3, 3.4 and 3.5). The supernatants from the diarrhoeal isolates were particularly toxic to Vero cells with all of them inducing monolayer destruction (Figure 3.1). In addition, 72.7% of the emetic strains and 70% of the food isolates were cytotoxic to Vero cells. No difference was noted in the extent of monolayer disruption between the three groups.

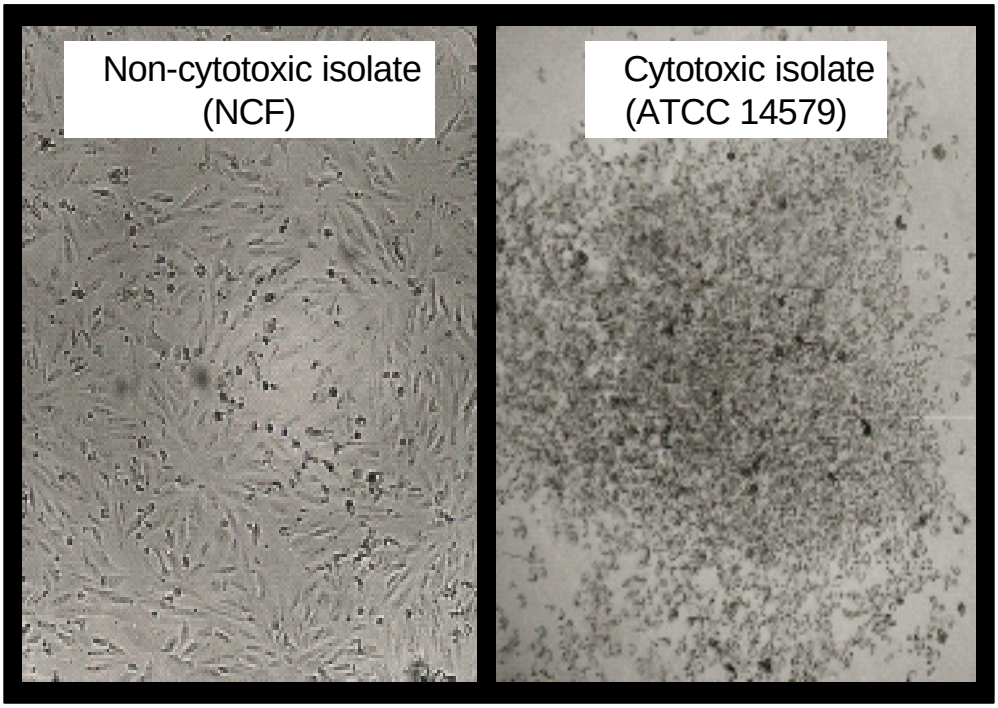
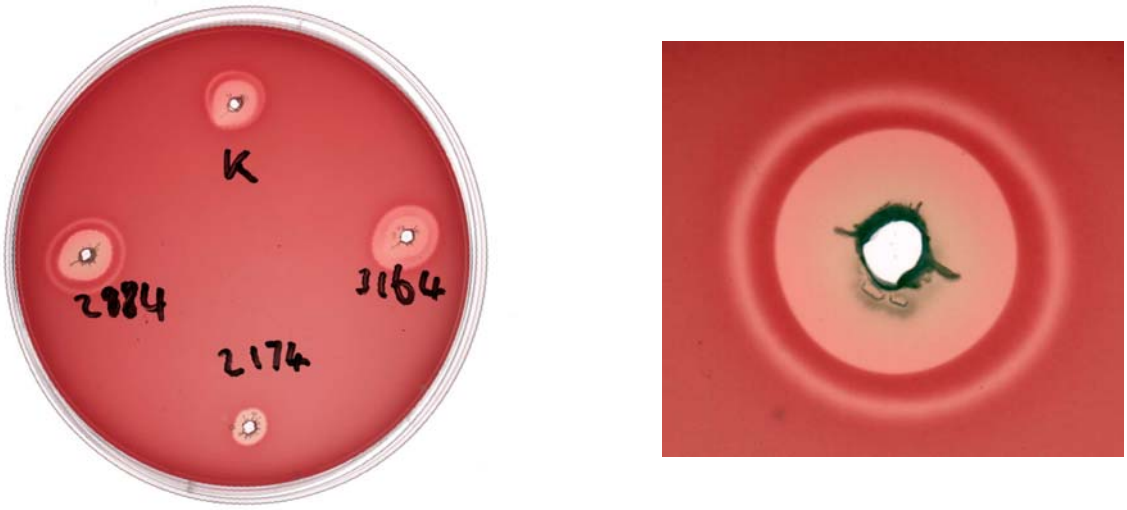


Figure 3.1 Vero cell cytotoxicity assay (magnification: 10X). The diarrhoeal toxins caused the Vero cells to lift off the plate and clump together.

3.3.2 Gel diffusion assay

Overall, 31.6% of *B. cereus* isolates produced the distinctive diffuse ring (Figure 3.2) when grown on HBL agar. None of the emetic strains and 44.4% of the diarrhoeal isolates were recorded as positive with this test (Table 3.3 and 3.4). Figure 3.2 displays the difficulties that sometimes occur in reading the gel diffusion assay. Strain 2884 is obviously positive and the results are easy to interpret. However, strain K (NVH1230/88) and strain 3164 display how the diffuse ring can sometimes be difficult to observe if the ring is incomplete or overlaps with the inner ring of haemolysis. Additionally the optimal time to observe the diffuse haemolysis was noted to vary between strains from two hours to greater than 12 hours.



A. **B.**

Figure 3.2 Gel diffusion assay. The unusual haemolysis ring is believe to be caused by the saturation of the B component of HBL. Haemolysis only occurs when the B component has diffused through the agar to a low enough concentration where haemolysis can occur.

A. A gel diffusion plate consisting of three positive isolates (K = NVH1230/88, 2884 and 3164) and one negative isolate (2174).

B. A close-up of the diffuse haemolysis caused by HBL (strain 2884).

3.3.3 Commercially available ELISA assays

3.3.3.1 Tecra BDE-VIA

The Tecra ELISA results are interpreted by a green colour change reaction (Figure 3.3). Although a colour identification card is included with the assay, results are sometimes difficult to differentiate. Overall 84.2% of isolates were recorded as positive with this assay (Table 3.3 and 3.4). A high proportion of both diarrhoeal (81.5%) and emetic isolates (90.9%) were positive with this assay.

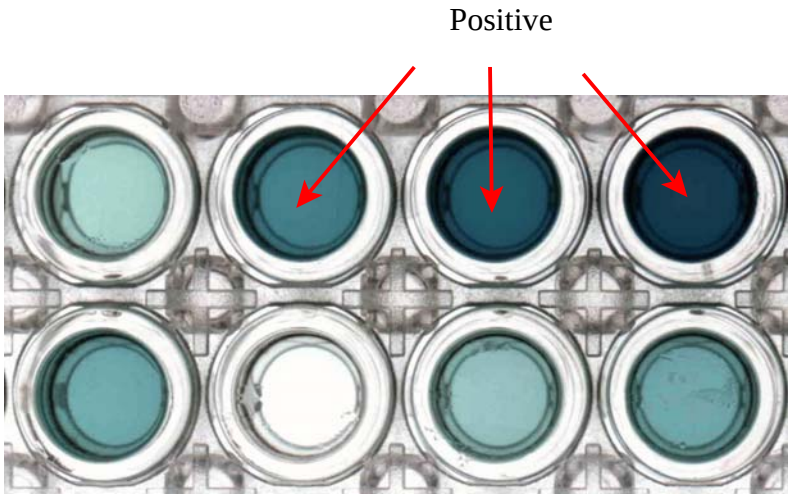


Figure 3.3 Tecra BDE-VIA. The TECRA BDE-VIA is a commercially available kit that detects the 45 kDa protein from NHE. A colour change to dark green indicates a positive result.

3.3.3.2 Oxoid BCET-RPLA

The Oxoid ELISA results are interpreted by an agglutination reaction (Figure 3.4). Overall, 36.8% of *B. cereus* isolates produced an agglutination reaction in the assay (Table 3.3 and 3.4). None of the emetic strains and 51.9% of the diarrhoeal isolates were recorded as positive.

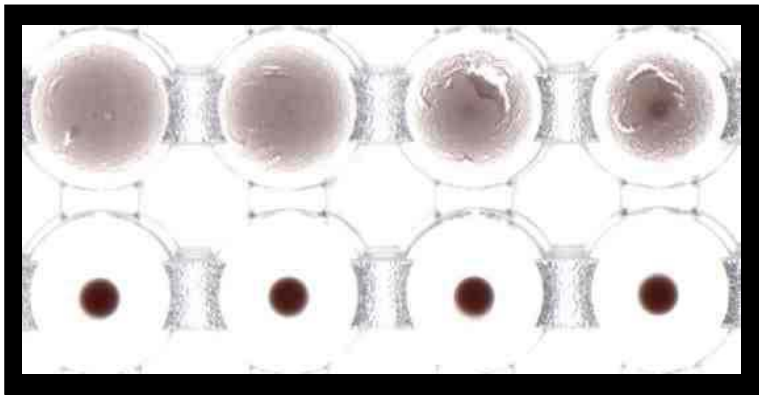


Figure 3.4 Oxoid BCET-RPLA. The Oxoid BCET-RPLA is a commercially available kit that detects the L_2 component of HBL. An agglutination reaction indicates a positive result (top row). Non-agglutinated material settles into a button on the bottom of the well (bottom row).

3.3.4 Enterotoxin PCR results

3.3.4.1 Haemolysin BL PCR

A 874 bp DNA product (Figure 3.5) was observed for 55.6% of diarrhoeal isolates, 0% of emetic isolates and 63.3% of food isolates (Table 3.3, 3.4 and 3.5). Overall, 50% of *B. cereus* isolates tested were positive for the gene *hblA* by PCR amplification.

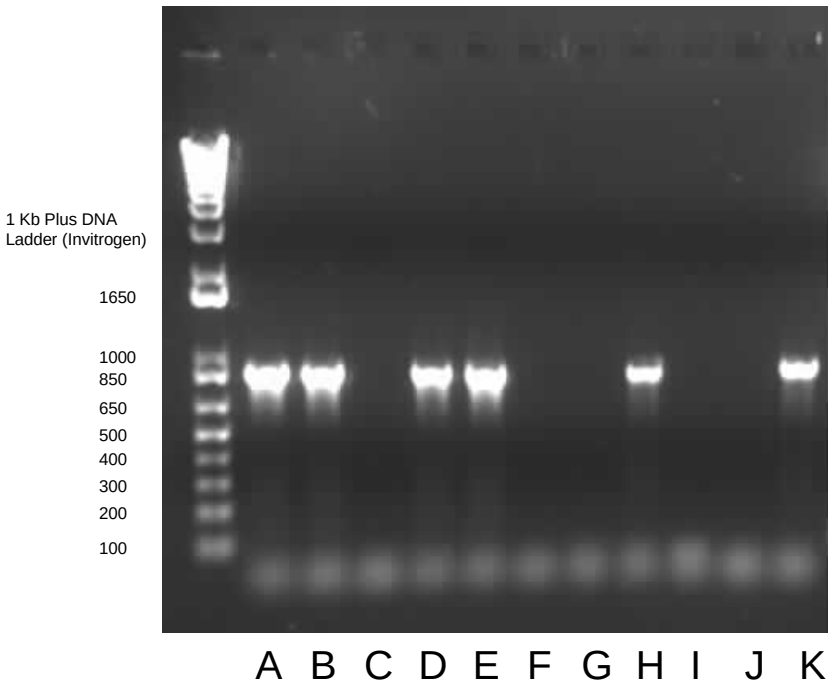


Figure 3.5 PCR to detect the *hblA* gene of haemolysin BL in strains of *Bacillus cereus*. The amplification results were resolved by 1% agarose gel electrophoresis.
(A) F4094/73; (B) ATCC14579; (C) F47; (D) F528; (E) F3453; (F) F4426; (G) F5881; (H) IH41064; (I) IH41385; (J) NVH0075/95; (K) NVH1230/88.

3.3.4.2 Enterotoxin T PCR

A 428 bp DNA product (Figure 3.6) was observed for 40.7% of diarrhoeal isolates, 0% of emetic isolates and 60% of food isolates (Table 3.3, 3.4 and 3.5). Overall, 42.6% of *B. cereus* isolates tested were positive for the gene *bceT* by PCR amplification.

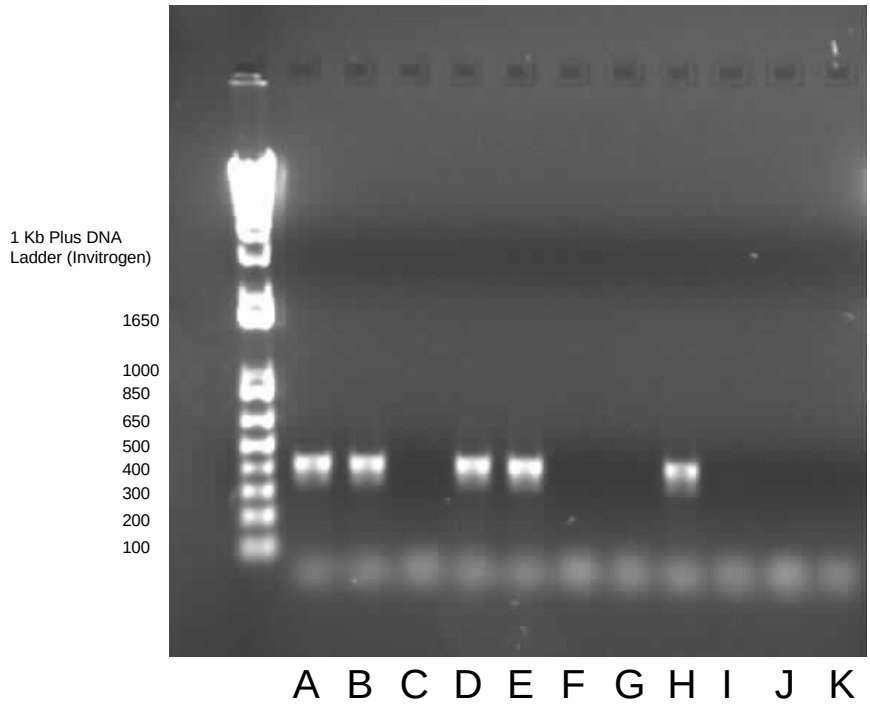


Figure 3.6 PCR to detect the *bceT* gene of enterotoxin T in strains of *Bacillus cereus*. The amplification results were resolved by 1% agarose gel electrophoresis
(A) F4094/73; (B) ATCC14579; (C) F47; (D) F528; (E) F3453; (F) F4426; (G) F5881; (H) IH41064; (I) IH41385; (J) NVH0075/95; (K) NVH1230/88.

3.3.4.3 Enterotoxin FM PCR

A 1269 bp DNA product (Figure 3.7) was observed for 86.8% of diarrhoeal isolates, 90.9% of emetic isolates and 76.7% of food isolates (Table 3.3, 3.4 and 3.5). Overall, 86.8% of *B. cereus* isolates tested were positive for the gene *entFM* by PCR amplification.

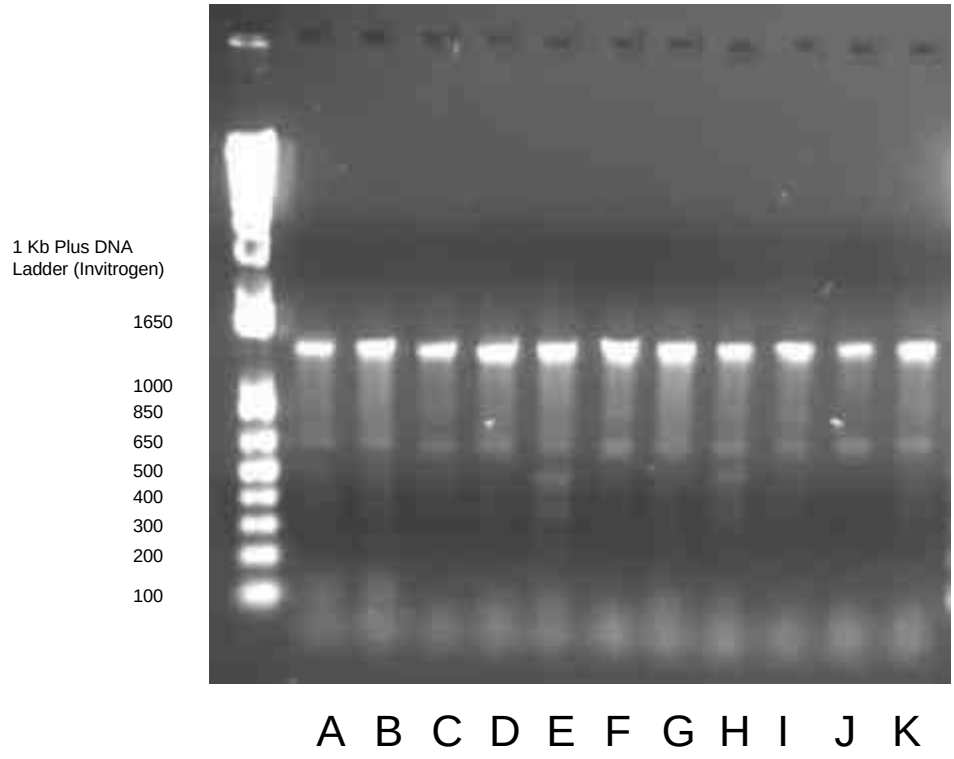


Figure 3.7 PCR to detect the *entFM* gene of enterotoxin FM in strains of *Bacillus cereus*. The amplification results were resolved by 1% agarose gel electrophoresis
(A) F4094/73; (B) ATCC14579; (C) F47; (D) F528; (E) F3453; (F) F4426; (G) F5881; (H) IH41064; (I) IH41385; (J) NVH0075/95; (K) NVH1230/88.

Table 3.3 Results from the enterotoxin survey for the diarrhoeal isolates

Strains	BDE-VIA	BCET-RPLA	*hblA* PCR	*bceT* PCR	*entFM* PCR	Vero cell assay	Gel diff. Assay
F4094/73	-	+	+	+	+	+	+
ATCC14579	-	+	+	+	+	+	+
F528	-	+	+	+	+	+	+
F3453	+	+	+	+	+	+	+
IH41064	+	+	+	+	+	+	+
IH41385	+	-	-	-	+	+	-
NVH0075/95	+	-	-	-	+	+	-
NVH1230/88	+	+	+	-	+	+	+
3153	+	-	-	-	+	+	-
3154	+	+	+	-	+	+	-
3155	+	-	-	-	+	+	-
3156	+	+	+	+	+	+	+
3259	-	-	-	-	+	+	-
3261	+	-	-	-	+	+	-
3262	+	-	-	-	+	+	-
3263	+	+	+	+	+	+	+
3264	+	+	+	+	+	+	+
1994	+	-	-	-	+	+	-
1996	-	-	-	-	+	+	-
1997	+	-	-	-	+	+	-
2174	+	-	-	-	+	+	-
2175	+	-	-	-	+	+	-
2884	+	+	+	+	+	+	+
3164	+	+	+	+	+	+	+
3166	+	+	+	+	+	+	-
CK1	+	-	+	-	+	+	-
CK2	+	+	+	-	-	+	+

Table 3.4 Results from the enterotoxin survey for the emetic isolates

Strains	BDE-VIA	BCET-RPLA	*hblA* PCR	*bceT* PCR	*entFM* PCR	Vero cell assay	Gel diff. Assay
F47	+	-	-	-	+	+	-
F4426	+	-	-	-	+	+	-
F5881	+	-	-	-	+	+	-
NC7401	+	-	-	-	+	+	-
F4810/72	+	-	-	-	+	-	-
NCF	-	-	-	-	+	-	-
NCG	+	-	-	-	+	+	-
NCY	+	-	-	-	+	+	-
NC954	+	-	-	-	+	+	-
NC1044	+	-	-	-	-	-	-
NC1078	+	-	-	-	+	+	-

Table 3.5 Results from the enterotoxin survey for the food isolates

Strains	*hblA* PCR	*bceT* PCR	*entFM* PCR	Vero cell assay
Bc1	+	+	+	-
Bc2	+	+	+	+
Bc3	-	-	-	-
Bc4	+	+	+	-
Bc5	+	+	+	-
Bc6	-	-	-	-
Bc7	+	-	+	+
Bc8	-	-	+	+
Bc9	+	+	-	+
Bc10	+	+	+	+
Bc11	+	+	+	+
Bc12	-	-	-	-
Bc13	+	+	+	-
Bc14	+	+	+	+
Bc15	-	-	+	+
Bc16	-	-	-	-
Bc17	-	-	+	+
Bc18	+	-	+	+
Bc19	-	-	-	+
Bc20	-	-	+	+
Bc21	-	-	-	-
Bc22	+	+	+	+
Bc23	+	+	+	+
Bc24	+	+	+	+
Bc25	-	+	+	+
Bc26	+	+	+	+
Bc27	+	+	+	+
Bc28	+	+	+	+
Bc29	+	+	+	+
Bc30	+	+	+	+

Table 3.6 Summary of results from the enterotoxin assays

Strains	**BDE-VIA**	**BCET-RPLA**	***hblA* PCR**	***bceT* PCR**	***entFM* PCR**	**Vero cell assay**	**Gel diff. assay**
Diarrhoeal	22/27 (81.5%)	14/27 (51.9%)	15/27 (55.6%)	11/27 (40.7%)	26/27 (96.3%)	27/27 (100%)	12/27 (44.4%)
Emetic	10/11 (90.9%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	10/11 (90.9%)	8/11 (72.7%)	0/11 (0%)
Food	NT	NT	19/30 (63.3%)	18/30 (60.0%)	23/30 (76.7%)	21/30 (70.0%)	NT
Overall	32/38 (84.2%)	14/38 (36.8%)	34/68 (50.0%)	29/68 (42.6%)	59/68 (86.8%)	56/68 (82.4%)	12/38 (31.6%)

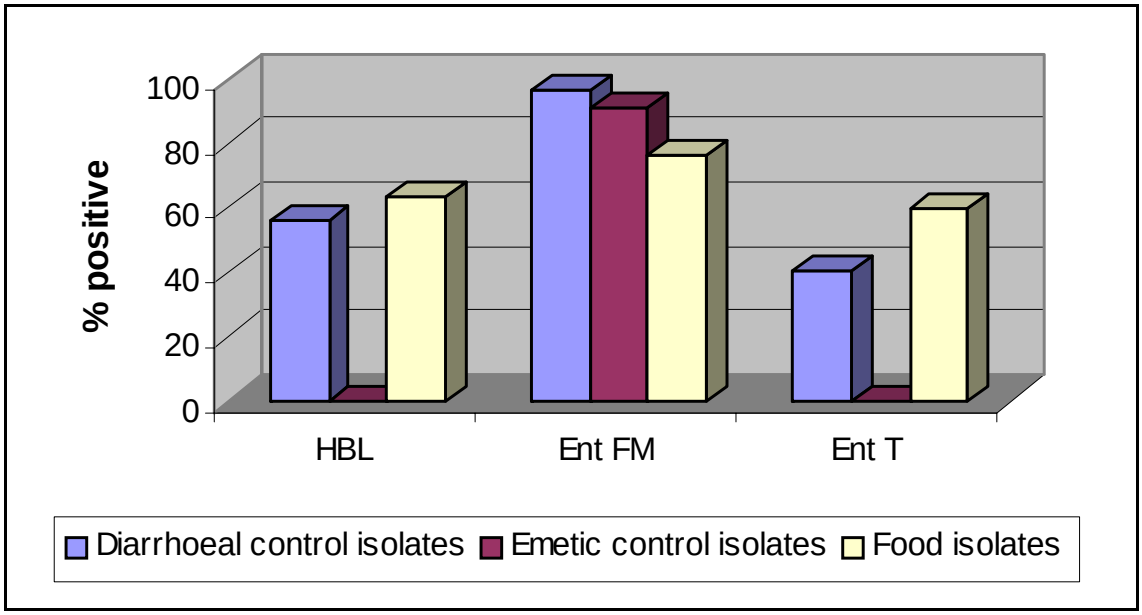


Figure 3.8 The prevalence of enterotoxin genes in *Bacillus cereus*. This figure shows the percentage of strains from each group that contained toxin genes (as identified by PCR).

3.4 Discussion

The Vero assay was probably the most useful of the toxin detection methods evaluated as it determined the actual toxicity of the *B. cereus* strain. The cell culture assay is also the only test that can detect all five of the diarrhoeal toxins (Agata *et al.,* 1995; Granum *et al.,* 1997). However, cell culture techniques are difficult to implement as quality control procedures due to the ongoing maintenance of cell lines and the extended time required before results can be obtained. This method cannot be used as conclusive evidence of enterotoxicity due to the large number of toxic compounds produced by strains of *B. cereus* that are potentially cytotoxic to Vero cells.

Haemolysin BL is the most comprehensively characterised of the toxins produced by *B. cereus*. Three assays are specific for the detection of this toxin (or the associated genes) - the gel diffusion assay, the Oxoid BCET-RPLA and the *hblA* PCR. These assays had a high correlation in their results with only three discrepancies between them. Two isolates (3154 and 3166) were positive with both the BCET-RPLA and *hblA* PCR but negative with the gel diffusion assay. The BCET-RPLA detects the L_2 component of HBL (Beecher and Wong, 1994), the *hblA* PCR detects the B component and the gel diffusion assay detects the action of the whole toxin complex. The differences in the results for these two isolates may be due to the L_1 component not being produced by these strains or the complex being produced at a level too low to be detected by the gel diffusion assay. One isolate (CK1) was positive with the *hblA* PCR but negative with the BCET-RPLA and gel diffusion assay. The negative BCET-RPLA result implies that this strain cannot produce the L_2 component and this explains why the diffuse haemolysis did not occur in the gel diffusion assay.

Enterotoxin FM (86.8%) was by far the most commonly detected toxin by PCR followed by haemolysin BL (50%) and enterotoxin T (42.6%). These results are comparable to the results obtained by Hsieh *et al*. (1997) using the same primers for a group of 84 strains of *B. cereus*. The most commonly detected toxin genes were those of enterotoxin FM (92.9%) followed by enterotoxin T (48.8%) and haemolysin BL (31%).

The prevalence of enterotoxin genes in the diarrhoeal and food isolates was very similar (Figure 3.8). Interestingly, the prevalence of *hblA* and *bceT* were slightly higher in the food isolates. This phenomenon was also noted by Guinebretiere *et al.* (2002) who found that haemolysin BL and enterotoxin T genes were more common in food isolates (92.2% and 70.6% for *hblA* and *bceT* respectively) than in diarrhoeal food poisoning isolates (73% and 56.8% for *hblA* and *bceT* respectively). An explanation for this may be that with less enterotoxin genes the clinical strains are able to devote more energy to producing only one or two toxins resulting in the production of a higher titre of toxins. This is supported by the results from the Vero assay where 100% of diarrhoeal isolates were cytotoxic compared to 70% of food isolates. None of the emetic isolates contained the genes *hblA* or *bceT*, suggesting that there may be broad genetic differences between emetic and non-emetic strains of *B. cereus*.

The majority of *B. cereus* isolates were able to produce at least one diarrhoeal toxin. Only six (8.8%) of the strains tested did not have the genes for any of the diarrhoeal toxins. Although PCR can only be regarded as an indication of the potential to produce toxins, it is apparent that enterotoxin genes are widespread throughout the species. Consequently, PCR is not suitable for a routine quality control method for the detection of enterotoxic strains of *B. cereus*. Following the survey conducted above it was planned that a multiplex PCR specific for all five enterotoxins would be developed. However, due to the high prevalence of enterotoxin genes in *B. cereus*, a multiplex PCR would not further elucidate the pathogenesis of the diarrhoeal syndrome.

Following an outbreak of food poisoning it is necessary to evaluate the clinical picture and epidemiological factors around the disease occurrence. A reliable method is required to determine if the bacterium associated with the disease outbreak is capable of inducing disease, or exhibits important virulence factors. With the enterotoxin detection methods that are currently available and the lack of a clear picture of the relative importance of the various diarrhoeal toxins produced by *B. cereus*, it is difficult to envisage a definitive assay that could be used in quality control procedures. Until a clearer understanding of the pathogenesis of the diarrhoeal disease is gained the conventional methods are not suitable due to the fact that the majority of isolates produce at least one of the diarrhoeal toxins.

Chapter 4

An Improved HEp-2 Cell Cytotoxicity Assay for the Detection of Emetic Strains of *Bacillus cereus*

4.1 Introduction

The emetic illness induced by *B. cereus* is probably the more dangerous food poisoning illness produced by this organism (Finlay *et al*., 2002). This acute illness is characterised by nausea and vomiting and in extreme cases may result in fulminant liver failure and death (Mahler *et al*., 1997). Despite the obvious importance of this bacterium as a food poisoning organism, a sensitive assay for the detection of emetic strains is not available. The remainder of this thesis is mostly focussed upon the development of a rapid and sensitive method for the detection of emetic strains of *B. cereus*. The first step in this process was the development of a cell culture method that could provide sensitive and non-subjective identification of emetic strains.

The emetic syndrome is caused by a peptide produced by *B. cereus*, which was termed the vacuolation factor (Shinigawa *et al*., 1992). It was shown to cause vacuolation of mitochondria when exposed to HEp-2 (human carcinoma of the larynx) cells (Hughes *et al*., 1988). The HEp-2 vacuolation factor was extracted and purified by Agata *et al*. (1994). The toxin responsible was a 1.2 kDa dodecadepsipeptide and was named cereulide. The structure of the toxin was shown to be similar to the potassium ionophore valinomycin, which, like cereulide, also causes mitochondrial damage when added to HEp-2 cells (Agata *et al*., 1995).

Due to difficulties in purifying cereulide very little is known about this peptide. Oral administration to monkeys has shown that the peptide is poorly antigenic (Melling and Capel, 1978). The most commonly used method for detection of cereulide is a HEp-2 cell cytotoxicity assay (Hughes *et al*., 1988). Cereulide induces vacuolation of the mitochondria of the HEp-2 cells. This effect is transitory and difficult to distinguish, making it somewhat subjective. Starch hydrolysis can also be used as a screening assay for emetic strains. Isolates of *B. cereus* that produce cereulide are unable to hydrolyse starch. This can be observed on nutrient agar supplemented with starch (Nishikawa *et al*., 1996). A review of the methods available for the detection of emetic strains of *B. cereus* is provided in Section 1.8.2 of this thesis.

In this phase of research, a HEp-2 cell cytotoxicity method that was developed by Finlay *et al*. (1999), was modified and evaluated using food and clinical isolates. The original method utilised the tetrazolium salt MTT to detect mitochondrial damage induced by cereulide. Tetrazolium assays are widely used to investigate cell damage and stability (Buttke *et al*., 1993; Goodwin *et al*., 1995). Commonly used tetrazolium salts include MTT, XTT and MTS (see List of Abbreviations). Previous studies have used the tetrazolium salt MTT to indicate cell damage from both the emetic and diarrhoeal toxins of *B. cereus* (Finlay *et al*., 1999; Fletcher and Logan, 1999). The MTT assay produces an insoluble, crystalline formazan end product and requires an additional step to solubilise the product before absorbance readings can be taken. MTS and XTT are bioreduced into an aqueous, soluble formazan end product which negates the need for a solubilisation step. The advantages of MTS over XTT include the rapidity of colour development and the storage stability of MTS (Buttke *et al*., 1993). Therefore, MTS was selected as the indicator agent in this assay due to the rapidity of colour development and ease of use, in comparison to other tetrazolium salts.

It is well documented that cereulide is only produced when *B. cereus* is grown upon certain substrates. Boiled and cooked rice, in particular, are notorious for being implicated in emetic food poisoning outbreaks. The titre for cereulide production has been reported as being much higher for rice based foods when compared to a range of other food products (Agata *et al*., 2002). Growth of *B. cereus* on most commercial media has resulted in only very low levels of cereulide production. Skim milk medium has been shown to be the most conducive commercially available substrate for cereulide production. Agata *et al*. (1999) found that *B. cereus* produced more than four-times more cereulide in skim milk medium in comparison to brain heart infusion broth, trypticase soy broth and nutrient broth. These findings are also supported by Szabo *et al*. (1991) and Finlay *et al*. (1999). Cereulide production has been documented over a range of temperatures, extending from 12°C to 37°C. The highest reported titres of cereulide production were achieved when *B. cereus* was grown at 15°C for 6 days (Finlay *et al*., 2000). However, this is not practical for a diagnostic test where time is critical. Skim milk medium and incubation temperature of 30°C was therefore utilised for *B. cereus* growth and toxin production in this study.

The aim of this phase of the project was to produce an improved cereulide detection assay that was non-subjective and was more sensitive than existing assays. Before the objective to produce a rapid molecular method for the detection of emetic strains could be pursued, it was necessary to confirm that all of the emetic strains received from other researchers were actually able to produce cereulide. Therefore, a modified cytotoxicity assay for the detection of cereulide was developed. Damage to HEp-2 cells was assessed by the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). The assay was evaluated using food and clinical isolates and compared to the starch hydrolysis assay and the original HEp-2 mitochondrial vacuolation assay.

4.2 Materials and Methods

4.2.1 Survey of food and clinical isolates using the HEp-2/MTS assay

Thirty strains of *B. cereus* isolated from Townsville supermarket salad bars (Horwood, 1998) were tested for cereulide production using the HEp-2/MTS assay. Emetic and non-emetic strains from Australia and overseas were also tested (Table 4.1). Each plate included a valinomycin (positive) and negative control series. All isolates were tested as outlined below.

Table 4.1 *Bacillus cereus* strains tested for cereulide production

Strain grouping [a]	Designation
Emetic isolates	F47, F5526, F5881, NC7401, F4810/72, NCF, NCG, NCY, NC954, NC1044, NC1078
Non-emetic isolates	F4094/73, ATCC14579, F528, F3453, IH41064, IH41385, 3153, 3154, 3155, 3156, 3259, 3261, 3262, 3263, 3264, 1994, 1996, 1997, 2174, 2175, 2884, 3164, 3166, NVH0075/95, NVH1230/88, CK1, CK2
Food isolates	Bc1 to Bc30

[a] See Tables 2.1, 2.2 and 2.3 for complete descriptions of *Bacillus* strains

4.2.2 Optimisation of skim milk medium concentration

Different concentrations of the skim milk medium were evaluated as higher levels had a toxic effect on the HEp-2 cells, this effect was also noted by Finlay *et al*. (1999). Concentrations of 10%, 5%, 3%, 2.5%, 2%, 1.5%, 1% and 0.5% w/v of skim milk medium were evaluated for toxin production with emetic strain F 48210/72 and skim milk toxicity. *B. cereus* culturing and the cell cytotoxicity assay were conducted as outlined below.

4.2.3 Culture of *Bacillus cereus*

B. cereus isolates were inoculated into skim milk medium (Difco, Becton Dickinson) and incubated at 30°C with shaking (100 rpm). Following optimisation of skim milk concentration, 25 g/L of skim milk powder in distilled water (Appendix 1) was used for all further experiments.

Following incubation, the samples were autoclaved to inactivate the heat labile diarrhoeal toxins to ensure that the assay was specific for the heat stable cereulide. The sample was then centrifuged at 10,000 *g* for 15 min and the supernatant was filtered through a 0.22 µm syringe filter (Millipore) to remove residual cell debris.

4.2.4 Cell cytotoxicity assay

A cell cytotoxicity assay was developed based on methods described by Finlay *et al*. (1999). The HEp-2 cells were grown in DMEM (Gibco) with 10% foetal bovine serum. Cells were diluted to 10^6 cells/ml, and 100 µl aliquots were used to seed a flat bottom 96-well mictotitre plate (Falcon, Becton Dickinson). After incubating the HEp-2 cells at 37°C overnight or until confluent, 100 µL of supernatant from each sample (as prepared above) was added to the cells. A two-fold serial dilution was then carried out across the plate. The plate was incubated at 37°C for 24 hrs.

Following incubation, the HEp-2 cells were examined under a light microscope for mitochondrial vacuolation (Hughes *et al*., 1988). Aliquots of 20 µl of MTS (CellTiter

96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay, Promega) were then added to each well. MTS is a yellow water-soluble tetrazolium salt that is converted to insoluble purple formazan by metabolising cells. The plates were returned to the incubator for a further 4 hrs incubation, after which the plates were examined for colour change i.e. cells that were damaged by cereulide were unable to convert the MTS to formazan and therefore the wells remained yellow.

4.2.5 Evaluation of the sensitivity of the HEp-2/MTS assay

Valinomycin is a potassium ionophore that is commonly used as a positive control for cereulide. The two peptides have similar structures and modes of action. Therefore, valinomycin was used in this preliminary study to ascertain the sensitivity of the HEp-2/MTS assay.

Valinomycin was solubilised in DMSO and added to DMEM to produce a stock solution of 400,000 ng/mL of valinomycin. The HEp-2 cells were grown in flat bottom microtitre trays as outlined above. Following two days incubation, 100 µL of the valinomycin stock solution was added to each well of the HEp-2 cells and two-fold serial dilutions were carried out across the plate. Three replicates of the series were tested and a control series of DMEM/DMSO was also included. The plate was then incubated at 37°C for 24 hrs.

Following incubation, 20 µL of MTS was added to each well and the plates were returned to the incubator for a further 4 hrs. The plates were then examined for colour change and the absorbance was read at 490 nm (Labsystems, Multiskan EX).

4.2.6 Reproducibility experiment

Six emetic strains of *B. cereus* (F4426, NC7401, NCG, NCF, NCY and NC1078) and a valinomycin/DMSO control (1000 ng/ml) were tested for cell cytotoxicity using the HEp-2/MTS assay. The assay was conducted in duplicate using different batches of media and the two plates were processed completely independently of each other to determine the reproducibility of the assay.

4.2.7 Starch hydrolysis assay

Isolates of *B. cereus* were inoculated into Brain Heart Infusion Agar (Acumedia) containing 1% w/v soluble starch (Sigma) (Appendix 1). Plates were incubated at 30°C for 24 hours. Colonies were scraped from the surface of the agar before the addition of iodine. Isolates exhibiting a zone of clearing surrounding bacterial colony sites were regarded as starch hydrolysis positive (Nishikawa *et al*., 1996).

4.3 Results

4.3.1 Determination of optimal skim milk concentration

Higher concentrations of skim milk medium for culturing of *B. cereus* resulted in toxicity to HEp-2 cells. The highest titres of cereulide production (as judged by HEp-2 toxicity) by the emetic strain F4810/72 were observed for skim milk concentrations of 10%, 5%, 3% and 2.5% w/v (Table 4.2). Lower concentrations of skim milk resulted in a reduced titre result. The optimal concentration for skim milk medium was determined to be 2.5% w/v (25 g/L). At this concentration, F4810/72 produced a high titre of cereulide and only minimal damage was caused by the skim milk toxicity. This concentration was used in all further HEp-2/MTS experiments in this thesis. It should be noted that the HEp-2 cells in the first well were always damaged by the higher concentration of skim milk medium and therefore only strains that induce a titre reading >2 were recorded as positive.

Table 4.2 Optimisation of skim milk medium for the HEp-2/MTS assay to reduce substrate toxicity

Skin milk concentrations (w/v)	Skim milk measurement	F4810/72[a] HEp-2/MTS titre	Skim milk[b] HEp-2/MTS titre
10%	100 g/L	512	8
5%	50 g/L	512	4
3%	30 g/L	512	4
2.5%	25 g/L	512	2
2%	20 g/L	256	2
1.5%	15 g/L	64	2
1%	10 g/L	32	0
0.5%	5 g/L	8	0

[a] toxicity titre induced by the emetic strain F4810/72

[b] toxicity titre induced by different skim milk concentrations

4.3.2 Detection of cereulide production by the HEp-2/MTS assay

Supernatant extracts from all of the known emetic strains produced cell damage when tested in the HEp-2/MTS and HEp-2 vacuolation assays (Table 4.2). None of the non-emetic strains nor the 30 food isolates produced cell damage in the HEp-2/MTS or HEp-2 vacuolation assays. Emetic and non-emetic strains were easily differentiated using the HEp-2/MTS assay, undamaged HEp-2 cells produced a distinct purple colour change in the medium which was easily distinguished from the yellow substrate of damaged cells (Figure 4.1). HEp-2 cells damaged by cereulide exhibited a ‘granular effect’ in the cytoplasm when analysed microscopically. Titres were recorded from the HEp-2 vacuolation assay and the HEp-2/MTS assay (Table 4.3).

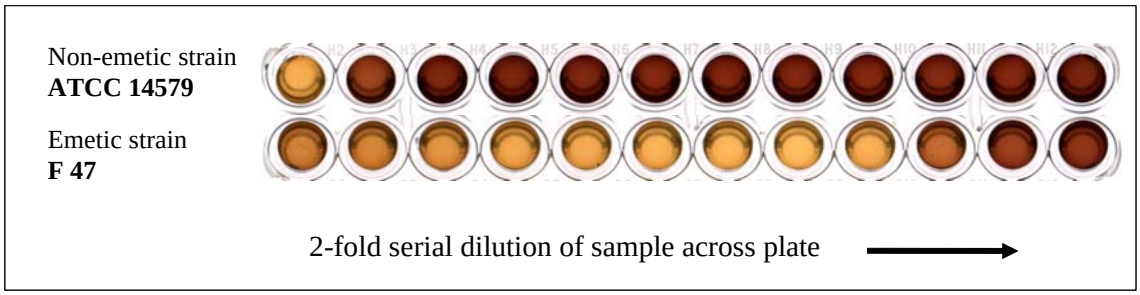


Figure 4.1 The HEp-2/MTS assay. Undamaged HEp-2 cells convert MTS to formazan (yellow to purple colour change). HEp-2 cells damaged by cereulide are unable to convert MTS to formazan and therefore the wells do not change colour. The first well for the non-emetic sample is yellow because of toxicity caused by the skim milk medium

Table 4.3 HEp-2/MTS titre results for emetic strains[a] of *Bacillus cereus*

Designation of strain	HEp-2 cell vacuolation titre	HEp-2/MTS titre
F47	512	1024
F4426	256	1024
F5881	64	128
NC7401	128	256
F4810/72	256	512
NCF	32	64
NCG	128	512
NCY	256	512
NC954	128	256
NC1044	128	256
NC1078	128	256

[a] Only emetic strains shown in table. The diarrhoeal and food isolates of *B. cereus* were not cytotoxic in either of the assays.

4.3.3 Valinomycin sensitivity test

The preliminary test with valinomycin was conducted to determine the sensitivity of the HEp-2/MTS assay. The plates were read visually according to colour change and the absorbance was also read at 490 nm (Figure 4.2). A very close correlation for visual and absorbance analysis was observed for the triplicate series. The detection limit of the assay for valinomycin varied between 0.8 ng/ml to 3.1 ng/ml (Appendix 2).

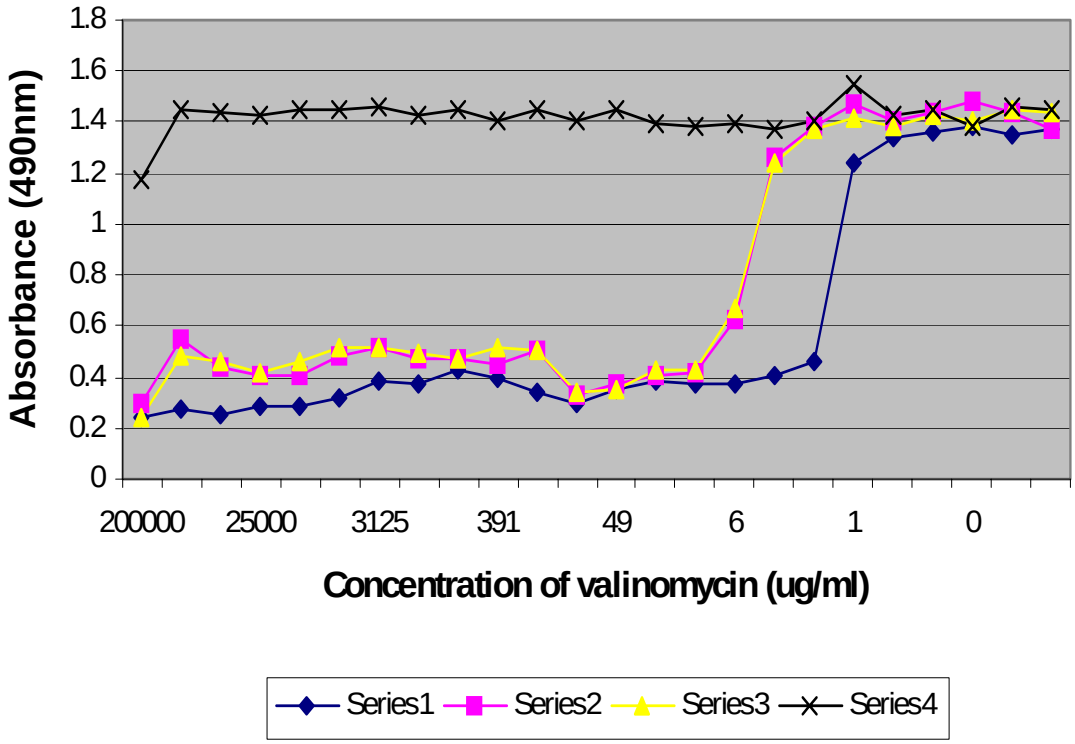


Figure 4.2 Experiment using valinomycin to determine sensitivity of the HEp-2/MTS assay. Series 1, 2 and 3 are a triplicated 2-fold serial dilution of valinomycin/DMSO; series 4 is a 2-fold serial dilution of DMSO.

4.3.4 Reproducibility of the HEp-2/MTS assay

The reproducibility of the HEp-2/MTS assay was found to be excellent. All six emetic strains and the valinomycin control produced identical titres for the duplicate series (Figure 4.3).

Figure 4.3 HEp-2/MTS assay reproducibility. Both plates were processed completely independently using different media batches etc. to determine the reproducibility of the assay.

1. F4426, **2.** NC7401, **3.** NCG, **4.** NCF, **5.** NCY, **6.** NC1078,

7. Valinomycin control (1000 ng/ml), **8.** Skim milk/DMSO control.

4.3.5 Starch hydrolysis screening assay

None of the emetic strains tested were able to hydrolyse starch (Figure 4.4) In contrast, 63% of the non-emetic strains and 76.7% of the food isolates exhibited clear zones of starch hydrolysis in the assay (Table 4.4).

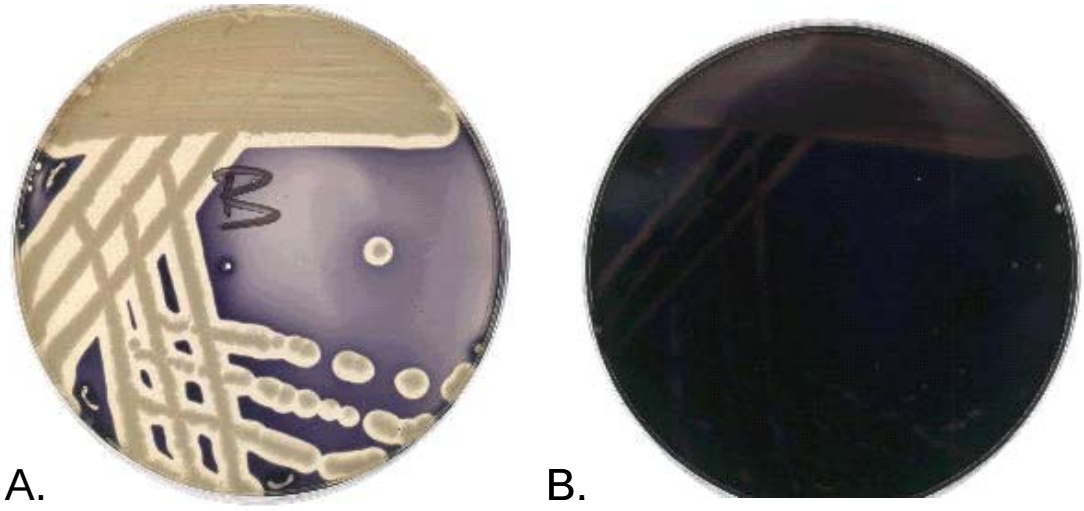


Figure 4.4 Starch hydrolysis assay. Isolates exhibiting a zone of clearing surrounding bacterial colony sites were regarded as starch hydrolysis positive.
A: ATCC14579 (starch hydrolysis - positive)
B: NC7401 (starch hydrolysis - negative)

Table 4.4 Starch hydrolysis groupings

Starch hydrolysis - positive	Starch hydrolysis - negative[a]
F4094/73, ATCC14579, F528, F3453, IH41064, NVH1230/88, 3153, 3154, 3156, 3259, 3263, 3264, 2884, 3164, 3166, CK1, CK2, Bc1, Bc2, Bc4, Bc5, Bc7, Bc9, Bc10, Bc11, Bc12, Bc13, Bc14, Bc18, Bc19, Bc20, Bc22, Bc23, Bc24, Bc25, Bc26, Bc27, Bc28, Bc29, Bc30	F47, F5526, F5881, NC7401, F4810/72, NCF, NCG, NCY, NC954, NC1044, NC1078, IH41385, NVH0075/95, 3155, 3261, 3262, 1994, 1996, 1997, 2174, 2175, Bc3, Bc6, Bc8, Bc15, Bc16, Bc17, Bc21

[a] emetic isolates are shown in red

4.4 Discussion

This research describes the development of a sensitive assay for the detection of cereulide. The assay produces easily read, non-subjective results, which is an improvement on the conventional mitochondrial vacuolation assay in which the transitory vacuolation effect is difficult to read and relies heavily upon the technical experience of the scientist. The HEp-2/MTS assay is read by observing an obvious colour change of yellow to purple, thus eliminating the need for measuring absorbance. Other cell lines such as CHO and INT 407 which exhibit similar vacuole damage as seen in HEp-2 cells (Szabo *et al*., 1991; Beattie and Williams 1999), could presumably also be used in this assay. The titres from the HEp-2/MTS assay were invariably higher than those recorded from the HEp-2 vacuolation assay (Table 4.2), indicating that mitochondrial damage has an effect on the metabolism of the cells that is not always visible by mitochondrial swelling. The mitochondrial swelling effect is characterised by a ‘granular’ effect appearing in the cytoplasm of affected cells. This characteristic is difficult to distinguish and requires a high level of expertise and experience to correctly identify. Therefore, damage to the HEp-2 cells was much easier to determine for the HEp-2/MTS assay in comparison to the HEp-2 mitochondrial assay.

Valinomycin has been used by many researchers as a 'positive control' for cereulide detection assays due to its similar structure, properties and toxic effect upon cell lines (Agata *et al*., 1994; Agata *et al*., 1995; Andersson *et al*., 1998; Mikkola *et al*., 1999). The toxic effects of cereulide and valinomycin are believed to be due to the ionophoretic uptake of potassium ions (Mikkola *et al*., 1999). Preliminary studies showed that the HEp-2/MTS assay could detect <3 ng/ml of valinomycin. The detection limit was comparable to the boar spermatozoa assay developed by Andersson *et al*. (1998), in which 2 ng/ml of valinomycin resulted in loss of motility and <400 ng/ml induced vacuolation of spermatozoa.

Overall 41.2% (28/40) of isolates were unable to hydrolyse starch, of these isolates only 39.3% (11/28) were emetic strains (according to the HEp-2/MTS assay). Therefore in a quality control laboratory further analysis would be required to determine if the starch negative strains were cereulide producers. All of the emetic isolates tested were starch hydrolysis negative. Shinagawa *et al*. (1979) also found that none of the emetic strains tested in that study were able to hydrolyse starch. This finding has been further substantiated by numerous other groups (Nishikawa *et al*., 1996; Agata *et al*., 1996). It is unknown why emetic strains are unable to hydrolyse starch.

To date very few studies have been conducted to determine the prevalence of emetic strains of *B. cereus* in food. This is probably due to the lack of a simple and effective cereulide detection assay. In this study all 30 food isolates were non-emetic according to both of the assays used. The work conducted here is the first reported survey of Australian *B. cereus* food isolates to be tested for cereulide production. Mikami *et al*. (1994) found 16 of 310 (5.2%) of Japanese *B. cereus* isolates from various foods were emetic strains, as shown by the HEp-2 cell vacuolation assay.

The HEp-2/MTS assay for *B. cereus* described here is an improvement upon existing cereulide detection assays. This assay is inexpensive, utilises common reagents, does not require a great high level of expertise and eliminates the subjectivity that is inherent in many of the existing cereulide detection assays. However, the assay takes at least three days before results can be read which is too long for the method to be used for quality control procedures in food factories. The assay will be useful for researchers and

may also be utilised following emetic food poisoning outbreaks. The 96-well format allows a high-throughput of samples with the possibility of semi-automation.

Chapter 5

Antibiotic Sensitivity Experiments

5.1 Introduction

The mitochondrial swelling observed when cereulide is added to HEp-2 cells (Hughes *et al*., 1988) and boar spermatazoa (Andersson *et al*., 1998) is believed to be due to the ionophoretic uptake of potassium ions (K^+) (Mikkola *et al*., 1999). Mikkola *et al.* (1999) found that cereulide exhibited a high selectivity for K^+ over sodium ions (Na^+), calcium ions (Ca^+) and hydrogen ions (H^+). This cation selectivity exhibited by cereulide was the same as that of valinomycin. The enterohaemorrhagic haemolysin of *Escherichia coli* and the insecticidal toxin (CrylA) of *Bacillus thuringiensis* are also believed to have ionophoretic effects (Schmidt *et al*., 1996; Grochulski *et al*., 1995). Valinomycin has been used extensively by researchers as a 'positive control' or simply for comparison in many cereulide detection assays (Agata *et al*., 1994; Agata *et al*., 1995; Andersson *et al*., 1998; Mikkola *et al*., 1999). The two peptides induce similar damage in cell cytotoxicity assays and both induce vomiting in the musk shrew, *Suncus murinus* (Agata *et al*., 1995).

Ionophores are compounds that form lipid-soluble complexes with polar cations, such as K^+, Na^+, Ca^{2+} and magnesium (Mg^{2+}) (Pressman, 1976). They facilitate the movement of these ions across the lipid bilayer of biological membranes (Steverding and Kadenbach, 1990). Ionophores show considerable ion specificity because the ion must be accommodated within a confined space inside the carrier (Figure 5.1). Therefore, there are potassium ionophores, calcium ionophores, carboxylic ionophores, etc. They can be divided further into channel forming ionophores (eg. gramicidin) which form a tiny pore through the membrane, and mobile carriers (eg. valinomycin) which diffuse backwards and forwards across the membrane (Figure 5.1). The rate of transport is extremely high, with some ionophores reported to carry >1,000 cations/molecule/second (Pressman, 1976). Although many ionophores are toxic to microbial organisms and have subsequently been characterised as antibiotics, they are often extremely toxic to higher organisms and thus their applications as antibiotics have been limited (Pressman, 1976).

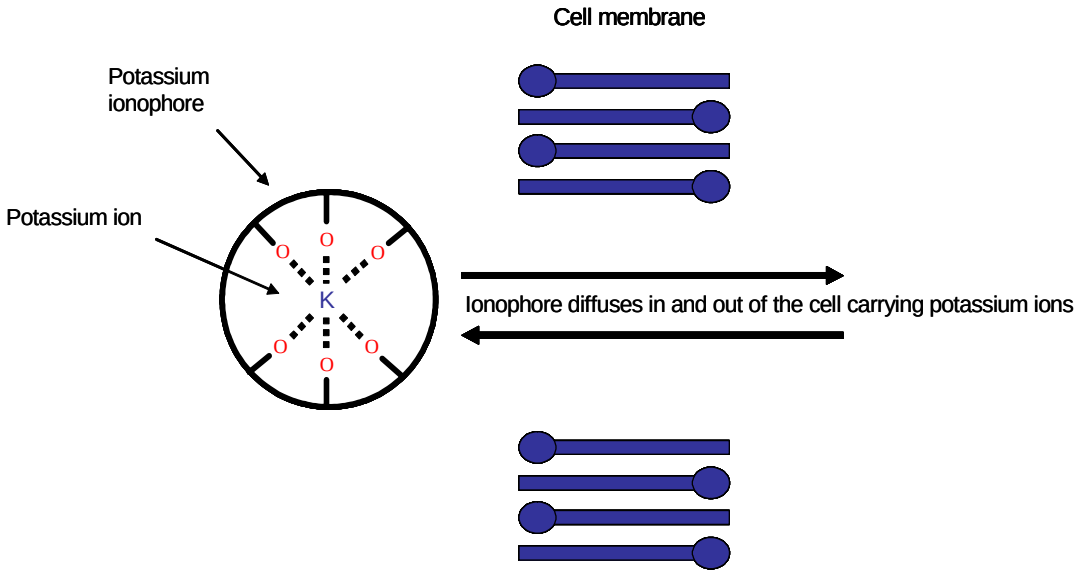


Figure 5.1 Diagrammatic representation of K^+ ionophore carrying a potassium ion into a cell

Researchers have found a linkage in many bacteria between genes encoding for resistance to antibiotics and antibiotic biosynthesis genes. These genes are commonly located in clusters which ensures their coordinated expression to avoid 'suicide' of the antibiotic-producing strains (Martin and Liras, 1989). An example of this system is seen with bacitracin which is a peptide antibiotic that inhibits cell wall biosynthesis and is most active against Gram positive bacteria. However, bacitracin is produced by the Gram positive bacterium *Bacillus licheniformis*. The *B. lichenifomis* strains that produce this antibiotic are not damaged by bacitracin due to the action of a specific resistance mechanism, in the form of an ABC transporter encoded by the *bcrABC* gene cluster (Neumuller *et al*., 2001). Antibiotic resistance mechanisms can sometimes confer resistance against closely related compounds. For example, blasticidin S-producing strains of *Streptomyces morookaensis* are resistant to the antibiotic puromycin (produced by *Streptomyces alboniger*) due to enzyme activity which hydrolyses the antibiotic (Nishimura *et al*., 1995).

This phase of the study was conducted to determine if emetic strains of *B. cereus* were more resistant to damage from related ionophores, antibiotics and cereulide itself than

non-emetic strains due to the presence of resistance genes which may confer protection from related compounds. Antibiotic sensitivity is commonly used in bacteriology laboratories and, if a correlation is observed, this could easily be implemented as a screening process for cereulide-producing strains following isolation of *B. cereus*. This could be achieved through impregnated discs or through the development of specialised growth media.

5.2 Materials and Methods

5.2.1 Antibiotic resistance experiments

5.2.1.1 Antibiotics

Ionophores and antibiotics were selected for use in this phase based upon their structural similarities to cereulide, relatedness of bacterial source to *B. cereus* and commercial availability. The compounds used were bacitracin, enniatin, gramicidin, nigericin, nonactin, surfactin and valinomycin (Table 5.1).

5.2.1.2 Production of antibiotic discs

Antibiotics were solubilised in DMSO to final concentrations of 2,500, 1,250, 625, 312.5, 156.25 and 78.13 µg/ml. A hole puncher (Punch No.300, Life Co. Ltd.) was used to create 6 mm discs from filter paper (Biorad). The discs were placed into glass Petri dishes and sterilised by autoclaving (121°C for 15 min). Following sterilisation, antibiotic discs were impregnated with antibiotic by adding 20 µl of each antibiotic/DMSO solution to the filter paper discs. The resulting concentrations of the valinomycin in the discs were 800, 400, 200, 100, 50 and 25 µg/disc. A DMSO control was also included for each bacterial strain tested. The discs were dried by overnight storage in a hot room at 37°C.

Table 5.1 Sources and characteristics of antibiotics used for antibiotic sensitivity experiments

Antibiotic	Bacterial source	Characteristics	Supplier
Bacitracin	*Bacillus licheniformis*	lipopeptide antibiotic	Sigma
Enniatin	*Fusarium* sp.	potassium ionophore	Sigma
Gramicidin	*Brevibacillus brevis*	channel-forming ionophore	Fluka
Nigericin	*Streptomyces hygroscopicus*	potassium ionophore	Sigma
Nonactin	*Streptomyces tsusimaensis*	potassium ionophore	Sigma
Surfactin	*Bacillus subtilis*	lipopeptide antibiotic	Sigma
Valinomycin	*Streptomyces* sp.	potassium ionophore	Sigma

5.2.1.3 Media and bacterial strains

Three emetic strains (NCG, F47 and F4810/72) and three non-emetic strains (F3453, 1997 ATCC14579) of *B. cereus* were each inoculated into 10 ml aliquots of nutrient broth (Oxoid) and incubated at 30°C for 18 hours. Overnight culture (100 µl) was spread plated onto nutrient agar that had been supplemented with 200 mM of potassium chloride (KCl). Plates were supplemented with additional potassium cations (in the form of KCl) to increase the toxicity of the potassium ionophores (Plater and Robinson, 1992). Antibiotic discs of each concentration were placed equidistantly around the plate. The plates were incubated at 30°C overnight. Following incubation, inhibition zone diameters were determined.

5.2.2 Resistance of *Bacillus cereus* to higher concentrations of valinomycin

Valinomycin discs were developed (as stated in section 5.2.1.2) with the final concentrations of 8,000, 4,000, 2,000 and 1,000 µg/disc. Plate streaking and disc placement were carried out as noted above (section 5.2.1.3). However, due to the high cost of valinomycin only two emetic (NCG and F47) and two non-emetic strains (1997

and F3453) were used. Following incubation at 30°C overnight, inhibition zone diameters were determined.

5.2.3 Resistance of *Bacillus cereus* to cereulide

5.2.3.1 Production of cereulide discs

The emetic strain F4810/72 and the non-emetic strain F3453 were inoculated into 10 ml skim milk medium (Difco, Becton Dickinson) and incubated at 30°C overnight. Following incubation, the samples were autoclaved at 121°C for 15 min to inactivate the diarrhoeal toxins and kill spores. The sample was then centrifuged at 10,000 *g* for 15 min. The supernatant was filtered through a 0.22 µm syringe filter (Millipore) to remove residual cell debris. Supernatant (20 µl) was added to discs as stated above (section 5.2.1.2).

The cereulide discs were added to spread plates (as per section 5.2.1.3) of NCG, F47, F4810/72, F3453, 1997 and ATCC14579. The plates were incubated at 30°C overnight. Following incubation plates were observed for zones antibiotic sensitivity. Measurements were taken with a desk ruler from the edge of the antibiotic disc to the outer limit of inhibition.

5.2.3.2 Modified CAMP test

A modified CAMP test was conducted on skim milk agar (Difco, Becton Dickinson). The original CAMP (named after the authors Christie, Atkins and Munch-Petersen) test was designed to detect beta toxin-producing strains of *Staphylococcus aureus*. The authors discovered that synergistic enhancement of beta haemolysis occurred when *S. aureus* was grown adjacent to haemolytic streptococci (Christie *et al*., 1944). This test method has been adapted for numerous other bacterial species including *Listeria mononcytogenes* (McKellar, 1994). The objective of the modified CAMP test described here is to determine if the production of cereulide from emetic strains of *B. cereus* results in growth inhibition to adjacent strains of non-emetic *B. cereus*. Emetic strains NCG, F47 and F4810/72 were streaked onto skim milk agar

perpendicular to the non-emetic strains F3453, 1997 and ATCC14579 (Figure 5.2), but retaining 1-2 mm between the streaked strains. Plates were incubated at 30°C overnight. Following incubation, plates were examined to determine if the extracellular products produced by the emetic strains inhibited the growth of non-emetic strains.

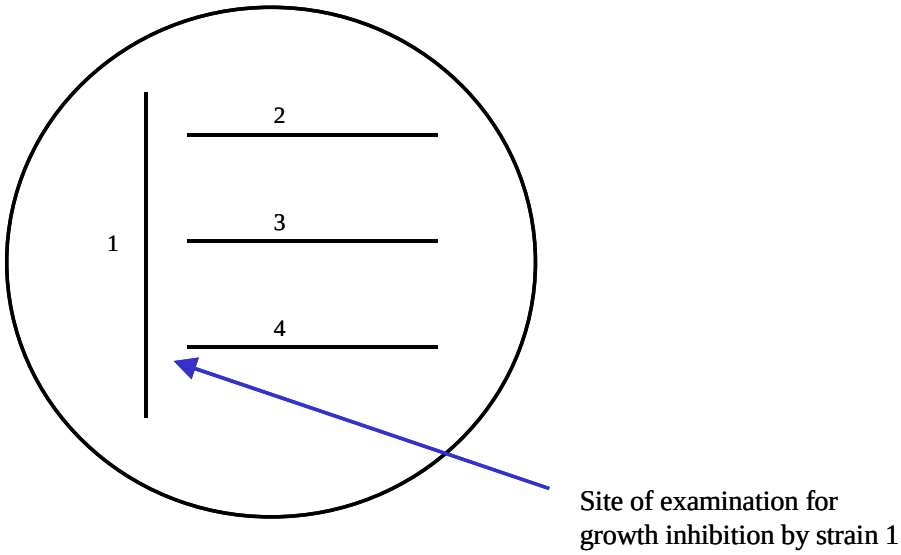


Figure 5.2 *Bacillus cereus* modified CAMP test. Non-emetic strains of *B. cereus* were streaked onto Skim Milk agar adjacent to an emetic strain to determine if the production of cereulide inhibited the growth of non-emetic strains.
1: Emetic strain; 2,3 and 4: Non-emetic strains.

5.3 Results

5.3.1 Measurement of antibiotic inhibition zones

The inhibitory effect of the antibiotics tested in this phase of the project differed greatly (Table 5.2). Varying from large clear zones surrounding the nigericin discs to almost non-existent zones surrounding the gramicidin discs. The comparative inhibitory effects of the antibiotics, with the exclusion of valinomycin, were:

nigericin>nonactin>enniatin>surfactin>bacitracin>gramicidin

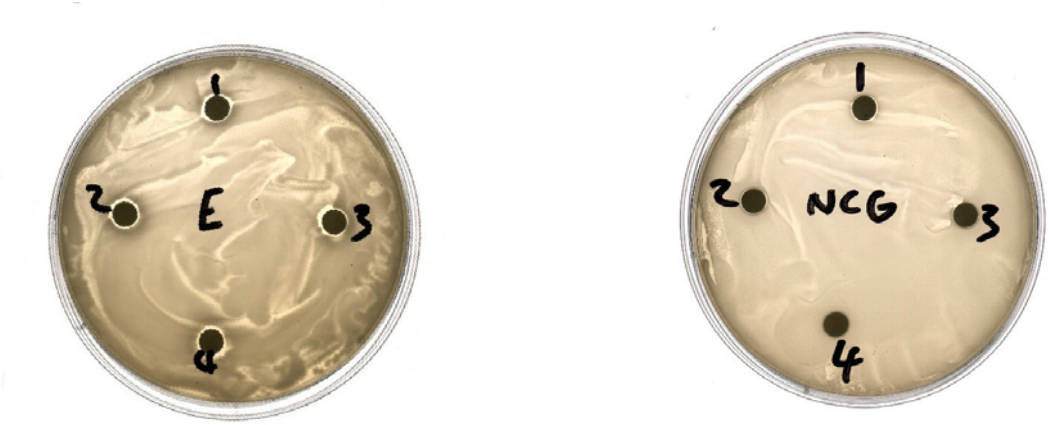
No discernable difference was noted in the extent or pattern of inhibition between emetic and non-emetic strains of *B. cereus* from the above antibiotics. Results are shown in Table 5.2.

A slight difference between the emetic and non-emetic strains was noted in the inhibition zones caused by valinomycin (Figure 5.3). Although the zones were not greatly different they were considered dissimilar enough for higher concentrations of valinomycin discs to be tested (section 5.2.2).

The differences between the emetic and non-emetic strains were more notable when the concentrations of the valinomycin discs were increased (Table 5.3). The inhibition zones for the emetic strains remained the same as those noted in Table 5.2, while the zones for the non-emetic strains increased slightly.

5.3.2 The effect of cereulide upon the growth of *Bacillus cereus*

The cereulide discs did not inhibit the growth of *B. cereus*. No zones of inhibition were observed. The growth of the emetic strains of *B. cereus* upon skim milk agar did not inhibit the growth of adjacent non-emetic strains.



Non-emetic strain F3453 (E)

Emetic strain NCG

Figure 5.3 Effect of valinomycin antibiotic discs upon the growth of *Bacillus cereus*. 1. 800 µg/disc; 2. 400 µg/disc; 3. 200 µg/disc; 4. 100 µg/disc.

Table 5.2 Measurement of antibiotic inhibition zones (mm)

		Emetic strains			Non-emetic strains		
Antibiotic	**µg/disc**	**NCG**	**F47**	**F4810/72**	**1997**	**ATCC14579**	**F3453**
Bacitracin	800	1	1.5	1	1	1.5	2
	400	1	1.5	0.5	0.5	1	1
	200	1	1	0.5	0.5	0.5	1
	100	0.5	1	0	0	0.5	1
	50	0	0.5	0	0	0.5	0.5
	25	0	0.5	0	0	0	0.5
Enniatin	800	4	3	3.5	4	3	3
	400	3.5	2.5	3.5	4	3	2
	200	3.5	2.5	3.5	4	2.5	2
	100	3.5	2	3.5	3.5	2	2.5
	50	3	2.5	3	3	2	2
	25	3	2	3	3	2	2
Gramicidin	800	0.5	1	0.5	1	1.5	0.5
	400	0.5	1	0	0.5	1	0.5
	200	0	1	0	0.5	1	0.5
	100	0	1	0	0.5	1	0.5
	50	0	1	0	0.5	1	0.5
	25	0	1	0	0.5	1	0.5
Nigericin	800	6	4	4	6	4.5	4
	400	5	3	4.5	5.5	4.5	4
	200	4.5	3	4.5	5.5	4.5	3.5
	100	4	2.5	4	5	3.5	3.5
	50	3.5	2	3.5	4	2.5	3
	25	2.5	1.5	2.5	4	2	1.5

		Emetic strains			Non-emetic strains		
Antibiotic	**µg/disc**	**NCG**	**F47**	**F4810/72**	**1997**	**ATCC14579**	**F3453**
Nonactin	800	4	4	4	3	5	4.5
	400	3	4	4	3.5	3	4
	200	3	3	4	3	4.5	4
	100	3	3	3.5	3	4.5	4
	50	3	3	3.5	3	4.5	3
	25	3	2	2.5	2.5	3	2.5
Surfactin	800	1	1	1	2	1	1
	400	1	1	1	1.5	1	1
	200	1	1	1	1	0.5	1
	100	1	1	0.5	0.5	0.5	0.5
	50	1	0.5	0.5	0.5	0.5	0.5
	25	0.5	0.5	0	0	0	0
Valinomycin	800	0.5	0.5	0.5	2.5	2.5	2
	400	0.5	0	0.5	2	2	2
	200	0.5	0	0	1.5	1.5	2
	100	0	0	0	1.5	1	1.5
	50	0	0	0	1	1	1
	25	0	0	0	1	0.5	1

Table 5.3 Measurement of inhibitory effect of valinomycin at higher concentrations (mm)

	Emetic strains		Non-emetic strains	
Valinomycin (µg/disc)	**NCG**	**F47**	**1997**	**F3453**
8,000	0.5	0.5	3.5	3
4,000	0.5	0.5	3	2.5
2,000	0.5	0.5	2.5	2
1,000	0.5	0.5	2.5	2

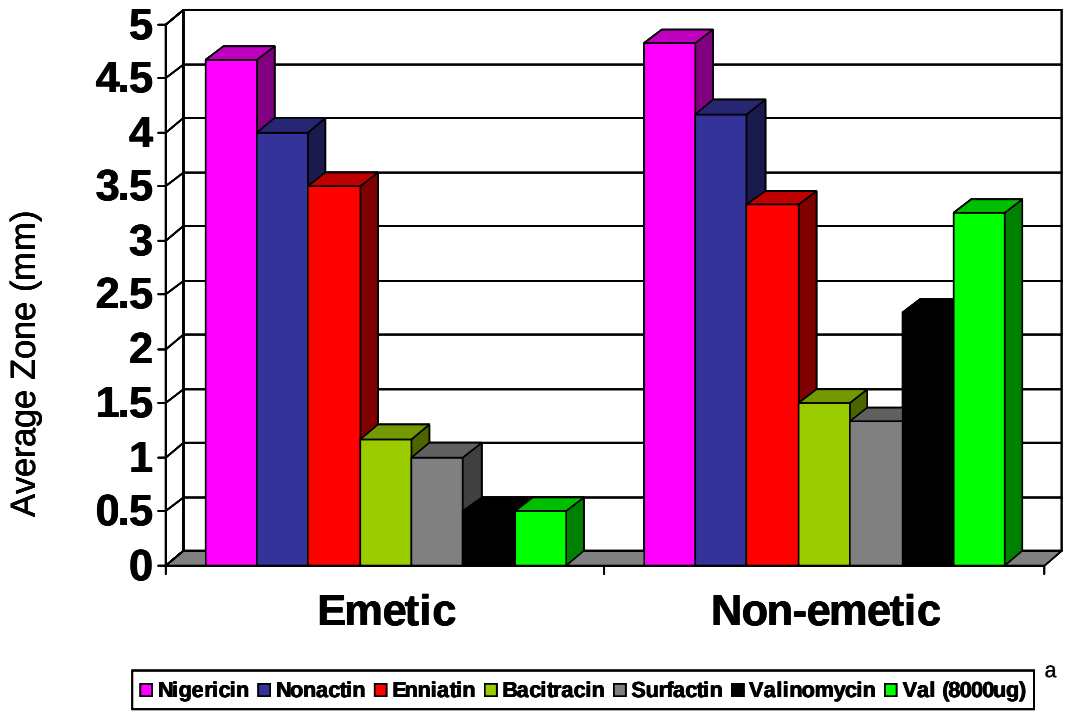


Figure 5.4 Average antibiotic (800 µg/disc)[a] inhibition zones for emetic and non-emetic strains of *Bacillus cereus*. Inhibition zones were comparable between emetic and non-emetic isolates, except for valinomycin where emetic strains were more resistant.

[a] All measurements were taken from 800 µg/discs except Val (8000µg) which was 8,000 µg/disc

5.4 Discussion

Organisms that produce antibiotics potentially active against the parent organism must have protective mechanisms to prevent self damage. Linkage between genes encoding for resistance to antibiotics and antibiotic biosynthesis genes have frequently been observed. Examples include methylenomycin, neomycin, thiostrepton, erythromycin, viomycin, hygromycin, kanamycin, oxytetracycline, paramomycin, streptomycin, puromycin, and chloramphenicol (Martin and Liras, 1989).

Non-emetic strains of *B. cereus* were observed to be more sensitive to valinomycin than emetic strains (Figure 5.3 and Table 5.3). It would have been interesting to increase the concentration of valinomycin used in Section 5.2.2 because the zone of inhibition for the emetic strains did not increase from 800 to 8,000 µg/disc, whereas the zones for the non-emetic strains were almost 50% larger (Figure 5.4). The inhibition zones were quite small for both emetic and non-emetic strains indicating that *B. cereus* is very resistant to the antibiotic effects of valinomycin. It would be difficult to determine if the zones were significant without a large increase in the number of *B. cereus* strains used in the study and a substantial increase in valinomycin concentration. However, the cost of such large amounts of valinomycin was prohibitive.

Self-toxicity experiments were conducted to determine if emetic strains were more resistant to damage from cereulide than non-emetic strains. This was to determine if cereulide resistance genes were present in emetic strains. The toxicity of cereulide has been tested extensively upon a variety of cell types, including HEp-2 cells (Hughes *et al*., 1988), CHO cells (Beattie and Williams, 1999), rat liver cells (Mahler *et al*., 1997) and boar spermatazoa (Andersson *et al*., 1998). However, all of these are eukaryotic cells. No reports could be found that investigated the toxic effect of cereulide upon prokaryotic cells. If cereulide does not damage bacterial cells then it is possible that *B. cereus* does not require an effective resistance mechanism against this peptide. It is possible that the concentration of cereulide in this study was too low to affect the bacterial cells. A study investigating higher concentrations of purified cereulide against emetic and non-emetic strains of *B. cereus* may determine if emetic strains are more

resistant to the effects of the toxin, and could that suggest emetic strains possess resistance mechanisms.

No difference in sensitivity to bacitracin, enniatin, gramicidin, nigericin, nonactin nor surfactin was noted between the emetic and non-emetic strains of *B. cereus*. This is not particularly surprising considering the high specificity of some antibiotic resistance mechanisms. Tetranactin resistance, for instance, is believed to involve the inactivation of the antibiotic through hydrolysis of ester linkages in the macrotetralide ring (Plater and Robinson, 1992). Therefore, only antibiotics that were very closely related structurally would conceivably be affected.

The antibiotics to which *B. cereus* was most sensitive were nigericin, nonactin, enniatin and valinomycin (non-emetic strains only). It is interesting to note that these four antibiotics encompass all of the potassium ionophores used in this study. The higher sensitivity of *B. cereus* to potassium ionophores in this study is probably due to the addition of 200 mM of KCl to the nutrient agar. Potassium ionophores kill cells by facilitating the diffusion of K^+ across the membrane, thus disrupting the ion gradient maintained by the cells. Therefore, the addition of K^+ (in the form of KCl) increases the damage that potassium ionophores can inflict (Plater and Robinson, 1992; Mikkola *et al*., 1999). Conversely, the antibiotics to which *B. cereus* was least sensitive were surfactin, bacitracin and gramicidin, which are all produced by species of *Bacillus*. It is possible that the mechanism for self protection against damage from *Bacillus* antibiotics is widespread throughout the genus.

A more extensive survey of related compounds and greater concentrations would greatly enhance the study conducted here. However, the cost for each of the antibiotics was too high to justify expanding the experiment for this thesis. When the difference between emetic and non-emetic sensitivity to valinomycin was observed, it was envisioned that the development of valinomycin media could differentiate between emetic and non-emetic strains of *B. cereus* based upon growth/no growth. However, further research showed that the cost of production of such media would be inhibitory for screening in routine food / public health microbiology laboratories.

Chapter 6

Non-Ribosomal Peptide Synthetase

Genes in *Bacillus cereus*

6.1 Introduction

Characterisation of the genes associated with the production of cereulide has defied scientists for years. The extremely small size of the peptide and its subsequent poor antigenicity, have resulted in difficulties in producing a reliable toxin detection method. Hence, research into the production of cereulide has been problematic. Although detection methods for cereulide have not significantly improved, molecular techniques and the understanding of peptide production have improved considerably.

Although the results from the work described in the previous chapter were inconclusive, a review of the literature lead to the realisation that cereulide was very similar in structure and function to many of the antibiotics investigated. The hypothesis was therefore formed that cereulide was synthesised in a similar manner as these antibiotics.

A wide variety of fungal and bacterial bioactive peptides are synthesised via a template-directed, non-ribosomal mechanism employing large enzyme complexes known as non-ribosomal peptide synthetases (NRPS) (Weber and Marahiel, 2001). These multienzyme complexes are associated with the production of a large range of important biological compounds such as the β-lactam antibiotics (e.g. penicillin), cyclosporin, gramicidin and surfactin (Chong *et al.,* 1998) (Table 6.1). Perkins *et al*. (1990) provided evidence that valinomycin was produced by *Streptomyces levoris* A-9 by peptide synthetases. The close structural and functional similarities between valinomycin and cereulide indicate that cereulide may be synthesised in a similar manner.

Peptide synthetases are organised in modules, one for each amino acid incorporated into the peptide product (Weber and Marahiel, 2001). Each module is responsible for the recognition, activation and, in some cases, modification of a single substrate in the final product. They are organised in co-linear arrangement with the primary sequence of the synthesised peptide. A minimal module of peptide synthesis must contain at least two domains, the adenylation domain and the thiolation domain (Mootz and Marahiel, 1997; Marahiel, 1997) (Figure 6.1). The flexibility of NRPS peptide production allows for a broad spectrum of possible products. The addition of non-proteinogenic substrates,

product cyclisation and various post-assembly modifications enables diverse and highly specific peptides to be produced through this system. NRPS can synthesise, in addition to the usual proteinogenic amino acids, unusual residues that are not present in proteins. These include D-amino acids, β-amino acids, and hydroxy- and *N*-methylated residues. Further modification can include glycosylation or heterocyclic ring formation (Marahiel, 1997; Mootz and Marahiel, 1997).

The heterocyclic structure of cereulide and the presence of D-amino acids (Figure 6.2) suggest that NRPS may be responsible for the production of cereulide. Many important peptides produced by members of the *Bacillus* genus are synthesised by NRPS, including surfactin, iturin, bacitracin, lichenysin, gramicidin and tyrocidine (Schneider *et al*., 1998; Tsuge *et al*., 2001; Konz *et al*., 1997; Konz *et al*., 1999; Krause and Marahiel, 1988). Analysis of the primary structure of NRPS genes has revealed the presence of highly homologous functional domains of about 600 amino acids. Universal PCR primers have been developed to detect the biosynthetic genes of peptide synthetases by targeting these conserved domains (Turgay and Marahiel, 1994). These primers were utilised to gain access to the biosynthetic genes associated with non-ribosomal peptide synthesis of cereulide.

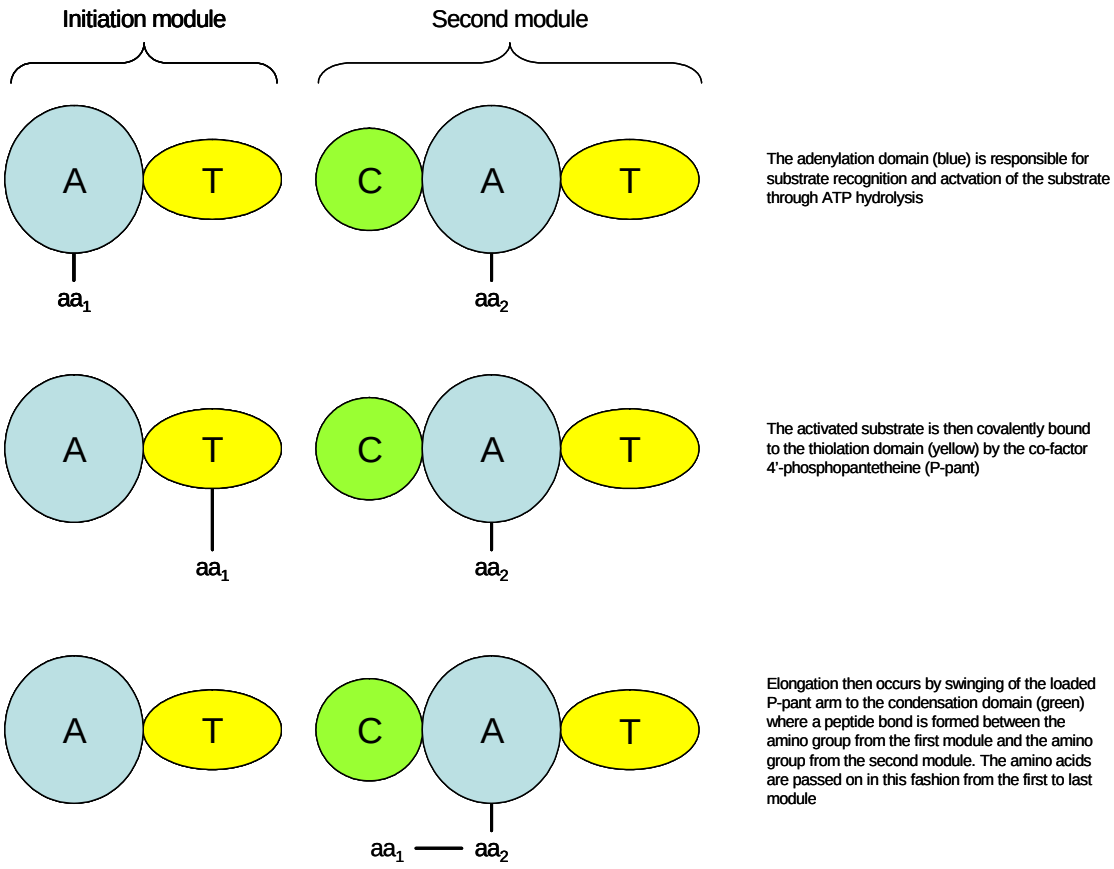


Figure 6.1 General organisation and operation of non-ribosomal peptide synthetases. An initiation module consists of an adenylation domain (blue circle) and a thiolation domain (yellow). Additional domains for substrate modification (such as epimerisation or heterocyclic ring formation) may also be present (not shown in diagram). During the synthesis, all intermediates remain covalently attached to the enzyme complex (Stachelhaus *et al*. 1998).

Table 6.1 A list of some important non-ribosomal peptide synthetase products

NRPS Product	Species	Characteristics
δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV)	Filamentous fungi, *Streptomyces* spp., some Gram-negative bacteria	Precursor to the penicillin and cephalosporin antibiotics which are the most important class of β-lactam antibiotics.
Surfactin	*Bacillus subtilis*	Lipopeptide antibiotic and biosurfactant
Iturin A	*Bacillus subtilis*	Antifungal peptide used for control of fungus on stone fruit
Bacitracin	*Bacillus licheniformis*	Antibiotic (skin ointments and eye drops) that interferes with bacteria's ability to form new cell walls
Lichenysin	*Bacillus licheniformis*	Powerful biosurfactant
Gramicidin S	*Brevibacillus brevis*	Cyclic decapeptide antibiotic (channel-forming ionophore) that is an ingredient in tyrothricin
Tyrocidine A	*Brevibacillus brevis*	Polypeptide antibiotic that is the major component of tyrothricin
Erythromycin	*Streptomyces* spp.	Macrolide antibiotic that inhibits ribosomal protein synthesis in bacteria
Calcium-dependent antibiotic (CDA)	*Streptomyces coelicolor*	Non-ribosomal peptide antibiotic
Enterobactin	*Escherichia coli*	Produced under conditions of iron limitation. Removes iron from iron-binding proteins and transports it into the bacterial cell
Enniatin	*Fusarium* spp.	Ionophore antibiotic. Contributes to virulence of *Fusarium* spp.
Cyclosporin	*Tolypocladium* spp.	Immunosupressive agent. Primary tool used to prevent rejection following solid organ and bone marrow transplants

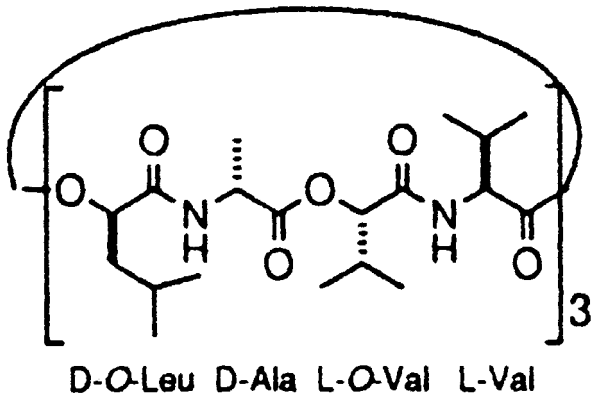


Figure 6.2 Molecular structure of cereulide (Agata *et al*., 1994). The heterocyclic structure and the presence of D- amino acids suggest that NRPS may be responsible for the production of cereulide.

6.2 Materials and Methods

6.2.1 Preparation of *Bacillus* DNA

Previously reported emetic strains of *B. cereus*, F5881 (Haggblom *et al*., 2002; Andersson *et al*., 1998) and NC7401 (Agata *et al*., 1994; Haggblom *et al*., 2002), were selected for sequencing. DNA was extracted using the protocol outlined in section 2.5.1 of this thesis.

6.2.2 Non-ribosomal peptide synthetase PCR

Primers (Table 6.2) designed to detect conserved domains of NRPS genes (Turgay and Marahiel, 1994) were utilised in this study. The final PCR reaction mixture (50 µl) contained sterile distilled water, 1x PCR buffer, 1.5 mM $MgCl_2$, 200 µM (each) of dNTP's, 1 U of *Taq* DNA polymerase (MBI Fermentas), 1 µM of each primer, TGD and LGG (Table 6.2), and 4 µl of template (as prepared in section 2.5.1). PCR amplification was performed in an Eppendorf Mastercycler. The PCR reaction conditions were as follows: 5 cycles at 95°C for 1 min, 45°C for 1 min and 72°C for 1 min; and 30 cycles at 95°C for 1 min, 50°C for 1 min and 72°C for 1 min (Turgay and

Marahiel, 1994). The amplification results were resolved by 1% agarose gel electrophoresis (section 2.5.2).

Table 6.2 Non-ribosomal peptide synthetase PCR primers (Turgay and Marahiel, 1994)

Primer Designation	Primer Sequence[a]
TGD	5' - TWY CGI ACI GGI GAY YKI GKI CG - 3'
LGG	5' - AWI GAR KSI CCI CCI SSR IMR AAR AA - 3'

[a] The primers were redundant/degenerated.
FASTA format - W: A or T; Y: T or C; K: G or T; S: G or C; R: G or A; M: A or C; I : inosine.

6.2.3 Secondary PCR

The DNA bands were considered too faint for sequencing. Therefore, the post-amplification PCR reaction was used as template material in a further PCR reaction, using the same primers as above. The product was diluted 1/100 and 1/10 in elution buffer for use as template. The reaction was then performed as outlined in Section 6.2.2.

6.2.4 Cloning and sequencing

The PCR products were purified using a PCR clean-up kit (Machery-Nagel) to remove residual reactants. PCR products were ligated in to a pGEM-T Vector System (Promega) according to the manufacturers instructions. Ligated plasmids were transformed into high efficiency *Escherichia coli* JM109 cells (Promega) according to the manufacturers instructions, and cloned by culture in Luria-Bertani (LB) medium (Appendix 1). Transformants were selected using blue-white screening and further amplified through culture in LB broth containing ampicillin (Appendix 1). Following overnight incubation the plasmids were extracted using the Wizard SV MiniPrep Kit (Promega). Plasmid yield was examined for purity and concentration using UV spectrometry. The plasmids were tested for the presence of the target insert by the PCR protocol outlined in Section 6.2.2. Three plasmids containing the NRPS amplicon were

selected for sequencing from each *B. cereus* strain. Sequencing was conducted using the CEQ DTCS - Quick Start Kit (Beckman Coulter) and capillary electrophoresis was carried out by the Advanced Analytical Centre of James Cook University, Queensland, Australia. Three replicates were analysed in each direction (i.e. forward and reverse primers). The resulting chromotograms were subsequently analysed using Sequencher and GeneDoc software to confirm the nucleotide sequence, align the sequence and create consensus data. The consensus sequence data was used in a BLASTn (nucleotide) and BLASTp (protein) search of the Genbank database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) to identify homologous sequences and to ascertain if the genes sequenced were related to other non-ribosomal peptide synthetases.

6.3 Results

6.3.1 NRPS PCR

The PCR products generated from Section 6.2.2 were not considered sufficient concentration for nucleotide sequencing. The PCR reactions were therefore used as a template in a secondary PCR at 1 in 100, and 1 in 10, dilutions. No amplicons were visible at 1 in 100, but visible amplification was observed from the 1 in 10 dilution of the primary PCR reaction (Figure 6.3). The emetic strains (F5881, NC7401 and NC954) all produced a single 500 bp product. Whereas the non-emetic strain ATCC14579 produced two bands, one slightly smaller than 500 bp and one at approximately 400 bp.

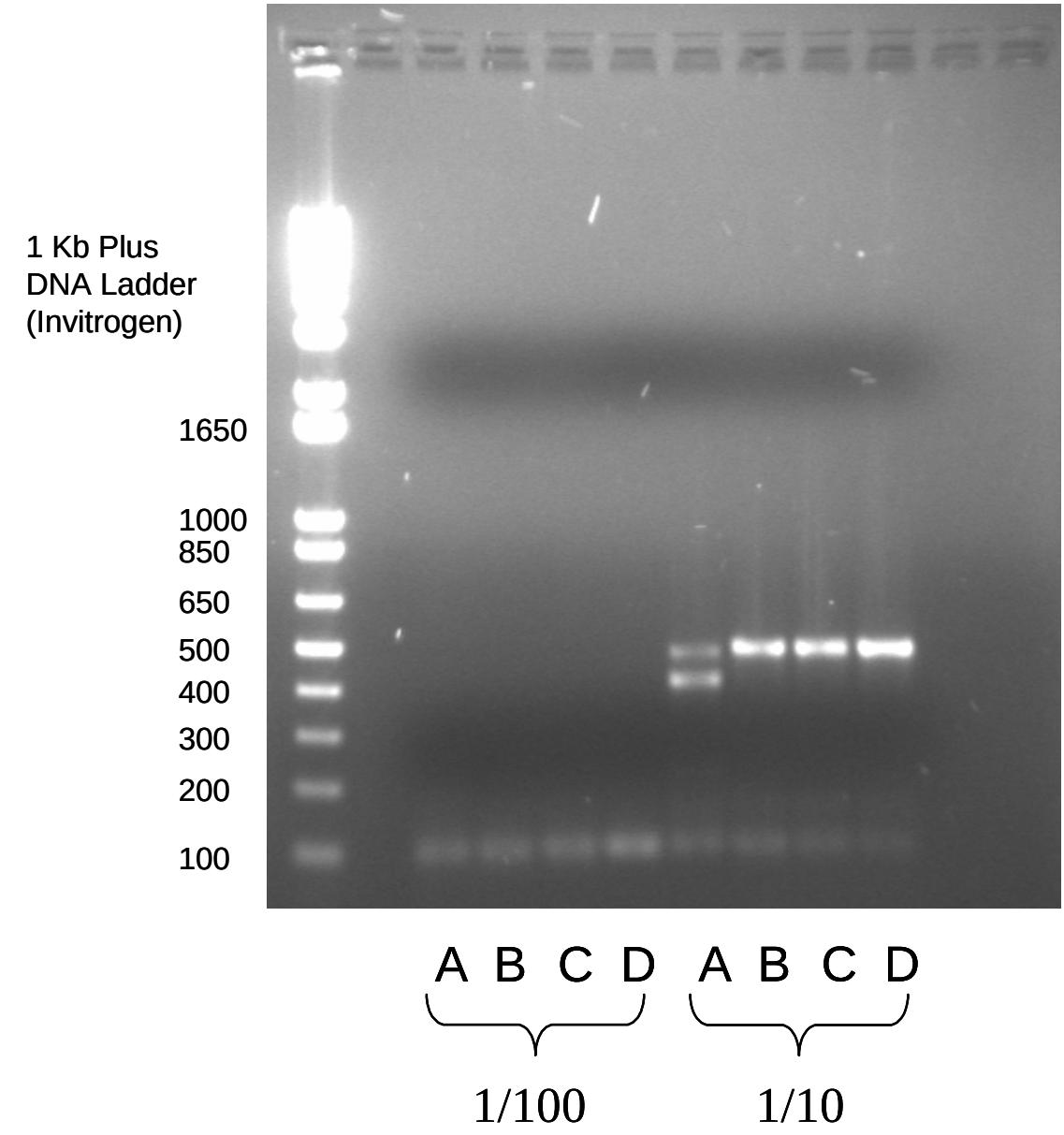


Figure 6.3 PCR to detect conserved regions of NRPS genes in *Bacillus cereus*. The primary PCR products were diluted (1/100 and 1/10) and then used as templates for a secondary PCR. The amplification results were resolved by 1% agarose gel electrophoresis.
A - ATCC14579; B - F5881; C - NC7401; D - NC954.

6.3.2 Sequencing from plasmid containing NRPS PCR amplicon

The PCR products for isolates F5881 and NC7401 were successfully ligated into the pGEM-T Vector and nucleotide sequences were subsequently determined. Analysis of

the forward and reverse sequence data with the computer programs Sequencher (GeneCodes) and GeneDoc (www.psc.edu/biomed/genedoc) resulted in a consensus sequence (Figure 6.4). The nucleotide sequences from F5881 and NC7401 matched exactly. The consensus NRPS sequence generated from this research was submitted to GenBank and assigned accession number - AY331260.

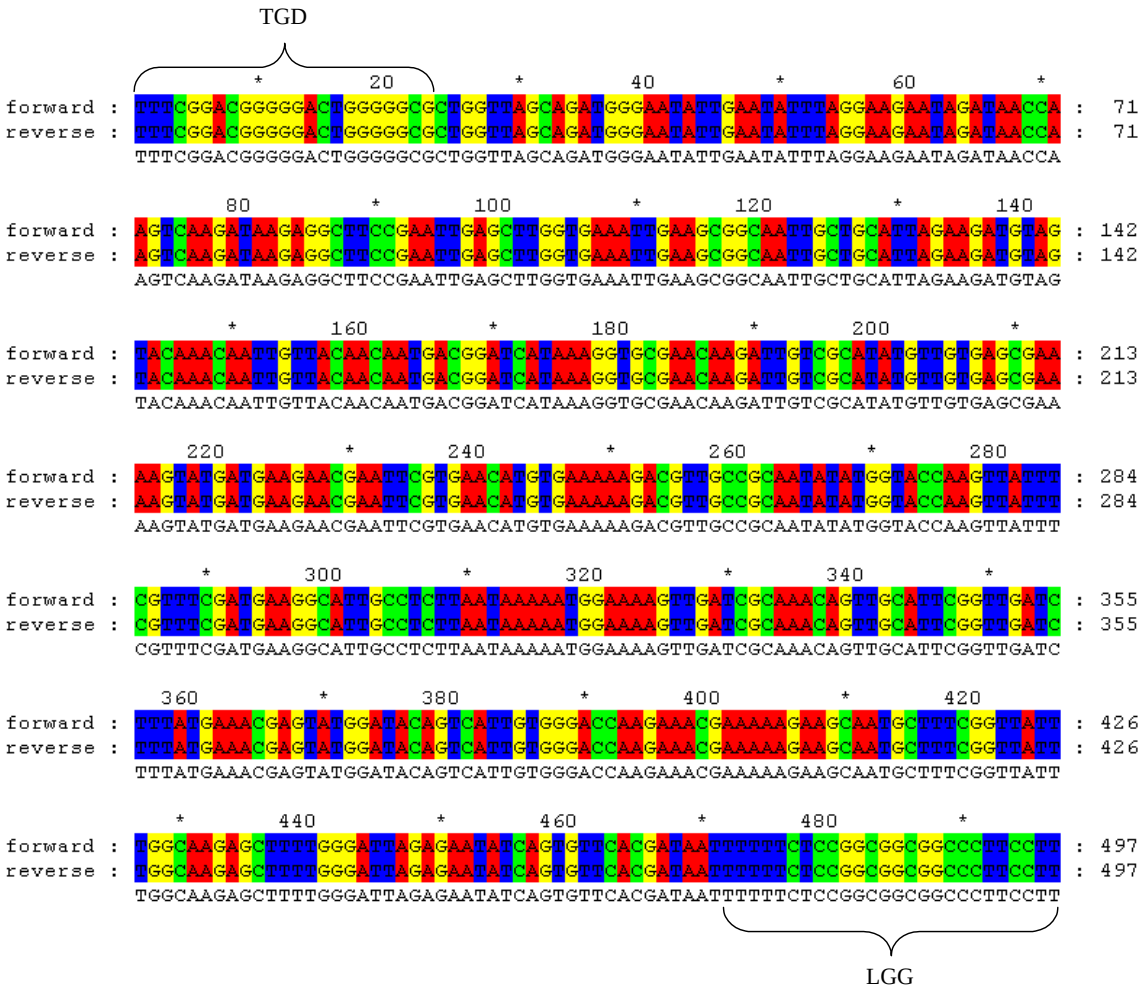


Figure 6.4 An alignment of the forward and reverse sequences generated from DNA sequencing (strains F5881 and NC7401).

Note: primer positions are marked on the figure

TGD: forward primer; LGG: reverse primer

6.3.3 BLASTn search analysis

The consensus sequence was analysed for homology with previously reported sequences using a BLASTn search of GenBank. The search showed that the NRPS PCR product had a conserved region of approximately 80 bp (Figure 6.5) that showed homology with other peptide synthetase genes (Table 6.3).

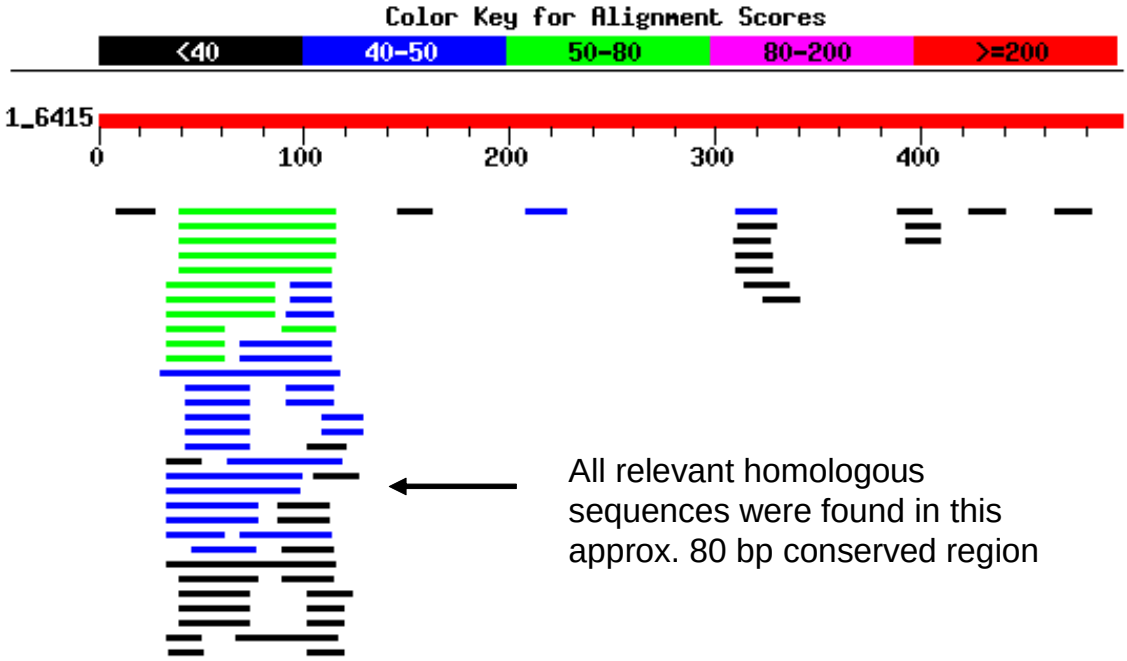


Figure 6.5 BLAST alignment for NRPS nucleotide sequence

Table 6.3 Results from BLASTn (GenBank) search of *Bacillus cereus* NRPS nucleotide sequence

Organism	Gene	Matching Bases	Accession Number and Authors	e-value[a]
Hypothetical protein (*Nostoc punctiforme*)	*nosA*	871-1037 ID: 77/168 (45%)	AAF15891.2 Hoffman *et al*., 2003	3e-35
Gramicidin S synthetase 1 (*Brevibacillus brevis*)	*grsA*	409-573 ID: 77/166 (46%)	P14687 Kratzshmar *et al*., 1989	1e-35
Tyrocidine synthetase 3 (*Brevibacillus brevis*)	*tycC*	3946-4110 ID: 75/166 (45%)	O30409 Mootz *et al*., 1997	5e-35
Syringopeptin synthetase B (*Pseudomonas syringae* pv. *syringae*)	*sypB*	4160-4327 ID: 76/169 (44%)	AAO72424.1 Scholz-Schroeder *et al*., 2001	3e-34
Fengycin synthetase (*Bacillus subtilis*)	*fenE*	1875-2037 ID: 74/166 (44%)	AAB80956.1 Chen *et al*., 1997	3e-34
Gramicidin S synthetase 2 (*Brevibacillus brevis*)	*grsB*	842-1006 ID: 75/167 (44%)	BAA06146.1 Saito *et al*., 1994	4e-34
Microcystin synthetase (*Microcystis viridis*)	*mcyB*	427-592 ID: 75/168 (44%)	BAC57997.1 Yoshida *et al*., 2003	6e-34
Surfactin synthetase (*Bacillus subtilis*)	*srfAA*	840-1006 ID: 69/168 (41%)	NP_388230.1 Kunst *et al*., 1997	5e-33
Iturin A synthetase B (*Bacillus subtilis*)	*ituB*	635-799 ID:71/166 (42%)	BAB69699.1 Tsuge *et al*., 2001	1e-32
Lichenysin synthetase A (*Bacillus licheniformis*)	*licA*	2909-3072 ID: 71/166 (42%)	AAD04757.1 Konz *et al*., 1999	1e-32
Bacitracin synthetase 2 (*Bacillus licheniformis*)	*bacB*	1930-2094 ID: 70/170 (41%)	O68007 Konz *et al*., 1997	1e-32
Mycosubtilin synthetase (*Bacillus subtilis*)	*mycB*	635-799 ID: 71/166 (42%)	T44807 Duitman *et al*., 1999	2e-32

[a] the Expect value (e) is a parameter that describes the number of hits one can "expect" to see just by chance. The lower the E-value, or the closer it is to "0" the more "significant" the match (http://www.ncbi.nlm.nih.gov/BLAST/blast_FAQs.shtml#Expect).

6.3.4 Conversion to protein sequence and BLASTp search

The nucleotide sequence was translated to amino acid sequence using Sequencher. The subsequent amino acid sequence was analysed for homology with previously reported sequences using a BLASTp search of GenBank (Figure 6.6). A large number of homologous sequences (244 bacterial and 144 fungal) were detected (Table 6.4). A selection of the sequences where the closest homology was observed are presented in Figure 6.8. The conserved putative domains detected in the BLASTp search are shown in Figure 6.7.

To provide further evidence that the hypothesised amino acid sequence was correct, the *B. cereus* NRPS nucleotide sequence was analysed using a BLASTx search, which searches the GenBank database for homology to the query sequence using all six possible reading frames. All of the sequences that exhibited homology were reading frame +1 and concurred with the results from the BLASTp search.

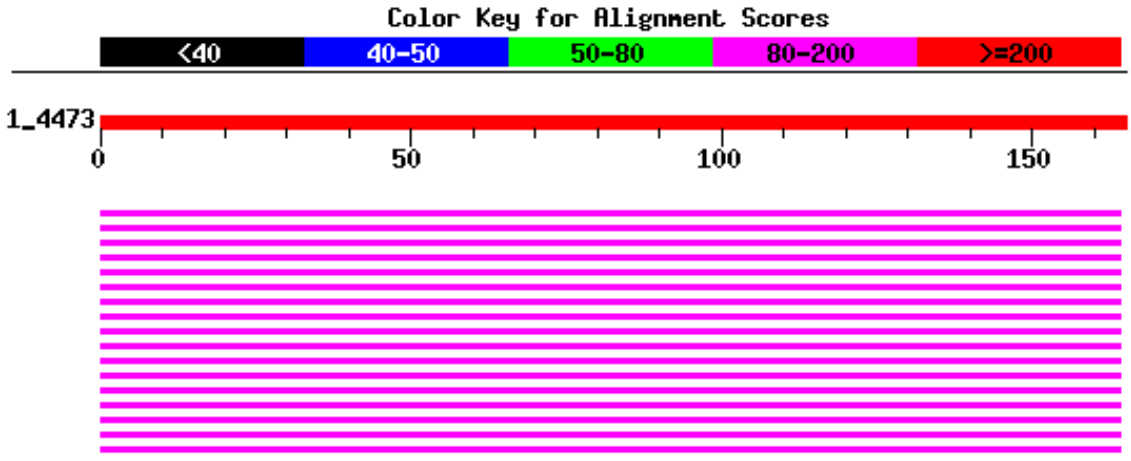


Figure 6.6 BLAST alignment for NRPS amino acid sequence

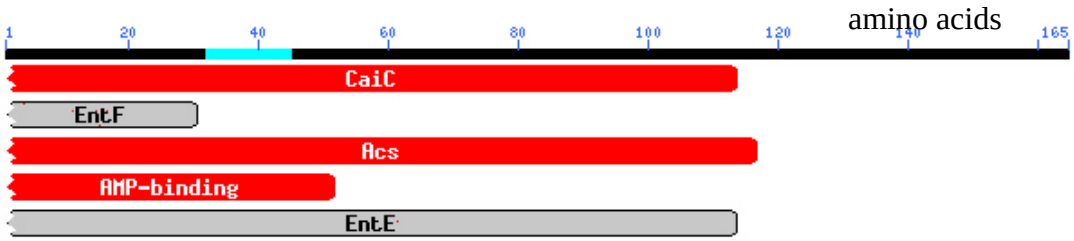


Figure 6.7 Diagram showing the conserved putative domains identified from the BLASTp search of GenBank. Conserved domains are identified by the Conserved Domain Database and Search Service (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) on the NCBI website. Protein domains are distinct units of molecular evolution, usually associated with particular aspects of molecular function such as catalysis or binding (Marchler-Bauer *et al.*, 2005). The hypothesised amino acid sequence for the *B. cereus* NRPS was related to the following protein domains:

CaiC: Acyl-CoA synthetases (AMP-forming)/AMP-acid ligases 2/secondary metabolites biosynthesis, transport and catabolism

EntF: Non-ribosomal peptide synthetase modules and related proteins

Acs: Acyl-coenzyme A synthetases/AMP ligases

Pfam: AMP-binding enzyme

EntE: Peptide arylation enzymes

Table 6.4 Results from BLASTp (GenBank) search of *Bacillus cereus* NRPS amino acid sequence (165 amino acids)

BLAST definition	Product (Gene)	Matching Bases	Accession Number and Authors	e-value
Bacillus cereus ATCC 14579 section 8 of 18 of the complete genome	Peptide synthetase (N/A)	291854-291924 ID: 61/71 (85%)	AE017005.1 Ivanova *et al*., 2003	1e-06
Bacillus subtilis srfA-srfP gene region for surfactin synthetase	Surfactin synthetase (*srfA2*)	13327-13401 ID: 64/75 (85%)	X70356.1 Cosmina *et al*., 1993	1e-06
Brevibacillus brevis gene for gramicidin S synthetase 1, complete cds	Gramicidin S synthetase 1 (*grsA*)	1825-1877 ID: 47/53 (88%)	D00519.1 Hori *et al*., 1989	1e-05
Bacillus subtilis yxjC, yxjD, yxjE, yxjF, ituD, ituA, ituB, ituC, xynD genes, partial and complete cds	iturin A synthetase A (*ituA*)	15210-15236 ID: 27/27 (100%)	AB0502629.1 Tsuge *et al*., 2001	2e-04
Staphylococcus aureus subsp. *aureus* N315 genomic DNA, complete genome	hypothetical protein, similar to surfactin synthetase (*sa0173*)	202657-202688 ID: 30/32 (93%)	AP003129.2 Kuroda *et al*., 2001	0.014
Candida albicans alpha-aminoadipate reductase large subunit	alpha-aminoadipate reductase (*lys2*)	2750-2800 ID:44/51 (86%)	U58133.1 Suvarna *et al*., 1998	0.057
Microcystis viridis mcyB gene for microcystin synthetase, partial cds	microcystin synthetase (*mcyB*)	1314-1358 ID: 39/45 (86%)	AB092907.1 Yoshida *et al*., 2003	0.89
Chlorogloeopsis fritschii partial cpsA gene for peptide synthetase	peptide synthetase (*cpsA*)	946-974 ID: 27/29 (93%)	AJ318785.1 Christiansen *et al*., 2001	0.89
Bacillus licheniformis bacitracin synthetase operon, complete sequence	bacitracin synthetase 3 (*bacC*)	35407-35438 ID:29/32 (90%)	AF007865 Konz *et al*., 1997	3.5

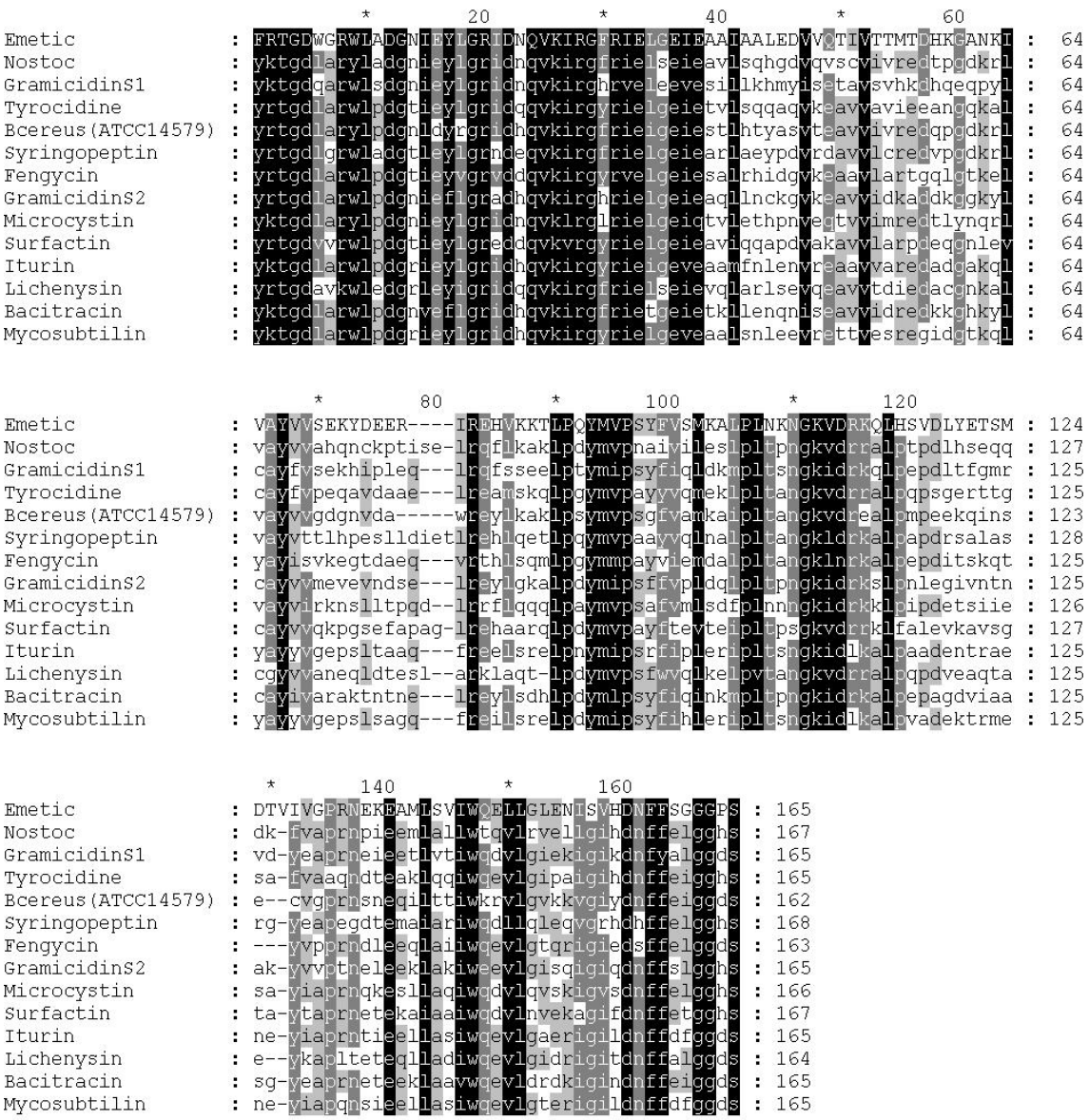


Figure 6.8 Protein alignment (GeneDoc) showing homology between NRPS products

Emetic - *Bacillus cereus* (AAQ97142); Nostopeptolide A - *Nostoc* sp. (AAF15891); Gramicidin S synthetase 1 - *Brevibacillus brevis* (P14687); Tyrocidine synthetase 3 - *Brevibacillus parabrevis* (O30409); Bcereus (ATCC14579) (AE017005); Syringopeptin synthetase B - *Pseudomonas syringae* (AAO72424); Fengycin synthetase - *Bacillus sunbtilis* (AAB80956); Gramicidin S synthetase 2 - *Brevibacillus brevis* (BAA06146); Microcystin synthetase - *Microcysitis viridis* (BAC57997); Surfactin synthetase - *Bacillus subtilis* (NP_388230); Iturin A synthetase B - *Bacillus subtilis* (BAB69699); Lichenysin synthetase A - *Bacillus licheniformis* (AAD04757); Bacitracin synthetase 2 - *Bacillus licheniformis* (O68007); Mycosubtilin synthetase - *Bacillus subtilis* (T44807)

6.4 Discussion

Non-ribosomal peptide synthetases have been detected in an increasingly wide variety of bacteria and fungi. In particular, members of the filamentous fungi and the bacterial genera *Streptomyces* and *Bacillus* are well known for their production of a large array of non-ribosomally synthesised bioactive peptides. Although NRPS production has been commonly reported in the genus *Bacillus*, this is only the second report of NRPS genes in *B. cereus*. The complete nucleotide sequence of the *B. cereus* type strain ATCC14579 was recently determined (Ivanova *et al.*, 2003). Analysis of the gene sequence showed that this strain of *B. cereus* contained NRPS genes that were highly homologous with previously reported NRPS genes. The nucleotide and amino acid sequences for the NRPS genes published by Ivanova *et al.* (2003) differed considerably from the sequence of the cereulide strains presented in this study (Figure 6.8). It is apparent that *B. cereus* is capable of producing more than one NRPS product. No research has been published that identifies the product of the ATCC14579 NRPS sequence. The sequence published by Ivanova *et al*. (2003) is probably one or both of the products that were produced by the strain ATCC14579 in the NRPS PCR in this study (Figure 6.3).

The PCR developed by Turgay and Marahiel (1994) is a useful tool for the identification of unknown NRPS genes. The primers are situated so that in between the two primers are five highly conserved regions (see Figure 6.8 and Table 6.5). Therefore, once sequencing has been conducted a sequence analysis can determine if the new sequence is in fact an NRPS gene sequence. The *B. cereus* NRPS sequence contained all of these conserved regions with only a small degree of difference from the reported consensus sequence (Table 6.5).

The 500 bp gene fragment sequenced from *B. cereus* strains F5881 and NC7401 was highly homologous with previously reported NRPS genes. All of the bacterial species that were utilised for the NRPS alignment (Figure 6.8) use the amino acid translation that is outlined by Codon Usage Table 11 - Eubacteria and Plant Plastid (www.ncbi.nlm.gov/Taxonomy/tax.html/). Analysis of the postulated amino acid sequence with the Genbank database showed that the *B. cereus* translated NRPS sequence was homologous with the superfamily of adenylate-forming enzymes.

Included in this gene family are all of the peptide synthetases so far sequenced, acyl-CoA synthetases and adenylating enzymes. All of these enzymes activate their substrate amino acids to form acyl adenylates through ATP hydrolysis and contain the highly conserved sequences, A1-A10 (Table 6.5 and Figure 6.9) (Marahiel, 1997; Stachelhaus and Marahiel, 1995). The high degree of homology that is observed in the conserved regions of peptide synthetases is probably due to the very specific and specialised functions of the enzyme products.

The gene fragment from the two emetic strains of *B. cereus* was very similar to other previously reported peptide synthetases. Although these results are encouraging, further evidence is required to establish a conclusive link between the NRPS genes and the production of cereulide.

Table 6.5 Conserved regions located between the PCR primers TGD and LGG (table adapted from Marahiel, 1997; Stachelhaus and Marahiel, 1995)

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Figure 6.9 Cereulide NRPS amino acid sequence displaying conserved regions

Chapter 7

Development of a PCR Method to Detect NRPS Genes Associated with the Synthesis of Cereulide

7.1 Introduction

The toxin detection assays that are currently available for cereulide are too insensitive and not rapid enough for them to be useful in the prevention of emetic food poisoning. The rice industry in particular would greatly benefit from the development of a viable cereulide detection method. Presently the most commonly used methods utilise cell cytotoxicity as an indicator of cereulide production. Cell culture maintenance is time consuming and expensive, therefore making it difficult for small food laboratories to employ these methods. In addition, cell cytotoxicity methods require two to three days before results can be obtained. This is too long for a quality assurance laboratory where the product is perishable. It is evident that there is an urgent need for a rapid, sensitive cereulide detection method to be developed.

The polymerase chain reaction was first described by Saiki *et al*. (1985) and was subsequently recognised as a powerful tool for the amplification of nucleic acids from a great range of organisms for a variety of purposes (Mullis and Faloona, 1987). In fact, the use of this molecular tool has become commonplace in most research and quality assurance laboratories. The mechanism of PCR is the amplification of a nucleic acid sequence by employing specific primers that anneal to the region flanking the target sequence. The primer annealing regions must be known, so that the oligonucleotide primers can be carefully designed to specifically anneal to the region flanking the target sequence. PCR methods would greatly facilitate the rapid detection of cereulide. However, despite much interest and conjecture about the genes associated with the production of this peptide, these genes have not been determined. Due to the similarities between cereulide and the peptide products of non-ribosomal peptide synthetases, the hypothesis was formed that cereulide is produced by these multi-enzyme complexes. To test this hypothesis, PCR primers were designed specifically for the *B. cereus* NRPS nucleotide sequence determined in Chapter 6 of this thesis. A large number of *B. cereus* isolates were subsequently tested using these primers.

7.2 Materials and Methods

7.2.1 Preparation of *Bacillus* DNA

Strains of *B. cereus* used in this study are presented in Table 7.1. DNA was extracted using the protocol outlined in section 2.5.1 of this thesis.

Table 7.1 *Bacillus cereus* strains used in the cereulide PCR

Description [a]	Strain Designation
Non-emetic isolates	F4094/73, ATCC14579, F528, F3453, IH41064, IH41385, NVH0075/95, NVH1230/88, CK1, CK2, 3153, 3154, 3155, 3156, 3259, 3261, 3262, 3263, 3264, 1994, 1996, 1997, 2174, 2175, 2884, 3164, 3166
Emetic isolates	F47, F4426, F5881, NC7401, F4810/72, NCF, NCG, NCY, NC954, NC1044, NC1078, NC88F, NC90T, NC1128, NC1149, NC1154, NC1184, NC1204, NC1219, NC1237, NC1240, NC1245, NC1246, NC1260, NC1287, NC1291, NC1310, NC1315, NC7401
Non-emetic food isolates	Bc1 to Bc30

[a] See Tables 2.1, 2.2 and 2.3 for complete descriptions of *Bacillus* strains

7.2.2 Design of *Bacillus cereus* NRPS PCR

Oligonucleotide primers were designed using the computer program OLIGO 6 (Molecular Biology Insights Inc.). Two variable regions identified from the nucleotide and translated amino acid alignments of previously reported NRPS sequences were selected for primer design. Within this region there is no discernible homology between different NRPS sequences. However, the two NRPS nucleotide sequences generated in

this study (from emetic strains F5881 and NC7401) were completely homologous within this region. Therefore, primers designed within this variable region would be specific for the *B. cereus* peptide synthetase nucleotide sequence.

The final PCR reaction mixture (50 µl) contained sterile distilled water, 1x PCR buffer, 1.5 mM $MgCl_2$, 200 µM (each) of dNTP's, 1 U of *Taq* DNA polymerase (MBI Fermentas), 1 µM of each primer, CER1 and EMT1 (Table 7.2), and 4 µl of template (as prepared in section 2.5.1). Amplification was performed in an Eppendorf Mastercycler. The PCR reaction conditions were as follows: 1 cycle at 94°C for 10 min. 35 cycles at 94°C for 1 min, 52°C for 1 min and 72°C for 1 min; and 1 cycle at 72°C for 7 min. The amplification results were resolved by electrophoresis on a 2% gel (section 2.5.2).

Table 7.2 Primers developed to detect the NRPS genes associated with the synthesis of cereulide

Primer designation	**Primer sequence**	**Expected size of product**
CER1	5'-ATC ATA AAG GTG CGA ACA AGA-3'	188 bp
EMT1	5'-AAG ATC AAC CGA ATG CAA CTG-3'	

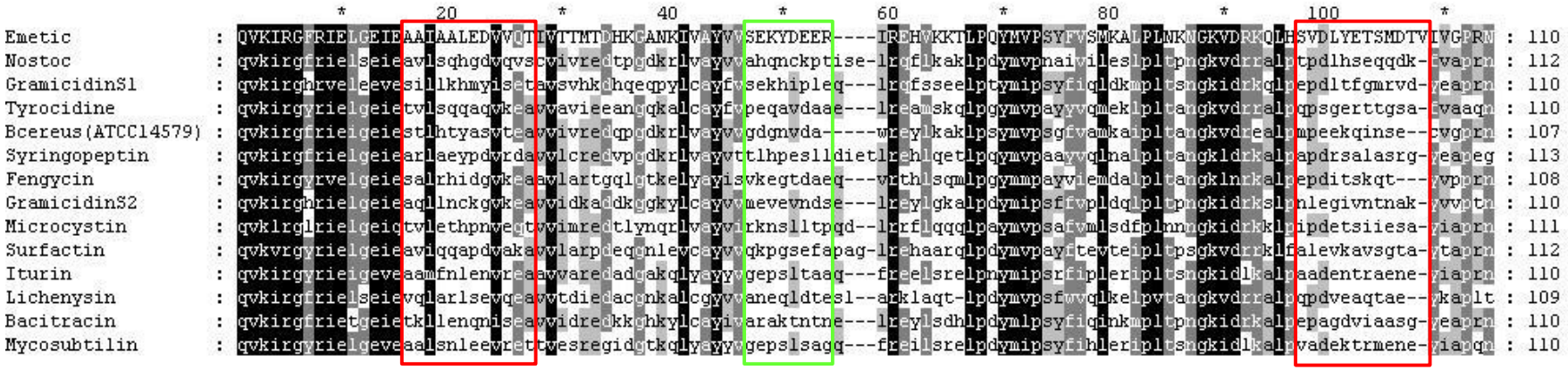

Figure 7.1 Amino acid alignment of peptide synthetases showing the emetic *Bacillus cereus*-specific regions that were targeted for primer and probe design. The red regions are where the primers (EMT1 and CER1) were designed and the green region was where the probe (SEKY) was designed.

7.2.3 Design and application of a PCR-ELISA detection system

A biotinylated probe was designed from the nucleotide sequence region that coincided with the SEKYDEER region of the NRPS protein sequence (Figure 7.1). This region was chosen because of its apparent specificity to emetic strains of *B. cereus*, in comparison to the other published NRPS sequences. The sequence of the probe (SEKY) was:
5'-biot-AAAATCGTTCATCATACTTTTGCTC-3'.

The following protocol was adapted from Muramatsu *et al*. (1997) by Mr. Ramon Layton (James Cook University, Townsville, Australia). Following PCR amplification, a subsequent labelling PCR was conducted using Dig-11-dUTP at a ratio of 19:1 (dTTP:Dig-11-dUTP). To make a 19:1 ratio the following solution was made using 100 mM dNTP's and 1 mM Dig-11-dUTP - 2.5 µl of dATP, 2.5 µl of dCTP, 2.5 µl of dGTP, 2.38 µl of dTTP, 12.5 µl of Dig-11-dUTP and 2.62 µl of H_2O (total volume of 25 µl).

PCR product (10 µl) was added to 90 µl of hybridisation buffer (4x SSC; 20 mM EDTA; 20 mM HEPES; 0.15% Tween 20) containing 1.3 nM biotinylated probe (SEKY). The solution was heated to 94°C for 5 minutes and annealed at 55°C for 5 minutes in an Eppendorf Mastercycler. The product was transferred to a streptavidin-coated microtitre plate (Thermo Labsystems, Finland) and incubated on a shaking platform (150 rpm) for 1 hour at 37°C. Following incubation the wells were washed three times with TBST ([100 mM Tris-HCL pH 7.5, 150 mM NaCl] with 0.1% Tween 20). Blocker solution (200 µl) (TropBio, Australia) was added and the plate was incubated at room temperature for 1 hour. Following incubation 200 µl (1:1000 dilution) of anti-digoxigenin-Pod (Roche) conjugated fragments in diluent (Roche) was added and the plates were incubated at 37°C for 30 minutes. The plates were subsequently washed twice with TBST, and 200 µl of ABTS single step solution (KPL, USA) was added. The plate was incubated at 37°C for 30 minutes. The plates were observed for a green colour change (Figure 7.2).

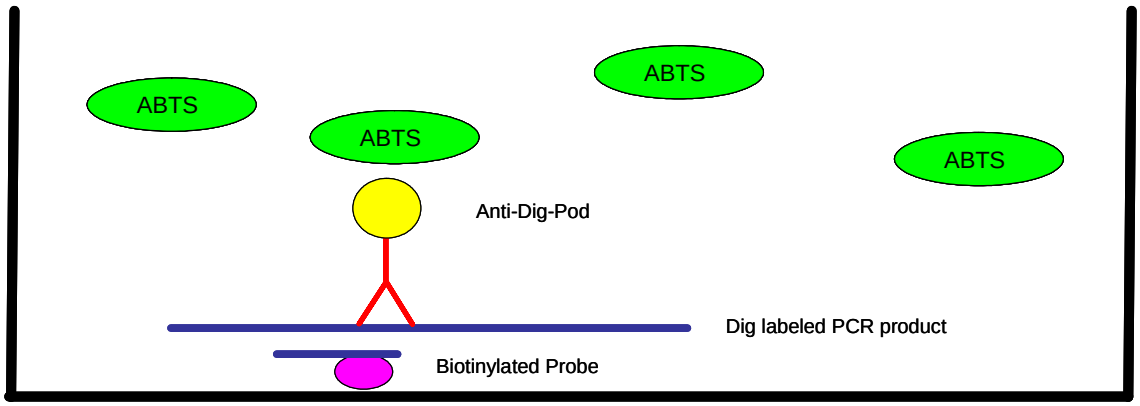

Figure 7.2 The PCR-ELISA detection system

7.2.4 Evaluation of PCR for detection of emetic strains of *Bacillus cereus*

A total of 86 *B. cereus* strains (Table 7.1) were tested using the PCR protocols described above. The results were compared to their known emetic or non-emetic activity (based upon reports from the source and the HEp-2/MTS assay) and the gel electrophoresis detection method was compared to the PCR-ELISA method. A total of 29 emetic strains and 57 non-emetic strains (according to the HEp-2/MTS assay) were tested using the PCR protocols

7.3 Results

7.3.1 PCR for the detection of emetic strains of *Bacillus cereus*

The PCR method developed in this study successfully distinguished between the emetic and non-emetic isolates tested (Figure 7.3). A 188 bp DNA product was observed for the 29 emetic strains following PCR amplification and subsequent electrophoresis. Only one of the non-emetic strains, IH41385, exhibited the DNA band after gel electrophoresis (Table 7.3). The PCR-ELISA correlated exactly with the PCR results obtained through gel electrophoresis. A dark green reaction was evident for all of the strains that were positive for the cereulide PCR (Figure 7.4).

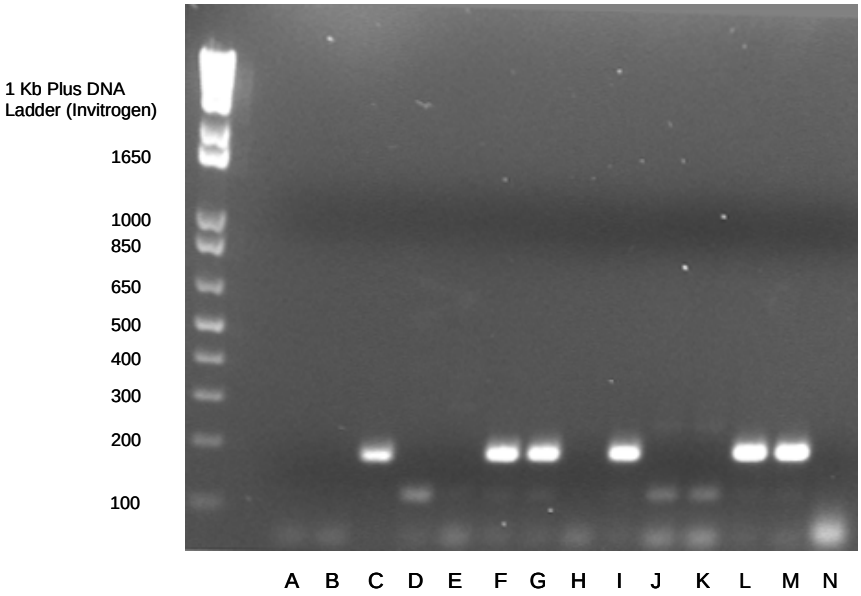

Figure 7.3 PCR gel differentiating between emetic and non-emetic strains of *Bacillus cereus*. The amplification results were resolved by 2% agarose gel electrophoresis.
(A) F4094/73; (B) ATCC14579; (C) F47; (D) F528; (E) F3453; (F) F4426; (G) F5881; (H) IH41064; (I) IH41385; (J) NVH0075/95; (K) NVH1230/88; (L) NC7401; (M) F4810/72; (N) no template control.

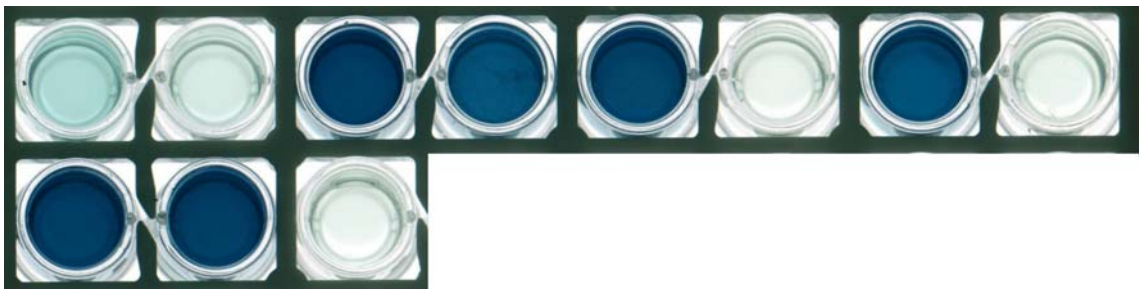

Figure 7.4 Cereulide PCR-ELISA. Dark green wells were observed for all of the strains positive by gel electrophoresis.
Strains: F4094/73, ATCC14579, F47, F4426, F5881, NVH0075/95, NC7401, NVH1230/88, F4810/72, NCG, no template control
Red = emetic strains

Table 7.3 Summary of the cereulide PCR results

Strain description	Strains	Cereulide PCR - agarose gel	Cereulide PCR - ELISA	HEp-2/MTS
Non-emetic	F4094/73, ATCC14579, F528, F3453, IH41064, NVH0075/95, NVH1230/88, CK1, CK2, 3153, 3154, 3155, 3156, 3259, 3261, 3262, 3263, 3264, 1994, 1996, 1997, 2174, 2175, 2884, 3164, 3166	-	-	-
Emetic	F47, F4426, F5881, NC7401, F4810/72, NCF, NCG, NCY, NC954, NC1044, NC1078	+	+	+
Emetic	NC88F, NC90T, NC1128, NC1149, NC1154, NC1184, NC1204, NC1219, NC1237, NC1240, NC1245, NC1246, NC1260, NC1287, NC1291, NC1310, NC1315, NC7401	+	+	NT[a]
Non-emetic	IH41385	+	+	-

[a] NT - not tested.

7.4 Discussion

To date, control of emetic strains of *B. cereus* in food has been a reactive approach, due to the unavailability of a rapid and reliable method of detecting toxigenic strains. The improvement of diagnostics for the detection of emetic strains of *B. cereus* is important because these strains are capable of producing food poisoning outbreaks with serious economic and health impacts. The development of a rapid cereulide-detection method would facilitate the identification of emetic strains in quality control procedures and epidemiological investigations following food poisoning outbreaks. The PCR method described here greatly increases the sensitivity and speed of detection of emetic strains. This method will enable food laboratories to take a preventative approach due to the greatly reduced time needed for the test.

The post-PCR detection methods correlated exactly. The agarose gel electrophoresis method is the most commonly used to evaluate a PCR reaction. The reagents and methods are more common to most laboratories, less expensive and gel electrophoresis is faster than ELISA for small numbers of samples (<1 hour to > 2 hours). In addition, gel electrophoresis allows the scientist to check product purity and determine the product size. Specific DNA bands can also be excised from the gel for further analysis such as sequencing. Theoretically, the ELISA detection system further enhances the specificity of PCR and reduces the chances of false positives due to the addition of a probe. Although the ELISA method is not as fast, the ability to adapt the method to automation means that large sample numbers can be analysed much faster than with gel electrophoresis. Sample numbers and the further application of the product need to be evaluated before selection of the method that best suites the laboratory in question.

All of the emetic strains tested and only one of the 57 non-emetic strains of *B. cereus* displayed a positive result with the PCR. This strain, IH 41385, has been reported as non-emetic by Andersson *et al.* (1998) through the boar spermatozoa assay and also tested negative with the HEp-2/MTS assay in Chapter 4 of this thesis (Table 7.3). However, recent results by Andersson *et al.* (pers. comm. 2003) using the newly developed chemical assay and a more sensitive generation of the boar spermatozoa test have shown that the strain IH 41358 produces very low amounts of cereulide (1/100th

of most cereulide producers). This shows that the cereulide PCR method is extremely sensitive and can detect strains that produce low titres of toxin that would be undetectable by conventional methods.

The high degree of homology between the translated *B. cereus* gene fragment and other NRPS amino acid sequences, coupled with the previously reported structural homology of cereulide with NRPS products (Agata *et al*., 1994) shows that there is compelling evidence to suggest that cereulide is produced by a system of peptide synthetases similar to other *Bacillus* peptide antibiotics such as gramicidin and surfactin. This would explain why researchers have had difficulties in discovering the genes responsible for the production of this peptide. Although it is evident that NRPS are prevalent in the *Bacillus* genus, the ability of the PCR primers developed in this study to consistently detect emetic strains of *B. cereus* indicates that the sequenced gene fragment is associated with the production of cereulide. However, further studies such as gene knockout mutagenesis will be required to conclusively show that these genes are directly involved in the production of cereulide.

Peptide synthetase genes were recently reported by Ivanova *et al*. (2003) when they sequenced the genome of the *B. cereus* strain ATCC 14579. This strain of *B. cereus* has been reported as non-emetic by a number of researchers and tests (Haggblom *et al*., 2002; Andersson *et al*., 1998), including the HEp-2/MTS assay in Chapter 4 of this thesis. This strain was tested using the primers CER1 and EMT1 and there was no cross-reactivity between these emetic primers and the NRPS genes from ATCC 14579. This provides further evidence that these primers are specific for emetic strains of *B. cereus*. The NRPS PCR of Turgay and Marahiel (1994) that was used in this study (Chapter 6) produced two DNA bands following amplification and gel electrophoresis of the DNA from ATCC 14579. Presumably one of these bands is the NRPS genes identified by Ivanova *et al*. (2003). The other DNA band is probably a shorter analogue of the same NRPS genes. The products of these NRPS genes have not been determined.

7.4.1 Proposed method for the identification of emetic strains of *Bacillus cereus* from food

At present, emetic strains of *B. cereus* are not identified in quality control food laboratories. Cell cytotoxicity is too labour intensive and the results take too long for such a method to be used routinely. Other methods require expensive or uncommon reagents. The cereulide PCR method developed in this study could be adapted for use in a quality control food laboratory. Following isolation of *B. cereus* from food (which takes approximately 24 hours), emetic strains could be identified in approximately 5 hours (1 hour contact time). This is a huge decrease in detection time compared to cell culture methods (>3 days). Confirmation of a strain's ability to produce cereulide could be provided by the HEp-2/MTS method. A proposed method is outlined below:

7.4.1.1 *Bacillus cereus* isolation from food (based upon Australian Standard procedures, reference AS 1766.2.6, 1991)

- Prepare 10 g of food test portion by weighing into a sterile stomacher bag
- Add 90 ml of maximum recovery diluent
- Blend using a stomacher for 2 min
- Perform a spread plate using PEMBA plates - duplicate each sample and dilution
- Incubate plates at 37°C for 24 hrs
- Examine plates for crenated peacock blue colonies ~5 mm surrounded by a similar blue egg yolk precipitate
- Further analyse suspect *B. cereus* colonies with the cereulide PCR method

7.4.1.2 Template preparation

- Transfer approximately 1-2 mm in diameter of colony to 200 µl of TE buffer in a 1.5 ml tube (Eppendorf)
- Place in a boiling water bath for 15 minutes to lyse the cells
- Remove cell debris by centrifugation at 15,0000 *g* for 5 minutes
- Transfer supernatant to a clean 1.5 ml tube and store at 4°C
- Type strains of *B. cereus* should be used for all reactions to maintain the integrity of the assay (e.g. ATCC14579 - non-emetic; F4810/72 - emetic)

7.4.1.3 Cereulide PCR method

- The PCR reaction mixture is outlined below:
  - Sterile distilled water (to a total volume of 50 µl)
  - 1x PCR buffer (MBI Fermentas)
  - 1.5 mM $MgCl_2$
  - 200 µM (each) of dNTP's
  - 1 U of *Taq* DNA polymerase (MBI Fermentas)
  - 1 µM of each primer, CER1 (5'-ATCATAAAGGTGCGAACAAGA-3') and EMT1 (5'-AAGATCAACCGAATGCAACTG-3')
  - 4 µl of template (as prepared above)
- The PCR reaction conditions are as follows:
  - 1 cycle at 94°C for 10 min
  - 35 cycles at 94°C for 1 min, 52°C for 1 min and 72°C for 1 min
  - 1 cycle at 72°C for 7 min
- Resolve the amplification results by 2% agarose gel electrophoresis
- A PCR product of 188 bp indicates that the strain is an emetic isolate
- Appropriate measures can be employed to ensure that emetic food poisoning cases are avoided.

Chapter 8

Prevalence of Toxin Genes in *Bacillus* spp.

8.1 Introduction

Bacillus cereus, B. anthracis and *B. thuringiensis* are genetically very closely related members of *Bacillus* subgroup 1. Comparative analysis of the 16s rRNA sequences of the members of *Bacillus* group 1 revealed high levels of sequence similarity (>99%), which was comparable to the intraspecies base differences for other Gram-positive bacteria (Ash *et al*., 1991). The high level of homogeneity between these organisms has been further demonstrated through 23S rRNA sequence analysis (Ash and Collins, 1992), multilocus enzyme electrophoresis (Helgason *et al*., 2000) and H-antigen cross-reactivity (Murakami *et al*., 1993). This has led to numerous proposals for their inclusion into a single species (Carlson *et al*., 1994; Helgason *et al*., 2000).

The rationale for the maintenance of individual species nomenclature is the differing phenotypes and pathological effects of the three *Bacillus* species: *B. cereus* is an ubiquitous soil organism that sometimes causes food poisoning, *B. thuringiensis* produces insecticidal toxins that allow it to be utilised as an important insecticide and *B. anthracis* is the cause of the acute and often fatal disease of mammals, anthrax. The *cry* genes that encode the insecticidal toxins of *B. thuringiensis* are carried upon large plasmids. Similarly, the genes for the toxin and protective capsule that are essential for *B. anthracis* to cause anthrax are carried upon two large plasmids (px01 and px02). Therefore the differences that are recognised between the bacteria in *Bacillus* subgroup 1 are mostly encoded by genes that are carried upon plasmids (Vilas-Boas *et al*., 2002). Recent findings have shown that the plasmids that are traditionally used for the species classification in *Bacillus* subgroup 1 are commonly found throughout the group. Carlson *et al*. (1994) found that the *cry* genes which are responsible for the formation of the toxic crystal inside *B. thuringiensis* can also be found in strains of *B. cereus*. Gonzalez *et al*. (1982) has previously demonstrated that the plasmid carrying the *cry* genes could be transferred to *B. cereus*. The crystal inside *B. thuringiensis* has been used as one of the most important criteria for distinguishing between the two species. On the basis of these findings Carlson *et al*. (1994) concluded that *B. cereus* and *B. thuringiensis* belong to the same species. A recent paper has described the isolation of a *B. cereus* strain (G9241) that was responsible for an illness resembling inhalation anthrax (Hoffmaster *et al*., 2004). This strain was identified as *B. cereus* by 16S rRNA

analysis, motility, haemolytic activity and other tests. The G9241 strain contained a circular plasmid (pBCX01) with 99.6% similarity with the toxin-encoding virulence plasmid (pX01) of *B. anthracis*. Challenge of A/J mice confirmed the virulence of this strain. The genome sequence of the *B. cereus* strain ATCC 10987 revealed a large plasmid related to the *B. anthracis* virulence plasmid pX01. Comparison of the chromosomes demonstrated that ATCC 10987 was more similar to *B. anthracis* Ames than to *B. cereus* ATCC 14579 (Rasko *et al*., 2004). The isolation of these strains highlights the transferability of the *Bacillus* subgroup 1 plasmids and strengthens the case for the reclassification of the bacteria in this group into one species.

Numerous species of *Bacillus* have been linked with food poisoning outbreaks including *B. licheniformis, B. subtilis, B. pumilus* and *B. thuringiensis* (Rowan *et al*., 2001; Jackson *et al*., 1995). In addition many species of *Bacillus*, including *B. thuringiensis, B. circulans, B. laterosporus, B. lentus, B. mycoides, B. subtilis, B. amyloliquefaciens, B. lentimorbis, B. pasteurii, B. sphaericus, B. megatarium* and *B. weihenstephanensis*, have exhibited enterotoxigenic activity or enterotoxin genes using a range of assays (Beattie and Williams, 1999; Phelps and McKillip, 2002; Rowan *et al*., 2001; Stenfors *et al*., 2002). Due to the high degree of phylogenetic relatedness between members of the *Bacillus* genus it is not surprising that enterotoxin genes have been discovered in many strains of *Bacillus*.

Salkinoja-Salonen *et al*. (1999) have reported toxigenic strains of *B. licheniformis* that were implicated in cases of food poisoning from raw milk and commercial baby food. The *B. licheniformis* toxin was physiochemically similar to cereulide but differed in biological activity (as determined by the boar spermatozoa assay).

The objective of this chapter was to utilise the previously reported enterotoxin PCR protocols and the cereulide PCR developed in this study to determine the prevalence of *B. cereus* toxin genes in other species of *Bacillus*.

8.2 Materials and Methods

8.2.1 Strains used in the study

A range of *Bacillus* spp. were tested for enterotoxin genes using previously published primers and primers designed in this thesis. The strains used are outlined below in Table 8.1. Positive and negative control isolates of *B. cereus* were also included in the tests. Strain ATCC14579 was positive control for HBL, enterotoxin T and enterotoxin FM. Strain F4810/72 was positive control for enterotoxin FM and cereulide. The primers and their properties are outlined in Table 8.2

Table 8.1 *Bacillus* species and strains used in the study

Bacterial Species	Number of Strains	Designation
Bacillus amyloliquefaciens	2	T376, T377
Bacillus anthracis	1	BA1
Bacillus circulans	1	T2715
Brevibacillus laterosporus	1	T3269
Bacillus licheniformis	10	T18, T368, T369, T370, T371, T372, T373, T374, T375, BL1
Bacillus megaterium	1	T16
Bacillus pumilus	1	T17
Bacillus sphaericus	2	T378, T379
Bacillus subtilis	7	T11, T12, T13, T14, T15, T745, BS1
Bacillus thuringiensis	2	BT4100, BT1
Bacillus cereus	2	ATCC14579, F4810/72

Table 8.2 *Bacillus cereus* toxin primers

Target Gene	Primers	Primer Sequences	Product Size	Reference
hblA	hblA1 hblA2	5'-GCTAATGTAGTTTCACCTGTAGCAAC-3' 5'-AATCATGCCACTGCGTGGACATATAA-3'	874 bp	Mantynen and Lindstrom, 1998
bceT	bceT1 bceT2	5'-TTACATTACCAGGACGTGCTT-3' 5'-TGTTTGTGATTGTAATTCAGG-3'	428 bp	Agata *et al*., 1995
entFM	entFM1 entFM2	5'-ATGAAAAAAGTAATTTGCAGG-3' 5'-TTAGTATGCTTTTGTGTAACC-3'	1269 bp	Asano *et al*., 1997
NRPS	EMT1 CER1	5'-ATCATAAGGTGCGAACAAGA-3' 5'-AAGATCAACCGAATGCA CTG-3'	188 bp	Horwood *et al*., 2004

8.2.2 DNA extraction

Samples were prepared as outlined previously in Section 2.5.1.

8.2.3 Haemolysin BL PCR

PCR parameters were followed as outlined in Section 3.2.6.1.

8.2.4 Enterotoxin T PCR

PCR parameters were followed as outlined in Section 3.2.6.2.

8.2.5 Enterotoxin FM PCR

PCR parameters were followed as outlined in Section 3.2.6.3.

8.2.6 Cereulide PCR

PCR parameters were followed as outlined in Section 7.2.2.

8.3 Results

8.3.1 Haemolysin BL PCR results

A 874 bp DNA product was observed for one strain of *B. thuringiensis* (BT1) and the *B. cereus* positive control strain (ATCC14579) following amplification and gel electrophoresis (Figure 8.1).

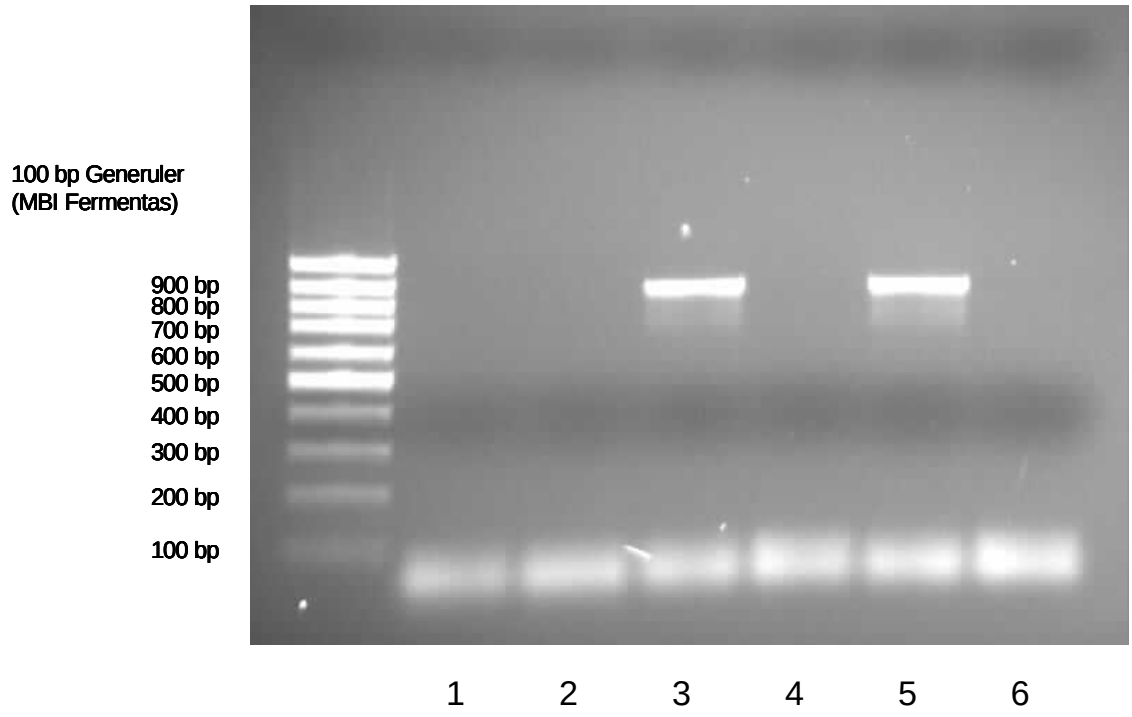

Figure 8.1 PCR to detect the *hblA* gene of haemolysin BL in strains of *Bacillus* spp. The amplification results were resolved by 1% agarose gel electrophoresis.

1: *Bacillus sphaericus* (T379)
2: *Bacillus subtilis* (T11)
3: *Bacillus thuringiensis* (BT1)
4: *Bacillus cereus* (F4810/72)
5: *Bacillus cereus* (ATCC14579)
6: No template control

8.3.2 Enterotoxin T PCR results

A 428 bp DNA product was observed for one strain of *B. thuringiensis* (BT1) and the *B. cereus* positive control strain (ATCC14579) following amplification and gel electrophoresis (Figure 8.2).

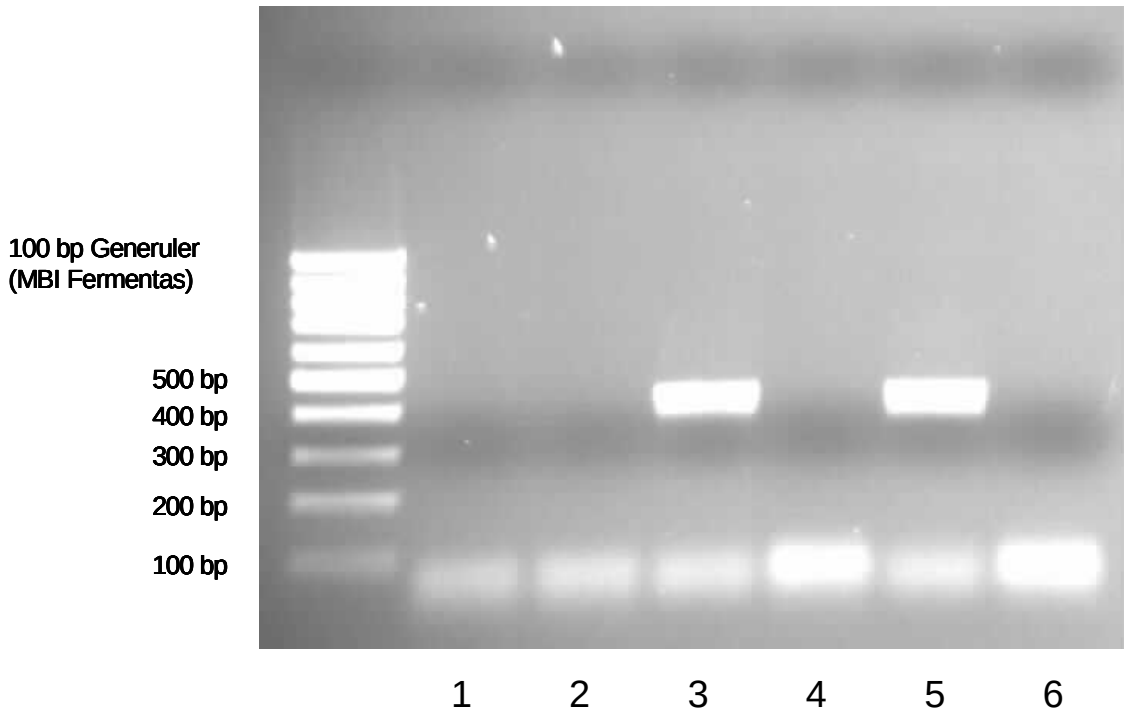

Figure 8.2 PCR to detect the *bceT* gene of enterotoxin T in strains of *Bacillus* spp. The amplification results were resolved by 1% agarose gel electrophoresis.

1: *Bacillus sphaericus* (T379)
2: *Bacillus subtilis* (T11)
3: *Bacillus thuringiensis* (BT1)
4: *Bacillus cereus* (F4810/72)
5: *Bacillus cereus* (ATCC14579)
6: No template control

8.3.3 Enterotoxin FM PCR results

A 1269 bp DNA product was observed for one strain of *B. thuringiensis* (BT1), one strain of *B. circulans* (2715) and the *B. cereus* positive control strains (ATCC14579 and F4810/72) following amplification and gel electrophoresis (Figure 8.3).

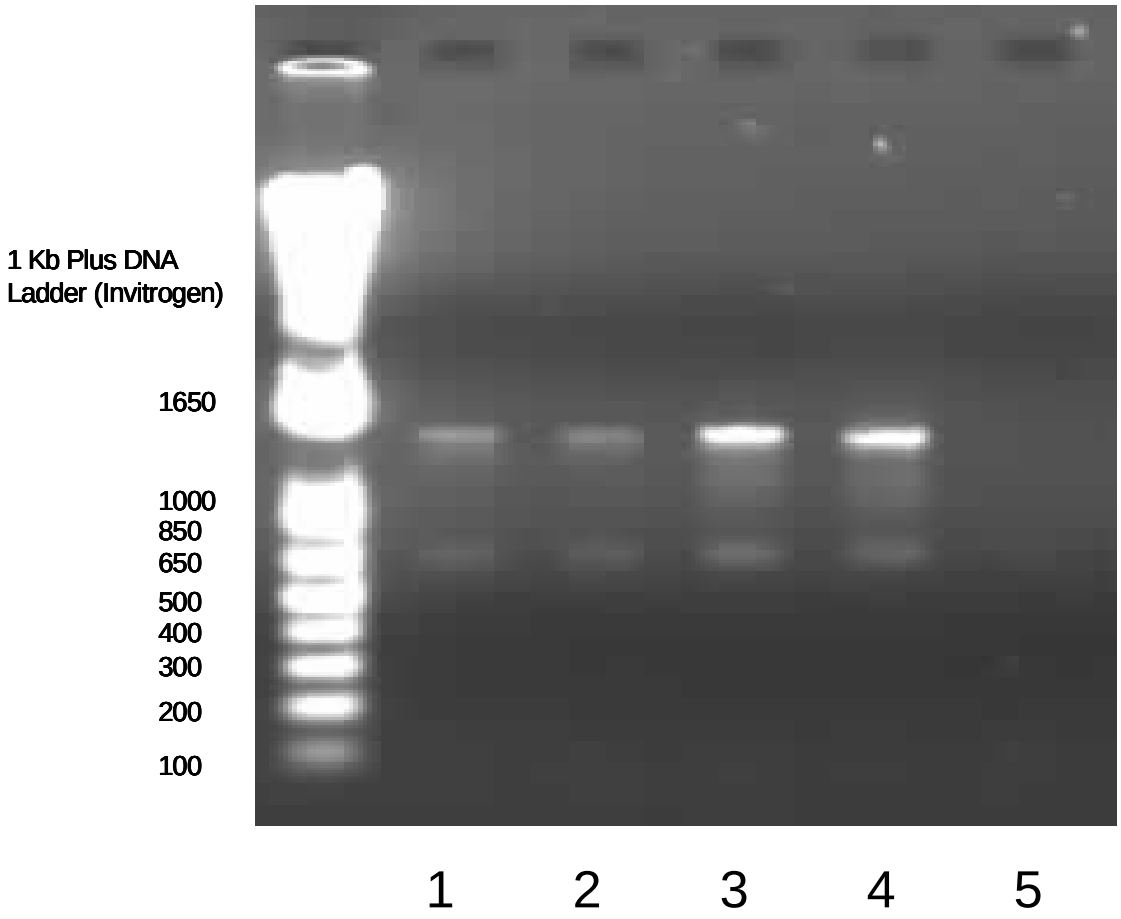

Figure 8.3 PCR to detect the *entFM* gene of enterotoxin FM in strains of *Bacillus* spp. The amplification results were resolved by 1% agarose gel electrophoresis.

1: *Bacillus thuringiensis* (BT1)

2: *Bacillus circulans* (T2715)

3: *Bacillus cereus* (ATCC14579)

4: *Bacillus cereus* (F4810/72)

5: No template control

8.3.4 Cereulide PCR results

A 188 bp DNA product was observed for one strain of *B. licheniformis* (BL1), one strain of *B. subtilis* (BS1) and the *B. cereus* positive control strain (F4810/72) following amplification and gel electrophoresis (Figure 8.4).

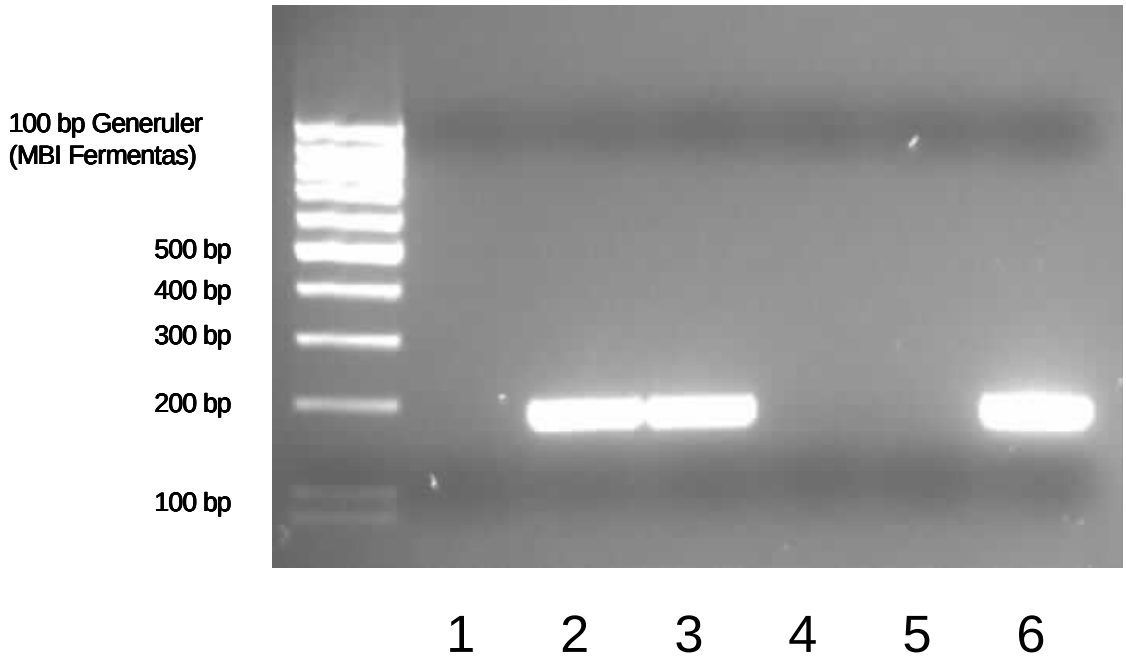

Figure 8.4 PCR to detect the NRPS genes associated with the production of cereulide in strains of *Bacillus* spp. The amplification results were resolved by 2% agarose gel electrophoresis.

1: *Bacillus circulans* (T2715)
2: *Bacillus licheniformis* (BL1)
3: *Bacillus subtilis* (BS1)
4: *Bacillus thuringiensis* (BT1)
5: *Bacillus cereus* (ATCC14579)
6: *Bacillus cereus* (F4810/72)

Table 8.3 Summary table of PCR results for toxin genes in *Bacillus* spp.

Bacterial species	Strain Designation	*hblA*	*bceT*	*entFM*	Cereulide
Bacillus amyloliquefaciens	T376	-	-	-	-
Bacillus amyloliquefaciens	T377	-	-	-	-
Bacillus anthracis	BA1	-	-	-	-
Bacillus circulans	T2715	-	-	+	-
Brevibacillus laterosporus	T3269	-	-	-	-
Bacillus licheniformis	T18	-	-	-	-
Bacillus licheniformis	T368	-	-	-	-
Bacillus licheniformis	T368	-	-	-	-
Bacillus licheniformis	T370	-	-	-	-
Bacillus licheniformis	T371	-	-	-	-
Bacillus licheniformis	T372	-	-	-	-
Bacillus licheniformis	T373	-	-	-	-
Bacillus licheniformis	T374	-	-	-	-
Bacillus licheniformis	T375	-	-	-	-
Bacillus licheniformis	BL1	-	-	-	+
Bacillus megaterium	T16	-	-	-	-
Bacillus pumilus	T17	-	-	-	-
Bacillus sphaericus	T378	-	-	-	-
Bacillus sphaericus	T379	-	-	-	-
Bacillus subtilis	T11	-	-	-	-
Bacillus subtilis	T12	-	-	-	-
Bacillus subtilis	T13	-	-	-	-
Bacillus subtilis	T14	-	-	-	-
Bacillus subtilis	T15	-	-	-	-
Bacillus subtilis	T745	-	-	-	-
Bacillus subtilis	BS1	-	-	-	+
Bacillus thuringiensis	BT4100	-	-	-	-
Bacillus thuringiensis	BT1	+	+	+	-
Bacillus cereus	ATCC14579	-	-	+	+
Bacillus cereus	F4810/72	+	+	+	-

8.4 Discussion

Enterotoxin genes traditionally found in *B. cereus* have previously been reported in other species of *Bacillus*. Yuan *et al*. (2002) found the enterotoxin genes *hblA* and *bceT* in all four of the *B. thuringiensis* strains they tested by PCR. Prub *et al*. (1999) found that 12 of 16 *B. mycoides* strains, 9 of 15 *B. weihenstephanensis* strains and all 8 *B. thuringiensis* strains tested contained the enterotoxin gene *hblA*. These reports correlate with the results found in this study where one strain (of two strains tested) of *B. thuringiensis* (BT1) contained all three of the enterotoxin genes that were targeted by PCR. The gene *entFM* was also found in a strain of *B. circulans* (2715). It is apparent that the *B. cereus* enterotoxin genes are widespread throughout the genus *Bacillus*.

Cereulide has not been previously reported in any other species of *Bacillus*. In this study a strain of *B. subtilis* (BS1) and a strain of *B. licheniformis* (BL1) produced a 188 bp DNA band following amplification with the cereulide PCR and subsequent gel electrophoresis. Targeting these strains with the primers developed by Turgay and Marahiel (1994) (used in Chapter 6) followed by cloning and sequencing would further determine the identity of the amplified sequences. Further assays would be required to determine if these strains produce cereulide. Its was planned that these isolates would be tested for cereulide production using the HEp-2/MTS assay, however unforseen technical problems occurred with the backup stocks of HEp-2 cells. The cell line was not able to be replaced.

The detection of toxin genes in other species of *Bacillus* is not necessarily indicative of their ability to induce food poisoning. Numerous strains of *B. licheniformis*, *B. pumilus*, *B. subtilis* and *B. thuringiensis* have been implicated in food poisoning cases, however the incidence is rare. Obviously other factors, besides containing toxin genes, are important for inducing food poisoning. Demonstrating the presence of toxin genes does not indicate whether the isolate is actually capable of producing the toxin in concentrations that are required to induce food poisoning. Although methods such as PCR are important for rapid identification of potentially toxigenic strains, assays that exhibit actual toxicity, such as the HEp-2/MTS assay, are required for confirmation.

This would give a clearer indication if the strains reported in this study are capable of inducing food poisoning illnesses similar to those caused by *B. cereus*.

It is evident that there is not a clear delineation between the respective species contained in *Bacillus* subgroup 1. The characteristics that are used to differentiate between the species in this group (i.e. the *cry* genes and the anthrax pathogenicity plasmids) have frequently been reported in the very species that they are supposed to differentiate. Consequently there have been suggestions that these species should be reclassified into one species. However, the classification of *Bacillus* subgroup 1 should probably be left in the current scheme due to the differing habitats and host preferences of the species in the group. If reclassified into one species concerns over inter-laboratory transfer of strains, the laboratory classification levels (i.e. PC2 to PC3) and the ability to continue the use of *B. thuringiensis* as a bio-pesticide would need to be addressed.

Chapter 9

General Discussion

9.1 Summary of Objectives

9.1.1 Objective 1: Evaluation of enterotoxin detection assays

It is evident from this study that the major obstacle inhibiting the development of an effective assay for the detection of diarrhoeal strains of *B. cereus* is the lack of a clear understanding of the factors that are necessary to confer virulence. None of the methods tested could be considered as a definitive method of detecting enterotoxigenic strains. Most methods were too specific (i.e. only detected a single toxin), and the remaining method, Vero cell cytotoxicity, was not specific enough (i.e. sensitive to too many compounds). The best method currently available for the detection of enterotoxigenic strains would be to use a combination of assays, such as PCR for screening followed by the Vero cell cytotoxicity assay. However, even using this approach, conclusive results cannot be obtained. Using the methods currently available it was impossible to determine the relative importance of the respective diarrhoeal toxins.

9.1.2 Objective 2: Development of a sensitive cell culture method for the detection of cereulide

The HEp-2/MTS cell cytotoxicity assay developed in this study is a sensitive assay, comparable in sensitivity to the boar spermatozoa bioassay (by valinomycin titration). The benefits of the HEp-2/MTS assay include the availability of the reagents, the simple interpretation of results, the reduction in subjectivity in the interpretation of results and the increased sensitivity (in comparison to the original HEp-2 method). This method is not suitable as a screening assay, due to the extended time needed for results. However, the method could be useful as a confirmatory assay following the cereulide PCR or another rapid detection method.

9.1.3 Objective 3: Characterisation of the genes associated with the synthesis of cereulide

The relationship between cereulide and other ionophores was investigated in the Antibiotic Sensitivity Experiments in Chapter 5. Although no conclusive results were generated, the review of the literature surrounding these compounds lead to the hypothesis that cereulide was related to other NRPS products. Through sequence analysis and the subsequent development of a PCR method, compelling evidence was generated to suggest that cereulide was also produced by peptide synthetases. The results of this study have lead to the first publication to provide evidence that cereulide is synthesised non-ribosomally (Horwood *et al*, 2004). This mode of production of cereulide is not particularly surprising considering the size, structure, substrate specificity and ionophoretic action of this peptide. The formation of the NRPS mode of production hypothesis, and the subsequent supporting evidence provided by the sequence homology and the cereulide PCR, were the major developments that this thesis contributed to the field of *B. cereus* biology.

9.1.4 Objective 4: Development of a rapid molecular detection method for emetic strains

The PCR method developed in this research successfully detected all of the emetic strains that were tested, including a strain of *B. cereus* (IH 41358) that is only detectable by the most sensitive cereulide detection assays (Andersson pers. comm., 2003). This PCR assay will allow food laboratories and health authorities to rapidly and sensitively detect emetic strains of *B. cereus*. The biotinylated probe and ELISA system provides another option for post-amplification analysis with the possibility of adaptation for automation.

9.2 Further Examination of Results and Recommendations for Future Research

9.2.1 The pathogenesis of the diarrhoeal syndrome and detection of enterotoxigenic strains

The research presented in Chapter 4 of this thesis indicates that the majority of *B. cereus* isolates are at least potentially capable of enterotoxigenicity. The PCR results showed that 91.2% of the isolates contained genes for at least one of the diarrhoeal toxins. The Vero cell cytotoxicity assay showed that 90.3% of these isolates (or a total 82.4% of isolates) produced enough toxin to be detectable by cell cytotoxicity. It is possible that the majority of *B. cereus* strains are capable of inducing the diarrhoeal disease and that the pathogenesis of this syndrome is simply reliant upon host susceptibility and the concentration of *B. cereus* in the contaminated food. If this is the case the best method for controlling diarrhoeal food poisoning will be to ensure that numbers of *B. cereus* are low in foods.

The majority of current enterotoxin detection assays are designed for the detection of only one of the five described diarrhoeal toxins. These assays include the gel diffusion assay, the two commercial ELISAs and PCR. The PCRs could conceivably be adapted to detect all of the enterotoxin genes in the one reaction by redesigning new primers to form a multiplex PCR. However, until a better understanding of the pathogenesis of *B. cereus* diarrhoeal food poisoning is gained, quality control screening of foods for the presence of enterotoxin genes using PCR is not feasible. Using the PCRs utilised in this study only six isolates did not contain at least one of the targeted enterotoxin genes. In addition, it should be noted that only three of the five reported diarrhoeal toxins were targeted by PCR. If adopted as a screening assay, quality control laboratories would therefore be required to further test >90% of *B. cereus* isolates for enterotoxin activity. This situation would be undesirable considering that *B. cereus* strains are very commonly isolated from the majority of foods. A multiplex PCR would provide no benefit to the food industry nor provide further insight into the pathogenesis of the diarrhoeal syndrome.

The pathogenesis of diarrhoeal food poisoning is still largely unknown. Many questions remain to be answered about the roles of the myriad of toxins that are produced by *B. cereus*. To date, five diarrhoeal toxins have been described, yet there is still not a clear understanding of the relative importance of each toxin. Until there is a more complete knowledge about the enterotoxins of *B. cereus* and their respective roles in producing the diarrhoeal syndrome of food poisoning, it is unlikely that a definitive enterotoxin detection assay will be developed. Although there is a dire need for more research to be conducted in this area, no further work was targeted at the diarrhoeal toxins in this study (except for testing other species of *Bacillus* with the enterotoxin PCRs). Funding commitments and an even greater need for clarity drove the research towards identifying the genes responsible for the production of cereulide and the subsequent development of a cereulide detection assay.

9.2.2 The genetic differences between emetic and non-emetic strains of *Bacillus cereus*

Emetic strains of *B. cereus* differ from non-emetic strains to a greater degree than would be considered normal within species, where the only grouping characteristic is the production/non-production of a toxin. Although, biochemical and physiochemical characteristics are indistinguishable (Jenson and Moir, 1997) there appear to be significant genetic differences that have not been thoroughly investigated.

None of the emetic strains tested carried the enterotoxin genes *hblA* nor *bceT*. These results are supported by the findings of Agata *et al*. (1996). In addition, all of the emetic strains tested in this study were unable to hydrolyse starch. This result has been noted by a number of researchers (Shinigawa *et al*., 1979; Nishikawa *et al*., 1996; Agata *et al*., 1996). Starch hydrolysis is mediated by β-amylases, which are commonly produced in the *Bacillus* genus (Mikami *et al*., 1999). The genetic basis for the consistent inability of emetic strains of *B. cereus* to hydrolyse starch or produce diarrhoeal toxins has not been determined. In all of the literature reviewed and communications with other researchers not a single emetic strain of *B. cereus* has been reported that produced HBL, enterotoxin T or β-amylase. The production of cereulide appears to preclude the isolate from the production of the other three compounds (Table 9.1).

Table 9.1 Table showing the inability of emetic strains to hydrolyse starch or produce HBL or enterotoxin T

Emetic strains	HEp-2/MTS assay	Cereulide PCR	Starch assay	HBL PCR	Enterotoxin T PCR
F47	+	+	-	-	-
F4426	+	+	-	-	-
F5881	+	+	-	-	-
NC7401	+	+	-	-	-
F4810/72	+	+	-	-	-
NCF	+	+	-	-	-
NCG	+	+	-	-	-
NCY	+	+	-	-	-
NC954	+	+	-	-	-
NC1044	+	+	-	-	-
NC1078	+	+	-	-	-
IH41385	-	+	-	-	-

Results compiled from Chapter 3, Chapter 4 and Chapter 7

Species of *Bacillus* that are further removed (taxonomically) than emetic strains of *B. cereus*, are commonly reported to hydrolyse starch, and contain the genes *hblA* and *bceT*. This indicates that there is another process, other than normal intraspecies differences, that is responsible for the observed differences between emetic and non-emetic strains of *B. cereus*. It is possible that the cereulide genes and the genes for HBL, enterotoxin T and β-amylase are carried on plasmids that cannot coexist inside the same *B. cereus* cell. Plasmids are commonly sorted into incompatibility groups as a form of classification. Plasmids of one incompatibility group are related to each other and usually share some sequence homology. The most common mechanism for this process is that similar plasmids with the same origin of replication cannot coexist in the same cell (Novick *et al*., 1987).

The plasmid hypothesis proposed here is mostly based upon circumstantial evidence. PCR analysis of *B. cereus* plasmids is required to determine if the genes for cereulide, haemolysin BL, enterotoxin T and β-amylase are carried upon plasmids or other transposable elements. It should be noted that strains of *B. cereus* have been reported to contain very large linear plasmids that are sometimes difficult to distinguish from chromosomal DNA (Beverley, 1988). Genome mapping has shown that the HBL genes are located on a variable region of the genome that is sometimes extrachromosomal. This region may be a large transposable plasmid (Carlson *et al*., 1996; Carlson and Kolstø, 1994).

Other mechanisms that could explain the genetic differences between emetic and non-emetic strains of *B. cereus* also need to be investigated. The majority of emetic strains are found in serotype H1 (Shinagawa *et al*., 1991; Mikami *et al*., 1994; Agata *et al*., 1996). This suggests that they are genetically removed from other strains of *B. cereus*. The mystery remains however, as to why species of *Bacillus* that are further removed in all other characteristics produce diarrhoeal toxins and hydrolyse starch yet emetic strains, without exception, do not. A further possibility is that emetic strains represent a clonal lineage that has recently evolved. It is believed that *B. anthracis* has evolved through a recent divergence due to such a mechanism (Henderson *et al*., 1994).

9.2.3 Non-ribosomal peptide synthetases and the potential applications

The complete mode of production of peptide synthetases is yet to be elucidated. The large size (<1.6 MDa) and complexity of peptide synthetases has made research into these enzymes and their genes particularly problematic. Research into these systems is important due to the possible production of a large array of novel compounds with medicinal and economic significance. The emergence of antibiotic resistance to the penicillins and other antibiotics has renewed interest towards the biosynthetic processes of these compounds. These production systems exhibit further potential because the biosynthetic pathway can be externally modified to produce novel compounds with a specific function (Symmank *et al*., 1999). The modular structure of peptide synthetases makes them suitable for rearranging or substituting modules. In the future this may lead to the rational design of novel antibiotics with a specific design and function (Moffitt

and Neilan, 2000). Although this application of peptide synthetases is only in its infancy the potential of this system is enormous. With the burgeoning need for a new generation of antibiotics to replace the overused and increasingly ineffectual antibiotics used today, peptide synthetases are likely to become the focus of increased scientific research.

The NRPS mode of production explains why *B. cereus* requires such a specific substrate before it can produce cereulide. Peptide synthetases incorporate amino acids from the substrate as the building blocks of the peptide product. Presumably, rice is one of the few food substrates that contains all of the necessary amino acids, in an accessible form, for incorporation into cereulide and is also a good growth medium for *B. cereus*. The manifestation of this is that fried or cooked rice has been implicated in approximately 95% of cases of *B. cereus* food poisoning with emetic symptoms (Jenson and Moir, 1997). Although most NRPS adenylation domains are extremely specific in their recognition and activation of substrates this is the first reported case where this is manifested by an extreme growth media specificity. The mechanism behind this is unknown however it could be due to NRPS gene regulation or a transport issue (Marahiel pers. comm., 2004).

Only a very small portion of the entire peptide synthetase genes for cereulide have been sequenced in this study. Experiments using techniques such as PCR gene walking need to be conducted to further characterise these genes. The biosynthetic genes of cereulide are likely to be very similar to NRPS genes found in other species of *Bacillus*. Further studies on the *B. licheniformis* (BL1) and *B. subtilis* (BS1) sequences amplified by the cereulide PCR need to be conducted to determine if they actually are the NRPS genes for cereulide. Comparative studies with the toxigenic strains of *B. licheniformis* isolated by Salkioja-Salonen *et al*. (1999) would also be interesting to determine if these strains produce a close analogue of cereulide.

Cereulide production has been suggested to be related to sporulation (Melling and Capel, 1978). Shinagawa *et al*. (1992) demonstrated that cereulide activity appeared only after spore formation in *B. cereus*. This relationship between sporulation and toxin production has previously been demonstrated in *Clostridium perfringens* (Labbe and Duncan, 1977). However, a recent paper by Finlay *et al*. (2002) suggested that cereulide

becomes detectable coincidentally with spore development, and that there is no direct association between the two processes. Starvation in other species of *Bacillus* has been linked to sporulation and the production of special metabolites such as tyrocidine, gramicidin S and surfactin (Marahiel *et al*., 1993). The relationship between cereulide and other peptide antibiotics supports the view that sporulation and the production of cereulide occur in parallel due to the onset of starvation and not due to a direct association between the two processes.

9.2.4 *Bacillus* spp. and the transfer of toxin genes

Debate currently exists over the classification of the *Bacillus* subgroup 1. Recent findings have shown that the plasmids that are traditionally used for the species classification in *Bacillus* subgroup 1 are commonly found throughout the group (Carlson *et al*., 1994; Hoffmaster *et al*., 2004) and therefore recommendations have been made that *B. anthracis, B. mycoides* and *B. thuringiensis* should all exist as subspecies of *B. cereus*. Although most of these arguments are purely academic, there is the possibility that *B. thuringiensis*, the world's most extensively used biopesticide, can acquire genes to allow it to produce anthrax and food poisoning toxins. The identification of a novel gene-swapping system that enables *B. thuringiensis* to exchange a wide variety of DNA with other *Bacillus* species make this an even more of a possibility (Anon., 1999). Recently, commercial preparations of *B. thuringiensis* have been identified that contain *B. cereus* toxin (HBL, enterotoxin T and non-haemolytic enterotoxin) genes and produce enterotoxins (according to commercial immunoassays). The true incidence of *B. thuringiensis* food poisoning is unknown because most medical laboratories do not discriminate between *B. cereus* and *B. thuringiensis* (Jensen *et al*., 2002).

Toxin genes from *B. cereus* have been found over a large number of species in the genus. The mechanism for the wide distribution of these genes is unknown. Species in *Bacillus* subgroup 1 are known to possess large virulence plasmids that are able to transfer to other closely related species of *Bacillus* through cell-to-cell exchange. Battisti *et al*. (1985) demonstrated that high levels of transfer of the tetracycline resistance plasmid pBC16 was achieved from *B. thuringiensis* to *B. cereus* and

B. anthracis in broth cultures. The transfer of plasmids in the *Bacillus* group 1 has also been experimentally observed in river water and insect larvae, although at a reduced rate compared to laboratory culture (Thomas *et al*., 2000; Thomas *et al*., 2001). Strains of *B. cereus* have also been reported that contain other mobile elements such as large insertion cassettes (Chen *et al*., 1999) and large linear plasmids (Carlson and Kolstø, 1994; Stromsten *et al*., 2003).

Beecher and Wong (2000) isolated a strain of *B. cereus* (MGBC 145) that contained two distinct homologous sets of genes for HBL. This may have arisen through horizontal transfer of these toxin genes. The genes for HBL have been mapped to a variable region of the *B. cereus* genome that is sometimes located on large extra-chromosomal DNA fragments that may be large mobile plasmids (Carlson *et al*., 1996; Carlson and Kolstø, 1994). Analysis of the genome surrounding the HBL genes revealed a 191 bp region that exhibited 78% identity to highly conserved transposase-encoding regions from two *B. thuringiensis* plasmids. This indicates that the HBL genes of ATCC14579 may have been involved in horizontal transfer processes (Okstad *et al*., 1999).

It is evident that the characteristics that are used for the differentiation of the members of *Bacillus* group 1 need to be investigated and criteria to differentiate the species (if they are indeed different species) need to be determined. In addition, the mechanism for the transfer of toxin genes throughout the *Bacillus* genus needs to be identified. Of particular concern is the potential threat of the biopesticide strains of *B. thuringiensis* acquiring virulence genes as the risk of these strains synthesising potential toxins in human foodstuffs could cause major problems to human health, and cause potential litigation against producers.

9.2.5 Implications for the prevention of *Bacillus cereus* food poisoning

It is impossible to effectively ensure that plant-based food products are free from soil bacteria such as *B. cereus*. However, using basic quality control procedures the incidence of food poisoning caused by this organism can be greatly reduced. The majority of cases occur where contaminated food has been prepared or stored in an

incorrect manner. Food should be washed free from soil prior to preparation and basic food hygiene practices should be employed by food handlers. Cooked food and processed salads and vegetables should be stored below 5°C or above 60°C. At these temperatures bacterial growth and toxin production are sufficiently reduced unless the food is stored for extended periods.

There are currently no assays available for the rapid identification of toxigenic strains of *B. cereus* for quality assurance purposes. In the case of the enterotoxigenic strains this will continue to be the case until further research has identified consistent characteristics that are common to pathogenic strains. The cereulide PCR developed in this study, however, will enable food laboratories to rapidly identify emetic strains from isolated *B. cereus*.

According to Food Standards Australia New Zealand (FSANZ) the satisfactory level of *B. cereus* and other pathogenic strains of *Bacillus* in food is <100 cfu/g (www.foodstandards.gov.au/). However, very few studies have been conducted to determine the prevalence of emetic strains of *B. cereus* in food products. The development of the cereulide PCR method will enable researchers to determine the prevalence of these strains and identify processing methods that are effective in reducing the numbers of these bacteria in food. Food surveys will enable regulatory authorities to decide the best course of action to reduce the incidence of emetic food poisoning i.e. quality control procedures such as PCR that are specific for emetic strains of *B. cereus* or improved food processing techniques that reduce bacterial numbers.

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Appendix 1.

Media Recipes

Brain Heart Infusion Agar (BHIA) with 1% w/v Soluble Starch

Dissolve 47 g of Brain Heart Infusion Agar (Oxoid) in 900 ml of distilled water
Adjust to pH 7.4±0.1
Sterilise by autoclaving at 121°C for 15 min
Allowing cooling to approx. 50°C in hot water bath

Dissolve 1 g of soluble starch (Sigma) in 100 ml of distilled water
Sterilise by syringe filtration (0.22 µl syringe filter - Millipore)
Add to warm BHIA to final concentration of 0.1% w/v of starch
Immediately pour (approx. 10 ml/plate) into agar plates
Store at 4°C until use

Brain Heart Infusion (BHI) Broth

Dissolve 37 g of Brain Heart Infusion (Oxoid) in1 L of distilled water
Adjust to pH 7.4±0.1
Mix well and distribute into final containers
Sterilise by autoclaving at 121°C for 15 min
Store at 4°C until use

Brain Heart Infusion with 0.1% Glucose (BHIG)

Dissolve 37 g of Brain Heart Infusion (Oxoid) in1 L of distilled water
Adjust to pH 7.4±0.1
Sterilise by autoclaving at 121°C for 15 min

Dissolve 100 mg of glucose (Sigma) in 10 ml of distilled water
Sterilise by syringe filtration (0.22 µl syringe filter - Millipore)
Add to BHI broth to final concentration of 0.1% w/v of glucose
Mix well and distribute into final containers
Store at 4°C until use

LB (Luria-Bertani) Agar with ampicillin/IPTG/X-Gal

Dissolve 40 g of LB agar (Difco) in 1 L of distilled water
Adjust to pH 7.0±0.1
Sterilise by autoclaving at 121°C for 15 min
Allowing cooling to approx. 50°C in hot water bath
Add ampicillin to a final concentration of 100 µg/ml
Add IPTG to a final concentration of 0.5 mM
Add X-Gal to a final concentration of 80 µg/ml
Mix well and pour (approx. 10ml/plate) into agar plates

IPTG stock solution

Dissolve 1.2 g of IPTG (Promega) in 50 ml of distilled water
Sterilise by syringe filtration (0.22 µl syringe filter - Millipore)
Store at 4°C until use

X-Gal stock solution

Dissolve 100 mg of X-Gal (Promega) in 2 ml of N,N'-dimethylformamide
Cover with aluminium foil and store at -20°C until use

Ampicillin stock solution

Dissolve 100 mg ampicillin (Sigma) in 1 ml of distilled water
Sterilise by syringe filtration (0.22 µl syringe filter - Millipore)
Store at 4°C until use

LB (Luria-Bertani) Broth with ampicillin

Dissolve 25 g of LB broth (Difco) in 1 L of distilled water
Adjust to pH 7.0±0.1
Sterilise by autoclaving at 121°C for 15 min
Allow cooling to room temperature
Add ampicillin to a final concentration of 100 µg/ml
Store at 4°C until use

Nutrient Agar (Slopes and Plates)

Dissolve 28 g of nutrient agar (Oxoid) in1 L of distilled water
Adjust to pH 7.4±0.1
Sterilise by autoclaving at 121°C for 15 min
Allowing cooling to approx. 50°C in hot water bath
Pour (approx. 10ml/plate) into agar plates, or
Pour (approx. 10ml/tube) into universal tubes and leave at angle of approx 45° until set
Store at 4°C until use

Nutrient Agar with 200 mM KCl

Dissolve 28 g of nutrient agar (Oxoid) in1 L of distilled water
Adjust to pH 7.4±0.1
Add KCL (Sigma) to a final concentration of 100 mM
Sterilise by autoclaving at 121°C for 15 min
Allowing cooling to approx. 50°C in hot water bath
Pour (approx. 10ml/plate) into agar plates
Store at 4°C until use

Skim Milk Agar

Dissolve 25 g of Skim Milk Medium (Difco) and 12 g Agar Technical (Oxoid) in1 L of distilled water
Sterilise by autoclaving at 121°C for 15 min
Allowing cooling to approx. 50°C in hot water bath
Pour (approx. 10ml/plate) into agar plates
Store at 4°C until use

Skim Milk Medium

Dissolve 25 g of Skim Milk Medium (Difco) in1 L of distilled water
Mix well and distribute into final containers
Sterilise by autoclaving at 121°C for 15 min
Store at 4°C until use

50x TAE Buffer

Measure approximately 900 ml pf distilled water
Add 242 g of Tris base (Sigma)
Add 57.1 ml of glacial acetic acid (Sigma)
Add 18.6 g of EDTA (Sigma)
Adjust volume to 1L with additional distilled water

TE Buffer

Measure 990 ml of distilled water
Add 100 ml of 1M Tris-HCl (pH 8.0)
Add 400 µl of 250 mM EDTA

Tryptone Soya Broth (TSB) with 10% Glycerol

Dissolve 30 g of Tryptone Soya Broth (Oxoid) in 900 ml of distilled water
Adjust to pH 7.3±0.1
Sterilise by autoclaving at 121°C for 15 min
Allowing cooling to room temperature (approx. 25°C)
Add 100 ml of sterile glycerol
Mix well and distribute into final containers
Store at 4°C until use

Appendix 2.

Absorbency Readings for Sensitivity

Determination of the HEp-2/MTS Assay

Using Valinomycin Dilutions

Absorbency readings (490 nm) to determine the sensitivity of the HEp-2/MTS assay

Well number	Concentration of valinomycin (ng/ml)	Valinomycin rep. no.1	Valinomycin rep. no.2	Valinomycin rep. no.3	DMSO control
1	200,000	0.245	0.291	0.244	1.179
2	100,000	0.276	0.547	0.482	1.445
3	50,000	0.248	0.440	0.466	1.436
4	25,000	0.285	0.409	0.417	1.431
5	12,500	0.289	0.404	0.464	1.444
6	6,250	0.321	0.478	0.513	1.451
7	3,125	0.387	0.518	0.511	1.463
8	1562.5	0.369	0.476	0.496	1.428
9	781.3	0.431	0.473	0.471	1.451
10	390.6	0.396	0.446	0.512	1.405
11	195.3	0.344	0.510	0.507	1.450
12	97.7	0.300	0.333	0.343	1.410
13	48.8	0.346	0.375	0.356	1.453
14	24.4	0.388	0.404	0.424	1.393
15	12.2	0.373	0.418	0.427	1.382
16	6.1	0.369	0.630	0.667	1.399
17	3.1	0.401	1.257	1.244	1.375
18	1.5	0.461	1.378	1.372	1.409
19	0.8	1.243	1.473	1.418	1.552
20	0.4	1.335	1.408	1.379	1.429
21	0.2	1.361	1.436	1.428	1.454
22	0.1	1.379	1.477	1.409	1.387
23	0.05	1.346	1.440	1.451	1.456
24	0.02	1.369	1.376	1.433	1.445

Appendix 3.

Publications from this project

Publications in refereed journals

Horwood, P.F., Burgess, G.W. & Oakey, H.J. 2004. Evidence for non-ribosomal peptide synthetase production of cereulide (the emetic toxin) in *Bacillus cereus*. *FEMS Microbiol. Lett.* 236: 319-324.

Publications in non-refereed journals

Horwood, P.F., Burgess, G.W. & Oakey, H.J. 2001. Development of improved molecular detection methods for *Bacillus cereus* toxin. *Ricegrowers Newsletter* 2001.

Horwood, P.F., Burgess, G.W. & Oakey, H.J. 2002. Detection of the food poisoning bacterium *Bacillus cereus* in rice. *Ricegrowers Newsletter* 2002.

Horwood, P.F., Burgess, G.W. & Oakey, H.J. 2003. Development of improved molecular detection methods for *Bacillus cereus* toxins. *Ricegrowers Newsletter* 2003.

Conference proceedings

Horwood, P.F., Burgess, G.W. & Oakey, H.J. 2002. Survey for toxigenic isolates of *Bacillus cereus* from North Queensland food, environmental and clinical sources. Presented to the Australian Society for Microbiology Symposium. Cairns, Queensland, 2000.

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