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JAMES COOK UNIVERSITY
AUSTRALIA

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

Thesis submitted by
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In July 2005

For the degree of Doctor of Philosophy in
Microbiology and Immunology
at James Cook University

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**P.F. Horwood**

July 2005

# Statement of Sources

### *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 Information derived from published or unpublished work of others has been acknowledged in the text and a list of references is given.

**P.F. Horwood**

July 2005

# 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<br>dATP = deoxyadenine triphosphate<br>dCTP = deoxycytosine triphosphate<br>dGTP = deoxyguanine triphosphate<br>dTTP = deoxythymine triphosphate<br>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 |
| w/v | weight/volume |
| XTT | sodium (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl])-2*H*-tetrazolium-5-carboxanilide |