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# Diversity and toxigenicity among members of the *Bacillus cereus* group

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ABSTRACT

Members of the *Bacillus cereus* group were isolated from rice products by centrifugation-plating and conventional spread-plating methods. Random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) results showed broad diversity among the strains and revealed some associations among isolates from raw and cooked rice samples, at the genotypic level. A comparatively greater diversity among strains was observed in isolates from raw rice than those from cooked rice and, generally, the RAPD profiles of isolates from raw and cooked rice were different, with only a few of them common to both types of rice. The toxigenic potential of the isolates was also determined by molecular and immunoassay analyses. The results revealed that most isolates from the *B. cereus* group were potentially or actually toxigenic, and some isolates could produce both diarrhoeal and emetic toxins. Generally, isolates belonging to the *B. cereus* group with the same RAPD pattern were shown to have a similar profile of enterotoxigenicity.



## 1. Introduction

Members of the genus *Bacillus* encompass a great diversity of bacterial species and are widespread in environment due to their ubiquitous nature. Of the genus *Bacillus*, the *Bacillus cereus* group has been widely recognised as causative agents of food-borne illness. A well-known member of the group, *B. cereus* is a food poisoning bacterium causing both emetic and diarrhoeal disease. Fatality as a result of intoxication from emetic-type *B. cereus* food poisoning has been recorded (Mahler et al., 1997) and severe forms of the diarrhoeal syndrome have been reported, including a necrotic enteritis causing three deaths (Lund et al., 2000). Recently, *Bacillus thuringensis* strains in this group have also been associated to outbreaks of foodborne poisoning (Beattie and Williams, 1999; Jackson et al., 1995).The *B. cereus* group includes members that demonstrate a wide diversity with respect to their physiology and ecological niche to DNA sequence and gene regulation (Schoeni and Wong, 2005). Therefore, typing techniques that have good discrimination power are needed for epidemiological investigation of outbreaks of foodborne disease involving microbial pathogens, including species in *B. cereus* group, or tracking of environmental sources of contamination for management of food safety and quality. The most common genotypic typing methods used and applied to isolates of *Bacillus* species include chromosomal DNA restriction analysis, plasmid typing, ribotyping, pulsed-field gel electrophoresis (PFGE) and PCR-based methods such as randomly amplified polymorphic DNA (RAPD) analysis. Recently, *B. cereus* groups were analysed by fluorescent amplified fragment length polymorphism (fAFLP) to characterise ecological diversification and a combination of multilocus sequence typing (MLST), AFLP, and multi-locus enzyme electrophoresis (MLEE) genotyping was employed to develop the phylogenetic perspective. Of these molecular typing methods, the randomly amplified polymorphic DNA (RAPD) PCR based method has been successfully applied to the differentiation, characterisation and identification of *Bacillus* species including *B. cereus*, *B. thuringiensis* and *Bacillus mycoides* (Brousseau et al., 1993; Choo et al., 2007; Daffonchio et al., 1999; Hansen et al., 1998; Kwon et al., 2009; Ronimus et al., 2003; Sorokulova et al., 2003; Thorsen et al., 2010; Woodburn et al., 1995; Zhang et al., 2002).

Further, while assessing genetic diversity has value as an ecological study, it is more valuable to assess the potential for and production of toxins, with respect to food safety. Also, the *Bacillus* species of most interest such as *B. cereus* tend to be known for their pathogenicity and are closely linked genetically (Schoeni and Wong, 2005). Traditionally, detection of *B. cereus* toxins has relied upon in vivo testing procedures (Jackson, 1993), however, these methods are time-consuming and labour intensive to perform. Thus, immunological assays were developed for the detection of enterotoxin(s) and two kits are available commercially which are the *B. cereus* enterotoxin-reversed passive latex agglutination (BCET-RPLA) test (Oxoid) and the sandwich enzyme-linked immunosorbent assay (ELISA) or *Bacillus* diarrhoeal enterotoxin visual immunoassay (BDE-VIA) test

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(3M). Although immunoassays exist for the detection of diarrhoeal toxins, development of an immunoassay for emetic toxin, cereulide, was considered difficult due to its low antigenicity (Mikami et al., 1994). However, Tecra International (3M) is developing such an assay, with a prototype currently available. An approach for assessing the potential toxigenicity of *Bacillus* strains is the detection of specific, unique DNA sequences of genes of enterotoxic and emetic toxins. PCR-based methods have been developed for detection of the genes encoding diarrhoeal toxins (Asano et al., 1997; Granum et al., 1996; Granum et al., 1999; Yuan et al., 2002), while two studies (Ehling-Schulz et al., 2004; Toh et al., 2004) have reported PCR-based detection of genes associated with cereulide production.

This study reports the diversity of strains of the *B. cereus* complex, isolated from raw and cooked rice samples using centrifugation-plating and conventional spread-plating methods, as well as their potential and actual toxigenicity, determined using molecular and immunological methods. This includes use of a PCR method for detection of a gene associated with cereulide production as well as a prototype ELISA for detection of cereulide.

## 2. Materials and methods

### 2.1. Reference Bacillus strains

The cultures used in this study were emetic strains of *B. cereus* NC 7401 and NC Y (Norio Agata, Nagoya city Public Health Research Institute, Nagoya, Japan), diarrheoal strains of *B. cereus* TICC 1994, TICC 1996 and 1997 (Culture Collection, Tecra International (TICC), Sydney, Australia), *B. thuringiensis* FDA 3, FDA 4, FDA 7, FDA 11 and FDA 12 (= ATCC10792) (supplied by Reginald Bennett, US FDA), and *B. mycoides* FS (School of Biotechnology and Biomolecular Sciences Culture Collection, University of New South Wales, Sydney, Australia).

### 2.2. Isolation of Bacillus species from rice products

#### 2.2.1. Rice samples

All raw and most cooked rice samples were purchased from several vendors to obtain a range of representative brands. Briefly, a variety of raw rice products including long grain rice, medium grain rice, wild rice, glutinous rice, sushi rice, Arborio rice, and flavour-added rice were purchased from 8 retail outlets in Australia. Cooked rice samples included Italian, Chinese, Indian, Japanese, and Korean foods purchased from 5 different restaurants, 10 take-away shops, and 1 retail outlet. Some of the cooked rice samples were prepared in the laboratory by cooking raw rice in a commercially available rice cooker. Raw rice was stored at ambient temperature until use, and cooked rice was kept at 4 °C for no longer than 4 h prior to analysis.

#### 2.2.2. Detection of Bacillus strains

*Bacillus* strains were isolated, using centrifugation-plating and conventional spread-plating methods, from 70 samples of raw and cooked rice products, purchased from several retail outlets and restaurants in Australia. The centrifugation-plating method was used according to Oh and Cox (2010). Briefly, the raw rice sample (10 g) was transferred into a sterile Stomacher bag and was mixed with 80 mL of sterile Buffered Peptone Water (BPW; CM0509B, Oxoid, Hampshire, England) and 10 mL of a 10% Tween 80 (Sigma, Sydney, Australia) in distilled water to facilitate dispersion of the food homogenates by Tween 80. For cooked rice, sample (10 g) was mixed with 80 mL of BPW, 1% enzyme (α-amylase, Sigma-Aldrich Fine chemicals, Castle Hill, NSW, Australia), and 0.1% Tween 80 in a sterile Stomacher bag with an internal mesh filter (K & R technologies, Frederick, USA), used to separate the cooked rice material from the liquid suspension. The mixture was then homogenised by stomaching for 2 min and the homogenate was centrifuged at 15,000×*g* for 12 min at 4 °C using a Beckman model J2-MC centrifuge (Global Medical Instrumentation, Inc., Minnesota, USA). The supernatant was carefully removed and discarded, then the pelleted material was resuspended in 1 mL of sterile BPW by vortex mixing. The entire suspended material was spread-inoculated over the surface of a large (150×20 mm) plate of Polymyxin Egg-yolk Mannitol Bromothymol blue Agar (PEMBA) (CM0617B, Oxoid). The plate was incubated aerobically for 48 h at 30 °C. Confirmation as *Bacillus* was determined according to phenotypic characteristics, namely colony morphology on the plate and determination of rod shaped cells with an endospore, using phase-contrast microscopy (Olympus, Olympus Australia Pty. Ltd, NSW, Australia). Several presumptive colonies of each morphology type were selected from each PEMBA plate. Colonies representative of a pure culture were identified by 16S rDNA sequencing, PCR detection of *gyr*B, and API systems as described below.

### 2.3. Identification and confirmation of Bacillus species

#### 2.3.1. DNA isolation

Cultures of *Bacillus* strains were grown in Tryptone Soya Broth (CM0129B, Oxoid) for 20 h at 30 °C, and a 1 mL aliquot was pelleted by centrifugation (5000×*g* for 10 min) using a Microfuge® 22R Centrifuge (Beckman Coulter™, Fullerton, CA, USA). DNA was isolated using the DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol.

#### 2.3.2. Identification of Bacillus species by 16S rDNA sequencing

PCR amplification was carried out as described by Lagacé et al. (2004) using the universal primers F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) (Chèneby et al., 2000) and R1492 (5′-TACGGYTACCTTGTTACGACTT-3′) (Wang and Wang, 1996). Sequencing reactions were prepared using PCR products and the ABI PRISM® BigDye™ Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, Forster City, CA, USA), then submitted to the Automated DNA Analysis Facility in the School of Biotechnology and Biomolecular Sciences (UNSW) to generate sequence data. The NCBI-BLAST (Basic Local Alignment Search Tool) programme was used to conduct genetic similarity searches against the GenBank sequence database following generation of sequencing data.

#### 2.3.3. Differentiation of B. cereus from B. thuringiensis

PCR amplification of the gyrase B (*gyr*B) gene of *B. cereus* was carried out as described by Yamada et al. (1999). The PCR primers used for differentiation of *B. cereus* from *B. thuringiensis* were BC1 (5′-ATTGGTGACACCGATCAAACA-3′) and BC2r (5′-TCATACGTATGGATGT-TATTC-3′). The amplification of *B. cereus*-specific fragments (365 bp) was performed using 30 cycles, each consisting of 60 s at 94 °C, 90 s at 58 °C, and 150 s at 72 °C, with a final extension step at 72 °C for 7 min.

#### 2.3.4. Biochemical identification of Bacillus species with API systems

The API 50CHB system (bioMérieux, Australia), combined with the API 20E kit (bioMérieux, Australia), were used for identification of *Bacillus* species according to the manufacturer's instructions. The results were analysed with the ATB Plus (bioMérieux) computer programme to identify isolates to species level.

### 2.4. Typing of isolated Bacillus strains by RAPD-PCR

#### 2.4.1. DNA extraction

A simplified DNA extraction method from pure cultures was an adaptation of the method of Ronimus et al. (2003). *Bacillus* isolates from rice samples were streaked on TSA plates and incubated at 30 °C for 20–24 h. Two or three well-isolated colonies were taken using a sterile disposable loop and suspended in 1.0 mL of 50 mM Tris–HCl, 100 mM Nacl, 20 mM EDTA, pH 8.0. This cell suspension was centrifuged at 4000×*g* for 10 min at ambient temperature and the pellet was resuspended in 0.1–1 mL of Milli-Q water (Millipore,