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Heat-stable toxin production by strains of *Bacillus cereus*, *Bacillus firmus*, *Bacillus megaterium*, *Bacillus simplex* and *Bacillus licheniformis*

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Abstract

Strains of *Bacillus cereus* can produce a heat-stable toxin (cereulide). In this study, 101 *Bacillus* strains representing 7 *Bacillus* species were tested for production of heat-stable toxins. Strains of *B. megaterium, B. firmus* and *B. simplex* were found to produce novel heat-stable toxins, which showed varying levels of toxicity. *B. cereus* strains (18 out of 54) were positive for toxin production. Thirteen were of serovar H1, and it was of interest that some were of clinical origin. Two were of serovars 17B and 20, which are not usually implicated in the emetic syndrome. Partial purification of the novel *B. megaterium, B. simplex* and *B. firmus* toxins showed they had similar physical characteristics to the *B. cereus* emetic toxin, cereulide.

Keywords: Heat stable toxins; Emetic food poisoning; *Bacillus*

1. Introduction

Bacillus cereus is the aetiological agent of two distinct types of food poisoning. One is the diarrhoeal syndrome, which is characterised by abdominal pain with diarrhoea and lasts for 12–24 h. Four different heat-labile enterotoxins have been implicated in the diarrhoeal syndrome and have been described from various strains: two protein complexes, haemolysin BL (HBL) and non-haemolytic enterotoxin (NHE) [1,2] and two single-gene products encoded by *ent*FM and *cyt*K [3].

The second kind of illness, the emetic syndrome, is characterised by nausea and vomiting occurring 1–5 h after ingestion of, predominantly, rice dishes [4] and is caused by a heat-stable dodecadepsipeptide. This emetic toxin, named cereulide [5], is produced during bacterial growth in contaminated foods and, being heat-stable, survives the cooking process to cause intoxication. The emetic syndrome is potentially more dangerous than the diarrhoeal syndrome, as fulminant liver failure, rhabdomyelitis and renal damage have been associated with its ingestion [6]. The emetic toxin was found to induce vacuolation [7] in Hep-2 cells, and subsequent examination of emetic toxin-treated Hep-2 cells by electron microscopy led to the conclusion that the vacuoles were swollen mitochondria [8]. A semi-automated tissue

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culture-based assay to detect the emetic toxin, utilising the cell viability indicator 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was described in 1999 [9]. This assay was used to identify heat-stable toxin producing strains in the present study.

It has already been shown that strains of *B. licheniformis* can produce a heat-stable toxin, and that this toxin is non-vacuolating [10]. The present study investigated the incidence of heat-stable toxin production in *B. cereus* strains that were of food, clinical and environmental origins and in strains of other *Bacillus* species that had not been examined previously.

2. Materials and methods

2.1. Bacillus *strains*

A total of 101 *Bacillus* strains representing seven *Bacillus* species were tested in triplicate for the production of heat-stable toxin(s) (Table 1). Strains were isolated from various sources including foods, clinical and environmental specimens and all were taken from the Logan *Bacillus* Collection in the Department of Biological and Biomedical Sciences, Glasgow Caledonian University.

Strain *B. cereus* F4810/72 (serotype 1, from vomit) which has been used for emetic toxin production [9,11] was used as the standard positive control. Strain *B. cereus* F4433/73 (serotype 2, from meatloaf) which did not produce emesis in monkeys [12] was used as the standard negative control. All of the strains examined were identified to species level by morphological and biochemical characteristics using the API 50 CHB gallery and API 20 E strip [13]. Single colonies were selected from pure cultures grown overnight at 30 °C on tryptone soya agar (TSA) (Oxoid, UK). All strains were maintained on TSA slopes supplemented with manganese sulphate (Sigma, UK) at 5 μg/ml to encourage sporulation.

2.2. *Toxicity testing*

Heat-stable toxin production was tested for by growing each strain, in triplicate, in 10% skim milk medium (SMM) (Oxoid, UK), [9,11] Serial logarithmic dilutions of heat-treated (autoclaved) supernatant fluids from SMM cultures were added to a tissue culture based toxicity assay in triplicate following the method of Finlay et al. [9]. The appearance of vacuolation in Hep-2 cells [7] in the toxicity assay was also monitored at regular intervals for up to 40 h using an Olympus CK2 inverted microscope (Olympus Optical Ltd, London, UK).

2.3. *Partial purification of heat-stable toxins*

Strains found to produce heat-stable toxin(s) were cultured in 100 ml of 10% SMM and the toxin partially purified following the method of Finlay et al. [14]. In brief, after centrifugation and autoclaving of the culture, the crude toxins present in the culture supernatants were partially purified using ammonium sulphate precipitation by adding an equal volume of 100% $(NH_4)_2SO_4$ solution to give a final concentration of 50% saturation. The resultant solution was held at 4 °C for 1 h, then centrifuged at 5000*g* for 30 min at 4 °C and the supernatant discarded. The pellet was then resuspended in 100% methanol by shaking at 30 °C until homogeneously dispersed. This suspension was then further centrifuged to deposit non-toxic, methanol-insoluble material and the pellet was discarded. Ten millillitre of the $(NH_4)_2SO_4$ supernatant was made 60:40 (v/v) in methanol:water and was applied to a column containing C18 SepPak reverse phase medium (Waters, UK). The column was washed with 2 bed volumes of 60:40 (v/v) methanol:water followed by 2.5 bed volumes of 80:20 (v/v) methanol:water to remove contaminants. The partially purified toxin was then eluted in 50 ml of 100% methanol. The column was washed further with 50 ml of 100% methanol and was regenerated with 2 bed volumes of 60:40 (v/v) methanol:water. All fractions from the column were tested for toxicity after replacing the methanol with phosphate buffered saline (PBS) (Oxoid, UK) by adding 1 ml of sterile PBS to 1 ml of the column fraction and reducing to a final volume of 1 ml by boiling on a hot plate.

Table 1
Mean reciprocal toxin titre of strains of *Bacillus* species tested for production of heat-stable toxin

Bacillus sp.[a]	Strain number	Serovar	Source	Mean reciprocal toxin titre
B. cereus	F4810/72 Positive control	1	Vomit, Melling	1024
B. cereus	F 4433/73 Negative control	1	Meat loaf	0
B. megaterium	F 98/3079		Blood culture pyrexia. Fibroid degeneration	128
B. simplex (received as *B. megaterium)*	95/1875	–	Sputum, cystic fibrosis patient	512
B. firmus	ATCC 14575^T	–	Type strain	512
B. firmus	ATCC 8247	–	Bredemann A strain, DSM	256
B. licheniformis	F 99/1105	–	Vomit	1024

[a] A further 17 of 52 strains of *B. cereus* tested positive for heat-stable toxin. The total number of other strains tested was *B. cereus* (54), *B. licheniformis* (23), *B. megaterium* (5), *B. firmus* (15) and *B. simplex* (1).