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FEMS Microbiology Letters 242 (2005) 313–317

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

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

Janice M.W. Taylor, Alastair D. Sutherland *, Kofi E. Aidoo, Niall A. Logan

Department of Biological and Biomedical Sciences, School of Life Sciences, Glasgow Caledonian University, Cowcaddens Road, Glasgow, Scotland G4 0BA, UK

Received 4 June 2004; received in revised form 11 August 2004; accepted 10 December 2004

First published online 24 November 2004

Edited by T.H. Birkbeck

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

* Corresponding author. Tel.: +44 141 331 3207; fax: +44 141 331 3208.

E-mail address: a.sutherland@gcal.ac.uk (A.D. Sutherland).

0378-1097/$22.00
doi:10.1016/j.femsle.2004.11.022

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).

2.4. PCR for B. cereus emetic toxin associated genes

DNA from strains found to produce heat stable toxin (plus *B. cereus* F4810/72 and F4433/73 as positive and negative emetic toxin producing controls, respectively) was prepared by the method of Pitcher et al. [15] with additional pre-incubation of bacteria with lysozyme (50 mg/ml final concentration) at 37 °C for 1 h. Samples were then tested for their reaction with PCR primers (EM1F and EM1R), which detect a putative DNA fragment associated with emetic strains of *B. cereus*. Primers and reaction conditions were as described in [16].

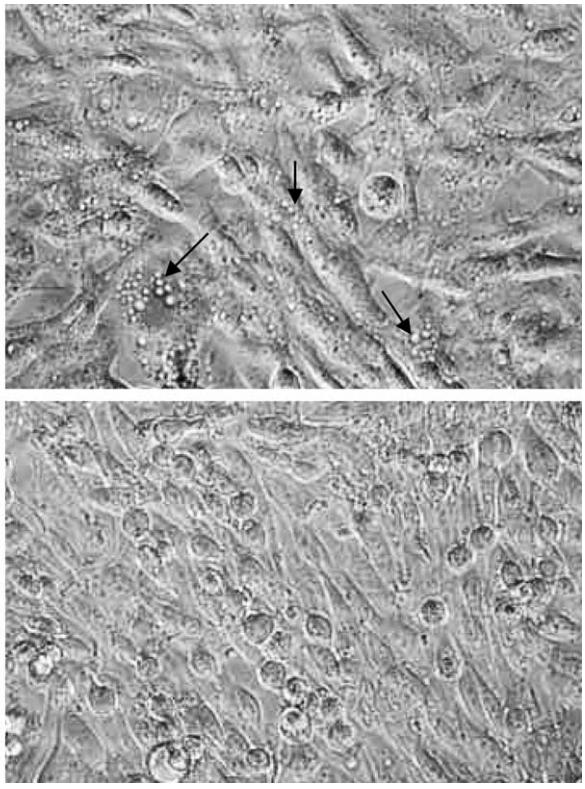

Fig. 1. (a) Hep-2 cells treated with *B. firmus* (ATCC 14575^T) culture supernatant at 1/8 dilution, 20 h after treatment. Arrows indicate vacuolation of cells (200× magnification). (b) Hep-2 cells control with no vacuolation present, 20 h after treatment (200× magnification).

3. Results

3.1. Screening of Bacillus strains for heat-stable toxin production

Table 1 shows the mean reciprocal toxin end-point titre [9] of *Bacillus* strains which were tested for heat-stable toxin production.

3.2. Vacuolation results

Two (of 15) strains of *B. firmus,* one (of one) *B. simplex.* one (of five) *B. megaterium* and one (of 23) strains of *B. licheniformis* were found to produce a heat-stable toxin (Table 1). The *B. firmus* and *B. simplex* toxigenic strains were also observed to cause vacuolation in Hep-2 cells. The toxic strains of *B. megaterium* and *B. licheniformis*, however, did not cause vacuolation.

Fig. 1 shows an example of vacuolation responses in Hep-2 cells after treatment with autoclaved supernatant of *B. firmus* (ATCC 14575^T), (Fig. 1a) compared to a control treated with tissue culture medium only (Fig. 1b).

3.3. Partial purification and characterisation of toxins

Fractions of partially purified toxins from *B. cereus* strain F4810/72; *B. firmus* strains ATCC 14575^T and ATCC 8247, *B. simplex* F 95/1875 and *B. megaterium* strain F 98/3079 were tested at various stages in the purification process. Results (Table 2) showed that the purification profiles obtained for these strains were comparable to the purification characteristics obtained with the standard emetic toxin producing strain F4810/72.

3.4. PCR for a B. cereus emetic toxin associated DNA fragment

None of the strains of *B. firmus*, *B. simplex*, *B. megaterium* or *B. licheniformis* which produce a heat-stable toxin (Table 1) were found to react in the PCR. The *B. cereus* strains F4810/72 and F4433/73 were, respectively, positive and negative emetic toxin producing

Table 2
Toxin titres of fractions recovered during partial purification of heat-stable toxins from *B. cereus*, *B. firmus*, *B. simplex* and *B. megaterium* strains (% recovery in parenthesis)

Mean reciprocal toxin titre				
Species	Strain	Culture supernatant (crude toxin)	$(NH_4)_2SO_4$ pellet resuspended in 100% methanol	Toxin eluted from Sep Pak C18 in 100% methanol
B. cereus	F4810/72 (positive control)	1024 (100%)	1024 (100%)	16 (1.56%)
B. firmus	ATCC 14575^T	512 (100%)	256 (50%)	32 (6.25%)
B. firmus	ATCC 8247	256 (100%)	128 (50%)	16 (6.25%)
B. simplex	F 95/1875	512 (100%)	256 (50%)	32 (6.25%)
B. megaterium	F 98/3079	128 (100%)	64 (50%)	8 (6.25%)

control strains in the PCR. Only F4810/72 gave the expected 635 bp PCR fragment (results not shown).

4. Discussion

Of the 101 strains of *Bacillus* species tested, the majority of those positive for heat-stable toxin production were *B. cereus* serotype H1. It is worthy of note that whilst about 95–98% of *B. cereus* strains contain NHE enterotoxin genes [17] emetic strains are less common, representing only 33% of *B. cereus* strains tested here, which were from diverse sources. It is assumed that the heat-stable toxin produced by *B. cereus* strains is the emetic toxin since this is the only heat-stable toxin reported from this species. *B. cereus* serovar H1 is also the predominant serovar implicated in the emetic food poisoning syndrome [18,19]. The majority of the serovar H1 strains examined in this study (where history was available) were isolated from food poisoning incidents where vomiting was reported. Two of the toxin-positive *B. cereus* strains were of serotypes H3 and H8. These serovars have also been reported as sources of emetic-type food poisoning previously [20].

However, some of the *B. cereus* strains found to produce heat-stable toxin in the present study were of serovars which are not usually implicated in the emetic syndrome, for instance serovars H17B (F 98/5462, titre 2048) and H20 (F 78/660, titre 2048) and these strains were not isolated from food poisoning incidents. Strain F98/5462 is from a sputum sample of a patient suffering from cystic fibrosis. Strain F78/660 was obtained from a patient where cellulitis had developed from facial burns. However, due to the limited histories provided with the strains it would be unreasonable to implicate *B. cereus* with the symptoms reported. It would be of interest, however, to determine further the incidence of emetic toxin producers in clinical isolates of *B. cereus*.

One of the 23 *B. licheniformis* strains tested was found to give a high toxin titre (F 99/1105, titre 1024). Toxigenic strains of *B. licheniformis* have been previously identified in relation to food poisoning incidents. This toxin has only been partially characterised, and though it is similar in physical characteristics to cereulide, it does not cause vacuolation of mitochondria [10]. Similarly, in this study vacuolation by culture supernatants was not demonstrated by the *B. licheniformis* strain found to show toxicity in the MTT dye assay. This toxin should be purified and further characterised.

Two strains of *B. firmus* (ATCC 14575^T and ATCC 8247) tested positive for toxin production (titres 512 and 256 respectively). Also, strain F 95/1875, which was initially provided as *B. megaterium*, gave a positive result for heat-stable toxin production (titre of 512). However, further analysis in this laboratory using API tests determined that this strain was *B. simplex*. The culture supernatant from these strains (ATCC 14575^T, ATCC 8247 and F 95/1875) caused vacuolation in Hep-2 cells. This is a characteristic of the emetic toxin suggesting that the *B. firmus*, and *B. simplex* heat-stable toxins are closely related. Partial purification of toxins from strains ATCC 14575^T, ATCC 8247, and F 95/1875 also resulted in similar physical characteristics to the emetic toxin. These novel heat-stable toxins were precipitable in $(NH_4)_2SO_4$, methanol-soluble, and highly hydrophobic, binding to a C18 reverse phase column and only being eluted in the 100% methanol wash fraction. There was a substantial loss of activity in all toxin preparations during C18 purification. This has been noted before during cereulide purification studies and it has been concluded that cereulide (and now similar toxins) is labile when semi-pure in methanol, especially when exposed to light (Sutherland, A.D., personal communication). The purification results suggests that strains of *B. firmus* and *B. simplex* are capable of producing a heat-stable toxin with physical characterisitics very similar to those of the emetic toxin which has been shown to be a highly hydrophobic dodecadepsipeptide [5].

One of five strains of *B. megaterium* was also shown to produce a heat-stable toxin (F 98/3079, titre 128) that gave a similar purification profile but was, however, non-vacuolating. This toxin should also be further characterised. There are no reports in the literature to date of heat-stable toxin-producing strains of *B. firmus*, *B. simplex* or *B. megaterium* or of food-borne illness caused by these strains.

These results suggest that the assay of Finlay et al., [9] may not be specific for *B. cereus* emetic toxin unless the isolate has been carefully speciated beforehand since it is now shown that heat-stable toxins from other *Bacillus* species may also be detected.

It can be speculated, based on the cyclic structure and presence of D and L forms of amino acids [5] that the emetic toxin, cereulide, at least, is non-ribosomally synthesised by a peptide synthetase complex. A recent publication [16] would support this in that PCR primers specific to emetic toxin strains have been deduced from peptide synthetase gene sequences. It should be noted however, that the specific fragment generated by this PCR shows no specific homology to NRPS gene sequences and in fact no homology to any current database sequence. The novel toxic *Bacillus* species described here were examined by this PCR and were found not to produce the specific 635 bp PCR fragment. This suggests that the specific DNA sequences recognised by the emetic toxin DNA specific primers does not exist in these species. This PCR therefore is still specific for emetic toxin strains of *B. cereus*.

Acknowledgment

We thank all those who donated strains used in this study.

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