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ORIGINAL ARTICLE

Bacillus cereus is common in the environment but emetic toxin producing isolates are rare

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Abstract

Aims: To determine the incidence of emetic toxin producing *Bacillus cereus* in soil, animal faeces and selected vegetable produce to compare the results with the previously reported high incidence in rice paddy fields. To examine whether the emetic toxin has antibiotic activity.

Methods and Results: The incidence of emetic toxin producing *B. cereus* was evaluated by plating on selective agar 271 samples of soils, animal faeces, raw and processed vegetables. Overall, 45·8% of samples were positive for *B. cereus*. One hundred and seventy-seven *B. cereus* isolates were recovered at 30°C with the grand mean spore count being $2{\cdot}6 \pm 1{\cdot}7$ $\log_{10}$ CFU g^{-1} and 148 *B. cereus* isolates were recovered at 7°C with the grand mean spore count being $2{\cdot}2 \pm 1{\cdot}2$ $\log_{10}$ CFU g^{-1} of the177 *B. cereus* isolated at 30°C, only 3 were positive for emetic toxin production at a titre of 1/64, 1/32, 1/16, respectively. Also, 1 of 148 *B. cereus* isolated at 7°C was positive for emetic toxin production to a titre of 1/128. All positive isolates came from washed or unwashed potato skins, one was psychrotrophic as determined by PCR and growth at 7°C on subculture. The emetic toxin was not shown to have any antibiotic effects in growth inhibition studies.

Conclusions: While *B. cereus* was a common isolate, the incidence of the emetic strain was rare. This is in contrast to previous findings of the high incidence in rice paddy fields and the processing environment, which may suggest rice is a selective area for growth of the emetic strain of *B. cereus*.

Significance and Impact of Study: The finding that a psychrotrophic isolate of *B. cereus* can produce emetic toxin is the first ever such observation and suggests the possibility that psychrotrophic isolates could grow in refrigerated fresh foods and cause emesis. The incidence of emetic *B. cereus* strains in rice paddy fields now requires further study for comparison with the low incidence found in other soils. The emetic toxin failed to inhibit the growth of other bacterial, fungal and yeast species. Whether the toxin (which is similar in structure to the antibiotic valinomycin) plays a competitive role in the environment therefore remains unclear.

Introduction

Bacillus cereus causes two distinct forms of food poisoning syndrome; the diarrhoeal syndrome and the emetic syndrome. The emetic syndrome, in which the main symptoms are nausea and vomiting, is largely (approximately 95% of cases) associated with the consumption of contaminated rice dishes. Other foods implicated include pasta dishes, vanilla slices, milk products and chicken dishes (Kramer and Gilbert 1989).

The emetic syndrome is caused by strains of *B. cereus* producing a low molecular size dodecadepsipeptide emetic toxin (cereulide) characterized by Agata *et al.* (1994).

It has been suggested that the large percentage of emetic cases associated with rice dishes, in particular fried rice dishes, is due to the manner in which the

rice is handled during cooking. There is also some suggestion that spores of the emetic strains are more heat resistant than others and so may be selected for during cooking (Gilbert and Parry 1977; Parry and Gilbert 1980). The emetic toxin on production is highly heat stable and survives cooking.

A number of previous studies have shown that *B. cereus* is commonly isolated from rice paddy field soils, rice plants and rice product (Gilbert and Parry 1977; Shinagawa *et al.* 1979; Blakey and Priest 1980; Bryan *et al.* 1981; Vijayalakshmi *et al.* 1981). These studies did not however determine whether the isolates were capable of emetic toxin production. Ueda and Kuwabara (1993) examined *B. cereus* isolates from rice paddy field soil, the processing environment and rice product, to determine whether these isolates were capable of producing the emetic toxin. A large percentage of isolates (overall 44%) from all areas were capable of producing the emetic toxin. This demonstrated that the emetic strains are commonly associated with rice and this therefore suggests that along with cooking practices, the common contamination of rice may be an additional reason why rice dishes rather than any other foods are mostly the cause of the emetic syndrome.

There has been no attempt however to determine the distribution of emetic toxin producing strains in soils other than paddy fields, this would establish whether there is preferential distribution of these strains in rice fields. *B. cereus* is a common bacterial isolate from soils, dust, natural waters (Watterson 1985) and many food types (Kramer and Gilbert 1989; Beecher 2001), and the question remains as to why other foods, especially root vegetables and salads are not associated with the emetic syndrome. One possibility we considered was that while *B. cereus* is common in the environment, emetic strains might be more restricted in their distribution. We therefore examined the incidence of *B. cereus*, and in particular emetic producing strains in soil samples, root and salad vegetables.

The organism is present in human faeces and although no evidence exists, it is possible that farm animals harbour the organism in their faeces and, as for other pathogens such as *Listeria monocytogenes*, may in this way contribute to the persistence of the organism in the soil environment by virtue of a continual cycle of faecal-oral enrichment (Fenlon 1999). Contamination of cow's milk with *Bacillus* species in general has been associated with faecal contamination of the udder of milking animals (McKinnon and Pettipher 1983). We therefore undertook to examine the presence of *B. cereus*, and emetic toxin producing strains in particular, in cow and horse faeces and vegetables including potato powder as a vegetable product.

Materials and methods

Sample collection

Soil samples

All samples were taken from areas within a 50-mile radius from Glasgow. All the soil samples were collected from entirely different locations on separate days within a 50 mile radius of Glasgow, Scotland, except for those farm samples which were taken from one farm (10 samples from fields growing crops and 10 samples from soils on which livestock were grazing); these were taken on the same day but each sample was taken from sites at least 500 m apart.

Samples were taken with a sterilized spoon down to a depth of 20 cm from the surface and put into sterile stomacher bags (Seward medical). All samples were treated on the same day of collection.

Samples were taken from three basic soil types:

i. Soil from parkland (20 samples taken from around grass roots).

ii. Soil from fields on which livestock were grazing (10 individual samples from 10 farms plus 10 samples all from a single farm, taken from around grass roots).

iii. Soil from fields growing crops (10 individual samples from 10 farms plus 10 samples all from a single farm, taken from around plant roots).

iv. Soil from woodlands (20 individual samples from areas unlikely to have been disturbed by livestock foraging).

Faecal samples

Cow faeces (56 samples) were collected into sterile containers from cattle at slaughter. Samples were taken from individual animals collected on seven separate occasions at least 1 week apart. Horse faeces (10 samples) were collected into sterile containers as fresh samples from individually stabled horses based at five separate riding establishments.

Root vegetable and lettuce samples

Samples were bought from five local retail outlets. When samples were taken on different occasions from the same outlet it was ensured that they were of a different batch and bought at least 2 weeks apart. Samples included: unwashed lettuce (25 samples), peeled skin from washed and unwashed potato (25 samples of each) and unwashed carrot (25 samples), and potato powder (25 cartons) each of which were bought in retail packages. Potatoes and carrots were skinned using a sterile potato peeler.

Microbiological analysis of samples

Five grams of each sample was added to 50 ml of sterile phosphate buffered saline (PBS) in a sterile stomacher

bag. The sample was then dispersed by stomaching for 5 min in a stomacher machine (Seward Medical). The sample was left to settle for 5 min and 20 ml of the supernatant was poured into a sterile universal bottle. The supernatant was heated for 10 min at 80°C in a water bath to kill vegetative bacteria and recover bacterial spores. The supernatant (500 μl volumes) was diluted in log dilutions to 10^{-4} in 4·5 ml volumes of sterile PBS. Volumes (200 μl) of each sample dilution (undiluted, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) were plated on PEMBA plates, which were *B. cereus* Selective Agar Base (CM617) (Oxoid) plus *B. cereus* Selective Supplement (SRR99) (Oxoid), and egg yolk Emulsion (SRO47) (Oxoid). Plates were left to dry then incubated at 30°C for 24–48 h to isolate *B. cereus* colonies. Also 200 μl of the undiluted, 10^{-2} and 10^{-4} dilutions of the same sample were spread plated onto PEMBA plates and incubated at 7°C for 14 days to selectively isolate psychrotrophic *B. cereus*.

Isolation and enumeration of *B. cereus*

The spore count, $\log_{10}$ colony forming units per gram ($\log_{10}$ CFU g^{-1}) of presumptive *B. cereus* in each sample was calculated from colonies recovered on PEMBA.

A single colony of each presumptive *B. cereus* isolate was streaked onto fresh PEMBA plates and incubated at the same temperature as they were isolated at to give a pure culture of each isolate.

Method of distinguishing *B. cereus*-group species

All colonies isolated on PEMBA agar, which were blue in colour and surrounded by an egg yolk precipitate were considered as presumptive *B. cereus* until further characterized.

Within the Genus Bacillus there is the *B. cereus* group, which consists of six species: *B. cereus*, *Bacillus weihenstephanensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus thuringiensis*, and *Bacillus anthracis*.

These species cannot be separated on the basis of biochemical characteristics. Additionally, these species have the same morphology on selective agar such as PEMBA (Oxoid). These species were therefore distinguished in this study in the following manner. *B. mycoides* was identified as having rhizoid growth on agar plate and being nonmotile. *B. pseudomycoides* is indistinguishable from *B. mycoides* by conventional characters. This organism was proposed on the basis of differences in fatty acid composition and DNA relatedness between strains of *B. mycoides* (Nakamura 1998). In this present paper isolates identified as *B. mycoides* were not characterized further.

Bacillus thuringiensis was differentiated by the production of parasporal crystals.

Bacillus cereus was identified as giving nonrhizoid colonial growth and lack of parasporal crystal production but was motile and haemolytic on horse blood agar.

Bacillus weihenstephanensis is phenotypically similar to *B. cereus* and only distinguishable from it by ability to grow at 7°C, inability to grow at 43°C, and by certain 16S rDNA signature sequences (Lechner *et al.* 1998), but not all psychrotolerant organisms resembling *B. cereus* are *B. weihenstephanensis*. There was no attempt to distinguish between *B. cereus* and *B. weihenstephanensis* in this present paper, all isolates capable of growth at 7°C were reported as *B. cereus*.

Bacillus anthracis was considered to be nonmotile, nonhaemolytic, not producing parasporal crystals and not showing rhizoid colonial growth on agar.

To detect parasporal crystal production, a single colony of each isolate was selected from a pure culture grown on PEMBA plates. This was sub cultured onto nutrient agar plates (NA) (Oxoid) supplemented with 5·0 mg l^{-1} $MnSO_4$ and incubated at 30°C for 24 h. The plates were then incubated at room temperature in the dark for up to 72 h. A colony of each isolate was emulsified in a loopfull of PBS on a microscope slide and dried. Smears were stained with 0·05% Carbol fuchsin, dried and then examined by microscopy at ×1000 under oil immersion for parasporal crystal production. To detect motility and sporulation, nutrient agar plates supplemented with 5 mg l^{-1} of $MnSO_4$ were inoculated with each isolate and incubated at 30°C for 24–48 h until endospore formation was observed. This was done by emulsifying a single colony with a drop of distilled water or sterile PBS on a clean microscope slide then covering with a cover slip and viewing with oil emersion at ×1000 using a phase-contrast microscope. To detect haemolysis on Blood agar medium, 10% horse blood agar was poured as sandwich plates (a 10 ml layer of blood agar base overlayed with 10 ml of blood agar base with 10% horse blood), thus allowing better visualization of weak haemolysis if present. For each isolate a single colony from a NA plate was used to inoculate the blood agar plates. The plates were incubated at 30°C for 24 h after which they were examined for haemolysis.

Presumptive *B. cereus* isolates were considered positively identified when they were shown to be motile, haemolytic and unable to produce parasporal crystals. The colonies of many of these *B. cereus* isolates were noted to have fimbriate edges.

Testing of *B. cereus* isolates for emetic toxin production

A single colony of each *B. cereus* isolate was picked from a PEMBA plate and inoculated into 10 ml of Tryptone

Soya broth (TSB) (Oxoid, UK) and incubated at 30°C for 24 h in an orbital shaker (Gallenkamp, UK). A colony of a know emetic toxin producing strain F4810/72 and a nonemetic toxin producing strain F4433/73, were grown in the same manner to act as a standard positive and negative control, respectively. A volume (100 μl) of each TSB culture was used as inoculum for 10 ml volumes of skim milk media (SMM), which were incubated at 30°C for 24 h in an orbital shaker for emetic toxin production. This medium has been shown to produce the highest titres of emetic toxin and it was found that the growth level in SMM had to be greater than 10^6 CFU ml^{-1} before toxin could be detected (Finlay *et al.* 1999). Growth of each sample in SMM was therefore confirmed by plate count to be to this level before testing for toxicity.

Each culture was centrifuged at 3000 *g* at 5°C for 30 min to pellet bacterial cells and large particulates. The supernatant was autoclaved at 121°C for 15 min to sterilize the sample and to destroy any heat labile toxins, thus leaving only the heat stable emetic toxin. The sample was then centrifuged again to remove any protein precipitate formed during autoclaving. The heated supernatant samples were tested in triplicate for toxicity using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a formazan blue salt] metabolic staining assay described previously by Finlay *et al.* (1999). The mean end point toxicity titre was determined as the highest dilution of each sample (tested in triplicate) giving an optical density less than that shown by the mean of control wells containing tissue culture cells alone.

Identification of mesophilic and psychrotrophic *B. cereus* by PCR

Random isolates (20) of *B. cereus* and all isolates that were found to be emetic toxin producers were examined by PCR using the method described by Francis *et al.* (1998). Taq enzyme was supplied in ready mix buffer (ABgene). Mesophilic and psychrotrophic isolates have been shown to give a single PCR band on gels of 284 bp when DNA is amplified with primers BcAPR1 and BcFF2, while only psychrotrophic isolates gave a band of 160 bp when DNA is amplified with primers BcAPR1 and BcAPF1.

BcAPR1 CTT (C/T) TT GGC CTT CTT CTA **A**

BcFF2 GAG ATT TAA ATG AGC TGT AA

BcAPF1 GAG GAA ATA ATT ATG ACA GT**T**

Discriminatory signature bases are shown in bold face, with a single degeneracy shown in parentheses.

Isolates identified as psychrotrophic by PCR were subsequently confirmed able to grow at 7°C by subculture on PEMBA.

Studies on the inhibitory action of emetic toxin on the growth of other microbial species

Skim milk medium (SMM) (100 g) and 10 g of bacteriological agar (Oxoid) were mixed together and dissolved with 1 l of distilled water. The medium was then autoclaved at 121°C for 20 min and after cooling was poured into sterile petri dishes. An overnight culture of *B. cereus* F4810/72 was streaked across the middle of the SMM plates and then incubated at 30°C for 24 h to grow and produce emetic toxin. In a preliminary study, the ability of the isolate to produce emetic toxin in the SMM agar was confirmed by cutting out the agar surrounding the growth, cutting it up into 5 ml of sterile PBS and autoclaving at 121°C for 20 min. After cooling the sample was centrifuged at 3000 *g* at 5°C for 30 min to pellet bacterial cells and large particulates. The supernatant was then tested in triplicate for emetic toxin as described above using the MTT assay.

For the inhibition studies, after *B. cereus* F4810/72 grew and produced the emetic toxin on SMM plates (as above) each test organism (Table 1) was streaked onto the plate at right angles to the streak of *B. cereus* growth. The plates were then incubated at 30°C for 24 h for growth of: *Bacillus* spp. (33 strains); soil bacteria (65 unidentified isolates of various colonial morphologies, not included in Table 1) and various laboratory strains of bacteria (six strains) or at 25°C for 24–48 h for the growth of fungi and yeast (17 strains). After incubation the plates were viewed under a plate microscope for any signs of inhibition of growth of the test organisms.

Results

The results of identification work on all presumptive *B. cereus* isolates showed that 23/209 (11%) and 15/165 (9%) of presumptive *B. cereus*, isolated at 30°C and 7°C respectively, were positive for producing parasporal crystal protein. These samples were therefore classified as *B. thuringiensis*.

Also, 9/209 (5·0%) and 2/165 (1·2%) of presumptive *B. cereus*, isolated at 30°C and 7°C respectively, were not motile. These nonmotile samples also were all of a rhizoid colonial morphology and were therefore classified as *B. mycoides*. None of the remaining isolates were considered as *B. anthracis* since they were all motile and haemolytic. The *B. thuringiensis* and *B. mycoides* isolates were not included in the results given for *B. cereus* isolates below. It is worth noting however that none of the *B. thuringiensis* and *B. mycoides* isolates had a heat stable toxin on testing in the emetic toxin assay.

All the samples examined for *B. cereus* were plated at both 30°C, to encourage the growth of mesophilic strains

Table 1 Identified microbial species tested for inhibition of growth by the emetic toxin

Samples number	Laboratory code number	Organism	Sample number	Laboratory code number	Organism
1	95/5802	*Bacillus licheniformis*	29	B0219	*Bacillus sphaericus*
2	95/7890	*B. licheniformis*	30	B0408	*B. sphaericus*
3	B0965	*B. licheniformis*	31	B0769	*B. sphaericus*
4	98/1897	*Bacillus megaterium*	32	B0770	*B. sphaericus*
5	B0909	*B. licheniformis*	33	K100	*Aspergillus carbonarius*
6	B0915	*B. licheniformis*	34	K001	*Aspergillus flavus*
7	98/3079	*Bacillus megaterium*	35	K002	*A. niger*
8	B2910	*B. licheniformis*	36	K003	*Aspergillus parasiticus*
9	96/6747	*B. licheniformis*	37	CBS588·68	*A. ochraceus*
10	B1408	*B. licheniformis*	38	K004	*A. ochraceus*
11	B0342	*B. maroccanus*	39	K005	*Fusarium graminearum*
12	B1421	*B. licheniformis*	40	K006	*Fusarium sporotrichoides*
13	B0168	*Bacillus amyloliquefaciens*	41	K007	*Mucor hiemalis*
14	B0173	*B. amyloliquefaciens*	44	K008	*M. hiemalis*
15	B0180	*Bacillus badius*	45	K009	*M. hiemalis*
16	B0201	*B. badius*	46	IMI285522	*Penicillium verrucosum*
17	B0241	*B. licheniformis*	47	K010	*Penicillium chrysogenum*
18	B0418	*B. licheniformis*	48	K011	*Sordaria Fimicola*
19	B0756	*B. licheniformis*	49	K012	*Saccharomyces cerevisiae*
20	B0407	*B. megaterium*	50	K013	*S. cerevisiae*
21	B0572	*B. megaterium*	51	K014	*Candida albicans*
22	B0759	*B. megaterium*	52	B0020	*B. cereus*
23	B0210	*Bacillus pumilus*	53	9001	*Escherichia coli*
24	B0233	*B. pumilus*	54	6751 oxbey	*Staphylococcus aureus*
25	B0409	*B. pumilus*	55	Lt_2	*Salmonella typhimurium*
26	B0220	*Bacillus subtilis*	56	W001	*Lactobacillus plantarum*
27	B0225	*B. subtilis*	57	W002	*Pseudomonas aeruginosa*
28	B0410	*B. subtilis*	58	TL 6750	*P. aeruginosa*

(defined here as not able to grow at 7°C) and at 7°C to selectively isolate psychrotrophic strains. However a random study of 20 isolates recovered at 30°C from soil or faeces showed that all gave a 160 bp PCR band (by the method of Francis *et al.* 1998), which identified them as psychrotrophic (results not shown). These were subsequently confirmed on subculture to be psychrotrophic by growth at 7°C on PEMBA.

With the exception of unwashed carrot skins more samples were found positive for *B. cereus* after plating at 30°C than at 7°C (in total 177/271 samples were positive at 30°C and 148 at 7°C). Three emetic toxin producing *B. cereus* isolates were recovered at 30°C and these were not psychrotrophic (Fig. 1 and see below). Together, these findings indicated plating samples at 30°C was beneficial.

The mean spore counts ($\log_{10}$ CFU g^{-1}) of *B. cereus* recovered from soil or faeces samples at 30°C are given in Table 2. In total, 101/146 (69%) of samples were positive for *B. cereus* spores. For individual samples the spore counts ($\log_{10}$ CFU g^{-1}) ranged between 2·3 and 6·0 with the grand mean for all samples being 3·0 ± 1·9.

All the 30°C isolates from soil and faeces were however negative for emetic toxin production: giving high optical density readings at 570 nm comparable to the cell control. The emetic toxin positive standard was toxic to a titre of 1/256.

The mean spore counts ($\log_{10}$ CFU g^{-1}) of *B. cereus* recovered from soil or faeces samples at 7°C are given in Table 2. In total, 95/146 (65%) of samples were positive. For individual samples, the spore counts ($\log_{10}$ CFU g^{-1}) ranged between 2·7 and 5·4 with the grand mean for all samples being 2·8 ± 1·7.

All the 7°C isolates from soil and faeces were however negative for emetic toxin production: giving high optical density readings at 570 nm comparable to the cell control. The emetic toxin positive standard was toxic to a titre of 1/256.

The mean spore counts ($\log_{10}$ CFU g^{-1}) of *B. cereus* recovered from the skins of washed and unwashed root vegetables, lettuce and potato powder at 30°C are given in Table 3. In total, 76/125 (61%) of samples were positive for *B. cereus* spores. For individual samples the spore counts ($\log_{10}$ CFU g^{-1}) ranged between 2·0 and 4·7 with the grand mean for all samples being 2·2 ± 1·4.

Three mesophilic *B. cereus* isolates, each recovered from washed potato skin samples at 30°C were positive for emetic toxin production (titres 1/64, 1/32, 1/16, respect-

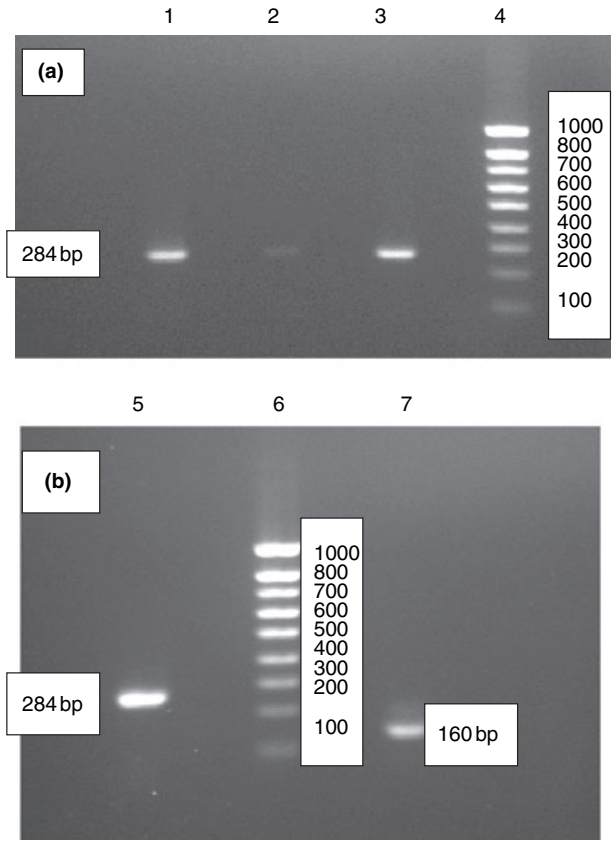

Figure 1 PCR of emetic toxin producing *Bacillus cereus* isolates. (a) Three 30°C isolates were shown to be mesophilic by production of a 284 bp band (lanes 1–3) and the absence of a 160 bp band (not shown). (b) One 7°C isolate was confirmed to be a psychrotroph by production of both a 284 bp band (lane 5) and a 160 bp band (lane 7). Lanes 4 and 6 are hyperladder I markers (Bioline).

ively). The emetic toxin positive standard F4810/72 was toxic to a titre of 1/256.

The mean spore counts ($\log_{10}$ CFU g^{-1}) of *B. cereus* recovered at 7°C from skins of root vegetables and potato powder are given in Table 3. In total, 53/125 (42%) of samples were positive for *B. cereus* spores. For individual samples the spore counts ($\log_{10}$ CFU g^{-1}) ranged between 2·0 and 4·3 with the grand mean for all samples being 1·5 ± 0·7.

One *B. cereus* isolate, recovered from an unwashed potato skin sample at 7°C was positive for emetic toxin production (titre 1/128). The emetic toxin positive standard F4810/72 was toxic to a titre of 1/256. This isolate was confirmed as a psychrotroph by PCR (Fig. 1).

The known emetic toxin producing *B. cereus* strain F4810/72 was found to produce emetic toxin detectable to a titre of 1/128 in SMM agar removed from plates after growth of the organism. In this study 33 strains of *Bacillus* species, 14 strains of fungi, 3 strains of yeast, 65 bacterial isolates from soil and 6 laboratory strains of various bacteria were studied to determine whether their growth was inhibited by the emetic toxin produced on SMM agar. The results showed that none of these organisms were inhibited by the emetic toxin. An example of this lack of inhibition is shown in Fig. 2 where *B. licheniformis* has grown right up to the area where *B. cereus* grew and emetic toxin was released into the agar.

Discussion

In total, 80 soil and 66 faeces samples were examined for *B. cereus*. Both were a common source of *B. cereus* with 76 and 65% of these respective samples being positive on plating at 30°C and 72 and 54% respectively, being positive on plating at 7°C. The grand mean spore counts for 30 and 7°C isolates ($\log_{10}$ CFU g^{-1} ± SD) were 3·0 ± 1·9 and 2·8 ± 1·7, respectively.

Both soil and faeces therefore represent common reservoirs for *B. cereus* and animal faeces can likely act as a vehicle for transfer to milk via udder contamination (McKinnon and Pettipher 1983) and meats at slaughter. However, none of the 196 isolates recovered from soil or faeces were emetic toxin producing strains, suggesting that these strains are rare in these environments. This is

Table 2 Mean spore counts of *Bacillus cereus* isolated at 30 and 7°C from soils and faeces

	30°C isolates		7°C isolates	
Sample	Number positive (%)	Mean counts $\log_{10}$ CFU g^{-1} ± SD	Number positive (%)	Mean counts $\log_{10}$ CFU g^{-1} ± SD
Park soil	15/20 (75)	3·2 (±1·9)	18/20 (90)	3·8 (±1·4)
Woodland soil	15/20 (75)	3·2 (±1·9)	5/20 (25)	1·0 (±1·7)
Soil from fields grazed by livestock	15/20 (75)	3·0 (±1·8)	15/20 (75)	3·1 (±1·9)
Soil from fields growing arable crops	16/20 (80)	3·6 (±1·9)	20/20 (100)	4·4 (±0·5)
Cow faeces	33/56 (59)	2·2 (±1·9)	32/56 (57)	1·8 (±1·6)
Horse faeces	7/10 (70)	2·7 (±1·9)	5/10 (50)	2·9 (±3·0)
	Total 101/146 (69)	Grand mean 3·0 (±1·9)	Total 95/146 (65)	Grand mean 2·8 (±1·7)

Table 3 Mean spore counts of *Bacillus cereus* isolated at 30°C and 7°C from root vegetables and potato powder

	30°C isolates		7°C isolates	
Sample	Number positive (%)	Mean counts $\log_{10}$ CFU g^{-1} ± SD	Number positive (%)	Mean counts $\log_{10}$ CFU g^{-1} ± SD
Unwashed potato	21/25 (84)	3·3 (±1·5)	21/25 (84)	2·9 (± 1·3)
Washed potato	8/25 (32)	1·1 (±1·7)	0/25 (0)	0
Potato powder	5/25 (20)	0·7 (±1·5)	0/25 (0)	0
Unwashed carrot	22/25 (88)	3·7 (±1·4)	23/25 (92)	3·5 (±1·1)
Unwashed lettuce	20/25 (80)	2·2 (±1·1)	9/25 (36)	0·9 (±1·2)
	Total 76/125 (61)	Grand mean 2·2 (±1·4)	Total 53/125 (42)	Grand mean 1·5 (±0·7)

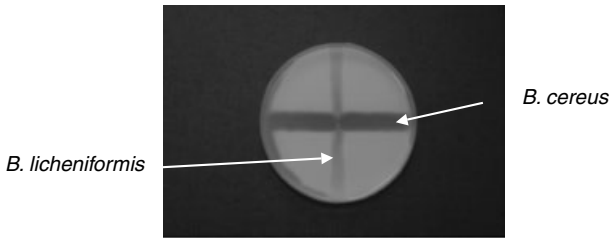

Figure 2 *Bacillus licheniformis* growth in the presence of emetic toxin produced by *Bacillus cereus* on SMM agar.

in contrast to the findings of Ueda and Kuwabara (1993) who found an average of 44% of *B. cereus* isolates from rice paddy fields and the processing environment were emetic strains. This perhaps suggests that the rice field is a selective area for enrichment of emetic strains and further studies should be conducted to confirm this.

An examination of vegetable produce showed that raw vegetables were also a common source of *B. cereus* spores with unwashed potato and carrot skins having a combined mean of 86 and 88% isolation rate on plating at 30 and 7°C, respectively. Washed potatoes had a reduced isolation rate of 32 and 0% at 30 and 7°C, respectively, and lower spore counts of 1·1 ± 1·7 and 0 $\log_{10}$ CFU g^{-1} ± SD, respectively. This presumably reflects the small amount of soil on the washed vegetable skins. Processing to potato powder further reduced the incidence and spore count of *B. cereus* in this product compared to raw potato (Table 3). Three emetic producing isolates of *B. cereus* were recovered from potato samples on plating at 30°C and these were subsequently shown by PCR to be mesophilic. A further psychrotrophic emetic toxin producing isolate was recovered at 7°C and confirmed psychrotrophic by PCR. The detection of a psychrotrophic emetic toxin producing isolate is an important finding. It is the first ever such isolation and suggests the possibility that psychrotrophic isolates could grow in refrigerated fresh foods, such as dairy products, and cause emesis.

In all, 3·2% of *B. cereus* isolates from vegetable produce were of the emetic toxin strain type. These findings together with the studies on soil and faeces isolates show that emetic toxin producing strains of *B. cereus* are very rare and this is in contrast to the incidence of the HBL enterotoxin where a mean of 63% of culture collection isolates are positive and the NHE enterotoxin where, in several studies, 83–93% of isolates have proven positive (Beecher 2001). This however tends to corroborate with the recent finding of Ehling-Schulz *et al.* (2005) who suggested upon polyphasic profiling that the emetic toxin strain may be an unusual clonal type of *B. cereus*.

In a recent review (Ehling-Schulz *et al.* 2004) evidence was given to show that of six emetic *B. cereus* isolates, five when tested, showed reduced lecithinase precipitation and peacock blue colouration compared to two nonemetic isolates tested. Also, three of the six emetic strains and one of the nonemetic strains showed reduced haemolysis on 5% sheep blood agar compared to the remaining three emetic and one nonemetic isolates. This small study should be repeated with a larger number of isolates. It suggests that care has to be taken to look for isolates displaying low characteristic diagnostic activities when isolating *B. cereus* from PEMBA, and that emetic isolates may be more prone to lower activity. In the present study, all isolates displaying even faint lecithinase activity and/or only slight colouration were further characterized and examined for emetic toxin. It is considered unlikely therefore that emetic strains would not have been isolated, if present. Also, double-layered blood agar plates were employed to identify strains exhibiting poor haemolysis.

It is interesting to note that emetic toxin strains of *B. cereus* have been isolated from rice (approximately 40% of samples) (Ueda and Kuwabara 1993) and milk (4·25% of samples) (Taylor 2005) and in this present study potato (16% of samples). Each of these products has been shown to support the production of emetic toxin, which very few foods have been shown capable of so far (Agata *et al.* 2002). This raises the question of whether the production of emetic toxin on or in these foods can offer a selective growth advantage to the emetic strains?

Valinomycin is an antibiotic and this may suggest that the emetic toxin, having a similar structure and iono-

phore activity (Andersson *et al.* 1998), may also act as an antibiotic. During the present studies on the effects of emetic toxin on other microbial species, the growth of all the organisms tested including; bacteria, fungi and yeast was not inhibited. The same methodology has however been used previously to demonstrate inhibition of growth between *Bacillus* species (Sutherland and Murdoch 1994). The emetic toxin, therefore, did not exhibit any apparent antibiotic activity. If the emetic toxin has a role as a competitive inhibitor and growth promoter for *B. cereus* in the natural environment it is a subtle feature and not bacteriostatic or bactericidal, at least in a nutrient rich environment such as SMM.

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