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Emetic toxin-producing strains of *Bacillus cereus* show distinct characteristics within the *Bacillus cereus* group

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Abstract

One hundred representative strains of *Bacillus cereus* were selected from a total collection of 372 *B. cereus* strains using two typing methods (RAPD and FT-IR) to investigate if emetic toxin-producing hazardous *B. cereus* strains possess characteristic growth and heat resistance profiles. The strains were classified into three groups: emetic toxin (cereulide)-producing strains (n = 17), strains connected to diarrheal foodborne outbreaks (n = 40) and food–environment strains (n = 43), these latter not producing the emetic toxin. Our study revealed a shift in growth limits towards higher temperatures for the emetic strains, regardless of their origin. None of the emetic toxin-producing strains were able to grow below 10 °C. In contrast, 11% (9 food–environment strains) out of the 83 non-emetic toxin-producing strains were able to grow at 4 °C and 49% at 7 °C (28 diarrheal and 13 food–environment strains). non-emetic toxin-producing strains. All emetic toxin-producing strains were able to grow at 48 °C, but only 39% (16 diarrheal and 16 food–environment strains) of the non-emetic toxin-producing strains grew at this temperature. Spores from the emetic toxin-producing strains showed, on average, a higher heat resistance at 90 °C and a lower germination, particularly at 7 °C, than spores from the other strains. No difference between the three groups in their growth kinetics at 24 °C, 37 °C, and pH 5.0, 7.0, and 8.0 was observed. Our survey shows that emetic toxin-producing strains of *B. cereus* have distinct characteristics, which could have important implication for the risk assessment of the emetic type of *B. cereus* caused food poisoning. For instance, emetic strains still represent a special risk in heat-processed foods or preheated foods that are kept warm (in restaurants and cafeterias), but should not pose a risk in refrigerated foods.

1. Introduction

The endospore-forming bacterium *Bacillus cereus*, which is commonly isolated from food, can cause foodborne illness, either an emetic or a diarrheal syndrome. The emetic syndrome is caused by the ingestion of the heat-stable emetic toxin (cereulide, a cyclic peptide of 1.2 kDa) produced in foods. The diarrheal syndrome is mainly due to the ingestion of *B. cereus* cells in the foods, followed by toxin production in the small intestine (Kramer and Gilbert, 1989 and Granum and Lund, 1997). A significant progress has recently been made in the biochemical

and biological detection of the *B. cereus* emetic toxin, and in the molecular detection of the emetic and of the diarrheal toxins genes. A LC–MS method has been developed to detect the emetic toxin production (Jääskeläinen et al., 2003 and Andersson et al., 2004) and the first molecular tools for the detection of emetic strains of *B. cereus* have been designed (Ehling-Schulz et al., 2004 and Ehling-Schulz et al., 2005a). Both methods allow a reliable detection of potential emetic toxin-producing strains within the *B. cereus* species.

The species *B. cereus* covers a huge diversity in genetic characters (Guinebretière and Sanchis, 2003, Helgason et al., 2004 and Hill et al., 2004), virulence factors (Beattie and Williams, 1999, Choma et al., 2000, Helgason et al., 2004 and Hill et al., 2004) and survival or growth characteristics (Dufrenne et al., 1994, Dufrenne et al., 1995, Choma et al., 2000 and Nguyen-the et al., 2003). However there is some evidence that emetic toxin-producing strains form a distinct cluster within *B. cereus* with some specific characters (such as the inability to hydrolyse starch) (Shinagawa, 1990, Agata et al., 1996, Pirttijarvi et al., 1999 and Ehling-Schulz et al., 2005b). The aim of this work is to determine whether emetic toxin-producing strains of *B. cereus* differ from the other strains of *B. cereus*, not producing the emetic toxin regarding their growth characteristics and their heat resistance to assess the risks of different types of food/ food preparation/food handling for emetic toxin production. For this purpose, a panel that comprises 100 strains of foodborne, environmental, and clinical strains of *B. cereus*, gathered by participants in an EU-funded project, was compiled.

2. Materials and methods

2.1. Strains

A collection of 100 *B. cereus* strains was compiled for this study (Table 1). The strain set includes clinical isolates and isolates from food remnants connected to both emetic (n = 10), and diarrheal foodborne outbreak (n = 40), and isolates from food (n = 35) and from the environment (n = 15).

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Table 1.

Origin of *B. cereus* strains

Type of *B. cereus* strains	Strain designation	Origin
Emetic toxin-producing strains	F3080B/87, F3351/87, F4810/72 (DSM4312), IH41385[a,b], MHI 1305, NC7401, RIVM BC 124, RIVM BC 67, UHDAM B315, UHDAM ML127	Emetic outbreak; clinical (*n* = 10)
	MHI 87, RIVM BC379, SDA GR177, UHDAM CIF1, UHDAM CIF3	Food (*n* = 5)
	UHDAM 3/pkl, UHDAM NS-115,	Environment (*n* = 2)
Strains not producing the emetic toxin	98HMPL63[c], F2081A/98, F2085/98, F3371/93, F3453/94, F352/90, F4430/73 (DSM 4384, B4ac), F4433/73, F528/94, F837/76 (DSM 4222), FH3502/72 (DSM 2301), IH41064[a], MHI 203, DSM 8438 (NRS 404), NVH 0075-95, NVH 0154-01, NVH 0165-99, NVH 0226-00, NVH 0230-00, NVH 0309-98, NVH 0391-98, NVH 0500-00, NVH 0597-99, NVH 0674-98, NVH 0784-00, NVH 0861-00, NVH 1104-98, NVH 1105-98, NVH 1230-88, NVH 141/1-01, NVH 1518-99, NVH 1519-00, NVH 1651-00, NVH 200, RIVM BC 122, RIVM BC 63, RIVM BC 90, UHDAM B106, UHDAM B154, UHDAM B217	Diarrhoeal outbreak (*n* = 40)
	INRA 1, INRA A3, INRA C24, INRA C3, INRA C57, INRA I20, INRA I21, INRA PA, MHI 124, MHI 13, MHI 32, NVH 445, NVH 449, NVH 506, RIVM BC 485, RIVM BC 934, RIVM BC 938, RIVM BC 964, SDA GR285, SDA KA96, SDA MA57, UHDAM 1IFI(13), UHDAM B102, WSBC10030, WSBC10204, WSBC10286, WSBC10377, WSBC10395, WSBC10466, WSBC10483	Food (*n* = 30)
	INRA SZ, NVH 460, NVH 512, NVH 655, SDA 1R177, SDA 1R183, SDA 1R72, SDA NFFE640, SDA NFFE647, SDA NFFE664, UHDAM TSP9, WSBC10310, WSBC10441	Environment (*n* = 13)

INRA, Institut National de la Recherche Agronomique, UMR408, Avignon, France; SDA, Svensk Mjolk, Swedish Dairy Association, Lund Sweden; WSBC: Weihenstephan *Bacillus cereus* collection, Weihenstephan, Germany; DSM, Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany; F strains were obtained from the Public Health Laboratory Service (PHLS), London, UK; NVH, The Norwegian School of Veterinary Science, Oslo, Norway; RIVM, Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven, The Netherlands; UHDAM, University of Helsinki, Department of Applied Chemistry and Microbiology, Helsinki, Finland; MIH strains were obtained from the Ludwig Maximilians-Universität, München, Germany.

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[b] Strain IH41385 is a clinical isolate from dialysis liquid connected to illness.

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To ensure a large diversity in these 100 strains, they were selected from a first set of 372 isolates provided by the various participants and isolated in various countries. These 372 strains included strains from various foods and food processing chains (dairy, vegetables, recipe dishes containing meat, sea foods, rice, pasta and bakery product), strains from the environment (soil and surface water), and strains implicated in foodborne poisoning cases. All strains were typed using two different typing methods, i.e. is random amplification of polymorphic DNA and Fourier transformed infrared spectroscopy, as described in Ehling-Schulz et al. (2005b). Isolates with similar profiles were eliminated. A Microsoft Access database was designed and used to generate a panel of 100 representative strains. Selection criteria were: (i) largest diversity in strain profiles, (ii) largest diversity in geographical origins, and (iii) keep 50 strains from clinical foodborne poisoning cases and 50 strains from both food and the environment.

2.2. Detection of *B. cereus* strains producing the emetic toxin

B. cereus emetic toxin (cereulide)-producing strains were detected by both production of cereulide, assayed with liquid chromatography–mass spectrometry (Jääskeläinen et al., 2003), and by detection of cereulide gene synthase (*ces*) using a recently developed PCR assay (Ehling-Schulz et al., 2005a). Both methods were applied to the entire test panel. *B. cereus* biomass (5 to 10 mg wet wt) grown on TSA plates (Difco, Becton Dickinson, Maryland USA) was extracted in 200 µl methanol in a capped tube (4 ml) in boiling water bath for 15 min. The cooled, filtered (0.45 µm) extract was used for LC–MS analysis, based on four molecular ions specific for cereulide (H^+, NH_4^+, K^+ and Na^+ adducts), performed as described elsewhere (Jääskeläinen et al., 2003). DNA extraction and PCR using the sequences of the cereulide synthetase specific primers were performed as described previously (Ehling-Schulz et al., 2005a).

2.3. Determination of temperature growth limits

Growth tests were performed in Plate count (PC) broth (5 g of casein peptone, 2.5 g of yeast extract, 1 g of glucose/l, with a pH adjusted to 7.0). Bacteria were grown for 18 h in 5 ml PC broth at 30 °C and 170 rpm. Fresh PC broth (5 ml) was inoculated with 10 µl of the overnight cultures (corresponding to approx. 10^5 cfu/ml), and incubated at different temperatures, at 170 rpm. Lower growth limits were tested at 4 °C, 7 °C and 10 °C, upper growth limits at 45 °C and 48 °C; in addition, strains that did not grow at 45 °C were also tested at 42 °C and those that grew at 48 °C were also tested at 52 °C. The tubes were sealed with parafilm to avoid evaporation and concentration of the liquid medium at higher temperatures. Growth was determined via optical density at 585 nm with a portable WinLab photometer LF2400 (Windaus Labortechnik, Germany) after 1, 2, 5, 7, 9 and 12 days. In addition, growth was measured with a Perkin-Elmer 550SE UV/VIS spectrophotometer at 600 nm (Perkin-Elmer, Germany) after 7 and 12 days. Growth was defined as an increase of OD_{600} by at least 0.1.

2.4. Growth kinetics at 24 °C, 37 °C and pH 5, pH 7, pH 8

Growth curves for the test strains were determined with a multiwell photometric plate reader Bioscreen C (Labsystems Corp., Helsinki, Finland). Overnight cultures of the test strains were grown in 5 ml PC broth, pH 7.0, at 30 °C and 170 rpm. A 10 µl aliquot of each culture was diluted in 190 µl of PC broth and a 10 µl aliquot of this dilution was used to inoculate 240 µl of PC broth (corresponding to approx. 10^5 cfu/ml) with different pH values in multiwell plates of the Bioscreen C. PC broth was adjusted to pH 5 with 1 M piperazin solution (Aldrich, Germany) and PC broth with a pH 7 or pH 8 was buffered with 1 M Tris–HCl solutions having the designated pH. The plates were incubated under continuous shaking in the plate reader at 24 °C and at 37 °C. OD was recorded every 60 min for 48 h with the computer software Biolink (Transgalactic Ltd., Finland, version 5.30). Data were exported to Microsoft Excel for statistical analysis.

2.5. Heat resistance of *B. cereus* spores

Purified *B. cereus* stock cultures were grown overnight in JB to produce spores (Claus and Berkeley, 1986) at 30 °C. An aliquot was then spread on duplicate plates of modified Fortified Nutrient Agar (FNA) (Fernández et al., 1999), and incubated at 30 °C for 3–7 days. Sporulation was checked daily by microscopic examination, and spores were harvested when at least 90% of the cells had produced spores. Strains WSBC10204 and WSBC10310 were unable to produce spores in several attempts. The agar surface was then flooded with 2 ml of sterile cold demineralized water. The spore suspension was twice washed in 2 ml sterile cold distilled water and finally suspended in 2 ml of an aqueous solution of glycerol at 30% (v/v). Spore suspensions were stored at – 20 °C until use. Number of survivors was evaluated after 0, 10, 20, 30, 60 and 120 min of heating at 90 °C. Samples were serially diluted in peptone water and surface spread on JA plates using a spiral plate apparatus (Spiral système, Intersciences, Saint-Nom la Bretèche, France). Cell concentrations were expressed as colony forming units per ml (cfu/ml) (Choma et al., 2000). Survival curves were fitted to a linear regression model to calculate decimal reduction times ($D_{90\,°C}$ values), and, as most survival

curves diverged from linearity, the non-linear model of Mafart et al. (2002) was used to calculate the time to first decimal reduction (*δ*).

2.6. Germination of *B. cereus* spores

The method was similar to that used by Dufrenne et al. (1994). Briefly *B. cereus* spores were produced and stored as previously described. A spore suspension containing ca 10^7–10^8 *B. cereus* spores/ml in 100 mM phosphate buffer at pH 6.8 was prepared and pasteurised at 80 °C for 5 min. Aliquots of 100 µl were inoculated in tubes containing 9.9 ml of J broth. The tubes were then incubated at 30 °C for 50 min and at 7 °C ± 0.5 °C for 7 days. After incubation tubes were pasteurised at 80 °C for 5 min. Spores were counted just after inoculation into tubes and after pasteurisation at the end of incubation, as described above. Two replicate tubes were performed for each incubation temperature. The extent of germination was expressed as the $\log_{10}(N_0/N)$, N_0 being the initial spore number and N the number of remaining spores after incubation.

2.7. Statistical analysis

Differences between emetic toxin-producing strains, diarrheal, and food–environment strains were tested (i) with the Pearson's Chi-square test for upper and lower growth temperatures and (ii) with the Kruskal–Wallis test for heat resistance and germination, using Systat version 9 (SPSS, Chicago, USA). Threshold values were kept for statistical analysis when no accurate value could have been determined.

The non-linear mixed model

$$y(t) = \frac{A}{1 + e^{-\frac{(t - t_{\mathrm{mid}})}{\mathrm{scale}}}} e^{-d_0 t} + B_0$$

was used to determine the horizontal final asymptote A (without background B_0 optical density) of the growth kinetics of all strains at 24 °C and 37 °C, and pH 5.0, 7.0 and 8.0 (Doucet and Sloep, 1992). In addition, t_{mid} (time at which 50% of A is reached) and the slope of each growth curve at t_{mid} (i.e. 1/scale) were estimated. The parameter d_0 is the rate of decay of the bacteria. The maximum likelihood method was used for statistical analysis (S-PLus 6.2 package with the nlme-library).

3. Results and discussion

Strains characteristics are shown in Table 2, Table 3 and Table 4. Seventeen out of the 100 strains of the collection both produced cereulide and possessed the *ces* gene (Table 1 and Table 2). None of the other strains (most importantly, the diarrheal strains) were positive for any of the tests, showing a perfect agreement between detection of the *ces* gene and cereulide production, although the expression of the *ces* gene varied 1000-fold between the strains. Table 2 shows that the cereulide productivity in TSA-plate grown (24 h) cultures ranged from very low (< 1 ng to 50 ng of cereulide per mg of bacterial biomass), medium (> 50 to 500 ng) and extremely high amounts (> 500 to 1600 ng of cereulide). The present results show that variation in cereulide production between individual strains is even higher than reported in an earlier study of 10 cereulide-producing strains (Andersson et al., 2004). The *ces* gene detection is hence usable for detecting strains capable of producing cereulide, but, as such, not useful as basis for risk assessment, because it does not account for the differences in the amounts of the emetic toxin produced by the tested strains under identical conditions. None of the 17 emetic toxin-producing strains of *B. cereus* were able to grow at 4 °C or 7 °C, while 50 (28 diarrheal and 22 food–environment strains) out of 83 non-emetic toxin-producing were able to grow at 4 °C or 7 °C (the difference in the frequency of strains growing at 7 °C and below in each category was significant at $P < 0.001$) (Table 5). This poor ability to grow at temperatures below 10 °C was linked to a low germination of spores at 7 °C (difference in the distribution of values for each category of strains significant at $P < 0.001$). At

30 °C, spores of the emetic toxin-producing strains also showed a lower ability to germinate than the strains belonging to the two other categories ($P < 0.01$) (Fig. 1). Germination of the spores of the diarrheal, and food–environment strains were highly similar for both temperatures (Fig. 1). Finlay et al. (2000) determined that the lower limit for growth of 7 tested emetic strains was 12 °C and Johnson et al., 1982 and Johnson et al., 1983 also observed a lower germination of spores of emetic strains (only three tested) in a range of temperatures and media in comparison to diarrheal strains.

Table 2.
Characteristics of the *B. cereus* emetic toxin-producing strains tested in this work

Strain no.	**Associated to emetic outbreak**	**Cereulide productivity (ng cereulide/mg biomass fresh wt[a])**	**Temperature growth limits (°C)**		**Heat resistance parameters of spores at 90 °C[b]**			**Germination of spores at[c]**	
			Lower[d]	**Upper[e]**	***D* (min)**	**δ (min)**	**Viability loss**	**7 °C**	**30 °C**
F3080B/87	Yes	500	> 10	48	372	130	0.80	0.20	2.03
F3351/87	Yes	20	10	48	537	> 120	0.61	0.23	2.47
F4810/72	Yes	240–600	10	48	763	> 120	0.35	0.10	1.92
IH41385	No	0.5–1	> 10	48	917	> 120	0.28	0.21	2.03
MHI 1305	Yes	170–200	> 10	48	672	> 120	0.80	0.29	1.17
MHI 87	No	70–110	> 10	48	318	23	1.24	0.28	1.44
NC7401	Yes	180–600	> 10	48	612	> 120	0.94	0.22	1.95
RIVM BC 124	Yes	60–90	10	48	369	149	1.00	0.24	1.19
RIVM BC 379	No	7–9	> 10	48	289	58	1.45	3.72	1.15
RIVM BC 67	Yes	20–30	10	48	270	120	0.93	1.32	1.79
SDA GR177	No	70	10	48	489	> 120	0.91	0.07	2.16
UHDAM CIF1	No	60–100	> 10	48	68	7	4.01	0.60	2.12
UHDAM CIF3	No	10–80	> 10	48	371	3	1.53	0.41	1.09
UHDAM 3/pkl	No	70–160	> 10	48	372	> 120	0.66	0.20	2.45
UHDAM B315	Yes	50–90	10	48	129	34	2.13	0.49	0.72
UHDAM ML127	Yes	130–230	10	48	318	6	1.40	0.48	1.12
UHDAM NS-115	No	1200–1600	> 10	48	341	148	1.00	0.25	1.45

nd: not determined.
[a] The range for results from three independent cultures are given.
[b] *D*, decimal reduction time at 90 °C; δ, time to first decimal reduction at 90 °C; Viability loss, $\log_{10}$ bacterial reduction after 120 min at 90 °C.
[c] Germination is expressed as $\log_{10}(N_0/N)$, where N_0 is the initial spore number and N is spore number after 7-day incubation at 7 °C and 50-min incubation at 30 °C in nutrient broth (see Materials and methods for details).
[d] Tested temperatures were 4 °C, 7 °C and 10 °C; > 10 °C indicates no growth observed at 10 °C.
[e] Tested temperatures were 42 °C, 45 °C, 48 °C and 52 °C.

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Table 3.

Characteristics of *B. cereus* strains connected to diarrheal foodborne outbreak tested in this work

Strain[a] no.	Temperature growth limits (°C)		Heat resistance parameters of spores at 90 °C[b]			Germination of spores at[b]	
	Lower[b]	Upper[b]	*D* (min)	*δ* (min)	Viability loss	7 °C	30 °C
98HMPL63	7	45	45	3	6.57	2.03	2.43
F2081A/98	7	48	29	5	> 3.42	2.00	2.91
F2085/98	10	48	52	3	5.58	2.10	2.67
F3371/93	7	45	47	23	5.24	3.94	3.47
F3453/94	10	45	115	28	2.40	0.18	1.91
F352/90	10	45	40	6	6.89	1.74	3.64
F4430/73	7	45	49	16	5.77	0.46	0.70
F4433/73	7	45	63	30	3.97	1.78	2.98
F528/94	7	45	40	2	6.91	2.88	2.00
F837/76	10	48	142	39	2.14	3.17	3.76
FH3502/72	7	48	300	120	0.93	2.32	4.44
IH41064	7	45	61	29	4.68	2.49	3.14
MHI 203	7	45	70	21	4.01	0.82	2.39
NRS 404	7	45	53	8	5.57	1.69	2.29
NVH 0075-95	> 10	48	185	53	1.77	0.26	1.37
NVH 0154-01	10	45	48	26	5.29	2.20	3.01
NVH 0165-99	7	48	69	1	4.55	1.84	1.57
NVH 0226-00	7	45	55	12	4.92	0.71	2.02
NVH 0230-00	7	48	92	20	2.86	3.40	3.63
NVH 0309-98	10	45	254	74	1.38	1.62	1.79
NVH 0391-98	> 10	45	60	41	4.34	0.70	2.44
NVH 0500-00	7	48	201	43	1.68	1.93	4.02
NVH 0597-99	7	48	41	29	6.10	1.79	2.44
NVH 0674-98	7	45	161	76	1.41	3.29	3.31
NVH 0784-00	7	45	92	57	2.84	2.97	3.81
NVH 0861-00	7	45	54	30	4.81	1.15	2.32
NVH 1104-98	7	42	74	12	3.76	3.00	3.90
NVH 1105-98	7	45	817	> 120	0.50	0.79	1.39
NVH 1230-88	7	45	49	41	> 2.79	1.10	3.59
NVH 141/1-01	7	45	79	15	3.95	2.43	3.69
NVH 1518-99	> 10	45	74	12	3.76	1.23	3.17
NVH 1519-00	> 10	48	93	67	3.43	0.39	2.30
NVH 1651-00	7	45	63	27	4.25	1.08	2.06
NVH 200	7	48	253	116	1.22	1.76	4.52
RIVM BC 122	10	45	81	2	4.10	2.35	2.16
RIVM BC 63	7	48	260	15	1.49	0.32	1.79
RIVM BC 90	> 10	48	162	35	1.96	0.23	0.64
UHDAM B106	7	48	301	106	1.04	2.14	3.16
UHDAM B154	7	48	48	4	5.91	4.04	3.82
UHDAM B217	7	48	131	43	2.43	0.55	2.11

[a] Strains not producing the emetic toxin.
[b] See Table 2 for legends.

Table 4.
Characteristics of *B. cereus* food and environment strains tested in this work

Strain[a] no.	Temperature growth limits (°C)		Heat resistance parameters of spores at 90 °C[b]			Germination of spores at[b]	
	Lower[b]	Upper[b]	*D* (min)	*δ* (min)	Viability loss	7 °C	30 °C
INRA 1	4	< 45	60	0	6.48	2.87	0.80
INRA A3	> 10	45	208	83	1.17	1.48	2.45
INRA C24	> 10	48	152	57	1.66	0.25	2.32
INRA C3	10	45	46	18	5.68	2.52	2.55
INRA C57	10	< 45	49	31	4.79	3.01	1.35
INRA I20	4	45	48	2	6.52	1.72	0.62
INRA I21	7	45	50	3	5.86	2.00	1.11
INRA PA	> 10	48	402	172	0.92	0.42	1.46
INRA SZ	7	45	61	0	6.48	2.09	1.07
MHI 124	10	48	140	9	2.27	0.75	2.32
MHI 13	10	45	796	104	0.83	1.90	1.54
MHI 32	7	45	396	148	1.05	0.48	2.09
NVH 445	10	48	241	70	1.33	0.46	1.70
NVH 449	10	45	178	32	2.00	2.51	4.23
NVH 460	7	48	68	49	3.43	1.34	3.55
NVH 506	10	52	328	> 120	0.76	0.22	0.02
NVH 512	7	45	66	6	4.41	2.89	3.02
NVH 655	7	42	44	4	6.28	3.14	2.47
RIVM BC 485	7	45	140	51	2.18	1.92	2.84
RIVM BC 934	> 10	48	113	16	2.68	2.47	2.65
RIVM BC 938	7	48	64	29	4.45	2.99	3.22
RIVM BC 964	7	42	115	27	2.48	2.10	3.01
SDA 1R177	4	45	75	11	4.12	2.48	1.88
SDA 1R183	10	45	52	22	4.99	2.05	1.90
SDA 1R72	4	45	99	7	3.10	0.69	1.73
SDA GR285	7	48	105	0	4.31	1.00	2.54
SDA KA96	10	45	41	3	6.89	2.52	2.80
SDA MA57	7	48	42	10	6.26	2.22	0.67
SDA NFFE640	4	45	74	2	4.90	2.00	1.46
SDA NFFE647	4	42	68	7	4.01	1.19	0.43
SDA NFFE664	4	45	78	12	3.44	2.20	1.47
UHDAM 1IFI(13)	10	48	152	87	1.91	0.60	0.12
UHDAM B102	10	48	92	3	3.71	1.51	1.76
UHDAM TSP9	10	45	66	26	3.96	2.09	2.57
WSBC10030	10	48	372	> 120	0.91	0.74	1.70
WSBC10204	4	ngo[c] at 42	nt[d]	nt	nt	nt	nt
WSBC10286	> 10	48	367	80	1.04	0.26	1.27
WSBC10310	> 10	48	nt	nt	nt	nt	nt
WSBC10377	4	ngo at 42	54	10	4.92	2.41	0.87
WSBC10395	7	ngo at 42	164	2	3.37	2.54	1.84
WSBC10441	10	45	64	31	3.95	2.54	3.36
WSBC10466	7	48	59	27	4.46	2.36	2.87
WSBC10483	10	45	56	36	5.15	0.82	3.83

[a] Strains not producing the emetic toxin.
[b] See Table 2 for legends.
[c] ngo: no growth observed.
[d] nt: not tested, these strains did not produce spores in our experimental conditions.

Table 5.

Temperature growth limits of emetic toxin-producing strains, diarrheal strains and food-environment strains of *B. cereus*

Character		Number of strains showing the character among:			Significance[a]
		Emetic toxin-producing strains (n = 17)	Diarrheal strains (n = 40)	Food-environment strains (n = 43)	
Lower temperature limit	Growth at 4 °C and/or 7 °C	0	28	22	$P < 0.001$
	No growth at 7 °C	17	12	21	
Upper temperature limit	Growth at 48 °C	17	16	16	$P < 0.001$
	No growth at 48 °C	0	24	27	

[a] Chi-square test.

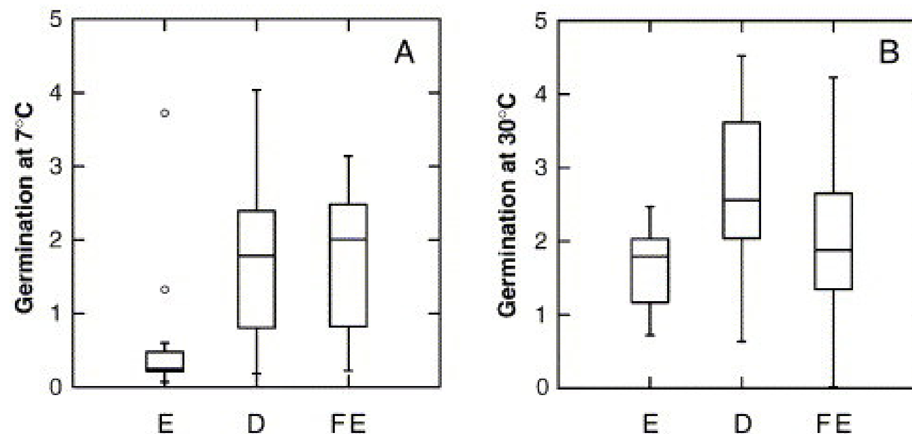

Fig. 1. Box-and-Whisker plot of the germination at 7 °C (A) and 30 °C (B) of spores of emetic toxin-producing (E) (n = 17), diarrheal (D) (n = 40), and food-environment (FE) (n = 41) *B. cereus* strains. Germination is expressed as $\log_{10}(N_0/N)$, where N_0 is the initial spore number and N is spore number after 7-day incubation at 7 °C and 50-min incubation at 30 °C in nutrient broth (see Materials and methods for details). In the “Box-and-Whisker” representation, the central “box” covers the middle 50% of the data values between the lower and the upper quartiles. The central line in the “box” is at the median. “Whiskers” extend 1.5 times the box length (interquartile range). External values are plotted.

All emetic toxin-producing strains were able to grow at 48 °C while only 39% of the non-emetic toxin-producing strains (16 diarrheal and 16 food–environment strains) grew at this temperature (the difference in the frequency of strains growing at 48 °C in each category was significant at $P < 0.001$) (Table 2, Table 3, Table 4 and Table 5). Spores of the emetic toxin-producing strains showed higher D-values ($P < 0.001$), δ values ($P < 0.01$), a higher survival

after 120 min at 90 °C ($P < 0.001$), and therefore a higher heat resistance, than spores of the non-emetic toxin-producing strains (Fig. 2). In contrast, spore germination patterns at both 7 °C and 30 °C and survival at 90 °C of diarrheal strains and food–environmental strains were similar. Parry and Gilbert (1980) also observed that the heat resistance of spores of 14 emetic strains was on average 6-fold higher than that of spores of 13 non-emetic strains isolated from rice. Quite recently, it has been shown that emetic toxin production is restricted to a single evolutionary lineage of *B. cereus* whereas diarrheal toxin producers are scattered throughout different phylogenetic clusters (Ehling-Schulz et al., 2005b). In particular emetic toxin-producing strains are unable to produce the Hbl toxin or don not carry the *hbl* genes, and only a few of them carry the *cytK* gene (Pirttijarvi et al., 1999 and Ehling-Schulz et al., 2005b). Our results are consistent with the phylogeny of *B. cereus*: all emetic toxin-producing strains rather thermophilic in contrast to diarrheal strains. Apart from the amount of cereulide produced by the strains, no other significant characteristics in growth profiles of diarrheal or food–environmental strains were observed. In particular none of the diarrheal strains produced the emetic toxin, without possible explanation at the present time of the significance of this observation.

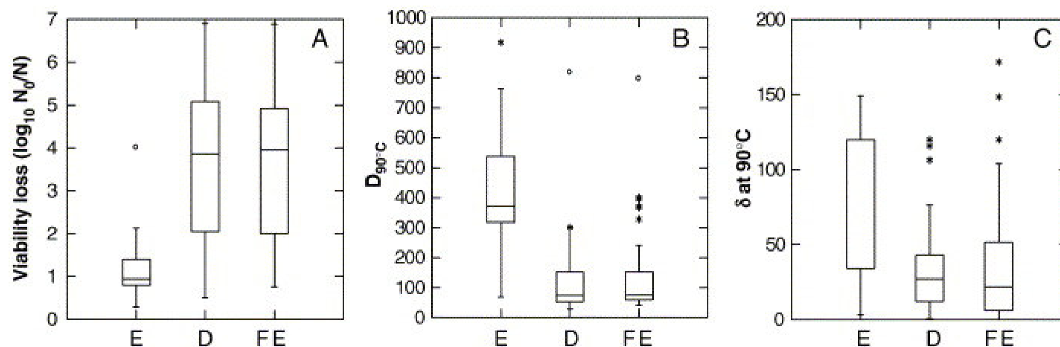

Fig. 2. Box-and-Whisker plots of the viability loss after 120 min at 90 °C (A), $D_{90\ °C}$ (B), and time to first decimal reduction (δ) (C) of spores of emetic toxin-producing (E) ($n = 17$), diarrheal (D) ($n = 40$), and food-environment (FE) ($n = 41$) *B. cereus* strains. Viability loss was expressed as the $\log_{10}(N_0/N)$ where N_0 is the initial spore number and N is spore number after 120-min heating at 120 °C (See Materials and methods for details).

Emetic toxin-producing, diarrheal, and food–environment strains did not show any significant difference ($P > 0.05$) in their estimated growth kinetics from experiments performed at 24 °C and 37 °C, and pH 5.0, 7.0 or 8.0 (data not shown).

In conclusion, our survey showed that emetic *B. cereus* strains possess specific growth characteristics (like a shift of growth limits towards higher temperatures combined with highly heat-resistant spores), which have to be taken into consideration if food safety issues are discussed. Our findings could help to assess the risks of special types of foods for emetic toxin production. For example, the poor ability to grow at low temperatures shows that emetic toxin-producing strains of *B. cereus* will pose a low risk in refrigerated foods. In contrast, the remarkable higher heat resistance of their spores favours their survival in heat-processed foods. Kramer and Gilbert (1989) proposed several recommendations to prevent outbreaks of *B. cereus* associated with cooked rice. These include either short time between preparation and consumption, keeping foods at high temperature (> 50 °C) or rapid cooling (favoured by preparation in small quantities) followed by storage in the refrigerator, or avoiding storage at room temperature for more than 2 h. Our study emphasized the importance of these recommendations and, in addition, showed that these advises should be extended to any kind of heat-processed foods and preheated foods that are kept warm, in restaurants and catering facilities for instance, to prevent the risk of emetic type of *B. cereus* food poisoning.

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