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Toxicological profile of cereulide, the *Bacillus cereus* emetic toxin, in functional assays with human, animal and bacterial cells

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Abstract

Some strains of the endospore-forming bacterium *Bacillus cereus* produce a heat-stable ionophoric peptide, cereulide, of high human toxicity. We assessed cell toxicity of cereulide by measuring the toxicities of crude extracts of cereulide producing and non-producing strains of *B. cereus,* and of pure cereulide, using cells of human, animal and bacterial origins. Hepatic cell lines and boar sperm, with cytotoxicity and sperm motility, respectively, as the end points, were inhibited by $\leqslant 1$ nM of cereulide present as *B. cereus* extract. RNA synthesis and cell proliferation in HepG2 cells was inhibited by 2 nM of cereulide. These toxic effects were explainable by the action of cereulide as a high-affinity mobile K^+ carrier. Exposure to cereulide containing extracts of *B. cereus* caused neither activation of CYP1A1 nor genotoxicity (comet assay, micronucleus test) at concentrations below those that were cytotoxic (0.6 nM cereulide). *Salmonella typhimurium* reverse mutation (Ames) test was negative. Exposure of *Vibrio fischeri* to extracts of *B. cereus* caused stimulated luminescence up to 600%, independent on the presence of cereulide, but purified cereulide inhibited the luminescence with an $IC_{50\%\ (30\,min)}$ of 170 nM. Thus the luminescence-stimulating *B. cereus* substance(s) masked the toxicity of cereulide in *B. cereus* extracts to *V. fischeri*.
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Keywords: Cereulide; Ionophore; Potassium carrier; *Bacillus cereus*; Genotoxicity; Cytotoxicity; RNA synthesis; Motility; Neutral red uptake (NRU); Micronucleus assay; Comet test (SCG); EROD; CYP1A1; Bioluminescence; Ames test; *Vibrio fischeri*; HEp-2; HepG2; Hepa-1; Boar sperm

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1. Introduction

Bacillus cereus is a Hazard group 2 classified gram positive bacterium (European Commission, 1993) and increasingly investigated as a safety risk in mass catering and convenience foods, including in infant food (Batt, 2000; Reiche, 2004; Becker et al., 1994; Duc et al., 2005; Pirhonen et al., 2005). *B. cereus* produces many toxins, and its ingestion usually links to mild illness, but the fatal and serious cases of human illness connected to this bacterium are believed to be caused by the toxin cereulide, produced by certain strains (Takabe and Oya, 1976; Mahler et al., 1997; Dierick et al., 2005). Cereulide is chemically and thermally stable, with molecular size of 1153 g/mol (Andersson et al., 1998a). Its structure has been proven by chemical synthesis as a cyclic peptide consisting of three repeats of two amino acids and two hydroxy acids, (D-Ala–D-*O*-Leu–L-Val–L-*O*-Val)$_3$ (Isobe et al., 1995). The chemical assay, based on liquid chromatography-ion trap mass spectrometry (LC-MS), allows measuring the exact contents of cereulide in bacteria, foods and environmental samples (Häggblom et al., 2002; Jääskeläinen et al., 2003a).

The toxicology of cereulide in mammalian cells is as yet poorly known. Primates and human, but not mice (Yokoyama et al., 1999), are highly sensitive to this toxin. The dose resulting into acute serious illness in healthy adult persons was reported to be $\leqslant 8\,\mu g$ of cereulide kg^{-1} d wt (Jääskeläinen et al., 2003b). This is similar to that reported earlier for rhesus monkey (Shinagawa et al., 1995) and musk shrew (*Suncus murinus*) (Agata et al., 1995). The in vitro toxicity assays that have been used so far to detect toxicity of cereulide were based on HEp-2 cell vacuolization (Hughes et al., 1998), the MTT metabolization test (Finlay et al., 1999), the boar spermatozoan motility inhibition assay (Andersson et al., 1998, 2004; Rajkovic et al., 2006) and an assay based on uncoupling of respiration of rat liver mitochondria (Kawamura-Sato et al., 2005).

In this paper we present the toxicological profile obtained for cereulide by applying a battery of selected in vitro toxicity assessment methods with eukaryotic and prokaryotic target cells and using inhibition endpoints ranging from macromolecular syntheses to energy metabolism and disruption of subcellular targets. The task was executed as multi-centre exercise with laboratories from three different countries.

2. Materials and methods

2.1. B. cereus strains and preparation of the bacterial extracts

The strain F4810/72 used is the most widely used emetic toxin (cereulide) producing strain of *B. cereus* (Turnbull et al., 1979; Andersson et al., 1998, 2004). The strain GR177, isolated from a dairy product in Sweden, was described in detail by Ehling-Schulz et al. (2005). The cereulide non-producing strains *B. cereus* NS61, isolated from a spruce tree (Hallaksela et al., 1991) and the strain P113 from raw potato, were confirmed by bioassay and by LC-MS as cereulide non-producing strains (Andersson et al., 2004) with a detection limit $0.9\,ng\,mg^{-1}$ cells fresh wt.

The *B. cereus* extracts were prepared from 3 to 4 g (fresh wt) of biomass grown on tryptic soy agar plates for 24 h at 28 °C plates. The bacterial biomass was extracted with methanol (100 mg wet wt ml^{-1}) as described in Andersson et al. (1998). The extracts were heated for 10 min at 100 °C, evaporated to dryness for measuring the concentration of extracted substance: 38.4 mg (strain F4810/72), 5.0 mg (strain GR177), 38.6 mg (strain NS 61) and 6.3 mg (strain P113) of dry substance ml^{-1}. Methanol was then added to restore the original volume and the cereulide concentration was determined by LC ion trap MS method. The assay is based on the cereulide specific mass ions with m/z of 1175 (Na^+ adduct), 1192 (K^+ adduct), 1171 (NH_4^+ adduct) and 1154 (H^+) and calibrated with valinomycin, described in detail by Jääskeläinen et al. (2003a). The extracts contained 90 μg (F4810/72), 24 μg (GR177) or $<0.01\,\mu g$ (NS61, P113) of cereulide ml^{-1} of extract. Purified cereulide was prepared from strain F4810/72 as described by Mikkola et al. (1999).

2.2. Cell toxicity assays

2.2.1. Tests with human larynx carcinoma cell line HEp-2

The cells were cultivated as described by Severin et al. (2005). Microplates of 96 wells were seeded with of 9×10^3 cells $well^{-1}$ in 250 μl of medium and treated, when semi-confluent, with the different dilutions of *B. cereus* methanol-extracts for 24 and 48 h. Alternatively, for the colony-forming ability assay, the cells were seeded in dishes (diameter 35 mm) at a density of 1.5×10^5 cells $dish^{-1}$ and, when semi-confluent, treated with the different

dilutions of *B. cereus* extracts for 24 and 48 h. The vehicle control was methanol alone (max. 1 vol%). The endpoints were used to evaluate toxicity were neutral red uptake (NRU), total protein content (TPC) and colony forming ability (CFA), performed as described in detail by Severin et al. (2005).

2.2.2. Assays for cytotoxicity and ethoxyresorufin-O-de-ethylase (EROD) activity in mouse hepatoma cell line Hepa-1

A mouse hepatoma cell line Hepa-1 that has retained the CYP1A1 activity (Hankinson, 1979) was used for testing compounds that could be metabolically transformed or induce the metabolizing enzyme system. Hepa-1 cells were grown as monolayer as described by Severin et al. (2005). When the cells were well attached and the culture was about 60% confluent, the cells were exposed to the test extracts by replacing the culture medium with a medium containing either serially diluted *B. cereus* culture extract or the positive control substances, 2,3,7,8-TCDD (synthesized in the State Institute of Agricultural Chemistry, Helsinki, Finland, TCDD content ca. 80%, Paasivirta et al., 1977) or β-naphtoflavone (β-NF, Sigma, St. Louis, USA) for the EROD activity, and 2,4- dinitrophenol (2,4-DNP) for the total protein content (TPC). Untreated cells were used as a negative control. After freezing of the cells at −70 °C and thawing, the EROD activity was measured as described by Koistinen et al. (1998). Briefly, 50 µl of sodium phosphate-buffer was added into the test wells and 100 µl to the reference wells. Ethoxyresorufin (0.4 mM in DMSO), diluted in sodium phosphate-buffer, was added to final concentration of 2 µM. Fluorescence of resorufin was measured using excitation/emission wavelengths of 530/590 nm and a resorufin standard curve was measured for each bioassay. The positive controls were 2,3,7,8-TCDD (1.25 nM) and β-NF (4.0 µM). The functionality of the assay was verified by measuring the EROD-activity of hepatic cells from a 2,3,7,8-TCDD-exposed rat. These were obtained from Department of Physiology, University of Kuopio (Kuopio, Finland), and they served as a historical control for the method. Subsequently the cytotoxicity was measured with TPC as an endpoint using the method of Kennedy and Jones (1994) as described by Severin et al. (2005). EROD-activity (pmoles of resorufin formed per minute per mg protein) was calculated per protein content of the cells.

2.2.3. RNA-synthesis inhibition test with HepG2 cells

The in vitro RNA synthesis inhibition was measured by automated test as a 30 min kinetic measurement uptake of tritiated uridine into the cellular macromolecules procedure, as described by Severin et al. (2005).

2.2.4. Inhibition of boar spermatozoan motility

The extended boar semen, a commercial product obtained from an artificial insemination station, contained 27×10^6 spermatozoa ml^{-1}. It contained 2–5 mM of extracellular potassium and 150–200 mM sodium. The extended semen was exposed to 1 vol% of the test extract or its dilutions in methanol for 24 h at room temperature. Inhibition of sperm motility post-exposure was assessed and the toxicity endpoint converted into cereulide equivalents as described by Hoornstra et al. (2003), using purified cereulide (Mikkola et al., 1999) for calibration.

2.3. Assays for genotoxicity

2.3.1. Salmonella/his reverse mutation test

The plate incorporation assay, based on the revised method of Maron and Ames (1983), was used. The testing was conducted with *Salmonella typhimurium* strains TA98 and TA100 which detect frameshift and base pair substitution mutations, respectively, and the strain TA102, which detects base pair mutations and small deletions. The external metabolic activation was done using rat liver S9 fraction, purchased in lyophilised form from Iffa Credo (Lyon, France), prepared from phenobarbital-methylcholantrene-induced rat livers. Benzo[a]pyrene (Sigma, St Louis MO USA), 1 µg (solved in DMSO) $plate^{-1}$ (for the strains TA98 and TA100), and methyl methanesulphonate (Sigma), 0.1 µl $plate^{-1}$ (for the strain TA102) were used as positive reference substances. Dilutions of the *B. cereus* extracts were done in methanol (Merck, Darmstadt, Germany). Replicate plates, with and without S9 mix, were used for each dose level.

2.3.2. Single Cell Gel (SCG)/comet assay

HepG2 cells were stored in ampoules in liquid nitrogen. For the test, the cells were thawed quickly, washed with medium, and grown in an incubator at 37 °C in a 5% CO_2 atmosphere. The culture medium was EMEM (Sigma) supplemented with 2 mM L-glutamine (Gibco; Paisley, UK), 1% non-essential

L-amino acids (NEAA; Sigma), and 10% heat-inactivated foetal bovine serum (FBS; Gibco). In the experiments always fresh cells were used.

The SCG assay was performed following the protocol of Valentin-Severin et al. (2003). Briefly, wells of a 96-well tissue culture plate were plated with 5×10^4 cells in 0.2 ml of the culture medium. Six wells per test concentration were established. After 28 h of incubation, the cells were treated with the *B. cereus* extract (dissolved in methanol), the positive control chemical (B[a]P, dissolved in DMSO) and the solvent controls (2.5 μl ml^{-1}). *B. cereus* extract concentrations for the tests based on preliminary experiments on cell counts and viability (Trypan blue exclusion) measured after exposure. The IC_{50} values from the RNA-synthesis inhibition assay with HepG2 were also considered. The cells were exposed for 20 h in complete EMEM supplemented with 0.5% FBS. At the end of the exposure period, the cells were washed with Ca^{2+} and Mg^{2+} -free phosphate-buffered salt solution (PBS; Gibco) and harvested with trypsin-EDTA solution (Gibco). Olive tail moment (a measure of length between the head and the tail and the amount of DNA in the tail) was used as the metric to characterize the DNA damage in individual cells.

2.3.3. Micronucleus assay

The micronucleus test was executed essentially as described by Valentin-Severin et al. (2003). Briefly, the cells were grown as described for the SCG test. Duplicates of 1.5×10^6 cells in 3 ml of the culture medium were established for each test concentration. After 24 h of incubation, the cells were exposed to the test substances and the vehicle (methanol) for 20 h in 2 ml complete EMEM supplemented with 0.5% FBS. Preliminary experiments were done to find the suitable test concentrations (0.039–0.313 μg ml^{-1}) for the *B. cereus* extracts so that with the highest concentration there were enough binucleated cells (BNC) for the scoring of micronuclei. After the exposure, the cells were washed with PBS (with Ca^{2+} and Mg^{2+}) and further incubated in EMEM with cytochalasin B (4.5 μg ml^{-1}, 30 h, Sigma), then swelled, harvested after trypsinisation and fixed for microscopy as described by Valentin-Severin et al. (2003). The fixed cells were spotted on glass slides and stained in acridine orange (AO; 62.5 μg ml^{-1} in Sørensen buffer pH 6.8) for 1 min and washed (2×2 min in Sørensen buffer pH 6.8) and covered in Sørensen buffer (pH 6.8) for the analysis. Micronuclei were scored in BNC by a fluorescence microscope using the following criteria: (1) the diameter of a micronucleus had to be less than 1/3 of that of the main nucleus, (2) the micronucleus had to be surrounded by a clear membrane, (3) without touching the main nucleus.

To assess the cytotoxicity, the proportions of mononucleated cells (MNC), binucleated cells (BNC) and polynucleated cells (PNC) were counted in 1000 cells and cell proliferation (cytokinesis-block proliferation index, CBPI) was calculated.

2.4. Luminescent bacteria test

The test was performed by the method of Jokinen et al. (2001), modified from the ISO standard 11348-1:1998(E). The modifications involved exposure temperature (25 °C instead of 15 °C used in ISO standard) and adapting the protocol for an automated instrument (BioOrbit 1251 Luminometry System, Turku, Finland). *Vibrio fischeri* (DSM 7151) was obtained from Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany). In brief, *V. fischeri* suspensions for the test were prepared from colonies grown on agar plates, made by adding agar in The Liquid Growth Medium (described in the ISO standard). Luminescent colonies from the plate were suspended into 2 ml of 2% (w/v) NaCl and pretested for luminescence as 10^{-4} dilution made in 2% NaCl. The bacterial test suspension used for the assay was $3–10 \times 10^{-3}$ dilution (depending on the desired light emission). For the test, 1 vol of each dilution of the test substances was blended with 1 vol of the bacterial test suspension. The test medium of ISO11348 contained 4 mM of K^+ and 341 mM of Na^+ in the final exposure mixture. NaCl solution (341 mM) was used as negative control, two at the beginning of the test and one at the end of the run. Cr^{VI} was used as the standard reference as recommended by ISO 11348. Light emission of the cuvettes measured at 1 min intervals. The EC_{50} values were computed with Excel software.

2.5. Statistical analysis of the data

NRU, TPC and CFA in HEp-2 cells and RNA synthesis assay in HepG2 cells were run in triplicate and repeated three times. The data are expressed as mean ± SD.

The SCG/comet assay results with the HepG2 cells are expressed as means of three independent experiments. One-way ANOVA followed by Dunnett's post hoc test (SPSS for Windows version

12.0.1; SPSS, Inc., Chicago, IL, USA) was used for the analysis of the data. The micronucleus data were analysed with χ^2-test.

3. Results

3.1. Preparation and distribution of the B. cereus extracts

Two strains of *B. cereus* were investigated for stable toxins with in vitro methods. One strain, F4810/72, is a known producer of cereulide and the other, NS61 a non-producer. To avoid interference by the numerous toxins other than cereulide, such as mammalian tissue damaging enzymes and other proteins (Batt, 2000), the toxicity assays were conducted with extracts prepared from the bacterial biomass in 100% methanol. Cereulide is methanol soluble (Andersson et al., 1998) whereas proteins mostly are not. The extracts were cleared of the methanol insoluble matter and the methanol extracts heat treated before use for toxicity assessment. In this way, the difference in the cell toxicity between the extracts prepared from the two strains should reflect the toxic contribution of cereulide.

The extracts were prepared by one laboratory (University of Helsinki) and simultaneously distributed to each of the 6 laboratories (b…g) participating in this project.

3.2. Toxicity of the B. cereus extracts and purified cereulide in the boar spermatozoan motility inhibition assay

The results in Table 1 show that the extract of from *B. cereus* strain NS61 (cereulide non-producing) affected boar sperm motility at concentrations of $>30\,\mu g\,ml^{-1}$. The motility inhibiting concentrations of similarly prepared extract from *B. cereus* F4810/72 were 0.1 to $0.2\,\mu g\,d\,wt\,ml^{-1}$ of boar semen. Thus the extract of strain F4810/72 was $150\times$ more toxic than that from *B. cereus* NS61. The toxicity endpoint of F4810/72 extract, expressed as its cereulide content ($0.23–0.45\,ng\,ml^{-1}$), was closely similar to that of purified cereulide ($0.2–0.5\,ng\,ml^{-1}$). This shows that the sperm motility inhibiting activity of F4810/72 extract was explained by its cereulide content alone.

The same *B. cereus* extracts as shown in Table 1 were subsequently distributed as coded aliquots to the participating laboratories to investigate their toxicities by 10 in vitro methods, employing for different mammalian cell lines/types and two bacterial assays.

3.3. Cytotoxicity of B. cereus extracts

3.3.1. Effects on HEp-2 cell viability and proliferation

Toxicity of the *B. cereus* extracts on HEp-2 cells was investigated by measuring effects on cell viability (NRU and TPC) and cell proliferation (CFA). Extract of the strain NS61 had no measurable effect within 48 h of exposure on any of these three parameters also at the highest tested concentration, $1.6\,\mu g\,d\,wt\,ml^{-1}$. The same concentration of substance from strain F4810/72 inhibited NRU by >95%, reduced the cell protein content (TPC) by 75%, inhibited cell proliferation at 48 h of exposure (Fig. 1). However, at 24 h of exposure cell proliferation was inhibited less than cell viability (data not shown). With lower doses the viability and cell

Table 1
Sperm toxicity of *Bacillus cereus* extracts and purified cereulide

Tested material	Toxicity endpoints in the sperm bioassay	
	EC_{50} ng ml^{-1} of the extended boar semen	
	Bacterial substance (dry wt)	Cereulide
Methanol extract of *B. cereus* NS 61 (cereulide non-producing strain)	>35,000	
Methanol extract of *B. cereus* F-4810/72 (cereulide producing strain)	96–192	0.23–0.45
HPLC purified cereulide from strain F-4810/72, solved in methanol		0.2–0.5

The toxicity endpoints of the test substances were measured by the boar spermatozoan motility inhibition assay, using 1:2 dilution steps. Purified cereulide was used as a reference substance and methanol (1 vol %) as the vehicle control. The bacterial extracts were prepared by soaking plate grown bacterial biomass overnight in 98% methanol. The insoluble debris were removed and the solved material, representing 6% of the total dry wt of the bacteria, was used for testing. Cereulide was purified from *B. cereus* strain F4810/72 as described by Mikkola et al., 1999.

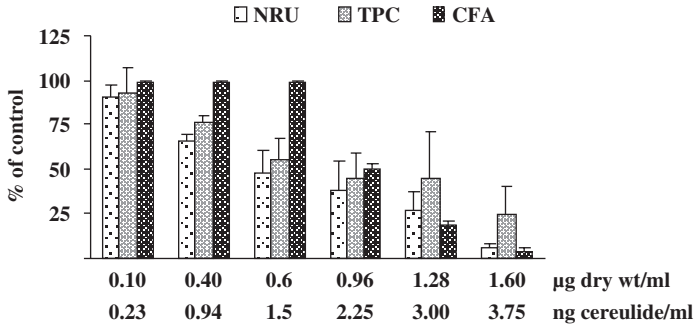

Fig. 1. Cytotoxicity of *B. cereus* extracts on HEp-2 cells. NRU (neutral red uptake) activity, total protein content (TPC) and proliferation (CFA) of the cells exposed for 48 h to different concentrations of methanol soluble substances from *B. cereus* F4810/72. The extract of strain F4810/72 contained 90 µg of cereulide ml^{-1}, and the extract of strain NS 61 $<<0.001$ µg ml^{-1}. Extract prepared from *B. cereus* strain NS61 caused no measurable effects in NRU, TPC or CFA at exposure concentration of 1.6 µg d.wt ml^{-1} (not included in the figure).

proliferation remained $\geqslant 50\%$ at exposure concentrations of $\geqslant 0.64$ and of $\geqslant 0.96$ µg ml^{-1} of F4810/72 extract, respectively. When HEp-2 cells, exposed to 1.60 µg ml^{-1} of extract of the strain F4810/72 (containing 4 ng of cereulide ml^{-1}), were inspected with microscope, a vacuolization of the cells was observed after 24 h exposure and it became massive at 48 h exposure (Fig. 2).

The cell viability and cell proliferation of HEp-2 cells thus were inhibited by the strain F4810/72. As there was no adverse effect observable by exposure to extracts of the cereulide non-producing *B. cereus* strain NS61, the toxicity to this human cell line must have been provoked by exposure to the cereulide present in strain F4810/72. At the IC_{50} concentration the extract contained 1–2 ng of cereulide ml^{-1} (= 1–2 nM, as cereulide molecular weight is 1153).

3.3.2. Cytotoxicity in Hepa-1 cells

Cytotoxicity towards a murine hepatoma cell line Hepa-1 was measured using total protein content (TPC) as the end point. Exposure to the highest tested concentration, 390 µg ml^{-1} of substances from *B. cereus* strain NS61, for 72 h had only slight effect (total protein content was 86% of vehicle control, Fig. 3B) and with 48 µg ml^{-1} there was no detectable toxicity at all (data not shown).

In a range-finding study (Fig. 3A) the extract of the strain F4810/72 proved $>50\%$ cytotoxic upon 24 h exposure up to 12 µg of *B. cereus* F4810/72 substance ml^{-1}. After 72 h exposure total cell death occurred upon exposure to 3 µg ml^{-1} (holding ca.7 ng of cereulide). Microscopic examination revealed that these cells were rounded and swollen, separated from each other and richly vacuolated. Since only slight effect (TPC was 86% of control, Fig. 3B) was seen when Hepa-1 cells were exposed to $\leqslant 390$ µg d wt ml^{-1} of substances of similarly extracted *B. cereus* NS 61 (cereulide non-producing), the devastating effect of the extract of *B. cereus* F4810/72 on the Hepa-1 cells must have been due to its contained cereulide.

When dilutions of extracts of the strain F4810/72 were tested, the TPC of Hepa-1 cells was 86% of control after exposure of 24 h–0.4 µg d.wt of the extract ml^{-1} (contains 0.9 ng cereulide ml^{-1}). Toxicity increased upon exposure of 72 h so that only 23% remained (Fig. 3B) indicating that the no-effect level was below this exposure concentration. Hepa-1 cells were thus >1000 times more sensitive to extract of *B. cereus* strain F4810/72 than to that from strain NS 61.

3.4. Metabolic assays

3.4.1. Cytochrome P_{450} (CYP1A1) activation

CYP1A1 inducing potential of the *B. cereus* extracts was investigating on the basis of EROD activity in exposed Hepa-1 cells. No activity was detected in extracts of the strain F4810/72 (cereulide producing) when tested at exposure concentrations of 0.4 and 0.8 µg d wt ml^{-1} for 24 h , while some transient EROD induction was found in extracts of strain *B. cereus* NS61 (cereulide non-producing) after exposure of 24 h at very high concentrations (180–390 µg ml^{-1})(data not shown). High concentrations or longer exposure times with F4810/72 extract could not be tested because of its high cytotoxicity. The positive reference substance, 2,3,7,8-TCDD, run in parallel, showed marked EROD induction (up to 44 pmol min^{-1} mg^{-1} protein) after a 72 h exposure, and β-naphtoflavone

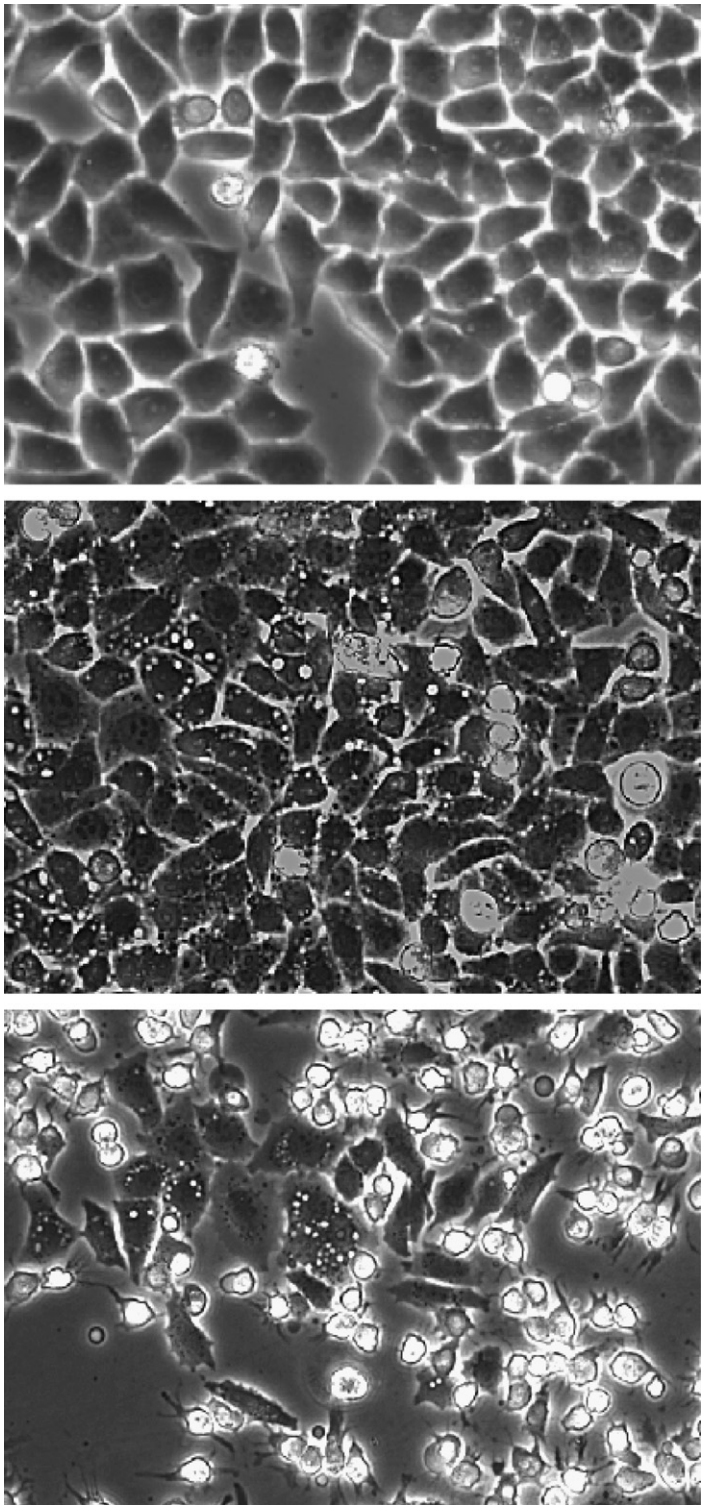

Fig. 2. Morphological alterations in HEp-2 cells exposed to methanol soluble substances from *B. cereus* F4810/72 (cereulide producing). Untreated (A); cells exposed to 1.60 μg of substance (d.wt.) ml^{-1} (contains 3.75 ng cereulide/ml) for 24 h (B) and 48 h (C). The images were taken with phase contrast microscope using 40 × objective.

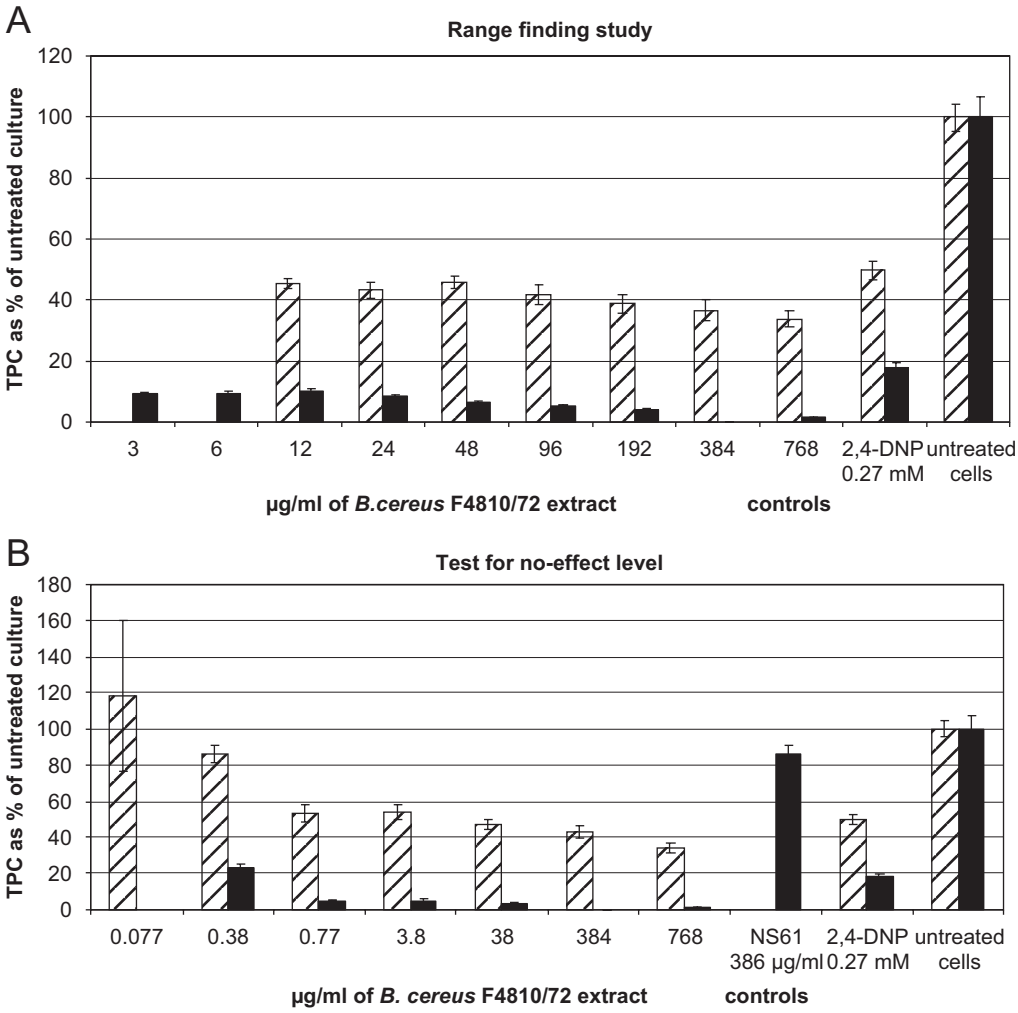

Fig. 3. The cytotoxicity of the *B. cereus* extracts to Hepa-1 cells. Extract from strain *B. cereus* F4810/72 in Hepa-1 cell line in a range finding experiment (3A), and in a subsequent test with higher dilutions and also including extract of the strain NS 61 (3B). Total protein content (TPC) was used as the toxicity endpoint and 2,4 -dinitrophenol (DNP) as the positive reference substance. The SD bars represent intraexperimental variation in the test and are based on 5–8 replicates per tested concentration ■ exposure time 72 h, ▨ exposure time 24 h.

induced EROD to the level of 49 pmol/min/mg protein after 24 h. The expected response of EROD activity to two different positive reference substances proves that the Hepa-1 cells were capable of expressing CYP1A1. The weakly positive EROD response to extract of *B. cereus* strain NS61 must be due to substances other than cereulide.

3.4.2. RNA-synthesis inhibition

HepG2 cells were used to assess the effects of *B. cereus* extracts on mammalian RNA synthesis. The bacterial extracts were sterile filtered before applying on HepG2 cells and the 30-min kinetics of incorporation of ^{3}H-uridine into the cellular macromolecules of HepG2 was measured after 20 h of exposure to the bacterial extract. Substances extracted from the *B. cereus* strain NS61 slowed down RNA synthesis only slightly (< 5%), when applied at the highest concentration (9.6 µg ml^{-1}) as compared to vehicle control (results not shown). In contrast, exposure to the extract from the cereulide-containing strain F4810/72 inhibited the RNA synthesis with an IC_{50} value 0.96 µg ml^{-1} medium. The value was determined as a non linear regression of three independent experiments (Fig. 4). The IC_{50} concentration corresponds to 2 ng of cereulide ml^{-1}.

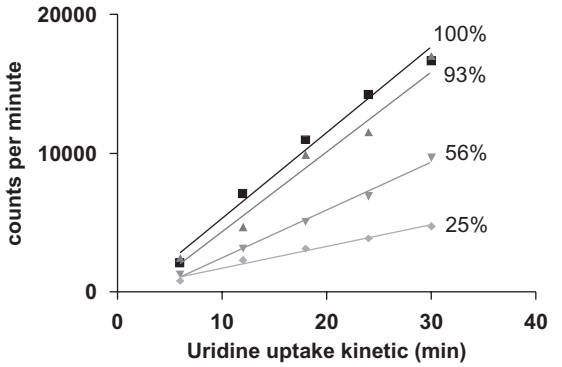

Fig. 4. Effect of *B. cereus* extracts on RNA synthesis in HepG2 cells. The figure shows 20 h exposure to different concentrations of the extract of strain *B. cereus* F4810/72. Percentages represent viability of cells treated with extract from strain F4810/72 compared to non treated cells, set as 100% (the raw data of the slope of the regression line is set as 100% of viability). Control regression line value, $y = 614.7x - 823.9$. Vehicle control, 100%; 0.096 μg d.wt ml^{-1}, 93%; 0.96 μg d.wt. ml^{-1} 56%; 9.6 μg d. wt.ml^{-1}, 25%. The extract of *B. cereus* F4810/72 contained 2.3 ng of cereulide μg^{-1} d. wt.

The IC_{50} values of F4810/72 extracts towards HepG2 cell RNA synthesis were thus approximately the same as those found cytotoxic (NRU, cell proliferation) to HEp-2 cells (Fig. 1).

3.5. Genotoxicity of the B. cereus extract

3.5.1. The SCG/comet assay

The SCG tests were carried out with HepG2 cells applying exposure concentrations of 0.3–5 μg of *B. cereus* F4810/72 methanol soluble substance per ml and benzo(a)pyrene (BaP, 30 μM) as the positive reference compound. The resulting Olive tail moments (OTM) induced by the F4810/72 extract were low, less than 2 × compared to vehicle control (methanol), and there was no dose response (Fig. 5A). The *B. cereus* F4810/72 extract thus appeared non-genotoxic in the comet assay at exposure concentrations $\leqslant 5$ μg ml^{-1}. The highest tested concentration of strain F4810/72 extract corresponded to exposure of 12 ng cereulide ml^{-1} (10 nM).

3.5.2. Cytotoxicity based on the test concentrations used in the micronucleus assay

Preliminary experiments were done to find the correct range of test concentrations for the highly cytotoxic *B. cereus* F4810/72 (see Sections 3.3.1, 3.3.2 and 3.4.2). The micronucleus assay slides contained enough binucleated cells for the scoring of micronuclei up to exposure to 0.313 μg of *B. cereus* F4810/72 extract d.wt ml^{-1}. This caused cytotoxicity of 50% ± 5% (Fig. 5B). The 0.3 μg d wt ml^{-1} concentration is close to the IC_{50} value observed with *B. cereus* F4810/72 extract for murine hepatoma cells (Hepa-1, 0.4 μg ml^{-1}), and lower that for the human epithelial carcinoma cells (HEp-2, 1–2 μg ml^{-1})and that observed in the RNA synthesis inhibition assay with HepG2 cells, 0.96 μg ml^{-1} (see Sections 3.3.1, 3.3.2, 3.4.2). The around 50% cytotoxicity observed in the micronucleus assay with the highest F4810/72 extract concentration is likely to be due to cereulide, of which the IC_{50} thus would be 1 ng ml^{-1}.

3.5.3. Micronucleus test

Micronucleus test was done by exposing HepG2 cells to methanolic extract of the *B. cereus* strain F4810/72, using benzo(a)pyrene (6.25 μM in DMSO) as the positive reference substance and methanol and DMSO as vehicle controls (Fig. 5B). The frequency of BNC with micronuclei (MNBNC) in HepG2 cells exposed to the *B. cereus* extract was not different from that observed in the vehicle control cultures, i.e. 6–7‰. The frequency of MNBNC after exposure to 0.156 μg d. wt of *B. cereus* F4810/72 ml^{-1} (containing 0.4 ng of cereulide ml^{-1}) was slightly elevated but $< 2 \times$ that of the controls. The effect was not dose-related nor statistically significant. The highest exposure concentration, 0.313 μg ml^{-1} (containing 0.7 ng of cereulide ml^{-1}) of the extract yielded fewer micronuclei than the second-highest concentration, possibly due to the high cytotoxicity of this exposure (Fig. 5B). It can be concluded that cereulide is not or only slightly genotoxic at exposures $\leqslant 0.4$ ng ml^{-1} and that genotoxicity for higher cereulide exposures cannot be measured with the cytokinesis block micronucleus test using HepG2 cells due to its high cytotoxicity towards these cells.

3.5.4. The S. typhimurium /his reverse mutation test

Genotoxicity of cereulide, the *B. cereus* emetic toxin, was evaluated also by the Ames test, targeted for detecting point mutations (base pair substitutions, frameshifts and small deletions). For this purpose *Salmonella typhimurium* strains TA 98, TA 100 and TA 102 were exposed to the *B. cereus* F4810/72 extract. Benzo(a)pyrene (strains TA98, TA100) and methyl methanesulphonate (strain TA102) were used as positive reference substances.

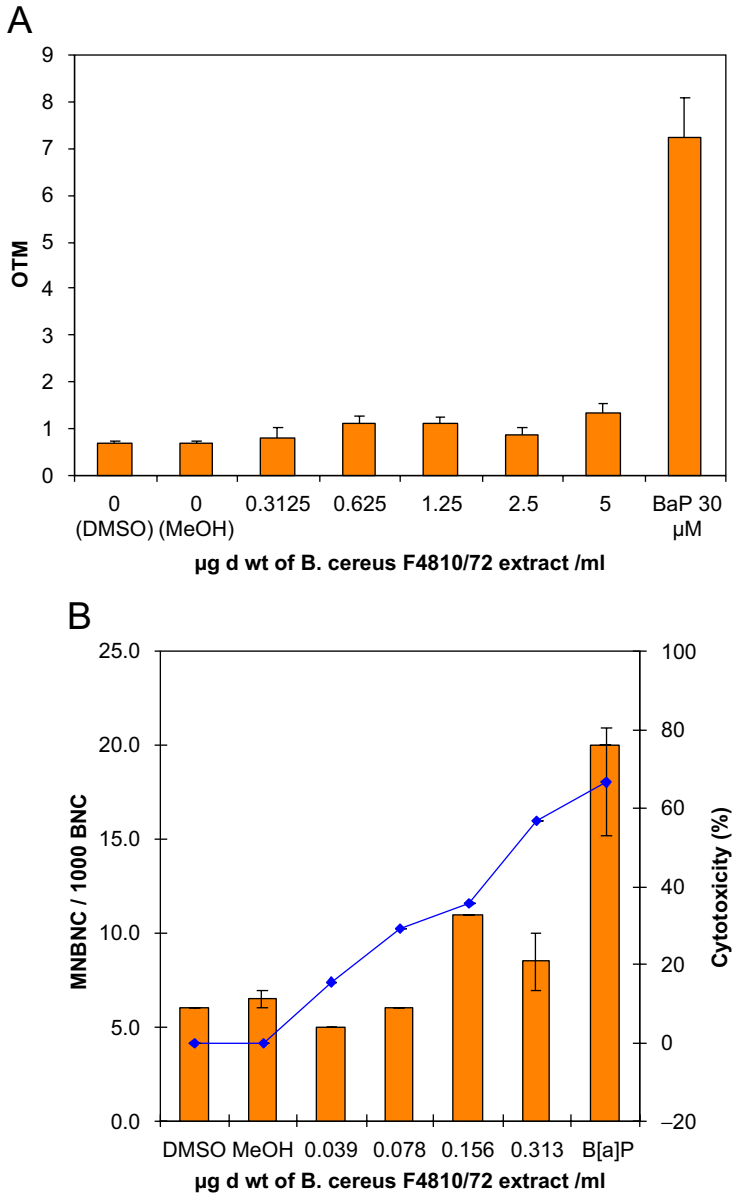

Fig. 5. Genotoxicity of *B. cereus* F4810/72 extract in HepG2 cells. (A, top). Comet assay genotoxicity expressed as Olive tail moment (OTM). Values are means ± S.E. of three experiments. (B, bottom). The figures shows the frequency of binucleated cells with micronuclei (MNBNC, columns) and cytotoxicity (continuous line, determined from cell proliferation). The B[a]P (benzo(a)pyrene) data are from a separate experiment.

The tests were carried out with and without external metabolic activation (rat liver microsome fraction S9). The exposure doses ranged from 10 to 160 µg plate^{-1} of substance extracted into methanol from the *B. cereus* strain. The numbers of mutants scored with the positive control substances were within the expected range. The scores obtained for the test doses of the *B. cereus* extract did not differ from the mutant scores obtained with solvent vehicles (methanol, DMSO), with and without metabolic activation with S9 (Fig. 6). It is thus concluded that the extract of *B. cereus* strain F4810/72 was negative in the *S. typhimurium* reverse mutation assay up to doses of 160 µg of methanol soluble substance per culture plate. This dose is equivalent to 370 ng of cereulide.

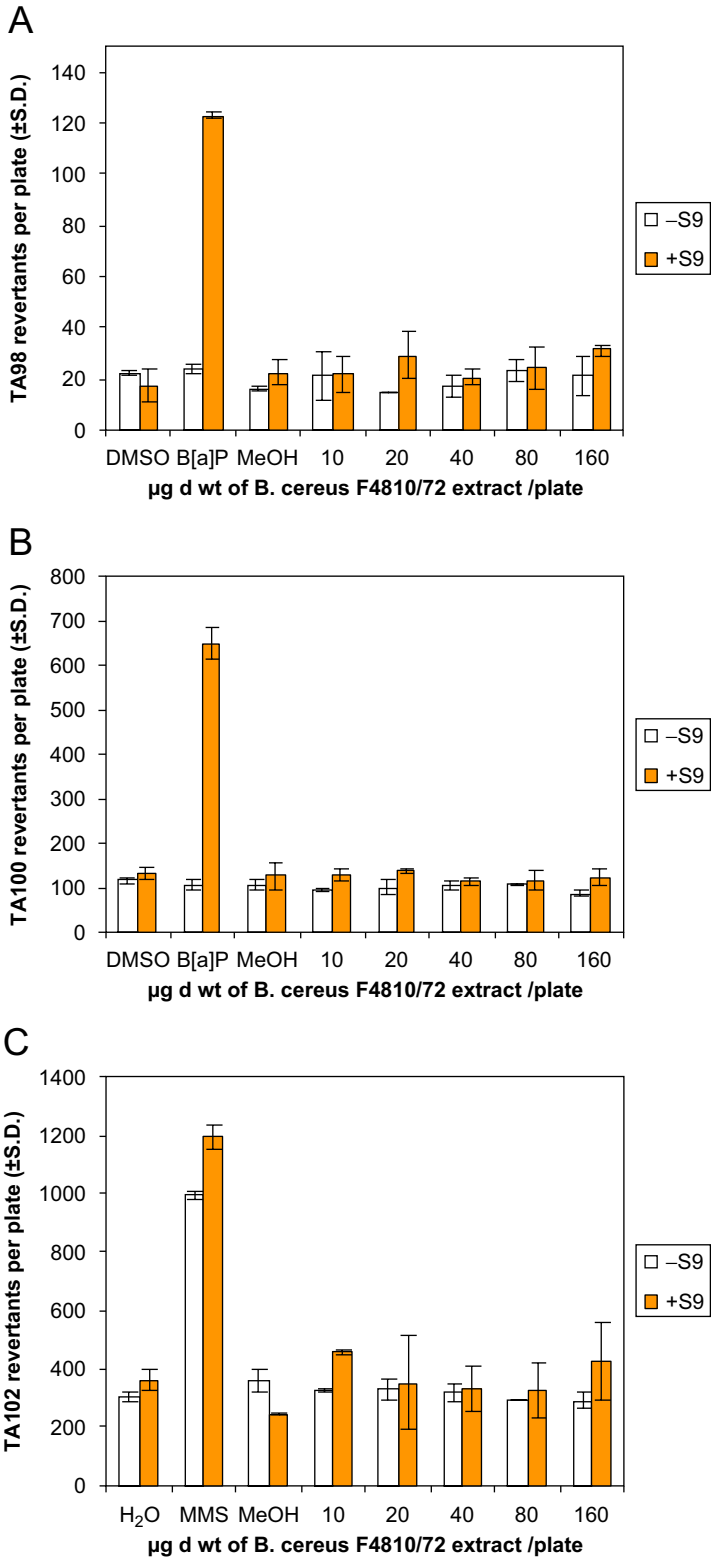

Fig. 6. Mutagenicity of *B. cereus* F4810/72 extract in the Salmonella/his reverse mutation (Ames) test without (−S9) and with (+S9) metabolic activation. The mutant (revertant) values are means of replicate plates. B[a]P (benzo(a)pyrene, 1 µg plate^{-1}) and methyl methane sulphonate (0.1 µl plate^{-1}) were used as positive reference substances. The solvent vehicles methanol and dimethylsulphoxide (DMSO) were used as negative controls. Panels: A, top; B, center; C, bottom.

3.6. Luminescent bacteria test (bioluminescence by *V. fischeri*)

The results obtained with the luminescent bacterium *V. fischeri* are shown in Fig. 7. The test was repeatable within each strain of *B. cereus*. The negative control substance, ethanol, was tolerated without any loss of light emission within the test concentrations 0.01–5 vol%. The tested range of exposure concentrations of the *B. cereus* extracts was 0.002–5 mg d wt ml^{-1}.

Fig. 7A shows the response of *V. fischeri* to the same extracts of *B cereus* strains F4810/72 and GR177(cereulide producing). An intense (up to 500%) stimulation of light emission up to 500% was seen, the intensity depending on the time and the dose of exposure. Inhibition of light emission was not observed below exposure concentrations higher than 0.1 or 2 mg biomass (dry wt) ml^{-1}, with the GR177 and F4810/72, respectively. To answer the question, whether the stimulation of *V. fischeri* was caused by cereulide, purified cereulide was used for exposure (Fig. 7B). The result showed that there was no stimulation—on the contrary, only inhibition of light emission was seen. The severity of inhibition increased with dose and exposure time: $IC_{50\%}$ of *V. fischeri* by cereulide was 1.3 μg (1100 nM) when the exposure time was 5 min, and decreased to 0.44 μg (380 nM) and to 0.2 μg (170 nM) ml^{-1} when the exposure time was extended from to 15 and 30 min, respectively. Fig 7C show the result obtained with extracts from *B. cereus* strains (NS61, P113) not producing cereulide. The result shows that also these strains contained heat stable substances strongly (100–600%) stimulating light emission of *V. fischeri*.

We conclude from the results that light emission of *V. fischeri* was inhibited by cereulide but that *V. fischeri* was less sensitive ($IC_{50\%,\ 30\,min}$ 170 nM) to cereulide than the functions of mammalian cells tested under Sections 3.1–3.5, where $IC_{50\%}$ values of 1 and 2 nM were measured. However, the inhibitory effect of cereulide (Fig. 7B) was masked by the stimulatory substances when extracts prepared from whole bacteria, rather than pure cereulide, was used for the test. This finding indicates that false negative results may be obtained when *V. fischeri* is used for assessing toxicity in samples that contain large amounts of bacteria.

4. Discussion

This study aimed at an overall picture of the toxicity of cereulide, known as a ionophoric peptide (Agata et al., 1994, Andersson et al., 1998a) with high affinity to K^+ (Mikkola et al., 1999; Teplova et al., 2006). To achieve this, three lines of somatic cells, two human (HEp-2, HepG2) and one of mouse (Hepa-1), sperm cells (boar) and bacteria (*S. typhimurium*, *V. fischeri*) were used as target cells. The test methods were chosen among those that are well established (Maron and Ames, 1983; Kaiser, 1998; Richardson, 1993; Tice et al., 2000; Kirsch-Volders et al., 2003), widely used and represented different cellular toxicity end points: cytotoxicity (cell viability and proliferation, RNA-synthesis), metabolic activation (CYP1A1 induction), genotoxicity (comet assay, micronucleus assay and *Salmonella* mutagenicity test) and bacterial bioluminescence. The results obtained by the different methods and laboratories are compiled in Table 2.

The sperm assay was reported as the most sensitive detection method for cereulide (Andersson et al.,1998, 2004; Hoornstra et al., 2003; Kawamura-Sato et al., 2005; Mikami et al., 2004; Rajkovic et al., 2006). This paper showed that the no observed effect level (NOEL) of cereulide towards the hepatic carcinoma cell lines Hepa-1 and HepG2 (with TPC 72 h or NRU 48 h as end points) was similarly low ($\leqslant$1 nM) as that towards boar spermatozoa (0.2–0.5 nM, motility inhibition as end point). The cytotoxicity of cereulide towards mammalian somatic cells was thus similar to that of the important mycotoxins deoxynivalenol (DON) and T2 toxin, of which 0.3–0.4 nM was cytotoxic to HEp2 cells upon exposure of 2 days (Calvert et al., 2005). The higher sensitivity of the hepatic cell lines (HepG2, Hepa-1) towards cereulide as compared to the epithelial cell line (HEp2), observed in the present study, was to be expected based on the high hydrophobicity of cereulide (log $K_{ow} = 5.96$. Teplova et al., 2006).

When RNA synthesis was measured as an endpoint for HepG2 cells, an IC_{50} of around 2 ng of cereulide ml^{-1} (i.e. 2 nM) was calculated (Table 2) for exposure of 20 h. The toxicity of cereulide towards this essential cellular process may thus be higher than that of *Fusarium* T2 toxin, for which inhibition was reported at 100 nM after an exposure of 4 h (Sorenson et al., 1986).

The vacuolization of mitochondria observed in HEp-2 cells exposed to the bacterial extract containing 4 ng ml^{-1} (3.5 nM) of cereulide (Fig. 2) confirms earlier reports on the same cell line carried out with cereulide containing extracts prepared from food (Sakurai et al., 1994), *B. cereus* culture (Mikami et al.,

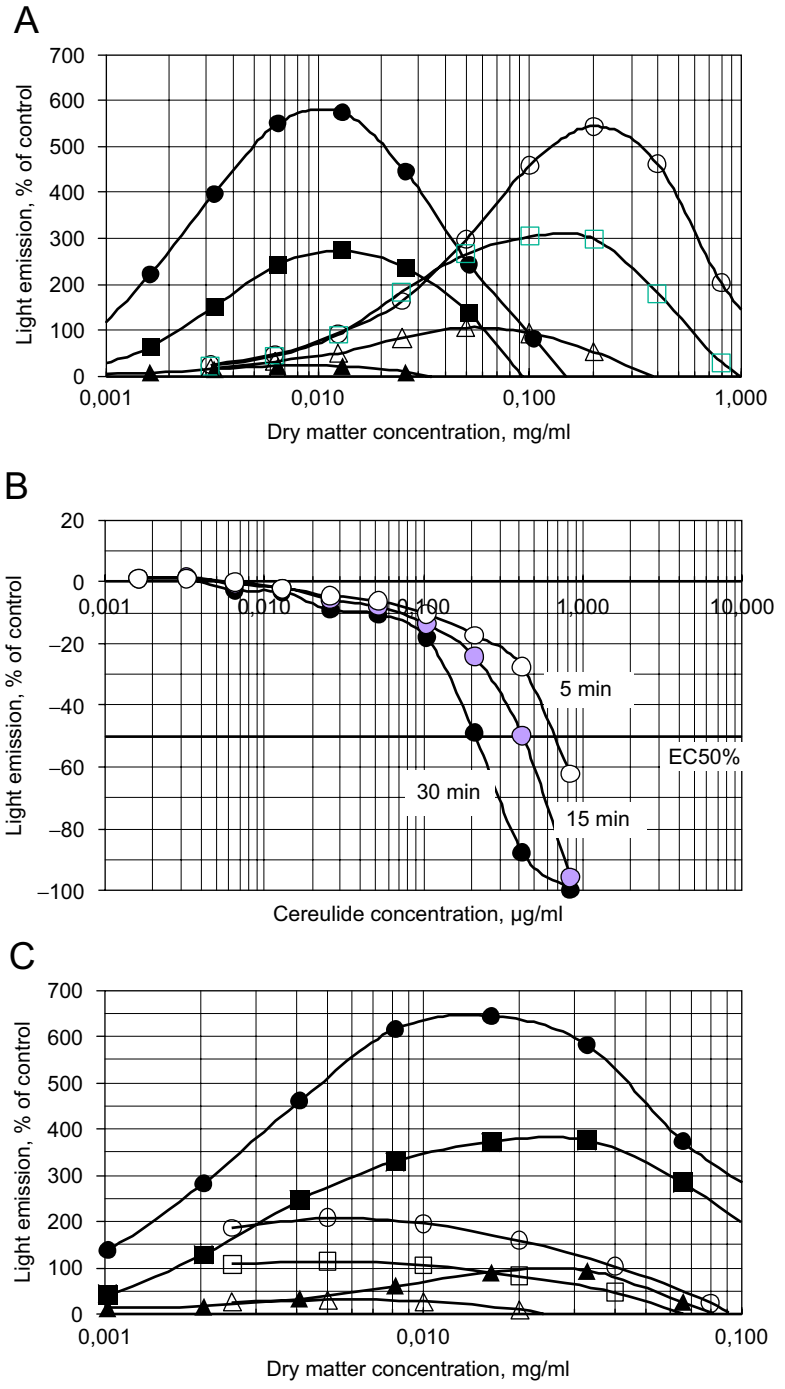

Fig. 7. Impacts of *Bacillus cereus* extracts and purified cereulide on light emission of *Vibrio fischeri*. Panel A shows the effects of extracts of strains *B. cereus* F4810/72 (open symbols) and GR177 (filled symbols), containing 2.3 and 4.8 µg of cereulide mg^{-1} d. wt, respectively. Panel B, purified cereulide from strain F4810/72. Symbols for exposure times: triangles, 5 min; squares, 15 min; circles, 30 min. Panel B, purified cereulide from strain F 4810/72. Exposure times are shown in the graph. Panel C, the effects of extracts of strains containing no cereulide (<0.0002 µg mg^{-1} d. wt). NS61 (open symbols), P113 (filled symbols). Exposure times: triangles, 5 min; squares, 15 min; circles, 30 min.

Table 2
Cell toxicities of extracts prepared from cereulide producing strain of *B. cereus* F4810/72 and cereulide, observed with different target cells and toxicity endpoints

Cellular system	Laboratory	Toxicity endpoints measurement		Effective exposure concentration[g]	
		Parameter	$[K^+]$ in medium (mM)	*B. cereus* F4810/72 extract, (d.wt ml^{-1}) (measured)	Cereulide (calculated)
HepG2	ENSBANA[a]	Inhibition of RNA synthesis	5.3	0.96 μg ml^{-1} (IC_{50})	2 nM
HEp-2	ISS[b]	Cytotoxicity (TPC)	5.3	0.6 μg ml (IC_{50})	1 nM
HEp-2	ISS	Cytotoxicity (NRU)	5.3	0.6 μg ml (IC_{50})	1 nM
HEp-2	ISS	Inhibition of cell proliferation (CFA)	5.3	1.0 μg ml (IC_{50})	2 nM
HEp-2	ISS	Vacuolisation	5.3	$EC_{50\%} < 1.6$ μg ml^{-1}	< 3.2 nM
Hepa-1	UKU[c]	Cytotoxicity (TPC)	5.3	LTC : 0.4 μg ml (24 h) LTC : $\ll 0.4$ μg ml (72 h)	0.8 nM (24 h) $\ll 0.8$ nM (72 h)
Hepa-1	UKU	CYP1A1 activation (EROD activity)	5.3	Not detected at 0.8 μg ml (the highest possible concentration)	1.6 nM
S. typhimurium $\geqslant 0.4$ nmol $plate^{-1}$	NPHI[d]	Direct mutagenicity (Ames)	115	160 μg $plate^{-1}$ or higher	
S. typhimurium $\geqslant 0.4$ nmol $plate^{-1}$	NPHI	Mutagenicity after metabolic activation (Ames)	115	160 μg $plate^{-1}$ or higher	
HepG2	NPHI	Genotoxicity (Comet)	5.3	5 μg ml^{-1} or higher	$\geqslant 10$ nM
HepG2	NPHI	Genotoxicity (Micronucleus)	5.3	0.3μg ml^{-1} or higher	$\geqslant 0.6$ nM
Boar spermatozoon	UHDACM[e]	Motility inhibition	2–5	$IC_{50\%}$: 0.1–0.2 μg ml^{-1} (24 h)	–0.4 nM (24 h) 0.2–0.4 nM[8] (24 h)
V. fischeri	KCL[f]	Inhibition of luminescence	4	$IC_{50,\%}$ 0.1–1.5 mg ml^{-1}(15 min); 0.15–0.4 mg ml^{-1} (30 min)	380 nM (15 min)[h] 170 nM (30 min)[h]

Toxicity of cereulide was calculated based on the excess of toxicity of extract from *B. cereus* F4810/72 (cereulide producing) as compared to the similarly prepared extract from *B. cereus* NS61 (cereulide non-producing). $[K^+]$ in exposure media were measured (extended boar semen) or calculated based on the compositions of the *V. fischeri* TestMedium (ISO 11348-1), *S. typhimurium* (Vogel-Bonner E) and Minimal Essential Medium (MEM, Sigma) used for exposures of cultured human and animal cells.

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[b]Istituto Superiore di Sanita Roma, Italy.

[c]Institute of Applied Biotechnology, University of Kuopio, Finland.

[d]National Public Health Institute Kuopio, Finland.

[e]Department of Applied Chemistry and Microbiology, University of Helsinki, Finland.

[f]Oy Keskuslaboratorio KCL, Espoo, Finland.

[g]IC_{50}, concentration causing inhibition of 50%; EC_{50}, concentration causing 50% of maximal effect. LTC, lowest toxic concentration, for TPC < 80% of the untreated cells.

[h]Measured with purified cereulide.

1994) or with purified natural and synthetic cereulide (Agata et al., 1994). Vacuolization was also shown in human natural killer (NK) cells by exposed to purified cereulide (Paananen et al., 2002).

Cereulide is a mobile K^+ carrier structurally and functionally related to valinomycin (Agata et al., 1994; Andersson et al., 1998; Mikkola et al., 1999). Valinomycin has been observed to induce drastic loss of [K^+] from the cytoplasm of rat hepatocytes (Bolken and Zierold, 2002). Exposure to cereulide was shown to induce uptake of K^+ in isolated, energized rat liver mitochondria independent on the external K^+ concentration (Teplova et al., 2006). Cereulide thus may induce efflux or influx of K^+, depending on the conditions: in nonenergized membranes cereulide causes leakage of K^+ down the concentration gradient and in the presence of sufficient electric transmembrane potential cereulide causes migration of K^+ towards the negatively charged side of the membrane.

Exposure to cereulide was shown to depolarise mitochondria of boar sperm cells (Hoornstra et al., 2003), and human epithelial (Jääskeläinen et al., 2003b), neural (Teplova et al., 2004) and NK cells (Paananen et al., 2002). Due to its extremely high affinity to K^+, cereulide is an active carrier of K^+ even in exposure media with low [K^+] (Teplova et al., 2006) such as foods or blood plasma (3.5–5 mM, Levine, 2002). The concentration of [K^+] in all exposure media used in this study (Table 2), excepting the *S. typhimurium* Ames test, was close to that in blood plasma. The depolarisation of mitochondria most likely results from the cereulide mediated influx of cytoplasmic K^+ ions to mitochondria. Depolarization deprives the mitochondrial ATP synthase of its driving force. This explains why cereulide, even though it does not transfer protons (Mikkola et al., 1999), causes uncoupling of ATP synthesis in respiring rat liver mitochondria, such as recently reported by Kawamura-Sato et al. (2005).

We propose that the cereulide induced impairment of the cellular K^+ gradients and of the mitochondrial function are the key pathway of the cytotoxicity, inhibition of cell proliferation, RNA synthesis and motility in human and animal cells, as observed in the present study. [K^+] gradients across membranes of cells and organelles are known as crucial regulators of cellular Ca^{2+} levels and metabolism (Nelson and Cox, 2005).

The EC_{50} values for the micronucleus test and for the induction of CYP1A1 (EROD) were not possible to measure because there was no measurable effect at concentrations below those that were cytotoxic to the test cells (HepG2). Also no genotoxicity was observed in the comet assay, micronucleus assay or *S. typhimurium* mutagenicity assay at any of the exposure concentrations tested.

The conditions for measuring effects of toxicants on the luminescence of *V. fischeri* are defined by ISO 11348 (a medium with no energy substrate and with 4 mM K^+). Under those conditions extracts of *B. cereus* strains massively stimulated luminescence of *V. fischeri* cells at low exposure concentrations (0.002–0.5 mg d wt ml^{-1}). Enhancement of light emission in response to toxic exposure has been reported in *V. fischeri* exposed to potassium cyanide, at concentrations sufficient to block respiration (Karatani et al., 2004). The stimulation observed in the present work by substance(s) produced by *B. cereus*, was not by the toxin cereulide. It may have been be caused other heat-stable substances, emitted by cereulide producing as well as nonproducing strains of *B. cereus*, e.g. quorum sensing signal molecules, that are known to be involved in regulation of bioluminescence by *V. fischeri*. The quorum sensing signal substances are known to be structurally related accross species and genera (Daniels et al., 2004). The purified cereulide strongly inhibited light emission of *V. fischeri*. The manyfold (up to 600%) but variable stimulation of light emission provoked by the substances emitted by *B. cereus* makes that the *V. fischeri* assay cannot reliably be used to detect toxicity in environments that may contain interfering bacterial substances. This should be taken into account when *V. fischeri* ISO 11348 method is used for detecting toxicity in natural materials, e.g. soils or composts. This aspect attention, considering the wide use of this test (Richardson, 1993; Kaiser, 1998) for ecotoxicological monitoring.

Summarizing, the four types of mammalian cells used in this study to assess toxicity, exhibited a toxic response upon exposure to cereulide containing *B. cereus* (Table 2). HepG2 cells, Hepa-1 cells and boar sperm cells, with TPC and motility inhibition, respectively, as the endpoints, were most sensitive (responding to $\leqslant$1 nM cereulide). The second most sensitive endpoints (1–2 nM) were obtained for RNA synthesis inhibition and cell proliferation, measured in human cell lines HepG2 and HEp2. The results described in this paper demonstrate that a mobile ion carrier substance like cereulide is likely to be detected as a highly toxic substance by many

different end points, in somatic cells as well as spermatozoa.

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