Potato Crop as a Source of Emetic *Bacillus cereus* and Cereulide-Induced Mammalian Cell Toxicity

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Bacillus cereus, aseptically isolated from potato tubers, were screened for cereulide production and for toxicity on human and other mammalian cells. The cereulide-producing isolates grew slowly, the colonies remained small ($\sim$1 mm), tested negative for starch hydrolysis, and varied in productivity from 1 to 100 ng of cereulide mg (wet weight)$^{-1}$ ($\sim$0.01 to 1 ng per 10^5 CFU). By DNA-fingerprint analysis, the isolates matched *B. cereus* F5881/94, connected to human food-borne illness, but were distinct from cereulide-producing endophytes of spruce tree (*Picea abies*). Exposure to cell extracts (1 to 10 µg of bacterial biomass ml^{-1}) and to purified cereulide (0.4 to 7 ng ml^{-1}) from the potato isolates caused mitochondrial depolarization (loss of $\Delta\Psi$m) in human peripheral blood mononuclear cells (PBMC) and keratinocytes (HaCaT), porcine spermatozoa and kidney tubular epithelial cells (PK-15), murine fibroblasts (L-929), and pancreatic insulin-producing cells (MIN-6). Cereulide (10 to 20 ng ml^{-1}) exposed pancreatic islets (MIN-6) disintegrated into small pyknotic cells, followed by necrotic death. Necrotic death in other test cells was observed only after a 2-log-higher exposure. Exposure to 30 to 60 ng of cereulide ml^{-1} induced K$^+$ translocation in intact, live PBMC, keratinocytes, and sperm cells within seconds of exposure, depleting 2 to 10% of the cellular K$^+$ stores within 10 min. The ability of cereulide to transfer K$^+$ ions across biological membranes may benefit the producer bacterium in K$^+$-deficient environments such as extracellular spaces inside plant tissue but is a pathogenic trait when in contact with mammalian cells.

Cereulide, the emetic toxin of *Bacillus cereus*, is most likely responsible for the severe cases of illness connected to the consumption of food contaminated with *B. cereus* (1–9). Cereulide-producing *B. cereus* isolates are frequently reported in processed foods, implicated (3–16) or not implicated (17–21) with food-borne illness, but appear infrequently in farming or natural outdoor environments (22–26).

B. cereus is known to occur in the rhizomicrobiota and endophytic community of plants, as well as in root vegetables, including potato (27), but these habitats have not been searched for cereulide producers (28). The extracellular spaces of plants, as well as natural waters, including soil water, contain <1 mM K$^+$ ions, whereas the concentration of K$^+$ in the interior of the cytoplasmic space of plant cells, as well as in bacteria, is >100 mM (29, 30). Bacteria living in the extracellular spaces of the tuber of a crop plant must thus compete for K$^+$ ions with its plant host and with other bacteria inside the crop plant.

Cereulide is known to be a heat-stable cyclic depsipeptide (6, 31, 32) with high affinity and selectivity for sequestering K$^+$ ions from a low-potassium environment (33–35). We recently found (36) that an endophytic, cereulide-producing *Bacillus cereus* strain (37) from *Picea abies* (Norway spruce), had a competitive advantage against nonproducers in potassium-deficient (<1 mM K$^+$) but not in potassium-rich (30 mM K$^+$) media. We hypothesized that the ability to produce cereulide might give a similar advantage to *B. cereus* in the intercellular spaces of root crops. Furthermore, we also sought to determine whether *B. cereus* could use its produced cereulide to withdraw K$^+$ ions from intact mammalian cells, with pathological consequences. We describe here the isolation of cereulide-producing endophytic *B. cereus* from potato tubers and show the mitochondrial toxicity and the potassium-translocating effects of the produced cereulide on human, porcine, and murine primary cells and cell lines.

MATERIALS AND METHODS

Isolation of cereulide-producing potato endophytic *B. cereus*. Healthy potatoes (*n* = 5, *Solanum tuberosum*) with no visible rot or scab lesions purchased from a local supermarket were washed with water, peeled, surface sterilized with 96% ethanol, and flamed. An aseptically cut piece from the tuber interior was streaked onto nonselective medium (tryptic soy agar [TSA]). After 7 to 10 days at 22°C, colonies with a *B. cereus*-like appearance (a few colonies per plate) were picked for pure culturing. The cultures were initially screened for heat-stable toxin production as follows. Biomass ($\sim$10 mg [wet weight]) looped from the culture plate was suspended in methanol (0.2 ml) in a screw-cap glass vial with Teflon lining and placed in boiling water for 15 min. After cooling, the vials were vortexed (2 min), and the obtained lysates were tested for sperm motility inhibition (100%) by a rapid assay as described previously (38). The methanol lysates that inhibited motility within 15 min in the rapid assay were serially diluted in methanol (10-fold dilution series), and no more than 20 µl of the dilutions was applied to boar spermatozoa (2 ml, 27 × 10^6 cells ml^{-1}) for 1 and 4 days at 22 ± 2°C. Corresponding volumes of methanol only served as a control. For reading the 100% effective concentration (EC$_{100}$), the exposed sperm suspension was prewarmed to 37°C

(Thermoblock), and the motility loss (taken as 100% of the immotile cells) was determined by phase-contrast microscopy (×40 objective lens) using a heated (37°C) stage.

Characterization of the potato isolates. DNA fingerprinting (ribopatterns) was performed using automated equipment (Riboprinter microbial characterization system; DuPont Qualicon, Wilmington, DE) with whole-cell lysates using EcoRI and Pvu2 for the cleavage. A commercial library (DuPont version 2.1.4216.0) amended with an in-house library (containing 120 well-characterized strains of *B. cereus*) was used for species identification as described previously (20). Starch hydrolysis was tested according to established methods (39) using *B. cereus* DSM31^T as the positive and strain F4810/72 as the negative reference.

Purification, identification, and quantitation of cereulide from the potato endophytes. Cereulide was purified from the lysates (isolate AB1A, strain NS-58) as described previously (33, 36, 40). The purified compound was identified with mass spectrometry (MS) and tandem MS (MS/MS) analyses. The concentration of cereulide was measured by liquid chromatography-electrospray ionization ion trap MS using an isocratic elution with 0.1% formic acid (solvent A) 6% and methanol (solvent B) 94% at a flow rate of 1 ml min^{-1} with an Atlantis C$_{18}$ column T3 (4.6 by 150 mm, 3 μm; Waters, Milford, MA) and valinomycin for calibration.

Target cells used for *in vitro* toxicity assessments. Porcine spermatozoa were retrieved from boars (ejaculates of eight individuals), delivered by a commercial supplier (Figen, Ltd., Tuomikylä, Finland) as suspensions of 27 × 10^6 cells ml^{-1} in a commercial extender (MR-A), and stored at 18 to 20°C until used (within 48 h) for assays (2). PK-15 is a porcine kidney tubular epithelial cell line (41), cultured as described earlier (40). It was used because cereulide is known to cause renal dysfunction of Na$^+$/K$^+$ homeostasis. Human monocyte-enriched peripheral blood mononuclear cells (PBMC) were purified from buffy coats of healthy individual donors (five separate batches) obtained (with ethical permission) from the Finnish Red Cross Blood Service (Helsinki, Finland) by centrifugation using the Ficoll gradient method of Bøyum (42) and Valmu et al. (43), washed four times in phosphate-buffered saline (PBS; Mg^{2+}- and Ca^{2+}-free) at 22°C, resuspended in RPMI 1640 (complete medium) to 5 × 10^6 cells ml^{-1}, and used within 36 h. The viability of each batch of PBMC was measured by trypan blue exclusion (Countess cell counter; Invitrogen) as described previously (44). The PBMC were used to model blood cells that become exposed when cereulide is sorbed from the gut into the arteries. The non-tumor-derived epithelial cell line, HaCaT, originates from adult human skin and exhibits normal differentiation (45). MIN-6 cells, kindly donated by J. Miyazaki (46), is a murine pancreatic beta cell line that grows as islets and retains glucose-inducible insulin secretion. The murine fibroblast cell line L929 was obtained from the American Type Culture Collection (ATCC) and used to show responses of a rodent cell line other than the beta MIN-6 cells.

All cells, except for the boar spermatozoa, were maintained in an atmosphere of 95% air, 5% CO$_2$, 37°C, and 95% relative humidity in a cell culture cabinet (Heracell 150i; Thermo Fisher Scientific, Vantaa, Finland). The HaCaT, PK-15, and L-929 cell lines were grown in RPMI 1640 (complete medium), and the MIN-6 cells were grown in Dulbecco modified Eagle medium (DMEM; with supplements [see below]). HaCaT, PK-15, and L-929 cells were adherently cultured. For passaging HaCaT and PK-15, the monolayers of ca. 70% confluence were incubated with 0.02% EDTA for 5 to 10 min, followed by 0.05% (wt/vol) trypsin in 0.02% (wt/vol) EDTA for 3 to 5 min. MIN-6 cells (grown as attached islets) were washed three times with PBS and treated with trypsin for 20 s; the flask was then turned upside down and allowed to drip for 50 to 70 s at 37°C, and the trypsin activity was stopped by rapidly adding 20 to 30 ml of freshly supplemented DMEM.

Toxicity assays. The boar spermatozoa in commercial extender (MR-A, containing 2 to 5 mM K$^+$ and 150 to 200 mM Na$^+$), was exposed as a suspension as described by Hoornstra et al. (47). PBMC (5 × 10^6 cells ml^{-1}) in suspension were exposed in RPMI 1640 medium. The HaCaT, L-929, PK-15, and MIN-6 cells were grown in 8-well flat-bottom chamber glass slides, seeded to a density of 4 × 10^4 cells ml^{-1}, in the respective medium for 48 h. MIN-6 cells were exposed as islet-like agglomerates, attached to the chamber slide bottom, while the other cells were exposed as monolayers. Assays were performed by adding the test substance (solved in methanol, <1 vol%) into the growth medium and incubated as indicated.

After exposure, the test cells were stained with the fluorogenic dye JC-1 (for membrane potential) or calcein AM combined with propidium iodide (live/dead staining) and the nucleus stain Hoechst 33342 as described previously (48, 49), either as slide cultures or as suspensions in microtiter plate wells. Free dye was removed by washing with PBS, and the slides were immediately analyzed with a fluorescence microscope (Zeiss Axiovert 200; Carl Zeiss, Inc., Jena, Germany) using a band-pass filter at 450- to 490-nm excitation and a long-pass filter at 515-nm emission of the JC-1 green and orange fluorescence and propidium iodide red fluorescence simultaneously. Methanol (≤1% [vol/vol]), used as the vehicle, gave no measurable effect alone. All assays were done in triplicate. The standard deviation within a culture and within a cereulide preparation was <20%. Variation between biological replicates (new lot of cultured cells, new preparation of cereulide) was ≤40%.

Assay of potassium efflux from intact cells. Monolayers of HaCaT keratinocytes were detached with trypsin and washed three to four times with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) for trypsin inhibition. The cells were pelleted by centrifugation at 10,000 rpm (Rotofix 32; Hettich, Germany) for 2 min (HaCaT cells), 5 min (PBMC), or 10 min (sperm cells). The cell pellet was resuspended in 0.2 ml of K$^+$-free medium and placed in a measurement cuvette (1.2 ml) provided with magnetic stirring, temperature control (24°C), and a potassium ion-selective electrode (Niko-Analit, Moscow, Russia) linked to PC recording software (Record 4; NIKO-ANALIT, Moscow, Russia). Efflux of K$^+$ from the cells was recorded (once per second) by determining the extracellular concentration of potassium. The electrode signal was calibrated by adding 100 μmol of KCl (100 mM stock) into the cuvette at the end of each run and recording the corresponding rise in the electrode response for extracellular K$^+$ concentration, as done in a previous study (40). An extracellular K$^+$ concentration of 0.9 mM saturates cereulide with K$^+$, whereas 0.3 mM K$^+$ saturates only partially (40).

Media, reagents, and disposables. Biosynthetic cereulide was purified from the *B. cereus* strains AB1A (potato endophyte) and NS58 (Norway spruce endophyte) as described previously (40).

K$^+$-free medium contained 150 mM NaCl, 5 mM NaH$_2$PO$_4$, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 5 mM glucose, and 10 mM HEPES (pH adjusted to 7.2 with Trizma base). RPMI 1640 (Lonza, Verviers, Belgium) was supplemented with 2 mM L-glutamine, 10% FBS, 50 IU of penicillin (Sigma, St. Louis, MO)/ml, and 50 μg of streptomycin (Sigma)/ml (complete medium). DMEM (Sigma) was supplemented with sterile-filtered solutions of 3.7 g NaHCO$_3$/liter, 100 μl of 50 mM β-mercaptoethanol (Gibco, Paisley United Kingdom)/liter, 15% FBS, 10 ml of 1 M HEPES (adjusted to pH 7.4), 1.25 ml of penicillin-streptomycin solution/liter (Sigma; 10,000 U and 10 mg/ml, respectively), and HEPES buffer, glutamine, valinomycin, and PBS (Mg^{2+}- and Ca^{2+}-free; Sigma). Ficoll-Paque Plus (endotoxin < 0.12 endotoxin units ml^{-1}) was from GE Healthcare Bioscience AB (Uppsala, Sweden), and TSA was from Scharlau Chemie (Barcelona, Spain). The fluorogenic dyes—JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide; a membrane-potential-responsive fluorogenic dye, dissolved in dimethyl sulfoxide [DMSO]), propidium iodide (dead stain propidium iodide, 2.4 mg ml^{-1} in water), and calcein AM (live stain, 1 mg ml^{-1} in DMSO)—were all obtained from Invitrogen (Carlsbad, CA). The other reagents were obtained from local suppliers and of analytical quality. The culture flasks and chamber slides were from Nunc (Roskilde, Denmark).

***B. cereus* reference strains and their ribopatterns.** Food poisoning isolates F4810/72 (HAMBI 2454/SMR-178/CWG52702) (20, 21, 50, 51, 52), F5881/94 (20, 21, 51), LMG17604 (51), B315 (52), and ML60 (54) and strains not connected to food-borne illness, including DSM31^T
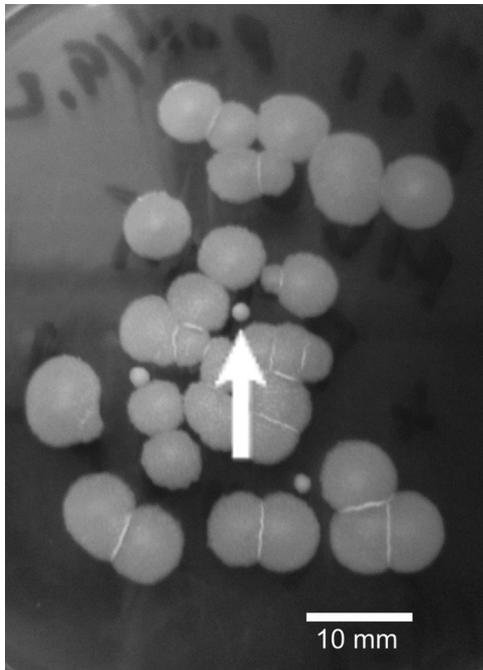
FIG 1 Typical appearance of cereulide-nonproducing (ML60, large colonies) and cereulide-producing (B315, small colonies) *B. cereus* isolates cultivated on the same tryptic soy agar plate.

(ATCC 14579) [51] and MIF1 [21], were obtained from the HAMBI laboratory collection (University of Helsinki). The ribopatterns (EcoRI and Pvu2) of reference strains can be found in references 20, 21, 50, and 51.

RESULTS

Heat-stable toxin-producing endophytic *B. cereus* from consumer potatoes. Bacteria were aseptically cultured from the interior of healthy potato tubers. Bacillus-like (gram plus rods) colonies (<10% of all colonies on the plate) were pure cultured and screened for the presence of heat-stable substances toxic to mammalian cells. To assess for the presence of toxic metabolites, the biomass of each potato isolate was dispersed into methanol, heat treated, and tested for the ability to inhibit cell motility using boar spermatozoa as test cells. The cultures yielding heat-stable extracts that, at a high dilution, inhibited the motility of sperm cells distinguished themselves from the major colony type (on the same plate) by their small size (Fig. 1). The TSA plates were incubated for 7 to 10 days, because after a 2-day incubation, as prescribed in the standard isolation protocols for food-borne *B. cereus* [55, 56], the small colonies were barely visible. After extended cultivation of the plates, the difference between small and large *B. cereus*-like colonies on potato tuber-seeded plates was easy to see. DNA-fingerprinting (ribopatterns obtained with EcoRI and Pvu2) and physiological tests showed that the pure cultures obtained from the small colonies (Fig. 1) represented *B. cereus* sensu stricto, whereas the large *B. cereus*-like colonies were identified as *B. cereus*, *B. mycoides*, *B. thuringiensis* (i.e., *B. cereus* sensu lato), or *B. subtilis* (Fig. 2).

Table 1 shows the toxic endpoints of lysates of 11 independent cultures of potato tuber endophytes. The toxic endpoint, EC_{100}, here indicates the amount of bacterial biomass (in mg [wet weight] ml^{-1}) required to inhibit the motility of 100% of the
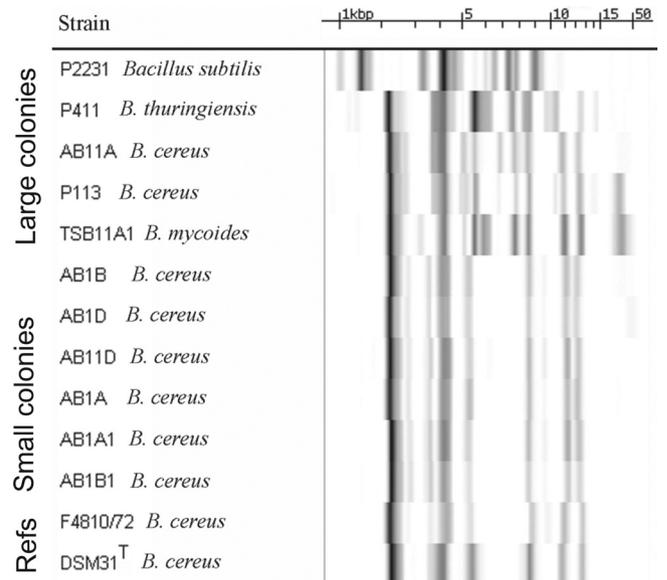
FIG 2 Fingerprints of the ribosomal operon area of *Bacillus* sp. isolated from potato tubers. Ribopatterns were generated with the automated Riboprinter using restriction enzyme EcoRI and visualized by hybridization to phosphorescence-labeled ribosomal operon of *E. coli*. The figure shows the identical ribopatterns of cereulide-producing, small colonies (Fig. 1), in contrast to the diversity of ribopatterns of the large-colony-forming *B. cereus* sensu lato that did not produce cereulide. The species names are indicated by the nearest matching ribopattern using a commercial library and an in-house database (120 well-characterized strains) of *B. cereus*. Patterns of strains *B. cereus* DSM31[T] (ATCC 14579[T]) and F4810/72 are shown as a reference. The measure bar shows the calibration of the fragment sizes in kilobases, obtained with molecular marker mixes of 1.1, 2.2, 3.2, 6.5, 9.6, and 48 kb.

exposed spermatozoa (27×10^6 ml^{-1}). Depolarization, i.e., loss of the mitochondrial transmembrane potential ($\Delta\Psi$m), was recorded as a shift in fluorescence emission of the JC-1-stained mitochondrial sheath, located in the midpiece of the sperm tail, from orange-yellow to green. As seen in Table 1 and Fig. 2, only the isolates with a small-colony type yielded methanol lysates that inhibited the motility and concomitantly depolarized the mitochondria of the sperm cells at the same exposure concentrations, ranging from 0.0001 to 0.01 mg of the bacterial lysate ml^{-1}, corresponding to 10^5 to 10^7 CFU equivalents.

Lysates of small-colony potato isolates (Fig. 2) and of *B. cereus* F4810/72 provoked the same effects in sperm cells, whereas the type strain of *B. cereus*, DSM31 (ATCC 14579, not producing cereulide), neither inhibited sperm motility nor depolarized the sperm mitochondria, with exposure concentrations up to 1 mg ml^{-1} (Table 1). General cytotoxic effects in the sperm cells, recorded as the emission of red fluorescence by propidium iodide, occurred only after exposure of up to 100- to 1,000-fold-higher concentrations of the same lysates (>1 mg ml^{-1}; Table 1).

Identification of the heat-stable substance produced by potato tuber *B. cereus* isolates. Figure 3 shows the results of liquid chromatography-MS (LC-MS) analysis and MS/MS of the toxic substance purified from the methanol extract of the potato isolate *B. cereus* AB1A. The total ion chromatogram of the toxic high-pressure liquid chromatography (HPLC) fractions is shown in Fig. 3A. The main peak at retention time 9.8 min had mass ions at m/z 1,153.8, m/z 1,171.1, m/z 1,175.8, and m/z 1,191.7 (Fig. 3B). These mass ions matched the $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$,

TABLE 1 Inhibition of motility, loss of mitochondrial membrane potential ($\Delta\Psi$m), and damage to cell membrane of boar spermatozoa by exposure to lysates prepared from plate-grown cultures of endophytic isolates from potato tuber and reference strains of *B. cereus*

Potato tuber isolate or reference *B. cereus* strain	Toxic dose (EC_{100}) in mg of lysed bacteria ml^{-1} categorized by toxicity endpoint[a]			
	Motility inhibition		Loss of $\Delta\Psi$m	Cell membrane damage
	1 day	4 days		
Potato tuber strains				
P2231	>1	>1	>1	>1
P411	>1	>1	>1	>1
AB11A	>1	>1	>1	>1
P113	>1	>1	>1	>1
TSB11A1	1	0.1	0.1	0.1
AB1B	≤0.01	≤0.01	≤0.01	>1
AB1D	≤0.1	0.001	0.001	>1
AB11D	≤0.01	≤0.01	≤0.01	>1
AB1A	0.001	≤0.001	≤0.001	>1
AB1A1	≤0.01	0.0001	≤0.001	>1
AB1B1	0.01	≤0.01	≤0.01	>1
Reference *B. cereus* strains				
F4810/72[b]	0.0001	0.0001	0.0001	>1
DSM31[Tc]	>1	1	1	>1

[a] EC_{100} indicates the concentration at which 100% of the exposed sperm cells (27×10^6 ml^{-1}) were affected. The dilution step was step 10. The $\Delta\Psi$m was measured by using the membrane potential sensor dye JC-1. Cell membrane damage was detected as relaxed permeation of the cells toward propidium iodide. The exposure time was 4 days, except as noted for the motility inhibition.
[b] Cereulide producing (53).
[c] Cereulide nonproducing.

and $[M+K]^+$ ions, respectively, of cereulide from the emetic reference strain *B. cereus* F4810/72 (2). This was verified by MS/MS fragmentation of the purified toxic peak with *m/z* 1,171.1 of the potato isolate *B. cereus* AB1A (Fig. 3B) as a precursor ion (Fig. 3C). The b ion series (b_1 to b_8) observed corresponded to the depsipeptide sequence of *O*-Val-Val-*O*-Leu-Ala-*O*-Val-Val-*O*-Leu-Ala, and the mass ion at *m/z* 385 (b_8) matched the protonated tetradepsipeptide *O*-Val-Val-*O*-Leu-Ala (at *m/z* 384) unit. Biosynthetic cereulide consists of three similar tetradepsipeptide (*O*-Val-Val-*O*-Leu-Ala) units forming a cyclic structure (*O*-Val-Val-*O*-Leu-Ala)$_3$. The MS and MS/MS spectra of the purified toxic compound of *B. cereus* AB1A were similar to the previously published MS data for cereulide produced by the endophytic *B. cereus* NS58 (36). Therefore, the LC-MS and MS/MS analysis showed that the main toxic peak (peak 1 in Fig. 3A) of *B. cereus* AB1A was cereulide.

The cereulide content of *B. cereus* AB1A and of the other five (Table 1) sperm toxic *B. cereus* isolates in Table 1 was quantified with the LC-MS using the area of mass ions $[M+NH_4]^+$ at *m/z* 1,171.1 and $[M+Na]^+$ at *m/z* 1,175.8, using the corresponding ions of valinomycin (at *m/z* 1,129.1 and 1,133.8) as a reference. The results (Table 2) show that six of the eight *B. cereus* isolates produced cereulide, but productivities (measured from cultures grown 4 days on TSA plates by the quantitative LC-MS method) were lower than that of the cereulide-producing reference strain F4810/72 and that of the Norway spruce endophyte NS58 grown under the same conditions. Furthermore, Table 2 shows that the

productivities of highest and lowest producers differed by 2 log units. The measured cereulide productivity divided potato isolates of *B. cereus* sensu stricto into three groups as follows: AB1A (medium) > AB1A1, AB1D, AB1B AB1D, and AB1B1 (low) and P113 and AB11A (no detectable cereulide). The measured toxicities (EC_{100} values, Table 1) divided the isolates similarly into three groups: highly toxic, EC_{100} = 0.001 mg/ml (AB1A); moderately toxic, EC_{100} = 0.01 to 0.1 mg/ml (AB1A1, AB11D, AB1B, AB1D, and AB1B1); and weakly toxic or none, $EC_{100} \geq 1$ (P113 and AB11A). The synchrony between the toxic potential (Table 1) and the cereulide contents (Table 2) of the potato tuber isolates over a 2-log range supports the view that the main toxic agent was cereulide, and the differences between the isolates show that they were not clonal.

Genotyping of the potato endophytic cereulide-producing *B. cereus*. Figure 2 shows the EcoRI cleavage ribopatterns of six cereulide-producing (toxic) isolates from five potato tubers. The six patterns were identical and distinct from nonproducers (strains P113 and AB11A). The patterns differed from that of *B. cereus* F4810/72 (cereulide-producing reference strain) in bands >10 kb in size. When the ribopatterns of the six cereulide-producing potato isolates were compared to those from 120 well-characterized *B. cereus* isolates in our laboratory database, a match was found (both in EcoRI and in Pvu2) with *B. cereus* F5881/94, a cereulide-producing isolate from a food-borne outbreak in United Kingdom (20, 50).

Detecting the toxicity of cereulide to somatic mammalian cells. Table 3 summarizes the toxic endpoints of cereulide against five different human, porcine, and murine primary cell lines. The toxic endpoints of mitochondrial depolarization (i.e., the loss of $\Delta\Psi$m) by 24 h of exposure to cereulide for human PBMC, human keratinocytes, porcine kidney proximal tubular epithelial cells, murine L-929 fibroblasts, and pancreatic β-cells (MIN-6) ranged from 0.0004 to 0.002 μg ml^{-1} (median, 0.002 μg ml^{-1}). This finding is similar to that for boar sperm cells (0.001 μg ml^{-1}), suggesting that boar spermatozoa, which are easy to handle and require no tissue culture facilities, have the potential of being a realistic surrogate toxicity sensor for observing cereulide-induced mammalian cell mitochondrial toxicity. In addition, boar sperm cell motility loss occurred at the same exposure concentration at which $\Delta\Psi$m was lost (Table 3). The motility loss (EC_{100}) can be observed with a phase-contrast or dark-field microscope, with no need for fluorogenic dyes and epifluorescence microscopy in a darkroom.

Table 3 further shows that necrotic cell death, visualized as relaxed cell membrane permeability to propidium iodide, was observed only at exposures 100- to 1,000-fold higher (>0.4 to 2 μg ml^{-1}) than for mitochondrial depolarization. Thus, exposure to cereulide caused mitochondrial damage in keratinocytes (HaCaT), PBMC, kidney epithelial cells (PK-15), spermatozoa, and fibroblasts (L-929), whereas the cells were alive and able to exclude the propidium iodide dye. This is illustrated in Fig. 4 for the keratinocytes and the PBMC, double stained with the membrane potential indicator dye JC-1 and propidium iodide. The orange-red fluorescence in JC-1-stained cells indicates high mitochondrial membrane potential ($\Delta\Psi$m > 140 mV), and green fluorescence indicates dissipated mitochondrial membrane potential ($\Delta\Psi$m < 100 mV). The unexposed cells (solvent only, 0 ng of cereulide, left panels) show the JC-1 orange-red fluorescence of highly energized mitochondria that fill the cytoplasm around the
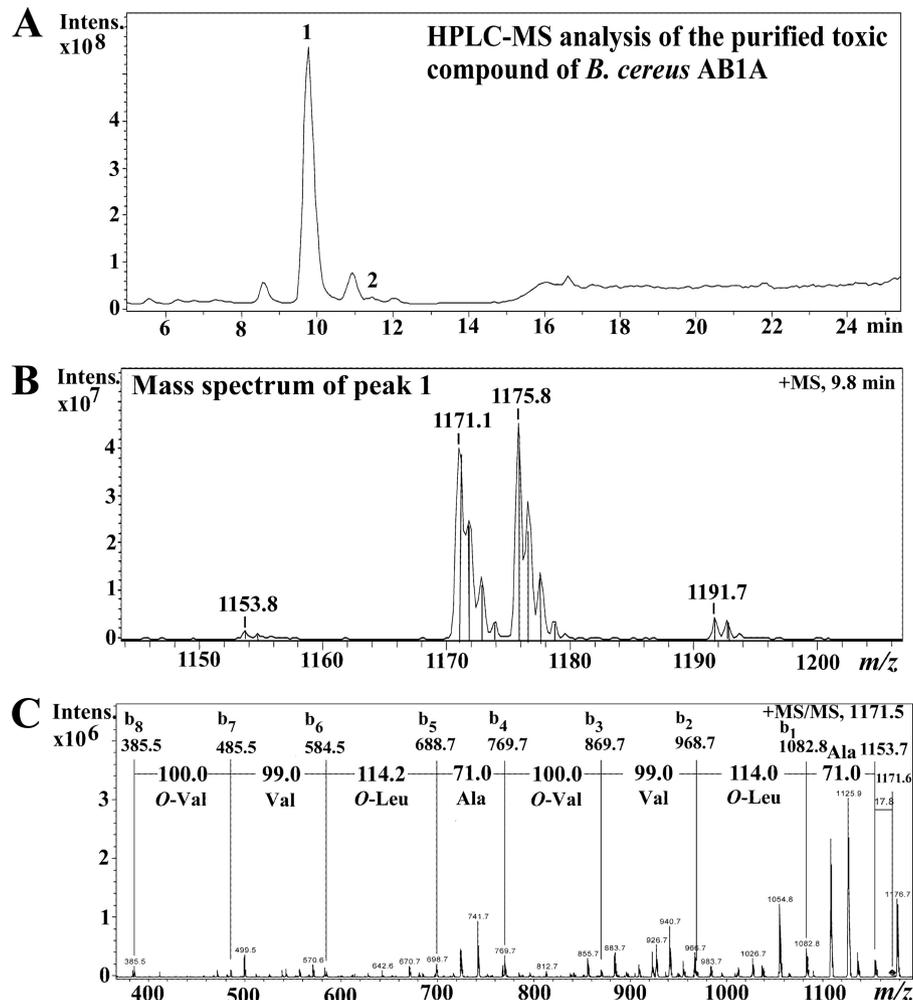
FIG 3 HPLC-MS analysis of the purified toxic compound of *B. cereus* AB1A. (A) Total ion chromatogram of the purified toxic compound (peak 1) of *B. cereus* AB1A. (B) The mass spectrum of the purified toxic compound of *B. cereus* AB1A at retention time 9.8 min. (C) Tandem mass spectrum of peak 1 using mass ion at m/z 1,171.5 as a precursor ion and obtained ion series with the interpreted sequences. *O*-Val is 2-hydroxyisovaleric acid, and *O*-Leu is 2-hydroxyisocaproic acid.

TABLE 2 Cereulide content analyzed by LC-MS in methanol extracts of potato tuber *Bacillus* isolates and reference strains[a]

Potato tuber isolate or *Bacillus* strain	Cereulide content (ng/mg of biomass [wet wt])[b]
Potato tuber isolates	
AB11A	<0.2
P113	<0.2
ABIB	9
AB1D	1
AB11D	21
AB1A	96
AB1A1	13
AB1B1	3
B. cereus reference strains	
F4810/72 (food poisoning)[c]	350
NS58 (endophyte of spruce tree)	900
DSM31[Td] (ATCC 14579)	<0.2

[a] See also Table 1.
[b] Tested with biomass harvested from tryptic soy agar plates at 7 days and 22°C. The variation between biological replicates was <40%.
[c] Reference strain for producing cereulide.
[d] Type strain; does not produce cereulide.

cell nuclei. The cells exposed for 30 min to 4 ng of cereulide ml^{-1} lost a major part of the orange fluorescence, indicating depolarization (i.e., a decrease in $\Delta\Psi$m) of the mitochondria. The cells exposed to 30 ng of cereulide ml^{-1} (right panels) have practically no energized mitochondria. However, none of the keratinocytes or PBMC cells showed any propidium iodide fluorescence staining (purple red), indicating that the cell membranes were intact and the cells were alive. This was confirmed using calcein AM (live stain) combined with propidium iodide (dead stain) (Fig. 4). The keratinocytes impermeable to propidium iodide exhibited fragmented nuclei visible by Hoechst 33342 staining after exposure to cereulide in amounts greater than 100 to 200 ng ml^{-1}. This indicated early apoptosis in response to cereulide exposure.

Pancreatic beta cells (MIN-6) responded to exposure to cereulide differently: the islets disintegrated, followed by necrotic cell death observed as a decrease in cell density, concomitant with the appearance of pyknotic cells and conversion to propidium iodide positivity (Fig. 4). This was observed after cereulide exposures as low as 10 ± 3 ng ml^{-1} (8 to 24 h exposure, Table 3), which is only $\leq$10-fold higher than that needed for depolarizing (loss of $\Delta\Psi$m) the mitochondria in these cells.

TABLE 3 Toxicity endpoints of exposure to cereulide for human, porcine, and murine primary cells and selected cell lines

Target cells[a]	Toxicity endpoint[b]			
	Loss on $\Delta\Psi m$		Relaxed permeability to PI	
	Exposure time	EC$_{100}$ (μg ml^{-1})	Exposure time	EC$_{100}$ (μg ml^{-1})
Human				
PBMC	30 min	0.004	24 h	>0.4[c]
Keratinocytes (HaCaT)	30 min	0.004		
	24 h	0.001	24 h	1.5
Porcine				
Spermatozoa[d]	20 min	0.007		
	24 h	0.001	24 h	>1.0
KPTE cells (PK-15)	30 min	0.005		
	24 h	0.0004	24 h	2.0
Murine				
Insulinoma, MIN-6 cells	20 min	0.014	20 min	>0.14
	8 h	0.001	8 h	0.013
	24 h	0.002	24 h	≤0.007
Fibroblast L-929 cells	24 h	0.001	24 h	>1.0

[a] PBMC and sperm cells were exposed as suspensions, and MIN-6 cells were exposed as islets. KPTE cells, kidney proximal tubular epithelial cells.

[b] $\Delta\Psi m$, mitochondrial membrane potential. The EC$_{100}$ indicates the exposure concentration causing an effect in 100% of the exposed cells. The variation between biological replicates was <40%. PI, propidium iodide.

[c] When PBMC were labeled with propidium iodide, the result was similar, i.e., with <20% of the cells fluorescing red, for both the nonexposed sample (vehicle only) and the highest test concentrations, i.e., 400 ng of purified cereulide ml^{-1}. A trypan blue assay showed 81 to 87% viability.

[d] Sperm motility ceased at the same exposure concentration at which $\Delta\Psi m$ was lost.

We conclude from the data shown in Table 3 and Fig. 4 that cereulide, at concentrations relevant for food-borne illness, induced specific damage to mitochondria rather than causing general cytotoxicity to the human (PBMC and keratinocytes), porcine (spermatozoa and kidney tubular epithelial cells [PK-15]), and murine fibroblasts. The pancreatic beta cells (MIN-6) underwent necrotic cell death following exposure at concentrations 2 logs lower than those for the other cells.

Cereulide-dependent efflux of potassium from human cells. Cereulide is known to act as a K$^+$ ionophore in isolated mitochondria, leading to mitochondrial dysfunction (34, 57). Given the findings in Table 3 and Fig. 4 showing mitochondrial depolarization inside intact, live cells, we asked the question whether the mitochondrial damage involved trafficking of K$^+$ ions from or to the intact mammalian cell. This was studied by monitoring cereulide imposed changes of the extracellular [K$^+$] in a cuvette fitted with a K$^+$ specific electrode and holding a suspension of live cells.

Figure 5 shows that exposing human keratinocytes (HaCaT) to 60 ng (Fig. 5A) and PBMC to 45 or 90 ng (Fig. 5B), respectively, of cereulide ml^{-1} provoked an efflux of K$^+$ within seconds that was measurable as an increase in [K$^+$] in the external medium. The efflux from 6 × 10^6 HaCaT cells into the external medium (1 ml) in the measurement cuvette raised the [K$^+$] by 8 μM (Fig. 5A) per 60 s. Efflux from 15 × 10^6 PBMC raised the extracellular [K$^+$] by 6 μM (trace 2 in Fig. 5B) per 60 s. These potassium fluxes were measured in a medium with [K$^+$] of ca. 300 μM (Fig. 5A and B).

When the efflux of K$^+$ was monitored in a medium with a [K$^+$]

of 850 to 950 μM, the flux of K$^+$ from HaCaT increased the extracellular [K$^+$] by 50 μM and from PBMC by 4 μM per 60 s (Fig. 5C and D). The summed cell volume of 6 × 10^6 cells HaCaT cells (cell diameter, 30 μm) is ~80 μl in the cuvette (1 ml) and that of the 15 × 10^6 PBMC (cell diameter, 7 μm) is ~3 μl. Assuming the intracellular [K$^+$] to be 150 mM, the summed potassium stores of HaCaT cells in the cuvette was 12 μmol. The efflux from HaCaT cells induced by exposure to 60 ng of cereulide ml^{-1} during the ~10-min observation time was 250 nmol (Fig. 5C), representing roughly 2% of the cellular K$^+$ stores. The summed cellular potassium stores of the PBMC in the cuvette were ~0.45 μmol; thus, a 10-min exposure to 30 ng of cereulide ml^{-1} induced an efflux of ~10% of the cellular K$^+$ stores of the PBMC.

The measured effluxes of K$^+$ from the PBMC were similar, whether the exposure dose of cereulide was 45 or 90 ng (Fig. 5B). This either indicates an on/off response or that the lower dose (~10^6 cereulide molecules per PBMC) was a saturating one (Fig. 5B). Cereulide (130 ng/ml) also induced an efflux of K$^+$ from porcine sperm cells (80 × 10^6 cells per ml) at similar exposure concentrations, i.e., ~10^6 cereulide molecules per sperm cell. The resulting increase in extracellular [K$^+$] was 50 μM in 8 min. This represents an efflux of ca. 10% of the cellular stores of the summed sperm cell volume (4 μl) in the cuvette (data not shown).

DISCUSSION

The extracellular spaces of vascular plants, including potato tubers, are not sterile (as animal tissues are) but contain microorganisms in the water channels and extracellular spaces (27, 28). In the present study we described the isolation and properties of *B. cereus* (sensu lato) from potato tubers. These environments are likely deficient in potassium for nonphytopathogens that do not penetrate into plant cells (27, 28). We recently showed that *B. cereus* benefits from its produced cereulide in a potassium-deficient environment (<1 mM) but not in potassium-rich environment (36). This may explain why potato endophytic *B. cereus* were frequently cereulide producers, similarly to what was earlier observed for endophytic *B. cereus* from Norway spruce (*Picea abies*), where four of the six *B. cereus* isolates were cereulide producers (NS58, NS88, NS115, and NS117 [20, 36, 50, 51]).

The potato tuber appeared not to suffer from its cereulide-producing endophytes. However, human, porcine, and murine cells the cereulides appeared highly toxic, indicating the endophyte isolates produced cereulide in its active, toxic form. Most of the *B. cereus* sensu stricto organisms from potato tubers were cereulide producers and displayed a genotype (both ribopatterns EcoRI and Pvu2) matching that reported for a cereulide-producing isolate from a food poisoning outbreak by *B. cereus* F5881/94 (fried rice, United Kingdom) (20, 50). The potato endophytic cereulide producers differed in ribopatterns from the nonproducers (Fig. 2). Endophytic cereulide-producing *B. cereus* organisms from spruce (*Picea abies*) were also reported to differ in ribopatterns from the nonproducers (NS61 and NS88 [20]). The potato isolates differed from the spruce tree producers in ribopatterns and modest cereulide production, 1 to 100 ng mg of biomass^{-1} compared 900 to 1,700 ng mg of biomass^{-1} for the NS strains (Norway spruce [51, 52, 54]). It might be that each plant species carries its own *B. cereus* cereulide-producing genotype, possibly adapted to the specific metabolic environment inside the host plant.

Vegetables may represent an emerging source of emetic food-
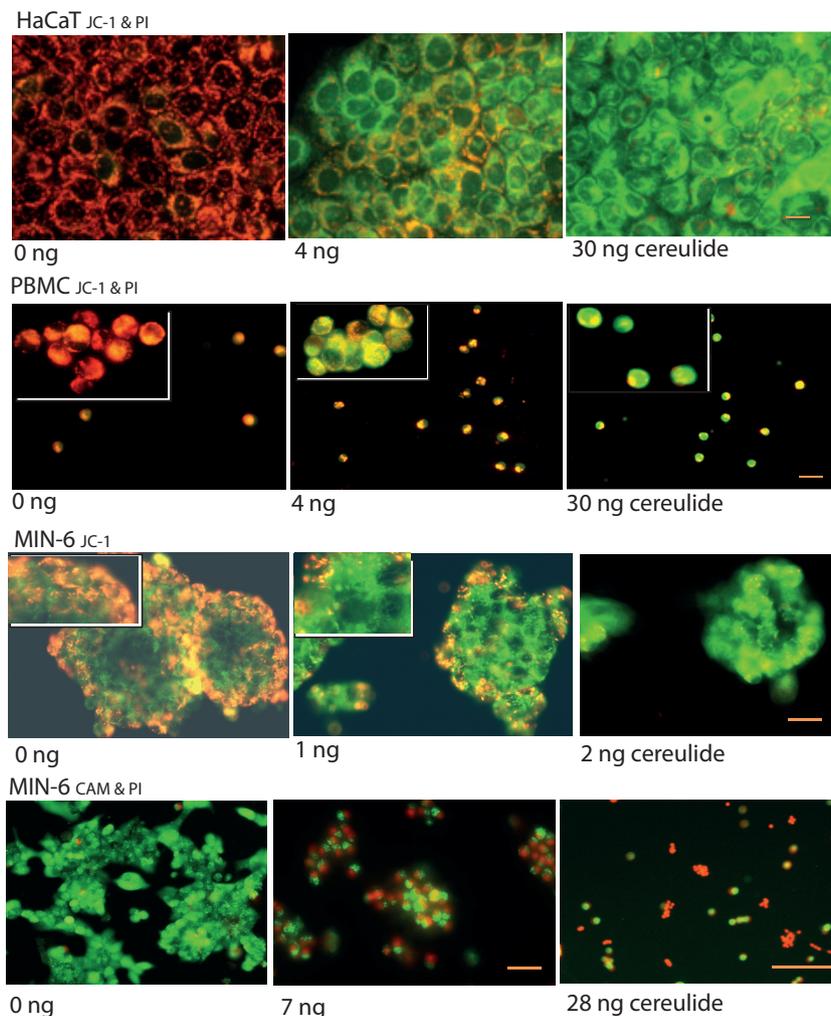
FIG 4 Epifluorescence micrographs of the effects of cereulide on human keratinocytes (HaCaT) and PBMC and murine pancreatic islet cells (insulin producing, MIN-6). The HaCaT and PBMC samples were double stained with the membrane potential responsive dye JC-1 and propidium iodide. The MIN-6 cells were stained with JC-1 (third row) or double stained with calcein AM (CAM) and propidium iodide (fourth row). The HaCaT and PBMC samples were exposed to cereulide for 30 min, and the MIN-6 cells were exposed to cereulide for 24 h. A value of "0 ng" indicates vehicle only. The orange-red fluorescence in JC-1-stained cells indicates a high membrane potential ($\Delta\Psi m > 140$ mV), and green fluorescence indicates a dissipated membrane potential ($\Delta\Psi m < 100$ mV). Unexposed pancreatic islets (MIN-6, 0 ng) stained with JC-1 fluoresce orange at the edges of the islet and green in the center. After exposure to cereulide, the islets fluoresce green (low $\Delta\Psi m$). Propidium iodide-positive cells were only seen in MIN-6 cells under these exposure conditions. The images are representative of three independent microscopic views. Scale bar, 30 μm.

borne illness by *B. cereus*, especially in mass catering. A large-scale food poisoning outbreak was reported from the Netherlands in which 116 students became ill with vomiting and nausea within 1.5 h after consuming a vegetarian meal. A total of 1,275 students participated in the meal, but only 120 vegetarian portions were served, and all of the students that became sick were from this group (57). Raw potato granules and potato flakes, constituents of many industrial foods, have been reported to contain log 1 to log 3.4 CFU of mesophilic aerobic spores g^{-1}, including *B. cereus* (58).

In the present study, we demonstrated that cereulide provoked cross-membrane flux of potassium ions within seconds of exposure in intact primary human and porcine cells. The potassium efflux from live, healthy cells, driven by exposure to nanomolar concentrations of cereulide (45 to 60 ng ml^{-1}) was observed to deplete the cells of 2 to 10% of the calculated cellular potassium stores within 10 min. This is a potential pathological trait, in ad-

dition to the observed mitochondrial toxicity (34, 47, 59), because transient cross cell-membrane gradients of potassium ions are known to operate as metabolic regulators in many mammalian cells, for example, the cells of innate and acquired immunity or voltage-gated potassium channel (hERG) heart muscle cells (60–64).

The mitochondrial toxic endpoints of cereulide were earlier shown in boar spermatozoa (30) and in many different tumor-derived cells lines (4, 48, 65). In the present study we showed that primary cells and nonmalignant immortalized cell lines of human and porcine origins were similarly sensitive to mitochondrial damage by cereulide, with EC$_{100}$ (i.e., the concentration affecting 100% of the exposed cells) values of a few nanograms per milliliter. Sperm motility ceased at the same exposure concentration of cereulide at which the mitochondria of spermatozoa, as well as somatic mammalian cells, were depolarized. Thus, the outcome of
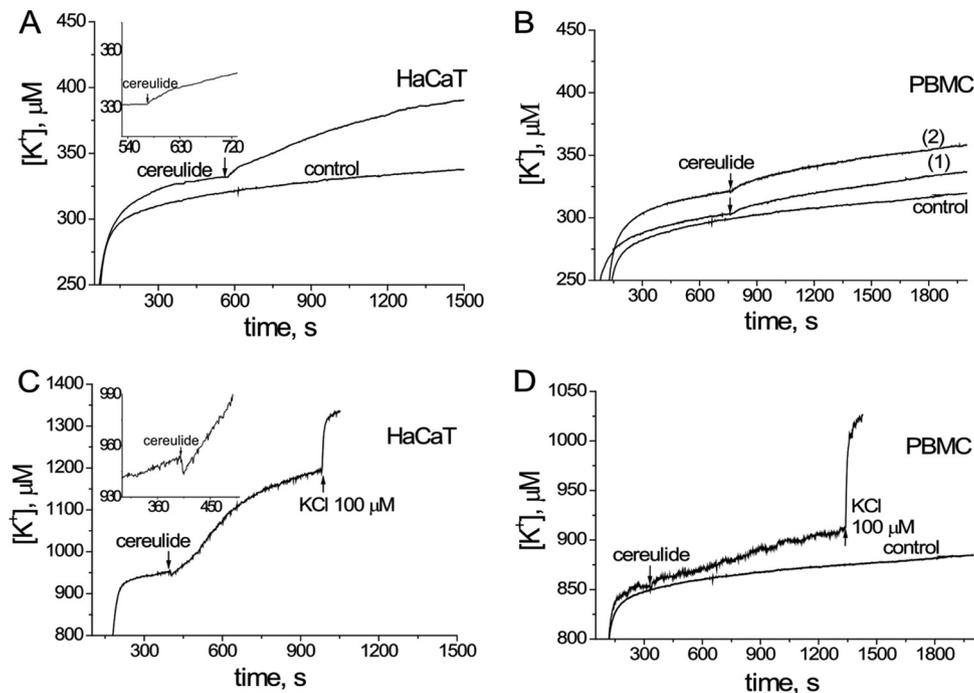
FIG 5 Cereulide-driven efflux of K^+ from human keratinocytes (HaCaT) and PBMC. Washed HaCaT cells (6×10^6 ml^{-1}) or PBMC (15×10^6 ml^{-1}) in 1 ml of K^+-free medium were placed in a measurement chamber, and the extracellular $[K^+]$ was recorded in real time with a K^+-selective electrode, with one reading per second. The effects of cereulide using 60 ng (panels A and C), 45 and 90 ng (traces 1 and 2, respectively, in panel B), and 30 ng (panel D) on the efflux of K^+ are shown. The "control" trace indicates data for vehicle only (methanol). The inset in panel C shows that the exposure to cereulide transiently initiated the influx of K^+, followed by a massive efflux of K^+. At the end of each run, the electrode response was verified by adding KCl to increase the concentration in the cuvette by 100 μM.

the spermatozoan motility assay is relevant to mammalian cells for initial estimation of mitochondrial toxic concentrations of cereulide in various samples (4, 19, 21, 24, 65).

The detection limit of the assay is 0.2 ng of cereulide mg of biomass^{-1}, and the response may be recorded within 30 min from sampling (38, 48). In a severe food poisoning case, the blood concentration of cereulide was reported as 4 ng ml^{-1} (3), which is in the range detectable by the sperm bioassay. One unit in the widely used Hep2 assay (66) was reported to correspond to 5 ng of biosynthetic cereulide (67). The sperm cell assay is therefore more sensitive; it also is simpler to execute since it requires no tissue culture facilities. The structurally related depsipeptides homocereulide (68) and paenilide (*Paenibacillus tundrae*) (40) are similarly toxic to cereulide and can be detected using the same method.

Mitochondrial damage by cereulide is presumed to impair the functioning of citric acid cycle and cell respiration, i.e., the aerobic pathways for the production of ATP. A consequence of dysfunctional mitochondria is that the body tissues must generate ATP from glycolysis, resulting in lactic acidosis. Where blood analysis data were available, all the serious cases of human emetic food poisoning by *B. cereus* displayed metabolic acidosis and increased plasma lactate levels and lowered the blood pH to 6.25 to 7.25 (3, 5, 7, 9, 10, 12). We recently showed that metabolic acidosis caused by mitochondrial toxicity from cereulide can be simulated by *in vitro* exposure using mammalian cultured cells (40). Human and murine insulin-producing β-cells are known *in vivo* and *in vitro* to be fully dependent on mitochondrial oxidative phosphorylation, since the beta cells do not possess a compensatory mechanism, e.g., glycolytic ATP from processing glucose to lactate (69, 70).

Here, we showed that insulin-producing pancreatic islet beta cells (MIN-6) differed from the other test cells by being highly sensitive to cytolytic killing by cereulide. Upon exposure, the pancreatic islets dispersed into pyknotic cells, followed by necrosis, at cereulide concentrations 2 log units below that required for other somatic human, porcine, or murine cell death. Earlier, we found that *in vitro* exposure of fetal porcine pancreatic islets to nanomolar concentration of cereulide from emetic potato isolates of *B. cereus* resulted in the necrosis of islet cells (71). Interestingly, a recent epidemiological survey from Finland indicated that the early introduction of root vegetables in infancy was associated with advanced β-cell autoimmunity in young children and increased susceptibility to type 1 diabetes (72). Cereulide-producing *B. cereus* is known to produce spores that are severalfold more resistant to heating at 90°C compared to *B. cereus* spores of cereulide-nonproducing *B. cereus* (52). Such spores are likely to survive and become enriched in foods processed by repeated heating. Endophytic *B. cereus* (strain NS117, spruce tree) and an isolate from a fatal food poisoning involving cereulide (strain 5964a) were shown to produce twice the amount of cereulide when inoculated in potato puree compared to that produced when inoculated into boiled rice (73). This finding indicates that potato is an environment favorable for cereulide production when cereulide producers are present.

The genotypic match between potato endophytes in the present study, strain F5881/94 (from a food-borne emetic outbreak involving fried rice in the United Kingdom [20, 50]), and cereulide-producing *B. cereus* from infant food formula (MIF1, not connected to food-borne illness [21]) suggests that vegetable crop

endophytes may be one of the points of entry of emetic *B. cereus* into foods.

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