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Cereulide produced by *Bacillus cereus* increases the fitness of the producer organism in low-potassium environments

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Cereulide, produced by certain *Bacillus cereus* strains, is a lipophilic cyclic peptide of 1152 Da that binds K^+ ions with high specificity and affinity. It is toxic to humans, but its role for the producer organism is not known. We report here that cereulide operates for *B. cereus* to scavenge potassium when the environment is growth limiting for this ion. Cereulide-producing *B. cereus* showed higher maximal growth rates (μ_{max}) than cereulide non-producing *B. cereus* in K^+-deficient medium (K^+ concentration ~1 mM). The cereulide-producing strains grew faster in K^+-deficient than in K^+-rich medium with or without added cereulide. Cereulide non-producing *B. cereus* neither increased μ_{max} in K^+-deficient medium compared with K^+-rich medium, nor benefited from added cereulide. Cereulide-producing strains outcompeted GFP-labelled *Bacillus thuringiensis* in potassium-deficient (K^+ concentration ~1 mM) but not in potassium-rich (K^+ concentration ~30 mM) medium. Exposure to 2 μM cereulide in potassium-free medium lacking an energy source caused, within seconds, a major efflux of cellular K^+ from *B. cereus* not producing cereulide as well as from *Bacillus subtilis*. Cereulide depleted the cereulide non-producing *B. cereus* and *B. subtilis* cells of a major part of their K^+ stores, but did not affect cereulide-producing *B. cereus* strains. Externally added 6–10 μM cereulide triggered the generation of biofilms and pellicles by *B. cereus*. The results indicate that both endogenous and externally accessible cereulide supports the fitness of cereulide-producing *B. cereus* in environments where the potassium concentration is low.

INTRODUCTION

Cereulide is a major metabolite of certain strains of *Bacillus cereus*, produced in quantities of up to 1 % of the dry weight of the bacterial cell (Jääskeläinen *et al.*, 2004; Apetroaie-Constantin *et al.*, 2008; Carlin *et al.*, 2006), suggesting that it may have an important function in the bacterium. Cereulide is the toxin that causes emetic illness connected to *B. cereus*-contaminated food. It has high human toxicity and is responsible for serious illness and fatalities in previously healthy persons (Mahler *et al.*, 1997; Dierick *et al.*, 2005; Pósfay-Barbe *et al.*, 2008; Ichikawa *et al.*, 2010; Shiota *et al.*, 2010). The toxic dose to humans is $\leqslant 8$ μg (kg body weight)$^{-1}$ (Jääskeläinen *et al.*, 2003b). Cereulide is toxic to mammalian cells by acting as a K^+ ion carrier across biological membranes and by depolarizing the electric transmembrane potentials of mitochondria (Hoornstra *et al.*, 2003; Teplova *et al.*, 2006; Andersson *et al.*, 2007), but it is not known what function cereulide has for the producer bacterium.

Cereulide is a cyclic peptide of 1152 Da consisting of six amino and six hydroxy acids (Agata *et al.*, 1994), and is produced by non-ribosomal peptide synthesis (Toh *et al.*, 2004; Horwood *et al.*, 2004; Ehling-Schulz *et al.*, 2005). It binds potassium ions with an affinity and selectivity higher than those of any other known potassium carrier, valinomycin included, at concentrations below 1 mM (Makarasen *et al.*, 2009; Teplova *et al.*, 2006; Mikkola *et al.*, 1999). A high intracellular potassium ion concentration (>100 mM) is

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Abbreviation: JC-1, 5,5′,6,6′,1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide.

required for transport and uptake of solutes involved in regulating cytoplasmic pH and cell turgor, essential for bacterial life (Booth *et al.*, 1985; Epstein, 2003; Corratgé-Faillie *et al.*, 2010). Extracellular non-protein binding molecules, comparable with the siderophores that facilitate iron uptake, have so far not been described for potassium uptake.

We hypothesized that *B. cereus* uses cereulide to exploit K^+ ions in environments where these ions are scarce. In this work we show direct evidence for the role of cereulide in maintaining the cellular K^+ homeostasis in *B. cereus*, and the competitive advantage gained by *B. cereus* strains producing this molecule. The results described in this paper suggest that cereulide is important for its producer, *B. cereus*, by increasing its fitness in environments low in K^+, such as natural waters or extracellular fluids of living organisms.

METHODS

Media and reagents. Tryptic soy broth (TSB; Scharlab) contained (per litre): casein peptone 17.0 g, soy peptone 3.0 g, NaCl 5.0 g, glucose 2.5 g, K_2HPO_4 2.5 g (pH 7.3, ~30 mM K^+). TSB-Na was similar to TSB except that K_2HPO_4 was replaced with an equimolar concentration of Na_2HPO_4. Tryptic soy agar (TSA; Scharlab) contained (per litre): casein peptone 15.0 g, soy peptone 5.0 g, NaCl 5.0 g, agar 15.0 g. Sporulation medium (Agaisse & Lereclus, 1994) contained 8.0 g nutrient broth powder l^{-1} (Becton Dickinson), 1 mM $MgSO_4$, 13 mM KCl and 1 μM $MnCl_2$. Sterile filtered ferric ammonium citrate, 17 μM, and $CaCl_2$, 0.5 mM, were added after autoclaving. SMH buffer contained 10 mM sucrose, 10 mM mannitol and 10 mM HEPES (pH 7.31, 35 mOsmol l^{-1}). JC-1 (5,5′,6,6′,1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide) was from Molecular Probes (Invitrogen) and valinomycin was from Sigma-Aldrich.

Cereulide preparations. Purified cereulide (molar mass 1152.7 g mol^{-1}), dissolved in methanol, was prepared as described in Teplova *et al.* (2006). Cereulide-containing crude extract was prepared from strains NC7401 and NS58 as follows. Biomass, harvested from TSA plates (3 days, 22 °C), was suspended in ethanol (96 vol%) at 10 ml (g wet weight)$^{-1}$, shaken overnight at 22 °C, heated in a water bath at 100 °C for 15 min, and cleared by centrifugation (15 min, 690 ***g***). The supernatant was evaporated to dryness with a flow of nitrogen gas and redissolved in methanol to 10 mg dry weight ml^{-1}. Quantification of cereulide was done by liquid chromatography-electrospray ionization ion trap MS using an MSD Trap XCT Plus ion trap mass spectrometer equipped with an Agilent electrospray ionization source and Agilent 1100 series liquid chromatograph (Agilent Technologies) and Atlantis C18 T3 4.6 × 150 mm, 3 μm column (Waters). Isocratic elution with 6 % of 0.1 % formic acid (A) and 94 % methanol (B) with a flow rate of 1 ml min^{-1} was used for the separation. Valinomycin was used as reference.

Bacterial strains. The strains were from the our laboratory collection unless otherwise stated. Origins of the strains are indicated below in parentheses. All cultivation was on TSA plates or in TSB at 28 °C, unless otherwise stated. Cereulide-producing *B. cereus* strains were as follows: F4810/72 (cooked rice, UK; Turnbull *et al.*, 1979), F5881/94 (fried rice, UK; Andersson *et al.*, 1998a), NS58 [live spruce tree (*Picea abies*), Finland; Hallarsela *et al.*, 1991], NC7401 (person with foodborne illness, Japan; Agata *et al.*, 1994), GR177 (milk, Sweden; Shaheen *et al.*, 2010), B318 (cake, Finland; this study). The measured cereulide contents of plate-grown biomass (3–4 days) were as follows [in μg cereulide (mg cell wet weight)$^{-1}$]: NS58 and NC7401, 0.6–1.8; F4810/72, F5881/94 and B318, 0.2–0.6; GR177, 0.01–0.09 (Jääskeläinen *et al.*, 2003a, 2004; Andersson *et al.*, 2004; Apetroaie, *et al.*, 2005; Carlin *et al.*, 2006; Shaheen *et al.*, 2006; Apetroaie-Constantin *et al.*, 2008; Shaheen, 2009).

Cereulide non-producing *B. cereus* and *Bacillus thuringiensis* strains were as follows: ATCC 14579^T (soil; Smith *et al.*, 1964), B319 (cake, Finland; Apetroaie *et al.*, 2005), B117 (meat pastry, Finland; Jääskeläinen *et al.*, 2003a), *B. thuringiensis* Bt 407Cry$^-$ [pHT315Ω(*papha3-gfp*)] (BT-1, Ekman *et al.*, 2009).

Strains (other than *B. cereus*) used as targets for assaying the antibacterial potential of cereulide strains were as follows: *Bacillus subtilis* DSM 347, *Escherichia coli* ATCC 51739, *Pseudomonas putida* C3024, *Bacillus megaterium* Ne10 (DSM 17641), *Dietzia* sp. MA147 (Andersson *et al.*, 1998b), *Sphingomonas aurantiaca* MA101b^T (DSM 14748, +18 °C; Busse *et al.*, 2003), *Ureibacillus thermosphaericus* P-11 (DSM 10633, formerly *Bacillus thermosphaericus*), +60 °C (Andersson *et al.*, 1995).

Measurement of potassium fluxes from and into bacterial cells. The test bacteria were grown in TSB overnight (120 r.p.m.), diluted in sterile TSB (1 : 50, v/v), incubated for 3 h (120 r.p.m.), harvested (10 min, 2000 ***g***), washed three times with sterile SMH buffer (pH 7.31) and suspended in 3–5 ml of the same buffer. The density (μg dry weight ml^{-1}) of the suspension was calculated from OD_{660} as described by Koch (1994). To record the kinetics of K^+ efflux from bacteria, washed cells (0.2 mg dry weight in 1.0 ml SMH buffer) were placed in a measurement cuvette (1.3 ml) provided with a potassium electrode. After 2 min equilibration, the test amendments were added. The K^+ concentration in the medium was measured online using a K^+ electrode (one reading per second) at 24 °C as described by Teplova *et al.* (2006). The signal was calibrated by adding a known quantity of KCl at the end of each run. The bulk efflux of K^+ from the bacterial cells after extended exposure was measured as follows: 0, 20 or 200 ng cereulide was added per millitre of cell suspension (0.2 mg dry weight ml^{-1} in SMH buffer). At indicated times (0–180 min), the cells were sedimented (10 min, 16 000 ***g***) and the potassium contents measured from 100 μl of the supernatant using the potassium electrode. The total content of K^+ in bacterial cells was similarly measured from lysates obtained after five cycles of freezing and thawing.

Observing the transmembrane electric potentials of *B. cereus* cells. Cells grown in TSB broth to exponential phase (OD_{660} ~0.3) were harvested by centrifugation (5 min, 900 ***g***) and washed with 1 mM sodium phosphate buffer (pH 7.3), supplemented as indicated. The cells were then stained with the membrane potential-responsive carbocyanine dye JC-1 and inspected with a fluorescence microscope. Green fluorescence of the JC-1-dyed cells indicates a membrane potential of <-100 mV and orange fluorescence indicates $\geqslant 140$ mV (Reers *et al.*, 1995).

Competition assays. The competitor strains were grown in TSB to late exponential phase (4–5 h), and 1 : 1000 dilutions, made in TSB or in TSB-Na, were mixed 1 : 1 (v/v) with a similarly diluted culture of the target strain *B. thuringiensis* BT-1 (fluorescently tagged with GFP; Ekman *et al.*, 2009). These co-cultures were dispensed into four replicate wells, at 300 μl per well, on polystyrene 96-well plates (Nunc), and incubated for 48 h (120 r.p.m.). Reference wells were inoculated with *B. thuringiensis* BT-1 alone. OD_{600} and fluorescence (excitation 485 nm, emission 535 nm) were recorded with a VICTOR^3 plate reader (Perkin-Elmer).

Effect of cereulide on growth rates of *B. cereus*. Test media seeded with overnight-grown cultures of the test strains (1 : 1000, v/v) were dispensed into the wells (300 μl per well) of Honeycomb 2 plates (Growth Curves Ltd). Cereulide-containing (280 μg ml^{-1}) extracts

(four replicates) or vehicle (methanol, five replicates) were added at 2 µl per well. OD_{600} was measured online with a Bioscreen C plate reader (Growth Curves Ltd) for 21 h (with shaking). Growth rates and their statistical significances (paired *t* test) were calculated using Microsoft Excel from optical density data obtained at 15 min intervals.

Assessment of antibacterial activity of cereulide. The target strains were inoculated with 16–48 h grown cultures (in TSB), at 10 µl per 2 ml TSB, followed by addition of 20 µl of cereulide-containing extract (final concentration 3 µg cereulide ml^{-1}) or vehicle alone (methanol). The final optical densities were recorded after 24 and 48 h (120 r.p.m.) at the growth temperature optimal for each strain.

Effect of cereulide on biofilm formation by *B. cereus*. Overnight cultures of the strains were diluted with 25 vols sterile TSB and grown for an additional 5 h (120 r.p.m.). Aliquots of these precultures and their 1 : 10 dilutions (in sterile tap water) were dispensed, at 200 µl per well, on 96-well plates, and cereulide was added (0 or 10 µM). After 21 h of shaking (120 r.p.m.), biofilms adhering to the walls of the wells were measured by the crystal violet staining method, as described by Kolari *et al.* (2002).

Pellicle formation. Overnight cultures of *B. cereus* were diluted with 50 vols sterile sporulation medium, grown for 3.5 h (120 r.p.m.) and dispensed into wells of a 96-well plate (200 µl per well). KCl or NaCl (0 or 150 mM final concentration) and cereulide (0 or 6 µM final concentration), or vehicle alone, was added to the wells. Pellicle formation was visually read after 22 h (120 r.p.m.).

RESULTS

Cereulide-driven efflux and influx of K^+ in *B. cereus*

We asked the question whether cereulide plays a role in potassium (K^+) homeostasis of *B. cereus*. Cereulide used for the experiments was isolated and purified from methanol extracts of the high-producing strains *B. cereus* NS58 and NC7401, and its concentration was measured based on the molecular ions shown in Fig. 1. Prior to measuring the potassium fluxes, the cells were washed with K^+-free buffer to remove K^+ ions emanating from the culture medium. The washed cells were resuspended (0.2 mg dry weight ml^{-1}) in K^+-free buffer and placed in a cuvette in which the extracellular concentration of K^+ ions was recorded in real-time by a potassium electrode. Fig. 2(a) shows that injection of cereulide into the cuvette initiated a burst of potassium ions from *B. cereus* ATCC 14579^T (cereulide non-producing) into the extracellular medium, ~23 µM within 6 min. When an energy source (glucose, 5 mM final concentration) was introduced, the efflux reversed: the K^+ concentration in the extracellular fluid fell within 3 min to the level that prevailed before cereulide was added. This indicates that the cells, when provided with an energy source, were able to pull the K^+, released in the absence of an energy source, back into the cells. When the same experiment was done with the cereulide-producing *B. cereus* strain F4810/72, spiking with cereulide did not change the spontaneous minor efflux of K^+ from the cells (Fig. 2b).

Table 1 shows that in K^+-free medium, in the absence of a utilizable carbon source, suspensions of intact cells of cereulide-producing *B. cereus* strains (NS-58, F4810/72) lost only a fraction (16 % in 30 min, 24–28 % in 180 min) of their cellular potassium stores, whether cereulide (0.02 or 0.2 µM) was added or not. In contrast to this, cereulide non-producing *B. cereus* (ATCC 14579^T, B319) responded to 0.02 µM added cereulide by a major efflux of potassium ions from the cells. Within 180 min, the efflux provoked by exposure to submicromolar concentrations of cereulide depleted 50–80 % of the cellular K^+ stores of the cereulide non-producing *B. cereus* strains and also of *B. subtilis* DSM347, whereas *E. coli* ATCC 51739 appeared insensitive to the effects of externally added cereulide. The cereulide insensitivity of *E. coli* cells may be explained by the Gram-negative outer membrane protecting against penetration by hydrophobic substances.

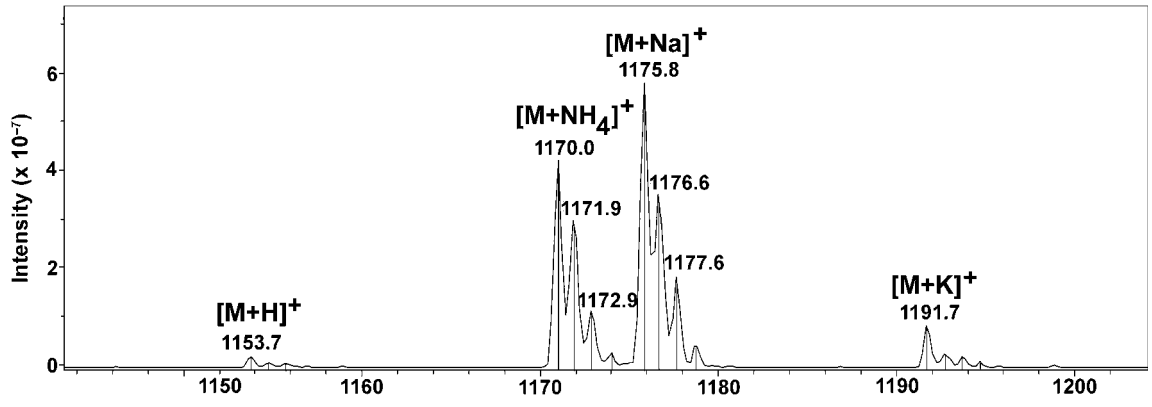

Fig. 1. Liquid chromatography-MS measurement of cereulide content in methanol extracts of *B. cereus* NS58. The compound at retention time 9.8 min had mass ions $[M+NH_4]^+$ at 1171.0 *m/z*, $[M+Na]^+$ at 1175.8 *m/z*, $[M+H]^+$ at 1153.7 *m/z* and $[M+K]^+$ at 1191.7 *m/z*.

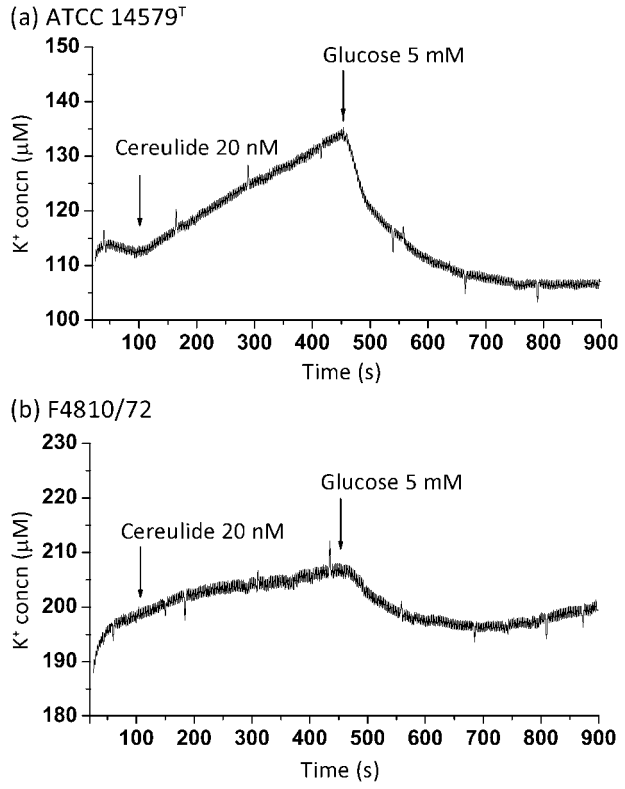

Fig. 2. Cereulide-driven efflux of potassium from *B. cereus* ATCC 14579^T (non-producer) and F4810/72 (cereulide producer). Pelleted cells were resuspended in SMH buffer (K^+ free) to 0.2 mg dry weight ml^{-1} in a cuvette provided with a K^+ electrode. Where indicated, 20 nM (=20 ng ml^{-1}) cereulide or 5 mM glucose was added. The extracellular K^+ concentration was monitored in real-time. The starting K^+ concentration of 0.1–0.2 mM represents remnants of the growth medium.

Effects of K^+ efflux on the membrane potential of *B. cereus* cells in a low-K^+ environment

Since access to an energy source appeared to be vital for the retention of intracellular K^+ by *B. cereus* in K^+-deficient medium (Fig. 2), this aspect was studied more closely, using the membrane potential-responsive carbocyanine dye JC-1 to probe the energy status of the cells. The fluorogenic dye JC-1 emits green fluorescence in membranes with potentials ($\Delta\Psi$) of $\leqslant -100$ mV and orange fluorescence at $\Delta\Psi \geqslant 140$ mV. Washed cells of the cereulide producer *B. cereus* NS58 in potassium-free buffer fluoresced green with JC-1, indicating low ($\Delta\Psi \leqslant -100$ mV) membrane potential. When an energy source (5 mM glucose) was added, the cells gained in $\Delta\Psi$, visible as orange fluorescence. Interestingly, a high $\Delta\Psi$ was also generated when a large amount of cereulide (70 μM) was added to a suspension of NS58 cells (cereulide producer) in K^+-free buffer (1 mM sodium phosphate, pH 7.3) in the absence of an organic energy source. However, when the same experiment was done in high-K^+ medium (120 mM KCl), the cells retained green fluorescence, indicating a low $\Delta\Psi$. We interpret these results to mean that the externally added high amount of cereulide provoked the efflux of the cytoplasmic K^+ ions from the cells into the buffer void of K^+, and that no energy source was available to draw the lost K^+ back into the cells, as shown in Fig. 2. Under these conditions, the efflux of the electropositive K^+ ions necessarily resulted in an increased net negative charge on the cytoplasmic side of the bacterial cell membrane, thus generating a high $\Delta\Psi$, visible as orange fluorescence from JC-1. Raising the extracellular K^+ concentration to 120 mM prevented the efflux of intracellular K^+.

As shown in Table 1, the measured cellular K^+ content of the *B. cereus* strains ranged from 1.1 to 2.0 nmol K^+ (μg cell dry weight)$^{-1}$. This corresponds to a cytoplasmic K^+ concentration of 150–250 mM. The $\Delta\Psi$ results described above thus indicated that externally added cereulide provoked the efflux of K^+ from the *B. cereus* cell when the K^+ concentration inside was much higher than the K^+ concentration outside the cell, but not when the K^+ concentrations inside and outside the cell membrane were of the same order of magnitude. We concluded that when the extracellular K^+ concentration was low ($\leqslant$0.2 mM), the externally added cereulide generated a high $\Delta\Psi$, negative inside, by provoking an efflux of K^+.

Does cereulide give a competitive benefit to *B. cereus* in a low-K^+ environment?

We investigated whether cereulide gives its producer strains a competitive advantage over non-producers. The success of cereulide-producing *B. cereus* strains in competition with GFP-labelled *B. thuringiensis* strain BT-1 was assessed by growing them in co-culture in K^+-rich and in K^+-deficient media. *B. thuringiensis* belongs to *B. cereus* (*sensu lato*) but does not produce cereulide. The GFP fluorescence emission of *B. thuringiensis* strain BT-1 was used as a marker of the growth of this strain in the co-cultures with the non-fluorescing *B. cereus* strains. The results in Table 2 show that in K^+-deficient medium, strain BT-1 grew poorly (as indicated by the fluorescence yield), and there was almost no growth when it was co-cultured with one of the cereulide-producing *B. cereus* strains (F5881/94, NS58, F4810/72). Co-culturing with the cereulide non-producer ATCC 14579 was much less inhibitory. In K^+-rich medium, cereulide producers (F5881/94, NS58, F4810/72) and a non-producer (ATCC 14579) (Table 2) only partially decreased the growth (fluorescence) yield of strain BT-1. We conclude from these data that cereulide-producing strains of *B. cereus* efficiently outcompete the cereulide non-producer *B. thuringiensis* BT-1 when co-cultured in medium with a growth-limiting K^+ concentration.

Cereulide-producing *B. cereus* profits from cereulide in low-K^+ environments

To see whether *B. cereus* strains gain an advantage or disadvantage from added cereulide in a medium where the

Table 1. Potassium traffic induced by cereulide in live bacteria suspended in K^+-free buffer

Strain	Response to cereulide			
	Intracellular K^+ stores [nmol (µg cell dry weight)$^{-1}$]	Added cereulide (µM)	Efflux of K^+ [nmol (µg cell dry weight)$^{-1}$]*	
			30 min	180 min
Cereulide producing				
B. cereus NS58	1.8	0	0.30 ± 0.029	0.42 ± 0.044
		0.02	0.29 ± 0.012	0.44 ± 0.012
		0.2	0.34	0.46
B. cereus F4810/72	2.0	0	0.33 ± 0.033	0.56 ± 0.092
		0.02	0.34 ± 0.036	0.56 ± 0.082
		0.2	0.48	0.60
Cereulide non-producing				
B. cereus ATCC 14579^T	1.5	0	0.33	0.67
		0.02	0.65	1.25
B. cereus B319	1.1	0	0.18	0.39
		0.02	0.30	0.57
B. subtilis DSM347	2.2	0	0.44	0.73
		0.02	1.3	1.9
		0.2	1.7	1.9
E. coli ATCC 51739	0.7	0	0.03	0.06
		0.02	0.03	0.07
		0.2	0.03	0.05

*After exposure the cell suspension was centrifuged at 15000 r.p.m. for 15 min and the K^+ concentration was measured in the supernatant. SD is given for experiments for which there were three or four replicates; for the others the averages of duplicates, deviating by less than 30 %, are given.

K^+ concentration is suboptimal for growth, maximal growth rates (μ_{max}) and OD_{600} (at 21 h) of different *B. cereus* strains were measured in media rich in (TSB) or deficient in K^+ (TSB-Na), in the presence and absence of added cereulide. The results in Table 3 show that the μ_{max} values of all cereulide-producing strains (n=5) of *B. cereus* were significantly higher in K^+-deficient medium (TSB-Na) with 2 µM cereulide added than in K^+-rich medium (no added cereulide). In K^+-deficient medium, the cereulide producers grew faster (P=0.02) in the presence

Table 2. Ability of cereulide-producing and non-producing strains of *B. cereus* to outcompete *B. thuringiensis* BT-1 grown in co-culture in K^+-rich and K^+-deficient media

Microplate wells (four parallel assays) were inoculated with a 1 : 1000 (v/v) dilution of growing cultures of *B. thuringiensis* BT-1 (fluorescently labelled with GFP) and one of the *B. cereus* strains. The fluorescence yields (± SD) obtained after 2 days of growth (at 120 r.p.m.) are shown. The fluorescence (relative fluorescence units; RFU) indicates the density of strain BT-1, OD_{600} the total biomass density.

Strain co-cultured with *B. thuringiensis* BT-1	Growth of *B. thuringiensis* BT-1 measured as fluorescence (RFU×10^4)	
	Yield in K^+-rich medium (TSB)*	Yield in K^+-deficient medium (TSB-Na)†
Cereulide non-producers		
B. thuringiensis BT-1 (=monoculture)	95 ± 3	10 ± 1
B. cereus ATCC 14579^T	56 ± 3	7 ± 1
Cereulide producers		
B. cereus F5881/94	33 ± 4	0.8 ± 0.5
B. cereus NS58	54 ± 7	2 ± 0.6
B. cereus F4810/72	88 ± 11	−0.3 ± 0.7

*Normalized to OD_{600} 0.9 (actual values 0.83–1.0).
†Normalized to OD_{600} 0.55 (actual values 0.45–0.69).

Table 3. Growth rates and culture densities of cereulide-producing and non-producing *B. cereus* strains in rich medium with high (TSB) and low (TSB-Na) concentrations of potassium ions

Values shown are mean ± SD.

Strain	Maximal growth rate, μ_{max} (h^{-1})*				Final OD_{600}			
	TSB (30 mM K^+)		TSB-Na (1 mM K^+)		TSB (30 mM K^+)		TSB-Na (1 mM K^+)	
Added cereulide concentration	0 μM	2 μM	0 μM	2 μM	0 μM	2 μM	0 μM	2 μM
Cereulide-producing *B. cereus* strains								
F4810/72	1.42 ± 0.11	1.64 ± 0.1	1.62 ± 0.06	1.72 ± 0.06	1.84 ± 0.012	1.88 ± 0.015	1.42 ± 0.031	1.40 ± 0.008
NS58	1.36 ± 0.08	1.23 ± 0.14	1.80 ± 0.13	1.90 ± 0.09	1.82 ± 0.095	1.89 ± 0.039	1.27 ± 0.048	1.26 ± 0.013
F5881/94	1.44 ± 0.26	1.26 ± 0.05	1.47 ± 0.21	1.85 ± 0.07	1.83 ± 0.016	1.89 ± 0.014	1.45 ± 0.003	1.44 ± 0.02
B318	1.28 ± 0.07	1.34 ± 0.07	1.47 ± 0.03	1.64 ± 0.09	1.84 ± 0.014	1.85 ± 0.018	1.40 ± 0.047	1.49 ± 0.017
GR177	1.27 ± 0.07	1.67 ± 0.08	1.43 ± 0.02	1.59 ± 0.06	1.86 ± 0.019	1.88 ± 0.01	1.42 ± 0.088	1.49 ± 0.01
Cereulide non-producing *B. cereus* strains								
ATCC 14579^T	0.96 ± 0.05	0.99 ± 0.01	1.26 ± 0.08	1.20 ± 0.02	2.00 ± 0.043	2.00 ± 0.027	1.68 ± 0.087	1.83 ± 0.114
B319	1.61 ± 0.54	1.71 ± 0.5	1.57 ± 0.09	1.63 ± 0.07	1.87 ± 0.009	1.87 ± 0.003	1.46 ± 0.016	1.49 ± 0.015
B117	1.16 ± 0.04	1.13 ± 0.01	1.23 ± 0.11	1.27 ± 0.01	1.75 ± 0.035	1.65 ± 0.024	1.39 ± 0.133	1.47 ± 0.016
BT-1	0.95 ± 0.03	0.88 ± 0.06	1.25 ± 0.09	1.02 ± 0.04	1.83 ± 0.031	1.76 ± 0.045	1.52 ± 0.024	1.50 ± 0.025
Other strains								
B. subtilis DSM 347	1.04 ± 0.02	1.02 ± 0.01	1.26 ± 0.22	1.09 ± 0.03	1.84 ± 0.013	1.79 ± 0.004	1.58 ± 0.006	1.66 ± 0.004
E. coli ATCC 51739	1.22 ± 0.05	1.05 ± 0.02	1.07 ± 0.03	1.02 ± 0.03	1.92 ± 0.021	1.94 ± 0.033	1.11 ± 0.007	1.11 ± 0.018
P. putida C3024	1.38 ± 0.05	1.36 ± 0.01	1.25 ± 0.07	1.30 ± 0.02	1.99 ± 0.033	1.98 ± 0.013	1.67 ± 0.013	1.69 ± 0.004

*Growth rates were calculated from OD_{600} data measured online in TSB and TSB-Na. TSB-Na contains sodium phosphate in place of potassium phosphate. SD values are from four (with added cereulide) or five (without cereulide) replicates, except for strain BT-1 (values from eight or 10 replicates).

of 2 μM added cereulide than when none was added. Despite these higher growth rates in TSB-Na broth, the final OD_{600} values of the *B. cereus* strains remained lower in TSB-Na than in TSB, proving that the K^+ concentration of TSB-Na (~1 mM) is indeed growth limiting. Cereulide non-producing *B. cereus* strains (n=4) did not display significantly higher μ_{max} values in the K^+-deficient medium than in the K^+-rich medium, regardless of whether 2 μM cereulide was added. *B. subtilis* DSM 347, *E. coli* ATCC 51739 and *P. putida* C3024 (Table 3) gave results similar to those of the cereulide non-producing *B. cereus*. The overall outcome of the results in Table 3 is that cereulide producers show better fitness in K^+-deficient broth culture than the non-producers, and mostly benefit from the presence of cereulide added to K^+-deficient medium.

Does cereulide inhibit bacterial growth?

As shown in Table 3, adding 2 μM (2 μg ml^{-1}) cereulide into rich medium (TSB) did not significantly reduce either the growth rate or the final OD_{600} of *B. cereus* strains (n=9) or of other species (n=3) of bacteria. We additionally tested *B. megaterium* Ne10, *Dietzia* sp. MA 147, *S. aurantiaca* MA 101b^T and *U. thermosphaericus* P-11^T to assess possible growth inhibition by cereulide-containing cell extracts prepared from *B. cereus* strain NC7401. No significant decrease (<10 %) of the final OD_{600} was observed (results not shown). The highest tested concentration corresponded to 3 μM cereulide.

We conclude from these results, and those presented in Table 3, that under the test conditions (TSB, pH 7.3, 2 days shaking), cereulide did not inhibit the growth of any of the test bacteria, including nine *B. cereus* strains and seven representatives of the phyla Alpha- and Gammaproteobacteria, Firmicutes and Actinobacteria.

Promotion of multicellular behaviour in *B. cereus* by cereulide

Biofilm formation of strains of *B. cereus* was measured on polystyrene microplates with and without added cereulide. Fig. 3 shows that in the presence of 10 μM cereulide, twofold more wall-attached growth was observed for the cereulide-producing strains (F4810/2, NS58, F5881/94) in TSB. The wall attachment of the cereulide non-producer ATCC 14579 also increased, although the increase was less than twofold.

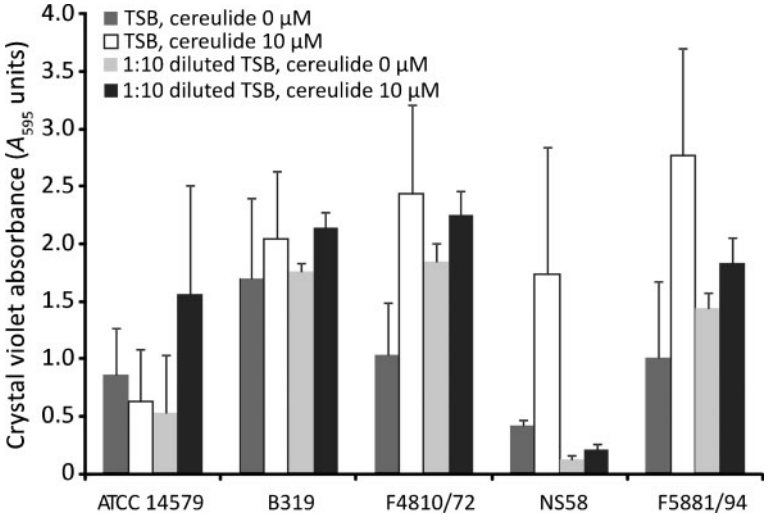

Fig. 3. Cereulide and biofilm growth of *B. cereus*. Cereulide non-producing (ATCC 14579, B319) and cereulide-producing strains (F4810/72, NS58, F5881/94) were grown in TSB or in 10× diluted TSB in wells of 96-well microplates amended with 10 μM cereulide or vehicle alone. After 21 h (120 r.p.m.), the wells were emptied and biomass adhering to the polystyrene walls was measured by the crystal violet method. Error bars, SD values of four biological replicates.

We inspected the formation of free-floating pellicles in broth cultures of *B. cereus* in TSB. We showed that when cereulide (6 μM) was added, the four cereulide-producing strains, F4810/72, B318, F5881/94 and NS58, formed massive pellicles in TSB and in sporulation medium (Fig. 4). The non-producer ATCC 14579 also formed surface-floating pellicles, but they were less massive. When cereulide (6 μM) was added together with 150 mM KCl to the TSB medium, the cereulide non-producer strain ATCC 14579^T stopped generating pellicles, although the cereulide producers continued to generate pellicles.

These experiments indicate that cereulide-producing strains form pellicles independently of the extracellular K^+ concentration, whereas a high extracellular concentration of K^+ prevents pellicle formation in the non-producing strain ATCC 14579^T.

DISCUSSION

Cereulide supports the fitness of *B. cereus* in a K^+-deficient environment

The experimental results presented in this paper indicate that cereulide, produced by certain strains of *B. cereus*, supports producer strains in potassium-deficient environments.

Potassium is the major intracellular cation (>100 mM) in the bacterial cytoplasm, but in soil and waters, potassium is often present in micromolar concentrations (Corratgé-Faillie *et al.*, 2010). *B. cereus*, colonizing versatile habitats, needs efficient tools to provide itself with potassium from fluctuating or dilute environments where potassium ions are in short supply. Most bacteria possess Trk and Ktr K^+ transporters and Na^+/K^+ or K^+/H^+ exchangers for K^+ uptake (Kröning *et al.*, 2007; Kuo *et al.*, 2005; Epstein, 2003; Corratgé-Faillie *et al.*, 2010). Cereulide thus represents an additional useful tool for potassium ion housekeeping in a K^+-deficient environment.

We found that cereulide-producing *B. cereus* strains had higher growth rates (μ_{max}) than non-producers in K^+-deficient medium and that externally added cereulide increased the growth rates of cereulide-producing *B. cereus* strains in such a medium. In this study, the potassium stores of *B. cereus* strains were assessed by direct measurement of K^+ released by cell lysis. The stores ranged from 1.1 to 2.0 nmol K^+ (μg cell dry weight)$^{-1}$. This is equivalent to a cytoplasmic K^+ concentration of ~150–250 mM. Similar

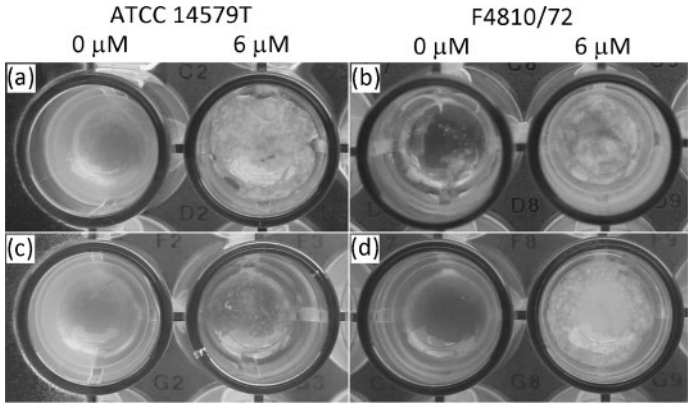

Fig. 4. Pellicle formation by *B. cereus* following exposure to cereulide. Undiluted and 10× diluted (with sterile water) TSB medium in wells of a 96-well microplate were amended with cereulide as indicated. K^+ (150 mM) was added to the wells in (c) and (d). Pellicle formation was read after 22 h with shaking (120 r.p.m.). The figure shows pellicles formed by the cereulide producer F4810/72 and by the type strain (ATCC 14579^T, non-producer). The results obtained with the cereulide producers B318, F5881/94 and NS58 were similar to those shown here for strain F4810/72.

values have been reported, using different methods, for other species of bacteria (Booth *et al.*, 1985; Epstein, 2003; Kuo *et al.*, 2005). In soil water, ground water and non-saline surface waters deficient in K^+ ($<<1$ mM), but rich in organic energy sources such as plant residues, cereulide could benefit the producer cell by facilitating uptake of K^+ ions into the cell. Cereulide may serve as the high-affinity mechanism for binding K^+, as it has been shown that cereulide can effectively bind K^+ ions at ambient concentrations as low as 0.2 mM (Teplova *et al.*, 2006; Saris *et al.*, 2009). The cereulide molecule binds K^+ in a molar ratio of 1 : 1. This lipophilic K complex is capable of migrating across lipid membranes (Tonshin *et al.*, 2010; Makarasen *et al.*, 2009). Whether the positively charged cereulide–K^+ complex undergoes influx into or efflux from the cell will depend on the concentration gradient and the net negative charge inside the bacterial cell. A cereulide-mediated transit of K^+ against a concentration gradient (high K^+ concentration inside, low outside) of, e.g. 100 mM, would, according to the Nernst equation:

$$\Delta\Psi = -59 \times \log([K_i^+]/[K_O^+])$$

require a $\Delta\Psi$ of up to -140 to -160 mV. This was achieved by *B. cereus* when glucose was present as an energy source.

Interestingly, it was found in the present work that in the absence of an organic energy source, in a low-K^+ environment the cereulide-producing *B. cereus* cells could utilize the concentration gradient of K^+ across the intra- and extracellular compartment to build up membrane potential ($\Delta\Psi$). This also occurred with the aid of cereulide at a high concentration similar to that in high cereulide producers (e.g. *B. cereus* strain NS58). The biomass of this strain, when grown on low-K^+ substrate (TSA, 4 mM K^+), was found to contain 0.6–1.8 μg cereulide (mg cell wet weight)$^{-1}$, equivalent to a cellular cereulide concentration of 500–1500 μM (Apetroaie *et al.*, 2005; Apetroaie-Constantin *et al.*, 2008). This method of generating $\Delta\Psi$ could serve as a temporary mechanism to energize vital transport functions such as the import of organic substrates for subsequent generation of proton motive force.

Tempelaars *et al.* (2011) recently reported that the $\Delta\Psi$ of *B. cereus* strain F4810/72 (cereulide producer) is dissipated by 9 μM of synthetic cereulide in 50 mM KCl in PBS. Their findings match our results, in showing that a cereulide-driven high $\Delta\Psi$ is only achieved when the external K^+ concentration is much lower than the internal K^+ concentration.

The current results indicate the role of cereulide in utilizing K^+ ions from low-K^+ environments, and the potential for generating $\Delta\Psi$ when access to organic energy sources is insufficient. This explains earlier observations that low K^+ concentration/low-energy environments may trigger increased cereulide production. Apetroaie-Constantin et al. (2008) reported that seven cereulide-producing strains, including NS58, F4810/72 and NC7401 used in the present study, produced high amounts of cereulide on TSA and blood agar (4–6 mM K^+). When grown on skimmed milk agar or raw milk agar (35–42 mM K^+) under otherwise identical conditions, the same strains produced five to ten times less cereulide. Furthermore, Shaheen *et al.* (2006) found that strain F4810/72 exponentially increased cereulide production when infant food formula was serially diluted with water, resulting in a decrease in K^+ concentration from 60 to 4 mM. It seems that a high K^+ concentration attenuates cereulide production.

Does cereulide inhibit or promote growth of cereulide non-producing bacteria?

We found no inhibitory action of purified cereulide or cereulide-containing crude extracts (2–3 μM) on the growth of non-producer strains of *B. cereus* or of other species when tested in rich medium (30 mM K^+) at pH 7.3. Altayar & Sutherland (2006) tested cell extracts prepared from *B. cereus* F4810/72 against 31 cereulide non-producing *Bacillus* strains, including *B. subtilis* and *B. cereus*, on skimmed milk medium, and found no antibacterial activity. Tempelaars *et al.* (2011) reported that synthetic cereulide (9 μM) added to brain heart infusion (BHI) medium, adjusted to pH 8.5, caused a transient growth delay of *B. subtilis* 168 and of *B. cereus* ATCC 10987 (cereulide non-producing), but not of *B. cereus* F4810/72 (cereulide producing). These apparently contradictory findings may be related to differences in the growth media. The skimmed milk medium, pH 7.3, used by Altayar & Sutherland (2006) is rich in energy sources and rich in potassium. The BHI used by Tempelaars *et al.* (2011) is rich in energy sources, but the pH was set at 8.5, a pH at which the pH gradient (ΔpH) across the membrane will not contribute to the $\Delta\Psi$ (negative inside), and thus the $\Delta\Psi$ will mainly depend on the K^+ gradient ($\Delta[K^+]$). As shown in the present study, *B. cereus* non-producers tend to lose their cytoplasmic K^+ in the presence of cereulide (see Fig. 2a, Table 1), whereas the cereulide producer F4810/72 will preserve its high intracellular K^+ concentration. When the cytoplasmic K^+ concentration is maintained at high levels, the $\Delta[K^+]$ across the plasma membrane is large, and, according to the Nernst equation, will contribute to the build-up of cellular $\Delta\Psi$. This explains why strain F4810/72 showed no growth delay in the presence of cereulide at pH 8.5.

Multicellular behaviour and cereulide

We observed that treatment with cereulide enhanced the multicellular behaviour of cereulide-producing *B. cereus* and also of the type strain ATCC 14579^T (cereulide non-producer). The cereulide-triggered pellicle formation by *B. cereus* ATCC 14579 was abolished by a high extracellular K^+ concentration, indicating that the efflux of potassium ions participates in biofilm signalling. López *et al.* (2009) showed that K^+ leakage induced by surfactin in *B. subtilis*

NCIB3610 triggered the generation of pellicles in broth culture. Our finding that cereulide induced a powerful K^+ efflux both from *B. cereus* ATCC 14579^T and from *B. subtilis* DSM 347, but not from the cereulide-producing strains F4810/72 and NS58, shows that the cereulide-producing *B. cereus* strains possess a means to prevent cereulide-driven efflux of K^+ ions. López *et al.* (2009) further showed that membrane histidine kinase KinC is critical for sensing the effects of K^+ leakage and that the PAS-PAC domain of KinC is needed for this. The publicly available sequenced genomes of *B. subtilis* NCIB3610, *B. cereus* ATCC 14579^T (Ivanova *et al.*, 2003) and the cereulide-producing strain F4810/72 (see the relevant Integrated Microbial Genomes pages at http://img.jgi.doe.gov/) show that *B. subtilis* NCIB3610 and *B. cereus* ATCC 14579^T possess the gene for the PAS-PAC sensor signal transduction histidine kinase, whereas the genome of the cereulide producer *B. cereus* F4810/72 (=AH187) does not contain this gene. This may explain why high extracellular K^+ concentrations prevented pellicle formation in strain ATCC 14579^T but did not do so in strains F4810/72, F5881/94, NS58 and B318.

Niches of cereulide producers

The natural reservoirs of cereulide producers are not known (Ceuppens *et al.*, 2011). Cereulide-producing isolates are rarely retrieved from soil (Altayar & Sutherland, 2006; Hoton *et al.*, 2009), with the exception of rice fields (Ueda & Kuwabara, 1993). The producers of the highest amounts of cereulide, NS58, NS88, NS115 and NS117 (this study and Pirttijärvi *et al.*, 1999; Carlin *et al.*, 2006; Shaheen, 2009; Jääskeläinen, 2008), so far reported were endophytes of a live conifer tree (Norway spruce, *Picea abies*; Hallaksela *et al.*, 1991). Cereulide-producing strains are also well represented among endophytes of potatoes (consumer product from local supermarket; Virtanen *et al.*, 2008). It is possible that the interstitial spaces and vascular ducts of live plants are a potassium-deficient microenvironment in which cereulide producers may benefit from their ability to scavenge K^+.

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