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Cereulide production by *Bacillus weihenstephanensis* strains during growth at different pH values and temperatures

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ABSTRACT

Besides *Bacillus cereus*, some strains of the psychrotolerant, potentially foodborne pathogen *Bacillus weihenstephanensis* can produce the emetic toxine (cereulide). This toxin is a heat- and acid-stable cyclic dodecadepsipeptide that causes food intoxication with vomiting. However, some severe clinical cases with lethal outcomes have been described. If cereulide can be produced during refrigerated storage, it will not be inactivated by reheating food, representing an important risk of food intoxication for consumers. In this paper, we determined the capacity of the *B. weihenstephanensis* strains BtB2-4 and MC67 to grow and produce cereulide on agar media at temperatures from 8 °C to 25 °C and at a pH from 5.4 to 7.0. At 8 °C, strain BtB2-4 produced quantifiable amounts of cereulide, whereas the limit of detection was reached for strain MC67. For BtB2-4, cereulide production increased 5-fold between 8 °C and 10–15 °C and by more than 100-fold between 15 °C and 25 °C. At temperatures of 10 °C and higher, cereulide concentrations were within the range of those reported by previous works in foods implicated in emetic poisoning. At 25 °C, decreasing the pH to 5.4 reduced cereulide production by strain BtB2-4 by at least 20-fold.

1. Introduction

Cereulide is the emetic toxin produced by some strains of the spore-forming pathogen *Bacillus cereus* (Ehling-Schulz et al., 2004; Stenfors Arnesen et al., 2008). Cereulide is a heat- and acid-stable cyclic dodecadepsipeptide, [D-*O*-Leu-D-Ala-*O*-Val-L-Val]3, with a molecular mass of 1153 Da (Agata et al., 1995; Teplova et al., 2006). Cereulide is synthesized by a non-ribosomal peptide synthase encoded by the cereulide synthase gene (*ces*) cluster (Ehling-Schulz et al., 2005). The cluster is located on the megaplasmid pCER270 which is related to the toxin plasmid pXO1 of *Bacillus anthracis* (Ehling-Schulz et al., 2006, 2015; Hoton et al., 2005). The effects of cereulide on the human body have not yet been completely elucidated. In the duodenum, this toxin acts on the 5-HT3 receptor and causes vomiting by stimulation of the afferent vagus nerve 0.5–6 h after the ingestion of contaminated food (Agata et al., 1995; Ehling-Schulz et al., 2004). It acts as a K^+ ionophore with a mechanism of action similar to that of valinomycin. It interferes with oxidative phosphorylation on mitochondrial membranes (Hoton et al., 2005; Mikkola et al., 1999); (Paananen et al., 2002; Teplova et al., 2006). Cereulide has been involved in severe clinical cases, leading in some cases to death of the patient (Dierick et al., 2005; Mahler et al., 1997; Naranjo et al., 2011).

The majority of the *B. cereus* strains producing cereulide are mesophilic (Carlin et al., 2006) and belong to phylogenetic group III, growing between 15 °C and 45 °C (Guinebretière et al., 2008). These strains are not able to grow and produce cereulide during cold storage of food. However, some psychrotolerant *Bacillus weihenstephanensis* strains, belonging to the *B. cereus* group and classified into phylogenetic group VI (Guinebretière et al., 2008; Guinebretiere et al., 2010), can produce cereulide (Hoton et al., 2009; Thorsen et al., 2006). These psychrotolerant cereulide-producing strains represent a potentially serious food safety risk

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for consumers of cooked-refrigerated foods. Indeed, if these strains grow and produce cereulide during food storage at low temperatures, the emetic toxin will not be inactivated by food reheating before consumption. However, the production of cereulide at low temperatures by these psychrotolerant strains is still debated. For instance, a recent study showed that *B. weihenstephanensis* MC67 grew at 8 °C and produced approximately 230 ppb of cereulide per bacterial wet biomass after 9 days on PCA (Rossvoll et al., 2014). In contrast, a previous study showed that this strain produced cereulide at a level close to the limit of detection at 8 °C after 1, 2 or 3 weeks on BHI-agar (Thorsen et al., 2009a). Furthermore, another cereulide-producing strain, *B. weihenstephanensis* M118, did not produce cereulide in cooked meat sausages stored at 8 °C (Thorsen et al., 2009b). At higher temperatures, the quantity of cereulide produced varied according to the food products and seemed to depend on the food pH. For example, a mesophilic emetic *B. cereus* strain produced 2.4 μg/g of cereulide in liver sausages at pH 6.2 and 1.0 μg/g in a quark dessert at pH 5.1 (Dommel et al., 2010).

All studies to date regarding cereulide production by *B. weihenstephanensis* have been performed using the psychrotolerant MC 67 and MC118 strains, which are phylogenetically very similar, both harbouring the *ces* genes on a comparable plasmid (Mei et al., 2014).

The goal of the present study was to determine the impact of growth temperature and pH on cereulide production by phylogenetically distant *B. weihenstephanensis* strains (Hoton et al., 2009; Rossvoll et al., 2014; Thorsen et al., 2006) (Hoton et al., 2009; Mei et al., 2014).

2. Materials and methods

2.1. Strains

The cereulide-producing strains used in this study were *B. weihenstephanensis* MC67, isolated from soil in a sandy loam on the island of Møn, Denmark, and *B. weihenstephanensis* BtB2-4, isolated from a forest soil (Hoton et al., 2009; Thorsen et al., 2006). Among the cereulide producing *B. weihenstephanensis*, these two strains are phylogenetically distant (Hoton et al., 2009; Mei et al., 2014). *B. weihenstephanensis* KBAB4, which was isolated from forest soil and belongs to phylogenetic group VI, was used as a non cereulide-producing strain (Sorokin et al., 2006; Vilas-Boas et al., 2002).

2.1.1. Growth conditions

Growth conditions were adapted from (Rossvoll et al., 2014): briefly, the stock cultures consisted of exponential phase cells (OD_{600} of 0.5) in 30% glycerol, stored at −80 °C. Frozen suspensions were streaked onto plate count agar (PCA; Oxoid) and incubated at 30 °C overnight. Three colonies were transferred to 10 ml Tryptic Soy Broth (TSB; Oxoid) in KIMAX tubes and cultured for 18–20 h at 20 °C while shaking at 200 rpm. The cultures were then incubated at 4 °C for 18–21 h for cold adaptation prior to growth (Rossvoll et al., 2014).

Bacterial growth occurred on PCA plates and on tryptic soy agar plates at 10 °C (TSA; Sigma-aldrich). The pH of the PCA plates was 7.0 ± 0.2 and was adjusted to 6.0, 5.6 or 5.4 with 1 N or 6 N HCl when necessary, 5.4 being the lowest pH permitting growth of *B. weihenstephanensis* at 8–10 °C (Guérin et al., 2016).

Each PCA plate was inoculated with 100 μl of a 10^3 CFU/ml dilution of the inoculum stored at 4 °C, which resulted in approximately 10^2 cells per plate. The agar plates were incubated at temperatures varying between 8 °C and 25 °C. For each condition, the test was performed three times with an independently prepared inoculum (biological triplicates). Plates were removed daily for the determination of viable cells. As 95% of cereulide is associated with the bacterial biomass (Thorsen et al., 2009a), the cells were collected from the agar surface using 2 ml of sterile water and a sterile Drigalski spatula. An aliquot of 100 μl of each suspension was used and plated in serial dilutions on Luria Bertani plates (LB; Biokar), and then the cells were incubated at 30 °C. The rest of the suspension was stored at −20 °C for cereulide extraction.

2.1.2. Extraction of cereulide

For cereulide extraction (Ronning et al., 2015), 1.4 ml of all samples were transferred into 2-ml non-skirted microtubes with O-ring seal caps (Dutscher) with 0.3 g of 0.1 mm silica spheres (MP Biomedical), and the cells were disrupted for 45 s at 1500 beats per min (bpm) using a Mini-G™ (SPEX®SAMPLEPREP). After centrifugation (3 min at 19,000 × g), 1 ml of each sample supernatant was recovered in 10 ml glass tubes. Ten nanograms of an internal standard, $^{13}C_6$-cereulide (>95% purity, 20 μg/ml in acetonitrile; Chiralix), and 1 ml of methanol LC-MS Grade (Fischer chemical) were added to each glass tubes. All samples were vigorously shaken for 30 s and evaporated to dryness by heating at 80 °C under a steady flow of nitrogen. Then, 1 ml of methanol was added, and all samples were vigorously shaken for 30 s and centrifuged 5 min at 5000 × g. The supernatant was recovered in a new glass tube before a second evaporation step at 80 °C under a nitrogen flow. When the samples were completely dry, 100 μl of methanol/sterile water (3/1 vol/vol) was added to the glass tubes, which were immediately vortexed for 30 s. The 100-μl samples were spun down and transferred through a 0.2-μm nylon Micro centrifuge filter (Sigma-Aldrich) and centrifuged 1 min at 10, 000 × g. Filtrates were then transferred into HPLC vials (screw vials 8-mm amber glass 12 × 32 mm with label; Interchim) containing a 0.1-ml micro insert (Sigma-Aldrich) and stored at −20 °C before analysis by LC-MS/MS.

2.1.3. Calibration curve

The calibration curve, for quantification of cereulide in the samples, was performed by adding cereulide (cereulide of >95% purity, 50 μg/ml in acetonitrile; Chiralix) (Biesta-Peters et al., 2010) to the fresh cell biomass of the non-cereulide producer *B. weihenstephanensis* KBAB4 at final concentrations of 0.1, 1.0, 5.0, 10.0, 50.0 and 100.0 ng/ml (Ronning et al., 2015). KBAB4 cells were grown on PCA plates at 30 °C overnight as described above. The cells were collected from the plates, disrupted and centrifuged as described above for the emetic strains. One millilitre of this supernatant was supplemented with cereulide and underwent the subsequent steps of the cereulide extraction protocol described above, including the addition of $^{13}C_6$-cereulide as an internal standard. The 18 samples of KBAB4 biomass were divided into the six cereulide concentration levels. Several controls were used for analysis, including extracts of KBAB4 cells alone (negative control) and extracts of KBAB4 cells with either cereulide at 10 ng/ml or $^{13}C_6$-cereulide.

2.1.4. Quantification of cereulide by LC-MSMS

The LC-MS/MS analysis was performed according to Ronning et al. (2015) using an Agilent 1200 SL LC-system (Agilent Technologies, Waldbronn, Germany) coupled with an Agilent G6460 MS/MS (Agilent Technologies, Santa Clara, CA, USA) (Ronning et al., 2015). The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as 3 times and 10 times the standard deviation of the signal obtained with the negative control, respectively (Ronning et al., 2015). They were determined as 0.1 ng/ml (LOD) and 0.33 ng/ml (LOQ).

2.1.5. Expression of results

The amount of cereulide was expressed as ng per ml of the