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# Phylogenetic and toxinogenic characteristics of *Bacillus cereus* group members isolated from spices and herbs

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ABSTRACT

The foodborne pathogen *Bacillus (B.) cereus* is a common contaminant in spices and herbs. To further characterise *B. cereus* and its closely related group members present in spices and herbs, we analysed presumptive *B. cereus* strains isolated from six different condiments with view to *B. cereus* group species, phylogenetic affiliation and toxinogenic potential.

Of a total of 59 isolates 44 were identified as *B. cereus* sensu stricto (s.s.), four as *B. toyonensis*-like, five as *B. thuringiensis*, one as *B. weihenstephanensis*, two as *B. pseudomycoides*/*B. mycoides* and three as undefined *B. cereus* group species. A maximum of three different species occurred simultaneously in the same spice sample. The isolates comprised 33 multilocus (ML) sequence types (STs), which can be assigned to three different phylogenetic groups. Except two *B. pseudomycoides*/*B. mycoides* strains, all isolates were able to produce enterotoxins and one strain the emetic toxin cereulide as detected by an immunoassay and LC-MS, respectively. The prevalence of toxin genes was 96.6% for *nheA*, 94.9% for *hblD*, 50.8% for *cytK-2* and 1.7% for *ces*. The emetic strain was characterised by ST 869, which for the first time was assigned to an emetic *B. cereus* (s.s.) strain and is not part of the previously known two emetic MLST clusters.

Our results demonstrate that not only *B. cereus* (s.s.) but also toxin producing *B. thuringiensis, B. weihenstephanensis* and *B. toyonensis*-like strains could be detected in condiments. For some isolates MLST revealed disagreements between phylogenetic relationship and the classification as *B. weihenstephanensis* and *B. mycoides* based on previously described species markers.



## 1. Introduction

The *Bacillus* (*B.*) *cereus* group, also referred to as *B. cereus* sensu lato (s.l.) or presumptive *B. cereus*, comprises Gram-positive spore forming bacteria of the species *B. cereus* sensu stricto (s.s.), *B. weihenstephanensis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides, B. anthracis, B. cytotoxicus* and *B. toyonensis*. Due to their close genetic relationship the individual group species are difficult to distinguish. Standard methods for the detection and enumeration of presumptive *B. cereus* are not discriminative. Thus, routine diagnostics as well as previous studies on the occurrence of *B. cereus* (s.s.) in food do not consistently differentiate individual *B. cereus* group species. Consequently, also the contribution of the different species to foodborne disease outbreaks is uncertain. Moreover, based on increasing whole genome sequence data the *B. cereus* group taxonomy is currently under revision and might turn out much more complex: 30 rather than eight species were recently suggested (Liu et al., 2015).

In our study we focus on those *B. cereus* group species that are validly published at the time of writing this report (see above). However, in consideration of putative taxonomic revisions and methodical difficulties of species determination, the assignment to a *B. cereus* group species has currently only a preliminary character. Thus, species names in our report address the most likely species based on colony morphology and the results of real-time polymerase chain reaction (PCR), microscopy and multilocus sequence typing (MLST). The uncertainty regarding the species applies to our own data as well as cited data of previous reports.

Due to the ubiquitous nature and the resistance of its endospores members of the *B. cereus* group are detectable in many kinds of food, among them frequently in spices and dried herbs (EFSA, 2013). Not uncommonly, contamination levels of $10^3$ to $10^5$cfu/g

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are reported for *B. cereus* (s.s.) in condiments (Banerjee & Sarkar, 2003; EFSA, 2005; Hariram & Labbe, 2015; Kneifel & Berger, 1994; Sagoo et al., 2009). Also a number of foodborne disease outbreaks caused by *B. cereus* (s.s.) could be associated with spices (EFSA, 2016; WHO, 2014).

*B. cereus* (s.l.) related foodborne disease is caused by enterotoxins or an emetic toxin resulting in a diarrheal or an emetic type of illness. In general, the enterotoxins are formed in the human gut after consumption of cells or spores in quantities of usually more than $10^5$ cfu/g of food (EFSA, 2016). In contrast, the emetic toxin is already produced in the food, while the onset of toxin production is also associated with high cell densities of $10^5$ to $10^6$ cfu/g (Ceuppens et al., 2011). Such cell concentrations may occur in food with preferable growth conditions after addition of contaminated seasonings, especially if these foods are cooled improperly after heat treatment.

Three different enterotoxins are recognised as the main cause for *B. cereus* (s.l.) related diarrhoea: the protein-complexes haemolysin BL (Hbl) and non-haemolytic enterotoxin (Nhe) and the single protein cytotoxin K (CytK) (Ceuppens et al., 2011). For CytK the two variants CytK-1 and CytK-2 are described with the first one showing higher cytotoxicity and being specific for *B. cytotoxicus* (Fagerlund, Ween, Lund, Hardy, & Granum, 2004; Guinebretiere et al., 2013). The causative agent for the emetic symptom, cereulide, is a heat stable cyclic peptide generated by the non-ribosomal cereulide synthetase (Ehling-Schulz & Vukov 2005).

The enterotoxin components are encoded in chromosomally located operons comprising *hblC*, *hblD*, *hblA* for Hbl (L2, L1 and B component) or *nheA*, *nheB*, *nheC* for Nhe (A, B and C component), and the single gene *cytK-1* or *cytK-2* for CytK1 or CytK2. The non-ribosomal cereulide synthetase is encoded in the megaplasmid located *cesHPTABCD* genes cluster (Ceuppens et al., 2011).

The ability to produce cereulide is so far reported for *B. cereus* (s.s.) and rarely for *B. weihenstephanensis* strains (Thorsen et al., 2006). In contrast, one or more enterotoxin genes (*nhe*, *hbl* and/or *cytK* genes) can be found in strains of all *B. cereus* group species (EFSA, 2014; Guinebretiere et al., 2010). Nevertheless, cytotoxicity is not evenly distributed among the different species and different phylogenetic groups (Guinebretiere et al., 2010).

Regarding *B. cereus* group isolates from spices and herbs data on the toxinogenic potential are limited and even more concerning phylogenetic information. However, these are important characteristics for assessing the risk associated with the occurrence of *B. cereus* group species in condiments or other food. Moreover, phylogenetic information could reveal links between phenotype and genotype and may further assist in epidemiological investigations.

The aim of our study was to enumerate and to characterise the *B. cereus* group population in spices and herbs in terms of species identification, phylogenetic affiliation and toxinogenic potential. Therefore, we analysed eight condiments on the presence of *B. cereus* group species. In total, 59 isolates were characterised through cultural, PCR based and microscopic determination of the *B. cereus* group species. In addition, MLST was applied to phylogenetically classify our strains and to assist in species identification. Further, we investigated the toxin gene profiles and the actual capability to produce toxins.

## 2. Material and methods

### 2.1. Control strains

As controls for the species determination and the toxinogenic analysis the following reference strains were used: *B. cereus* (s.s.) strain DSM 31, emetic *B. cereus* (s.s.) strain DSM 4312, *B. thuringiensis* strain DSM 2046, *B. pseudomycoides* strain DSM 12442, *B. weihenstephanensis* strain DSM 11821, *B. cytotoxicus* strain DSM 22905 and *B. anthracis* strain 081101RA0367-13/01/2014 provided by the German Federal Research Institute for Animal Health (FLI).

### 2.2. Enumeration and isolation of presumptive B. cereus in spices and herbs

In order to quantify and isolate members of the *B. cereus* group in condiments the following representatives were analysed: allspice, cinnamon, nutmeg, paprika and pepper for spices and basil, oregano and parsley for herbs. All spices and herbs were provided for research purposes by FUCHS Gewürze GmbH, Dissen, Germany as dried and ground powders. Except oregano and parsley all matrices were processed by heat steam treatment by the manufacturer for microbiological decontamination.

For presumptive *B. cereus* enumeration 10 g triplicates of each matrix were dissolved in 190 ml peptone water (0.1%, w/v; Merck, Darmstadt, Germany) and shaken by hand for 1 min. Using peptone water tenfold dilutions were prepared and 100 μl of the dilutions were plated on polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA; Oxoid, Wesel, Germany) in duplicate. Typical colonies were counted after incubation at 37 °C for 24 h to determine the cfu/g. If no typical colonies were countable, samples were again diluted as described above and 1 ml of the first dilution was split plated on three PEMBA plates in duplicate. The theoretical limit of detection (LOD) was therefore 20 cfu/g. Randomly picked typical colonies were confirmed on mannitol egg yolk polymyxin agar (MYP; Merck) and sheep blood agar (SBA; Mast Diagnostica, Reinfeld, Germany) plates. Confirmed strains were kept at −80 °C in glycerine culture. Calculation and declaration of cfu/g was carried out in accordance with ISO 7218:2014-09 (Anonymous, 2014a).

### 2.3. Species identification within the B. cereus group

Species determination was based on colony morphology, PCR, microscopy and MLST. For the attribution of isolates to individual species we made use of the following parameters: *B. mycoides* = rhizoid growth (*B. mycoides* may in addition also be PCR positive for *motB* using MotB_1 or MotB_2 probe) (Oliwa-Stasiak, Kolaj-Robin, & Adley, 2011); *B. pseudo*mycoides = rhizoid growth and PCR positive only for *bpm*; *B. weihenstephanensis* = non-rhizoid growth and PCR positive only for *motB* using the MotB_2 probe (Oliwa-Stasiak et al., 2011); *B. anthracis* = PCR positive only for *motB* using the MotB_1 probe and for PL3 (Oliwa-Stasiak et al., 2011; Wielinga et al., 2011); *B. thuringiensis* = PCR positive only for *motB* using the MotB_1 probe and for *cry1* or microscopic positive for parasporal crystals (Oliwa-Stasiak et al., 2011; Wielinga et al., 2011); *B. cytotoxicus* = PCR positive only for *cytK1* (Guinebretiere, Fagerlund, Granum, & Nguyen-The, 2006, 2013); *B. cereus* (s.s.) PCR positive only for *motB* using the MotB_1 probe and exclusion of alternative *B. cereus* group species based on the parameters mentioned above and the results of MLST (below).

The species *B. toyonensis* is formally only represented by the type strain NCIMB 14858$^T$ (previously BCT-7112$^T$). This strain was so far only differentiated from *B. cereus* (s.s.) by digital DNA:DNA hybridization (dDDH) based on whole genome sequences and supported by MLST using the housekeeping genes *adk*, *ccpA*, *glpT*, *pyrE*, *recF* and *sucC* (Jimenez et al., 2013). Both of these datatypes - whole genome sequences and MLST using the outlined genes - were not generated for our isolates. Instead, we made use of the publically available sequence types (STs) (http://pubMLST.org/bcereus/(Jolley & Maiden, 2010)) of NCIMB 14858$^T$ as well as three other strains belonging to the same genomospecies (VD148,

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