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Thermal inactivation parameters of spores from different phylogenetic groups of *Bacillus cereus*

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ABSTRACT

The thermal inactivation kinetics of spores from 39 *Bacillus cereus* strains belonging to six different phylogenetic groups (group II to VII) was studied. Fresh spore suspensions in glass capillaries were heated in an oil bath at three or more different temperatures for five different times. Survival curves and thermal death curves were established and the kinetic parameters D_T (decimal reduction time at temperature T) and z (temperature dependence of D_T) were derived by linear regression. Most strains (38/39) had survival curves without a pronounced shoulder or tail, as reflected by linear regression coefficients R^2 generally higher than 0.95. The heat resistance of the strains and groups of strains was then compared by determining the temperature (°C) at which logD = 0.8 ($T_{logD = 0.8}$). Spores from group VI strains showed significantly lower heat resistance than all other groups except group II, with $T_{logD = 0.8}$ ranging between 82.7 °C and 92.8 °C. Spores from groups III and VII, on the other hand, were generally most heat resistant, with $T_{logD = 0.8}$ between 91.9 °C and 101.8 °C. Further analysis revealed a positive correlation between spore heat resistance and both minimal and maximal growth temperatures of the strains. In contrast, the z value was negatively correlated with the minimal and maximal growth temperatures. The availability of genetic group-specific heat resistance data will contribute to a more accurate risk assessment of *B. cereus*.

1. Introduction

Bacillus cereus sensu lato is a large and diverse group of facultatively anaerobic, Gram-positive spore-forming bacteria belonging to the Firmicutes phylum. They are widely distributed in nature, being commonly found in the soil but also in the digestive tracts of vertebrate and invertebrate animals. As such, they also frequently contaminate and spoil raw and processed foodstuffs, including starchy food, vegetables, meat and dairy products (Samapundo et al., 2011). Furthermore, some members of the *B. cereus* group are food-borne pathogens, causing emetic or diarrheal disease (Arnesen et al., 2008). The emetic syndrome is caused by cereulide, a heat-stable, acid and protease-resistant cyclic peptide toxin that is produced in the food before ingestion. Cereulide intoxication is highly associated with rice and rice products, has a short incubation time of 30 min to 6 h, and generally lasts for 6 to 24 h with symptoms of nausea and vomiting, similar to those of *Staphylococcus aureus* intoxication (Arnesen et al., 2008; Ehling-Schulz et al., 2006; Mahler et al., 1997). The diarrheal syndrome is an infection with an incubation time of 8–16 h resulting from the production of heat-labile protein enterotoxins in the small intestine. Diarrheal strains can produce several enterotoxin complexes, like hemolysin BL (Hbl) and nonhemolytic enterotoxin (Nhe), or single protein enterotoxins such as cytotoxin K and enterotoxin T (Michelet et al., 2006; Schoeni and Wong, 2005). The contribution of each of these toxins to the disease is not exactly known, and it remains to be investigated whether they act separately or in combination. Diarrheal disease symptoms resemble those caused by *Clostridium perfringens* and include abdominal pain, watery diarrhea and, occasionally, nausea and emesis.

Six species are commonly distinguished within the *B. cereus* group based on specific phenotypic properties, and the foodborne pathogens are designated as *B. cereus* sensu stricto (Arnesen et al., 2008; Pirttijärvi et al., 2000). However, more recent DNA-based methods have revealed that these phenotypically delineated species do not form homogeneous genetic groups. Using a polyphasic approach based on both molecular (Fluorescent Amplified Fragment Length Polymorphism, ribosomal gene sequences, partial *panC* gene sequences and 'psychrotolerant' DNA sequence signatures in *cspA*), and phenotypic properties (growth temperature range), Guinebretière et al. (2008) proposed a classification of *B. cereus* sensu lato into seven phylogenetic groups (Table 1).

This analysis shows that *B. cereus* sensu stricto, which groups the strains that can cause foodborne illness, is genetically and phenotypically heterogeneous, being dispersed over four phylogenetic groups.

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Table 1
Species composition and growth temperature range of seven phylogenetic groups of *B. cereus* sensu lato as proposed by Guinebretière et al. (2008).

Phylogenetic group	Growth temperature	Species
I	10–43 °C	*B. pseudomycoides*
II	7–40 °C	*B. thuringiensis* II or *B. cereus* II
III	15–45 °C	*B. thuringiensis* III, *B. cereus* III or *B. anthracis*
IV	10–45 °C	*B. thuringiensis* IV or *B. cereus* IV
V	8–40 °C	*B. thuringiensis* V or *B. cereus* V
VI	5–37 °C	*B. weihenstephanensis*, *B. mycoides* or *B. thuringiensis* VI
VII	20–50 °C	*B. cytotoxicus*

Moreover, strains from phylogenetic group VII, which have been recently assigned to the novel species *Bacillus cytotoxicus*, are also foodborne pathogens (Guinebretière et al., 2013). The study of the ecological and phenotypic properties of the different phylogenetic groups will provide a better insight into the causes of *B. cereus* group foodborne illness, and allow the development of more effective control measures.

Since *B. cereus* is a sporeformer, foods that are mildly heated and subsequently refrigerated are a particular concern, not only with regard to foodborne illness but also food spoilage. In particular the psychrotolerant strains of group VI are known to cause spoilage of foods like milk or ready-to-eat meals (Carlin et al., 2000; Larsen and Jørgensen, 1997). Several studies have shown a large variability in spore thermal resistance within the *B. cereus* group (Choma et al., 2000; Fernández et al., 1999; Wijnands et al., 2005). A large comparative study of hundred *B. cereus* strains from various sources revealed that strains producing emetic toxin had higher heat resistance than diarrheal and environmental strains (Carlin et al., 2006). When the same strains were later typed it was concluded that spore heat resistance was lowest for group VI, highest for groups III and VII, and intermediate for groups II, IV and V (Afchain et al., 2008; Carlin et al., 2010). However, even this large study leaves some questions unanswered. For example, the comparison between phylogenetic groups was based on analysis of the heat resistance of the spores at only one single temperature (90 °C), and it is therefore not known whether spores from the different genetic groups have different z values (parameter reflecting temperature sensitivity of the heat resistance). Knowledge of the z value is essential to correctly estimate spore inactivation at temperatures different from those at which heat resistance (decimal reduction time, D) has been experimentally determined. Further, spores from the most heat resistant strains were hardly inactivated at 90 °C, leading to high experimental errors in the reported $D_{90\ °C}$ values.

Therefore, the aim of this study was to document spore heat resistance of *B. cereus* strains from different phylogenetic groups at different temperatures, in order to allow a more detailed analysis of the difference in heat resistance between these groups as well as a comparison of the z values.

2. Materials and methods

2.1. Microorganisms

A collection of 39 *B. cereus* strains belonging to different phylogenetic groups was used in this work. The origin and relevant properties of these strains are listed in Table S1 (Supplementary data). Thirty strains of this collection were obtained from M.-H. Guinebretière (INRA, Avignon, France), while the other nine are psychrotrophic strains isolated from soil as follows. Different samples containing 10 g of soil were suspended (1:10) in sterile 10 mM potassium phosphate buffer pH 7.0, heated at 70 °C for 10 min to eliminate vegetative cells, and 1 ml was surface-plated on Plate Count Agar (PCA—Oxoid, Basingstoke, U.K.) with 50 μg/l polymyxin. Colonies emerging after 21 days of incubation at 6 °C were restreaked twice on PCA for purification. Purified isolates were stored at −80 °C as 24-h cultures in Lysogeny Broth (LB, 30 °C) with 25% glycerol.

Further identification and characterization of the isolates were done by PCR amplification and (partial) sequence analysis of the 16S rRNA and *panC* genes. The primer pairs used were GGTTACCTTGTTACGACTT (1492R) and AGAGTTTGATCCTGGCTCAG (27 F) for the 16S rRNA gene, and TYGGTTTTGTYCCAACRATGG and CATAATCTACAGTGCCTTTCG for the *panC* gene. This allowed for identifying *B. cereus* sensu lato strains and to assign them to phylogenetic groups I–VII using an online algorithm specifically developed for this purpose (Guinebretière, 2011). Finally, we used a diagnostic PCR method to distinguish psychrotrophic from mesophilic strains based on the presence of specific sequence signature in the *cspA* cold shock gene of the former (Francis et al., 1998).

2.2. Spore production

To induce sporulation, 100 μl of a culture grown at 30 °C in Brain Heart Infusion (BHI) broth (Oxoid) was diluted into 1 ml of deionized water, surface-plated on Nutrient Agar CM0003 (Oxoid) and incubated at 30 °C. This medium contains added $CaCl_2$ (0.125 g/l), $MgCl_2$ (0.125 g/l) and $MnSO_4$ (0.0025 g/l), which promote sporulation and spore heat resistance. The plates were checked regularly and harvested only when more than 95% of the cells were phase bright spores. Depending on the strain, this took 4–21 days. Spores were harvested from the plates in deionized water, washed three times by centrifugation at 4000 ×*g* for 10 min at 4 °C, resuspended in deionized water to a concentration of approximately 10^8 spores/ml and stored at −20 °C.

2.3. Thermal treatment

Spore suspensions were used within one week after harvesting. They were reexamined by phase contrast microscopy and only used when the fraction of phase bright spores was >90%. Spores were diluted in potassium phosphate buffer (10 mM, pH 7.0) to reach a concentration of approximately 10^7–10^8 cfu/ml. The suspension was vortexed well and microscopic examination confirmed there was no clumping of spores. The initial concentration of heat resistant spores in the suspension (N_0) was then exactly determined by plate count of a portion of the suspension that had been heated at 70 °C for 15 min to kill any remaining vegetative cells.

For the kinetic heat inactivation experiments, 80 μl portions of spore suspension were transferred into glass capillaries, which were heat sealed and immersed in an oil bath at at least three different temperatures (chosen depending on the heat resistance of the strain) for 5, 10, 15, 20, and 25 min. After heating, the capillaries were quickly cooled in an ice–water mixture and aseptically opened, and the number of surviving spores was determined by plate count on Trypton Soy Agar (TSA) plates (Oxoid). Decimal reduction times at each temperature (D_T) were calculated as the negative reciprocal of the regression line fitted to the data (log cfu/ml versus time–survivor curve). We also calculated for each strain the temperature (°C) at which logD equals 0.8, further denoted as $T_{logD\ =\ 0.8}$, because this allowed better comparison between strains (see results). z values of each strain were determined as the negative reciprocal of the regression line fitted to the thermal death time curve ($logD_T$ versus temperature). All heat treatments were done in triplicate using the same spore suspension. The number of survivors (log cfu/ml) for each treatment was calculated as the average of the triplicate data. Significant differences among $T_{logD\ =\ 0.8}$ and z values of different groups were analyzed by using the one-way Analysis of Variance (ANOVA), followed by Tukey–Kramer's post-hoc test for multiple comparison with a 5% level of significance ($p < 0.05$).

2.4. Determination of cardinal growth temperatures

For each of the 39 *B. cereus* strains, three colonies from 24-h culture on BHI agar at 30 °C were each streaked for single colonies on a fresh