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Absence of oxygen affects the capacity to sporulate and the spore properties of *Bacillus cereus*

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ABSTRACT

This study was performed to evaluate the effect of anaerobiosis on the formation of *Bacillus cereus* spores and their resulting properties. For this purpose, an appropriate sporulation medium was developed (MODs). Sporulation of 18 strains from different phylogenetic groups of *B. cereus* was studied in MODs medium in aerobiosis and anaerobiosis. In anaerobiosis, sporulation ability was weaker and more heterogeneous than in aerobiosis. Among tested strains, *B. cereus* AH187 produced the highest level of spores in anaerobiosis. This strain was therefore chosen to study spore properties. Spores produced in anaerobiosis were more resistant to wet heat at 90 °C, 92.5 °C, 95 °C, 97.5 °C and 100 °C. For example, D_{90} were 21,09 ± 1.70 and 81.87 ± 2.00 for aerobiosis and anaerobiosis conditions, respectively. Spores produced in anaerobiosis have a *z*-value of 7.70 °C compared with 10.52 °C for spores produced in aerobiosis. Spores produced in anaerobiosis were also more resistant to 1 M NaOH, 1 M nitrous acid and pulsed light at fluences of 0.34 J cm^{-2} and 0.49 J cm^{-2}. No difference in resistance to UV-C, 5% hydrogen peroxide or 0.25 mM formaldehyde was observed between these two conditions. In the presence of L-alanine, spores produced in anaerobiosis germinated more efficiently than spore produced in aerobiosis. No difference in germination was observed with inosine as inducer. No difference in the size of spores produced in the different conditions was observed by transmission electron microscopy. However, spores obtained under anaerobic conditions had a damaged exosporium, or in some cases a completely detached exosporium, unlike spores produced under aerobic conditions.

This study shows that few spores are formed under anaerobic condition; nevertheless, this condition has an impact on the spore properties of *B. cereus* AH 187 strain. Spores obtained under anaerobic condition were more resistant to heat and to some chemical compounds. This is an important feature, considering the risk associated with the presence of this pathogen in thermally processed and packaged food in absence of oxygen.

1. Introduction

The *Bacillus cereus* group is formed of ubiquitous Gram-positive facultative anaerobic endospore-forming bacteria. It comprises seven phylogenetically close species: *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis*, *Bacillus cytotoxicus* and *B. cereus sensu stricto*. Seven major phylogenetic groups (I–VII) have been identified, with ecological differences that are evidence for a multiemergence of psychrotolerance in the *B. cereus* group. A moderate thermotolerant group (VII) was basal to the mesophilic group I, from which in turn distinct thermal lineages have emerged, comprising two mesophilic groups (III, IV), an intermediate group (V) and two psychrotolerant groups (II, VI) (Guinebretiere et al., 2008, 2013). *B. cereus* is frequently isolated from foods and it can cause two types of foodborne disease, described as emetic and diarrheal. The emetic syndrome results from toxins produced in the ingested food. Diarrheal toxi-infection results from ingesting food contaminated by *B. cereus*, whose cells transit through the digestive tract and produce the diarrheal toxins in the small intestine (Perez Portuondo, 2012; Ceuppens et al., 2013).

Under environmental stress conditions such as nutrient deprivation, *B. cereus* cells are able to produce spores (Moir et al., 2002; Setlow, 2003; Setlow and Johnson, 2007). Sporulation can take

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place (i) in soil (considered the natural habitat of spores), (ii) insects, and animal gut, and (iii) food processing lines or food such as ready-to-eat foods, dairy products and vegetables. It is well known that spores are able to resist chemical and physical stresses such as air-drying, high temperature, high pressure, UV light and acidity (Clavel et al., 2004; Setlow, 2006; Tam et al., 2006; Nguyen Thi Minh et al., 2011). This high resistance ability is in some extent due to the presence of calcium dipicolinate and the dehydration state of the spore core (de Vries et al., 2005). These properties favor their survival through food processing and their long-term persistence in foods, where they cause serious problems (Andersson et al., 1995; Carlin, 2011). Under suitable conditions, *B. cereus* spores are able to germinate and revert to life as vegetative cells responsible for toxins production (Paredes-Sabja et al., 2011). Germination may take place within minutes; this depends on strains and conditions under which spores have been produced. In addition, germination can be initiated by some amino acids, sugars or other compounds found in complex culture media in bacilli (van der Voort et al., 2010; McKenney et al., 2013).

The different environments where sporulation take place present numerous temperatures and nutrient conditions affecting various spore properties, including resistance to many different stress factors, structure and composition (Margulis et al., 1998; Nicholson, 2002; Cazemier et al., 2001; Melly et al., 2002; Rose et al., 2007; Nguyen Thi Minh et al., 2008; van der Voort et al., 2010; van der Voort and Abee, 2013). In *B. cereus* and *Bacillus subtilis*, the effect of temperature and nutrient conditions on spore resistance to heat, UV and chemicals and germination has been extensively studied (Melly et al., 2002; Faille et al., 2007; Gounina-Allouane et al., 2008; Planchon et al., 2011). Few studies on the effect of anaerobiosis on sporulation of the *B. cereus* group have been published except the effect of oxygen on the sporulation capacity of *B. thuringiensis* but spore properties were not studied (Avignone-Rossa et al., 1992; Finlay et al., 2002; Boniolo et al., 2012). Most studies on sporulation in anaerobic condition were performed with the anaerobic clostridia species (Paredes et al., 2005). However, *B. cereus* vegetative cells can be found in a large variety of natural environments with low oxygen level (intestine, soil or on food processing line) where sporulation can take place. Then, spores of *B. cereus* can be found in various food processing intermediates and foods products. Spores produced in these anaerobic environments could have particular properties and cause a problem of food safety.

The aim of this work was to investigate the sporulation capacity of *B. cereus* in an anaerobiosis environment. In this work, a panel of strains belonging to *B. cereus* phylogenetic groups II–VII was studied for their capacity to sporulate in anaerobiosis. In addition, spores of the strain AH187 from group III were study for heat resistance, chemical resistance, germination capacity, spore size and structure.

2. Materials and methods

2.1. Strains and media

A collection of 18 *B. cereus* strains was selected for this study (Table 1). These strains belong to phylogenetic groups II, III, IV, V, VI and VII as defined by Guinebretiere et al. (2008). The chemically defined medium MOD, previously described by Glatz and Goepfert (1977) was optimized for the growth and sporulation of *B. cereus* strains. MOD medium was first supplemented with 5.74 mM K_2HPO_4, 45.4 mM $(NH_4)_2SO_4$, and 0.16 mM $MgSO_4$ (Duport et al., 2004). This medium (MODs) was then quarter-diluted and supplemented with 0.5 mM $MgCl_2$, 0.01 mM $MnCl_2$, 0.05 mM $ZnCl_2$, and 0.2 mM $CaCl_2$. MODs was finally adjusted to pH 7.2 with KOH.

Table 1
Characteristics of the studied strains.

Strain designation	Origin	Temperature growth limits (C°)		Phylogenetic group[a]
		Lower	Upper	
D15	Diarrhoeal outbreak			II
INRA KBAA5	Soil	7	40	II
INRA 15	Food			II
AH187	Emetic outbreak			III-5
F837/76	Clinical infection	15	45	III-8
F4433/73	Diarrhoeal outbreak			III-12
F4430/73	Diarrhoeal outbreak			IV-3
ATCC14579	Type strain *B. cereus*	10	40/45	IV-3
NVH1230				
F2769/77	Diarrhoeal outbreak			V
NVH 141/1-01	Diarrhoeal outbreak	10	40	V
UHDAM TSP9	Environment			V
INRA KBAB4	Environment			VI-1
WSBC 10688	Environment	5/7	37	VI-2
WSBC 10204T	Type strain *B. weihens.*			VI-1
NVH883/00	Diarrhoeal outbreak			VII
AFSSA 08CEB44bac	Diarrhoeal outbreak	20	50	VII
NVH 391-98	Diarrhoeal outbreak			VII

[a] The phylogenetic groups to which they belong in the *B. cereus* group as defined by Guinebretiere et al. (2008).

For the growth of group VII strains, 4.8 mM tryptophan was added to the medium because of their auxotrophy for this amino acid. After autoclave sterilization, MODs was supplemented with 10 mM glucose as a fermentative carbon source. For uncontrolled batch culture, 80 mM K_2HPO_4 and 20 mM KH_2PO_4 were used to buffer the medium.

2.2. Uncontrolled batch cultures

Overnight cultures of *B. cereus* strains were run in MOD supplemented with 30 mM glucose with stirring (200 rpm). Incubation was performed at 30 °C for strains belonging to phylogenetic groups II, V and VI, and 37 °C for strains belonging to phylogenetic groups III, IV and VII. After incubation for 18 h, each culture was centrifuged at 7000*g* for 5 min at room temperature, and cells were suspended in MODs medium. For anaerobic experiments, cultures were run in Hungate tubes equipped with open-top caps and rubber septa, and filled with 12 ml MODs. To eliminate any traces of oxygen, a flow of N_2 sent through a Hungate column was sparged into the medium. Aerobic cultures were performed in 250 ml conical flasks containing 50 ml MODs. Cultures were performed in triplicate.

2.3. Controlled batch cultures

B. cereus AH187 cells from overnight cultures in MOD medium, were centrifuged at 7000*g* for 5 min at room temperature, washed twice and resuspended in MODs medium. A 2 L bioreactor (Inceltech Discovery 100 (Toulouse, France) containing 1.3 L of MODs medium was inoculated at an initial A_{600} value of 0.05 in MODs with this suspension. Temperature was regulated at 37 °C. For aerobic conditions, agitation was adjusted to maintain the partial pressure of oxygen (pO_2) above 60%. Bioreactors were kept under airflow to obtain aerobiosis, or nitrogen flow to obtain anaerobiosis. The pH was regulated at 7.2 by adding 2 M KOH or 2 M HCl. The regulated batch was equipped with a polarographic oxygen electrode (Mettler Toledo) calibrated in a medium flushed with air