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Comparative evaluation of DNA extraction methods for amplification by qPCR of superficial vs intracellular DNA from *Bacillus* spores

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ABSTRACT

This study was designed to assess the efficiency of eight extraction methods regarding their ability to release superficial (exogenous) and intracellular (endogenous) DNA from *B. cereus* spores for subsequent analysis by quantitative PCR (qPCR). *B. cereus* spore suspensions were subjected to both commercial DNA extraction kits and mechanical DNA extraction methods. The spores were observed by transmission electron microscopy to evaluate any damage caused during extraction. The efficiency of both extraction and purification were assessed using a qPCR assay targeting the *bclA* gene. Most of the extraction methods assessed, except the passage through the French press or the use of the QIAamp DNA Blood Mini kit without 95 °C treatment, allowed the amplification of significant amounts of DNA. By using propidium monoazide, which is a photoreactive DNA-binding dye, the presence of non-negligible amounts of amplifiable DNA at the spore surface was highlighted. A further set of extraction assays was then performed on spores previously treated with PMA. The results of this study show that both superficial and intracellular spore DNA can be released by extraction methods to a greater or lesser extent and then further amplified by qPCR. The Precellys extraction allowed the detection of both intracellular and superficial DNA, the DNeasy Blood & Tissue kit the specific detection of intracellular DNA, while the Instagene kit detected only superficial DNA. Of the methods tested in this study, the Precellys extraction was the most efficient in terms of further DNA detection.

Significance and impact of the study: In order to verify the presence or absence of *B. cereus* spores in food or on surfaces in the food environment, the use of an efficient extraction method is required, followed by a qPCR analysis on the DNA released. Conversely, in order to quantify the population of *Bacillus* spores, any superficial DNA must be blocked, e.g. with PMA, prior to intracellular DNA extraction and further amplification.

1. Introduction

Bacillus cereus is a spore-forming bacteria often associated with food products such as meat, fish, vegetables, rice, and dairy products. *B. cereus* is of great concern to the food industry since many *B. cereus* strains produce enzymes responsible for food spoilage (Flach et al., 2014) and/or toxins responsible for foodborne illness (Bennett et al., 2013). *B. cereus* is also frequently isolated as adherent spores or as biofilms from various environments including food processing lines and workshops (Cappitelli et al., 2014; Shi and Zhu, 2009). Biofilms are particularly difficult to eradicate, partly because of the intense sporulation often occurring within *B. cereus* biofilms (Faille et al., 2014).

It is thus crucial that accurate methods be provided for the detection of *B. cereus* cells and spores on contaminated surfaces. Indeed current detection methods including conventional culturing and biochemical or serological analysis (Encinas et al., 1996; Wong et al., 1988), take at least two days to deliver results. In addition, these methods would be much less sensitive than DNA-based methods (Fricker et al., 2007). Therefore, molecular methods and in particular quantitative polymerase chain reaction (qPCR) techniques have been developed either with the TaqMan® probe design (Fernandez-No et al., 2011; Reekmans et al., 2009), or with SYBR green technology (Hanabara and Ueda, 2016; Ueda et al., 2013; Wehrle et al., 2010), which have reduced the time and cost of detection of *B. cereus*.

Since *Bacillus* spores are commonly found in food or environmental isolates, an efficient and reproducible spore lysis is a prerequisite for the enumeration of *Bacillus* populations. Various methods have been devised to make spore DNA accessible for further amplification,

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including 1) heat treatment (Drago et al., 2002; Luna et al., 2003; Ryu et al., 2003), 2) treatment with hot detergent (Buttner et al., 2001; Kabir et al., 2003), 3) mechanical disruption, e.g. sonication (Belgrader et al., 1999; Luna et al., 2003), FastPrep FP120 (Priha et al., 2004) or liquid nitrogen grinding (Kabir et al., 2003), or 4) DNA commercial extraction kits (Mertens et al., 2014). However, most approaches have not been evaluated against spore lysis efficacy. Indeed, it is widely admitted that *Bacillus* spores carry extracellular DNA. This superficial DNA, not readily removed by multiple wash steps, can be amplified during PCR, leading to an over-estimation of the spore concentration (Belgrader et al., 1999; Vandeventer et al., 2011). On the contrary, previous observations have suggested that most extraction kits do not efficiently lyse the *Bacillus* spores, thereby resulting in an under-estimation of the number of spores (Dauphin et al., 2009; Priha et al., 2004).

In this study, commercial DNA extraction kits and mechanical DNA extraction methods were evaluated with regards to their ability to lyse spores and extract intracellular amplifiable DNA from *B. cereus* spores. The efficiency of spore lysis was verified by transmission electron microscopy. Extra- and intra-cellular DNA were analyzed by a qPCR assay, targeting the *bclA* gene with or without a PMA treatment.

2. Materials and methods

2.1. Strain, culture conditions and spore production

Bacillus cereus CUETM 98/4 (subsequently named Bc 98/4) isolated from a dairy processing line, was used in this study. Cultures were stored in brain-heart infusion (BHI) medium (AES, Combourg, France) supplemented with glycerol (18% v/v) at − 20 °C. Bc 98/4 was grown on nutrient agar (NA) at 30 °C for 24 h. Spores were produced at 30 °C on Spo8-agar (Faille et al., 2013). When over 95% of spores were obtained, they were harvested by scraping the surface, washed five times by centrifugation at 1500*g* for 15 min. The purity of spore batches was verified by observation under optical microscopy after staining with malachite green. Once reached to < 1% of vegetative cells observed, spores were stored at 4 °C in sterile water until use. Before each experiment, a further set of two washes was performed and spores were subjected to a 2.5-min ultra-sonication step in an ultrasonic cleaner (Bransonic 2510E–MT, 42 kHz, 100 W, Branson Ultrasonics Corporation, USA) to limit the presence of aggregates. An example of the spore batches used throughout this study is given in Fig. 1 (spores were stained with Acridine Orange, as described in Section 2.6).

2.2. Spore treatment with propidium monoazide (PMA)

PMA, a photo-reactive dye with a high affinity to DNA, was used to block accessible (superficial) DNA. Spores (± 10^8 spores/ml) were treated with PMA at 0.05 µM for 5 min in the dark at room temperature. The tubes were then exposed to light for 10 min using a PhAST Blue lamp at 80% (GenIUL, Barcelona, Spain). Spores were recovered by centrifugation (5000*g*, 10 min), and subjected to DNA extraction.

2.3. Extraction of DNA using commercial kits

The following commercial kits were used in this study: DNeasy Blood & Tissue (Qiagen, Hilden, Germany) with or without lysozyme at 20 mg/ml; QIAamp DNA Minikit (Qiagen) and InstaGene Genomic DNA Kit (Bio-Rad, Marne-La-Coquette, France). To determine the DNA-extraction efficiency of the kits, 1 ml of a suspension of around 10^8 spores/ml (determined by plating on nutrient agar) was treated as recommended by the suppliers, with minor modifications.

2.4. Physical extraction of the DNA from B. cereus spores

One milliliter of spore suspensions (around 10^8 spores/ml) were subjected to the following physical treatments: 1) five successive passages through a French press at 20.000 psi (SLM Instruments, Urbana, USA) as previously described (Faille et al., 2010); 2) 3 rounds of 30 s of bead-beating at 6000 rpm, at 4 °C with Zirconia-Silica beads (0.1 mm diameter, BioSpec Products, Bartlesville, OK, USA), using a Precellys 24 homogenizer (Bertin Technologies, Aix en Provence, France); 3) 3 rounds of 60 s of the same bead-beating treatment. After treatments, spore and exosporium fragments were pelleted by centrifugation (3000 g, 30 min, 4 °C) and supernatants were kept at − 20 °C until use. The spore pellets were washed twice in water and kept at 4 °C until use.

2.5. Primer design and qPCR assay

The *bclA* gene coding for the protein backbone of the major surface glycoprotein of *B. cereus* spores was chosen for the following reasons: 1) the *bclA* gene is present in all *B. cereus* strains contrarily to the toxin

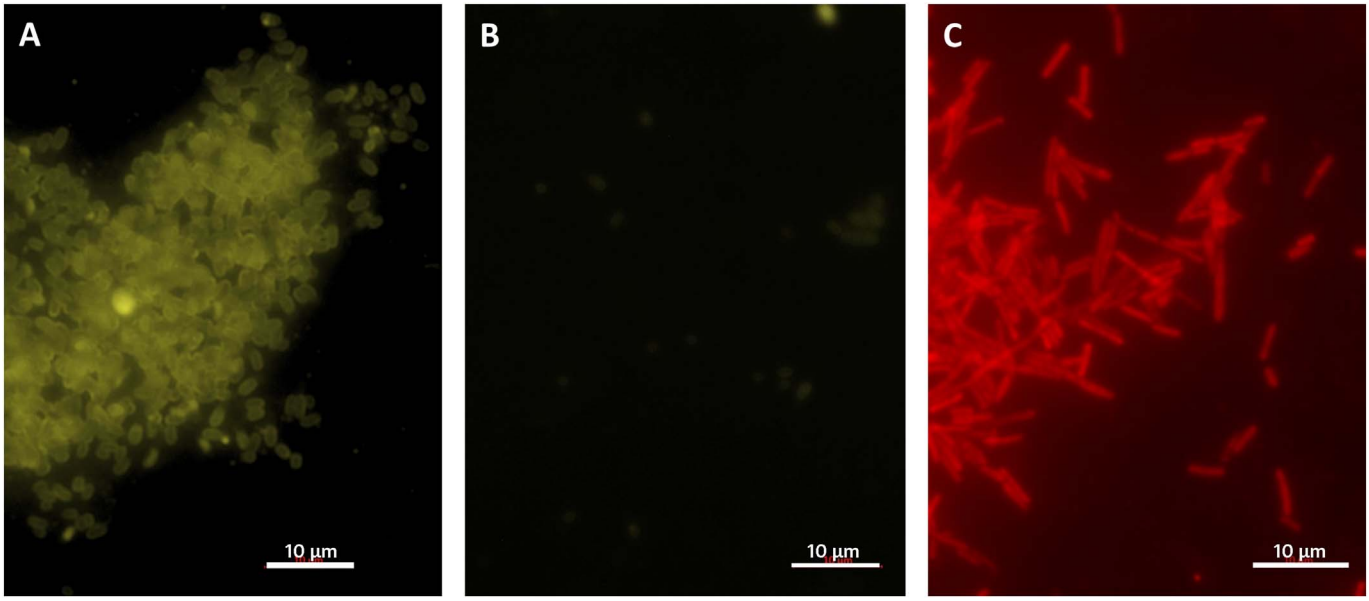

Fig. 1. Microscopic observations of *B. cereus* CUETM 98/4 spores pretreated with PMA (A) or without (B) with PMA, and of vegetative cells (C) stained with Acridine Orange (magnification × 1000). (For interpretation of the references to color, the reader is referred to the web version of this article.)