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Characteristics and phylogeny of *Bacillus cereus* strains isolated from Maari, a traditional West African food condiment

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ABSTRACT

Maari is a spontaneously fermented food condiment made from baobab tree seeds in West African countries. This type of product is considered to be safe, being consumed by millions of people on a daily basis. However, due to the spontaneous nature of the fermentation the human pathogen *Bacillus cereus* occasionally occurs in Maari. This study characterizes succession patterns and pathogenic potential of *B. cereus* isolated from the raw materials (ash, water from a drilled well (DW) and potash), seed mash throughout fermentation (0-96 h), after steam cooking and sun drying (final product) from two production sites of Maari. Aerobic mesophilic bacterial (AMB) counts in raw materials were of 10^5 cfu/ml in DW, and ranged between 6.5×10^3 and 1.2×10^4 cfu/g in potash, 10^9–10^{10} cfu/g in seed mash during fermentation and 10^7 – 10^9 after sun drying. Fifty three out of total 290 AMB isolates were identified as *B. cereus sensu lato* by use of ITS-PCR and grouped into 3 groups using PCR fingerprinting based on *Escherichia coli* phage-M13 primer (M13-PCR). As determined by *panC* gene sequencing, the isolates of *B. cereus* belonged to PanC types III and IV with potential for high cytotoxicity. Phylogenetic analysis of concatenated sequences of *glpF, gmk, ilvD, pta, pur, pycA* and *tpi* revealed that the M13-PCR group 1 isolates were related to *B. cereus* biovar *anthracis* CI, while the M13-PCR group 2 isolates were identical to cereulide (emetic toxin) producing *B. cereus* strains. The M13-PCR group 1 isolates harboured poly-γ-D-glutamic acid capsule biosynthesis genes *capA, capB* and *capC* showing 99-100% identity with the environmental *B. cereus* isolate 03BB108. Presence of *cesB* of the cereulide synthetase gene cluster was confirmed by PCR in M13-PCR group 2 isolates. The *B. cereus* harbouring the *cap* genes were found in potash, DW, cooking water and at 8 h fermentation. The "emetic" type *B. cereus* were present in DW, the seed mash at 48–72 h of fermentation and in the final product, while the remaining isolates (PanC type IV) were detected in ash, at 48–72 h fermentation and in the final product. This work sheds light on the succession and pathogenic potential of *B. cereus* species in traditional West African food condiment and clarifies their phylogenetic relatedness to *B. cereus* biovar *anthracis*. Future implementation of GMP and HACCP and development of starter cultures for controlled Maari fermentations will help to ensure a safe product.

1. Introduction

Maari is a spontaneously fermented alkaline food condiment produced from Baobab tree seeds (*Adansonia digitata L.*). Maari and similar products are produced in West African countries, especially in rural areas; they constitute an important part of the diet, as a nutritious protein rich supplement to various soups and sauces (Parkouda et al., 2009). Daily consumption of Maari in villages of Burkina Faso is commonly between 10 and 100 g per person (Kando C. K., oral communication). The microorganisms occurring in the spontaneous seeds fermentations are suggested to originate from handling, raw seeds, utensils, and containers (Odunfa, 1981). *Bacillus* species, notably *Bacillus subtilis*, are considered as the main fermenting microorganisms in these alkaline fermentations, while *Staphylococcus* spp. and lactic acid bacteria have been detected as well (Parkouda et al., 2009, 2010). Bacterial species belonging to *Bacillus cereus sensu lato (sl)* group may occur in high numbers, or even be dominant in traditional fermented African foods (Agbobatinkpo et al., 2013; Azokpota et al., 2007; Oguntoyinbo and Oni, 2004). It has been reported that *B. cereus* in a final Maari product comprised op to 43% of the total counts (10 $\log_{10}$ cfu/g) of aerobic mesophilic bacteria (Parkouda et al., 2010).

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B. cereus sl group includes the closely related species *B. cereus sensu stricto (ss), B. thuringiensis, B. anthracis, B. weihenstephanensis* (Lechner et al., 1998), *B. mycoides, B. pseudomycoides* (Nakamura, 1998) and *B. cytotoxicus* (Guinebretiere et al., 2012). *B. cereus* is of special concern as it may cause food poisoning, diarrhoea and emesis, through the production of enterotoxins or cereulide (Stenfors Arnesen et al., 2008), as well as various systemic and local infections (Kotiranta et al., 2000). Some *B. cereus ss* strains are used as human and animal probiotics, underlining the highly variable impact on human and animal health of the species (Cutting, 2011). Some *B. thuringiensis* strains may also cause human infections (Hernandez et al., 1998), but the species is mainly known for its production of insecticidal toxins and its use as a bio-pesticide (Aronson, 2002). *B. anthracis* is the cause of lethal anthrax in mammals, and has been used as a bio-weapon (Rasko et al., 2005). The ability of *B. anthracis*, emetic *B. cereus ss*, and *B. thuringiensis* to cause disease in mammals and insects relies on the presence of specific virulence plasmids (Ehling-Schulz et al., 2006; Rasko et al., 2005). In *B. anthracis* presence of two plasmids are essential for full virulence, among them, pXO1 carrying the virulence genes *cya* (edema factor), *lef* (lethal factor) and *pagA* (protective antigen) and pXO2 carrying genes for the poly-γ-D- glutamic acid capsule biosynthetic operon (*capB, capC, capA*, and *capD*) (Levy et al., 2014). Emetic *B. cereus* carries a plasmid with high similarity to pXO1 that harbours the cereulide synthetase gene cluster necessary for the production of the emetic toxin cereulide (Ehling-Schulz et al., 2006).

Although currently separated into three different species, *B. anthracis* appears to be genetically indistinguishable from members of the *B. cereus-B. thuringiensis* group (Helgason et al., 2000). Recent studies using amplified fragment length polymorphism (AFLP) and multiple locus sequence typing (MLST) analysis have showed that a number of *B. cereus* and *B. thuringiensis* strains, including e.g. *B. thuringiensis* serovar *konkukian* strain 97–27, *B. cereus* E33L and *B. cereus* biovar *anthracis* CI, are closely related to the highly monomorphic species of *B. anthracis* (Han et al., 2006; Klee et al., 2006, 2010). Some strains, as *B. cereus* G9241 and *B. cereus* biovar *anthracis* CI, harbour plasmids that are highly similar to the *B. anthracis* pXO1 and pXO2 virulence plasmids, including the anthrax-causing virulence genes (Hoffmaster et al., 2004; Klee et al., 2010). It has been shown that plasmids can be transferred between the different species of *B. cereus sl* group (Hu et al., 2009; Van der Auwera et al., 2007). Thus, phylogeny, species delineation and pathogenic potential of *B. cereus sl* group is currently a subject of extensive scientific discussion (Helgason et al., 2000; Hill et al., 2004; Tourasse et al., 2006, 2011; Zwick et al., 2012; Økstad and Kolstø, 2011).

Several studies have applied MLST of chromosomally encoded housekeeping genes to analyse phylogenetic relationships (Barker et al., 2005; Helgason et al., 2004; Hoffmaster et al., 2004; Klee et al., 2006; Priest et al., 2004; Sorokin et al., 2006; Tourasse et al., 2011). These analyses show that the *B. cereus sl* can be grouped into three main phylogenetic clusters (Økstad and Kolstø, 2011) which can be further divided into seven clusters (Guinebretiere et al., 2008; Tourasse et al., 2011). Genome analysis of sequenced *B. cereus sl* strains confirmed this phylogeny (Zwick et al., 2012). Guinebretiere et al. (2010) showed that *B. cereus sl* strains could be affiliated to these seven groups by partial sequencing of *panC*, and that the groups differed with regard to their growth temperature and cytoxicity.

The objective of the present study was to describe the characteristics and phylogeny of *B. cereus sl* during traditional production of Maari at two different production sites in Burkina Faso by use of M13-PCR. DNA typing by M13-PCR previously showed to be applicable to differentiate between *B. cereus* strains and *Bacillus* species isolated from fermented Sudanese bread (Ehling-Schulz et al., 2005; Thorsen et al., 2011). Furthermore, the study analysed the phylogenetic relationship of *B. cereus* isolates by *panC* sequencing and MLST, and determined their pathogenic potential by PCR analysis for specific virulence genes.

2. Materials and methods

2.1. Microbial analysis

Baobab seeds for Maari production were purchased at the market at Orodara and Pousghin in Burkina Faso and were brought to two different production sites (A and B) within Pousghin. The producers used the same ash and water from the same drilled well (DW) for potash production. A flow diagram for Maari production and the sampling points are shown in Fig. 1. Samples were collected from ash, DW, potash (ash mixed with DW), water after cooking (CW), fermenting seed mash throughout the first fermentation (8, 12, 24, and 48 h) and second fermentation (96 h), fermenting mash after pounding (72 h + P), after steam cooking (96 h + SC) and in the final product after sun drying (SD). Sampling was performed in two replicates collected from each production site (A and B). For all samples, except water samples, 10 g was homogenized in 90 ml sterile diluent (1% (w/v) peptone (Difco, Detroit, Michigan, USA), 0.9% (w/v) NaCl, pH 7.0) by use of a stomacher (Masticor IUL, Barcelona, Spain) for 2 min. To quantify aerobic mesophilic bacteria (AMB), 1 ml of ten-fold sample dilutions were plated into plate count agar (PCA) (Liofilchem, Roseto degli Abruzzi, Italy) and incubated at 30 °C for 72 h. For purification the isolates were successively streaked on nutrient agar (NA, Merck, Germany) (30 °C, 24 h). For long term maintenance of isolates, stock cultures were stored at −80 °C in 20% (v/v) glycerol and 80% (v/v) nutrient broth (Merck). After purification, isolates were examined for Gram reaction and catalase production. Micro-morphology, motility and spore formation were observed by phase contrast microscopy (magnification × 1000, Olympus 40, Olympus, Japan).

2.2. Identification of B. cereus

Gram positive, catalase positive and spore forming isolates were spotted on Brilliance *Bacillus cereus* Agar (CM1036B, supplement SR0230E, Oxoid, Denmark) and incubated at 30 °C for 24 h. Colonies with a blue/green appearance (a result of the enzymatic cleavage of 5-bromo-4-chloro-3-indolyl-ß-glucopyranoside by ß-glucosidase present in *Bacillus cereus*) were considered as presumptive *B. cereus sl* species (Fricker et al., 2008). The isolates were subjected to inter transcribed spacer polymerase chain reaction (ITS-PCR) profiling in order to verify them as *B. cereus sl* (Willumsen et al., 2005). The isolates were checked for the ability to produce hemolysis on TSA-sheep blood agar (Oxoid). Hemolytic activity was determined from the radii of the clarified zones around the *B. cereus* colonies as (++) high ($r > 2$ mm), (+) weak ($r < 2$ mm) and (-) nonhemolytic.

2.3. DNA isolation

DNA was isolated from bacterial colonies by boiling in Tris-EDTA buffer as previously described (Hansen and Hendriksen, 2001). Shortly, bacteria were transferred to 200 μl of Tris-EDTA buffer and lysed by incubation at 102 °C for 10 min. Cell debris was removed by centrifugation and the DNA-containing supernatant was kept at -20 °C until use.

2.4. M13-PCR typing

The *B. cereus* isolates were subjected to M13-PCR fingerprinting adopted from Ehling-Schulz et al. (2005). Shortly, PCR with the use of random primer PM13 (GAGGGTGGCGGCTCT) included denaturation step at 94 °C for 3 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 40 °C, 8 min at 65 °C and an elongation step at 65 °C for 16 min. Cluster analysis of the obtained profiles was performed