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Short communication

Identification, genetic diversity and cereulide producing ability of *Bacillus cereus* group strains isolated from Beninese traditional fermented food condiments

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ABSTRACT

Bacillus cereus sensu lato is often detected in spontaneously fermented African foods but is rarely identified to species level. Only some of the *B. cereus* group species are reported to be pathogenic to humans and identification to species level is necessary to estimate the safety of these products. In the present study, a total of 19 *Bacillus cereus* group spp. isolated from *afitin*, *iru* and *sonru*, three spontaneously fermented African locust *(Parkia biglobosa)* bean based condiments produced in Benin, were investigated. The strains were isolated at 6, 12, 18, 24 and 48 h fermentation time. By using phenotypic and genotypic methods all of the isolates could be identified as *B. cereus* sensu stricto. The isolates were grouped according to their PM13 PCR (random amplification of polymorphic DNA PCR) fingerprint and formed two major clusters, one of which contained eight strains isolated from *afitin* (cluster 1). Highly similar PM13 profiles were obtained for seven of the isolates, one from *afitin*, one from *iru* and five from *sonru* (cluster 2). Four of the isolates, one from *afitin* and three from *sonru*, did not form any particular cluster. The PM13 profiles of cluster 2 isolates were identical to those which are specific to emetic toxin producers. Cereulide production of these isolates was confirmed by liquid chromatography mass spectrometry/mass spectrometry. This is the first report on cereulide producing *B. cereus* in African fermented foods. Occurrence of the opportunistic human pathogen *B. cereus*, which is able to produce emetic toxin in *afitin*, *iru* and *sonru*, could impose a health hazard. Interestingly, no reports on food poisoning from the consumption of the fermented condiments exist.
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1. Introduction

The *Bacillus cereus* sensu lato group comprises six closely related Gram positive spore forming species; *B. cereus* sensu stricto, *B. thuringiensis*, *B. anthracis*, *B. weihenstephanensis* (Lechner et al., 1998), *B. mycoides* and *B. pseudomycoides* (Nakamura, 1998; Nakamura and Jackson, 1995). Of these six species *B. cereus* is recognized as a food borne pathogen capable of causing vomiting through the production of cereulide, and diarrhea through the production of various enterotoxins (Agata et al., 1994; Granum, 2001). Identification of *B. cereus* sensu lato to species level in connection with food poisoning outbreaks is not routinely performed (Anonymous, 1999). Recently, food poisoning strains previously identified as *B. cereus* were reclassified as *B. thuringiensis* and *B. mycoides* (McIntyre et al., 2008). Increased focus on identification at species level is needed in order to map the prevalence in foods and elucidate the role in food poisonings of the six species.

African alkaline fermented foods are known to be dominated by various *Bacillus* species (often *B. subtilis* group spp) that are responsible for biochemical changes, flavor development and increases in pH (Parkouda et al., 2009). *B. cereus* is frequently identified in these foods, but only few studies identify the bacterium to species level (Padonou et al., 2009; Parkouda et al., 2009). As an example *B. cereus* (sensu lato) has been isolated at levels of 6.3 to 8.3 $\log_{10}$ CFU/g sample of the Nigerian condiments *iru* and *ogiri* (Oguntoyinbo and Oni, 2004). In Europe, *B. cereus* at levels above 10^3–10^5 CFU/g or ml food is considered unsafe (EFSA, 2005). The high levels of *B. cereus* occurring in foods sold for human consumption in Africa gives rise to concern.

Molecular methods often used to identify bacteria at species level, i.e. 16S rRNA and *gyrB* PCR followed by restriction endonuclease digestion, are not sufficient to distinguish the *B. cereus* group species (Jensen et al., 2005). To identify at species level, additional phenotypic tests and species specific PCR are needed (Hendriksen et al., 2006).

Afitin, *iru* and *sonru* are traditional alkaline fermented African locust bean based condiments that are used as flavor enhancers in various soups and dishes (Azokpota et al., 2006). The aim of this study was to identify, at species level, previously identified *Bacillus cereus* sensu lato strains from these products at different fermentation times, using phenotypic and genotypic approaches. Randomly amplified

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polymorphic DNA PCR typing, with the *Escherichia coli* M13 phage derived primer PM13 (Henderson et al., 1994), was used to evaluate genetic diversity. The results are discussed in relation to the safety of *afitin*, *iru* and *sonru*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A total of 19 *Bacillus* isolates identified as belonging to the *B. cereus* group (Azokpota et al., 2007) were used in the current study (Table 1). The 19 *B. cereus* group isolates had been collected at different stages of the spontaneous fermentation of African locust beans in the production of *afitin*, *iru* and *sonru*. The cereulide producing *B. cereus* strains used were NC7401 (Agata et al., 1994), NS117 (Hallaksella et al., 1991), F4810/72 (Turnbull et al., 1979) and RIVM-BC68 (Andersson et al., 2004), which were kindly provided by Maria A. Andersson, Department of Applied Chemistry and Microbiology, Division of Microbiology, University of Helsinki, Helsinki, Finland. The isolates were either maintained in 30% glycerol at −40 °C, or on Luria Bertani-agar (LB-A) slants (Bertani, 1951) at an ambient temperature of 21–25 °C.

2.2. Isolation of DNA for PCR analysis and PCR reaction mixtures

Genomic DNA was isolated from cultures grown overnight on LB-A by boiling in Tris–EDTA buffer as described previously (Hansen and Hendriksen, 2001). Unless otherwise stated, the PCR reaction mixtures (25.0 µl) used included 2.5 µl 10× *Taq* Buffer containing 200 mM $(NH_4)_2SO_4$ and 0.1% Tween 20, 0.25 mM dNTP mix, 2.5 mM $MgCl_2$, 10 pM of each of the forward and reverse primer, 0.5 U Taq recombinant DNA Polymerase and 1.5 µl template DNA. All chemicals were purchased from Fermentas (St. Leon-Rot, Germany).

2.3. Identification to species level by phenotypic and genotypic methods

Initially, as previously described, the 19 *B. cereus* group isolates were observed for rhizoid colony growth, and growth/non growth over 28 days at 6 °C and 4 days at 42 °C, in order to identify *B. mycoides* and *B. weihenstephanensis* (Hendriksen et al., 2006; Lechner et al., 1998). To identify *B. thuringiensis*, the isolates were screened for crystal toxin formation by phase contrast microscopy. In addition, six primer sets; cry1, cry3, cry8, cry11, nem and cyt were used to detect various groups of insecticide encoding toxin genes of *B. thuringiensis* by PCR using protocols and primers described by Jensen et al. (2002), and genomic DNA from *B. thuringiensis* subsp. *israelensis* (cry11$^+$) and *B. thuringiensis* subsp. *kurstaki* (cyt$^+$, cry1$^+$) were used as positive controls. To identify *B. anthracis*, the *B. anthracis* specific primers; *SLU2/SLUD* and *MO11/MO12* (Cheun et al., 2001) and pagA (forward-GTGTTAATACCATTAATGGC, reverse-TAAATCCTGCAGATACACTCCCAC), were used to perform PCR using methods described by Hoffmaster et al. (2006).

Table 1
Bacillus cereus group strains isolated from *aftin*, *iru* and *sonru*.

B. cereus group strains	Origin
F6H1, F6H3, F6H5, F6H7, F12H3, F12H4, F12H6, F18H2, F24H4, F24H5	*Afitin*
Y48H10	*Iru*
Ba18H1, Ba18H2, Ba18H8, Ba24H8, Ba48H3, Ba48H6, Ba48H7, Ba48H8	*Sonru*

[a]The first letters indicate the condiments from which the bacteria were isolated and the technology used for the production (F = *afitin*, Y = *iru* and Ba = *sonru*) (Azokpota et al., 2006); the following digits indicate the sampling time (6, 12, 18, 24 or 48 h) and the second digit, the number of the isolate.

2.4. Randomly amplified polymorphic DNA PCR (RAPD-PCR)

A randomly amplified polymorphic DNA PCR (RAPD-PCR) technique, based on the use of the *Escherichia coli* M13 phage derived primer PM13 (5′-GAGGGTGGCGGCTCT-3′ (Henderson et al., 1994), was used for clustering of the 19 *B. cereus* group isolates (Table 1). The method has previously been used for describing the genetic variation between strains of the same species (*B. cereus*) (Ehling-Schulz et al., 2005). Furthermore, the method has been used to identify cereulide producing *B. cereus*, as these show identical PM13 RAPD patterns regardless of geographical origin (Ehling-Schulz et al., 2005). The PM13 RAPD-PCR conditions were as previously described(Ehling-Schulz et al., 2005). Briefly, 20 pM of the PM13 primer was used for the PCR mixture described above. The PCR amplification products were run on a 2% agarose gel. After staining with ethidium–bromide, bands were visualized by UV-light (302 nm) and photographed using a Kodak EDAS 290 system (Eastman Kodak, New Haven, CT). Cluster analysis was performed using Bio-Numerics 2.50 (Applied Maths, Sint-Martens-Latam, Belgium), by calculating the dendrograms on the basis of Dice's coefficient of similarity, using the Unweighted Pair Group Method with Arithmetic averages clustering algorithm (UPGMA) as previously described (Sawadogo-Lingani et al., 2007). PM13 RAPD profiles of the cereulide producing *B. cereus* strains NC7401 and NS117 were included as controls in the experiments.

2.5. Verification of cereulide production

Cereulide production by *B. cereus*, incubated for 24 h and 48 h on Brain Heart Infusion-agar (Oxoid, Denmark) at 30 °C, was verified using liquid chromatography mass spectrometry/mass spectrometry (LC–MS/MS) as previously described (Thorsen et al., 2009b). As previously described (Thorsen et al., 2009a), the cereulide was extracted from the biomass using 96% ethanol. The cereulide producing reference strains used were *B. cereus* RVIM-BC68 and *B. cereus* F4810/72.

3. Results and discussion

The phenotypic and genotypic analyses performed in this study showed that the 19 investigated *B. cereus* group isolates from *afitin*, *iru* and *sonru* (Table 1) could be identified as mesophilic variants of *B. cereus* sensu stricto. As expected, none of the isolates were psychrotolerant (no growth at 6 °C), since psychrotolerant *B. cereus* group species occur infrequently in tropical climates (von Stetten et al., 1999). Furthermore, none harbored the genes specific for *B. anthracis* or *B. thuringiensis* and none of the isolates showed a rhizoid colony morphology typical of *B. mycoides*. In the present study, *B. anthracis*, which is the cause of lethal anthrax in humans (Schmid and Kaufmann, 2002), was absent. The fact that *B. cereus* sensu stricto is the sole *Bacillus cereus* group spp. in *afitin*, *iru* and *sonru* is different from the results obtained for a similar product Soumbala, where *B. mycoides* and *B. thuringiensis* were also identified (Sarkar et al., 2002).

A RAPD assay, based on the PM13 primer, was used to investigate the genetic diversity of the *B. cereus* isolates from *afitin*, *iru* and *sonru* (Fig. 1). Three different PM13 RAPD-types coexisted in *sonru* throughout the fermentation time from 18 h. *B. cereus* Ba18H1, isolated at 18 h of fermentation, is conceivably the same strain as *B. cereus* Ba48H3, isolated at the end of fermentation of *sonru* at 48 h. Similarly, Ba18H2 and Ba18H8 (cluster 2), isolated at 18 h, are probably the same strain as Ba24H8 isolated at 24 h, and Ba48H6 and Ba48H7 isolated at 48 h of fermentation. The latter 5 *B. cereus* isolates from *sonru* are very similar to two isolates Y48H10 and F6H3 from *iru* and *afitin*, respectively (Fig. 1).

Previously, mesophilic cereulide producing *B. cereus* has been shown to have identical or very similar PM13 RAPD profiles regardless of geographical origin and food (Ehling-Schulz et al., 2005). The PM13

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