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Genotypes and the persistence survival phenotypes of *Bacillus cereus* isolated from UHT milk processing lines

Yingying Lin [a, b], Fazheng Ren [a, c], Liang Zhao [a, d], Huiyuan Guo [a, b, *]

[a] Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Food Science & Nutritional Engineering, China Agricultural University, Beijing 100083, China
[b] Key Laboratory of Functional Dairy, China Agricultural University, Beijing 100083, China
[c] Beijing Laboratory of Food Quality and Safety, China Agricultural University, Beijing 100083, China
[d] Hebei Engineering Research Center of Animal Product, Sanhe 065200, China

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ABSTRACT:

Bacillus cereus is an important foodborne pathogen that can cause emesis and diarrhea. The aim of this study was to find the phenotypic properties of *Bacillus cereus* enabling its persistence in dairy plants, and identify the corresponding genotypic features. The toxin gene profile, multilocus sequence typing (MLST), biofilm-forming capability and the alkali and acid tolerance of the biofilms of the strains were analyzed. All strains were positive for one or more toxin genes tested, except for A20. The three main toxin genes were *nheABC* (74.07%), *bceT* (73.37%), and *ces* (48.15%), followed by *entFM* (40.74%) and *cytK* (33.33%), but no strain harbored *hblABD*. A total of 17 ST-types were generated among 27 isolates by MLST, and clustered into four groups. Although no significant difference was found in the tolerance of biofilm to acid and alkali among four MLST groups, the strains of MLST groupIhad stronger biofilm formation capability. The biofilm was formed on stainless steel coupons, of which the OD values of A1, B16 and B18 were more than 1.0, and close to each other in the phylogenetic tree. Persistence survival strategies analysis showed that strains can be divided into three groups: 15 strains without persistence strategies; two strains were capable of forming biofilm may be effectively inactivated by hot-acid or hot-alkali; ten isolates were capable of forming biofilm and possessing the tolerance of biofilm to acid and alkali, which should be under strict supervision and control in the future.

1. Introduction

Bacillus cereus is a Gram-positive, spore-forming bacterium and ubiquitous in environment. As its heat stable characteristic, *Bacillus cereus* is commonly contaminating raw milk and dairy products, but an underestimated pathogen in the dairy industry. It can cause two types of gastrointestinal disease, emesis and diarrhea (Bremer, Fillery, & McQuillan, 2006; Ceuppens, Boon, & Uyttendaele, 2013; Kim et al., 2009), and its growth may result in various dairy defects. Several studies performed in Ethiopia, Irish, Tunisa, and Turkey found that the prevalence of *Bacillus cereus* in raw milk reached 12.86%, 23%, 47.5%, and 90.0%, respectively (Aouadhi, Maaroufi, & Mejri, 2014; Gundogan & Avci, 2014; Garedew, Mengesha, Birhanu, & Mohammed, 2015; O'Connell et al., 2016). The occurrance of the pathogen in dairy products in different studies in USA, Turkey and Brazil reached 17.64%, 20% and 24.23%, respectively (Gundogan & Avci, 2014; Montanhini & Bersot, 2013; Reis, Montanhini, Bittencourt, Destro, & Bersot, 2013). A study conducted in 10 local dairy farms in Beijing showed that the total occurrance of *Bacillus cereus* in raw milks was as high as 9.8%, and the *nhe, hbl,* and *ces* genes were detected at the rate of 100%, 55.0%, and 5.0%, respectively(Cui et al., 2016). In pasteurized milk, the contamination rate of *Bacillus cereus* was 33.3–71.4% in China (Zhou, Liu, He, Yuan, & Yuan, 2008). These previous data provided information that occurrence of *Bacillus cereus* in raw milk and final products was high, especially in China. This is a matter of concern as it can lead to foodborne outbreaks. However, the genotype diversity of *Bacillus cereus* isolates from dairy plants in China remains unclear, especially the ultra-high temperature sterilization (UHT) milk.

In modern dairy plant environment, Bacterium can negatively

* Corresponding author. College of Food Science and Nutritional Engineering, China Agricultural University, P.O. Box 287, No. 17 Qinghua East Road, Haidian, Beijing 100083, China.
E-mail address: guohuiyuan@cau.edu.cn (H. Guo).

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survive and colonise. Indeed, the endospores and biofilm formation properties of *Bacillus cereus* are considered as its survival strategies (Ehling-Schulz, Fricker, & Scherer, 2004a), which is the main cause of *Bacillus cereus* contamination in dairy industry (Flach, Grzybowski, Toniazzo, & Corção, 2014). The resistance towards heat and disinfectants of *Bacillus cereus* endospores allow them survive cleaning procedures, and then they attach to equipment surface depending on their hydrophobic properties (Ryu & Beuchat, 2005). Adherence to stainless steel surfaces of dairy plants can result in biofilm formation, which is more resistant to antimicrobials and cleaning regimes compared to planktonic cells. This makes elimination of *Bacillus cereus* from dairy industry a big challenge, and induces recurrent contamination of dairy products (Kumari & Sarkar, 2014; Shaheen, Svensson, Andersson, Christiansson, & Salkinoja-Salonen, 2010). In recent year, the structure and the mechanism of resistance exhibited by Bacillus spores are investigated (Soni, Oey, Silcock, & Bremer, 2016), and a variety of sanitizers and biocide are evaluated for use in CIP systems to promote its biofilm removal (Lemos, Gomes, Mergulhao, Melo, & Simoes, 2015). Besides, biofilm-producing ability of *Staphylococcus aureus* and *Listeria monocytogenes* isolated from diary plants were investigated (In Lee et al., 2017; Lee et al., 2014). Thus hygiene practices must be improved to prevent biofilm formation, and appropriate treatment should be taken for removing biofilms (Lee, Cappato, Corassin, Cruz, & Oliveira, 2016).However, to develop novel control strategy of *Bacillus cereus* in dairy processing environment, there is a strong need to gain a better insight into the phenotypic properties enabling its persistence under the dairy conditions, and identify the corresponding genotypic features.

Therefore, the aim of this study was to isolate the persistent *Bacillus cereus* from the UHT milk processing lines, and reveal the connections of the genotypes and survival phenotypes of the strains. First, the intraspecific genotype diversity of 27 *Bacillus cereus* strains isolated from UHT milk processing lines were characterized by Multilocus sequence typing (MLST)-based phylogenetic analysis, and the potential hazard based on toxin genes traits of the strains were evaluated. Then, the abilities of the *Bacillus cereus* strains to form biofilms were investigated, and the three-dimensional structure of the biofilms were observed by confocal laser scanning microscope. Third, we exposed the *Bacillus* strains biofilms to highly alkaline and acid liquids at high temperature applied during the cleaning-in-place (CIP) procedures, to assess the alkali and acid tolerance of the biofilms These data is of importance for further understanding the persistent mechanism of *Bacillus cereus* and might then be used for providing effective strategies to minimize it.

2. Materials and methods

2.1. Sample collection and Bacillus cereus isolation

In this study, two local dairy plants in China were under surveillance, and investigated at the critical control points throughout the production chain every production day, from November 2014 to May 2015. The isolates were all obtained in UHT milk processing lines, including silo tanks, ingredients tanks, pasteurization tanks, post-pasteurization (air, homogenizer), filling room air and UHT milk products. *Bacillus cereus* was isolated according to the standard procedures described in the National Standards of the People's Republic of China (GB.4798.14-2014). Briefly, for each serial dilution (10^1 to 10^4), 100 μL were plated on MYP agar (Qingdao Hope Bio-Technology Co., Ltd, China), a selective medium for isolating members of the *Bacillus cereus* group, and incubated at 37 °C for 48 h. Those bacteria forming rough and dry colonies with a violet pink background surrounded by egg yolk precipitation on the MYP agar and with parasporal crystals observed under phase-contrast microscopy, were identified as presumptive *Bacillus cereus*. These presumptive colonies were transferred into 2 mL brain heart infusion (BHI, AOBOX Technology) broth and incubated at 37 °C for 16 h. Then, the bacterial cultures were spread on BHI agar, and incubated at 37 °C for another 24 h Single colony was chosen for further study, and all isolates were stored at −80 °C until use.

2.2. DNA extraction and identification of Bacillus cereus-like strains

Genomic DNA was extracted from overnight cultures grown in Luria–Bertani (LB) broth using the TIANamp Bacteria DNA Kit (Tiangen Bio-Tech Co., Ltd, China) in accordance with the manufacturer's instructions. 16S rRNA sequence analysis was used to further characterize the *Bacillus cereus*-like strains, using primers 27F and 1492R (Lane, 1991). The amplified sequences of each strain were analyzed by nucleotide blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The strains with high similarity to 16S rRNA sequence of the *Bacillus cereus* reference strain (Evalue = 0 and Max identity ≥ 98%) were regarded as *Bacillus cereus* strains (Zhou, Zheng, Dou, Cai, & Yuan, 2010).

2.3. Detection of toxin genes

For detection of *Bacillus cereus* ten toxin genes (*hblA*, *hblB*, *hblC*, *nheA*, *nheC*, *nheD*, *cytK*, *bceT*, *emtF*, *ces*), the primer pairs and PCR reaction conditions were in accordance with a previous report (Seong, Lim, Lee, Lee, & Hong, 2008). *Bacillus cereus* ATCC 14579 provided by CGMCC was used as control strain.

2.4. MLST-based phylogenetic analysis

All the isolates were characterized by the MLST scheme using primers and conditions reported in the primers section of the *Bacillus cereus* MLST database (http://pubmlst.org/bcereus/). Portions of *glpF*, *gmk*, *ilvD*, *pta*, *pur*, *pycA*, and *tpi* housekeeping genes were amplified. The PCR programs were performed by using the given conditions and cycle.

Amplification products were purified using the QiaAmp PCR purification kit (Qiagen, Germany). The sequences of the seven housekeeping genes of each stain were assigned allele numbers based on the locus queries at http://pubmlst.org/bcereus/, and sequence types (ST) were numbered based on the combination of seven alleles. To assess the relationship of the *Bacillus cereus* isolates under this study, a phylogenetic tree was generated from their allelic profiles using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm with the aid of MEGA7.0 software. Genetic distances, based on the nucleotide polymorphism in housekeeping alleles, were calculated using the UPGMA method and Kimura 2-parameter mathematical model. The correctness of the results was evaluated using a 1000-step bootstrap test.

2.5. Assay of biofilms formation on stainless steel coupons

Biofilms were grown on stainless steel coupons of AISI 304 with 2B finished (1 × 1 cm^2) vertically placed in 24-well polystyrene plates which were half filled with 2 ml broth and inoculated with 1.5% pre-culture, following the method described by Hayrapetyan (Hayrapetyan, Abee, & Nierop Groot, 2016). The plates were incubated at 30 °C for 48 h. Biofilm on the coupons were quantified using the crystal violet (CV) assay for total biofilm biomass formed as described in Hayrapetyan (Hayrapetyan, Muller, Tempelaars,