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Multilocus sequence type profiles of *Bacillus cereus* isolates from infant formula in China

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ABSTRACT

Bacillus cereus sensu stricto is an opportunistic foodborne pathogen. The multilocus sequence type (MLST) of 74 *B. cereus* isolated from 513 non-random infant formula in China was analyzed. Of 64 sequence types (STs) detected, 50 STs and 6 alleles were newly found in PubMLST database. All isolates except for one singleton (ST-1049), were classified into 7 clonal complexes (CC) by BURST (n-4), in which CC1 with core ancestral clone ST-26 was the largest group including 86% isolates, and CC2, 3, 9, 10 and 13 were first reported in China. MLST profiles of the isolates from 8 infant formula brands were compared. It was found the brands might be potentially tracked by the variety of STs, such as ST-1049 of singleton and ST-1062 of isolate from goat milk source, though they could not be easily tracked just by clonal complex types of the isolates.

1. Introduction

Bacillus cereus sensu lato is a Gram-positive spore-forming bacterial group, consisting of *B. anthracis, B. cereus, B. cytotoxicus, B. mycoides, B. pseudomycoides, B. thuringiensis,* and *B. weihenstephanensis*. It has a great diversity of phylogenesis and occupies widespread ecological niches (Ceuppens et al., 2013). *B. cereus sensu stricto* (*B. cereus* in short) is an opportunistic foodborne pathogen, produces toxins such as cereulide, cytotoxin K, hemolysin BL (HBL) and non-hemolytic enterotoxin (NHE), and causes food poisoning or even severe human infectious diseases (Rowan and Anderson, 1998; Ehling-Schulz et al., 2005; Arnesen et al., 2008; Bottone, 2010). It was suggested that *B. cereus* should be controlled and might be a suitable microbiological safety indicator for food products, especially for infant formula (De Jonghe et al., 2008; Lund, 2015).

Infant formula is considered as one of high risk foods on account of its high protein contents and its vulnerable consumers. Great attention has been paid to infant formula safety around the world, especially after melamine incidence in China (Newell et al., 2010). The surveillance of *B. cereus* along infant formula production chains was carried out (Carlin, 2011). It was discovered that *B. cereus* isolates mostly occurred in infant formula with other thermophilic spore-forming species such as *B. licheniformis* and *Geobacillus stearothermophilus* (Becker et al., 1994; Bartoszewicz et al., 2008; Haughton et al., 2010; Sadiq et al., 2016). *B. cereus* might come from farm lands, endure ultrahigh-temperature (UHT) pasteurization and concentration, survive from spray drying tower and appear in final products (McAuley et al., 2014). Toxin genes such as *ces, cytK, hbl* and *nhe* were detected in *B. cereus* isolates from lots of food (Svensson et al., 2007; Lücking et al., 2013; Hwang and Park, 2015; Organji et al., 2015). It was suggested that the *B. cereus* pathogens should be intensively monitored in infant formula (Shaheen et al., 2006; Di Pinto et al., 2013).

Multilocus sequence type (MLST) is a housekeeping gene-based molecular typing technique, first developed for *Neisseria meningitidis* typing (Maiden et al., 1998; Spratt, 1999; Urwin and Maiden, 2003). It is not only a portable and comparable method for characterizing bacteria at a nucleic acid molecule level, but also a powerful tool for pathogenic microorganism tracing and genetic evolution exploration (van Belkum, 2003; Cardazzo et al., 2008; Pérez-Losada et al., 2013). MLST has been intensively used to type over 70 bacterial species, 7 eukaryotes, 1 bacteriophage

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Table 1
The PCR amplifying conditions for housekeeping genes of *B. cereus*.

Gene	Protein	PCR primers	Annealing temp. (°C)
glpF	glycerol uptake facilitator protein	[a]F-GCGTTTGTGCTGGTGTAAGT [a]R-CTGCAATCGGAAGGAAGAAG	59
gmk	guanylate kinase, (putative)	F-ATTTAAGTGAGGAAGGGTAGG R-GCAATGTTCACCAACCACAA	56
ilvD	dihydroxy-acid dehydratase	F-CGGGGCAAACATTAAGAGAA R-GGTTCTGGTCGTTTCCATTC	58
ilvD_2[b]		F-AGATCGTATTACTGCTACGG R-GTTACCATTTGTGCATAACGC	58
pta	phosphate acetyltransferase	F-GCAGAGCGTTTAGCAAAAGAA R-TGCAATGCGAGTTGCTTCTA	56
pur	phosphoribosyl-amino-imidazole carboxamide	F-CTGCTGCGAAAAATCACAAA R-CTCACGATTCGCTGCAATAA	56
pycA	pyruvate carboxylase	F-GCGTTAGGTGGAAACGAAAG R-CGCGTCCAAGTTTATGGAAT	57
tpi	triosephosphate isomerase	F-GCCCAGTAGCACTTAGCGAC R-CCGAAACCGTCAAGAATGAT	58
Recycling procedure	step 1: 95 °C 3 min. step 2: 94 °C,30 s. step 3: annealing temperature (above), 60–90 s. step 4: 72 °C, 60 s. step 2–4, 30 cycles. step 5: 72 °C, 7 min.		

[a] F- and R-were referred to forward and reverse primers with direction from 5′ to 3′, respectively.
[b] *ilvD_2** was alternative to *ilvD* as *B. cereus* was emetic toxin producing strain. In this study, if the amplification of *ilvD* was failed, then *ilvD_2* was used.

(*Lactococcus lactis* 936-like bacteriophages) and 4 plasmids (Chan et al., 2001; Cooper and Feil, 2004; Maiden, 2006).

Since the MLST scheme for *B. cereus sensu lato* was built in 2004, the genetic diversity of clinical and foodborne isolates was extensively studied with MLST (Helgason et al., 2004; Vassileva et al., 2006; Cardazzo et al., 2008; Hoffmaster et al., 2008). However, the MLST of *B. cereus sensu stricto* isolates from infant formula were not systematically estimated both in China and abroad. The exploration of molecular character of *B. cereus* in infant formula is in great need for foodborne disease prevention and control (Ehling-Schulz and Messelhäusser, 2013).

The aim of this study was to provide the first overview of MLST profiles of *B. cereus* isolates from infant formula in China. The results would enrich database of *B. cereus* in PubMLST (http://pubmlst.org/bcereus) and might be used in tracking of infant formula brands in the future.

2. Materials and methods

2.1. Infant formula samples and B. cereus isolates

74 *B. cereus* isolates in this study were isolated from 513 non-random infant formula samples of 23 brands in Chinese market between 2012 and 2013 by using mannitol, yolk and polymyxin agar plate (MYP) (LUQIAO, Beijing, China) (GB 4789.14, 2014), and re-identified by both morphological observation and biochemical test with VITEK 2 Systems and BCL TEST KIT (bioMerieux, Inc, USA).

2.2. Genome DNA extract

Each strain was streaked on nutrient agar plates and incubated at 37 °C for 16–18 h. 5–10 colonies were collected into 200 μl sterilized purified water, centrifuged at 12,000 rpm for 5 min. The precipitate was used to extract genome DNA with Bacterial DNA kit (OMEGA, bio-tek) according to operating instruction.

2.3. Housekeeping gene amplification

Seven housekeeping genes *glp, gmk, ilv,pta, pur, pyc* and *tpi* were to be sequenced according to MLST scheme for *B. cereus* in PubMLST (http://pubmlst.org/bcereus/info/primers.shtml). PCR amplification conditions for the genes were briefly showed in Table 1 (Hoffmaster et al., 2008). PCR kit (TaKaRa, China), Mastercycler proS (Eppendorf, Germany), Electrophoresis and Gel Imaging System (BIO-RAD, Milan, Italy) were used in the study. All primers were synthesized by Sangon, China.

2.4. Housekeeping gene sequencing

PCR product was purified using the kit SK1131 (Sangon, China) according to manufacturer's instructions, then 10 ng purified DNA was sequenced in 3730XL Genetic Analyser (Applied Biosystems, California, USA) with PCR forward primer firstly. The reverse primer was used when the forward primer did not work and the *gmk* (504bp) was sequenced with both forward and reverse primers in case of sequencing data distortion.

2.5. Database interrogation for allele sequence and ST identification

The allele numbers were provided after submission of housekeeping gene sequences to PubMLST. If there were no exact allele sequences for submitted sequences, novel allele numbers would be assigned. The multilocus sequence type of each isolate was the arrangement of 7 housekeeping gene allele numbers. If there was no same arrangement for submitted ST, a new ST number would also be assigned. All novel allele sequences and STs detected in the study has been submitted to PubMLST database, and endowed new numbers.

2.6. Clonal complexes clustering

Online BURST (based upon related sequence types) algorithm, simple and speedy for epidemiological surveillance disregarding much of the evolutionary information contained in the nucleotide sequence, was used for analysis (Urwin and Maiden, 2003; Vassileva et al., 2006). The isolates in this study together with isolates of *B. cereus sensu stricto* in PubMLST database were clustered into separate clonal complexes by BURST (n-4, 3 or more