Typing and evaluating heat resistance of *Bacillus cereus sensu stricto* isolated from the processing environment of powdered infant formula

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ABSTRACT

Bacillus cereus sensu lato is one of the most harmful bacterial groups affecting the quality and safety of powdered infant formula (PIF). In this study, samples were collected from the raw materials and processing environments of PIF. A total of 84 isolates were identified as *Bacillus cereus sensu stricto* (*B. cereus s. s.*) by 16S rRNA analysis, molecular typing technology, and physiological and biochemical tests. The 84 *B. cereus s. s.* strains were assigned to *panC* group II, group III, and group IV. Then, the 7 housekeeping genes *glpF*, *gmk*, *ilvD*, *pta*, *pur*, *pycA*, and *tpi* were selected for multilocus sequence typing. Results showed that the 84 isolates were clustered into 24 sequence types (ST), and 14 novel ST were detected. Among the 24 ST, ST999 (19/84, 22.62%) and ST1343 (13/84, 15.48%) predominated. The correlation between processing areas and ST showed that the processing environments of the production and packing areas were the most susceptible to contamination by *B. cereus s. s.* Spores of these ST showed different heat resistance phenotypes evaluated by the analysis of D_T (time in minutes of spore decimal reduction at each temperature) and Z values (temperature increase required to reduce the D_T value to one-tenth of the original). Spores from group III according to *panC* gene analysis were the most heat resistant. These findings will help us to better understand *B. cereus s. s.* contamination and control in PIF processing environments.

Key words: powdered infant formula, *Bacillus cereus sensu stricto*, processing environments, heat resistance

INTRODUCTION

Powdered infant formula (PIF) contains the nutrients necessary for the growth and development of infants and it is an excellent substitute for human milk (Kent et al., 2015). In recent years, the quality and safety of PIF are increasing concerns because they are closely related to infant health. Several researchers have reported that pathogenic microorganisms are present in PIF (Fei et al., 2015; Liu et al., 2018).

Processing PIF involves high-temperature sterilization and spray drying, with the vegetative cells of pathogenic bacteria being killed during processing. However, because *Bacillus cereus* shows strong tolerance or resistance to heat, dryness, and disinfectants, heat-resistant spores produced by *B. cereus* group species may not be eliminated completely (Kumari and Sarkar, 2016). These spores may germinate into vegetative cells and form biofilms that produce toxins or spoilage enzymes (Scheldeman et al., 2006; Esteban et al., 2013). Additionally, biofilms may lead to prolonged presence of *B. cereus* group strains in the processing environment, and produce heat-stable enzymes, which may have negative implications for product quality (Teh et al., 2012). Therefore, the presence of sporeformers with strong heat resistance could be an important factor in deterioration of PIF quality, leading to defects in flavor and function and causing spoilage of PIF or food poisoning (Hayrapetyan et al., 2016). These properties allow *B. cereus* to be introduced into PIF from raw materials and processing environments, which cause foodborne illness and have negative effects on human health (McKillop et al., 2014; Patiño-Navarrete and Sanchis, 2017). Thus, the contamination of *B. cereus* in PIF should be controlled strictly.

The *B. cereus* group, also known as *B. cereus sensu lato*, is composed of gram-positive, rod-shaped, spore-forming aerobic bacteria that exist widely in the environment (Luu-Thi et al., 2014). This group comprises 11 species (Liu et al., 2017), *B. cereus sensu stricto*

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(*B. cereus s. s.*), *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis* (Lechner et al., 1998), *B. cytotoxicus*, *B. toyonensis*, *B. gaemokensis*, *B. manliponensis*, and *B. bingmayongensis* (Giffel and Beumer, 1999). The identification and typing of the *B. cereus* group can provide useful insights into the contamination routes of PIF. However, species of the *B. cereus* group cannot be confirmed based on traditional detection methods and phenotyping technologies, because the physiological-biological properties and 16S rRNA sequences of species of the *B. cereus* group are similar (Liu et al., 2018). Therefore, molecular typing methods, such as *panC* gene analysis (Guinebrière et al., 2013) and multilocus sequence typing (MLST; Maiden et al., 2013) should be combined with traditional methods to differentiate diverse species of the *B. cereus* group (Jung et al., 2011; Otlewska et al., 2013; Castiaux et al., 2014).

The *panC* gene is a housekeeping gene of the *B. cereus* group that encodes pantothenate- β -alanine ligase. This gene is associated with the growth temperature and heat resistance of the *B. cereus* group. The *panC* gene has been used to distinguish different species of the *B. cereus* group with similar genetic and physiological-biochemical characteristics (Guinebrière et al., 2008). According to *panC* genotyping, different isolates in the *B. cereus* group can be divided into 7 groups (I to VII) with different phenotypic characteristics (Guinebrière et al., 2008). Candelon et al. (2004) identified 12 isolates from the *B. cereus* group by *gdpD*, *panC*, and *plcR* gene typing and demonstrated that the *panC* gene was more effective than *gdpD* or *plcR* to classify *B. cereus* strains.

Multilocus sequence typing is one of the most popular typing methods for bacterial species (Santos et al., 2012), and it is a powerful method for tracing analysis as well (Yang et al., 2017). Genetic evolutions can be determined by comparative analysis of alleles (Forsythe et al., 2014; Hammerum et al., 2015); MLST facilitates gene analysis and the study of genetic evolution of bacteria by amplifying housekeeping genes (Drewnowska and Swiecicka, 2013; Lin et al., 2017). Unique sequences of each housekeeping gene are assigned an allele number (allele), the combination of which relates to a specific sequence type (ST). These alleles and ST genotypes are curated and the alleles can be compared online (Pérez-Losada et al., 2013).

In this study, we combined *panC* gene typing with MLST analysis to characterize *B. cereus s. s.* isolated from the raw materials and processing environments of PIF facilities. The heat resistance of *B. cereus s. s.* in different ST and phylogenetic groups was explored to enhance our understanding of heat resistance in *B.*

cereus s. s. subgroups, which can inform strategies to reduce the contamination of PIF.

MATERIALS AND METHODS

Culture Media and Chemical Reagents

Phosphate-buffered saline, mannitol-egg yolk-polymyxin (MYP) agar, tryptose soy agar (TSA), Luria-Bertani (LB) broth medium, nutrient agar (NA) medium, casein agar, alkaline bromide, peptone, beef extract powder, and agar were purchased from Hope Bio-Technology (Qingdao, China). Sheep blood was obtained from Elite Media Co., Ltd. (Shanghai, China). Methanol, NaCl, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, KCl, and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ were of analytical grade and purchased from Aladdin Reagent Co. Ltd. (Shanghai, China).

Sample Collection

Sample collection was conducted in a typical wet-processing PIF factory located in northeast China. Samples were collected once a month from November 2014 to June 2015. Based on the PIF processing workflow, 6 sampling locations were selected for sample collection: (A) raw materials area, (B) pretreatment area, (C) production area, (D) big bag filling area, (E) can filling area, and (F) packaging area. Samples of raw materials were collected from 14 categories. Samples collected from the processing environments (B to F) included air samples and samples from the surface of production equipment, the floor, and the hands, shoes, and clothes of operators. The details of raw materials and numbers of samples collected from each sampling locations are summarized in Table 1.

Around 200 g of each type of raw material was collected and stored in low-temperature sampling boxes. Samples were returned to the laboratory for analysis as quickly as possible. All raw material samples were analyzed in triplicate. A microbe air sampler fitted with an aseptic Millipore filter (JWL-SWI, Sennon Technologies, Beijing, China) was used to collect bacteria in the air of processing environments (B to F). The number of air samples was determined according to the sampling area, and air samples were taken per 10 m^2 . The sampler was placed on the tripod about 1 m above each sampling point (the center and 4 corners to the east, west, north, and south of sampling area), the sampling time was about 2.5 min, and the inlet flow velocity was 200 L/min. Three samples were taken at each sampling point for determination of bacteria. A total of 15 air samples were collected from processing environments B

Table 1. Basic information of samples collection

Sampling point	Sampling area	Type of samples	No. of samples
A	Raw materials	Raw milk, α -lactalbumin, whey protein concentrate, casein phosphopeptide, calcium carbonate, nutrients, lactose, potassium chloride, vitamins, nucleotides, minerals, fructo-oligosaccharide, lactoferrin, palm oil	42
B	Pre-treatment	Air samples and surface of production equipment, floor, and hand, shoes and clothes of operators	48
C	Production		72
D	Big bag filling		54
E	Can filling		60
F	Packaging		72

to F. After sampling, the filters that absorbed bacteria in the samplers were transferred into sterile chambers and stored at 4°C.

Sterile cotton swabs moistened with sterile PBS were used to collect bacteria on the production equipment, floor, and hand, shoes, and clothes of operators. The areas most likely to be contaminated on the surface of equipment and the floor were chosen as sampling points. The area of each sampling point was $10 \times 10 \text{ cm}^2$. The sampling area of hands, shoes, and clothes of each operator was $5 \times 10 \text{ cm}^2$. After sampling, the swabs were placed into sterile containers and stored at 4°C.

Bacterial Isolation

Samples from raw materials and the surfaces of production equipment were serially diluted several times with PBS and spread onto MYP agar. Then, the plates were inverted and bacteria were incubated at 30°C for 24 h. In general, TSA and LB broth medium are universal media for bacterial culture. Therefore, isolates from air samples were collected on TSA. Afterward, colonies were cultured in LB broth medium for 12 h, followed by plating and incubation on MYP agar. Rough or dry colonies surrounded by egg yolk precipitation on a pink MYP agar background were considered to be presumptive positive *B. cereus* group isolates.

DNA Extraction

We extracted DNA from overnight cultures grown in LB liquid medium using a TIANamp Bacteria DNA Extraction Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions for gram-positive bacteria.

Identification of Isolates

16S rRNA Analysis. The DNA was amplified using 16S rRNA sequencing with the universal prim-

ers 515F (5'-GTGCCAGCMGCCGCGG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') (Wu et al., 2018). The PCR products were purified using a PCR clean-up kit (TaKaRa, Dalian, China). Then, the purified PCR products were directly sequenced by SinoGenoMax Co. Ltd. (Beijing, China). The 16S rRNA sequences were aligned by the BLASTN algorithm in the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>).

panC Gene Sequence Analysis. The PCR amplification of the *panC* gene was performed using the primers TYGGTTTTGTGCCAACRATGG and CATA-ATCTACAGTGCCTTTCG, which were provided by an online typing tool (<https://www.tools.symphonius.org/bcereus/english.php>). The PCR reaction mixture and conditions were described by Guinebrethière et al. (2013). The PCR products were sequenced by SinoGenoMax Co. Ltd.

Hemolysis and Caseinase Tests. To further confirm these isolates, a single colony on NA medium was picked for physiological and biochemical tests (hemolysis, caseinase, and parasporal crystal tests). Because *B. cereus* s. s. has β -hemolytic activity and the ability to hydrolyze casein, whereas *B. anthracis* does not have these abilities, the hemolysis and caseinase tests can be used to distinguish between *B. cereus* s. s. and *B. anthracis*. The enzymatic activity of hemolysis and caseinase were screened using selective medium, TSA with 5% sheep blood and casein agar, and incubated at 37°C for 24 h.

Parasporal Crystal Test. Compared with *B. cereus* s. s., *B. thuringiensis* can produce parasporal crystals. Thus, *B. cereus* s. s. and *B. thuringiensis* can be distinguished by parasporal crystal test. The bacterial culture was spread onto NA medium supplemented with $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.0308 g/L) and incubated at 30°C for 24 h. The plates were stored at room temperature for 3 d. The culture was picked up on a glass slide with distilled water. After drying and fixing the slide, methanol was added for 30 s. The bacteria were stained with 0.5% alkaline bromide and put on the flame for

1 min. The alkaline bromide was washed off and the bacteria were stained again. The slides were examined for the presence of parasporal crystals using an optical microscope (BA200, Motic China Group Co., Ltd., Xiamen, China).

MLST Analysis

Seven housekeeping genes, *glpF* (glycerol uptake facilitator protein), *gmk* (putative guanylate kinase), *ilvD* (dihydroxy-acid dehydratase), *pta* (phosphate acetyltransferase), *pur* (phosphoribosylamino-imidazolecarboxamide), *pycA* (pyruvate carboxylase), and *tpi* (triosephosphate isomerase), were chosen according to the *B. cereus* MLST database (<http://pubmlst.org/bcereus/>). These 7 housekeeping genes are used to classify the *B. cereus* group because they are single copy, have high discriminatory power, and are not subject to lateral transfer or genetic recombination (Priest et al., 2004; Zahner et al., 2013; Liu et al., 2017). The PCR products were sequenced by SinoGenoMax Co. Ltd. The sequences of the 7 housekeeping genes were assigned to specific allele numbers by referencing the *B. cereus* MLST database, and ST were numbered based on the combination of 7 housekeeping genes allele numbers. If there were no exact allele numbers or exact matches for submitted sequence or ST, a new allele number or ST was assigned.

The nucleotide diversity indices of 7 loci, including the number of alleles, the number of polymorphic sites, nucleotide diversity per site (π), the mean G+C content, the Ka/Ks ratio (where Ka represents the number of nonsynonymous substitutions per nonsynonymous site, and Ks represents the number of synonymous substitutions per synonymous), and Tajima's D (the deviation from the standard neutral model of evolution) were calculated with DnaSP version 5.0 software (Fei et al., 2015).

Phylogenetic Analysis

The phylogenetic relationship of the concatenated sequences (2,829 bp) of the 7 housekeeping genes was analyzed using the maximum-likelihood algorithm in MEGA (Molecular Evolutionary Genetic Analysis) 7.0 software, with 1,000 bootstrap replicates test. To determine the interspecific phylogenetic relationship, 11 reference strains of *B. cereus* group were included (*B. cereus* ATCC 14579^T, *B. anthracis* ATCC 14578^T, *B. thuringiensis* ATCC 10792^T, *B. mycoides* ATCC 6462^T, *B. pseudomycoides* DSM 12442^T, *B. weihenstephanensis* DSM 11821^T, *B. cytotoxicus* NVH 391-98^T, *B. toyonensis* BCT-7112^T, *B. gaemokensis* BL 3-6^T, *B.*

manliponensis BL 4-6^T, and *B. bingmayongensis* FJAT-13831^T).

Population Genetics Analysis

Genetic relationship and population structure among isolates were analyzed by the eBURST online tool (<http://pubmlst.org/bcereus/>). Single locus variant (SLV) and double locus variants (DLV) were determined by the eBURST online tool to analyze the relationship of *B. cereus* s. s. ST. All ST could be divided into different clusters or singletons. The clusters of identical alleles from 6 of the 7 loci were identified as a clonal complex (CC). The ST with the highest SLV value among a CC was considered the central ST of that CC (Liu et al., 2017).

Tracing Analysis

The correlations between different ST and sample sources were analyzed by using SPSS 22.0 software (SPSS Inc./IBM Corp., Chicago, IL) to trace the isolates from the raw materials and processing environments of PIF.

Heat Resistance of Different ST of *B. cereus*

All isolates of each ST were evaluated for heat resistance characteristics. One hundred microliters of each culture in LB was spread on sporulation agar and incubated for 3 to 7 d at 37°C to prepare a spore suspension. The final pH of the sporulation agar was 7.3 ± 0.1 and included the following ingredients (L⁻¹): peptone (3.3 g), beef extract powder (1 g), NaCl (1.7 g), MnSO₄·H₂O (0.003 g), MgSO₄·7H₂O (0.51 g), FeSO₄·7H₂O (0.1 g), KCl (0.97 g), CaCl₂·2H₂O (0.2 g), and agar (15 g). The plates were observed regularly by microscope and harvested when more than 90% of the cells sporulated. The spores were collected with cold deionized water and the entire growth surface was scraped from the plate using sterile hockey spreaders and transferred to a 15-mL centrifuge tube for centrifugation at $4,000 \times g$ for 10 min at 4°C (Multifuge X3R, Thermo Fisher Scientific, Waltham, MA). The supernatant was washed 3 times. Finally, the spores were resuspended in deionized water to a concentration of approximately 10^8 spores/mL and stored at 4°C for 30 to 40 d to obtain mature spores.

To enumerate spores, the spore suspension was heated at 80°C for 10 min to kill the remaining vegetative cells. One hundred milliliters of the spore suspension was then heated in an oil bath at 115, 118, or 121°C for 5, 10, 15, or 20 min. After heating, the spore suspension was quickly cooled and the number of surviving spores

was determined by plate count on TSA (Luu-Thi et al., 2014). We calculated the time (min) of spore decimal reduction at each temperature (D_T ; Ziane et al., 2014) using the following formula (Mafart et al., 2002):

$$\left(\frac{t}{D_T}\right)^K = \log N_0 - \log N,$$

where N_0 is the number of spores before heat, N is the number of surviving spores after heating for t minutes, and K is the slope of the curve of heating time (abscissa) and number of surviving spores (ordinate). The Z value represents the temperature increase required to reduce the D_T value to one-tenth of the original. Therefore, the Z value was obtained from the negative reciprocal of the slope of the regression line by plotting $\log D_T$ versus temperature. All heat treatments were carried out in triplicate using the same spore suspension.

Statistical Analysis

The data were expressed as mean $\pm$ standard deviation. The significance of differences was determined by one-way ANOVA of SPSS 22.0 software (SPSS Inc./IBM Corp.).

RESULTS

Isolation and Identification of *B. cereus sensu lato*

Based on the highest similarity with known sequences of *B. cereus* group strains presented in GenBank of the National Center for Biotechnology Information, 84 isolates were identified as *B. cereus* group strains using BLAST alignment. Then, the *panC* gene sequence confirmed that the 84 strains of the *B. cereus* group belonged to *panC* groups II, III, and IV. Of these, 7 isolates were assigned to group II (consisting of *B. thuringiensis* II and *B. cereus s. s.* II), 38 isolates to group III (a mixture group of *B. thuringiensis* III, *B. cereus s. s.* III, or *B. anthracis*), and 39 isolates to group IV (a mesophilic group of *B. thuringiensis* IV or *B. cereus s. s.* IV). We confirmed that the 84 *B. cereus* group isolates could be assigned to *B. cereus s. s.*, *B. thuringiensis*, or *B. anthracis*.

Biochemical tests were performed to further identify the isolates (results are shown in Supplemental Figure S1; <https://doi.org/10.3168/jds.2019-16392>). A clear hemolysis circle around the colonies on blood agar medium (Supplemental Figure S1) indicated that the isolated strains had β -hemolytic activity. Furthermore, transparent hydrolyzed circles could be observed around colonies on casein medium, indicating that isolates

could hydrolyze casein. *Bacillus cereus s. s.* possesses β -hemolytic and casein hydrolytic activities, whereas *B. anthracis* does not share these properties. Therefore, *B. anthracis* was deemed absent from the 84 isolates of the *B. cereus* group. Colonies on the manganese sulfate NA medium were then stained and observed under the microscope. Results showed that all isolates produced spores and none produced toxin crystals (Supplemental Figure S1). This indicates that these isolates did not belong to *B. thuringiensis*, which can produce parasporal crystals. In summary, the 84 isolates were thus identified as *B. cereus s. s.*

MLST Analysis

The sequences of 7 housekeeping genes of the *B. cereus* group were determined and referenced to the MLST database, with 3 novel alleles (*pur*-242, *pyc*-198, *ilv*-276) detected. According to the ST of the isolates, which were determined based on the allelic coding of 7 housekeeping genes, these isolates clustered into 24 ST, including 14 novel ST, as shown in Table 2. The new ST were numbered from 1333 to 1345 and 1347, with corresponding ID from 2003 to 2015 and 2110. All of the new allele profiles and ST detected have been submitted to the *B. cereus* PubMLST database. The predominant ST was ST999 (19/84, 22.62%), followed by ST1343 (13/84, 15.48%), ST1335 (6/84, 7.14%), and ST1345 (6/84, 7.14%). The isolates of ST999 were mainly detected in the can filling and production areas, whereas ST1343 was most frequently associated with the big bag filling and production areas, as shown in Table 2.

Polymorphism Analysis

Polymorphism analysis of the 7 housekeeping genes for the 84 *B. cereus s. s.* isolates is summarized in Table 3. The number of unique housekeeping genes alleles varied from 8 (*gmk*) to 16 (*glpF*), and the number of polymorphic sites ranged from 21 (*tpi*) to 53 (*pur*). The nucleotide diversity (π ; the average number of nucleotide differences for each site between randomly selected sequences) varied from 0.0144 (*tpi*) to 0.0726 (*ilvD*). The G+C content of the 7 housekeeping genes (from 38.2 to 44.7%) was higher than the average G+C content (35%) of the whole-genome sequence of *B. cereus* group in the GenBank database (Liu et al., 2017). The value of Tajima's D ranged from -1.4997 to -0.7908 and the ratios of K_a/K_s were much less than 1 for all loci, indicating that the sequence of housekeeping genes was conservative and stable. Thus, these genes were suitable for MLST analysis (Fei et al., 2015).

Table 2. Multilocus sequence typing and traceability information of 84 *Bacillus cereus sensu stricto* isolates

<i>panC</i> group	Sequence type	Source	No. of strains	Allele						
				<i>glpF</i>	<i>gmk</i>	<i>ilvD</i>	<i>pta</i>	<i>pur</i>	<i>pycA</i>	<i>tpi</i>
II	1342	Pretreatment	1	229	5	271	126	16	183	190
	1345	Pretreatment	6	229	5	271	240	11	183	190
III	26	Production	2	3	2	31	5	16	3	4
	32	Raw materials	1	5	4	3	4	15	6	16
		Can filling	4							
	62	Packaging	1	38	1	1	1	18	33	24
	144	Production	2	67	2	63	5	36	3	4
	374	Production	1	29	2	24	5	32	3	2
	1119	Big bag filling	1	34	1	71	16	33	33	24
		Can filling	2							
	1284	Raw materials	1	247	1	83	1	239	37	43
	1334	Raw materials	1	3	2	8	5	36	3	4
	1338	Packaging	1	3	2	8	5	16	3	4
	1340	Big bag filling	1	3	2	9	5	36	3	4
	1343	Production	5	67	2	8	5	36	3	4
		Big bag filling	8							
	1344	Production	2	67	2	9	5	36	3	4
		Packaging	3							
	1347	Production	2	67	8	8	5	36	3	4
	24	Big bag filling	2	12	8	9	14	11	12	10
IV	999	Pretreatment	1	13	8	8	11	11	12	10
		Production	6							
		Can filling	8							
		Packaging	4							
	1243	Production	1	11	9	14	29	232	27	7
	1333	Pretreatment	1	241	8	8	12	2	17	17
		Big bag filling	1							
		Packaging	3							
	1335	Pretreatment	1	14	8	8	19	29	17	10
		Big bag filling	2							
		Packaging	3							
	1336	Production	1	13	2	8	11	9	12	7
	1337	Raw materials	1	242	2	8	12	2	17	17
		Big bag filling	1							
	1339	Packaging	2	93	6	23	9	242	198	15
	1341	Packaging	1	127	27	276	126	131	42	100

Phylogenetic Analysis

A maximum likelihood phylogenetic tree was constructed based on the concatenated sequences of the 7 housekeeping genes (total length 2,829 bp) for all 84 *B. cereus s. s.* isolates in this study and an additional 11 reference strains. The phylogenetic tree showed a

clear phylogenetic relationship for the isolates (Figure 1). The 24 ST clustered to the clade of *B. cereus s. s.* ATCC 14579^T, *B. anthracis* ATCC 14578^T, and *B. thuringiensis* ATCC 10792^T and separately from the other 8 reference strains. As shown in Table 2, isolates with the same ST were classified into the same *panC* group, with some *panC* groups containing mul-

Table 3. Polymorphism of 7 housekeeping genes for 84 *Bacillus cereus sensu stricto* strains¹

Gene	Size (bp)	No. of alleles	No. of polymorphic sites (%)	π	G+C content (%)	Tajima's D	Ka/Ks
<i>glpF</i>	372	16	33	0.0177	38.3	-1.4997	0.0759
<i>gmk</i>	504	8	35	0.0313	38.2	-1.3989	0.0289
<i>ilvD</i>	393	13	39	0.0726	44.7	-1.3784	0.0129
<i>pta</i>	414	13	27	0.0168	42.3	-1.2807	0.0243
<i>pur</i>	348	15	53	0.0502	38.6	-1.0732	0.0086
<i>pycA</i>	363	10	44	0.0694	41.1	-1.3292	0.0234
<i>tpi</i>	435	11	21	0.0144	44.0	-0.7908	0.1193

¹ π = nucleotide diversity (per site); Tajima's D = deviation from the standard neutral model of evolution; Ka = the number of nonsynonymous substitutions per nonsynonymous site; Ks = the number of synonymous substitutions per synonymous site.

multiple ST. The 24 ST were divided into 3 *panC* gene groups. Group III was the largest group and included 13 ST (ST1119, ST62, ST1284, ST374, ST26, ST32, ST144, ST1338, ST1340, ST1334, ST1347, ST1343,

and ST1344). Group II contained 2 ST: ST1342 and ST1345. Group IV included 9 ST (ST1341, ST1336, ST1337, ST1339, ST1243, ST1333, ST1335, ST24, and ST999).

Figure 1. Maximum likelihood phylogenetic tree of the concatenated sequences (2,829 bp) of 7 housekeeping genes of the 84 *Bacillus cereus sensu stricto* isolates collected from raw materials and processing environments of the powdered infant formula (PIF) facility, and the *Bacillus cereus* group type strains (*B. cereus* ATCC 14579^T, *B. anthracis* ATCC 14578^T, *B. thuringiensis* ATCC 10792^T, *B. mycoides* ATCC 6462^T, *B. pseudomycoides* DSM 12442^T, *B. weihenstephanensis* DSM 11821^T, *B. cytotoxicus* NVH 391-98^T, *B. toyonensis* BCT-7112^T, *B. gaemokensis* BL3-6^T, *B. manliponensis* BL 4-6^T and *B. bingmayongensis* FJAT-13831^T). Bootstrap values were calculated based on 1,000 replicates. ST = sequence type.

Figure 2. The clonal complexes (CC1343) classified by eBURST (<http://pubmlst.org/bcereus/>). Sequence type (ST)1343 was the founding ST of this CC, with the subgroup that contained ST1334 being closely related to this main group.

Population Genetics Analysis

Single and double locus variants were calculated using the eBURST online tool to investigate the relationship of the 24 ST identified. These 24 ST were divided into 4 clusters and 5 singletons. Cluster 1 included 6 ST (ST24, ST999, ST1333, ST1335, ST1336, and ST1337). Cluster 2 comprised 9 ST (ST26, ST144, ST374, ST1334, ST1338, ST1340, ST1343, ST1344, and ST1347). Two ST each belonged to cluster 3 (ST62, ST1119) and cluster 4 (ST1342 and ST1345). In addition, 5 ST (ST1284, ST32, ST1341, ST1339, and ST1243) were singletons. Among the 4 clusters, cluster 2 was a CC, with ST1343 being the central ST based on having the highest number of SLV (4 SLV and 2 DLV in total). As shown in Figure 2, ST1343 was the common ancestor ST of the CC, indicating that the other ST diverged from this ST. In addition, ST1334 included 3 SLV and 4 DLV and was assigned as the founder of a subgroup connected with the main CC group.

Tracing Analysis

With the exception of 4 isolates (0.05%) from the raw materials, all others were isolated from the processing environment (Table 2). Among these 80 isolates, 10 were collected from the pretreatment area, 22 from the

production area, 16 from big bag filling area, 14 from the can filling area, and the remaining 18 isolates from the packaging area. The correlations between sample sources and different ST of *B. cereus s. s.* are shown in Figure 3. Sequence type 32 was associated with the can filling area; ST1336, ST1343, ST1344, and ST1347 were associated with the production area; and ST1333, ST1334, ST1335, and ST1337 were associated with the pretreatment, packaging, big bag filling area, and raw material areas.

Heat Resistance of Different ST of *B. cereus sensu stricto*

In some cases, *B. cereus s. s.* can produce spores with high heat resistance. Results in Table 4 show that the D_T values of different ST varied under the same temperature conditions, and the D_T values of the same ST decreased with increasing temperature. Variation was also observed when comparing ST in individual *panC* gene groups. On average, the spores of group III were more heat resistant than those of the other 2 groups ($P < 0.05$). Among the 24 ST, the 9 with the greatest heat resistance phenotype for all 3 temperatures tested (ST62, ST1338, ST144, ST1284, ST26, ST1343,

Table 4. Heat resistance (mean $\pm$ SD) of different sequence types (ST) for *Bacillus cereus sensu stricto* strains¹

panC' group	ST	D _T (min)						Z (°C)	
		115°C		118°C		121°C			
		Value	Average value	Value	Average value	Value	Average value		
Group II	1345	3.21 ± 0.06 ^{bc}	3.19 ± 0.03 ^a	1.79 ± 0.12 ^c	1.73 ± 0.07 ^a	0.96 ± 0.19 ^b	11.45	0.96 ± 0.01 ^a	11.47 ± 0.03 ^b
	1342	3.16 ± 0.12 ^{bc}		1.66 ± 0.05 ^c		0.95 ± 0.13 ^b	11.50		
Group III	62	4.99 ± 0.11 ^a	4.61 ± 0.21 ^b	2.92 ± 0.14 ^a	2.51 ± 0.21 ^b	1.45 ± 0.21 ^a	11.18	1.29 ± 0.12 ^b	10.89 ± 0.50 ^a
	1338	4.87 ± 0.22 ^a		2.82 ± 0.15 ^a		1.47 ± 0.31 ^a	11.53		
	144	4.81 ± 0.07 ^a		2.74 ± 0.02 ^a		1.48 ± 0.19 ^a	11.72		
	1284	4.76 ± 0.12 ^a		2.48 ± 0.09 ^a		1.43 ± 0.17 ^a	11.49		
	26	4.73 ± 0.04 ^a		2.53 ± 0.23 ^a		1.30 ± 0.10 ^a	10.70		
	1343	4.62 ± 0.08 ^{ab}		2.61 ± 0.16 ^a		1.31 ± 0.27 ^a	10.96		
Group IV	1119	4.61 ± 0.23 ^{ab}		2.44 ± 0.32 ^a		1.21 ± 0.02 ^a	10.33		
	1347	4.55 ± 0.16 ^{ab}		2.49 ± 0.29 ^a		1.25 ± 0.25 ^a	10.69		
	1340	4.54 ± 0.05 ^{ab}		2.47 ± 0.24 ^a		1.32 ± 0.21 ^a	11.18		
	374	4.44 ± 0.20 ^b		2.34 ± 0.08 ^{ab}		1.21 ± 0.09 ^a	10.63		
	1334	4.39 ± 0.09 ^b		2.39 ± 0.21 ^a		1.17 ± 0.20 ^a	10.45		
	32	4.38 ± 0.15 ^b		2.17 ± 0.06 ^b		1.12 ± 0.16 ^{ab}	10.13		
	1344	4.26 ± 0.14 ^b		2.23 ± 0.14 ^{ab}		1.15 ± 0.14 ^{ab}	10.55		
	1333	4.02 ± 0.15 ^b	3.43 ± 0.32 ^a	2.39 ± 0.11 ^a	2.04 ± 0.19 ^a	1.01 ± 0.12 ^b	10.00	1.04 ± 0.06 ^a	11.70 ± 0.91 ^b
	1341	3.73 ± 0.17 ^b		2.14 ± 0.41 ^b		1.02 ± 0.03 ^b	10.66		
	1337	3.69 ± 0.02 ^b		2.28 ± 0.17 ^{ab}		1.07 ± 0.16 ^b	11.16		
	1336	3.57 ± 0.08 ^b		1.96 ± 0.20 ^{bc}		1.15 ± 0.09 ^{ab}	12.20		
	1339	3.34 ± 0.26 ^{bc}		2.06 ± 0.05 ^b		1.08 ± 0.18 ^b	12.24		
	1335	3.31 ± 0.21 ^{bc}		1.87 ± 0.18 ^c		1.12 ± 0.10 ^{ab}	12.75		
	1243	3.13 ± 0.13 ^c		1.99 ± 0.08 ^{bc}		0.97 ± 0.27 ^b	11.79		
24	3.08 ± 0.20 ^c		1.79 ± 0.29 ^c		1.01 ± 0.06 ^b	12.39			
	999	3.01 ± 0.09 ^c		1.87 ± 0.13 ^c		0.96 ± 0.04 ^b	12.09		

^{a-c}Different lowercase letters within a column represent differences between different groups at the same temperature ($P < 0.05$).¹D_T = time (min) of spore decimal reduction at each temperature; Z = temperature increase required to reduce the D_T value to one-tenth of the original.

ST1119, ST1347, and ST1340) belonged to group III. In contrast, D_T values of ST999, ST24, and ST1243 (attributed to group IV) were lowest, followed by ST1342 and ST1345 (members of group II). The heat resistance of group IV, although higher, on average, than that of group II, was not significantly different.

The spores from different phylogenetic groups had varying Z values. With the exception of ST1333, ST1341, and ST1337, spores of ST in group IV had higher Z values than those in the other 2 groups; ST1335, ST24, and ST1339 from group IV had the highest Z values among ST from all groups in our study. Although Z values between group IV and group II were not significantly different from each other, they were significantly higher than that of group III ($P < 0.05$).

DISCUSSION

The *B. cereus* group is an important foodborne pathogen that can be found in food products and the environment. The *B. cereus* group contains 11 species with high similarity in physiological and biochemical characteristics. Therefore, it is difficult to distinguish different species by a single phenotypic method or simple molecular method. Pink or white ring-like precipitation formed around colonies on MYP agar is used as a presumptive positive phenotype for typical strains of *B. cereus* group; however, this phenotype may be difficult to detect under a yellow background generated by other species present that utilize mannitol. Thus, suspected colonies on MYP agar should be confirmed and identified by molecular methods to avoid inaccurate detection results (Oliwa-Stasiak et al., 2011; Chon et al., 2015). Analysis of the 16S rRNA gene is a commonly used molecular tool for bacterial species identification, with BLAST comparison of the sequences demonstrating a close match to known *B. cereus* group sequences in the NCBI database confirming *B. cereus* group species. If the homology of sequences is greater than 99%, the strain is highly likely to be the same species as the known sequence (McHugh et al., 2017). However, the genetic characteristics of various species in the *B. cereus* group are highly similar. For example, the 16S rRNA sequences of *B. cereus s. s.*, *B. thuringiensis*, and *B. anthracis* may differ by only a few bases, with an associated homology as high as 99% (Liu et al., 2015). Therefore, 16S rRNA gene analysis, while useful for confirmation at the *B. cereus* group level, may not distinguish at the species level.

The *panC* gene is one of the housekeeping genes for the *B. cereus* group, and is used in a classical taxonomy method for *B. cereus* group division (Ziane et al., 2014; Heini et al., 2018; Porcellato et al., 2019) to divide isolates into 7 groups (I to VII) according to different

physiological and biochemical characteristics (Guinebrière et al., 2008). In this study, the 84 isolates were divided into 3 groups by *panC* gene analysis, including group II, III, and IV. These 3 groups consisted of *B. cereus s. s.*, *B. thuringiensis*, and *B. anthracis*, allowing exclusion of the other 8 species of *B. cereus* group as potentially among the isolate cohort. We further distinguished these 3 species by the protease, hemolysis, and parasporal crystal tests. The results showed that all 84 stains were *B. cereus s. s.* In this study, molecular identification methods combined with physiological and biochemical tests eliminated the need for additional biochemical identification and reduced the time required for definitive speciation of isolates.

By using MLST, the 84 strains of *B. cereus s. s.* were divided into 24 ST, 14 of which were novel ST. This indicated that the *B. cereus s. s.* isolated from the raw materials and processing environments of PIF showed high genetic diversity. So far, the *B. cereus* MLST typing database contains more than 2,300 strains of *B. cereus* group, in 1,487 ST. In our study, 14 new ST contained 47 strains, accounting for 56% of the isolated strains, indicating that additional genotyping data should be collected for the MLST database.

Among the 24 ST in this study, ST999 was the most frequent type, shared by 19 isolates collected from the pretreatment, production, can filling, and packaging areas. This indicated that ST999 existed in most processing areas in this PIF facility. Based on eBURST analysis, ST999 and ST1336 belonged to the same cluster, which suggests that ST999 is closely related to ST1336 in phenotypic characteristics. Other ST have been reported in some studies, including those associated with carriage of the emetic toxin virulence gene *ces*. This toxin, a small and highly stable cyclic peptide, may be produced by *B. cereus s. s.* strains harboring the *ces* gene (Kumari and Sarkar, 2016). They are difficult to inactivate by standard food processing approaches or human gastric digestion once cereulide has formed in contaminated foods or associated ingredients (Ehling-Schulz et al., 2004; Han et al., 2015). Sequence type 26 and ST144, which have been associated with *ces* carriage, have been increasingly isolated from infant foods in recent years (Zhang et al., 2016; Yang et al., 2017). In this study, these 2 ST were part of clonal complex ST1343 and isolated from the production area and big bag filling area, belonging to *panC* gene group III. Sequence type 26 and ST144 were also isolated from infant formula, with previous work demonstrating that ST26 is the most common ancestor clone of *B. cereus* in infant formula from China (Yang et al., 2017).

In this study, eBURST analysis separated the 84 isolates in this study into 4 clusters and 5 singletons, with one of these clusters comprising a CC of which ST1343

was inferred as the founder ST (Figure 2). This CC (CC1343) was the main group of *B. cereus* s. s. associated with the PIF processing environment and suggests these strains have a closer phylogenetic relationship. Clonal complex 1343 contained 9 ST and was one of the largest clusters represented in the MLST database. In addition, CC1434 (with ST1434 as core ST) may be of particular public health significance because it harbored isolates of the cereulide-associated subgroups ST26 and ST144. The sanitary conditions of processing environments must be strictly controlled in accordance with GMP (good manufacturing practices) and HAC-CP (hazard analysis critical control point) regulations, particularly the spray drying, fluidized bed, and packing processes, which are vulnerable to contamination. Secondary contamination of raw materials in processing environments should also be prevented. In addition, it is necessary to strengthen the supervision of infant formula production to prevent cross-contamination in different processing areas to ensure the quality and safety of products.

The phylogenetic tree based on the 7 housekeeping genes divided the 24 ST into 2 phylogenetic branches with high bootstrap values (Figure 1). These 2 main branches were further divided into smaller phylogenetic groups. Of the species included in this phylogenetic analysis, *B. cereus* s. s., *B. anthracis*, and *B. thuringiensis* showed a closer phylogenetic relationship; ST1340, ST1334, ST1347, ST1343, and ST1344 clustered relatively closely. These were mainly isolated from the production, big bag filling, and packaging areas, and all represent novel ST. Therefore, we speculate that there may be a risk of cross-contamination during the processing of infant formula in these 3 processing areas. Isolates from each of the *panC* groups identified among isolates in this study clustered separately into individual clades.

Tracing analysis can identify the possible contamination pathways of *B. cereus* s. s. during PIF processing and help direct efforts to reduce the contamination burden. The 84 *B. cereus* s. s. isolates were unevenly distributed through the raw materials and PIF processing environments. Most of the *B. cereus* s. s. isolates (80/84, 95.24%) were from processing environments. Of these, most (27.5%, 22/80) were obtained from the production area and the packing area (18/80, 22.5%). This may indicate that the bacteria colonized niches in these areas such as the surface of the equipment, and human activities may be more frequent in these processing areas (Jacobs et al., 2011). Some studies have indicated that microorganisms can be associated with the shoes of employees, external roofs above spray driers, powder-packaging rooms, air treatment areas, and areas with high foot traffic in the processing environ-

ments of PIF facilities, leading to cross-contamination into spray-drying facilities and the entry of pathogens into products (Craven et al., 2010; Sonbol et al., 2013). Previous tracing analysis of a UHT milk processing facility showed that most *B. cereus* isolates were associated with ingredient tanks, pasteurization tanks, and silo tanks, whereas few isolates were detected in final products (Lin et al., 2017). This suggested that *B. cereus* was inactivated during processing, with a smaller number of heat-resistant strains persisting.

The *panC* gene grouping scheme can be associated with the growth temperature range and heat resistance of *B. cereus* group isolates. In our study, the spores with the highest D_T value belonged to group III, and the average D_T value of this group was the highest of the 3 groups. This suggests that the heat resistance of group III was stronger than that of groups II and IV, which is in agreement with the results obtained by Luu-Thi et al. (2014). We also reviewed Z value ranges of *B. cereus* spores from previous studies, which ranged from 8.3 to 12.8°C; all of the Z values of spores from the 24 ST in this study were within this range. Comparing the average Z value of 3 groups, group IV showed the highest value and group III the lowest, again mirroring results reported by Luu-Thi et al. (2014). Fiedoruk et al. (2017) found that the ribosomal proteins and heat resistance of *B. cereus* were relevant and well correlated with phylogenetic groups. Taken together, these results indicate that the heat resistance of spores from group III is higher and, as such, measures implemented for their control must account for this higher temperature resistance to reduce the contamination burden in dairy processing systems such as PIF manufacturing.

CONCLUSIONS

Samples collected from the raw materials and processing environments of PIF production facilities were analyzed by molecular biological and physiological and biochemical tests. Eighty-four *B. cereus* s. s. were detected. Only 4 of the 84 *B. cereus* s. s. isolates were isolated from raw materials, indicating that the contamination of raw materials was lower than that in processing environments. The relationship between ST and processing environments can determine possible contamination pathways of *B. cereus* s. s. during PIF production. The spores from different *panC* gene groups varied in heat resistance, with the spores from group III showing higher resistance. *PanC* group III includes strains from all sampling areas with the exception of pretreatment. This demonstrates that temperature should be strictly controlled during all processing procedures to avoid the formation of spores. These results improve our understanding of *B. cereus* s.

s. strains associated with dairy production systems and provide insights into strategies to control their presence in processing environments, including those associated with PIF production.

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