



Prevalence, antimicrobial susceptibility, and antibiotic resistance gene transfer of *Bacillus* strains isolated from pasteurized milk

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ABSTRACT

Pasteurization is carried out in dairy industries to kill harmful bacteria present in raw milk. However, endospore-forming bacteria, such as *Bacillus*, cannot be completely eliminated by pasteurization. In this study, a total of 114 *Bacillus* strains were isolated from 133 pasteurized milk samples. Antibiotic susceptibility tests showed that the percentage of *Bacillus* with intrinsic resistance to ampicillin and penicillin were 80 and 86%, respectively. Meanwhile, some *Bacillus* isolates had acquired resistance, including trimethoprim-sulfamethoxazole resistance (10 isolates), clindamycin resistance (8 isolates), erythromycin resistance (2 isolates), and tetracycline resistance (1 isolate). To further locate these acquired resistance genes, the plasmids were investigated in these 16 *Bacillus* strains. The plasmid profile indicated that *Bacillus cereus* BA008, BA117, and BA119 harbored plasmids, respectively. Subsequently, the Illumina Novaseq PE150 was applied for the genomic and plasmid DNA sequencing. Notably, the gene *tetL* encoding tetracycline efflux protein was found to be located on plasmid pBC46-TL of *B. cereus* BA117. In vitro conjugative transfer indicated that pBC46-TL can be transferred into *Bacillus invictae* BA142, *Bacillus safensis* BA143, and *Bacillus licheniformis* BA130. The frequencies were of 1.5×10^{-7} to 1.7×10^{-5} transconjugants per donor cells. Therefore, *Bacillus* strains with acquired antibiotic resistance may represent a potential risk for the spread of antibiotic resistance between *Bacillus* and other clinical pathogens via horizontal gene transfer.

Key words: *Bacillus*, antimicrobial susceptibility, antimicrobial resistance gene, conjugative transfer, pasteurized milk

INTRODUCTION

Bovine milk is highly nutritious and has a near-neutral pH (6.6–6.8), which can be a suitable growth medium for various bacteria including *Enterobacteriaceae*, *Streptococcaceae*, and *Bacillaceae* (Bartoszewicz et al., 2008; Quigley et al., 2013). These bacteria can influence the quality and safety of fluid milk or fermented dairy products. During the dairy product manufacturing, most of these bacteria can be destroyed by pasteurization. However, endospore-forming bacteria, such as *Bacillus* cannot be completely eliminated (Gopal et al., 2015). It has been reported that *Bacillus* from pasteurized milk showed resistance to some antibiotics including ampicillin, lincomycin, erythromycin, and tetracycline (Liu et al., 2020; Zhao et al., 2020). For example, *Bacillus* strains were isolated from pasteurized milk samples collected from dairies in France and showed resistance to penicillin or erythromycin (Perrin-Guyomard et al., 2005). Seventy *Bacillus cereus* strains were isolated from 258 pasteurized milk samples collected from 32 cities in China and most of the isolates were resistant to ampicillin (99%), penicillin (99%), and cefoxitin (95%; Gao et al., 2018). Thus, it is necessary to assess the prevalence and antimicrobial susceptibility of *Bacillus* strains in pasteurized milk.

In *Bacillus*, the resistance to antibiotics could be either intrinsic or acquired. Generally, genes encoding acquired antibiotic resistance are located on mobile genetic elements such as plasmids or transposons, which could lead to the spread of antibiotic resistance among *Bacillus* and other clinical pathogens via horizontal gene transfer (Navaneethan and Effarizah, 2021). The erythromycin resistance gene on plasmid pHT73 can be transferred from *Bacillus thuringiensis* ssp. *kurstaki*

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KT0 to *B. cereus* and *Bacillus mycoides* by conjugation (Hu et al., 2004). The tetracycline resistance gene *tet(M)* located on a Tn916-like transposon can be transferred from *B. cereus* R89 to *Enterococcus faecalis* JH2-2 and *Staphylococcus aureus* 8794RF by filter mating (Agersø et al., 2002). Therefore, *Bacillus* strains in pasteurized milk could act as donors or reservoirs for antibiotic resistance genes (**ARG**), which can be transferred to pathogenic bacteria (von Wintersdorff et al., 2016). Moreover, European Food Safety Authority recommends that bacterial strains harboring transferable ARG should not be present in foods for human (European Food Safety Authority, 2007). Thus, it is necessary to determine the transferability of ARG from *Bacillus* strains in pasteurized milk, which is crucial for preventing the spread of ARG via food chain.

To assess the prevalence and antimicrobial susceptibility of *Bacillus* strains in pasteurized milk, 114 *Bacillus* strains were isolated and identified from the pasteurized milk collected from different regions in China between May 2019 and March 2020. Subsequently, the broth microdilution method was used to assess the antibiotic susceptibility profiles of these *Bacillus* strains. Some *Bacillus* isolates showed acquired resistance to trimethoprim-sulfamethoxazole, clindamycin, erythromycin, and tetracycline, respectively. Genomic and plasmid DNA sequencing indicated that a tetracycline resistance gene *tetL* was located on plasmid pBC46-TL in *B. cereus* BA117. Moreover, the plasmid pBC46-TL can be transferred into *Bacillus invictae* BA142, *Bacillus safensis* BA143, and *Bacillus licheniformis* BA130 by filter mating. Altogether, our results indicated that the prevalence and antimicrobial susceptibility of *Bacillus* strains isolated from pasteurized milk should be paid more attention to prevent the spread of ARGs.

MATERIALS AND METHODS

Because no live human or animal subjects were used in this study, no approval from IRB or IACUC was required.

Sample Collection and Preparation

To assess the prevalence of *Bacillus* in pasteurized milk products, 30 pasteurized milks (stored at 4°C and within the shelf life) were purchased from retail markets and supermarkets in Beijing, and 30 samples of pasteurized milk were provided by local dairy factories in Beijing between May 2019 and March 2020. Meanwhile, 73 raw milk samples were collected from local dairy farms in Beijing, Tianjin, Hebei, Henan, Jiangsu, Heilongjiang, and Inner Mongolia. All raw milk samples were collected from bulk tanks by staff with permission

from the quality manager and filled into 50-mL sterile plastic tubes (Corning). These samples were kept on ice and transported to the laboratory within 24 h. To address the spore-forming *Bacillus*, the raw milks were pasteurized at 80°C for 10 min in the laboratory and kept below 4°C until analysis (Griffiths and Phillips, 1990).

Isolation and Identification of *Bacillus* Strains

Bacillus strains were isolated according to National Food Safety Standard (China, 2014) with minor modifications (Kwon et al., 2021). Briefly, 25-g samples were randomly collected from each pasteurized milk sample and put into a sterile blender jar with 225 mL of 0.85% sterile saline buffer, then homogenized for 2 min at high speed (10,000 to 12,000 rpm), followed by dilution until 10^{-5} . An aliquot of 0.2 mL of each dilution was plated onto Mannitol-Egg-Yolk-Polymyxin Agar (AOBOX) and incubated at 30°C for 24 to 48 h. Pink colonies surrounded by a zone of precipitation were considered as presumptive *B. cereus* group strains, whereas yellow colonies without a zone of precipitation were considered as other *Bacillus* strains. Colonies on MYP plates were streaked onto Nutritional Agar plate and incubated at 30°C for 24 h before identification.

Genomic DNA of *Bacillus* strains was extracted using a TIANamp Bacteria DNA Kit (TIANGEN) according to the manufacturer's instructions. Concentration and purity of the DNA were measured by a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). A 16S rDNA fragment was amplified by PCR using Q5 High-Fidelity DNA polymerase (NEB) and universal bacterial primers 27F and 1492R (Lane, 1991). The PCR products were sequenced by Sangon Biotech and analyzed by nucleotide blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The strains with high similarity to 16S rRNA sequence of the *Bacillus* reference strain (E-value = 0 and Max identity $\geq 98\%$) were regarded as *Bacillus* strains.

Antibiotic Susceptibility Test

The antimicrobial susceptibility of 114 *Bacillus* isolates were determined by the broth microdilution method using the Biofosun gram-positive panel (Fosun Diagnostics). The antimicrobial susceptibility test was carried out and interpreted according to the criteria of the Clinical and Laboratory Standards Institute CLSI-M45 (Hindler and Richter, 2016). The 13 antibiotic agents included ampicillin (0.12–4 µg/mL), penicillin (0.06–8 µg/mL), meropenem (0.12–16 µg/mL), erythromycin (0.12–16 µg/mL), clindamycin (0.12–16 µg/mL), ciprofloxacin (0.06–8 µg/mL), rifampicin (0.06–8 µg/mL)

mL), sulfamethoxazole-trimethoprim (0.06/1.15–8/152 µg/mL), vancomycin (0.12–16 µg/mL), tetracycline (0.25–32 µg/mL), chloramphenicol (0.5–64 µg/mL), gentamicin (0.5–64 µg/mL). *Staphylococcus aureus* ATCC 29213 was used as a quality control in Minimum Inhibitory Concentration determination.

Plasmid DNA Extraction

Plasmid DNA of *Bacillus* strains that exhibited acquired antibiotic resistance was extracted using EZNA Plasmid DNA Mini Kit (Omega Bio-Tek Inc.) according to the manufacturer's instructions. Overnight cultures were inoculated (1% vol/vol) into 10 mL of Nutritional Broth and incubated at 30°C and 180 rpm. When cell density reached an OD_{600 nm} of 0.8, bacterial cells were collected by centrifugation at 6,000 × *g* for 10 min at 4°C for plasmid DNA extraction. Concentration and purity of the DNA were measured by a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). Plasmid DNA samples were analyzed by electrophoresis on 1% wt/vol agarose gel (Pan et al., 2011). A supercoiled DNA ladder (catalog no. 3585A, Takara) was used to estimate the size of supercoiled plasmid. Agarose gel was stained with Gel Red (Biotium) in 1 × TAE buffer for 15 min. Then the plasmid DNA on gel was visualized with UV light (302 nm wavelength) and photographed by the gel documentation system (UVP).

Genome Sequencing and Detection of Antibiotic Resistance Genes

Genomic and plasmid DNA were sequenced using Illumina NovaSeq PE150 at the Beijing Novogene Bioinformatics Technology Co. Ltd., respectively. Briefly, 1 µg of DNA per sample was fragmented by sonication to a size of 350 bp, then DNA fragments were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. At last, PCR products were purified (AMPure XP system) and libraries were analyzed for size distribution by Agilent2100 Bioanalyzer and quantified using real-time PCR. The library was sequenced by Illumina NovaSeq PE150. The raw data obtained by sequencing was filtered to obtain Clean Data. The genome assembly with Clean Data was performed by SOAP denovo software. The functions of the predicted protein-coding genes were annotated with the Clusters of Orthologous Groups database using the WebMGA. Antibiotic Resistance Genes Database and Comprehensive Antibiotic Resistance Database were used for the prediction of antibiotic resistance genes (Liu and Pop, 2009; Alcock et al., 2020).

Conjugative Plasmid Transfer by Filter Mating

All bacterial strains used for conjugative plasmid transfer were listed in Table 1. *Bacillus* was grown in nutritional broth at 30°C. *Lactobacillus* was grown under anaerobic conditions at 37°C in a De Man-Rogosa-Sharpe medium. *Escherichia coli* was grown at 37°C in Luria Bertani broth. Transfer of tetracycline resistance gene *tetL* was determined by filter mating as described previously (Hinnekens et al., 2019) with slight modification. Both donor and recipient strains were incubated at 30 or 37°C to reach OD_{600 nm} of 0.8, respectively. Subsequently, 500 µL of donor and 500 µL of recipient were mixed well and passed through a 0.45-µm cellulose-nitrate membrane filter (Xingya). The filter was then collected and placed on a nonselective agar plate for 24 h. After mating, the filter was washed by 10 mL PBS. Cells were collected by centrifugation at 6,000 × *g* for 10 min at 4°C. Transconjugants were screened by plating serial dilutions of the mating cells on appropriate selection agar plates (Table 4). Antibiotics (Sigma-Aldrich) were added at the following final concentrations: 20 µg/mL of tetracycline for the donor strain BA117; 16 µg/mL of erythromycin for *Bacillus* recipient; 4 µg/mL of clindamycin for *Bacillus* recipient BA102, BA142, and BA143; 16 µg/mL of clindamycin for *Bacillus* recipient BA107, BA130, and BA131; 34 µg/mL of chloramphenicol for *E. coli* recipient; 8 µg/mL of chloramphenicol for *Lactobacillus* recipient, respectively. Each mating experiment included a plating control of donor and recipient cells on selective medium to assess the presence of potential spontaneous mutants. The transfer frequencies were calculated as the ratio of transconjugants per donor cells (T/D) at the end of the conjugation time. The transfer of tetracycline resistance gene *tetL* was confirmed by plasmid profiling and PCR.

Nucleotide Sequence Accession Numbers

The genome sequence of *B. cereus* BA008, BA117, and BA119 was deposited at GenBank under the accession number PRJNA774561.

RESULTS AND DISCUSSION

Prevalence Analysis of *Bacillus* in Pasteurized Milk

In this study, a total of 114 *Bacillus* strains were isolated from 133 pasteurized milk samples. These 114 isolates included 46 *Bacillus cereus*, 4 *Bacillus licheniformis*, 3 *Bacillus subtilis*, 2 *Bacillus pumilus*, 1 *Bacillus invictae*, 1 *Bacillus paralicheniformis*, 1 *Bacillus safensis*, 2 *Bacillus toyonensis*, and 54 other *Bacillus*

Table 1. Bacterial strains used for conjugative plasmid transfer in this study

Strain	Relevant phenotype or genotype ¹	Role in conjugative transfer	Reference or source
<i>Bacillus cereus</i> BA117	Pasteurized milk isolate, TET ^r	Donor	This study
<i>Bacillus invictae</i> BA142	Pasteurized milk isolate, CLI ^r	Recipient	This study
<i>Bacillus safensis</i> BA143	Pasteurized milk isolate, CLI ^r	Recipient	This study
<i>Bacillus paralicheniformis</i> BA103	Pasteurized milk isolate, CLI ^r , ERY ^r	Recipient	This study
<i>Bacillus licheniformis</i> BA130	Pasteurized milk isolate, CLI ^r	Recipient	This study
<i>Bacillus licheniformis</i> BA107	Pasteurized milk isolate, CLI ^r	Recipient	This study
<i>Bacillus</i> spp. BA131	Pasteurized milk isolate, CLI ^r	Recipient	This study
<i>Bacillus pumilus</i> BA102	Pasteurized milk isolate, CLI ^r	Recipient	This study
<i>Bacillus licheniformis</i> BA123	Pasteurized milk isolate, CLI ^r , ERY ^r	Recipient	This study
<i>Escherichia coli</i> Rosetta (DE3)	F ^r <i>ompT hsdS_B(_{FB}⁻ m_B⁻) gal dcm</i> (DE3) pRARE (Cam ^R)	Recipient	Novagen, Germany
<i>Lactobacillus paracasei</i> sCAUH35-2	Kefir isolate, CHL ^r	Recipient	Laboratory stock

¹TET^r = tetracycline resistance; CLI^r = clindamycin resistance; ERY^r = erythromycin resistance; CHL^r = chloramphenicol resistance.

strains as shown in Table 2. The isolation rate of *Bacillus* was over 93.33% in pasteurized milk samples from market and dairy factory in Beijing. This agrees with the findings that the prevalence of *Bacillus* was high (~100%) in the pasteurized milk collected in China and Thailand (Chitov et al., 2008; Zhou et al., 2008). However, the prevalence of *Bacillus* was much lower in the milk samples pasteurized in the laboratory, which was 32.87% (24/73 samples). These findings were reasonable because the heat treatment of these raw milk samples was 80°C for 10 min, which was more sufficient than commercial pasteurization. In addition, post-pasteurization contamination along the milk-processing lines was possibly a source of *Bacillus* in pasteurized milk. For instance, *B. cereus* showed outstanding ability to adhere to stainless steel surfaces of dairy plant and form biofilm (Kumari and Sarkar, 2016; Silva et al., 2018).

In this study, the isolate rate of *Bacillus cereus* was 41.23% (46/114), indicating that *B. cereus* is a common contaminant of pasteurized dairy products. It has been reported that the environments for milk production,

handling, and processing could introduce *B. cereus* into dairy products (Cui et al., 2016). The heat-stable *B. cereus* spores in raw milk and the post-pasteurization contamination along the milk-processing lines were major sources of *B. cereus* contamination in pasteurized milk (Saleh-Lakha et al., 2017; Gao et al., 2018). The growth of *B. cereus* limits the shelf life of pasteurized milk. In addition to the risk of causing spoilage, *Bacillus* strains from pasteurized milk showed resistance to some antibiotics, such as ampicillin, erythromycin, and tetracycline (Liu et al., 2020; Zhao et al., 2020). Therefore, *Bacillus* strains not only influence the shelf life of pasteurized milk, but also might be reservoir of ARG.

Antimicrobial Susceptibility Tests of *Bacillus* Isolates

The 114 *Bacillus* isolates were tested for susceptibilities to 13 antibiotics using the broth microdilution method. All tested isolates were susceptible to the remaining 5 antibiotics, including meropenem, vancomycin, gentamicin, ciprofloxacin, and rifampin. A high percentage of the isolates were resistant to ampicillin (80%, 91/114) and penicillin (86%, 98/114). This is consistent with previous studies that most isolates of *Bacillus cereus* group were intrinsically resistant to β -lactam antibiotics (da Silva Fernandes et al., 2014; Yibar et al., 2017) owing to β -lactamase production (King et al., 2017). However, the isolates identified as *B. pumilus*, *B. safensis*, and *B. invictae* were sensitive to both ampicillin and penicillin. These findings agree with 4 *B. pumilus*, 27 *B. safensis*, and 9 *B. invictae* isolated from different terrestrial sources and geographic locations were sensitive to both ampicillin and penicillin (Branquinho et al., 2015). It is important to note that a few *Bacillus* isolates displayed acquired resistance to some antibiotics. Ten *Bacillus* isolates (9%) were resistant to trimethoprim-sulfamethoxazole

Table 2. Prevalence of *Bacillus* isolates in pasteurized milk

Isolate	Type		
	From the factory (n = 30)	From the market (n = 30)	Made in the laboratory (n = 73)
<i>Bacillus cereus</i>	22	15	9
<i>Bacillus licheniformis</i>	0	0	4
<i>Bacillus paralicheniformis</i>	0	0	1
<i>Bacillus pumilus</i>	0	0	2
<i>Bacillus safensis</i>	0	0	1
<i>Bacillus subtilis</i>	0	0	3
<i>Bacillus toyonensis</i>	0	1	1
<i>Bacillus invictae</i>	0	0	1
Other <i>Bacillus</i> ¹	6	22	26
Total	28	38	48

¹Strain cannot be identified at species level by 16s rDNA sequencing.

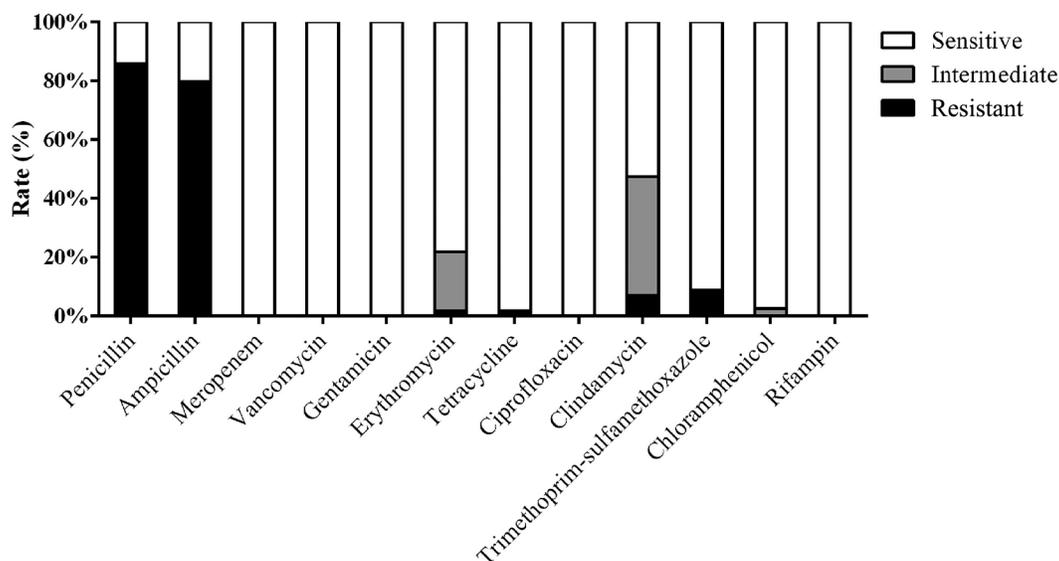


Figure 1. The proportions of susceptible, intermediate, and resistant strains among 114 isolated *Bacillus* strains tested against 13 antibiotics.

(Figure 1). Eight strains were observed to be resistant to clindamycin. In addition, 2 isolates were resistant to erythromycin and 1 isolate named *B. cereus* BA117 was resistant to tetracycline (Table 3). Clindamycin, tetracycline, and erythromycin are frequently used for treatment of bovine mastitis in China (Gao et al., 2012). These antibiotics have been frequently found in cattle manure and wastewater (Zhou et al., 2013), which could create selective pressure for antibiotic-

resistant *Bacillus* in dairy farms. The spores of these antibiotic-resistant *Bacillus* could be sources of contamination of raw milk. Notably, 14% (16/114) of the isolates exhibited multiple antibiotic resistance, which agrees with previous studies (Cui et al., 2016; Gao et al., 2018). The antibiotic resistance of *Bacillus* isolates, especially acquired resistance, should be paid more attention to, as the acquired antibiotic resistance might

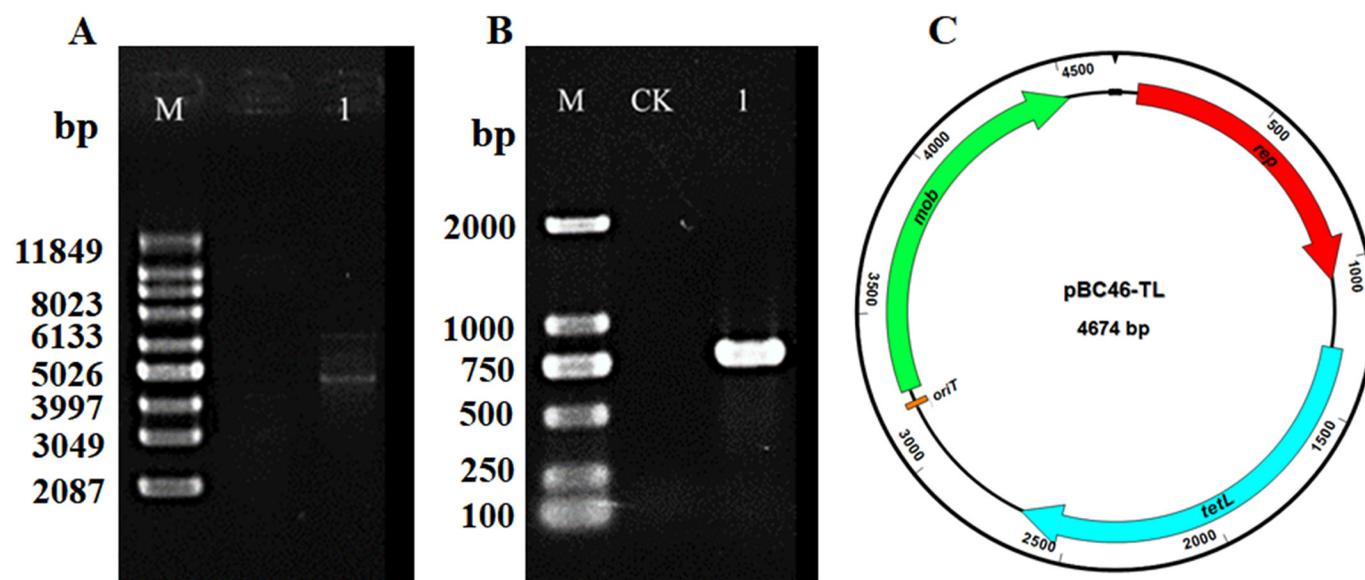


Figure 2. (A) Agarose gel electrophoresis of plasmid pBC46-TL purified by gel extraction kit. Lane M: supercoiled DNA ladder (Takara). Lane 1: pBC46-TL. (B) Agarose gel electrophoresis of the PCR product of gene *tetL*. Lane M: DL2000 DNA ladder marker (Takara). Lane 1: DNA fragment of *tetL*, obtained by PCR with purified pBC46-TL as template. (C) The physical maps of plasmid pBC46-TL. Plasmid map was generated by SeqBuilder program of Lasergene v7.1 (DNASTAR Inc.). Closed arrows indicated open reading frames (orfs).

Table 3. Antibiotic resistance of 114 *Bacillus* isolates in the study¹

Category	Antibiotic	MIC ($\mu\text{g/mL}$) interpretive criteria ²		<i>Bacillus cereus</i> (n = 47)	<i>Bacillus licheniformis</i> (n = 4)	<i>Bacillus paralicheniformis</i> (n = 1)	<i>Bacillus pumilus</i> (n = 2)	<i>Bacillus safensis</i> (n = 1)	<i>Bacillus subtilis</i> (n = 3)	<i>Bacillus toyonensis</i> (n = 1)	<i>Bacillus invictae</i> (n = 1)	Other <i>Bacillus</i> ³ (n = 54)	
		S	I										R
Penicillins	Penicillin	<0.12	—	>0.25	4	1	0	0	1	1	0	44	
	Ampicillin	<0.25	—	>0.5	2	1	0	0	1	1	0	40	
Carbapenems	Meropenem	<4	8	>16	0	0	0	0	0	0	0	0	
Glycopeptides	Vancomycin	<4	—	—	0	0	0	0	0	0	0	0	
Aminoglycosides	Gentamicin	<4	8	>16	0	0	0	0	0	0	0	0	
Macrolides	Erythromycin	<0.5	1-4	>8	0	1	0	0	0	0	0	1	
Tetracyclines	Tetracycline	<4	8	>16	0	0	0	0	0	0	0	0	
Fluoroquinolones	Ciprofloxacin	<1	2	>4	0	0	0	0	0	0	0	0	
Lincosamides	Clindamycin	<0.5	1-2	>4	2	1	1	1	0	0	1	2	
Folate pathway inhibitors	Trimethoprim-sulfamethoxazole	<2/38	—	>4/76	0	0	0	0	0	0	0	5	
Phenicol	Chloramphenicol	<8	16	>32	0	0	0	0	0	0	0	0	
Ansamycins	Rifampin	<1	2	>4	0	0	0	0	0	0	0	0	

¹For some organism or antimicrobial combinations, the absence or rare occurrence of resistant strains precludes defining any results categories other than "susceptible" (Hindler and Richter, 2016).

²S = susceptible; I = intermediate; R = resistant.

³Strains identified as *Bacillus* spp. with BLAST search of 16S rRNA.

be further transferred to other pathogens via horizontal gene transfer (Zhu et al., 2016).

Plasmid Profile of *Bacillus* Strains with Multiple Antibiotic Resistance

Antimicrobial resistance in bacteria is frequently encoded by genes carried on mobile genetic elements, in particular by plasmids (Partridge et al., 2018). The 16 *Bacillus* strains with acquired antibiotic resistance were tested for the presence of plasmids. The plasmid profile showed that 3 strains contained plasmids. *B. cereus* BA119, which was resistant to trimethoprim-sulfamethoxazole, had a 3.5 kb plasmid. Another trimethoprim-sulfamethoxazole resistant strain, *B. cereus* BA008, had a large plasmid with an apparent size of 10 kb. Notably, *B. cereus* BA117, which was resistant to tetracycline, had at least 5 plasmids differing in size from 3.5 to 10 kb. To further identify and locate these ARG, both genomic and plasmid sequencing were carried out using Illumina NovaSeq PE150.

Detection of Antibiotic Resistance Genes by Genome and Plasmid Sequencing

The draft genome of *B. cereus* BA008 was ~5.2 Mb with a G + C content of 34.98%. A total of 5,436 genes were predicted with functional assignments. Moreover, 91 RNA genes were detected, consisting of 73 tRNAs, 13 rRNAs, and 5 sRNAs. The ARG were predicted by the ARG databases Antibiotic Resistance Genes Database and Comprehensive Antibiotic Resistance Database. Genes *blaA*, *blaC*, or *bla1* encoding class-A β -lactamase and *pbp2B* encoding penicillin-binding protein 2B could confer BA008 resistance to β -lactam. Gene *dfrA* encoding drug-insensitive dihydrofolate reductase could confer BA008 resistance to trimethoprim. These were consistent with the results of the antimicrobial susceptibility test. The draft genome of *B. cereus* BA119 was ~5.4 Mb with a G + C content of 35.29%. A total of 5,630 protein-coding genes, 12 rRNA genes, 95 tRNAs, and 17 genomic islands were predicted in the genome of BA119. The presence of various ARG were observed, including for β -lactam resistance (*bla1*, *mecA*, *mecC*, and *pbp2B*), trimethoprim resistance (*dfrA*), and sulfamethoxazole resistance (*sul3*). The draft genome of *B. cereus* BA117 was ~6.5 Mb with a G + C content of 35.44%. We observed 7,078 predicted genes, including 106 tRNAs, 36 rRNAs, and 9 sRNA as well as 28 genomic islands. The ARG prediction indicated β -lactam resistance genes *blaA* and *mecA*, tetracycline resistance gene *tetM*, and *tetL* were present in *B. cereus* BA117.

If these ARG are located on mobile genetic elements such as plasmids or integrative conjugative elements

Table 4. Frequencies of conjugative transfer with *Bacillus cereus* BA117 as donor

Recipient strain	Antibiotic for selection ($\mu\text{g}/\text{mL}$)	Frequency ¹
<i>Bacillus paralicheniformis</i> BA103	Tetracycline (20) + Erythromycin (16)	1.7×10^{-5}
<i>Bacillus invictae</i> BA142	Tetracycline (20) + Clindamycin (4)	1.5×10^{-7}
<i>Bacillus safensis</i> BA143	Tetracycline (20) + Clindamycin (4)	2.0×10^{-6}
<i>Bacillus licheniformis</i> BA130	Tetracycline (20) + Clindamycin (16)	1.0×10^{-6}
<i>Bacillus licheniformis</i> BA107	Tetracycline (20) + Clindamycin (16)	0
<i>Bacillus</i> spp. BA131	Tetracycline (20) + Clindamycin (16)	0
<i>Bacillus pumilus</i> BA102	Tetracycline (20) + Clindamycin (4)	0
<i>Bacillus licheniformis</i> BA123	Tetracycline (20) + Erythromycin (16)	0
<i>Escherichia coli</i> Rosetta (DE3)	Tetracycline (20) + Chloramphenicol (34)	0
<i>Lactobacillus paracasei</i> sCAUH35-2	Tetracycline (20) + Chloramphenicol (8)	0

¹The transfer frequencies were calculated as the ratio of transconjugants per donor cells (T/D) at the end of the conjugation time.

(ICEs), these genes will be easily transferred into other bacteria via horizontal gene transfer (Huddleston, 2014). Bioinformatics analysis indicated that the β -lactam resistance genes and trimethoprim or sulfamethoxazole resistance genes in BA008, BA117, and BA119 were located on the chromosome DNA. In addition, the tetracycline resistance gene *tetM* was also on the chromosome DNA of BA117. Moreover, ICE prediction with ICEberg 2.0 database indicated that these genes were not on mobile genetic elements (Liu et al., 2019). It is important to note that gene *tetL* was located on the 4.6 kb plasmid, which was designated as pBC46-TL. To further confirm the location of gene *tetL*, plasmid pBC46-TL was separated from other plasmids in *B. cereus* BA117 and purified by gel extraction (Figure 2A). A partial of *tetL* was obtained by PCR with purified pBC46-TL as a template, indicating that gene *tetL* was inside plasmid pBC46-TL (Figure 2B). The complete sequence of pBC46-TL was obtained by PCR amplification with the primer pair (TL-F: 5'- GCTGGTGCTGGAATGAGTTTG -3' and TL-R: 5'- TTGGTTGTGTCGTAATTCG -3') and DNA sequencing. The sequence analysis showed that the gene encoding the Mob protein and a putative transfer origin sequence *oriT* were present in pBC46-TL (Figure 2C), indicating that this plasmid might be transferable via conjugation.

Conjugative Transfer of the *tetL* Gene by Filter Mating

The conjugative transfer capacities of the plasmid pBC46-TL were determined by filter mating using the tetracycline-resistant strain BA117 as donor. Eight *Bacillus* strains, 1 *Escherichia coli* strain, and 1 *Lactobacillus paracasei* strain were selected as recipients in this study (Table 1). Filter mating assays with BA117 (donor) and BA103 (recipient) revealed an average frequency of conjugation of 1.7×10^{-5} (Table 4). When comparing the plasmid profile of the transconjugant

with the donor strain, we have observed that the transconjugant harbored a small plasmid (~ 4.6 kb) of similar size to that of the donor strain (Figure 3). The *tetL* in the transconjugant was further confirmed by PCR amplification combined with DNA sequencing (data not shown).

In addition, transfer of pBC46-TL to *B. invictae* BA142, *B. safensis* BA143, and *B. licheniformis* BA130 occurred at a frequency of 1.5×10^{-7} to 2.0×10^{-6} transconjugants per donor cells (T/D). However, no transconjugant was detected when the conjugative transfer of pBC46-TL was performed with *B. licheniformis* BA107, *Bacillus* spp. BA131, *B. pumilus* BA102, *B. licheniformis* BA123, *Escherichia coli* Rosetta (DE3), and *Lactobacillus paracasei* sCAUH35-2. These results revealed highly significant associations between transfer frequency and donor or recipient species, which

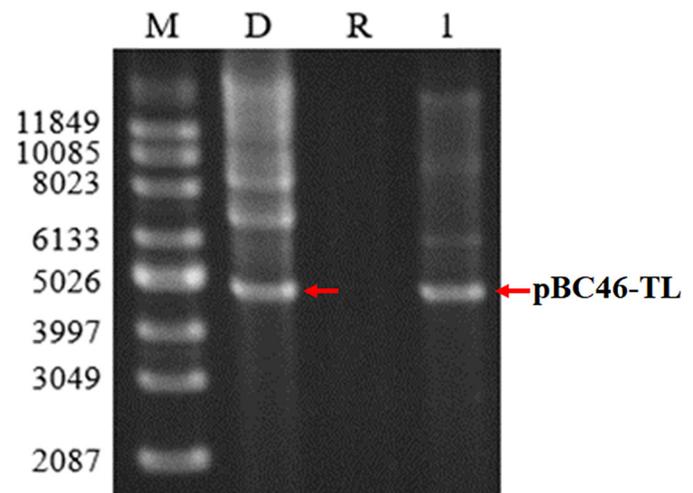


Figure 3. Plasmid profiles of donor, recipient, and transconjugant strains in experiment with *Bacillus cereus* BA117 as donor and *Bacillus paralicheniformis* BA103 as recipient. Lane M: supercoiled DNA ladder. Lane D: plasmid extracted from *B. cereus* BA117. Lane R: plasmid extracted from *B. paralicheniformis* BA103. Lane 1: plasmid extracted from the transconjugant.

was in accordance with previous studies (Leroy et al., 2019). Altogether, the filter mating experiments confirmed the transferability of *tetL* on plasmid pBC46-TL across some *Bacillus* strains, which might lead to the production of a new tetracycline-resistant strain in a natural environment.

CONCLUSIONS

A total of 114 *Bacillus* strains were isolated from 133 pasteurized milk samples. The antimicrobial susceptibility of these strains indicated that a high percentage of the isolates were intrinsically resistant to ampicillin and penicillin. Meanwhile, some *Bacillus* isolates displayed acquired resistance, including trimethoprim-sulfamethoxazole, clindamycin, erythromycin, and tetracycline. Subsequently, genomic and plasmid DNA sequencing were carried out to determine the ARG in plasmid-containing strains *B. cereus* BA008, BA117, and BA119. It is important to note that gene *tetL* encoding tetracycline efflux protein was located on the plasmid pBC46-TL of *B. cereus* BA117. This plasmid can be transferred into other *Bacillus* strains by filter mating. Therefore, *Bacillus* strains in pasteurized milk is not only of concern with regard to the spoilage of the products, but also might be a reservoir of ARG. Although the detection of *Bacillus* is not currently required for the dairy microorganism test of Chinese food security standards, prudent use of antibiotics, improved hygienic conditions during milking, and removing spores with bacterofugation should be taken into consideration to reduce the antibiotic resistance of *Bacillus* in pasteurized milk.

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