Genetic Diversity and The Presence of Circular Plasmids in *Bacillus Cereus* Isolates of Clinical and Food Origin

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Abstract

The diversity of 61 *Bacillus cereus* strains isolated from different clinical specimens, food including raw milk and milk products, and water was evaluated. PFGE analysis could discriminate 61 distinct pulsotypes with similarity levels from 25 to 82%, which were divided into 13 clonal complexes. The similarity between clonal complexes was at least 40%. Clinical strains were divided into 10 clonal complexes, while the strains, isolated from milk, food and water were included in 9, 6 and 6 clonal complexes, respectively. Three clonal complexes were dominated by clinical isolates, while they were absent in two complexes. Bacterial isolates from foods, being a probable source of alimentary toxoinfection, showed low similarity to isolates from stool specimens. The isolates from both sources were classified together in only 4 out of 13 clonal complexes. The large circular and linear plasmids with the sizes between 50 and 200 kb were detected in 24 (39.3%) and 14 (23%) *B. cereus* strains, respectively. Thirteen (21.3%) strains contained only one plasmid, two plasmids were found in 6 (9.8%) of strains, and three or more plasmids were obtained in 5 (8.2%) of tested strains. The plasmids were confirmed in 30.8% and 40% of isolates from clinical specimens and food and milk samples, respectively. No clear correlation between the PFGE profiles, the source as well as plasmid content among all tested strains was observed.

Introduction

Bacillus cereus is a ubiquitous Gram-positive, motile, aerobic, non-encapsulated, endospore-forming rod-shaped bacterium (Chen et al. 2003).

The natural environmental reservoir for *B. cereus* consists of decaying organic matter, fresh and marine waters, vegetables and fomites, and the intestinal tract of invertebrates, from which soil and food products may become contaminated, leading to the transient colonisation of the human intestine (Jensen et al. 2003). This bacteria is associated with foodborne poisoning expressed as diarrhoea and emesis, serious wound infections, pneumonia, bacteriemia, meningitis, endophthalmitis, necrotising fasciitis, osteomyelitis, and endocarditis (Chen et al. 2003). The *B. cereus* spores are thermal resistant and cause post-processing contamination of ready-to-eat food, milk powder, and sausage (Ehling-Schulz et al. 2006).

Bacillus cereus sensu lato is a group of bacteria displaying close phylogenetic relationships but a high ecological diversity. The three most studied species are *Bacillus anthracis*, *Bacillus cereus sensu stricto* and *Bacillus thuringiensis*. However, the species delineation between *B. thuringiensis* and *B. cereus sensu stricto* has been problematic despite the various approaches and techniques used. It has been suggested that plasmids, especially large toxin-carrying plasmids, play a crucial role in the phenotypical heterogeneity of the *B. cereus* group (Fayad et al. 2019; Patino-Navarrete and Sanchis 2017), which might be one of the reasons that *B. cereus sensu stricto* itself often demonstrated high genetic heterogeneity between the strains (Vassileva et al. 2007; Yang 2017). There were also still no definite conclusions on whether the *B. cereus* emetic strains belonged to a single clone or a diversiform complex (Vassileva et al. 2007; Yang 2017).

Many of the species-specific phenotypes of the *B. cereus sensu lato* group are encoded by plasmid genes. The major virulence factors are located extrachromosomally on large plasmids. For *B. thuringiensis* strains, typical plasmid-encoded crystalline inclusions contain *Cry* and *Cyt* proteins, some of which are toxic against a wide range of insect orders, nematodes and human-cancer cells (Palma et al. 2014). The pXO1 and pXO2 plasmids, found in *B. anthracis* strains, are responsible for producing anthrax exotoxin and the capsule, respectively. The genes for emetic toxins are located at *B. cereus* on plasmids, and they have already been sequenced and analysed (Rasko et al. 2007).

Meanwhile, the diarrhoeal haemolytic, nonhaemolytic enterotoxins and cytotoxin K are encoded chromosomally (Griffiths and Schraft 2017). Some acrySTALLIFEROUS *B. cereus* also carry conjugative plasmids with *Cry* genes native to *B. thuringiensis*, the pXO1 and pXO2-like plasmids were confirmed in some *B. cereus* strains as well (Rasko et al. 2004). Plasmids are vectors for redundant or similar chromosomal genes in the *B. cereus* group (Zheng et al. 2015). Therefore, the presence of these plasmids cannot serve as signatures for species identification (Liu et al. 2015), and it is more useful to consider the *B. cereus* group as a unique species comprised of extremely diverse strains whose properties differ due to plasmid content or because of gene expression associated with key regulatory genes (Helgason et al. 2000; Rasko et al. 2005).

The purpose of our study was to determine the diversity of *B. cereus* strains, obtained from patients' specimens, food and water samples. The number and the length of plasmids that could contribute to the genetic heterogeneity of isolates were also investigated.

Materials And Methods

Bacterial isolates

A total of 61 isolates of *B. cereus* collected between 2007 and 2015 were analysed in the study. Twenty-six of them originated from patients hospitalised in different clinical wards in Ljubljana hospitals. Isolates were obtained from various specimens such as, wounds, burns, faeces, other excreta, ear ducts, nose mucosa swabs, etc. Another thirty were isolated from food samples. Their sources were raw milk from individual farms, pasteurised milk, cream, ice-cream, skim milk powder, ultra-high temperature-treated milk produced by a Slovenian dairy (21 strains), and salad, rice meal, pudding, infant food, selected sauces, dumplings, spices, beefsteaks, etc. prepared in public catering plants (9 strains). Five isolates were obtained from drinking and underground water. Identification of the strains was carried out by conventional methods including colony morphology, cell morphological and physiological characteristics and haemolytic activity (ISO 7932 2004).

The isolates were biochemically identified with the API 50CHB and API10 S test systems using the API WEB identification programme Vitek 2.1 (bioMérieux, Marcy-l'Étoile, France) and by multiplex PCR according to Park et al. (2007) and Leski et al. (2009). Total DNA was extracted using the SDS method, followed by purification using the phenol-chloroform-isoamyl alcohol protocol, as previously described (Mäntynen and Lindström 1998; Moore et al. 2004; Sambrook et al. 1989).

Reference strains used for the quality control: *B. cereus* ATCC 14579^T, *B. mycoides* IAM 1190, *B. thuringiensis* ATCC 10792 (CCM, Brno, Czech Republic), *B. cereus* ATCC 11778 (Oxoid, Cambridge, UK) and *B. subtilis* BGA (Merck, Darmstadt, Germany).

DNA extraction and PFGE for detection of genetic diversity of *B. cereus* strains

PFGE analysis of the *B. cereus* isolates was performed according to Liu et al. (1997) and Sjölund et al. (2005) with some modifications.

All strains were cultured on blood agar (BD BBLTM) with the ampicillin discs (AM-10 mg, BD BBLTM) placed on the surface of each plate. After incubation at 37 °C for 24 h, the colonies were transferred into 2 ml of the BHI broth (Merck, Germany) and incubated with shaking at 37 °C for 4 h. Cells were harvested by centrifugation and the cell pellet was resuspended in 0.5 ml of SE buffer (75 mM NaCl, 25 mM EDTA [pH 7.5], both Sigma-Aldrich, Germany) and the centrifugation was repeated. The supernatants were removed, and the cell lysis took place in 500 mL of SE buffer containing 105 mg of lysozyme (Sigma-Aldrich, Germany) and 10 U of lysostaphin (Sigma-Aldrich, Germany) at 37 °C for 1 h.

The bacterial suspension was then mixed with 500 mL of 1% Low-Melting agarose (Invitrogen, USA), dispensed in a plug mould (Bio-Rad Laboratories, USA), and allowed to solidify at 4 °C.

For lysis, the resulting plugs were then placed in a mixture of 6 mM/L Tris base (Sigma-Aldrich, Germany), 100 mM/L EDTA [pH 7.5], Sigma-Aldrich, Germany), NaCl 58,4 g (Merck, Germany), 0.5% Brij®58 (Merck, Germany), 0.5% sodium lauryl sarcosine (Sigma-Aldrich, Germany), 0.2% sodium deoxy-cholate (Merck, Germany), 1.5 mg of lysozyme (our modification instead 1 mg) per mL, and 5 U of lysostaphin per mL. Following 72 h incubation at 37 °C, the plugs were transferred to a solution which contained 1% sodium lauryl sarcosine, 0.5 M EDTA [pH 9.5], and 200 mg of proteinase K per mL, and the mixture was incubated for 24 h at 50 °C under gentle shaking. The plugs were washed in TE buffer (10 mM Tris-HCl [pH 7.5], 10 mM EDTA) six times for 30 min at room temperature.

A slice of each plug (2.5 mm) was cut out and incubated 1 h at 37 °C with 25 U of *Sma*I restriction endonuclease (Roche, Switzerland) in the relevant buffer. The enzyme solution was removed, and a fresh one was added afterwards for further 24 h incubation at 37 °C. The slices were then loaded into the wells of 1% Pulsed Field Certified Agarose (Bio-Rad Laboratories, USA) in 0.5 × Tris borate EDTA (TBE) buffer.

Electrophoresis was done in a contour-clamped homogeneous electric field apparatus (CHEF-DR[®]III, Bio-Rad Technologies, USA) for 30 h at 11 °C, with an electric field of 6 V/cm at an angle of 120 °; the pulse time was increased from 5.3 to 34.9 s. After electrophoresis the gel was stained with ethidium bromide (0.5 µg/mL) for 30 min and destained in distilled water for 1 h, afterwards, DNA was visualised under UV light. A low range Lambda Ladder PFGE Marker 50-1000 kb (BioLabs, New England) was used as the molecular weight marker (Liu et al. 1997; Sjölund et al. 2005).

Isolation of large circular and linear plasmids by PFGE

A method for detecting and estimating the sizes of large bacterial plasmids in the presence of genomic DNA by pulsed-field gel electrophoresis (PFGE) was used according to Barton et al. (1995) with a few modifications. The agarose plugs with DNA, prepared in the same way as for studying the genetic diversity between the isolates, were cut into two slices with a sterile glass coverslip. The first one was soaked in the buffer S1 for 1 h at 37 °C, while to the second one appr. 450 U (0.3 mL) of endonuclease S1 in 1 × S1 buffer (100 mL) (Thermo Fisher Scientific, ZDA) was added and incubated for 30 min at room temperature. Digested and undigested slices were applied to wells in 1% agarose gel, prepared in 0.5 × TBE buffer (45 mM Tris-OH [pH 8.0], 45 mM boric acid, 1 mM EDTA), and run in a CHEF-DR®III apparatus for 24 h at 11 °C, with an electric field of 6 V/cm at an angle of 120 °, the pulse time was increased from 1 to 12 s. The presence of linear plasmids was demonstrated in undigested slices, while the circular plasmids were detected in digested slices.

Statistical analysis

Macrorestriction profile analysis was made in BioNumerics 7.1 (Applied Maths, Saint-Martens, Belgium) using the Dice coefficient, and represented by unweighted pair grouping by mathematical averaging (UPGMA) with 0.5% band tolerance and 0.5% optimisation settings. Images of *B. cereus* ATCC 14579^T were used as a marker to calibrate images' position, with manual correction if necessary.

The SPSS software (version 25.0; IBM, USA) was used for statistical analyses. The statistical difference of the PFGE clonal complexes with the source of the strains and the presence of plasmids was calculated using the Kullbach 2 $\bar{I}$ (Likelihood Ratio) and Pearson chi-square tests. A *p*-value lower than 0.05 was considered statistically significant.

Results

Diversity of pulsed-field gel electrophoresis patterns of *B. cereus* strains

Genomic DNA of 61 *B. cereus* strains analysed by PFGE with *Sma*I restriction enzyme yielded 10 to 23 bands of approximately 145.5 kb to 727.5 kb (Fig. 1).

A total of 61 distinct pulsotypes was obtained from the PFGE banding patterns with a similarity level from 25 to 82%, which were divided into 13 clonal complexes. The similarity between clonal complexes was at least 40%, and they contained from 1 (clonal complex no. 2) to 7 pulsotypes (clonal complexes no. 3 and 4) (Fig. 2)

These results showed a high genetic polymorphism existing among isolates with a diversity index of 0.62. Clinical strains were deployed into 10 clonal complexes, while the strains isolated from milk, food and water were included in 9, 6 and 6 clonal complexes, respectively. Three clonal complexes no. 9, 10, 13, were dominated by clinical isolates, while they were absent in complexes no. 5 and 8.

Bacterial isolates from foods, being probable sources of alimentary toxoinfection, showed very low similarity to isolates from faeces and other clinical specimens. The isolates from both sources were classified together in only 4 out of 13 clonal complexes.

Eleven out of 21 strains, obtained from milk and milk products, were isolated from raw milk. They were classified into 6 complexes, and they dominated in the complex no. 3 (4 strains), while the strains from milk products were mostly present in the fourth complex (4 strains). The standard strain ATCC 14579 was classified into the twelfth clonal complex.

A significant difference was observed in the distribution of the strains of different origin between the clonal complexes ($p = 0.008$).

Characterisation of large plasmids

Large circular and linear plasmids were detected in 24 (39.3%) and 14 (23%) *B. cereus* strains, respectively. Thirteen (21.3%) strains contained only one plasmid, two plasmids were found in six (9.8%) of strains, and three or more plasmids were obtained in five (8.2%) of the tested strains. The sizes of plasmids were between 50 and 200 kb. Ten out of 45 plasmids found had 100 kb, seven plasmids each had the sizes 80 kb and 100 kb, respectively, while only one was 200 kb in size. Plasmids were present in 40% of strains isolated from food and milk, as well as in 30.8% of clinical specimens. Four out of five water isolates also contained one or more plasmids. The exceptions were the fourth and eleventh clonal complexes, where only one and no plasmid-containing strains were classified, respectively. The plasmids were detected in all strains of the second and sixth complex (Table 1). A significant difference was detected between the origin and the linear plasmid content of the isolates ($p = 0.006$) as well as between the strains possessing linear and circular plasmids ($p < 0.001$).

Furthermore, no significant difference was determined between strains containing plasmids and their distribution in different clonal complexes ($p > 0.05$).

Discussion

Comparison of PFGE patterns has been extensively used in epidemiological studies to confirm or to discriminate the sources of disease, but also to evaluate the genetic diversity among a group of closely related strains from the same species (Castiaux et al. 2014). One aspect of this diversity may be explained by the dynamic repertoire of plasmids found in the *B. cereus* group as well as in individual strains of the *B. cereus sensu stricto* (Rasko et al. 2004; Rasko et al. 2005; Rasko et al. 2007).

We determined the relationships between the *B. cereus* strain, isolated from clinical, food and water sources using PFGE analysis using *Sma*I restriction endonuclease (Akamatsu et al. 2019; Liu et al. 2016; Yavuz et al. 2004). The fingerprints generated by macrorestriction of the DNA comprised approximately 10 to 23 bands of 48.5 to 727.5 kb. The number of bands obtained in our study was quite similar to Yavuz et al. (2004) findings, but they were slightly longer (Fig. 1).

The profiles showed a remarkable polymorphism existing among all strains, which was also reported by Merzougui et al. (2013). These authors also confirmed a visible correlation between PFGE types and the sources of *B. cereus* food isolates, which cannot be claimed for the strains in a recent study. Each out of 61 tested strains was included in the individual pulsotype, which were classified in 13 clonal complexes. Most complexes contained from four to seven pulsotypes. The second and the sixths complex account only one and two strains, respectively, both contained water isolates. The water isolates were distributed in different clonal complexes. In only 4 (33.3%) out of 12 complexes, in which the strains from food or faecal specimens were classified, were strains from both origins present together. These data show that there was no significant similarity between strains that cause gastrointestinal problems, and those present in food samples, as reported by Liu et al. (2016). It should be emphasised that food and stool samples were not epidemiologically or temporally related, despite the fact that they were obtained in the same period from 2013 to the first half of 2014. The milk and food isolates together were present in 5 (50%) out of 10 complexes, which involved isolates from these two sources and showed only partial aggregation. The strains from raw milk were present mostly in the third and eighth complexes, while the isolates from milk products prevailed in the fourth and eleventh complexes. The source of *B. cereus* strains could be the milking cows themselves, the environment in farms or bulk milk tanks. It could be presumed, that the reason for the presence of *B. cereus* in milk products is more common post-processed contamination from the production line in dairy and not from the raw milk (Fig 2, Table 1). Milk and clinical isolates were grouped in 7 out of 13 clonal complexes, and we could not classify them into separate groups, as was reported by Helgason et al. (2000), who compared the genetic diversity of the periodontal *B. cereus* and *B. thuringiensis* isolates to isolates from dairies using PFGE analysis. Cluster analysis revealed two major groups, one cluster included solely isolates from dairies, while the other cluster, included all human isolates as well as the isolates from dairies (Helgason et al. 2000).

The degree in PFGE patterns higher than 70% was yielded between the strains Bc37 (pepper) and Bc38 (risotto) both sampled in the same restaurant, between Bc44 (raw milk) and Bc46 (pasteurised milk), between Bc42 (pasteurised milk) and Bc12 (faeces), between Bc14 (wound) and Bc17 (acoustic duct) as well as between Bc 47 and Bc 65 with the origin in raw bulk milk, transferred in January and July to the same dairy, respectively, which suggested possible connections in origin as well as some deficiencies in cleaning and disinfection of the equipment.

Adesetan et al. (2020) reported, that the RAPD profile of *B. cereus* isolates from some retailed foods showed that all the strains are closely related, with a similarity coefficient of 70%. Tourasse et al. (2011) concluded that isolates from food and dairy-related sources frequently share identical genotypes with strains of diverse environmental origins.

We could not confirm these findings because we did not include enough environmental samples in the study, but we can assume that the environment (i.e. water) and animals (i.e. raw milk) represent a potential common origin of the pathogenic *B. cereus* strains caused food contamination as well as clinical infections. Therefore Castiaux et al. (2014) recommended that animals should be a focus of attention in the process of identifying a potential common origin of food contamination by emetic *B.*

cereus strains. Chang et al. (2018) determined that the *B. cereus* strains involved in skin infection tended to form a distinct genetic cluster compared to isolates associated with invasive diseases like bacteriemia, which also had unique genetic features. The strains causing the same types of illnesses were also classified in different clonal complexes in our study. For example, the clonal complex no. 10 included only isolates from clinical specimens, but from different sources, such as faeces, haemoculture and nasal mucosa.

Large circular plasmids have been described in a variety of microorganisms. Many of them are responsible for distinctive and significant bacterial traits, including virulence, nitrogen fixation, root nodulation, antibiotic and heavy metal resistance, conjugation and other metabolic transformations. Their closed-circular supercoiled forms move very slowly in pulsed-field gels and relaxed or nicked open-circular forms remain trapped in the sample wells (Barton et al. 1995)

Some authors considered that among the representatives of *B. cereus sensu lato* plasmids are essential for defining only the *B. anthracis*, *B. cereus sensu stricto* and *B. thuringiensis* species (Ehling-Schulz et al. 2006; Vilas-Bôas et al. 2007; Zheng et al. 2015); however, Andrup et al. (2008) confirmed that *B. mycoides* also harboured large plasmids. *B. anthracis*, *B. thuringiensis*, and the emetic *B. cereus* major virulence factors are located extrachromosomally on large plasmids (Rasko et al. 2005). The plasmids in this group display strain-dependent distribution, with some strains containing no plasmids, whereas others have many (more than 10). Some of these plasmids have small genome size, only 2 kb, whereas others are very large, up to 600 kb (He et al. 2010; Liu et al. 2013; Zheng et al. 2015). Fayad et al. (2019) also confirmed the most striking difference between species resides at the level of their plasmid content.

In contrast, the authors Helgason et al. (2000) and Rasko et al. (2005) claimed, that the plasmid profile of *B. cereus sensu lato* was extremely variable, and no well-defined conserved members have been identified that could delineate the species. These plasmid-based species definitions have resulted in the classification of members of the *B. cereus* group that are not valid when molecular typing is applied and the suggestion that these three species should be regarded as a single species.

With the PFGE method using restriction endonuclease S1 we confirmed only 39.3% of *B. cereus* isolates harbouring one or more plasmids, while Helgason et al. (2000) obtained the plasmids in size 15 to 600 kb in 82% of periodontal and dairy isolates. One large circular plasmid was found in 21.3% of our tested strains, while three and more of them in 8.2% of strains, mostly with the sizes from 50 to 200 kb. Rasko et al. (2007) reported about plasmids in *B. cereus* strains ranged from 54 to 466 kb including for the *B. anthracis* characteristic pXO1-like plasmids in size from 181 to 272 kb, while Fayad et al. (2019) studied *B. cereus sensu stricto* strains with an average of two plasmids per strain with the size from 2931–715,614 bp. In present study the maximum size of plasmids was 200 kb, which was probably the consequence of the limited methodology we used. We have to highlight that we could not detect smaller plasmids with the regular gel electrophoresis according to Andrup et al. (2007), because we were not able to perform the gel electrophoresis the predicted time at enough low temperature to obtain useful results.

The problem of chromosomal and large plasmid DNA preparation for PFGE is also the sporulation of *B. cereus* (Liu et al. 2016). However, to prevent this, we pre-incubated the fresh culture for DNA extraction for only 4 hours, such that only a small number of spores formed. *B. cereus* cells have cell walls, which is difficult to be lysed, so the time of incubation with lysozyme, lysostaphin, and proteinase K was longer than for some other Gram-positive bacteria (Liu et al. 2016; Samapundo et al. 2011).

68. *B. cereus* is well known as an intrinsic metallo- β -lactamase producer with chromosomal resistance to penicillins and cephalosporins (Chen et al. 2003). Some *B. cereus* isolates, resistant not only to β -lactams, but also to cotrimoxazole, clindamycin, erythromycin, tetracyclines, and carbapenems have been identified recently (Bottone 2010; Savini et al. 2009). The genes for resistance to some of these antimicrobials, i.e. erythromycin or tetracyclines might be located on plasmids (Barbosa et al. 2014; Rather et al. 2012). In our previous work, we studied the antibiotic resistance at the same *B. cereus* isolates as in the present study. The resistance to kanamycin, bacitracin, gentamicin, ciprofloxacin, tetracyclin or carbapenems was observed in only a few strains, while the amplicons of the family *bla*_{CTX-M}, *bla*_{TEM} and *bla*_{VIM-like} genes were confirmed among 68.2%, 34.8% and 21.2% of the samples, respectively (Godič Torkar and Bedenić 2018). We could not confirm any significant differences between the presence of plasmids, PFGE patterns or the source of studied *B. cereus* isolates and their content of resistance genes ($p > 0.05$).

Conclusions

The PFGE profiles showed a remarkable polymorphism existing among all *B. cereus* strains, a visible correlation between profiles and the sources of isolates was not confirmed. Bacterial isolates from foods, being the probable source of alimentary toxoinfection, showed very low similarity to isolates from stool specimens and were not epidemiologically or temporally related. The environmental water isolates were each distributed in different clonal complexes. The distribution of the strains of different origin between the clonal complexes was significantly different. Only 39.3% of *B. cereus* isolates harboured one or more plasmids in sizes of 50 to 200 kb. The strains with the plasmids were spread evenly across all PFGE clonal complexes

Declarations

Conflict of interests

The authors do not have any conflict of interest to declare. No competing financial interests exist.

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Tables

Table 1 The number of *B. cereus* strains from different origins and those containing plasmids, classified in individual clonal complexes

Clonal complex	Origin of the strains					Number of strains (pulsotypes) containing plasmids
	Specimen /feces	Food	Milk and milk products	Water	Total no. of pulsotypes	
1	4/2	1	1	0	6	2
2	0	0	0	1	1	1
3	2/1	0	5	0	7	3
4	1/0	1	4	1	7	1
5	0	3	2	1	6	3
6	1/0	0	0	1	2	2
7	1/1	1	3	0	5	2
8	0	2	2	1	5	3
9	5/2	0	1	0	6	3
10	4/2	0	0	0	4	2
11	3/2	0	2	0	5	0
12	2/1	0	1	0	3	1
13	3/1	1	0	0	4	1
Σ	26/12	9	21	5	61	24

Figures

Figure 1

PFGE of *Smal* digested DNA from *B. cereus* isolates. Lane M: bacteriophage lambda ladder; Lanes: 28-33: *B. cereus* strains no. 28, 29, 30, 31, 32, 33.

Figure 2

PFGE profile of *Smal* digested DNA of 61 *Bacillus cereus* isolates. Dendrogram of similarities between strains was calculated using the UPGMA algorithm and Dice coefficient. S: clinical specimen; F: food, sampled on suspicion of infection; M: raw milk and milk products; W: water; BcX: *B. cereus* strain number; 1-13: the number of clonal complex.