



Differentiation of *Bacillus anthracis* and other *Bacillus cereus* group bacterial strains using multilocus sequence typing method

GRZEGORZ GRANIAK ¹, ALINA OLENDER ², KATARZYNA NAYLOR ³

¹ General Karol Kaczkowski Military Institute of Hygiene and Epidemiology, Biological Threats Identification and Countermeasure Centre, Lubelska 4, 24-100 Pulawy, Poland

² Medical University in Lublin, Department of Medical Microbiology, Chódzki 1, 20-093 Lublin, Poland

³ Medical University in Lublin, Department of Didactics and Medical Simulation, Chódzki 4, 20-093 Lublin, Poland

E-mail: grzegorz.graniak@gmail.com

ABSTRACT

The study describes the preparation of the phylogenetic differentiation of *Bacillus cereus* strains. The *Bacillus cereus* group of bacteria is very important for human and animal health. The multilocus sequence typing scheme has been used to present this group of bacteria's phylogenetic relationship and structure. The MLST system was established using 60 isolates of *B. anthracis*, *B. cereus sensu stricto*, *B. thuringiensis*, and transitional environment strains of *Bacillus spp.* As a negative control, five strains of *B. subtilis* and *B. megaterium* were used. Primers for amplification and sequencing were designed to target highly conserved internal fragment of seven housekeeping genes: *glpF*, *gmk*, *ilvD*, *pta*, *pur*, *pycA*, and *tpi*. A total of 22 different sequence types (STs) were distinguished. Analysis of the sequence data showed that all of the *Bacillus cereus* strains are very closely related. The MLST scheme exhibited a high level of resolution that can be used as an excellent tool for studying the phylogenetic relationship, epidemiology, and population structure of the *Bacillus cereus* group strains. The MLST method additionally allows us to define the phylogenetic relationship between very closely related strains based on a combination of the sequences of all seven alleles fragments and each of them separately. Thus, this genetic investigation tool is very useful in epidemiological investigation of potential military/ bioterrorist use of *B. anthracis*.

KEYWORDS: *Bacillus cereus* group, multilocus sequence typing, sequencing, house-keeping genes, phylogenetic differentiation, BioNumerics

Introduction

Bacterial strains from the *Bacillus cereus* group belong to the Bacillaceae family (Ludwig *et al.* 2009). This group

comprises six species of bacteria: *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides*, and

B. pseudomycooides, which are very important for medical (biological) and economic reasons. The *Bacillus cereus* group contains nonpathogenic strains found in humans and animals (saprophytic environmental strains), industrial use strains, and very pathogenic strains, which can be used as a biological weapon agent. *B. cereus* is an opportunistic human pathogen commonly present in soil and water. Due to its ubiquitous nature, *B. cereus* can cause contamination in the pulp and paper and the dairy industry. Additionally, *B. cereus* strains can cause various infections in immunodeficient patients and patients after surgery, including endocarditis, cutaneous infections, septicemia, pneumonia (Hoffmaster *et al.* 2006), or emetic and diarrheal food poisoning (Kim *et al.* 2010). *B. thuringiensis* is a pathogen for insects *Diptera sp.*, *Lepidoptera*, and *Coleoptera sp.* (Elleuch *et al.* 2014) and an opportunistic pathogen for human. *B. thuringiensis* is used in agriculture as a source of natural insecticides in tomatoes, cucumbers, or cabbages cultivation (Frederiksen *et al.* 2006). *B. weihenstephanensis* has been described recently due to its ability to grow in very low temperatures (+4°C to +7°C). As a separate species in the *Bacillus cereus* group, it was identified based on the 16S rRNA and cold-shock protein gene *cspA* sequences (Thorsen *et al.* 2006). Additionally, some strains of *B. weihenstephanensis* possess genes for enterotoxin *NheA* and emetic toxin cereulide (Svensson *et al.* 2007; Økstad and Kolstø 2011), like *B. cereus*. For *B. mycooides* and *B. pseudomycooides* strains, a different rhizoidal type of growth was observed (Sorokin *et al.* 2006). *B. anthracis* is a Gram-positive, spore-forming, aerobic, rod-shaped bacterium. It is the most dangerous human and animal pathogen, causes the very high lethality zoonotic disease anthrax,

which can be acquired in four ways: inhalation (50–80% mortality even with treatment), cutaneous, gastrointestinal, and injection. The pathogenicity of *B. anthracis* depends on two virulent factors encoded by genes located on two plasmids pXO1 (anthrax toxin) and pXO2 (poly- γ -d-glutamic acid capsule) (Brossier and Mock 2001; Gierczyński 2010). Changes in the genome (addition or loss of the pathogenicity) can result from the conjugation (horizontal gene transfer) process, which has been described in *Bacillus* strains (Singh *et al.* 2013). Owing to its spores being very easy to produce, transport, and disperse, *B. anthracis* is potentially useful as a biological warfare agent, classified as a group of dangerous pathogens by the Centers for Disease Control and Prevention (2017).

Numerous methods have been used to perform genetic differentiation of *Bacillus cereus* group strains. Recently, the most popular were: restriction enzyme analysis – pulsed-field gel electrophoresis (REA-PFGE) (Helgason *et al.* 2000b; Tenover *et al.* 1995, 1997), amplified fragment length polymorphism (AFLP) (DeVos *et al.* 1995; Ticknoe *et al.* 2001), 16S and 23S rRNA analysis (Cherif *et al.* 2003; Soufiane and Côté 2013), and multilocus enzyme electrophoresis (MEE) (Helgason *et al.* 2000a; Selander *et al.* 1986). Even though those methods are advantageous, the results are very arduous to standardise and compare among different laboratories. In 1998 Maiden's team developed a novel molecular method based on sequencing internal fragments of the chromosome, enabling genetic characterisation of multiple bacterial strains in one experiment (Maiden *et al.* 1998).

Multilocus sequence typing is based on the analysis of nucleotide sequences of highly conserved fragments of a few housekeeping genes. Since 1998, the

MLST scheme was developed for several human bacterial pathogens including: *Clostridium botulinum* (Olsen *et al.* 2014), *Clostridium difficile* (Zhou Y. *et al.* 2014), *Corynebacterium diphtheriae* (Bolt *et al.* 2010), *Vibrio cholera* (Zhou H. *et al.* 2014), *Yersinia sp.* (Duan *et al.* 2014; Souza *et al.* 2013), *Bordetella sp.* (Diavatopoulos *et al.* 2005), *Haemophilus influenzae* (Skaare *et al.* 2014), and *Streptococcus pneumoniae* (Chang *et al.* 2014).

In this study, the MLST scheme for genetic differentiation and characterization of *Bacillus cereus* group strains from the internal collection of the Biological Threats Identification and Countermeasure Centre of Military Institute of Hygiene and Epidemiology in Pulawy (BTICC MIHE) was performed.

Materials and methods

A total of 65 bacterial strains were analysed. Sixty of them were from the *Bacillus cereus* group strains, and five strains of *B. subtilis* and *B. megaterium* were used as negative control samples. All of them were part of an internal collection of the BTICC MIHE (obtained from the Institute of Medicine and Molecular Biology (IMBM) at the University of Scranton, Pennsylvania, USA; Veterinary Inspection, Łomża, Poland, Medical University of Lublin, Poland, and from the environment isolated by BTICC specialists) and have been identified before by various methods (microscopy, biochemical, immunological, PCR, real-time PCR, etc.) (Ciešlik *et al.* 2015). The strains were isolated from animals, people, and the worldwide environment (Germany, China, France, USA). Tested groups of bacterial strains included 10 strains of *B. cereus*, 17 *B. thuringiensis*, seven *B. anthracis*, and 26 transitional environmental *Bacillus sp.* strains. Transitional strains gave positive results in the PCR

reaction for *B. anthracis* chromosomal marker *Ba 813+*. The analysed strains are shown in Table 1.

This study includes selected fragments of seven housekeeping genes. The following genes were chosen: *glpF* (encoding glycerol uptake facilitator protein), *gmk* (guanylate kinase), *ilvD* (dihydroxy-acid dehydratase), *pta* (phosphate acetyltransferase), *pur* (AICAR transformylase), *pycA* (pyruvate carboxylase), and *tpi* (triosephosphate isomerase) (Priest *et al.* 2004). The primers used for amplification and sequencing of these fragments are shown in Table 2.

The primers were synthesised by Genomed JSC Poland, based on the sequence from the PubMLST Database (primers used for MLST of *Bacillus* genes) (PubMLST 2018).

The genetic material was isolated from fresh bacterial culture using the automatic isolation station QIAcube® (QiaGene Corp.®) using the QIAamp DNA Mini Kit (QiaGene Corp.®), according to the manufacturer's instructions. Fresh cultures of tested strains were prepared on Columbia Agar plates containing 5% sheep blood (Graso Biotech, Poland), incubated at 37°C for 24 hours under aerobic conditions. After incubation, the selected colonies (after morphological and microscopy evaluation) were transferred to the buffer from the isolation kit.

The amplification reactions were performed in a volume of 50 µl for 45 cycles. Reaction mixtures consisted of 5 µl of dNTP mix (starting concentration 2 mM each dNTP), 1 µl of each primer (starting concentration 10 mM), 5 µl of 10x PfuUltra II reaction buffer, 1 µl of PfuUltra II fusion HS DNA polymerase (Agilent Inc.®), and 50 ng of genomic DNA. The thermal profile comprised of the following steps: initial denaturation at 94°C for 1 min, denaturation at 94°C for

Table 1. The analysed strains of *Bacillus* sp.

No.	Species	Strain
1.	<i>B. cereus</i>	ATCC 10876
2.	<i>B. cereus</i>	ATCC 13472
3.	<i>B. cereus</i>	ATCC 19637
4.	<i>B. cereus</i>	ATCC 23261
5.	<i>B. cereus</i>	F 17202
6.	<i>B. cereus</i>	F 17285
7.	<i>B. cereus</i>	T 7-101
8.	<i>B. cereus</i>	UM1
9.	<i>B. cereus</i>	UM2
10.	<i>B. cereus</i>	UW 85
11.	<i>B. thuringiensis</i>	ATCC 10792T
12.	<i>B. thuringiensis</i>	ATCC 33679
13.	<i>B. thuringiensis</i>	T07-128
14.	<i>B. thuringiensis</i>	H36
15.	<i>B. thuringiensis</i>	T 07-001
16.	<i>B. thuringiensis</i>	T 07-005
17.	<i>B. thuringiensis</i>	T 07-019
18.	<i>B. thuringiensis</i>	T 07-113
19.	<i>B. thuringiensis</i>	T 07-146
20.	<i>B. thuringiensis</i>	T 07-148
21.	<i>B. thuringiensis</i>	T 07-153
22.	<i>B. thuringiensis</i>	T 7-030
23.	<i>B. thuringiensis</i>	T 7-055
24.	<i>B. thuringiensis</i>	T 7-101
25.	<i>B. thuringiensis</i>	T 7-128
26.	<i>B. thuringiensis</i>	T07-151
27.	<i>B. thuringiensis</i>	T07-155
28.	<i>B. anthracis</i>	1153
29.	<i>B. anthracis</i>	1582
30.	<i>B. anthracis</i>	1583
31.	<i>B. anthracis</i>	1584
32.	<i>B. anthracis</i>	211
33.	<i>B. anthracis</i>	211Y

No.	Species	Strain
34.	<i>B. anthracis</i>	34F2
35.	<i>B. sp. Ba 813+</i>	#11 (9594(3))
36.	<i>B. sp. Ba 813+</i>	#12/S8553(2)
37.	<i>B. sp. Ba 813+</i>	#13 (PC1)
38.	<i>B. sp. Ba 813+</i>	#14
39.	<i>B. sp. Ba 813+</i>	#15 (11614-2)
40.	<i>B. sp. Ba 813+</i>	#16 (PJ572)
41.	<i>B. sp. Ba 813+</i>	#17 (094)
42.	<i>B. sp. Ba 813+</i>	#19 (T579-77)
43.	<i>B. sp. Ba 813+</i>	#21 (TH97-3)
44.	<i>B. sp. Ba 813+</i>	#22
45.	<i>B. sp. Ba 813+</i>	#22 (Br-13)
46.	<i>B. sp. Ba 813+</i>	#23 (III-4)
47.	<i>B. sp. Ba 813+</i>	#24 (II/B5)
48.	<i>B. sp. Ba 813+</i>	#25 (97-27)
49.	<i>B. sp. Ba 813+</i>	#28 (II)
50.	<i>B. sp. Ba 813+</i>	#28 (III)
51.	<i>B. sp. Ba 813+</i>	#29 (IV)
52.	<i>B. sp. Ba 813+</i>	#30 (1B)
53.	<i>B. sp. Ba 813+</i>	#31
54.	<i>B. sp. Ba 813+</i>	#25 (97-24)
55.	<i>B. sp. Ba 813+</i>	#6
56.	<i>B. sp. Ba 813+</i>	#S8553/2
57.	<i>B. sp. Ba 813+</i>	#7 (II/3)
58.	<i>B. sp. Ba 813+</i>	C9A
59.	<i>B. sp. Ba 813+</i>	Z 5 (1) Miazga
60.	<i>B. sp. Ba 813+</i>	ZL 2 B
61.	<i>B. subtilis</i>	ATCC 6633
62.	<i>B. subtilis</i>	UM1
63.	<i>B. subtilis</i>	UM2
64.	<i>B. megaterium</i>	UM1
65.	<i>B. megaterium</i>	UM2

Table 2. The primers used for amplification and sequencing chosen genes

<i>glpF</i> :	F - 5'GCGTTTGTGCTGGTGTAAGT3',	R - 5'CTGCAATCGGAAGGAAGAAG3';
<i>gmk</i> :	F - 5'ATTTAAGTGAGGAAGGGTAGG3',	R - 5'GCAATGTTCCACCAACCACAA3';
<i>ilvD</i> :	F - 5'CGGGGCAAACATTAAGAGAA3',	R - 5'GGTTCTGGTCGTTTCCATTC3';
<i>pta</i> :	F - 5'GCAGAGCGTTTAGCAAAAAGAA3',	R - 5'TGCAATGCGAGTTGCTTCTA3';
<i>pur</i> :	F - 5'CTGCTGCGAAAAATCACAAA3',	R - 5'CTCACGATTGCTGCAATAA3';
<i>pycA</i> :	F - 5'GCGTTAGGTGGAAACGAAAAG3',	R - 5'CGCGTCCAAGTTTATGGAAT3';
<i>tpi</i> :	F - 5'GCCAGTAGCACTTAGCGAC3';	R - 5'CCGAAACCGTCAAGAATGAT3'.

20 sec, annealing for 30 sec at 60°C, and elongation for 20 sec at 72°C. After 45 cycles, the reaction was completed by the final elongation at 72°C for 5 min. PCR products were prepared for sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit and BigDye® XTerminator™ Purification Kit (Applied Biosystems™). DNA sequencing reac-

tions were finalised using an ABI Prism® Genetic Analyzer 3100 sequencer (Applied Biosystems™).

Chromatograms and sequences were exported to BioNumerics Software (Applied Maths, BioMérieux Company) where they were edited, aligned, and compared with the *Bacillus cereus* group MLST profiles database [35] using the

recommended tools. Sequences of every fragment of the analysed gene were edited to a suitable length (*glpF* 381 bp, *gmk* 504 bp, *ilvD* 393 bp, *pta* 414 bp, *pur* 348 bp, *pycA* 363 bp, and *tpi* 435 bp). The allelic profile was arbitrarily assigned for all different sequences, and all identical sequences got the same value. Based on the characteristic profiles of the combination of seven loci, each strain was described by multilocus sequence type (MLST). The dendrograms, showing the genetic relationship between the *Bacillus cereus* group strains, are based on separate allelic profiles and were constructed using the unweighted pair group method with arithmetic mean (UPGMA).

Results

The length of the analysed fragments ranged between 348 bp (*pur*) and 504 bp (*gmk*). The average amount of GC bases in the analysed genes fragments ranged from 38.15% to 44.71%.

As an effect of the analysis obtained by the BioNumerics software, 22 different STs profiles were observed, which had been previously identified in the pubmlst.org database (Table 1). Only once 10 STs (ST1, ST10, ST26, ST34, ST53, ST105, ST206, ST245, ST665, ST972) were identified, 13 strains were recognised to possess STs profile 15 (12 strains of *B. thuringiensis* and one of *B. cereus*), nine strains of transitional environmental strains of *Bacillus sp. Ba 813+* possess STs profile 109. All of the analysed full pathogenic *B. anthracis* strains have the same STs profile (ST3). However, the vaccination strain *B. anthracis 34F2* had a different one (ST1). The difference between them resides only in the *glpF* gene sequence. Furthermore, some of the transitional environmental strains of *Bacillus sp. Ba 813+* and *B. anthracis* have the same

allelic types of sequences for *gmk* and *pta*.

After UPGMA clustering, four major groups were revealed (Figure 1). Cluster I is the most interesting because it includes all analysed *B. anthracis* isolates, both fully pathogenic, and vaccination strains (ST 3 and ST 1) and 13 transitional environmental *B. sp. Ba813+* strains (nine with ST109, two with ST163, and two with ST113). All the environmental strains had the same *gmk* and *pta* (excluding ST113 and ST163) genes like the analysed *B. anthracis* strains. Cluster II and III contains a mixture of *B. cereus*, *B. thuringiensis*, and transitional environmental strains, gained from reference collections and isolated from the environment. Cluster III is very interesting because it includes the *B. thuringiensis* strain from the ATCC collection (ST10), two *B. cereus* strains isolated from humans (ST56), and two environmental strains isolated from tooth pulp of dead horses (ST144). In Cluster IV, the one strain of *B. cereus* from the ATCC collection and eight isolates from the environment of *B. sp. Ba813+* were grouped.

Discussion

For many years, standard microbiology was the only bacterial identification method and bacteriological diagnostic in medicine. Biological tests with laboratory animals are still recommended by the World Health Organization as a reference method in diagnosis of anthrax (Turnbull 2008). The identification of *B. anthracis*, based on phenotypic features of strains isolated from material derived from the anthrax regarding affirmed cases, never caused a problem. However, after the 1960s, doubtful cases of anthrax diagnosis were described. In those cases, isolated strains from wounds considered anthrax related,

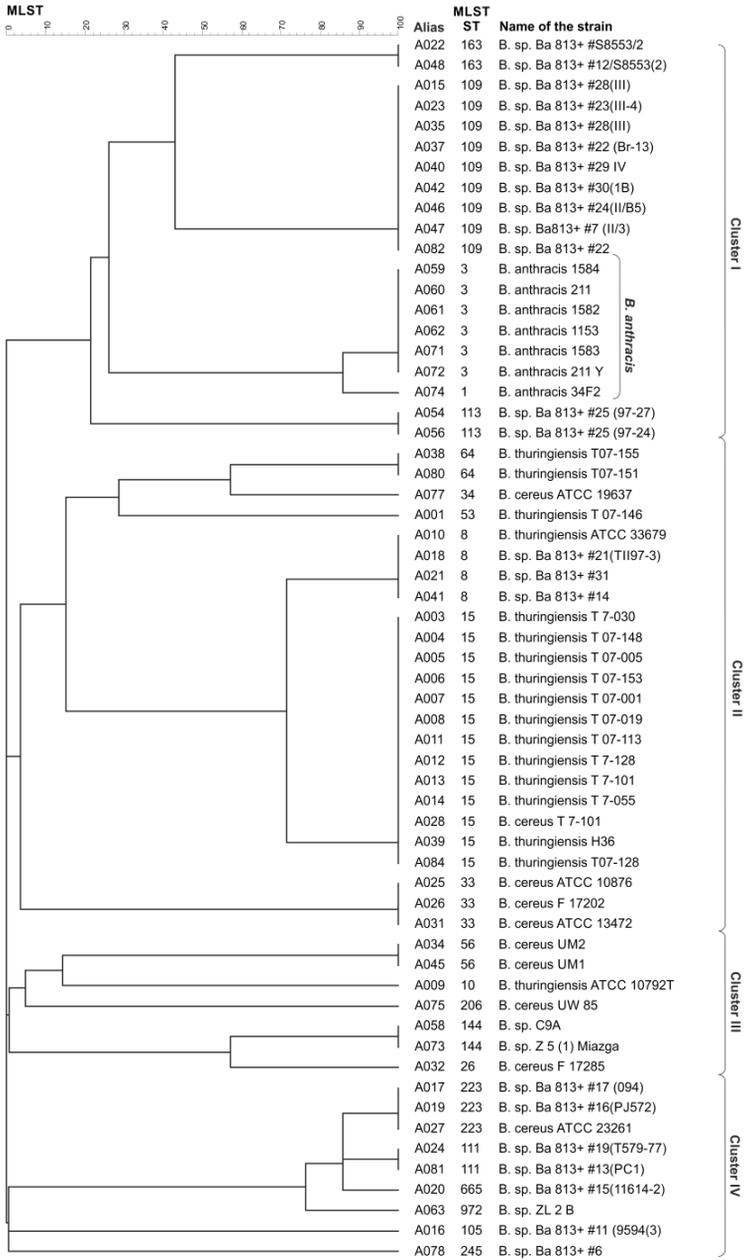


Figure 1. Phylogenetic differentiation of Bacillus cereus group strains clustered by UPGMA method

were identified as *B. cereus*. Those cases were interpreted as an environmental contamination, as *B. cereus* is present in the natural environment (Turnbull 1999).

Owing to performed genetic studies, this hypothesis has been refuted.

The greater phenotypic identification problem is related to strains isolated from

the environment, which presents larger biochemical differentiation. Predisposing factors may include a long period of remaining in the spore form and a low protein content in the environment. The occurrence of the cell capsule, which is the characteristic structural element of *B. anthracis*, depended on the origin of the strains, was also observed. Strains without capsules were isolated from the environment more often than from living organisms (Turnbull 2008).

In relation to the advanced development of molecular biology techniques, phenotypic methods are increasingly being replaced by modern genetic tools. They allow for a rapid and sure analysis and reduce the risk of mistakes in the data interpretation. Furthermore, advanced bioinformatics programmes (e.g., BioNumerics®) are very useful in interpreting sequencing data from an experiment with a vast group of microorganisms.

In this paper, an MLST experiment based on seven conservative housekeeping genes (*glpF*, *gmk*, *ilvD*, *pta*, *pur*, *pycA* and *tpi*) was performed. Molecular typing and phylogenetic investigation concerned 60 *Bacillus cereus* group strains, including *B. cereus*, *B. thuringiensis*, *B. anthracis*, and transitional environmental *Bacillus spp.* strains. This study aimed to identify the exact relationships among the various strains belonging to the common group of *Bacillus cereus*, including *B. anthracis*. Close relationships among these strains carry the large risk of their modification from the strains originally nonpathogenic form into a virulent form. In 2003, Radnegde et al. analysed the genetic homology between *B. anthracis*, *B. cereus*, and *B. thuringiensis* strains isolated from several diverse places (environment, infected people and animals, and contaminated food) using amplified fragment length polymorphism

(Radnedge et al. 2003). This study, employing the MLST technique, confirmed the large genetic homology of the strains comprehended by tests, which also characterised the various places of origin. Tests also showed very large genetic homology among *B. anthracis*, *B. cereus*, and *B. thuringiensis*. Very similar effects were obtained by Helgason's group in their studies on the different housekeeping genes (*adh*, *ccpA*, *ftsA*, *glpT*, *pyrE*, *recF*, and *sucC*) (Helgason et al. 2004).

Cluster I is very interesting from the epidemiological point due to its consistency in including all analysed *B. anthracis* strains and transitional strains isolated from the environment. The fact that all *B. anthracis* strains were grouped in one cluster has confirmed a very high level of genetic monomorphism. All of the full pathogenic strains showed the same sequence of analysed gene fragments and as a result, the same sequencing type. The vaccination strain *B. anthracis* 34F2 varied only in the sequence of the fragment of glycerol uptake facilitator protein gene. Unfolded differences caused the other ST profile classification.

Analysis of the individual alleles provided the information concerning full homology in the sequences of the *gmk* and *pta* genes among *B. anthracis*, and some transitional environment *Bacillus sp.* strains. This fact can support the contention that they possess a common ancestor (Patra et al. 1998; Ramisse et al. 1999). Full homology of the sequence of these genes was affirmed by Kim's team (Kim et al. 2005), which provided additional identifying sequences of these genes in some *B. cereus* and *B. thuringiensis* strains. However, similar to our results, Kim's team did not affirm the compatibility of the sequences in all analysed alleles among *B. anthracis* and other representatives of the *Bacillus*

cerus group. The examined group in the study included strains, which gave false-positive results in identifying *B. anthracis*. These results confirmed that MLST is a useful method to differentiate *Bacillus cereus* strains.

Multilocus sequence typing allows us to define the phylogenetic relationship between closely related strains based on the combination of the sequences of all seven alleles fragments and each of them separately. This characteristic is very important in epidemiological investigations on the potential military/bioterrorist use of *B. anthracis*. Using this method, the differentiation of *B. anthracis* from other *B. cereus* group strains after genetic modification, e.g., after cloning pXO1 and pXO2 plasmids, is possible. The MLST method is also very useful when the sequence of virulent genes (localised on plasmids) in material from people with anthrax symptoms can't be detected using typical molecular techniques (e.g., PCR). The false-negative results of PCR sometimes can be caused by the mutation changes in the target spaces during annealing (Turnbull *et al.* 1992).

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