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ORIGINAL CONTRIBUTION

Determination of the Most Closely Related *Bacillus* Isolates to *Bacillus anthracis* by Multilocus Sequence Typing

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There have been many efforts to develop *Bacillus anthracis* detection assays, but the problem of false-positive results has often been encountered. Therefore, to validate an assay for *B. anthracis* detection, it is critical to examine its specificity with the most closely related *Bacillus* isolates that are available. To define the most closely related *Bacillus* isolates to *B. anthracis* in our *Bacillus* collections, we analyzed by multilocus sequence typing (MLST) the phylogeny of 77 closely related *Bacillus* isolates selected from 264 *Bacillus* isolates. The selection includes all the *Bacillus* isolates that have been shown in our previous studies to produce false-positive results by some anthrax-detection assays. The MLST phylogenetic analyses revealed that 27 of the non-*B. anthracis* isolates clustered within the *B. anthracis* clade, and four of them (three sequence types, STs) had the highest degree of genetic relatedness with *B. anthracis,* 18 (11 STs) had the second highest, and five (five STs) had the third highest. We anticipate that the inclusion of the 19 ST isolates when analyzing *B. anthracis* detection assays will prove to be useful for screening for their specificity to detect *B. anthracis*.

INTRODUCTION

Bacillus anthracis is the etiological agent of anthrax, the acute and often fatal infectious disease of herbivores and humans and is a threatening agent used in bioterrorism. It is a member of the *B. cereus* group, which includes other spore-forming soil bacteria commonly isolated in nature, *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. weihenstephanensis* [1] and *B. pseudomycoides* [2]. *B. cereus* causes food-borne gastroenteritis and opportunistic infections in immunocompromised patients [3-6], and other members have been reported to be potentially enteropath-

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†Abbreviations: *glpF*, glycerol uptake facilitator protein; *gmk*, guanylate kinase; *ilvD*, dihydroxy-acid dehydratase; MLST, multilocus sequence typing; *pta*, phosphate acetyltransferase; *pur*, phosphoribosylaminoimidazolecarboxamide; *pycA*, pyruvate carboxylase; *rpoβ*, the β subunit of RNA polymerase; ST, sequence type; *tpi*, triosephosphate isomerase; UPGMA, unweighted pair group method with arithmetic means.

ogenic [7-11]. Amongst this group, *B. cereus* and *B. thuringiensis* are genetically and phenotypically closely related to *B. anthracis,* and the unambiguous identification of these organisms has been difficult [12]. However, the unambiguous and rapid diagnostic detection of *B. anthracis* is important for early treatment and control of anthrax due to its highly pathogenic nature to animals and humans. Many methods have been developed to detect *B. anthracis*, but these methods have revealed some undesirable problems. The early reported PCR-based methods [13-17], which target only virulence genes located on the plasmids of *B. anthracis*, can readily differentiate vaccine or fully virulent *B. anthracis* plasmid genotypes. But when using these methods, plasmid-cured *B. anthracis* [18-21], species containing the transferred plasmids or virulence genes [22-31], or near-neighbor species containing closely-related *B. anthracis* plasmids [32] are very difficult to distinguish from *B. anthracis*. Therefore, PCR-based methods targeting the *B. anthracis* chromosome for *B. anthracis* detection have been developed but suffer from lack of assay specificity; Ba813 [16, 33, 34], *vrrA* gene [35-37], gyrase B gene (*gyrB*) [38], SG-850 [39], the β subunit of RNA polymerase gene (*rpoβ*) [40] and the gyrase A (*gyrA*) gene [41].

When these assays were originally developed, the most closely related *Bacillus* species were not examined, and afterwards the assays were revealed not to be specific by further examination of other closely related *Bacillus* species. In our previous studies on developing highly specific PCR detection methods for *B. anthracis* (manuscript in preparation and [12]), we used 272 isolates of bacteria to verify the specificity of our detection assay. We compared the specificity of our detection assay to the specificity of previously reported *B. anthracis* detection assays, and the results revealed that as many as 42 isolates showed false-positive results when using one of the previous detection assays. This result motivated us to study and define the most closely related *Bacillus* isolates to *B. anthracis*. Once we identify *Bacillus* isolates that are the most closely related to *B. anthracis* and include these isolates first when examining *B. anthracis* detection assays, we can readily screen the specificity of these assays and search for the highly specific ones.

For this purpose, we have determined the most closely related *Bacillus* isolates among 77 isolates selected from 264 *Bacillus* isolates, many of which have shown false-positive results by the previously reported assays, by analyzing their phylogenetic relatedness to *B. anthracis* using multilocus sequence typing (MLST)† with seven house keeping genes.

MATERIALS AND METHODS

Bacterial strains and DNA isolation

A total of 77 strains used in this study are shown in Table 1. The strains, except for *B. anthracis*, were acquired from the American Type Culture collection (Manassas, Virginia), *Bacillus* Genetic Stock Center (Department of Biochemistry, The Ohio State University, Columbus, Ohio), Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), Korean Collection for Type Cultures (Dajeon, Korea) and Korean Culture Center of Microorganisms (Seoul, Korea), and Dr. Terrance Leighton (Children's Hospital Oakland Research Institute, Oakland, California). *B. anthracis* genomic DNA preparations were a gift from Chung-Ang University Medical College. Genomic DNA from other strains was isolated using the Easy-DNA kit (Invitrogen Corporation, Carlsbad, California) according to the manufacturer's protocol. DNA concentrations were determined spectrophotometrically [42].

Table 1. Bacterial strains used in this study.

Species	Strain ID	Source[a]	*Species*	Strain ID	Source[a]
B. anthracis	14578^T	ATCC	*B. cereus*	6E1	BGSC
B. anthracis	CAU-1	Korea	*B. cereus*	6E2	BGSC
B. anthracis	CAU-2	Korea	*B. cereus*	1012	KCTC
B. anthracis	CAU-3	Korea	*B. cereus*	1014	KCTC
B. anthracis	CN1	Korea	*B. cereus*	1094	KCTC
B. anthracis	CN2	Korea	*B. cereus*	1661	KCTC
B. anthracis	14185	ATCC	*B. cereus*	3062	KCTC
B. anthracis	14186	ATCC	*B. cereus*	3624	KCTC
B. anthracis	Sterne 34-F2	NVRQS	*B. mycoides*	10206	ATCC
B. anthracis	BC	China	*B. mycoides*	19647	ATCC
B. anthracis	Pasteur #2	NVRQS	*B. mycoides*	21929	ATCC
B. thuringiensis	4AB1	BGSC	*B. mycoides*	23258	ATCC
B. thuringiensis	4AF1	BGSC	*B. mycoides*	6462^T	ATCC
B. thuringiensis	4AJ1	BGSC	*B. mycoides*	40260	ATCC
B. thuringiensis	4AN1	BGSC	*B. mycoides*	3453	ATCC
B. thuringiensis	4AQ1	BGSC	*B. pseudomycoides*	12442^T	DSM
B. thuringiensis	4AS1	BGSC	*B. weihenstephanensis*	11821^T	DSM
B. thuringiensis	4AW1	BGSC	*Bacillus sp.*	IB	Rogers J.E.
B. thuringiensis	4AX1	BGSC	*Bacillus sp.*	2A6	Pasteur Institute
B. thuringiensis	4AY1	BGSC	*Bacillus sp.*	2C1	Pasteur Institute
B. thuringiensis	4BA1	BGSC	*Bacillus sp.*	003	Rogers J.E.
B. thuringiensis	4BD1	BGSC	*Bacillus sp.*	3466-8.1	Pasteur Institute
B. thuringiensis	4BG1	BGSC	*Bacillus sp.*	9594/3	Patra G.
B. thuringiensis	4BN1	BGSC	*Bacillus sp.*	DM55	El-Helow E.
B. thuringiensis	4BY1	BGSC	*Bacillus sp.*	S8553/2	Pasteur Institute
B. thuringiensis	4CB1	BGSC	*Bacillus sp.*	I2	Patra G.
B. thuringiensis	4CC1	BGSC	*Bacillus sp.*	IV	Rogers J.E.
B. thuringiensis	4CD1	BGSC	*Bacillus sp.*	III	Rogers J.E.
B. thuringiensis	4U1	BGSC	*Bacillus sp.*	III BL	Ramisse V.
B. thuringiensis	4Y1	BGSC	*Bacillus sp.*	III BS	Ramisse V.
B. thuringiensis	2046^T	DSM	*Bacillus sp.*	Peyr 6	Pasteur Institute
B. thuringiensis	1034	KCTC	*Bacillus sp.*	Peyr 8	Pasteur Institute
B. thuringiensis	1507	KCTC	*Bacillus sp.*	Peyr 9	Pasteur Institute
B. thuringiensis	1509	KCTC	*Bacillus sp.*	PT030101-01	Dean D.
B. thuringiensis	3452	KCTC	*Bacillus sp.*	PT030101-02	Dean D.
B. thuringiensis	97-27	Hernandez E.	*Bacillus sp.*	Rho	Korea
B. cereus	14579^T	ATCC	*Bacillus sp.*	S10A	Korea
B. cereus	6A7	BGSC	*Bacillus sp.*	11R-1	Korea
B. cereus	6A8	BGSC			

[a]ATCC, American Type Culture Collection, Manassas, Virginia; NVRQS, National Veterinary Research and Quarantine Service, Anyang-si, Kyeonggi-do, South Korea; BGSC, Bacillus Genetic Stock Center, Department of Biochemistry, The Ohio State University, Columbus, Ohio; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

Table 2. Genetic diversity at the seven loci examined in this study.

Locus	Fragment length (bp)[a]	Average G + C (%)	No. of alleles	Conserved nucleotide site (%)	Variable nucleotide site (%)	Average dN/dS[b]
glpF[c]	381 (549)	38.82%	35	314/381 (82.4)	67/381 (17.6)	0.0654
gmk	504 (600)	38.10%	17	409/504 (81.2)	95/504 (18.8)	0.0222
ilvD	393 (556)	45.20%	35	287/393 (73)	106/393 (27)	0.0152
pta	414 (579)	40.54%	32	352/414 (85)	62/414 (23.3)	0.0107
pur	348 (536)	38.52%	29	267/348 (76.7)	81/348 (24.2)	0.0124
pycA	363 (550)	40.38%	28	275/363 (75.8)	88/363 (24.2)	0.0268
tpi	435 (558)	44.01%	29	395/435 (90.8)	40/435 (9.2)	0.0791

[a]The numbers in parentheses are total lengths of PCR products.
[b]dN/dS, pairwise ratios of non-synonymous substitutions to synonymous substitutions.
[c]Abberviations: *glpF*, glycerol uptake facilitator protein; *gmk*, guanylate kinase; *ilvD*, dihydroxy-acid dehydratase; *pta*, phosphate acetyltransferase; *pur*, phosphoribosylaminoimidazolecarboxamide; *pycA*, pyruvate carboxylase; *tpi*, triosephosphate isomerase.

MLST and data analysis

We amplified fragments of seven housekeeping genes (*glpF*, *gmk*, *ilvD*, *pta*, *pur*, *pycA,* and *tpi*) by PCR as described on the website for MLST of *B. cereus* (www.pubmlst.org/bcereus) using the standard primers. The PCR fragments were sequenced in both directions using the amplification primers to provide unambiguous sequence data.

The allele sequences were compared with existing allele sequences on the *B. cereus* MLST website using Sequence Comparator software (www.pubmlst.org/ bcereus) [43] and given new allele numbers if they differed from known alleles. The seven allele numbers define a sequence type (ST) a number of which was assigned to each new allele combination. To improve the discrimination between divergent lineages, analyses were conducted after each gene was split into three segments as described by Helgason et al [44]. Allele numbers were assigned independently for each gene region, and the allelic profiles were then combinations of 21 numbers. To assess the genetic relatedness among the isolates, a dendrogram based on the allelic profiles was constructed by using the unweighted pair group method with arithmetic means (UPGMA) applied to a matrix of pairwise distances, defined as the percentages of allelic differences between profiles, by means of the MEGA 2.1 analysis package [45]. In addition, the concatenated sequences for the seven gene fragments were used for building neighbor-joining trees using the MEGA software. The two phylogenentic dendrograms were compared to determine the most closely related isolates to *B. anthracis*.

Standard MLST statistics such as number of polymorphic sites and allele frequencies were computed by using the START v1.0.5 (www.pubmlst.org/ bcereus) [43].

RESULTS

Allelic profiles and sequence types

The lengths of the fragments analyzed ranged from 348 bp (*pur*) to 504 bp (*gmk*). The sequences of the seven gene fragments amplified from the isolates had variable sites from 9.2 percent (*tpi*) to 27 percent (*ilvD*), which resulted in 17 to 35 distinct alleles for the different loci (Table 2). The average number of alleles per locus was 29.3. No insertions or deletions were observed in any of the sequences. The average dN/dS values ranged from 0.0107 (*pta*) to 0.0791 (*tpi*), indicating that most

of the sequence variability identified is selectively neutral.

Altogether, 48 unique STs were observed in the 77 isolates (Table 3), and 32 of the STs (67 percent) were identified only once. Three STs were observed in *B. anthracis* isolates, and ST 1 was the most frequent type of *B. anthracis*. Interestingly, even though *B. anthracis* isolates did not have the same ST as any other *B. cereus* group isolates, some *B. cereus* group strains shared one or more alleles with *B. anthracis*. *B. thuringiensis* BGSC 4AJ1 had four alleles (*gmk*, *pta*, *pur,* and *tpi*) that were the same as *B. anthracis*; *B. thuringiensis* BGSC 4AB1 and 4BA1 had three same alleles (*gmk*, *pta*, and *pur*); *B. thuringiensis* strains BGSC 4AY1, 4CC1, *Bacillus* species 1B, III, 003, III BL, III BS, IV, DM55, and PT030101-01 had two same alleles (*gmk* and *pta*); and *B. thuringiensis* strains BGSC 4BY1, 4CB1, 4AS1, 97-27, *B. cereus* strains BGSC 6E1, 6E2, and KCTC 1661 had the same *gmk* allele.

Phylogenetic relatedness

To estimate the degree of genetic relatedness of the closely related *Bacillus* isolates to *B. anthracis*, we constructed two dendrograms using the improved MLST methods; one of them was built in the similar way to the standard MLST but instead used split profiles of the gene fragments (Table 4) and the other one was done by the neighbor-joining method using the concatenated sequences for the seven gene fragments. Clustering of the *Bacillus* isolates in both dendrograms revealed three major clades (Figures 1 and 2); within clade I, *B. anthracis* clustered with many of the *B. cereus* and *B. thuringiensis* isolates; within clade II, *B. cereus* and *B. thuringiensis*, unidentified *Bacillus* isolates, and one isolate of *B. mycoides* clustered together; and within clade III, the former dendrogram (Figure 1) showed that many of the *B. mycoides* isolates clustered with *B. thuringiensis* and *B. cereus* isolates but the latter one showed with more reliability that all the *B. mycoides* isolates except one clustered within clade III, separately from the *B. cereus* and *B. thuringiensis* isolates (Figure 2). However, the *Bacillus* isolates clustering with *Bacillus anthracis* within the clade I were grouped in a very similar way in both dendrograms, but in the latter dendrogram, clade I had six more isolates than the clade I of the former one; these isolates clustered within clade II separately from the clade I of the former one. Twenty-seven non-*B. anthracis* isolates clustered with *B. anthracis* in clade I of both dendrograms: *B. thuringiensis* BGSC strains 4AJ1, 4AB1, 4BA1, 4CC1, 4AS1, 4AY1, 4BY1, 4CB1, 4CD1, 4Y1, 4BG1, *B. thuringiensis* 97-27, *B. thuringiensis* KCTC 1014, *B. cereus* BGSC strains 6E1 and 6E2, *B. cereus* KCTC 1661, *Bacillus* isolates DM55, III, III BL, III BS, IV, 003, 1B, S8553/2, PT030101-01, PT030101-02, 9594/3. All of these *B. cereus* group isolates showed false-positive results in one or more of the previously reported assays used for *B. anthracis* detection in our previous studies (manuscript in preparation and [12]). Among these, 4AJ1, 4AB1, 4BA1, and 4CC1 were proximate to *B. anthracis* in both dendrograms. 4AJ1 and 4CC1 showed false-positives in two to three assays used for *B. anthracis* detection in our previous studies. The six isolates additionally clustered in clade I of the second dendrogram include three *Bacillus* isolates Rho, S10A, and 11R-1, which were isolated from the patients and soils of an unidentified epidemic disease in Korea and were once the isolates of controversy due to their connection with anthrax.

DISCUSSION

Of the species in the genus *Bacillus*, *B. anthracis*, *B. cereus*, and *B. thuringiensis* are in the group referred as the *B. cereus* group. These species are genetically very closely related to one another and have con-

Table 3. Allele numbers at the seven loci assigned for entire gene fragments.

No.	Strain	Allele no. at the following loci							
		ST[a]	*glpF*	*gmk*	*ilvD*	*pta*	*pur*	*pycA*	*tpi*
1	*B. anthracis* ATCC 14578	1	1	1	1	1	1	1	1
2	*B. anthracis* CAU-1	1	1	1	1	1	1	1	1
3	*B. anthracis* CAU-2	1	1	1	1	1	1	1	1
4	*B. anthracis* CAU-3	1	1	1	1	1	1	1	1
5	*B. anthracis* Sterne 34-F2	1	1	1	1	1	1	1	1
6	*B. anthracis* BC	1	1	1	1	1	1	1	1
7	*B. anthracis* Pasteur No.2	1	1	1	1	1	1	1	1
8	*B. anthracis* CN1	2	2	1	1	1	1	1	1
9	*B. anthracis* CN2	2	2	1	1	1	1	1	1
10	*B. anthracis* ATCC 14185	3	1	1	2	1	1	2	1
11	*B. anthracis* ATCC 14186	3	1	1	2	1	1	2	1
12	*B. thuringiensis* BGSC 4AJ1	4	57	1	52	1	1	52	1
13	*B. thuringiensis* BGSC 4AB1	5	57	1	56	1	1	54	42
14	*B. thuringiensis* BGSC 4BA1	5	57	1	56	1	1	54	42
15	*B. thuringiensis* BGSC 4CC1	6	61	38	55	1	1	53	19
16	*Bacillus sp.* 1B	7	34	1	32	1	51	37	24
17	*Bacillus sp.* III	7	34	1	32	1	51	37	24
18	*Bacillus sp.* 003	7	34	1	32	1	51	37	24
19	*Bacillus sp.* III BL	7	34	1	32	1	51	37	24
20	*Bacillus sp.* III BS	7	34	1	32	1	51	37	24
21	*Bacillus sp.* IV	7	34	1	32	1	51	37	24
22	*B. thuringiensis* BGSC 4AY1	8	56	1	51	1	50	52	24
23	*Bacillus sp.* DM55	9	44	1	32	1	18	33	24
24	*Bacillus sp.* PT030101-01	10	76	1	66	1	18	52	24
25	*B. cereus* BGSC 6E1	11	38	1	32	16	18	33	24
26	*B. cereus* BGSC 6E2	11	38	1	32	16	18	33	24
27	*B. thuringiensis* BGSC 4BY1	12	70	1	66	60	55	37	51
28	*B. thuringiensis* BGSC 4CB1	12	70	1	66	60	55	37	51
29	*B. thuringiensis* 97-27	13	62	1	57	52	55	37	43
30	*B. thuringiensis* BGSC 4AS1	14	56	1	64	58	18	53	48
31	*B. cereus* KCTC 1661	15	34	1	68	16	18	33	53
32	*B. cereus* BGSC 6A7	16	43	26	35	42	39	41	30
33	*Bacillus sp.* PEYR 9	16	43	26	35	42	39	41	30
34	*B. cereus* BGSC 6A8	16	43	26	35	42	39	41	30
35	*Bacillus sp.* 3466-8.1	16	43	26	35	42	39	41	30
36	*B. mycoides* ATCC 21929	17	73	42	71	64	66	64	55
37	*B. mycoides* ATCC 23258	17	73	42	71	64	66	64	55
38	*B. mycoides* KCCM 40260	17	73	42	71	64	66	64	55
39	*Bacillus sp.* 11R-1	18	78	45	74	67	70	67	58
40	*Bacillus sp.* Rho	18	78	45	74	67	70	67	58
41	*Bacillus sp.* S10A	18	78	45	74	67	70	67	58
42	*B. cereus* ATCC 14579	19	13	8	8	11	11	12	7
43	*B. cereus* KCTC 3624	19	13	8	8	11	11	12	7
44	*B. thuringiensis* DSM 2046	20	15	6	10	8	3	7	14
45	*B. thuringiensis* KCTC 3452	20	15	6	10	8	3	7	14
46	*B. mycoides* ATCC 6462	21	25	10	22	53	57	23	44
47	*B. mycoides* KCTC 3453	21	25	10	22	53	57	23	44
48	*B. thuringiensis* BGSC 4AW1	22	69	40	65	59	62	60	49
49	*B. thuringiensis* BGSC 4AX1	22	69	40	65	59	62	60	49
50	*B. thuringiensis* KCTC 1034	23	33	8	13	19	2	17	17
51	*B. cereus* KCTC 1012	23	33	8	13	19	2	17	17
52	*Bacillus sp.* 2A6	24	43	26	35	42	39	41	50
53	*Bacillus sp.* 2C1	24	43	26	35	42	39	41	50
54	*B. thuringiensis* BGSC 4BG1	25	24	12	50	21	23	31	40
55	*B. thuringiensis* BGSC 4Y1	26	24	12	33	37	48	31	19
56	*Bacillus sp.* 9594/3	27	55	12	33	47	49	31	41
57	*B. pseudomycoides* DSM 12442	2 8	63	13	58	23	25	44	35
58	*B. weihenstephanensis* DSM 11821	29	64	10	9	36	56	22	11
59	*B. thuringiensis* BGSC 4AF1	30	66	39	62	56	60	41	30
60	*B. thuringiensis* BGSC 4AN1	31	67	26	63	57	61	59	30

Table 3. Allele numbers at the seven loci assigned for entire gene fragments (contd).

No.	Strain	Allele no. at the following loci							
		ST[a]	*glpF*	*gmk*	*ilvD*	*pta*	*pur*	*pycA*	*tpi*
61	*B. thuringiensis* BGSC 4AQ1	32	68	39	62	42	60	41	30
62	*B. thuringiensis* BGSC 4BD1	33	14	8	48	45	58	61	7
63	*B. thuringiensis* BGSC 4BN1	34	67	26	35	42	39	41	50
64	*B. thuringiensis* BGSC 4CD1	35	71	22	67	61	63	62	52
65	*B. thuringiensis* BGSC 4U1	36	67	39	62	42	64	41	30
66	*B. thuringiensis* KCTC 1507	37	8	8	16	13	2	16	7
67	*B. thuringiensis* KCTC 1509	38	15	6	7	2	6	8	13
68	*B. cereus* KCTC 1014	39	3	2	31	5	16	3	4
69	*B. cereus* KCTC 1094	40	8	41	14	12	2	25	7
70	*B. cereus* KCTC 3062	41	12	8	9	14	11	12	10
71	*B. mycoides* ATCC 10206	42	72	13	69	62	65	63	54
72	*B. mycoides* ATCC 19647	43	67	26	70	63	39	41	30
73	*Bacillus sp. S8553/2*	44	44	26	32	16	18	33	24
74	*Bacillus sp. I2*	45	74	43	72	65	67	65	32
75	*Bacillus sp. PEYR 6*	46	75	26	35	40	68	41	30
76	*Bacillus sp.* PEYR 8	47	43	26	35	42	39	41	56
77	*Bacillus sp.* PT030101-02	48	77	44	73	66	69	66	57

[a]Abbreviations: ST, sequence type; *glpF*, glycerol uptake facilitator protein; *gmk*, guanylate kinase; *ilvD*, dihydroxy-acid dehydratase; *pta*, phosphate acetyltransferase; *pur*, phosphoribosylaminoimidazolecarboxamide; *pycA*, pyruvate carboxylase; *tpi*, triosephosphate isomerase; Sp., species

stantly provoked a nomenclatural controversy. In fact, a high level of genetic relatedness amongst these species has been demonstrated by whole-genome DNA hybridization [46, 47], 16S and 23S gene rRNA sequence analysis [48, 49], sequence analysis of 16S-23S operons [50, 51], sequence analysis of the *gyrB-gyrA* intergenic spacer region [50], pulsed-field gel electrophoresis [52, 53], and restriction fragmentation pattern analysis of the genome [54]. All of these methods failed to completely discriminate the species of the *B. cereus* group. There is so little difference amongst these species that some investigators have suggested that all these organisms should be classified as a single species [12, 53]. However, the clinical aspects of the diseases caused by these species are distinctively different. *B. anthracis* causes the highly fatal disease in humans and animals, *B. cereus* causes food-poisoning and opportunistic infections, and *B. thuringiensis* is an insect pathogen. Therefore, despite the nomenclatural controversy that is encountered due to the high level of genetic relatedness amongst these species, a specific diagnostic assay to identify *B. anthracis* is clearly required.

There have been many efforts to develop a simple, rapid, and accurate detection assay for *B. anthracis* using advanced molecular biology tools. All the previously developed methods are good at detecting *B. anthracis*, but, however, many of the developed methods have the problem of lack of specificity, which can often lead to false-positive results. If these detection assays are used for the detection of *B. anthracis* in society, the possible occurrence of false-positives can bring about a false alarm of anthrax to the community. The potential consequences resulting from the shock and anxiety of a false-positive result on a community should never be underestimated, particularly under the current threat of increased bioterrorism. Thus, when an assay for *B. anthracis* detection is developed or is being developed, the validation of its specificity is important.

For the specificity analysis, a number of similar, but not *B. anthracis* isolates are required for the test. The more isolates that

Table 4. Allele numbers at the seven loci assigned after gene fragments were split into three equal-length parts.

	Allele no. at the following loci						
ST[a]	*glpF*	*gmk*	*ilvD*	*pta*	*pur*	*pucA*	*tpi*
1	1, 1, 1	1, 1, 1	1, 1, 1	1, 1, 1	1, 1, 1	1, 1, 1	1, 1, 1
2	1, 2, 1	1, 1, 1	1, 1, 1	1, 1, 1	1, 1, 1	1, 1, 1	1, 1, 1
3	1, 1, 1	1, 1, 1	1, 1, 2	1, 1, 1	1, 1, 1	2, 1, 1	1, 1, 1
4	36, 23, 1	1, 1, 1	1, 29, 1	1, 1, 1	1, 1, 1	20, 28, 27	1, 1, 1
5	36, 23, 1	1, 1, 1	33, 28, 23	1, 1, 1	1, 1, 1	35, 29, 29	1, 1, 1
6	39, 9, 1	1, 1, 21	1, 31, 15	1, 1, 1	1, 1, 1	35, 29, 28	3, 4, 6
7	24, 16, 1	1, 1, 1	20, 19, 15	1, 1, 1	32, 26, 1	20, 1, 1	1, 15, 6
8	36, 8, 1	1, 1, 1	31, 28, 23	1, 1, 1	9, 32, 1	20, 28, 27	1, 15, 6
9	31, 16, 1	1, 1, 1	20, 19, 15	1, 1, 1	9, 13, 1	24, 1, 1	1, 15, 6
10	46, 8, 1	1, 1, 1	20, 36, 15	1, 1, 1	9, 13, 1	20, 28, 27	1, 15, 6
11	27, 1, 1	1, 1, 1	20, 19, 15	8, 8, 11	9, 13, 1	24, 1, 1,	1, 15, 6
12	40, 8, 1	1, 1, 1	20, 36, 15	31, 17, 32	9, 26, 1	20, 1, 1	1, 23, 6
13	40, 8, 14	1, 1, 1	34, 32, 23	27, 1, 1	9, 26, 1	20, 1, 1	1, 4, 6
14	36, 8, 1	1, 1, 1	34, 28, 23	1, 1, 31	9, 13, 1	35, 29, 28	2, 4, 14
15	24, 16, 1	1, 1, 1	14, 38, 38	8, 8, 11	9, 13, 1	24, 1, 1	21, 15, 6
16	30, 19, 11	18, 3, 14	23, 21, 21	20, 22, 22	25, 27, 22	27, 24, 21	6, 18, 5
17	44, 30, 2	24, 19, 23	43, 39, 40	33, 28, 36	25, 37, 34	43, 33, 34	22, 8, 5
18	48, 1, 4	26, 21, 25	46, 42, 43	34, 31, 37	42, 40, 26	46, 35, 35	2, 25, 6
19	10, 1, 2	7, 3, 3	7, 6, 8	5, 1, 8	1, 7, 6	8, 10, 6	2, 4, 3
20	12, 1, 2	5, 4, 3	8, 8, 9	5, 4, 7	1, 3, 3	5, 5, 3	8, 10, 3
21	18, 10, 4	8, 6, 6	4, 3, 17	28, 7, 10	12, 25, 13	15, 7, 13	5, 21, 5
22	43, 1, 4	23, 3, 18	39, 35, 36	11, 9, 15	27, 36, 32	13, 32, 11	20, 4, 15
23	11, 1, 9	7, 3, 3	11, 8, 11	9, 1, 7	1, 2, 2	11, 13, 8	9, 4, 4
24	30, 19, 11	18, 3, 14	23, 21, 21	20, 22, 22	25, 27, 22	27, 24, 21	6, 22, 5
25	4, 9, 7	2, 7, 7	21, 27, 31	5, 1, 14	14, 17, 12	22, 18, 16	3, 17, 6
26	4, 9, 7	2, 7, 7	21, 20, 20	5, 19, 20	30, 17, 12	22, 18, 16	3, 4, 6
27	4, 9, 1	2, 7, 7	21, 20, 20	24, 19, 20	31, 14, 12	22, 18, 16	19, 4, 1
28	41, 24, 15	10, 8, 8	35, 33, 33	12, 11, 16	15, 18, 15	30, 17, 15	16, 20, 10
29	18, 7, 4	8, 6, 6	7, 7, 8	17, 10, 10	35, 12, 29	14, 15, 13	5, 7, 5
30	30, 25, 11	18, 18, 14	23, 21, 35	30, 1, 29	38, 27, 30	27, 24, 21	6, 18, 5
31	30, 26, 11	18, 3, 14	38, 21, 21	20, 22, 30	25, 27, 31	27, 24, 33	6, 18, 5
32	42, 27, 11	18, 18, 14	23, 21, 35	20, 22, 22	38, 27, 30	27, 24, 21	6, 18, 5
33	11, 1, 2	7, 3, 3	30, 26, 29	5, 24, 25	36, 14, 3	40, 27, 26	2, 4, 3
34	30, 26, 11	18, 3, 14	23, 21, 21	20, 22, 22	25, 27, 22	27, 24, 21	6, 22, 5
35	4, 28, 2	2, 7, 1	40, 37, 37	5, 1, 33	7, 6, 33	41, 21, 20	3, 24, 5
36	30, 26, 11	18, 18, 14	23, 21, 35	20, 22, 22	25, 27, 30	27, 24, 21	6, 18, 5
37	6, 1, 2	7, 3, 3	12, 12, 13	6, 6, 8	1, 2, 2	8, 9, 9	2, 4, 3
38	12, 1, 2	5, 4, 3	6, 5, 7	2, 2, 2	3, 5, 5	5, 6, 3	7, 9, 5
39	2, 1, 1	2, 2, 1	19, 14, 15	4, 2, 5	7, 11, 10	3, 2, 2	2, 2, 1
40	6, 1, 2	7, 3, 22	12, 8, 12	5, 5, 8	1, 2, 2	17, 9, 8	2, 4, 3
41	9, 1, 2	7, 3, 3	7, 7, 8	4, 1, 6	1, 7, 6	8, 10, 6	2, 4, 4
42	41, 29, 16	10, 8, 8	41, 33, 39	32, 27, 34	39, 18, 15	42, 17, 15	11, 20, 10
43	30, 26, 11	18, 3, 14	42, 21, 21	20, 1, 35	25, 27, 22	27, 24, 21	6, 18, 5
44	31, 16, 1	18, 3, 14	20, 19, 15	8, 8, 11	9, 13, 1	24, 1, 1	1, 15, 6
45	45, 31, 17	25, 20, 18	44, 40, 41	20, 29, 27	40, 38, 35	44, 32, 11	13, 4, 5
46	30, 32, 11	18, 3, 14	23, 21, 21	20, 21, 22	41, 27, 36	27, 24, 21	6, 18, 5
47	30, 19, 11	18, 3, 14	23, 21, 21	20, 22, 22	25, 27, 22	27, 24, 21	23, 18, 5
48	47, 9, 1	1, 1, 24	45, 41, 42	19, 30, 32	9, 39, 1	45, 34, 1	2, 4, 6

[a]Abbreviations: ST, sequence type; *glpF*, glycerol uptake facilitator protein; *gmk*, guanylate kinase; *ilvD*, dihydroxy-acid dehydratase; *pta*, phosphate acetyltransferase; *pur*, phosphorribosylaminoimidazolecarboxamide; *pycA*, pyruvate carboxylase; *tpi*, triosephosphate isomerase; Sp, species.

are tested, the more reliable the test will be. However, the test isolates should be closely related to *B. anthracis* because false-positive results often occur in tests with bacteria that are similar to *B. anthracis*. The selection of a limited number of the most closely related isolates to *B. anthracis* will be useful and economical, especially when time, labor and materials are limited. In addition, the assays showing low specificity will be detected early and discarded, avoiding the possible wasting of time and resources.

To select these isolates among a number of *Bacillus* isolates, their genetic relatedness to *B. anthracis* is usually investigated by analysis of the genetic diversity amongst them. For this purpose, various methods including multilocus enzyme electrophoresis, pulsed-field gel electrophoresis, and amplified fragment length polymorphism have been used. However, these methods are difficult to standardize between laboratories, and it is difficult to compare the results for large strain collections. MLST has been developed and has been used successfully when exploring the population structure of many important bacterial pathogens [53, 55-66]. This method is based on sequencing a number of essential or housekeeping genes spread around the bacterial chromosome and is a method that is unambiguous and truly portable among laboratories [67]. However, the standard MLST method, which is based on the allelic profiles of seven gene fragments, was known to have low resolving power when constructing a dendrogram [53]. To improve the resolution, MLST based on split allele profiles or the concatenated sequences for the seven gene fragments was attempted, and the resulting dendrograms are highly resolved [53, 68].

In this study, we applied the standard MLST as well as two other improved MLST methods to construct phylogenetic dendrograms. The dendrogram constructed by the standard MLST was lowly resolved (data not presented). The other two improved MLST methods provided dendrograms that were highly resolved to the similar degree, but the MLST using the concatenated sequences appeared better and phylogenetically more reliable than the gene split MLST because it showed good clustering of *B. mycoides* isolates out of the *B. thuringiensis* and *B. cereus* cluster. The dendrograms constructed by these two methods revealed three major clades among the *Bacillus* isolates tested. The composition of the *Bacillus* isolates clustering in clade I of both dendrograms, within which all the *B. anthracis* isolates clustered, were almost similar except for the fact that the dendrogram of the second method showed that clade I had six more isolates than the clade I of the first dendrogram. Clade I appeared as a *B. anthracis* clade, and within this clade many *Bacillus* isolates were proximate to *B. anthracis*. This clade was composed of the isolates that gave false-positive results by previously reported assays for *B. anthracis* detection, and the degree of proximity to *B. anthracis* and the frequency of false-positive results appeared correlated. For instance, *B. thuringiensis* BGSC 4AJ1, which was shown to give false-positive results by three of the reported assays in our previous studies was the most proximate to *B. anthracis*.

In conclusion, we determined 27 *Bacillus* isolates that are the most closely related to *B. anthracis* amongst 77 *Bacillus* isolates as shown in the dendrograms. We believe that considering these strains when testing the specificity of newly developing or already developed assays for the detection of *B. anthracis* will prove to be very useful and will help to lead to the development of more highly specific detection assays.

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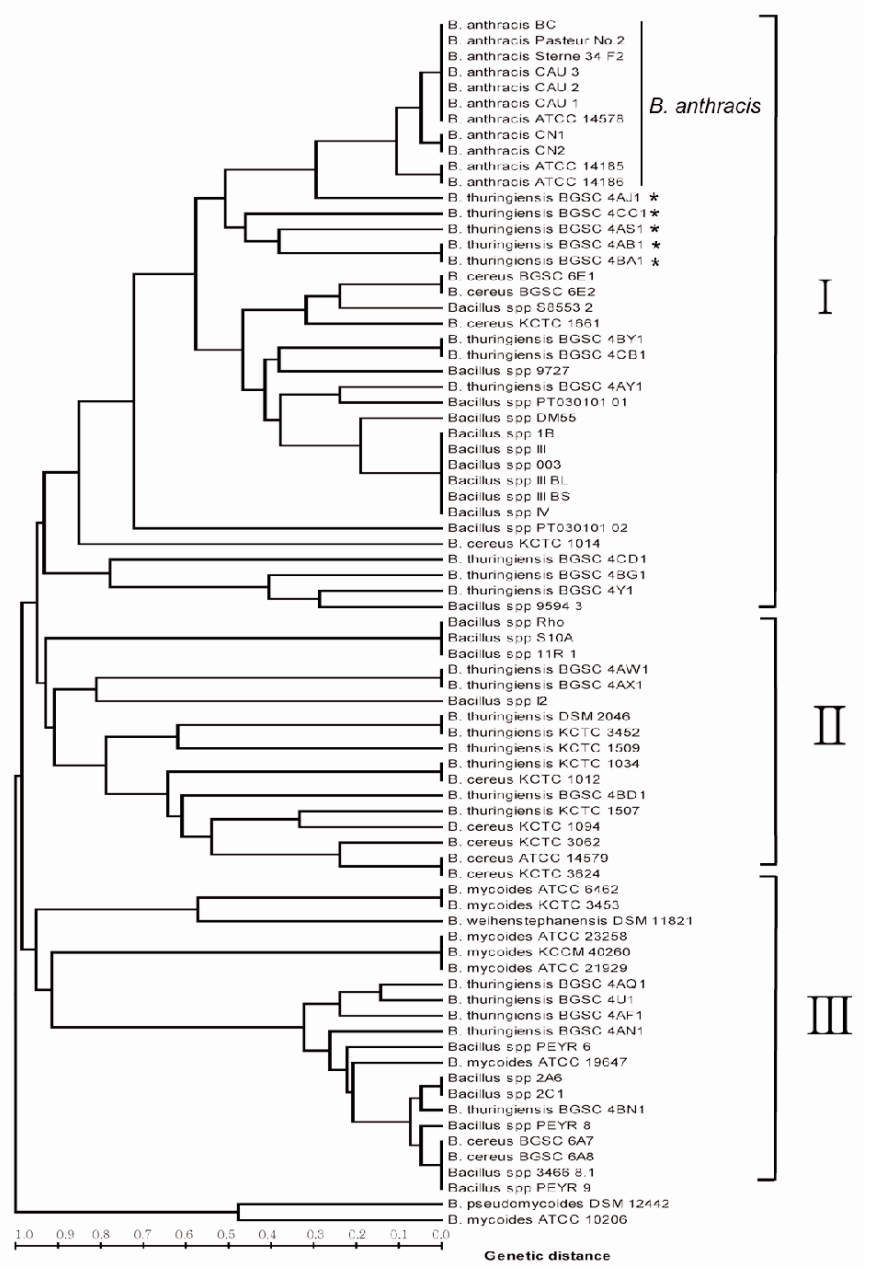

Figure. 1. Phylogenetic dendrogram of 77 isolates of the *B. cereus* group constructed by UPGMA MLST based on the differences between allelic profiles. Alleles were identified after gene fragments were split into three equal-length parts (Table 3). The population comprised three major clades. The clades are indicated by Roman numerals and lineages by names. Clade I encompassed all the *B. anthracis* isolates and 27 isolates of other *B. cereus* group species. *B. thuringiensis* BGSC 4AJ1, 4CC1, 4AS1, 4AB1 and 4BA1 (asterisks) were the most proximate to *B. anthracis*. The scale bars indicate the percentages of mismatches between allelic profiles.

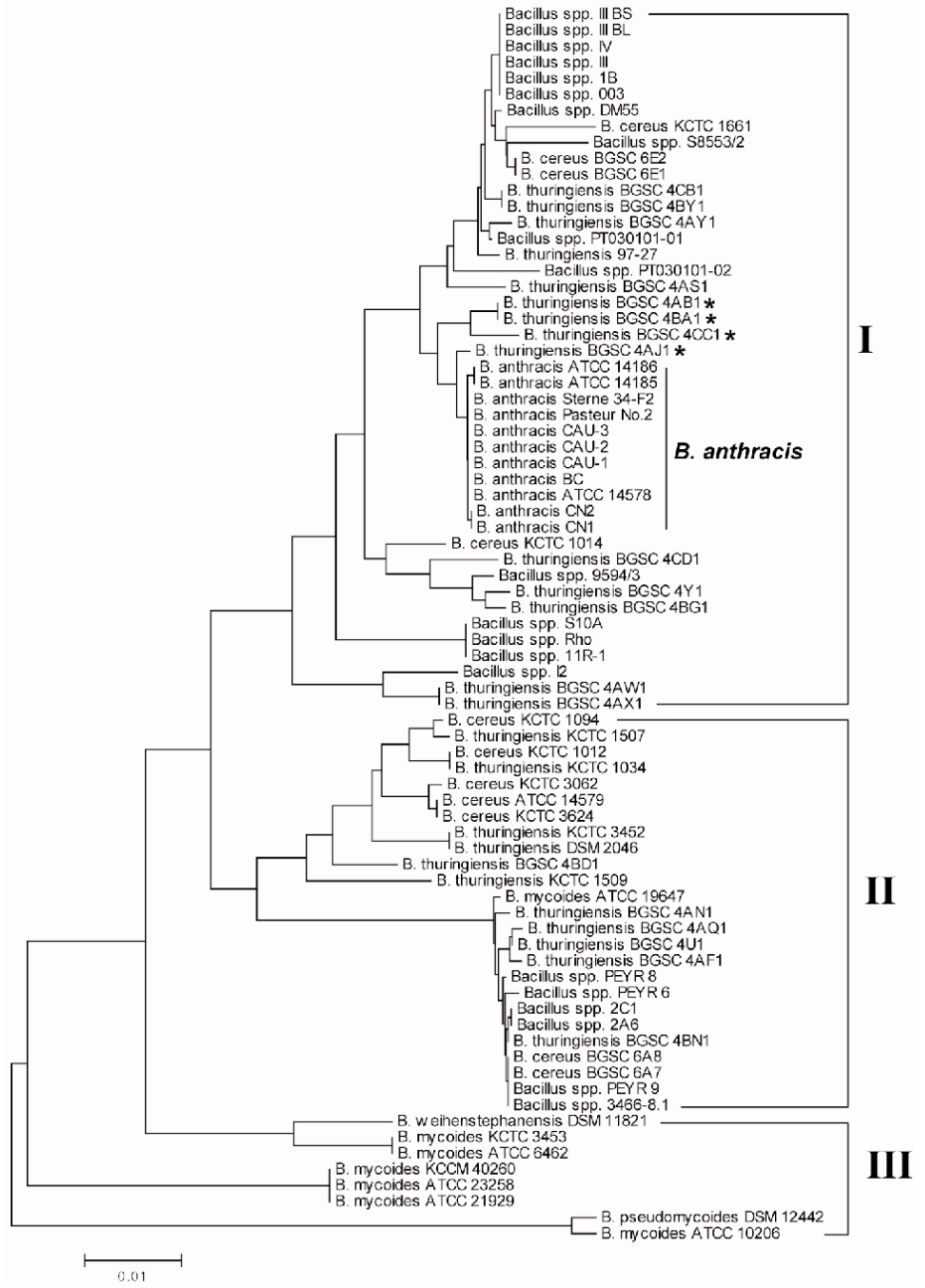

Figure 2. Phylogenetic dendrogram of 77 isolates of the *B. cereus* group constructed by MLST neighbor-joining method based on the concatenated sequences of the seven gene fragments. The population comprised three major clades. The clades are indicated by Roman numerals and lineages by names. Clade I encompassed all the *B. anthracis* isolates and 33 isolates of other *B. cereus* group species. *B. thuringiensis* BGSC 4AJ1, 4CC1, 4AB1 and 4BA1 (asterisks) were the most proximate to *B. anthracis*. Bar is substitutions/site.

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