Molecular Characterization of Korean *Bacillus anthracis* Isolates by Amplified Fragment Length Polymorphism Analysis and Multilocus Variable-Number Tandem Repeat Analysis

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We analyzed the genetic relationships and molecular characteristics of 34 *Bacillus anthracis* isolates from soil and clinical samples in various regions of Korea and 17 related *Bacillus* species, using the amplified fragment length polymorphism (AFLP) and multilocus variable-number tandem repeat (MLVA) approaches. Triplicate AFLP profiles of these strains showed high reproducibility and identified 376 polymorphisms. AFLP phylogenetic analysis of *B. anthracis* isolates showed a high level of similarity, 0.93, and this monomorphic fragment profile proved to be useful to differentiate *B. anthracis* strains from other *Bacillus* species. The *B. cereus* group was separated from other *Bacillus* species at a level of similarity of 0.68. Among them, some *B. cereus* strains showed genetic interspersions with *B. thuringiensis* strains. The evolutionary pattern of nucleotide differences among *B. anthracis* strains with the eight MLVA markers showed nine MLVA types. Three MLVA types, M1 to M3, were pathogenic *B. anthracis* isolates and were assigned as new genotypes belonging to the A4 and B3 clusters, compared with 89 genotypes deduced from previous data. This indicates that differences in cluster prevalence and distribution may be influenced more by MLVA markers on two plasmids loci and human activity. Consequently, we suggest that the novel MLVA type may represent significant evidence for historic adaptation to environmental conditions of the Asian continent, particularly Korea. Therefore, MLVA techniques may be available for molecular monitoring on anthrax-release-related bioterrorism and further study is required for the continuous epidemiological study of variable anthrax collections.

Bacillus anthracis is a causative agent of anthrax, a serious and often fatal infection of livestock and humans. Humans may contract the disease via three routes: cutaneous inoculation via a cut or abrasion, ingestion of contaminated meat, or inhalation of spores (2, 3, 7, 39). The spores of this organism can remain stable for decades and can be used as a powerful bioterrorism agent (17, 20). Recent bioterrorism-associated anthrax outbreaks demonstrated the need for the rapid molecular typing of *B. anthracis* isolates (11, 14, 17, 18, 28).

B. anthracis is a member of the *B. cereus* group, which includes *B. cereus* and *B. thuringiensis*. This is one of the most taxonomically ambiguous groups of bacilli (16, 32). These species are indistinguishable by using DNA-DNA hybridization, 16S-23S rDNA sequences, and *gyrB*-*gyrA* intergenic spacer regions (1, 5, 12, 25, 38). It has been hypothesized that the members of this group evolved from a common ancestor (15, 40).

Molecular typing of pathogens has long been a part of pathogen identification and control. Pulsed-field gel electrophoresis data sets are not easily standardized for transfer throughout the public health community. Ribotyping is generally applicable to all bacteria but is limited by the number of ribosomal loci in the genome. These methods often may not

distinguish between closely related species or may misidentify species, or several strains within a particular species may show identical patterns (6, 11–13). As the emergence of new technologies gains momentum, effects on the diversity studies within each of these *Bacillus* species have been reported. Using multilocus enzyme electrophoresis analysis, Helgason et al. (15) revealed little molecular diversity of *B. anthracis* in contrast to the diverse nature of *B. cereus* and *B. thuringiensis*. Recently, this result was confirmed by multiple-locus sequencing typing, which is based on sequencing a number of essential or housekeeping genes spread around the bacterial chromosome (14, 30). Amplified fragment length polymorphism (AFLP) markers are a recent innovation in genetic marker technology, providing a greater capacity for genome coverage and more reproducible results than previous technologies (23, 42), and have been used to examine phylogenetic relationships between *B. anthracis* and its close relatives, *B. cereus* and *B. thuringiensis* (17, 22, 26, 39). Moreover, to study the diversity, evolution, and molecular epidemiology of pathogens, multilocus variable-number tandem repeat analysis (MLVA) markers, short nucleotide sequences that are repeated multiple times, create length polymorphisms that can be detected easily by PCR using flanking primers (10, 41) and so have been used to subtype monomorphic *B. anthracis* strains (9, 27, 28, 31, 37).

For a basic molecular epidemiological study of Korean *B. anthracis* isolates, we analyzed the genetic diversity of 51 strains, including 38 *B. anthracis* strains and 13 related *Bacillus* species, by using both the AFLP and MLVA approaches.

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TABLE 1. Strains used in this study

Taxon	Strain no. ^a	Source ^b	pXO1	pXO2
<i>Bacillus anthracis</i> (Korean isolates)	H0001	Blood	+	+
	H0002	Vesicle	+	+
	M0001	Meat	+	+
	S0001, S0204-16,	Soil	+	+
	S0302, S0309,	Soil	+	+
	S0312-14			
	S0303-8, S0310-11, S0315-16	Soil	—	+
	S0201-2	Soil	—	—
Type and reference strains				
<i>Bacillus anthracis</i>	14578 ^T	ATCC	+	+
	14185	ATCC	+	—
	14186	ATCC	+	—
	Sterne	NVRQS	+	—
<i>Bacillus cereus</i>	14579 ^T	ATCC		
	11778	ATCC		
	27348	ATCC		
	53522	ATCC		
	11950	ATCC		
<i>Bacillus thuringiensis</i>	10792 ^T	ATCC		
<i>Bacillus mycoides</i>	6462 ^T	ATCC		
<i>Bacillus licheniformis</i>	14580 ^T	ATCC		
<i>Bacillus coagulans</i>	7050 ^T	ATCC		
<i>Bacillus subtilis</i>	6633 ^T	ATCC		
<i>Bacillus megaterium</i>	9885 ^T	ATCC		
<i>Bacillus polymyxa</i>	21830 ^T	ATCC		
<i>Bacillus sphaericus</i>	14577 ^T	ATCC		

^a Type strains are indicated by a superior T.

^b ATCC, American Type Culture Collection (Manassas, Va.); NVRQS, National Veterinary Research and Quarantine Service (Anyang, Korea).

MATERIALS AND METHODS

Bacterial strains. Seventeen *Bacillus* type and reference strains and 34 strains from *B. anthracis* isolates were used in this study (Table 1). *Bacillus anthracis* strains were isolated from 12 different farm estates located in various regions of Korea during 2000, 2002, and 2003; these isolates were mostly from soils, but three isolates were from meat, blood, and vesicle samples of the outbreak that occurred in Changnyeong province in 2000, which resulted in five patients developing cutaneous anthrax (with two deaths) after contact with the meat of a dead cow. When we collected soil samples from 12 loci, we dug out about 10 to 15 cm from the ground surface and collected approximately 30 to 50 g of soil. We mixed sterilized and distilled water and samples at a ratio of 1:1 or 1:2. Samples were treated at 62.5°C for 15 min. We incubated 10-fold serially diluted samples on sheep blood agar (SBA; Bionerieux, Inc., Paris, France) plates at 37°C for 24 h. Also, we collected blood and vesicular fluid samples from cutaneous patients who were infected during illegal butchery and meat that they stored in a refrigerator and incubated these samples on SBA plates at 37°C for 24 h. We selected colonies showing nonhemolysis, medusa heads, and comet tails on an SBA plate. We performed a Gram stain, motility test, and biochemical test using API 50CHB and 20E. Also, we confirmed the presence of pXO1 and pXO2 by TaqMan real-time PCR using the *pagA* and *cap* genes (34). The pure cultures were prepared on brain heart infusion (Difco Laboratories, Detroit, MI) agar plates, collected with brain heart infusion broth and 50% glycerol solution at a ratio of 1:1, and stored as stock cultures at -70°C for future examination.

DNA preparation. Genomic DNA was isolated from the pure cultures following the procedures of Beyer et al. (4) with some modification. Briefly, about 50 ng of cell mass harvested from the culture was suspended in 500 µl Tris-EDTA (TE) buffer (pH 8.0). Lysozyme (5 mg/ml) and mutanolysin (30 µg/ml) were added to the suspension, which was then incubated at 37°C for 1 h. The bacteria were broken down by the addition of 5 µl proteinase K (200 µg/ml) and 60 µl 10% sodium dodecyl sulfate at 60°C for 30 min. To this mixture, 120 µl of 5 M NaCl was added, and the mixture was refrigerated at 4°C for 45 to 60 min. The bacterial debris was pelleted for 10 min at 5,000 × g at 4°C. One volume of equilibrated phenol was added to the supernatant, and the mixture was emulsified by agitation; subsequently, the same volume of chloroform was added. After the mixture had been centrifuged at 4°C for 10 min at 5,000 × g, the aqueous supernatant was transferred to a fresh tube and incubated with RNase A (200 µg/ml) at 37°C for 15 min. The DNA was precipitated by the addition of 2.5

volumes of ice-cold absolute ethanol and centrifugation at 4°C for 10 min at 5,000 × g. The DNA was then washed with 70% ethanol, dried in air, resuspended in 100 µl of TE buffer (pH 8.0), and used as a template for AFLP and MLVA.

AFLP analysis of DNA samples. AFLP analysis was accomplished as previously described (17, 26, 39). Briefly, genomic DNA was digested with EcoRI and MseI, and the resulting fragments were ligated to EcoRI and MseI double-stranded adapters (Table 2). The composition of the restriction and ligation reactions was as follows: 2 µl of 10× T4 DNA ligase buffer, 1 µl of 0.5 M NaCl, 0.5 µl of bovine serum albumin (1 mg/ml), 1 µl of EcoRI adapter, 1 µl of MseI adapter, 2 µl of enzyme master mix, and 1 µl of DNA in a total volume of 11 µl. The restriction and ligation reactions were incubated at 37°C for 2 h. The enzyme master mix was as follows: 1× T4 DNA ligase buffer, 0.05 M NaCl, 500 U EcoRI, 100 U MseI, and 100 U T4 DNA ligase in a total volume of 100 µl. When the preselective PCR template was used, the digested and ligated DNA was added to 189 µl of TE buffer. For preselective PCR, the 20-µl reaction mixture contained 0.5 µl of EcoRI preselective primer and MseI preselective primer (Table 2), 2.0 mM concentrations of deoxynucleoside triphosphates (dNTPs), 2.5 mM MgCl₂, 0.5 U *Taq* polymerase (TaKaRa Bio Inc., Otsu, Shiga, Japan), and 4 µl of diluted digestion and ligation DNA. PCRs were performed in a model 9700 thermal cycler (PE Applied Biosystems Inc.). Thermal cycling was achieved according to the following program: initial denaturation at 72°C for 2 min and 20 cycles at 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min, followed by a final extension step at 60°C for 30 min. Following the addition of 190 µl of TE buffer, the preselective PCR product was used as the template for selective PCR. For selective PCR, the 10-µl reaction mixture contained 0.5 µl of EcoRI and MseI selective primers (Table 2), 2.0 mM concentrations of dNTPs, 2.5 mM MgCl₂, 0.25 U *Taq* polymerase, and 1.5 µl of diluted preselective PCR template. The selective PCRs were performed in an ABI PRISM 7900HT sequence detection system (PE Applied Biosystems Inc.). Thermal cycling was performed with the following program: initial denaturation at 94°C for 2 min and 10 cycles at 94°C for 20 s, 66°C for 30 s, and 72°C for 2 min, decreasing the annealing temperature by 1°C/cycle, followed by 20 cycles at 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min, with a final extension at 60°C for 30 min. One microliter of the selective PCR products was mixed with 12.0 µl of formamide (PE Applied Biosystems Inc.) and 0.5 µl of GeneScan 500 size standards (PE Applied Biosystems Inc.) and denatured at 95°C for 5 min. The samples were then analyzed on an ABI PRISM 310 automatic sequencer (PE Applied Biosystems Inc.) as follows: 5 s of injection time, 15 kV of injection voltage, 15 kV of run voltage, and 30 min of running time.

AFLP profiles were analyzed using Genescan analysis software (version 3.1; PE Applied Biosystems Inc.) to determine the fragment length by comparison to the size standards included with each sample. Genescan analysis was as follows: analysis range, 2,400 to 6,500 bases; size call range, 50 to 500 bases; data processing, light; size calling method, local Southern method. A binary matrix was generated by the presence (1) or absence (0) of selected peaks shown to have a fluorescence intensity of more than 100 in the peak height of the fingerprint and more than twice in the triplicate samples. Cluster analysis for the AFLPs was performed by using the unweighted-pair group method arithmetic average (UPGMA; F. J. Rohlf, NTSYS-PC numerical taxonomy and multivariate analysis system, version 2.10b; Exeter Software, Setauket, NY). The similarity among *Bacillus* species was expressed with a simple matching coefficient.

TABLE 2. Adapters and PCR primers used for AFLP analysis

Adapter or primer	Oligonucleotide sequence (5'-3')
Adapters	
EcoRI.....	CTC GTA GAC TGC GTA CC CAT CTG ACG CAT GGT TAA
MseI	GAC GAT GAG TCC TGA G TAC TCA GGA CTC AT
Preselective primers	
EcoRI.....	GAC TGC GTA CCA ATT C
MseI	GAT GAG TCC TGA GTA A
Selective primers	
EcoRI-0	FAM ^a -GAC TGC GTA CCA ATT C
MseI-C	GAT GAG TCC TGA GTA AC

^a FAM, 6-carboxyfluorescein.

TABLE 3. PCR primers used for MLVA analysis

Marker locus	Primer	Oligonucleotide sequence (5'-3')
<i>vrnA</i>	<i>vrnA</i> -f	CAC AAC TAC CAC CGA TGG CAC A
	<i>vrnA</i> -r	GCG CGT TTC GTT TGA TTC ATA C
<i>vrnB</i> ₁	<i>vrnB</i> ₁ -f	ATA GGT GGT TTT CCG CAA GTT ATT C
	<i>vrnB</i> ₁ -r	GAT GAG TTT GAT AAA GAA TAG CCT GTG
<i>vrnB</i> ₂	<i>vrnB</i> ₂ -f	CAC AGG CTA TTC TTT ATC AAA CTC ATC
	<i>vrnB</i> ₂ -r	CCC AAG GTG AAG ATT GTT GTT GA
<i>vrnC</i> ₁	<i>vrnC</i> ₁ -f	GAA GCA AGA AAG TGA TGT AGT GGA C
	<i>vrnC</i> ₁ -r	CAT TTC CTC AAG TGC TAC AGG TTC
<i>vrnC</i> ₂	<i>vrnC</i> ₂ -f	CCA GAA GAA GCG GAA CCT GTA GCA C
	<i>vrnC</i> ₂ -r	GTC TTT CCA TTA ATC GTG CTC TAT C
CG3	CG3-f	TGT CGT TTT ACT TCT CTC TCC AAT AC
	CG3-r	AGT CAT TGT TCT GTA TAA AGG GCA T
pXO1-aat	pXO1-aat-f	CAA TTT ATT AAC GAT CAG ATT AAG TTC A
	pXO1-aat-r	TCT AGA ATT AGT TCA TTC ATA ATG GC
pXO2-at	pXO2-at-f	TCA TCC TCT TTT AAG TCT TGG GT
	pXO2-at-r	GTG TGA TGA ACT CCG ACG ACA

Quality evaluation of AFLP data. We compared AFLP patterns using three separate DNA amounts (10, 100, and 500 ng) and examined the reproducibility among triplicate AFLP data, using nine type strains of *Bacillus* species and three *B. anthracis* isolates: *B. anthracis* ATCC 14578^T, *B. anthracis* S0001, *B. anthracis* S0201, *B. anthracis* S0204, *B. cereus* ATCC 14579^T, *B. thuringiensis* ATCC 10792^T, *B. mycoides* ATCC 6462^T, *Bacillus licheniformis* ATCC 14580^T, *Bacillus coagulans* ATCC 7050^T, *Bacillus megaterium* ATCC 9885^T, *Bacillus polymyxa* ATCC 21830^T, and *Bacillus sphaericus* ATCC 14577^T.

MLVA of DNA samples. The three reactions were designed so that *vrnB*₁ was grouped with *vrnC*₁, CG3, and pXO1-aat; *vrnB*₂ was grouped with *vrnC*₂ and pXO2-at; and *vrnA* was amplified alone. Each reaction mixture contained 1 µl of 100 ng template, 2.0 mM concentrations of dNTPs, 2.5 mM MgCl₂, and 2 U of *Taq* polymerase in a total volume of 25 µl. Primer sets were used in each reaction as follows (Table 3): in reaction 1, 1 µl of *vrnB*₁-f1, *vrnB*₁-r1, *vrnC*₁-f1, *vrnC*₁-r1, and CG3 each at 10 pmol and 4 µl of pXO1-aat-f3 and pXO1-aat-r3 primers each at 10 pmol; in reaction 2, 1 µl of *vrnB*₂-f1, *vrnB*₂-r1, *vrnC*₂-f1, and *vrnC*₂-r1 each at 10 pmol and 2 µl of pXO2-at-f3 and pXO2-at-r3 primers each at 10 pmol; in reaction 3, 2 µl of *vrnA*-f1 and *vrnA*-r1 primers each at 10 pmol. PCRs were performed in a model 9700 thermal cycler. Thermal cycling was performed according to the following program: initial denaturation at 95°C for 2 min and 35 cycles at 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s, followed by a final extension at 72°C for 5 min. Amplicons were analyzed on 3.5% metaphor agarose gels (Cambrex Bio Science Inc., Rockland, Maine) containing ethidium bromide.

Amplicons were purified using a QIAquick PCR purification kit (QIAGEN, Inc., Valencia, CA) and cloned in T-Vector plasmids by using the pGEM-T Easy vector system (Promega Corporation, Madison, WI). The plasmid was purified with the QIAprep Spin Miniprep kit (QIAGEN, Inc., Valencia, CA). The purified plasmid was sequenced using the BigDye Terminator cycle sequencing reaction kit (Applied Biosystems Inc., Foster City, CA). Sequence analysis was performed with SeqManII and MEGALIGN in the DNASTAR software package (version 4.0; DNASTAR, Inc., Madison, WI). Cluster analysis was performed by UPGMA in the NTSYS package. The similarity among *B. anthracis* strains was expressed by using a simple matching coefficient. Individual marker diversity (*D*) was calculated as equal to $1 - \sum(\text{allele frequency})^2$ (43) and based on allele frequencies in the pathogenic *B. anthracis* strains.

RESULTS

We examined the quality of AFLP patterns according to DNA amount and the reproducibility of triplicate AFLP patterns. Amplified fragment patterns of selective PCR using different amounts of DNA (10, 100, and 500 ng) were similar, but the intensity of each banding pattern was dependent on the DNA amount (Fig. 1). Peak height of the amplified fragments using 10 ng of DNA could not be differentiated from the baseline, whereas the AFLP pattern using 500 ng DNA displayed very high peak heights. Peak heights of the AFLP using

FIG. 1. Amplified fragment patterns of selective PCRs using different DNA concentrations. Lanes: M, 100-bp ladder; 1 through 4, 500 ng DNA; 5 through 8, 100 ng DNA; 9 through 12, 10 ng DNA; 1, 5, and 9, *B. anthracis* ATCC 14578^T; 2, 6, and 10, *B. anthracis* S0001; 3, 7, and 11, *B. anthracis* S0201; 4, 8, and 12, *B. anthracis* S0204.

100 ng DNA displayed good resolution, with intensities of 100 to 2,000 relative fluorescent units.

Triplicate AFLP profiles of other species within the *B. cereus* group represented a similar range of peak number and fluorescence intensity, but *B. mycoides* ATCC 6462^T showed relatively low fluorescence intensity (Fig. 2). We could confirm high reproducibility in triplicate AFLP patterns. The AFLP fingerprint for *B. anthracis* and related *Bacillus* taxa produced about 100 fragments with peak heights of more than 100 relative fluorescent units and fragment sizes ranging from 50 to 500 bp. A total of 376 polymorphisms were generated from binary data made by the presence and absence of fragments presented more than twice among triplicates. AFLP profiles showed specific markers for the *B. anthracis* and *B. cereus* groups that differentiated them from other *Bacillus* species (384.09 ± 0.5 and 203.82 ± 0.5 of the peak range, respectively) (Fig. 2).

In the phylogenetic analysis of the AFLP data, *B. anthracis* isolates showed a high level of similarity (0.93) and, regardless of the presence of two plasmids, the sampling location and the sample type, *B. anthracis* strains were intermixed within one another (Fig. 3). The *B. cereus* group was separated from other *Bacillus* species at a level of similarity of 0.68. Among them, *B. cereus* ATCC 53522 was closely related to the *B. anthracis* strains and *B. cereus* ATCC 11950 was related to the *B. thuringiensis* strain. *B. cereus* strains showed a high genetic diversity from other species in the *B. cereus* group.

To better understand the dynamics of anthrax in domestic regions, we examined the genetic diversity of four *B. anthracis* reference and type strains and 34 *B. anthracis* isolates by MLVA. We found nine MLVA types (M1 to M9) in this collection of 38 *B. anthracis* strains (Table 4). Three MLVA types (M1 to M3) were pathogenic *B. anthracis* strains, and another four types (M7 to M9) were nonpathogenic isolates. Among them, M1 was the major MLVA type of the Korean isolates. Multiple alignments of the hypervariable regions at eight MLVA loci (*vrnA*, *vrnB*₁, *vrnB*₂, *vrnC*₁, *vrnC*₂, CG3, pXO2, and pXO1) were observed with a range of two to three alleles per locus. *vrnA*, CG3, pXO1, and pXO2 were repeats of the same sequences, but the repeating sequences of *vrnB*₁, *vrnB*₂, *vrnC*₁, and *vrnC*₂ were not all identical, including multiple different nucleotides. This result is consistent with previous data (21, 28).

FIG. 2. Triplicate AFLP profiles and specific marker of *Bacillus anthracis* strains and related *Bacillus* taxa. Specific AFLP markers of the *B. anthracis* and *B. cereus* groups were indicated at 384.09 bp and 203.82 bp, respectively, by the dotted lines.

The number of alleles and their frequency were assigned as MLVA markers to differentiate *B. anthracis* isolates at the strain level. In pathogenic *B. anthracis* isolates, a diversity index value range of 0.35 to 0.4 was observed, with the exception of CG3. Although nonpathogenic isolates generated a few alleles, only *vrB*₂ and *vrC*₁ displayed a low diversity index. Compared with 89 genotypes reported by Keim et al. (28), these three MLVA types were divided into two dominant branches, A and B, and designated as new genotypes: M1 was classified into the A4 cluster and M2 and M3 into a new B3 diversity cluster (Fig. 4).

Thirty *Bacillus anthracis* isolates originated from soil in various regions of Korea during 2002 and 2003, but they did not show a correlation between their MLVA type and isolated region. In addition, four isolates (H0001, H0002, M0001, and S0001), originating from the Changnyeong outbreak in 2000, were the identical MLVA type, M2 (Fig. 5).

DISCUSSION

We analyzed the genetic diversity and molecular characteristics of 51 strains, including Korean *B. anthracis* isolates and related *Bacillus* species, by using AFLP and MLVA.

The AFLP data generated were high quality, because of the appropriate selection of DNA concentration, and showed reproducibility of triplicate samples (Fig. 1 and 2). Previous AFLP variability studies reported that the AFLP technique is insensitive to different DNA sources of the same isolates, different extraction methods, and different reagent and DNA concentration and showed identical banding patterns (22, 23, 33). However, our results indicate that an appropriate DNA concentration is required for the standardization and availability of AFLP fingerprints because the fluorescence peak height may be influenced by the amount of product in the peak and changes in relative product concentrations within different AFLP reactions. When an additional nucleotide is added to each fingerprinting primer to increase specificity, the AFLP fragment number drops considerably with a typical range of 50 to 100 fragments detected from a bacterial genome (19). In the *Bacillus* species, the quality of AFLP fragments was lowest with high A+T primer combinations, where the most fragments were observed (23, 26). Therefore, the complexity of the banding pattern was always reciprocal to the number of selective bases used. In addition, specific markers obtained from AFLP profiles can be useful for the detection of *B. anthracis* or *B.*

FIG. 3. Dendrogram based on the AFLP profiles by using Korean *Bacillus anthracis* isolates and related *Bacillus* taxa. Similarity between pattern patterns was calculated by using a simple matching coefficient. The data were analyzed by using the UPGMA clustering method (NTSYS package, version 2.10b).

TABLE 4. MLVA types of *Bacillus anthracis* isolates and related strains

MLVA type	Isolate(s) (reference strain)	Amplicon size (bp) for:							
		<i>vrrA</i>	<i>vrkB</i> ₁	<i>vrkB</i> ₂	<i>vrnC</i> ₁	<i>vrnC</i> ₂	<i>CG3</i>	pXO1	pXO2
M1	S0204-16, S0302, S0313, S0314 (ATCC 14578 ^T)	289	229	153	538	604	158	135	139
M2	S0309, H0001-2, M0001, S0001	301	256	171	583	532	158	129	137
M3	S0312	301	229	171	583	532	158	129	137
M4	(ATCC 14185)	313	229	162	613	532	158	132	— ^b
M5	(ATCC 14186)	313	229	162	613	532	153	129	—
M6	S0303-4, (Sterne)	313	229	162	583	532	158	132	—
M7	S0307-8, S0310-11, S0315-16	313	229	162	583	532	158	—	137
M8	S0201-2	313	229	171	583	532	158	—	—
M9	S0305, S0306	313	229	162	583	532	158	—	—
<i>D</i> ^a		0.40	0.35	0.40	0.40	0.40	0.00	0.40	0.40

^a *D* is Nei's marker diversity, which is calculated as $1 - \sum(\text{allele frequency})^2$ and based solely upon the three unique MLVA types (M1 through M3) of Korean *B. anthracis* isolates.

^b —, no amplicon detected because at least one of the two plasmids was absent from the *B. anthracis* strains.

cereus groups from related *Bacillus* species. Recently, these oligonucleotides, distinguishable at the level of species or strains, have been developed by sequence analysis of these AFLP fragments by using ligation-mediated suppression PCR (8, 24, 35, 36).

On AFLP data, the presence or absence of both virulent plasmids was not considered a genetic characteristic for subgroup discrimination in this study, as previously reported by Keim et al. (26). The polymorphism associated with the presence or absence of pXO1 and pXO2 plasmid fragments was ignored for many of the AFLP profiles because of the ephemeral nature of these plasmids; that is, we analyzed only fragments within the 50- to 500-bp size range and so regarded plasmid-generated fragments as negligible on AFLP profiles.

AFLP-based phylogenetic data had high monomorphic diversity between *B. anthracis* type and reference strains and 34

isolates, whereas *B. cereus* and *B. thuringiensis* exhibited polymorphic diversity. These results are consistent with previous data (17, 22, 26, 39). This genetic similarity of *B. anthracis* strains corresponds with the previous suggestion that ongoing epidemic anthrax is the result of a single introduction, changing very little with the spread of the disease, and that AFLP alleles appear to be stable over the time period, important for tracking an epidemic (17, 26, 39).

The evolutionary pattern of nucleotide differences among *B. anthracis* strains is consistent among the eight MLVA markers. Compared with the diversity index value reported previously, the mean diversity index value of the eight MLVA markers used in this study was 0.34, which is lower than that for the French and worldwide study, where the averages were 0.50 (9) and 0.52 (28), respectively. However, our diversity index value was higher than that reported in the Kruger National Park

FIG. 4. Genetic relationships of *Bacillus anthracis* strains by MLVA. The dendrogram is based on comparative analysis between three novel types in this study and the genotypes of Keim et al. (28). The three types are observed from pathogenic *B. anthracis* strains used in this study and are labeled with asterisks. Number (N) of isolates on each genotype (GT) is shown to the right of the dendrogram. The eight variable-number tandem repeat (VNTR) marker loci were used to calculate a simple matching coefficient. The data were analyzed by using the UPGMA clustering method (NTSYS package, version 2.10b).

FIG. 5. Locations of Korean *B. anthracis* isolates. This map shows the regions where *B. anthracis* isolates were obtained for this genetic study. The actual collection sites are indicated by the small black dots. The labels indicate the number of isolates from a location, MLVA type (M), and the year the isolate was collected (in parentheses).

Study, where the average was 0.23 (37). Six chromosomal MLVA markers had a similar range of diversity index values (0.3 to 0.5), and so these marker alleles appeared stable to routine and even long-term handling in the laboratory while the MLVA markers on two plasmids had various array sizes among the *B. anthracis* strains collected around the world (9, 18, 28, 29, 37). This is because genetic variation in the short nucleotide repeat at the pXO1 and pXO2 loci occurs more frequently with a slippage during the replication of the repeat region by DNA polymerase (9, 18, 28, 37).

In the dendrogram based on MLVA profiles, pathogenic Korean *B. anthracis* isolates were split into A and B branches. Korean isolates showed the major MLVA type of M1 and two different types (Fig. 4). The M1 MLVA type belongs to the A4 cluster, which is notable for the Vollum strain (genotype 77) used in the British biological warfare program. This new genotype had one more allele number on the pXO1 locus than genotype 77. Another two MLVA types, M2 and M3, were assigned to the new B3 cluster, showing different allele frequencies in *vrB*₁, *vrB*₂, pXO1, and pXO2 from the B1 and B2 clusters, which are found mostly in France and South Africa.

B. anthracis strains originating from several geographic regions of Africa, Europe, and America reflect the genetic dissimilarity range belonging to the A and B branches. Considering that *B. anthracis* isolates originating from Asia, Turkey, and China have been assigned only to branch A, it is remarkable that Korean collections included both A and B branches in this study. In a previous molecular epidemiological study, 42 Ko-

rean *B. anthracis* strains were collected from environmental soils and several outbreaks from the 1960s through the 1990s and belonged to branch A exclusively (unpublished data). At the time when the Changnyeong outbreak collections had been identified as branch B in 2000, we assumed that the outbreak had occurred as a result of the recent introduction of the causative organism via international human activities. However, based on the fact that two strains originating from soil samples in 2003 have been classified into the same B3 cluster as the Changnyeong outbreak collections, the introduction of a new cluster could be considered a result of divergent evolution in Korea over a long period of time rather than recent dispersal of *B. anthracis* into this area. Consequently, the Korean *B. anthracis* isolates have doubtlessly been influenced by human activity and through modern international commerce (28), and the novel MLVA type introduction may imply that *B. anthracis* has independently adapted to environmental conditions in Asia as well as in Korea. Further study of the continuous evolutionary and epidemiological impact on variable anthrax collections in Korea is required.

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