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Geographical Distribution of Genotypic and Phenotypic Markers Among *Bacillus anthracis* Isolates and Related Species by Historical Movement and Horizontal Transfer

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ABSTRACT. The geographical distribution of *Bacillus anthracis* strains and isolates bearing some of the same genetic markers as the Amerithrax Ames isolate was examined and evaluated. At least one mechanism for the horizontal movement of genetic markers was shown amongst isolates and closely related species and the effect of such mixing was demonstrated on phenotype. The results provided potential mechanisms by which attempts to attribute isolates of *Bacillus anthracis* to certain geographical and isolate sources may be disrupted.

Bacillus anthracis appears to be a bacterium that has unique qualities that might allow using the genotype alone to determine the geographical distribution of genetic markers. It is extremely genetically homogeneous across its worldwide distributed strains (Keim *et al.* 1997, 2000, 2002). However, there are enough single-nucleotide polymorphisms (SNPs) and variable tandem repeat sequence number differences among various isolates to appear to distinguish *B. anthracis* from various locales. This lack of substantial variability may be attributed to the low-passage number of natural anthrax bacteria and their relative isolation to very limited areas because they are purported to reside in soil as spores for decades before infecting a host and completing another cycle of replication (Keim *et al*. 1997). The laboratory mutation rate of *B. anthracis* is $\approx 2 \times 10^{-5}$ (Keim *et al*. 2001) and the natural mutation rate is $\approx 2 \times 10^{-10}$ (Van Ert *et al*. 2007), assuming little or no recombination, which may not be the case (Ko *et al*. 2004); however, we have previously shown that nitration conditions *in vitro* easily generate profound mutations (Kiel *et al*. 2002). Such conditions mimic the inflammatory activity of nitric-oxide synthase, NADPH oxidase and peroxidase in leukocytes against microbes *in vivo*. The sheer number of organisms produced in the terminal infection of a large animal host, such as a cow, should easily produce mutations well represented in the final spore yield based on the number of spores produced alone. A 550-kg cow could yield 60 mL/kg blood containing $\approx 10^8$ spores per mL (Swartz 2001), totaling 3.3×10^{12} spores per carcass. This yield could contain from 3.3×10^2 to 3.3×10^7 mutants.

MATERIALS AND METHODS

Microbiological methods. β-Hemolysis was observed on tryptic soy agar plates with 5 % sheep blood (BAP; *Remel*) after 18 h of growth at 37 °C. Cherry γ phage spot tests were performed on host lawns derived from tryptic soy broth (TSB) cultures, incubated for 18 h at 37 °C with shaking (150 rpm). Plates were swabbed bidirectionally with host lawn, incubated for 2 h at 37 °C, spot-inoculated with 20 μL of undiluted Cherry γ phage, and incubated for 18 h at 37 °C. Antibiotic disk sensitivities were performed with disks placed directly on lawns omitting the 2-h incubation step used for the phage tests. Cherry γ phage rescue used 500-μL aliquots of host culture (host-inoculated TSB incubated for 18 h at 37 °C with shaking) seeded with 20 μL of serially diluted Cherry γ phage (10^0, 10^{-1}, 10^{-2}, and 10^{-3}), vortexed briefly, and incubated for 20 min at 37 °C. Molten tryptic soy top agar was inoculated with phage and/or host mixture, vortexed, and poured onto tryptic soy agar plates (TSA; *Remel*). The plates were incubated for 18 h at 37 °C. TSA pour plates showing well isolated and/or delineated plaques were read as positive. The horse serum on TSA tests were

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performed with TSA plates flooded with 250 μL of sterile horse serum, swabbed to coat entire surface, and streaked with a loop for microbial colony isolation. Plates were incubated in a candle jar placed for 18 h at 37 °C. Highly glossy colony morphology was recorded as a positive.

Molecular biological methods. A total of 53 isolates of *B. anthracis* were analyzed. Suttex samples were isolated from the remains of a deer carcass and surrounding soil, resulting from the July 2005 outbreak of anthrax in Sutton County (Texas). Samples designated as Vito 1-20 refer to isolates described by Brumlik *et al.* (2001). Polymerase chain reactions (PCRs) were performed using a *Biometra*® T3000 thermocycler (Germany). A typical mixture (50 μL) included 1× PCR buffer, Me_2SO, primers (2 μmol/L each), deoxynucleoside triphosphates (0.2 μmol/L), DNA template, and *Taq* DNA polymerase (2.5 U). The reactions were run with an initial 2-min denaturation at 94 °C, followed by 30 cycles (30 s at 94 °C, 35 s at 60 °C, 2 min at 72 °C) and a final extension (6 min at 72 °C). PCR products (5 μL) were run on a 0.8 % agarose gel with *New England Biolabs* φX174 DNA–*Hae*III digest (USA). DNA was precipitated from the remaining PCR products using 1× volume 5 μmol/L ammonium acetate and 2× volume of 2-propanol, with products stored at –20 °C overnight. The next day, products were spun (16467 ***g***, 10 min, 4 °C); pellets were rinsed with 70 % ethanol and spun again under the same conditions for 2 min. Pellets dried on the bench were resuspended in 10 μL TE buffer.

Sequence chemistry methods. The precipitated PCR products were used in a sequence reaction with *Applied Biosystems* BigDye® Terminator v3.1 chemistry (USA). The resulting precipitated sequencing products were resuspended in 50 μL formamide and 25 μL of each was loaded to an *Applied Biosystems* MicroAmp® Optical 96-well reaction plate. The reactions were run on an *Applied Biosystems* 3100 Automated Sequencer and the results were analyzed with *Applied Biosystems* SeqScape program software. Reference sequences used in the analysis were the *B. anthracis* plasmids pX01 and pX02 (Okinaka *et al.* 1999). All the results were of samples with at least 4× coverage.

Five sets of primers (PS-32, PS-33, PS-34, PS-39, PS-51) were designed using DNASIS® Max (*MiraiBio*, USA) for the regions of interest designated by Read *et al*. (2002) that exhibited single nucleotide polymorphisms (SNPs) on both pX01 and pX02 of the Florida outbreak isolate, A2012. The forward and reverse primers designed by our laboratory and manufactured by *Invitrogen*™ (USA) were labeled in accordance with Read *et al*. (2002).

Sequence results obtained with primer set PS-32 were compared with the reference sequence reported by Okinaka *et al*. (1999*a*,*b*) (NCBI AF065404.1), while sequence results obtained with primer sets PS-33, PS-34, PS-39, and PS-51 were compared with the reference sequence (NCBI AF 188935; Okinaka *et al.* 1999*a*,*b*).

For PS-32 analysis, the region of interest amplified and sequenced corresponded with a 514-bp read designated as nucleotide position 180460–180973 on the reference strain; for PS-33, a 539-bp read designated as nucleotide position 8828–9366; for PS-34, a 517-bp read designated as nucleotide position 9335–9851; for PS-39, a 841-bp read designated as nucleotide position 37814–38654; for PS-51, a 421-bp read designated as nucleotide position 67209–67629.

RESULTS AND DISCUSSION

Reported sources of strains associated with genotypes. Certain genotypes, related to the Florida isolate (A2012) by selected markers, have been linked to geographical regions (Table I). Based on Keim *et al*. (2001) multiple-locus variable-number tandem repeat analysis, an A3b type (genotype 57) has been isolated from both China and the USA. Ames is also an A3b (genotype 62) and differs by two markers from these two sources of genotype 57. The 53 isolates and strains were examined for similarities and differences based on published polymorphisms for the Amerithrax (Florida isolate A2012) *B. anthracis* Ames-like strain (Read *et al*. 2002; Van Ert *et al*. 2007).

Sequencing results of the PCR product SNPs. In the PS-32 (pXO1) region of interest, one difference was noted in only the Keim 57 (China/USA) and Florida (A2012) isolates – an A ⟶ G SNP (single nucleotide polymorphism) observed at reference nucleotide position 180855.

In the PS-33 (pX02) region of interest, a T ⟶ C SNP at reference nucleotide position 9133 was observed in five isolates – A2012, Keim 57, Vito 1, Vito 12, 8002A.

In the PS-34 (pX02) area of interest, a G ⟶ T SNP was observed in the following samples at reference nucleotide position 9600: A2012, Keim samples 25, 35, 38, 41, 55, 57, and 68, Vito samples 1, 5, 12, and 15, and 8002A. Also observed in this region of interest was the insertion of 90 bps in the sequences of

Table I. Sequence comparisons[a] among the most closely related PCR products of reported marker sets for the 53 isolates of *Bacillus anthracis* analyzed

Isolate/strain	PS-32	PS-33	PS-34			PS-39		PS-51	
	A → G at position 180 855	T → C at position 9133	G → T at position 9600	90 bp insert at position 276	180 bp insert at position 276	C → T at position 37 994	T → C at position 38 448	A → C at position 67 335	T deletion at position 67 495
A2012 (Florida isolate)	+	+	+	–	–	+	+	+	+
NCBI AF188935	n/a	–	–	–	–	–	–	–	–
Sterne (NCBI AF065404.1)	–	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Vito 1 (North America)	–	+	+	–	–	+	+	+	+
Vito 3 (Europe)	–	–	–	+	–	–	–	–	–
Vito 5 (Europe)	–	–	+	–	–	+	–	–	+
Vito 12 (Europe)	–	+	+	–	–	+	+	+	+
Vito 15 (Europe)	n/d	n/d	+	–	–	–	–	–	–
Vito 20 (Europe)	–	–	–	+	–	–	–	–	+
Keim 25 (USA)	–	–	+	–	–	+	–	–	+
Keim 35 (Turkey, UK, USA)	–	–	+	–	–	+	–	–	+
Keim 38 (Germany)	–	–	+	–	–	+	–	–	+
Keim 41 (Turkey)	–	–	+	–	–	+	–	–	+
Keim 51(USA)	–	–	–	–	–	+	–	–	+
Keim 55 (Australia)	–	–	+	–	–	+	–	–	–
Keim 57 (China, USA)	+	+	+	–	–	+	+	+	–
Keim 68 (USA)	–	–	+	–	–	–	–	–	–
Keim 80 (France)	–	–	–	+	–	–	–	–	–
Keim 87 (South Africa)	–	–	–	–	+	–	–	–	–
8002A (*Iowa State University*)	–	+	+	–	–	+	+	+	–

[a]Single-nucleotide polymorphism and/or mutation observed (+) or not observed (–); n/a – not used in analysis; n/d – not done.

3 samples and 180 bps in 1 sample. In samples Keim 80, Vito 3, and Vito 20 the following sequence was observed inserted between reference positions 276 and 277:

5´-276 – CTT CCT GTA TTT CTT AGT TTG TTT TGT AAT TCG ATT TCT CGT TCC GCT
GTT TGT CTT TCG CTT TTC ACA TTA CCC TGT TGC CCT TCT TCT – 277-3´

In Keim 87, a 180 bp insert between positions 276 and 277 was observed and appears to be the above sequence repeated twice in succession.

In the PS-39 (pX02) area of interest, a C ⟶ T SNP at reference nucleotide position 37994 was observed in a large number of samples as listed in Table I. Also in the PS-39 region of interest, a T ⟶ C SNP was observed at reference position 38 448 in the following samples A2012, Vito 1, Vito 12, Keim 57, 8002A. Interestingly, this mutation had not been previously reported by Read *et al.* (2002).

In the PS-51 area of interest, two differences were noted: an A ⟶ C SNP at reference nucleotide position 67 335 and a nucleotide deletion (T) observed at reference position 67 495. As there are a number of samples in this study that correlate with the above-mentioned differences, please refer to Table I for more detail.

In the entire battery of samples analyzed, the only samples that very closely resembled the Florida strain in the afore-mentioned sequence differences are Vito 1, Vito 12, Keim 57, and 8002A. Samples Vito 1 and Vito 12 are designated 0074 (North America) and A32 (Europe), respectively, in Brumlik *et al*. (2001). It is noteworthy that the A3b type from China (designated as Keim 57) is the same isolate for which Anthrax Vaccine Adsorbed (AVA, the only licensed human vaccine) affords no protection in guinea pigs (Coker *et al*. 2003). Isolate 8002A is of unknown geographical origin and was part of the recently destroyed *Iowa State University* collection.

Cross species and strain horizontal transfer of genes by temperate phages could confound genetic marker association with geographical distribution. Besides the natural mutation rate or one driven by nitration from the inflammatory process in the host, other causes of genetic variation have been noted in *B. anthracis* and its close relatives *B. cereus* and *B. thuringiensis* (Thorne 1968; Helgason *et al*. 2000; Hoffmaster *et al.* 2004). These bacteria share transducing bacteriophages that may become temperate and/or exist as plasmidal prophages and yield lysogenic strains that bear significantly different genotypes and, subsequently, phenotypes, further confusing the lineage and geographical origins of particular strains and isolates (Brown *et al*. 1955). They also possess transducing plasmids that can pass among these species (Battisti *et al.* 1985). *Bacillus* species, in general, can take up, and therefore share, free DNA as well (Lopez *et al.* 1982). An example of horizontal transfer was seen in an isolate ("K strain") from the *Bacterial Research Laboratory, Zoonoses Unit of the Baghdad Veterinary College*, which was originally a gift from *Al-Kindi Co*. for *Production of Veterinary Vaccines & Drugs*, and collected by the *United Nations Monitoring, Verification and Inspection Commission* (UNMOVIC) in March 2003. It was reported to be the 34 F2 Sterne spore vaccine strain.

Table II contains a list of the phenotypes and other bacillus species found in the K strain mixture and related species and strains from other sources for comparison. Isolates KM-10A and KM-10B were isolated from the livers of mice (*data not shown*) first inoculated with sublethal concentrations of Sterne strain anthrax spores (recovered from infection) followed by inoculation with anthrax spores derived from the K strain mixture (Kalns *et al*. 2002). Isolate KM-12 was collected from eschar skin lesions on the same mice. KM-10B is a hemolytic *B. anthracis*. PCR products of protective antigen and capsular gene primers were observed (*data not shown*). The K strain *B. anthracis* contains the pXO1, but not the pXO2 plasmid, but does contain a truncated capsular gene (probably chromosomally located). Two types of *B. cereus*, KJ and KJ Ery Halo, were isolated from the original mixture of the K strain. KJ Ery Halo was identical with the ATCC 4342 *Bacillus cereus* strain (Schuch *et al*. 2002). This latter one is of particular interest because it can be lysed by γ bacteriophages whereas other strains of *B. cereus* cannot (as KJ). The 17JB strain is a Pasteur Vaccine *2* strain, which contains both pX01 and pXO2 plasmids, and therefore, is fully pathogenic, although somewhat attenuated (Uchida *et al.* 1985). It differs from Pasteur Strain *1*, which is pXO2 positive used in Table I, Sterne vaccine strain which is pXO1 positive, and δ strains which lack both pathogenic plasmids. Table II illustrates the fact that some isolates from the K strain mixture contain bacteriophages that can be rescued by co-culture with Cherry γ phage under an agar overlay. Cherry γ phage is a genotype distinct from two other γ phages used in the diagnosis of anthrax (Fouts *et al.* 2006).

When MLVA-15 allelic patterns were generated from K5A and the two K *B. cereus* isolates (KJ and KJ Ery Halo) by PCR amplification, blindly in Paul Keim's laboratory, and compared to the Keim Genetics Laboratory MLVA-15 database, only a small subset of the VNTR loci were generated and they could not be identified as representing a particular *B. anthracis* strain. However, the other isolates from the K strain mixture (12 out of 19) were found to be genotypically identical to Sterne (Keim *et al*. 2004). The *B. cereus*

Table II. Phenotypic comparison of K strain isolates and related control strains of *Bacillus* species[a]

Isolates	β-Hemolysis	Cherry γ phage-spot test	Cherry γ phage-rescued	Penicillin G 10 U	Erythromycin 15 µg	Horse serum on TSB[b]
Controls						
Bacillus anthracis strain Sterne (spore vaccine)	–	+	–	S	S	–
Bacillus anthracis strain Sterne "A" γ-phage resistant[c]	–	–	–	S	S	–
Bacillus anthracis strain 17JB	–	+	+	S	S	+
Bacillus cereus strain 569	++	–	–	R	S	±
Bacillus cereus ATCC 4342	++	+	+	S	S	±
Bacillus cereus FRI 13[d]	++	–	–	R	S	–
Bacillus thuringiensis ssp. *Kurstaki*	+	–	–	R	S	–
Bacillus thuringiensis strain Al-Hakam	++	–	–	R	S	–
Bacillus thuringiensis sv. konkukian 97-27	++	–	–	R	S	–
Samples						
Bacillus cereus strain KJ	++	–	–	R	S	–
Bacillus cereus strain KJ Ery Halo	++	+	+	S	S	–
Bacillus strain K spore	–	±	–	S	S	–
Bacillus anthracis strain K5A	–	–	+	S	S	±
Bacillus anthracis strain KM-10A (liver)	–	±	+	S	S	–
Bacillus anthracis strain KM-10B (liver)	+[e]	+	+	S	S	–
Bacillus anthracis strain KM-12 (skin)	–	±	–	S	S	–

[a](++) – strongly positive, (+) – positive, (±) – weakly positive, (–) – negative; S – sensitive, R– resistant.
[b]Horse serum on trypticase soy agar facilitates capsule formation; highly glossy colony recorded as positive.
[c]In-house isolate cloned January 19, 2001.
[d]Obtained from *Food Research Institute, University of Wisconsin*.
[e]Exhibits temperature-sensitive hemolysis after overnight storage at 4 °C.

isolates were rightfully negative for *B. anthracis* genotypes but the K5A which should have been nearly identical to the other Sterne-like isolates (the afore-mentioned 12), especially since it was derived from the same culture, was not. K5A is phenotypically *B. anthracis* (Table II), even though, like the *B. cereus* components of the K strain mixture, it did not appear to be like any known anthrax bacillus. Also, limited sequencing of nitration survival genes of *B. anthracis* showed distinct differences between K and various reference strains (Parker and Kiel 2005). In Table III, these six strains were compared along an ≈1800 nucleotide sequence. There were 16 observed SNPs unique to the K strain, one inserted nucleotide unique to the K strain, and one instance where the K strain and the Pasteur 17 JB strain of anthrax shared a common nucleotide deletion.

Anthrax spreads very slowly from one site of spore deposition to another contiguous location, through infected animals that die during the periods when animals congregate around limited water and food supplies due to adverse weather conditions, such as drought. The spread is limited by how far animals can travel while sick and dying. Modern outbreak cycles seem to support this view. Human activity seems to have facilitated the worldwide distribution of anthrax. Since the number of spores in a location where an animal dies is so large, it is likely to always contain mutants, but be dominated by a singly selected clone based on host susceptibility and strain propagation in that particular host. Because other species of *Bacillus*, such as *B. thuringiensis* and *B. cereus*, are available in the same soil as *B. anthracis* and can share temperate bacteriophages and transducing plasmids, they can also share genetic material that not only causes the mixing of strain DNA but also confuses the speciation. Based on these observations and assumptions, it seems reasonable to conclude that geographical association with genetic markers, in itself, cannot determine, with any reasonable degree of certainty, the origin of a given isolate of *B. anthracis*.

Table III. Sequence data[a] of K strain compared with other *Bacillus anthracis* strains, *Bacillus cereus*, and *Bacillus thuringiensis*

Isolate/strain	Sequence (5´→3´)[b]
Section 1[c]	
Bacillus strain K spore	C S G G A A G T A A G T C T G
B. anthracis strain Sterne	G T G A T A G T A A G T - T G
B. anthracis strain 17JB	G T G A T A G T A A G T - T G
B. anthracis strain Ames	G T G A T A G T A A G T - T G
B. thuringiensis sv. konkukian (97-27)	G T G A T A G T A A G T - T G
B. cereus ATCC 14579	G T G A T A G T A A G T - T G
Section 2[d]	
Bacillus strain K spore	T G A T T T C C A T C C C G A C C T T T
B. anthracis strain Sterne	T G A T T T C C A T C C C G A C T T T T
B. anthracis strain 17JB	T G A T T T C C A T C C C G A C T T T T
B. anthracis strain Ames	T G A T T T C C A T C C C G A C T T T T
B. thuringiensis sv. konkukian (97-27)	T G A T T T C C A T T T C G A C T T T T
B. cereus ATCC 14579	T G A T T T C T A T C T C G A C T T T T
Section 3[e]	
Bacillus strain K spore	A A T T A G C G A A A A A - G T T A T
B. anthracis strain Sterne	A A T T A G C G A A A A A A A G T T A T
B. anthracis strain 17JB	A A T T A G C G A A A A A - G T T A T
B. anthracis strain Ames	A A T T A G C G A A A A A A A G T T A T
B. thuringiensis sv. konkukian (97-27)	A A T T A G C G A A A A A A A G T T A T
B. cereus ATCC 14579	A A T T A G C A A A A A A A A G T T A T

[a]Nucleotide differences as compared with reference – *Bacillus anthracis* strain Sterne, complete genome (AE017225.1).
[b]S – cytosine or guanine, according to the IUPAC rule.
Nucleotide range on reference strain: [c]3184269–3184256, [d]3183264–3183245, [e]3182909–3182891.

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