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Identification of *Bacillus anthracis* specific chromosomal sequences by suppressive subtractive hybridization

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Abstract

Background: *Bacillus anthracis*, *Bacillus thuringiensis* and *Bacillus cereus* are closely related members of the *B. cereus*-group of bacilli. Suppressive subtractive hybridization (SSH) was used to identify specific chromosomal sequences unique to *B. anthracis*.

Results: Two SSH libraries were generated. Genomic DNA from plasmid-cured *B. anthracis* was used as the tester DNA in both libraries, while genomic DNA from either *B. cereus* or *B. thuringiensis* served as the driver DNA. Progressive screening of the libraries by colony filter and Southern blot analyses identified 29 different clones that were specific for the *B. anthracis* chromosome relative not only to the respective driver DNAs, but also to seven other different strains of *B. cereus* and *B. thuringiensis* included in the process. The nucleotide sequences of the clones were compared with those found in genomic databases, revealing that over half of the clones were located into 2 regions on the *B. anthracis* chromosome.

Conclusions: Genes encoding potential cell wall synthesis proteins dominated one region, while bacteriophage-related sequences dominated the other region. The latter supports the hypothesis that acquisition of these bacteriophage sequences occurred during or after speciation of *B. anthracis* relative to *B. cereus* and *B. thuringiensis*. This study provides insight into the chromosomal differences between *B. anthracis* and its closest phylogenetic relatives.

Background

Bacillus anthracis, the etiological agent of anthrax, is responsible for a serious and often fatal disease of mammalian livestock and humans [8,22]. Its spore-forming capability and highly pathogenic nature have made it one

of the most effective bioterrorism agents. Pathogenic strains of *B. anthracis* contain two plasmids that confer virulence; the toxin-encoding 181.6-kb pXO1 [21,27,29] and the capsule-encoding 94.8-kb pXO2 [12,27,42]. Loss of either plasmid attenuates virulence. Environmental

isolates of virulent *B. anthracis* have been found to contain some pXO2⁻ cells indicating that spontaneous loss of this plasmid may occur outside the selective pressures of the host [11,41].

Bacillus cereus is commonly isolated from soil and has been implicated in food-related diseases [9]. *Bacillus thuringiensis* contains plasmids that encode an array of gene products including the insecticidal crystal proteins [39]. *B. anthracis*, *B. cereus* and *B. thuringiensis* are closely-related members of what has been referred to as the *Bacillus cereus*-group. Comparison of their 16S rRNA sequences place them within a range expected for members of the same species [3]. In addition, DNA-DNA hybridization [19,37] and pulsed field gel electrophoresis [14] studies have revealed great homology between their chromosomal DNAs. Recent data obtained from multilocus enzyme electrophoresis (MEE) and by multiple-locus sequence typing suggested that *B. anthracis*, *B. cereus*, and *B. thuringiensis* belong to one and the same species, and that salient distinguishing of each member is due to plasmid-borne genes [15].

Suppressive subtractive hybridization (SSH) is a PCR-based DNA subtraction method that enables the identification of genomic sequence differences between various strains or species of bacteria. Tester DNA is defined as that in which the differences are being sought relative to a driver DNA. For example, SSH has been successfully used to identify unique genomic sequences for specific strains of *Helicobacter pylori* [2], uropathogenic *E. coli* [18,43], and colonizing *Campylobacter jejuni* [1]. This technique has also been used to detect unique genomic sequences found in *E. coli* but not in *Salmonella typhimurium* [4], in pathogenic *Burkholderia pseudomallei* but not in nonpathogenic *B. thailandensis* [36], in pathogenic *Burkholderia mallei* but not in *B. thailandensis* [7] and in *B. anthracis* but not in *B. cereus* 14579T, *B. thuringiensis* Al Hakam, or *B. cereus* 3A [32]. This methodology has not only identified genetic differences among different strains or species of bacteria but also defined genes that contribute to the virulence of an organism. These virulence-related genes are often localized on the chromosome. Such clusters have been referred to as pathogenicity islands [13].

Herein we report the application of SSH to elucidate genomic differences of *B. anthracis* in comparison to *B. cereus* and *B. thuringiensis*, thereby identifying DNA sequences that are unique to the *B. anthracis* chromosome. In contrast with *B. anthracis* described as one of the most monomorphic species within the *B. cereus* group, *B. cereus* and *B. thuringiensis* present a vast range of genetic diversity. For this study we used the genomic DNA of *B. cereus* 14579T or *B. thuringiensis* 10792T as driver DNA because these are representative of the respective species. In

the subsequent screening of the *B. anthracis*-specific chromosomal sequences, we used several *Bacillus* sp. strains more closely-related to *B. anthracis* [15,31,33]. The *B. anthracis*-specific chromosomal sequences found elucidate genomic differences of these three closely-related bacteria. These sequences can be exploited for the development of more stable and robust chromosome-specific markers important for accurate identification of *B. anthracis* and unique from those previously reported [30-32].

Results

Screening of *B. anthracis*/*B. cereus* and *B. anthracis*/*B. thuringiensis* SSH libraries

SSH libraries were generated using *RsaI*-digested genomic DNA from *B. anthracis* strain 7700 (pXO1⁻, pXO2⁻) as the tester and either *RsaI*-digested genomic DNA from *B. cereus* 14579T or *B. thuringiensis* 10792T as the drivers. All clones derived from the *B. anthracis*/*B. cereus* (*Ba/Bc*) library were given a numerical designation, whereas those of the *B. anthracis*/*B. thuringiensis* (*Ba/Bt*) library commenced with an alphabetical designation. The subsequent screening process used for selection of clones containing *B. anthracis*-specific chromosomal DNA is outlined in Table 1.

Forty four hundred *Ba/Bc* and *Ba/Bt* clones were pre-screened for *B. anthracis*-specific sequences by comparing duplicate sets of colony filters. One set was probed with ³²P-labeled tester sequences, while the other set was probed with the corresponding ³²P-labeled driver sequences. Two hundred and twenty-four *Ba/Bc* and 189 *Ba/Bt* clones displayed more intense hybridization when probed with *B. anthracis* genomic DNA than with that of *B. cereus* or *B. thuringiensis*. Recombinant plasmid DNA of the 413 SSH clones selected was digested with *RsaI*, electrophoresed on agarose gels, Southern blotted, and then probed with the corresponding ³²P-labeled driver genomic DNA. Insert DNA sizes ranged between 100–2000 bp, as predicted from analysis of the frequency with which *RsaI* digests each of these *Bacillus* genomic DNAs. Ninety-six *Ba/Bc* and 59 *Ba/Bt* clones had inserts that were undetected by the driver DNA probe.

The insert DNA from each of these 155 candidate tester-specific clones was isolated, ³²P-labeled and used to probe DNA blots containing the *HindIII*-digested genomic DNAs of (1) *B. anthracis* strain 7700, (2) *B. thuringiensis* strain 97-27, (3) *B. cereus* strain 14579T and (4) *B. thuringiensis* strain 10792T. *B. thuringiensis* strain 97-27 genomic DNA was used because it is extremely similar to that of *B. anthracis* [15,16,28,32,33]. Thirty-two of the *Ba/Bc* clones and 20 *Ba/Bt* clones detected sequences of *B. anthracis* 7700 DNA only (data not shown).

Table 1: Progressive screening of SSH libraries.

SSH library	<i>B. anthracis</i> 7700 vs. <i>B. cereus</i> 14579T	<i>B. anthracis</i> 7700 vs. <i>B. thuringiensis</i> 10792T
Number SSH clones tested	2400	2000
Comparative colony filters selection (detection by tester probe > detection by driver probe)	224	189
Clone DNA blot analysis (selection of clones not detected by driver probe)	96	59
Initial genomic blot selection (blot contained <i>B. anthracis</i> 7700, <i>B. cereus</i> 14579T, <i>B. thuringiensis</i> 10792T and <i>B. thuringiensis</i> 97-27 genomic DNA probed with SSH clone insert DNA)	32	20
Survey genomic DNA blot selection of <i>B. anthracis</i>-specific clones (blot contained 4 strains of <i>B. anthracis</i> and 8 other strains of <i>B. cereus</i> and <i>B. thuringiensis</i> – see Figure 1 and 1)	27	12

Survey Southern blots containing samples of the HindIII-digested genomic DNAs of *B. anthracis* 7700, *B. anthracis* AC1, *B. anthracis* RA3, *B. anthracis* 7611, *B. thuringiensis* 97-27, *Bacillus* Ba813+ S8553/2, *Bacillus* Ba813+ III-BL, *B. cereus* F17289, *B. cereus* 14579T, *B. thuringiensis* CT43, *B. thuringiensis* huazhongensis, and *B. thuringiensis* 10792T were probed with the ³²P-labeled insert DNA of each of the 52 clones found to be *B. anthracis*-specific after initial genomic DNA blot screening. An example of this more extensive analysis is provided in Figure 1, demonstrating detection of sequence homology in the four *B. anthracis* genomic DNA samples only when probed with the insert of *Ba/Bt* clone E4. Clone E4 did not detect any homology in any of the *B. cereus*, *B. thuringiensis*, or other *Bacillus* species included on this blot.

A summary of the 52 clones tested in this manner is presented in Table 2 (see Additional file 1). Thirty-nine of the 52 clones were observed to be completely specific for *B. anthracis* (highlighted in bold type). All probes but one consistently hybridized and detected a similar pattern of restriction fragments found in all four *B. anthracis* genomic DNA samples which included pathogenic *B. anthracis* strains RA3 and 7611. Clone 140 was the exception in that it detected some polymorphism in the restriction fragment pattern displayed in the *B. anthracis* genomic DNA samples (data not shown). Thirteen SSH clones did detect nucleotide sequences in strains other than *B. anthracis*. Clones 14, 34, 140, O4, Z6, L7, and O7 hybridized with one other; clones 151, 227, G and R3 hybridized with two other; and clones A4 and K4 hybridized with three other *B. cereus* and / or *B. thuringiensis* genomic DNAs on the survey blot, exclusive of the driver strains.

Sequence analysis of *B. anthracis*-specific clones

Sequence determination and chromosomal location of *B. anthracis*-specific clones

The nucleotide sequence was determined for each insert of the 39 clones that were found to be *B. anthracis*-specific. Seventeen of the 39 clones demonstrated double, triple and as much as quadruple redundancy, finally sorting into 7 different *RsaI*-fragments (Table 2 and 3, see Additional file 1 and 2). Therefore, these 39 clones delineate 29 different *RsaI* fragments of the *B. anthracis* 7700 chromosome. BLAST analysis [24] sorted these 29 *RsaI* fragments into 6 different regions on the A2012 strain [34] chromosome (Figure 2). Areas on the chromosome delineated either by 13 or more clones, 3 clones, or one clone were designated as regions, clusters, or orphans, respectively. Regions I and II encompassed eight and thirteen different *B. anthracis*-specific *RsaI* fragments that were identified by thirteen and sixteen clones, respectively. Three other locations were found to encompass two or three different *B. anthracis*-specific *RsaI* fragments. Clones 81/231 and F7 define Cluster I; clones 230, 84 and 135 define Cluster II; and clones 9/49 and 221 define Cluster III. Single *B. anthracis*-specific orphan clone M7 identified the remaining chromosomal area.

Annotation of Region I and II delineated by the *B. anthracis*-specific clones

Open reading frames (ORFs) overlapping and/or bordering *B. anthracis*-specific sequences were identified [26] and the amino acid sequences derived from these ORFs were compared to other protein sequences [25]. The results of these analyses are summarized in Table 3 (see Additional file 2).

Region I, delineated by 8 *B. anthracis*-specific *RsaI*-fragments and consisting of an area of 14.5 kb, contains 10 potential ORFs. Nine of these ORFs demonstrated

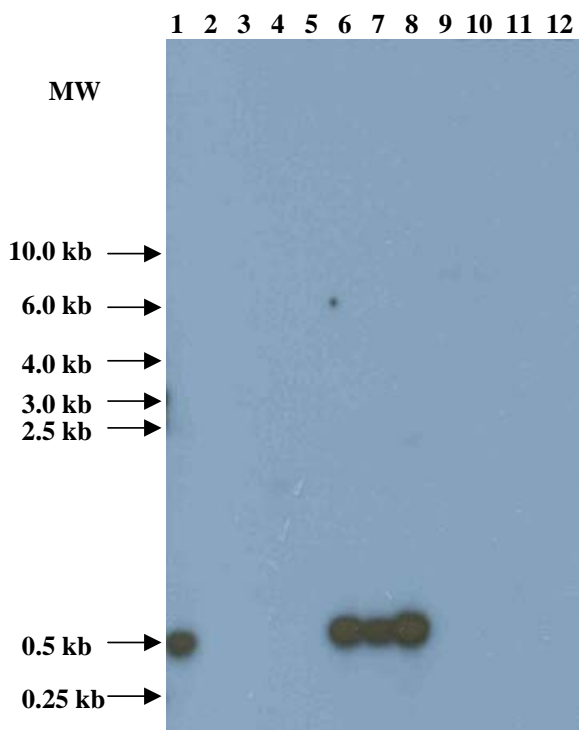


Figure 1
Survey genomic DNA blot probed with clone E4 insert DNA. A total of 1 µg of genomic DNA digested with 70 U of *Hind*III to completion was used per lane. Lanes 1, *Bacillus anthracis* 7700; 2, *Bacillus thuringiensis* (97-27); 3, *Bacillus cereus* F17289; 4, *Bacillus thuringiensis huazhongensis*; 5, *Bacillus thuringiensis* CT43; 6, *Bacillus anthracis* AC1; 7, *Bacillus anthracis* RA3; 8, *Bacillus anthracis* 7611; 9, *Bacillus* spp. Ba813 (S8553/2); 10, *Bacillus* spp. Ba813 (III-BL); 11, *Bacillus cereus* 14579; 12, *Bacillus thuringiensis* 10792. The positions of molecular weight markers are indicated on the left side.

significant sequence similarity with genes encoding enzymes and proteins involved in cell wall biosynthesis or export of polysaccharides in other bacterial species. The G + C content of the DNA encompassing the SSH-selected sequences in this region was 32.1% which is not significantly different from that of the entire *B. anthracis* chromosome, reported to be 35.4% [35].

Region II, which encompassed an area of 24.5 kb flanked by clones H4 and 150b, contains 40 potential ORFs. Nineteen of these ORFs had sequence homology with a

variety of bacteriophage-related gene products. Ten of the 13 *B. anthracis*-specific *Rsa*I-fragments overlapped with these bacteriophage-related sequences. No functional match could be assigned to 21 of the potential ORFs interspersed with the 19 bacteriophage-related ORFs. However, detection of 13 of these potential ORFs by overlapping SSH clones suggests they contain *B. anthracis*-specific DNA. Analysis of the flanking chromosomal sequences reveals other bacteriophage-related ORFs 7.5 kb upstream and 5 kb downstream of this region, including a third ORF homologous to phage integrase gene sequences. Outside of these limits, potential ORFs had homology with housekeeping genes found to be conserved in other *Bacillus* species. For example, ORF BA_1004, homologous with the *YjfO* gene (RNA methyltransferase) of *B. subtilis*, was found closest upstream, while ORF BA_1064 homologous to the *RocR* gene (transcriptional activator of the arginine utilization operon) of *B. subtilis* was found downstream. The G + C content of the chromosomal region defined by these *B. anthracis*-specific clones was 35.3%. Again this is not significantly different from the average G + C content of the *B. anthracis* chromosomal DNA. In the recently published genome sequence of *B. anthracis* Ames strain Region II is contained within the area designated "*B. anthracis* phage lambda Ba01" [35].

Annotation of clustered and isolated genes delineated by the *B. anthracis*-specific clones

Cluster III, encompassing an area of 30 kb, was defined by 2 *B. anthracis*-specific *Rsa*I-fragments. This region contains 43 potential ORFs. Nineteen of the ORFs had sequence homology with bacteriophage-related gene products. *Bacillus anthracis*-specific *Rsa*I-fragments derived from SSH clones 9/49 and 221, overlapped 2 of these bacteriophage-related sequences. No sequence similarity could be assigned to 17 of the 43 potential ORFs located in this region, three of which had sequences that overlapped with SSH clones. Also found in this cluster were 3 ORFs (BA_4580/ *YndN* gene *B. subtilis*; BA_4583/ *dUTPase*; BA_4585/ *RspF* gene *B. subtilis*) encoding *B. anthracis* homologues for gene functions probably conserved in most species of *Bacillus*. No SSH-selected clones mapped to these genes. Analysis of the chromosomal sequences bordering Cluster III revealed other bacteriophage-related ORFs up to 2.6 kb upstream and 7 kb downstream. Two ORFs that were homologous to a phage integrase gene sequences were found, one in each flank. Open reading frames outside of these limits demonstrated homology with housekeeping genes found conserved in other *Bacillus* species. For instance, ORF BA_4538, the *FtsK_SpoIIIE* gene, was found closest upstream; while ORF BA_4601, homologous to the *YlbM* gene of *B. subtilis* was situated closest downstream. The G + C content of the DNA encompassing the SSH clone sequences mapped to this

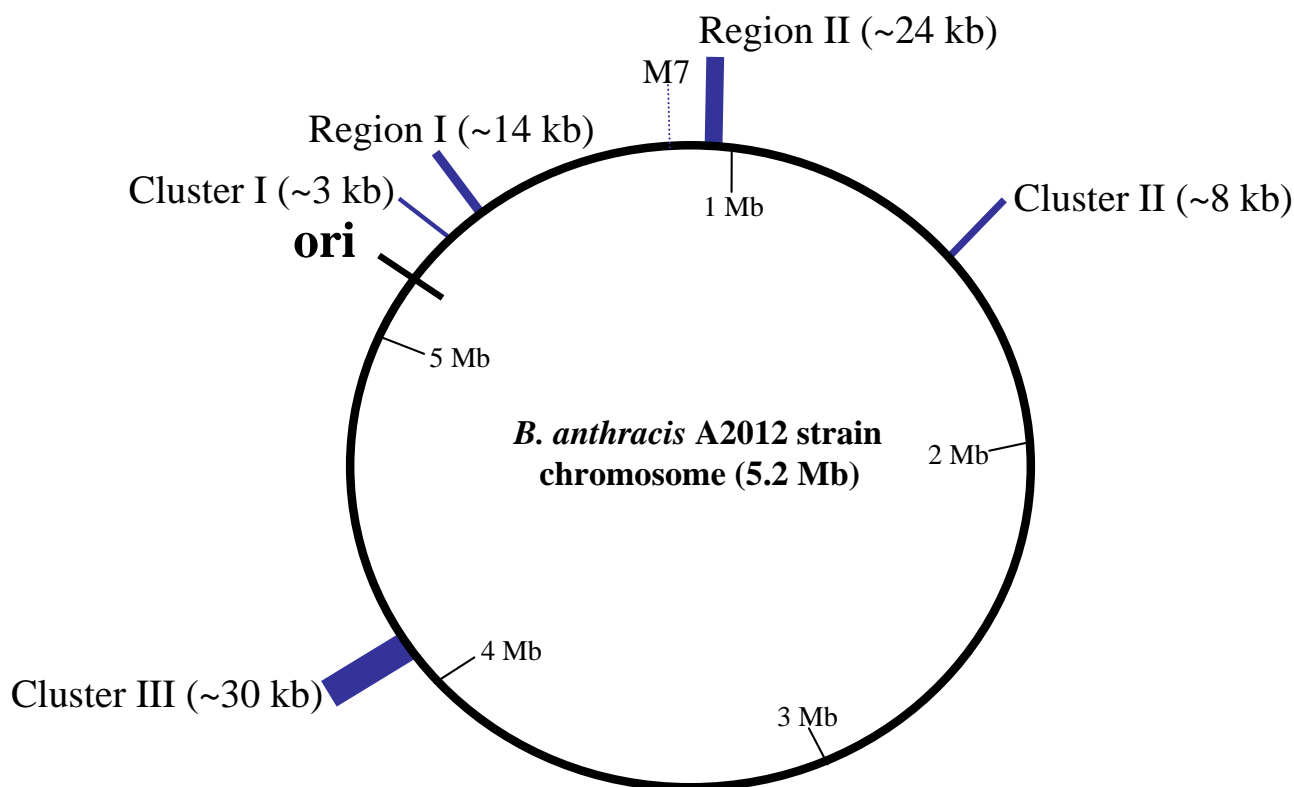


Figure 2

Distribution of the SSH-selected region, cluster and orphan areas on the *B. anthracis* A2012 strain chromosome. Megabase (Mb) distances are estimated on the inside of the circular chromosome.

third cluster was determined to be 35.0%. Again, this is not significantly different from the average G + C content of the remainder of the *B. anthracis* chromosome. Cluster III is contained within the area designated "*B. anthracis* phage lambda Ba03" of the *B. anthracis* Ames strain genome [35].

Cluster I contains a bacteriophage-related sequence that was detected by *B. anthracis*-specific clones 81/231, while clone F7 sequences overlapped with 3 potential ORFs encoding hypothetical proteins found to have no homology with any known proteins. These clones are contained within the area designated "*B. anthracis* phage lambda Ba02" of the *B. anthracis* Ames strain genome [35].

In Cluster II clone 230 was derived from an ORF displaying full-length homology with the *wapA* gene of *B. subtilis*

encoding a cell wall-associated protein. Clones 84 and 135, also located in Cluster II, overlapped potential ORFs encoding hypothetical proteins with no known function.

Finally, orphan clone M7 overlapped with an ORF demonstrating homology to a gene encoding a Nitrogen regulation protein in *B. subtilis*.

Comparison with published *B. anthracis*-specific SSH-selected clones

A comparable SSH analysis was recently reported using the genomic DNAs of *B. anthracis* as the tester and of either *B. cereus* 14579T, *B. thuringiensis* Al Hakam or *B. cereus* 3A as the driver [32]. Of the 28 *B. anthracis*-specific clones reported only 3 share nucleotide sequences with 3 of the 29 *B. anthracis*-specific *RsaI* fragments presented in this study (clones M. Ctg032, dhp61.183, and dhp64.188 with clones 55/67/212/235, 81/231 and 9/49,

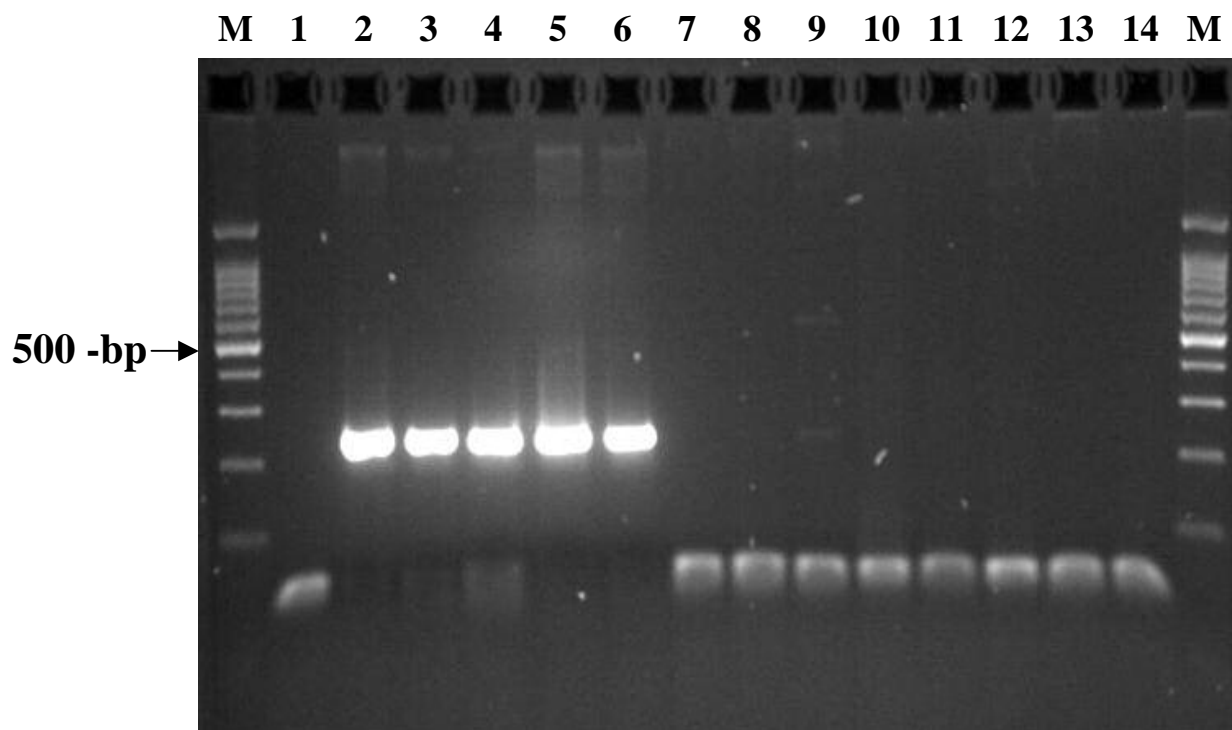


Figure 3

Gel electrophoresis analysis of PCR products generated after reaction of various *Bacillus* genomic DNAs with clone E4 derived primers. Lanes, M: Molecular weight markers, 100-bp DNA ladder; 1, Negative Control (no DNA); The samples are: 2, *Bacillus anthracis* A5; 3, *Bacillus anthracis* Sterne; 4, *Bacillus anthracis* A58; 5, *Bacillus anthracis* A16; 6, *Bacillus anthracis* 0074; 7, *Bacillus cereus* 14579; 8, *Bacillus cereus* 23261; 9, *Bacillus thuringiensis* 10792; 10, *Bacillus thuringiensis* B8; 11, *Bacillus* spp. Ba813 (9594/3); 12, *Bacillus* spp. Ba813 (S8553/2); 13, *Bacillus mycooides* 21929; 14, *Bacillus thuringiensis* 35646.

respectively). Therefore, 26 of the SSH clones reported here offer newly defined *B. anthracis*-specific chromosomal sequences. Five clones found in the other study (dhp61.178, dhp77.002, M. Ctg037, R. Ctg177, and dhp73.022) were proximal to (within the same ORF) 5 of the *B. anthracis*-specific *RsaI* fragments listed in this paper (clones 03, 5,198,150b, and 9/49, respectively). This comparison is summarized in the last column of Table 3 (see Additional file 2), as well as the letter designation of the corresponding genomic island. Thus, the genomic islands designated as A, B, C, and E [32] correlate with the locations of Cluster I, Region I, Region II and Cluster III, respectively. In addition, clone dhp77.047 is located in Region II upstream of clone H4, but within the confines of the bacteriophage-related sequences (phage lambda Ba01). Finally, clones dhp61.181 and R. Ctg152 are found

within the area of Cluster III with phage lambda Ba03 sequences [35].

Diagnostic screening of *Bacillus* strains by PCR amplification using clone E4-specific primers

B. anthracis-specific clone E4 sequences were used to design oligonucleotide primers for PCR screening of a larger sampling of *Bacillus* strains. The expected size of the anticipated PCR product was 235 bp. PCR amplifications were performed on (i) 14 *B. anthracis* strains isolated from different geographical locations and harboring both, one, or neither of the pX01 and pX02 plasmids, (ii) 34 closely-related *B. cereus* and *B. thuringiensis* strains, including 14 *Bacillus* sp. Ba813⁺ transition strains, and (iii) a *B. mycooides* strain (see Table 2). The same set of strains was used to evaluate a *B. anthracis* chromosome-specific PCR assay that exploited polymorphisms found in the *rpoB*

Table 4: *Bacillus* species used for SSH, DNA blot and PCR screening, respectively.

<i>Bacillus</i> Species	Strain ID	pX01	pX02
<i>Bacillus anthracis</i>	7700	-	-
<i>Bacillus cereus</i>	14579T*	N/A	N/A
<i>Bacillus thuringiensis</i>	10792T*	N/A	N/A
<i>Bacillus anthracis</i>	AC1	+	-
<i>Bacillus anthracis</i>	RA3	+	+
<i>Bacillus anthracis</i>	7611	+	+
<i>Bacillus cereus</i>	F17289*	-	-
<i>Bacillus thuringiensis</i>	<i>huazhongensis</i> *	N/A	N/A
<i>Bacillus thuringiensis</i>	CT43*	N/A	N/A
<i>Bacillus thuringiensis</i>	97-27*	-	-
<i>Bacillus</i> sp. Ba813 ⁺	S8553/2*	-	-
<i>Bacillus</i> sp. Ba813 ⁺	III-BL	-	-
<i>Bacillus anthracis</i>	Sterne	+	-
<i>Bacillus anthracis</i>	A5	+	+
<i>Bacillus anthracis</i>	A12	+	+
<i>Bacillus anthracis</i>	A16	+	+
<i>Bacillus anthracis</i>	A18	-	+
<i>Bacillus anthracis</i>	A19	-	+
<i>Bacillus anthracis</i>	A30	+	+
<i>Bacillus anthracis</i>	A32	+	+
<i>Bacillus anthracis</i>	A33	+	+
<i>Bacillus anthracis</i>	A39	+	+
<i>Bacillus anthracis</i>	A43	+	+
<i>Bacillus anthracis</i>	A58	-	-
<i>Bacillus anthracis</i>	9066	+	+
<i>Bacillus anthracis</i>	0074	+	-
<i>Bacillus cereus</i>	13472	N/A	N/A
<i>Bacillus cereus</i>	19637	N/A	N/A
<i>Bacillus cereus</i>	23261	N/A	N/A
<i>Bacillus cereus</i>	F17202	-	-
<i>Bacillus cereus</i>	F16959	-	-
<i>Bacillus cereus</i>	UW-85	N/A	N/A
<i>Bacillus thuringiensis</i>	35646	N/A	N/A
<i>Bacillus thuringiensis</i>	B8	N/A	N/A
<i>Bacillus thuringiensis</i>	H1	N/A	N/A
<i>Bacillus thuringiensis</i>	H3	N/A	N/A
<i>Bacillus thuringiensis</i>	H4	N/A	N/A
<i>Bacillus thuringiensis</i>	H13	N/A	N/A
<i>Bacillus thuringiensis</i>	H29	N/A	N/A
<i>Bacillus thuringiensis</i>	H39	N/A	N/A
<i>Bacillus thuringiensis</i>	H43	N/A	N/A
<i>Bacillus</i> sp. Ba813 ⁺	#4	-	-
<i>Bacillus</i> sp. Ba813 ⁺	#13	-	-
<i>Bacillus</i> sp. Ba813 ⁺	#17	-	-
<i>Bacillus</i> sp. Ba813 ⁺	#18	-	-
<i>Bacillus</i> sp. Ba813 ⁺	#21	-	-
<i>Bacillus</i> sp. Ba813 ⁺	#29	-	-
<i>Bacillus</i> sp. Ba813 ⁺	#3403	-	-
<i>Bacillus</i> sp. Ba813 ⁺	#6681	-	-
<i>Bacillus</i> sp. Ba813 ⁺	9594/3	-	-
<i>Bacillus</i> sp. Ba813 ⁺	11614/2	-	-
<i>Bacillus</i> sp. Ba813 ⁻	IB/A	-	-
<i>Bacillus</i> sp. Ba813 ⁻	Zimbabwe	-	-
<i>Bacillus mycoides</i>	21929	-	-

*used for both DNA blot and PCR assay; N/A – not applicable

gene [31]. Without exception, all 14 *B. anthracis* strains analyzed resulted in the amplification of the expected size PCR product, while all 35 closely-related *B. cereus*, *B. thuringiensis* and *B. mycoides* strains analyzed did not yield any significant amplification product. An electrophoretic representation of these results is presented in Figure 3.

Discussion

Two SSH libraries were generated that identified chromosomal DNA sequences found in *B. anthracis* and not in *B. cereus* or *B. thuringiensis*. *B. anthracis* 7700 DNA was used as the tester DNA, and total genomic DNA of either *B. cereus* 14579T or *B. thuringiensis* 10792T served as the drivers. Progressive screening by colony filter and Southern blot analyses identified 39 clones that were specific for the *B. anthracis* chromosome relative to the driver strains of *B. cereus* and *B. thuringiensis* and seven other more closely-related strains of *B. cereus* and *B. thuringiensis*. Since *B. anthracis* 7700 is cured of all plasmids, only chromosomal sequences were identified. The *B. anthracis*-specific clones' insert DNAs were sequenced, mapped to the *B. anthracis* chromosome, and annotated. Seventeen clones shared insert sequence, resulting in a final count of 29 *B. anthracis*-specific *RsaI*-fragment inserts. Interestingly, four of these seven redundant inserts were derived from both driver libraries. The 29 insert sequences are distributed over 6 different areas on the *B. anthracis* chromosome, with 8 and 13 of them being found in Regions I and II, respectively. Seven insert sequences were distributed into 3 clusters. The remaining clone was located singly on the chromosome.

Region I contains ORFs that had significant similarity with genes encoding enzymes and proteins involved in cell wall polysaccharide biosynthesis. Typically in this process, phosphorylated sugar molecules are first activated by conversion into nucleotide sugar derivatives. Enzymes, such as epimerases and dehydrogenases, may then modify these nucleotide sugar precursors into the distinctive sugar subunit(s) characteristic of the cell wall of a particular bacterial species. Transferases may then catalyze the sequential transfers of the sugar residues from their nucleotide carriers to a membrane-bound acceptor, usually a lipid carrier, forming the characteristic carbohydrate repeating unit. This first stage of cell wall polysaccharide biosynthesis usually occurs in the cytoplasm at the cytoplasmic side of the plasma membrane. At this point, an ABC transporter cassette may permit the lipid-linked carbohydrate-repeating unit to pass through the membrane. This two-component system consists of a translocation permease protein with membrane-spanning domains and an associated ATP-binding protein that energizes the permease.

In Region I potential ORFs BA-0356/7/8 and BA_0361/2, demonstrating sequence homology to genes encoding an epimerase and a dehydrogenase, respectively, were identified by SSH clones 190/N, Y4 and 142/16. Potential ORFs BA_0365/6 and BA_0371, detected by SSH clones 55/67/212/235, 66 and P3, demonstrated sequence similarity to genes encoding different types of transferases. Clone 190/N and Y4 inserts also overlapped with potential ORFs BA_0359 and BA_0360, which displayed significant sequence similarity to other known ABC transporter cassette proteins. The best match was with *B. subtilis* genes *tagG* and *tagH*, involved in the translocation of cell wall component teichoic acid. However, the corresponding *B. anthracis* potential ORFs cannot encode equivalent functional homologs. *B. subtilis* cell wall-associated teichoic (TA) and teichuronic acid (TUA) are absent in *B. anthracis* [23]. The remaining potential ORFs (BA_0363/4 and BA_0368/9 and BA_0370 detected by clones 201, 55/67/212/235, O3, G and P3) in this region were found to share homology with proteins involved in cell wall synthesis whose specific functions are currently unknown. These same ORFs were also found to be specific for *B. anthracis* A2012 by whole genome comparison with *B. cereus* 14579T [17].

The Gal-NAG polysaccharide, derived from sugar components D-galactose and N-acetyl-D-glucosamine, has been demonstrated by monoclonal antibodies to be uniquely associated with the cell wall of *B. anthracis* and a few *B. cereus* strains [10]. This result was also obtained for *B. anthracis* pX01- and pX02-strains, indicating that the enzymes and proteins necessary for synthesis and export of this *B. anthracis*-specific polysaccharide are located on the chromosome. It is possible that the Gal-NAG polysaccharide, or other components associated uniquely with the cell wall of *B. anthracis*, are synthesized by enzymes and exported by proteins encoded by genes specific to *B. anthracis*. Such specific genes might be detected by SSH as the genes associated with Region I have been. Substantiation of this hypothesis will require functional analysis of the Region I genes. It is interesting to note though that genes encoding cell wall biosynthesis proteins have been selected in many other subtractive hybridization experiments [1,4,5,7,18,36].

Bacteriophage-related sequences dominated *B. anthracis*-specific Regions II and Cluster III delineated by 15 SSH-selected *RsaI* fragments. Potential ORFs within and flanking Regions II and Cluster III included some identified as bacteriophage integrases/recombinases. Such enzymes could have facilitated the integration of these sequences into the chromosome of *B. anthracis*. The putative ORFs of Regions II and Cluster III encoding proteins of unknown function may be bacteriophage sequences that have degenerated beyond recognition by database comparison.

PCR analysis indicated that the clone E4 sequence of Region II is conserved in and is specific for *B. anthracis*. Also, phage lambda Ba01 and Ba03 sequences, which include Regions II and Cluster III, respectively, were found by comparative genome hybridization between the *B. anthracis* Ames strain and 19 other members of the *B. cereus* group to be absent in all but *B. anthracis* [35]. These results suggest that acquisition of bacteriophage-related Region II and Cluster III occurred during or after speciation of *B. anthracis* relative to *B. cereus* and *B. thuringiensis*, *i.e.* during or after the acquisition of plasmids pX01 and pX02, the distinguishing attributes of *B. anthracis*. The G + C content of Region II (35.3 %) and Cluster III (35.0%) do not differ significantly from the genome-wide average of 35.4%. This indicates that these regions may have been (i) acquired from bacteriophage with similar G + C content or (ii) acquired so long ago that they amalgamated with the *B. anthracis* genome, attaining an indistinguishable G + C content by amelioration [20].

For the most part the remaining *B. anthracis*-specific clones correlated again with ORFs encoding (i) cell surface molecules (detected by clone 230), (ii) other bacteriophage-related sequences (detected by clone 81/231), (iii) conserved hypothetical proteins (detected by clones 135 and M7) or (iv) hypothetical proteins of unknown function (detected by clones 81/231, F7, 84,135, and M7).

In conclusion, the present study revealed differences between the chromosome of *B. anthracis* and those of eight strains of *B. cereus* and *B. thuringiensis*. The general differences were seen in genes that may encode proteins related to cell wall synthesis and bacteriophages as well as ORFs encoding proteins of unknown function.

Methods

Bacterial strains and genomic DNA preparation

The *B. anthracis*, *B. cereus* and *B. thuringiensis* strains used in this study are listed in Table 2. The bacterial genomic DNA was extracted by a method described elsewhere [6].

Suppressive subtractive hybridization

SSH was performed using the PCR-Select Bacterial Genome Subtraction Kit as per manufacturer's instructions (Clontech, Palo Alto, CA). Generation of SSH libraries also required the use of the TA Cloning Kit (Invitrogen, Carlsbad, CA) with vector pCR2.1 as per manufacturers instructions.

Screening of suppressive subtractive libraries

Transformed *E. coli* TOP10F' cells were plated out on selective LB (Luria-Bertani) medium containing X-Gal, IPTG and either ampicillin (100 µg/ml) or kanamycin (50 µg/ml) [38]. White colonies were streaked onto master

plates and two sets of nitrocellulose filters (Millipore, Billerica, MA) on LB/kanamycin plates. Filters were treated with 10% (w/v) SDS, denature (0.4 N NaOH, 0.6 M NaCl), neutralize (0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl) and 2X SSPE (20 mM EDTA, 0.2 M sodium phosphate, pH 7.4, 3.6 M NaCl) solutions for 5 min. each, air dried and baked at 80°C under vacuum for 2 hrs. Filters were prehybridized in 5 × Denhardt solution at 65°C for at least four hours before hybridization in 5 × SET (0.75 M NaCl, 0.1 M Tris-HCl, pH 7.8, 5 mM EDTA), 1 × Denhardt, 0.5 % (w/v) SDS, 12.5% dextran sulfate, and 25 µg/ml denatured salmon sperm DNA at 65°C overnight. *Rsa*I-digested *B. anthracis*, *B. cereus*, and *B. thuringiensis* genomic DNAs were labeled with ³²P (Random-Primer Labeling System, Roche Molecular Biochemicals, Indianapolis, IN) and used to probe the two sets of colony filters for each SSH library.

Plasmid DNA preparation and insert fragment isolation

Plasmid DNA was isolated from 1–5 ml LB or TB/kanamycin cultures by either the boiling miniprep method [38] or the Plasmid Mini Kit (Qiagen, Valencia, CA). Insert fragments to be used as probes for genomic DNA blots were separated from vector sequences on 0.7% agarose gels and extracted by electroelution [38].

DNA blot analysis

One microgram of DNA was digested with the appropriate restriction enzyme (Life Technologies, Rockville, Md.), subjected to electrophoresis in 0.7% (w/v) agarose gels and transferred to Genescreen Plus nylon membranes (New England Nuclear Life Science Products, Inc., Boston, MA) as per manufacturer's instructions. DNA blots were prehybridized and hybridized at 65°C in 330 mM sodium phosphate, pH 7, 10 mM EDTA, 5% (w/v) SDS, 10% (w/v) dextran sulfate and salmon sperm DNA at 25 µg/ml. Filters were washed twice for 30 min. intervals at 65°C in 2 × SET, 0.5% SDS and autoradiographed.

Sequencing and bioinformatics

Each clone was sequenced in duplicate with the M13 forward and reverse primers using an ABI377 automated sequencer (Applied Biosystems, Boston, MA) and the BigDye terminator ready reaction kit (Perkin-Elmer Applied Biosystems, Foster City, CA). The nucleotide sequences were edited and assembled with the Sequencing Analysis 3.0 and AutoAssembler 3.1.2 programs. The SSH DNA sequences were subsequently localized on the A2012 strain chromosome [24] and annotated [25,26].

PCR amplification of the sequences identified by SSH clone E4

The sequences of primer E4F (5'-AGC AAA AGC TAA CTG CTC GGG-3') and E4R (5'-TCT ACC CCT TCA GGA TTA GCG-3') were derived from SSH clone E4 sequences

found 235 bp apart. The PCR reaction of 25 µl consisted of 10 mM Tris-HCl (pH 9.2), 75 mM KCl, 1.5 mM MgCl₂, 0.2 mM of dNTPs (Boehringer Mannheim Corp., Indianapolis, IN), 1 µM of each of primers E4F and E4R, 0.05 U/µl of AmpliTaq[®] DNA polymerase (Perkin Elmer Corp., Foster City, CA), and 100 ng of genomic DNA template. Amplification was performed in a GeneAmp PCR System 2400 (Perkin Elmer) and the cycling conditions were: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec., 64 °C for 30 sec., and 72 °C for 30 sec., and finishing with a final extension at 72 °C for 7 min. The PCR assays were performed on 14 *B. anthracis* strains isolated from different geographical locations and harboring any combination of the pXO1, pXO2 plasmids, as well as 35 closely-related *Bacillus* strains, including 14 *Bacillus* spp. Ba813⁺ strains (Table 2). The amplicons were electrophoresed in 2% (w/v) SeaKem GTG agarose (FMC Bio-products, Rockland, ME) with 40 mM Tris-acetate-1 mM EDTA (pH 8.3) running buffer and visualized by ethidium bromide staining.

Authors' contributions

KGD solely made both SSH libraries, screened the first 800 clones, did all ³²P hybridizations for colony filters and Southern blots, performed all of the annotation analysis, and served as primary author on the text of this paper. JAS was the primary co-researcher for screening of the second 800 SSH clones batch. AL acted as primary co-researcher for screening of the third 800 SSH clones batch. JML acted as primary co-researcher for screening of the last 2000 SSH clones. JB performed SSH clone E4 PCR detection analysis of 49 *Bacillus* strains. GP provided the *Bacillus* DNA with the help of LW. RR served as consultant early in the project. GP and VGD supervised the sequencing and acted as consultants.

Additional material

Additional File 1

Table 2. Survey genomic DNA blot results. Indication of detection (+) or no detection (-) by each ³²P-labeled insert of the 52 SSH-selected clones used to probe survey Southern blots containing samples of HindIII-digested genomic DNAs of *B. anthracis* 7700, *B. anthracis* AC1, *B. anthracis* RA3, *B. anthracis* 7611, *B. thuringiensis* 97-27, *Bacillus* Ba813⁺ S8553/2, *Bacillus* Ba813⁺ III-BL, *B. cereus* F17289, *B. cereus* 14579T, *B. thuringiensis* CT43, *B. thuringiensis* huazhongensis, and *B. thuringiensis* 10792T.

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Additional File 2

Table 3. Annotation of the 29 *B. anthracis*-specific SSH clones. For each of the 29 *B. anthracis*-specific SSH clones reported in this paper this table provides their *B. anthracis* A2012 chromosome coordinates, overlapping ORF designations, closest homolog genes with putative function, e value, accession number and comparative results with other recently published *B. anthracis* SSH study [32].

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