Glycosyltransferase – a specific marker for the discrimination of *Bacillus anthracis* from the *Bacillus cereus* group

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Bacillus anthracis, the aetiological agent of anthrax, has been taxonomically classified with the *Bacillus cereus* group, which comprises *B. cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycooides* and *Bacillus weihenstephanensis*. Although the pathogenesis and ecological manifestations may be different, *B. anthracis* shares a high degree of DNA sequence similarity with its group member species. As a result, the discrimination of *B. anthracis* from its close relatives in the *B. cereus* group is still quite difficult. Suppression subtractive hybridization (SSH) was performed to search for genomic differences between a *B. anthracis* Korean isolate CR and the most closely related *B. cereus* type strain KCTC 3624^T. Two-hundred and five *B. anthracis* CR clones obtained by SSH underwent Southern hybridization, and comparative sequences were analysed using the BLAST program from the National Center for Biotechnology Information (NCBI). Subsequently, primer sets based on the glycosyltransferase group 1 family protein gene specific to *B. anthracis* were designed from the sequences of subtracted clones, and their specificities were evaluated using eight *B. anthracis*, 33 *B. cereus*, 10 *B. thuringiensis*, six *B. mycoides*, one *B. pseudomycooides*, one *B. weihenstephanensis* and 19 strains from 11 other representative *Bacillus* species. PCR primers specific for the glycosyltransferase group 1 family protein gene did not amplify the desired products from any of the *Bacillus* strains under examination, except *B. anthracis* alone. These findings may be useful in the future development of efficient diagnostic tools for the rapid identification of *B. anthracis* from other members of the *B. cereus* group.

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INTRODUCTION

The *Bacillus cereus* group comprises *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycooides* and *Bacillus weihenstephanensis* (Ash *et al.*, 1991; Lechner *et al.*, 1998; Nakamura, 1998). *B. anthracis*, *B. cereus* and *B. thuringiensis* were the first three species described (Priest *et al.*, 2004), and they are not easily distinguishable from one other on the basis of phenotypic or genotypic traits. Methods used to discriminate *B. cereus*

group species based on phenotypic traits include motility, biochemical testing (Logan *et al.*, 1985; Odendaal *et al.*, 1991), antibiotic susceptibility testing (Mohammed *et al.*, 2002), direct fluorescent antibody staining of capsule and cell wall polysaccharide (Sastry *et al.*, 2003), lysis of colonies by gamma phage (Logan *et al.*, 1985; Schuch *et al.*, 2002), and animal virulence testing (Odendaal *et al.*, 1991). Although these species share a high degree of genetic relatedness, attempts have been made to distinguish *B. cereus* group member strains as demonstrated by whole-genome DNA–DNA hybridization (Read *et al.*, 2003), 16S and 23S rRNA gene sequencing (Ash *et al.*, 1991; Ash &

Abbreviations: AFLP, amplified fragment length polymorphism; SSH, suppression subtractive hybridization.

Collins, 1992), *gyrB*–*gyrA* (Harrell *et al.*, 1995) or 16S–23S rDNA intergenic spacer region comparisons (Daffonchio *et al.*, 1998; Cherif *et al.*, 2003), PFGE analysis (Carlson *et al.*, 1994), variable number tandem repeat mapping (VNTR; Andersen *et al.*, 1996; Keim & Smith, 2002), BOX-PCR fingerprinting (Kim *et al.*, 2002), and multilocus sequence typing (MLST) (Helgason *et al.*, 2000, 2004; Priest *et al.*, 2004).

This diverse range of methods has failed to reveal consistent differences between isolates to warrant segregating *B. anthracis*, *B. cereus* and *B. thuringiensis* into discrete species (Read *et al.*, 2003); separate species status is maintained because of their pathogenic traits (Priest *et al.*, 2004). Although PCR-based methods can differentiate vaccine or fully virulent *B. anthracis* genotypes, there is difficulty in distinguishing plasmid-cured *B. anthracis* or near-neighbour species. Furthermore, these techniques lack assay specificity for the detection of the *B. anthracis* chromosome (Daffonchio *et al.*, 1999) and can yield false-positive results (Ellerbrok *et al.*, 2002).

Suppression subtractive hybridization (SSH) has been used to analyse genomic differences (Radnedge *et al.*, 2003) and compare pathogenic and non-pathogenic bacterial strains to identify possible virulence factors among bacterial strains (Agron *et al.*, 2002). The advantage that SSH has over microarray DNA–DNA hybridization (Volokhov *et al.*, 2004) is that the latter identifies only those genes present in the reference genome and not genes which are absent (Jones *et al.*, 2006). The objective of this study was to use

SSH to search for genomic differences between the *B. anthracis* Korean isolate CR and the most closely related *B. cereus* type strain KCTC 3624^T, and to identify sequences useful for rapid *B. anthracis* identification and diagnosis.

METHODS

Bacterial strains. A total of 78 bacterial strains were used in this study and are listed in Table 1. *B. anthracis* ATCC 14578^T, ATCC 14185 and ATCC 14186 were obtained from the American Type Culture Collection (ATCC), and Korean isolates of *B. anthracis* CR and BC were provided by the National Veterinary Research and Quarantine Service (Anyang, Korea). The recent outbreak strain of *B. anthracis* CR was isolated from beef eaten by a patient who died from gastrointestinal anthrax in 2000, and *B. anthracis* CAU 1, CAU 2 and CAU 3 were isolated in our laboratory from patients in 1994. The remaining 70 *Bacillus* strains comprised isolates from our laboratory and strains obtained from the Korean Collection of Type Cultures (KCTC; Taejeon, Korea), the Korean Culture Center of Microorganisms (KCCM; Seoul, Korea) or the ATCC.

Genomic DNA preparation. Each bacterial strain was grown on brain heart infusion agar (Difco) at 28 °C for 16 h, and bacterial genomic DNA was prepared by employing the cetyltrimethylammonium bromide method (Ausubel *et al.*, 1993). Purified DNA was dissolved in sterile water with 40 µg ml^{−1} RNase, and quantified using an MBA 2000 spectrophotometer (Perkin-Elmer) at a wavelength of 260 nm.

Construction of subtracted genomic DNA libraries by SSH. Genes unique to *B. anthracis* were identified by SSH using *B. anthracis* CR as tester DNA and *B. cereus* KCTC 3624^T as driver DNA. SSH was

Table 1. *Bacillus* species (n=78) used in this study

Group	Species	Strain
<i>B. cereus</i> (n=59)	<i>B. anthracis</i>	ATCC 14578 ^T , ATCC 14185, ATCC 14186, Korean isolates CR, BC, CAU 1, CAU 2, CAU 3
	<i>B. cereus</i>	KCTC 3624 ^T , KCTC 1012, KCTC 1013, KCTC 1014, KCTC 1094, KCTC 1661, KCTC 3062, ATCC 10702, ATCC 13061, ATCC 21769, ATCC 21771, ATCC 25621, ATCC 27348, Korean isolates WY1, WY2, WY3, WY4, WY5, WY6, WY7, WY8, WY9, WY10, WY11, WY12, WY13, WY14, WY15, WY16, WY17, WY18, WY19, WY20
	<i>B. thuringiensis</i>	KCTC 1034 ^T , KCTC 1507, KCTC 1509, KCTC 1519, KCTC 3452, Korean isolates C1, C2, C3, C4, C5
	<i>B. mycoides</i>	KCTC 3453 ^T , ATCC 10206, ATCC 19647, ATCC 21929, ATCC 23258, KCCM 40260
	<i>B. pseudomycoloides</i>	KCTC 3862 ^T
	<i>B. weihenstephanensis</i>	KCTC 3975 ^T
Other	<i>Bacillus circulans</i>	KCTC 3347 ^T
<i>Bacillus</i> spp. (n=19)	<i>Bacillus coagulans</i>	KCTC 3625 ^T , KCTC 1015
	<i>Bacillus licheniformis</i>	KCTC 1918 ^T
	<i>Bacillus pumilus</i>	KCTC 3348 ^T
	<i>Bacillus sphaericus</i>	KCTC 3346 ^T
	<i>Bacillus subtilis</i>	KCTC 3135 ^T , KCTC 1021, KCTC 1022, KCTC 1028, KCTC 1691, KCTC 3014
	<i>Bacillus atrophaeus</i>	KCTC 3701 ^T
	<i>Bacillus mojavensis</i>	KCTC 3706 ^T
	<i>Bacillus vallismortis</i>	KCTC 3707 ^T
	<i>Bacillus amyloliquefaciens</i>	KCTC 1660 ^T , KCTC 3002, KCCM 40764
	<i>Bacillus stearothermophilus</i>	KCTC 1752 ^T

performed using the PCR-Select Bacterial Genome Subtraction kit (Clontech) according to the manufacturer's instructions, with some modifications. Tester and driver genomic DNA (1 µg) were digested separately with the restriction enzyme *RsaI* (Roche Diagnostics), generating fragments between 500 and 2000 bp in size (data not shown). Then, digested tester DNA was subtracted and amplified by PCR. Tester-specific fragments were subsequently cloned into a pCR2.1-TOPO vector using the TOPO TA Cloning kit (Invitrogen), and transformed into *E. coli* TOPO10F⁺ competent cells. The transformed culture was plated onto Luria-Bertani (LB) medium containing ampicillin/IPTG/X-Gal, and white colonies were screened for the insert fragment. Recombinant cultures were maintained in LB broth with glycerol at -80 °C before screening.

Isolation of insert fragment by Southern blot analysis. To verify the presence of cloned inserts, white colonies were cultured in LB broth, and recombinant plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen). Two-hundred and five inserts were amplified using primers directed at tester-ligated adaptor sequences in a GeneAmp PCR System 2700 (Applied Biosystems). Electrophoresis of each PCR product in 1.0% SeaKem LE agarose gel (FMC Bioproducts) was performed, and following ethidium bromide staining, the results were viewed under the Gel Doc 2000 image-analysis system (Bio-Rad). PCR products were purified using a QIAquick PCR Purification kit (Qiagen), resuspended in 50 µl distilled water, and denatured at 100 °C for 5 min. Then, denatured DNA from each clone was dispensed into the separate wells of a Bio-Dot apparatus (Bio-Rad) and transferred under vacuum onto Hybond-N+ nylon membranes (Amersham Biosciences) which had been pre-equilibrated in 6× standard sodium citrate (SSC) (USB). The individual wells and membranes were washed with 2× SSC, and the membranes exposed to 1000 mJ UV for 3 min 30 s in a Stratalinker (Stratagene). All hybridizations were performed at 42 °C after pre-hybridization in hybridization buffer at 42 °C for 2 h. ECL-labelled DNA probes were prepared by the digestion of either *B. anthracis* CR or *B. cereus* KCTC 3624^T genomic DNA with *AluI*/*HaeIII*/*RsaI* in accordance with the ECL Direct nucleic acid labelling and detection system instructions (Amersham Biosciences). The membranes were washed twice at 20 min intervals in 0.5× SSC+0.4% SDS, and 2× SSC at room temperature. Following high-stringency washing, the membranes were exposed to Kodak X-ray film MXG-1 (Kodak).

DNA sequencing analyses. Each clone was sequenced in duplicate with the M13 forward (-20) and/or reverse primers using the BigDye Terminator Cycle Sequencing kit and ABI PRISM 310 automated DNA sequencer (Applied Biosystems). Nucleotide sequence homologies of the genomic subtracted fragments of *B. anthracis* CR were determined using the BLAST suite of programs (Altschul *et al.*, 1997) against the National Center for Biotechnology Information (NCBI) GenBank nonredundant database (<http://www.ncbi.nlm.nih.gov>).

PCR conditions for species identification. In order to develop rapid identification and diagnosis tools for *B. anthracis*, *B. anthracis* species-specific oligonucleotide primers based on the chromosomal glycosyltransferase group 1 family protein gene were designed using Primer 3 software (Rozen & Skaletsky, 2000) and default settings. The glycosyltransferase group 1 family protein gene-based PCR was performed with 100 ng genomic DNA template in 25 µl reaction mixtures containing 1 µM each 20 bp primer, 2.5 µl reaction buffer, 0.2 mM dNTPs, 1.5 mM MgCl₂ and 2.5 U *Taq* polymerase (Roche Diagnostics). Amplification was carried out in a GeneAmp PCR system 2700 (Applied Biosystems) and primary PCR cycling conditions were: one cycle of initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 30 s, and one final extension at 72 °C for 7 min. The amplicons were electrophoresed in 1.2% SeaKem LE agarose gel (FMC

Bioproducts) and visualized following ethidium bromide staining. The PCR assays were performed on eight *B. anthracis*, 33 *B. cereus*, 10 *B. thuringiensis*, six *B. mycoides*, one *B. pseudomycoides*, one *B. weihenstephanensis* and 19 closely related *Bacillus* strains.

RESULTS AND DISCUSSION

Construction and screening of *B. anthracis* SSH libraries

The search for genetic markers that enable the unambiguous differentiation of *B. anthracis* from other *B. cereus* group members is an important and challenging task. While there is extensive genetic diversity within *B. cereus* and *B. thuringiensis*, *B. anthracis* appears to be genetically clonal (Keim *et al.*, 1997; Helgason *et al.*, 2000). Unique genomic differences may assist in the understanding of genetic relationships between *B. anthracis* and its phylogenetic neighbours, and such differences may also function as DNA signatures that select for *B. anthracis* (Radnedge *et al.*, 2003). This study has shown that *B. anthracis* can be discriminated from other *B. cereus* group taxa using a strategy based on SSH to develop primers specific to *B. anthracis*.

Two-hundred and five *B. anthracis* clones were obtained and screened for *B. anthracis*-specific sequences. Since the most important criteria for determining effective DNA signatures are their absence from closely related organisms (false-positive results) and their occurrence in all isolates of the target (false-negative results; Radnedge *et al.*, 2003), clone libraries generated by SSH were tested by reverse Southern blot analysis using *B. anthracis* CR as the tester probe and *B. cereus* KCTC 3264^T as the driver probe. Of these, 46 clones contained inserts that were undetectable using the driver probe but detectable using the tester probe, and dot-blot results of seven of the 46 positive clones are presented in Fig. 1. A comparison of the seven encircled wells shows that low levels of hybridization between the clones and the driver probe (Fig. 1a), indicated by faint signals, correspond with dense signals between the clones and the tester probe (Fig. 1b), where hybridization was high. These seven signals demonstrate the specificity of the *B. anthracis* CR probe and reflect the likelihood that these clones specifically contain chromosomal *B. anthracis* DNA. Similarly dense signals appearing in corresponding tester and driver wells are indicative of hybridization with both probes. They represent clones that harbour not only *B. anthracis* chromosomal DNA but also DNA inserts from *B. cereus*.

Only two of the 46 clones examined, 4.3%, possessed sequences that were completely specific to chromosomal *B. anthracis*, a percentage that is slightly higher than the 1.1% determined by Dwyer *et al.* (2004) using SSH. It is possible that a pre-amplification step for both the driver and tester probes, prior to the initiation of the SSH procedure, results in the loss of some sequences. Also, if long DNA fragments are abundant in a restriction enzyme digest, they may form

Fig. 1. Reverse Southern blot of PCR-amplified clones identified from SSH as specific to *B. anthracis* and hybridized to (a) the *B. cereus* KCTC 3624^T probe and (b) the *B. anthracis* CR probe. Strong and weak signals represent high and low levels of hybridization, respectively, between clone and probe. Encircled wells represent seven clones that may contain *B. anthracis*-specific chromosomal DNA.

complex networks that prevent the formation of appropriate hybrids. In this study, the PCR-Select Bacterial Genome Subtractive Hybridization kit (Clontech) was employed, which uses a single enzyme, *RsaI*, for genomic digestion. It is possible that a more tailored SSH protocol that combines data from parallel SSH experiments using different restriction enzymes would have the potential to significantly increase the coverage of the genome compared with the use of a single enzyme. Nonetheless, it is likely that the low number of clones is simply a reflection of the homology that exists between *B. anthracis* and *B. cereus* group members. The remaining 44 clones contained sequences that either corresponded to *B. anthracis*-specific plasmids (pXO1, pXO2) or showed high nucleotide similarities, in excess of 90%, with *B. cereus* and/or *B. thuringiensis*.

Identification and annotation of *B. anthracis*-specific clones

The results of the nucleotide sequence alignment analysis using NCBI BLAST are detailed in Table 2. Three non-synthetic nucleotide sequence alignments were found, with GenBank accession numbers AE017334 (*B. anthracis* str. 'Ames Ancestor'), AE017225 (*B. anthracis* str. Sterne) and AE016879 (*B. anthracis* str. Ames). It appears that the 265 bp nucleotide sequence of the first *B. anthracis*-specific clone (clone 2/205) corresponds to part of the full 1389 bp sequence of a conserved domain protein identified as belonging to a putative lysogenic prophage lambda Ba03. As would be expected of successful cloning, this sequence is unique to *B. anthracis* and is absent from all other members of the *B. cereus* group (Read *et al.*, 2003). Indeed, Read *et al.* (2003) reported that 141 proteins in *B. anthracis* had no match to the protein set of the *B. cereus* ATCC 10987 sequence. It was suggested that these proteins were encoded by genes of unknown function and were possibly transposases or, as appears to be the case for the 265 bp sequence of clone 2, were present in phage regions.

Comparative genome hybridization of *B. anthracis* and 19 members of the *B. cereus* group showed that they had 66–92% of chromosomal genes in common (Read *et al.*,

2003). Six small regions were identified as unique to the *B. anthracis* Ames strain genome, since they were absent from all other *B. cereus* group strains. Regions I–IV corresponded to *B. anthracis* prophages and, more specifically, region III contained the area designated as phage lambda Ba03. It is thought that bacteriophage sequences were acquired during or after the separation of *B. anthracis* from *B. cereus* and *B. thuringiensis* (Dwyer *et al.*, 2004).

For clone 2, query coverage for all four significant sequence alignments versus the query sequence was 87%, and the percentage identity between the query sequence and the aligned sub-sequences was 99%, with expected values indicating that these four alignments were not a random occurrence (Table 2). This non-random occurrence also held true for all four significant sequence alignments observed with the second *B. anthracis*-specific clone (clone 5/205). Compared with clone 2, clone 5 query coverage was much greater at 100%, and maximum identities, 100%, were expressed.

Clone 5 possessed a 617 bp sequence that corresponded to a portion of the full 1242 bp sequence of the *B. anthracis* glycosyltransferase group I family protein gene. Table 2 shows that the three non-synthetic nucleotide sequence alignments identified in clone 5, GenBank accession numbers AE017334 (*B. anthracis* str. 'Ames Ancestor'), AE017225 (*B. anthracis* str. Sterne) and AE016879 (*B. anthracis* str. Ames) were present in clone 2 also. However, the synthetic nucleotide sequence alignments differed between clones 2 and 5. For clone 5, the synthetic construct *B. anthracis* clone FLH240894.01L (GenBank accession no. EF037225) was associated with the BA5519 gene for glycosyltransferase, and for clone 2, the synthetic construct *B. anthracis* clone FLH247209.01L (GenBank accession no. EF040076) was related to the BA4081 gene for a hypothetical protein.

An additional gene sequence appeared only upon full alignment of the 1242 bp nucleotide sequence, and it corresponded to *B. cereus* E33L (GenBank accession no. CP000001; data not shown). In this instance, the identity was much lower, 84 %, than for other sequence alignments. This level of dissimilarity was reinforced by the 203 base

Table 2. Nucleotide sequence alignment analysis using NCBI BLAST of *B. anthracis*-specific clones 2 and 5

GenBank accession no.	Clone 2			Clone 5		
	EF040076	AE017334	AE017225	AE016879	EF037255	AE017334
Description	Synthetic construct <i>B. anthracis</i> clone FLH247209.01L BA4081 gene	<i>B. anthracis</i> str. 'Ames Ancestor', complete genome	<i>B. anthracis</i> str. Sterne, complete genome	<i>B. anthracis</i> str. Ames, complete genome	Synthetic construct <i>B. anthracis</i> clone FLH240894.01L BA5519 gene	<i>B. anthracis</i> str. 'Ames Ancestor', complete genome
Identity	264/265	264/265	264/265	264/265	617/617	617/617
Query coverage (%)	87	87	87	87	100	100
E-value	6e-134	6e-134	6e-134	6e-134	0	0
Maximum identity (%)	99	99	99	99	100	100
Fragment alignment		265 bp of 138 bp conserved domain protein			617 bp of 1242 bp glycosyltransferase group I family protein gene	

substitutions observed when both sequences were compared, and is less supportive of the reported high homology between *B. anthracis* and other *B. cereus* strains (Read *et al.*, 2003; Bell *et al.*, 2002; Helgason *et al.*, 2000; Keim *et al.*, 1997; Andersen *et al.*, 1996). Interestingly, Han *et al.* (2006) sequenced the genomes of two isolates, *B. thuringiensis* 97-27 subsp. *konkukian* serotype H34, from a necrotic human wound, and *B. cereus* E33L, from a swab of a Zebra carcass in Namibia. It was shown by comparative amplified fragment length polymorphism (AFLP) analysis that of the two isolates, *B. cereus* E33L was more closely related to *B. anthracis* than to *B. cereus* and *B. thuringiensis*. This is in keeping with the concept that the *B. cereus* group has evolved as asexually derived clonal populations (Helgason *et al.*, 2000; Priest *et al.*, 2004), although *B. thuringiensis* 97-27 subsp. *konkukian* serotype H34 and *B. cereus* E33L are members of the anthracis lineage (Han *et al.*, 2006; Priest *et al.*, 2004). This lineage also supports the molecular-based distinction between *B. thuringiensis* strains of commercial value and pathogenic strains of *B. anthracis*.

The 413 aa of the glycosyltransferase group I family protein of *B. anthracis* CR were aligned using NCBI BLASTP, and with expect values of 0, shared 100% identity with amino acid sequences from *B. anthracis* str. Ames (GenBank accession no. AAP29164), *B. anthracis* str. 'Ames Ancestor' (GenBank accession no. AAT34661) and *B. anthracis* str. Sterne (GenBank accession no. AAT57416). Much lower amino acid sequence identities, for the expected value $0 - 2e^{-35}$, were observed between *B. anthracis* CR and other bacteria, namely, *B. cereus* E33L (GenBank accession no. AAU15307; 80%), *Exiguobacterium sibiricum* (GenBank accession no. EAM88130; 42%), *Thermoanaerobacter tengcongensis* (GenBank accession no. AAM23928; 32%), *Clostridium botulinum* (GenBank accession no. ABS34160; 29%), *Bacteroides fragilis* (GenBank accession no. AAG26480; 29%), and *Geobacter metallireducens* (GenBank accession no. EDJ80581; 27%). Data reliability is enhanced because these measures ensure that more accurate E-values are generated that in turn distinguish more accurately between false and true matches (Gertz *et al.*, 2006).

In an examination of genomic differences that distinguish *B. anthracis* from non-anthrax-causing *Bacillus* species, Radnedge *et al.* (2003) used a combination of AFLP and SSH and mapped *B. anthracis* sequences to five regions with distinct loci that were within 50 kb of each other on the 5.23 Mb *B. anthracis* A2012 genome. However, none of the sequences detailed was purported to correspond to a glycosyltransferase group I family protein gene. More recently, Dwyer *et al.* (2004), identifying *B. anthracis* specific chromosomal sequences through the use of two SSH libraries, have found that half of the clones generated are located across regions I and II on the *B. anthracis* chromosome. Region I contains ORFs that share significant similarity with genes that encode the enzymes and proteins associated with cell wall polysaccharide biosynthesis. Typically, this process involves transferases that catalyse

the sequential transfer of sugar residues forming the carbohydrate repeating structure of the cell wall.

Glycosyltransferases are involved in carbohydrate modification of the cell envelope, and this in turn may allow *B. anthracis* to modify its cell-surface characteristics. However, comparatively little is known of the carbohydrates in the vegetative cell walls of *B. anthracis* (Choudhury *et al.*, 2006). Using aqueous hydrogen fluoride to isolate and structurally characterize Ames, Sterne and Pasteur strains of *B. anthracis* versus *B. cereus* ATCC 14579 and ATCC 10987, Choudhury *et al.* (2006) have shown that the cell wall structure of *B. anthracis* is clearly different from that of both *B. cereus* strains, and may have potential use as a target marker for the development of antimicrobials against anthrax.

Reviewing a comparative genome analysis of *B. cereus* group genomes with that of *B. subtilis*, Anderson *et al.* (2005) highlighted one species of UDP-glucose dehydrogenase in *B. anthracis* that is located in the same chromosomal cluster (BA5519–BA5512) as two genes that are homologous to a teichoic acid ATP binding cassette (ABC) transporter gene and, more significantly, a glycosyltransferase gene. This chromosomal cluster may encode the neutral cell wall polysaccharide characterized by Choudhury *et al.* (2006) that consists of an amino sugar backbone substituted with galactose residues, reflecting the more complex nature of this glycan mostly found in *B. anthracis*. In the current study, the clone 5 nucleotide sequence was aligned with GenBank accession number EF037225, a *B. anthracis* synthetic construct of the BA5519 gene. Therefore, the function of the glycosyltransferase sequence uncovered in this study may be associated with a capsular polysaccharide biosynthesis protein.

Diagnostic screening of *Bacillus* strains using *B. anthracis*-specific, clone 2-specific primers

Since the objective of this study was to use SSH to develop discriminating primers based on natural environmental strains of *B. anthracis*, attention focused on the sequence alignments with *B. anthracis* str. 'Ames Ancestor' (GenBank accession no. AE017334), *B. anthracis* str. Sterne (GenBank accession no. AE017225) and *B. anthracis* str. Ames (GenBank accession no. AE016879). Oligonucleotide primer construction for the species-specific discrimination of *B. anthracis* from other *B. cereus* group strains was based on information deduced from clone 5 and the full nucleotide sequence of the glycosyltransferase group I family protein gene. Two 20 bp primers, Ba-G206F (5'-TCTTCAG-TGACAAAACCACA-3') and Ba-G1013R (5'-CAAGAA-ATCTTTTTCGAAGG-3'), were generated, and subsequently their specificities were tested against all 78 strains of the *Bacillus* species listed in Table 1.

Of the *B. anthracis* strains included in this analysis, six strains, ATCC 14578^T and Korean isolates CR, BC, CAU 1, CAU 2 and CAU 3, harboured the pXO1 and pXO2

plasmids, and are therefore pathogenic to humans and animals. *B. anthracis* strains ATCC 14185 and ATCC 14186 harboured the pXO1 plasmid alone, rendering these strains avirulent but useful for vaccines. As seen in Fig. 2, the expected PCR product of 808 bp was common to all *B. anthracis* strains (lanes 1–8), regardless of virulence. This product was efficiently and distinctly detected, because no amplification of PCR products from any other closely related *Bacillus* strain occurred with primers Ba-G206F and Ba-G1013R.

The value of the SSH-derived primers used in this study is that they are discriminatory and are directed to the *B. anthracis* chromosome. This confers an advantage over primers that select for fragments of DNA located on plasmids pXO1 and pXO2, which can be lost from *B. anthracis* both in nature and in the laboratory. Bell *et al.* (2002) favoured the use of LightCycler PCR to select for the *B. anthracis* gene targets *pagA* and *pagB* on both virulence plasmids, and discouraged targeting chromosomal nucleic acid owing to the chromosomal homology among members of the *B. cereus* group. This contrasts with the decision of Keim *et al.* (1997), while assessing the molecular diversity of *B. anthracis* using AFLP, to ignore the presence or absence of plasmids pXO1 and pXO2 as discriminating genetic markers because of their ephemeral nature. Thus, reliance upon extrachromosomal markers alone may result in a failure to distinguish avirulent *B. anthracis* strains lacking both plasmids from near-neighbour *B. cereus* group strains, and therefore might lead to misidentification. Restriction digestion with *AluI* of an amplified SG-749 fragment revealed a restriction profile, of two DNA fragments, that was unique to *B. anthracis*, differentiating it from other strains of the *B. cereus* group (Daffonchio *et al.*, 1999). The SG-850 fragment from which this marker was derived is common to *B. cereus*, *B. thuringiensis* and *B. mycoides*, as well as *B. anthracis*.

While arguments persist concerning reliance upon extrachromosomal markers to differentiate *B. anthracis* from other *B. cereus* group member strains, an interesting report by Hoffmaster *et al.* (2004) draws attention to the unusual *B. cereus* isolate G9241, confirmed by 16S rRNA analysis, which can cause a severe inhalation anthrax-like illness. This isolate carries anthrax toxin genes on a circular plasmid, pBCXO1, and they share 99.6 % homology with pXO2. In the current study, 20 *B. cereus* Korean strains (WY 1–20) were isolated from patients with food poisoning, and all were negative when tested by PCR for the presence of *B. anthracis* plasmids. Although there were no atypical *B. cereus* strains among these Korean isolates, if *B. cereus* G9241 were shown to cause an anthrax-like gastrointestinal infection, it would be interesting to evaluate this and similar atypical *B. cereus* strains using the SSH-derived primers, Ba-G206F and Ba-G1013R.

SSH is the ideal, versatile method for two reasons. First, SSH allows for the swift cloning of cDNAs of differentially expressed genes because of their high level of enrichment,

Fig. 2. Electrophoretic examination of *B. anthracis*-specific PCR products generated with primers Ba-G206F and Ba-G1013R based on the glycosyltransferase family 1 protein gene. Lanes: 1, GeneRuler 1 kb DNA Ladder (Fermentas); 2, *B. anthracis* ATCC 14578^T; 3, *B. anthracis* ATCC 14185; 4, *B. anthracis* ATCC 14186; 5, *B. anthracis* CR; 6, *B. anthracis* BC; 7, *B. anthracis* CAU 1; 8, *B. anthracis* CAU 2; 9, *B. anthracis* CAU 3; 10, *B. cereus* KCTC 3624^T; 11, *B. cereus* KCTC 1012; 12, *B. cereus* KCTC 1013; 13, *B. cereus* KCTC 1014; 14, *B. cereus* KCTC 1094; 15, *B. cereus* KCTC 1661; 16, *B. cereus* KCTC 3062; 17, *B. cereus* ATCC 10702; 18, *B. cereus* ATCC 13061; 19, *B. cereus* ATCC 21769; 20, *B. cereus* ATCC 21771; 21, *B. cereus* ATCC 25621; 22, *B. cereus* ATCC 27348; 23, *B. cereus* WY1; 24, *B. cereus* WY2. Data for the remaining 54 strains ($n=78$), including isolates from the *B. cereus* group and other *Bacillus* species, are not shown because the results for these strains are the same as those seen in lanes 9–24; no *B. anthracis*-specific DNA is present. The arrow shows the expected PCR product of 808 bp common to all *B. anthracis* strains (lanes 1–8).

low background and normalized abundance of cDNAs in the subtracted mixture. Second, it is possible to use uncloned subtracted cDNA mixtures as hybridization probes (Diatchenko *et al.*, 1996). In addition, the application of SSH is wide-ranging. Although it has been used to identify genetic differences between virulent and avirulent pathogens such as *Mycobacterium ulcerans* (Jenkin *et al.*, 2003), *Mycoplasma agalactiae* and *Mycoplasma bovis* (Marenda *et al.*, 2004, 2005), and *Helicobacter pylori* (Oleastro *et al.*, 2006), it has also been used as a tool to identify genetic diversity in environmental microbial communities (Galbraith *et al.*, 2004).

This study used SSH to develop primers Ba-G206F and Ba-G1013R for the successful discrimination of *B. anthracis* from other *B. cereus* group members. These new primers offer the opportunity for rapid, optimal screening in the routine diagnosis of presumptive *B. anthracis*. The time and cost advantages over other DNA-based methods have been outlined previously, but work is still needed to elucidate the nature and role of the glycosyltransferase group I family protein gene identified in this study.

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REFERENCES

Agron, P. G., Macht, M., Radnedge, L., Skowronski, E. W., Miller, W. & Andersen, G. L. (2002). Use of subtractive hybridization for

comprehensive surveys of prokaryotic genome differences. *FEMS Microbiol Lett* **211**, 175–182.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Andersen, G. L., Simchock, J. M. & Wilson, K. H. (1996). Identification of a region of genetic variability among *Bacillus anthracis* strains and related species. *J Bacteriol* **178**, 377–384.

Anderson, I., Sorokin, A., Kapatral, K., Reznik, G., Bhattacharya, A., Mikhailova, N., Burd, H., Joukov, V., Kaznadzey, D. & other authors (2005). Comparative genome analysis of *Bacillus cereus* group genomes and *Bacillus subtilis*. *FEMS Microbiol Lett* **250**, 175–184.

Ash, C. & Collins, M. D. (1992). Comparative analysis of 23S ribosomal RNA gene sequences of *Bacillus anthracis* and emetic *Bacillus cereus* determined by PCR-direct sequencing. *FEMS Microbiol Lett* **73**, 75–80.

Ash, C., Farrow, J. A., Dorsch, M., Stackebrandt, E. & Collins, M. D. (1991). Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. *Int J Syst Bacteriol* **41**, 343–346.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G. & Struhl, K. (1993). *Current Protocols in Molecular Biology*, Section 2.4. New York: John Wiley and Sons.

Bell, C. A., Uhl, J. A., Hadfield, T. L., David, J. C., Meyer, R. F., Smith, T. F. & Cockerill, F. R., III (2002). Detection of *Bacillus anthracis* DNA by LightCycler PCR. *J Clin Microbiol* **40**, 2897–2902.

Carlson, C. R., Caugant, D. A. & Kolsto, A. B. (1994). Genotypic diversity among *Bacillus cereus* and *Bacillus thuringiensis* strains. *Appl Environ Microbiol* **60**, 1719–1725.

Cherif, A., Borin, S., Rizzi, A., Ouzari, H., Boudabous, A. & Daffonchio, D. (2003). *Bacillus anthracis* diverges from related clades of the *Bacillus cereus* group in 16S–23S ribosomal DNA intergenic transcribed spacers containing tRNA genes. *Appl Environ Microbiol* **69**, 33–40.

Choudhury, B., Leoff, C., Saile, E., Wilkins, P., Quinn, C. P., Kannenberg, E. L. & Carlson, R. W. (2006). The structure of the major cell wall polysaccharide of *Bacillus anthracis* is species-specific. *J Biol Chem* **281**, 27932–27941.

- Daffonchio, D., Borin, S., Consolandi, A., Mora, D., Manachini, P. L. & Sorlini, C. (1998). 16S–23S rRNA internal transcribed spacers as molecular markers for the species of the 16S rRNA group I of the genus *Bacillus*. *FEMS Microbiol Lett* **163**, 229–236.
- Daffonchio, D., Borin, S., Frova, G., Gallo, R., Mori, E., Fani, R. & Sorlini, C. (1999). A randomly amplified polymorphic DNA marker specific for the *Bacillus cereus* group is diagnostic for *Bacillus anthracis*. *Appl Environ Microbiol* **65**, 1298–1303.
- Diatchenko, L., Lau, Y. F., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N. & other authors (1996). Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc Natl Acad Sci U S A* **93**, 6025–6030.
- Dwyer, K. G., Lamonica, J. M., Schumacher, J. A., Williams, L. E., Bishara, J., Lewandowski, A., Redkar, R., Patra, G. & DelVecchio, V. G. (2004). Identification of *Bacillus anthracis* specific chromosomal sequences by suppressive subtractive hybridization. *BMC Genomics* **5**, 15.
- Ellerbrok, H., Nattermann, H., Ozel, M., Beutin, L., Appel, B. & Pauli, G. (2002). Rapid and sensitive identification of pathogenic and apathogenic *Bacillus anthracis* by real-time PCR. *FEMS Microbiol Lett* **214**, 51–59.
- Galbraith, E. A., Antonopoulos, D. A. & White, B. A. (2004). Suppressive subtractive hybridization as a tool for identifying genetic diversity in an environmental metagenome: the rumen as a model. *Environ Microbiol* **6**, 928–937.
- Gertz, E. M., Yu, Y.-K., Agarwala, R., Schaffer, A. C. & Altschul, S. F. (2006). Composition-based statistics and translated nucleotide searches: improving the TBLASTN module of BLAST. *BMC Biol* **4**, 41.
- Han, C. S., Xie, G., Challacombe, J. F., Altherr, M. R., Bhotika, S. S., Brown, N., Bruce, B., Campbell, C. S., Campbell, M. L. & other authors (2006). Pathogenomic sequence analysis of *Bacillus cereus* and *Bacillus thuringiensis* isolates closely related to *Bacillus anthracis*. *J Bacteriol* **188**, 3382–3390.
- Harrell, L. J., Andersen, G. L. & Wilson, K. H. (1995). Genetic variability of *Bacillus anthracis* and related species. *J Clin Microbiol* **33**, 1847–1850.
- Helgason, E., Økstad, O. A., Caugant, D. A., Johansen, H. A., Fouet, A., Mock, M., Hegna, I. & Kolst, A. B. (2000). *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* – one species on the basis of genetic evidence. *Appl Environ Microbiol* **66**, 2627–2630.
- Helgason, E., Tourasse, N. J., Meisal, R., Caugant, D. A. & Kolst, A. B. (2004). Multilocus sequence typing for bacteria of the *Bacillus cereus* group. *Appl Environ Microbiol* **70**, 191–201.
- Hoffmaster, A. R., Ravel, J., Rasko, D. R., Chapman, G. D., Chute, M. D., Marston, C. K., De, K. B., Sacchi, C. T., Fitzgerald, C. & other authors (2004). Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax. *Proc Natl Acad Sci U S A* **101**, 8449–8454.
- Jenkin, G. A., Stinear, T. P., Johnson, D. R. & Davies, J. K. (2003). Subtractive hybridization reveals a type I polyketide synthase locus specific to *Mycobacterium ulcerans*. *J Bacteriol* **185**, 6870–6882.
- Jones, H., Ostrowski, M. & Scanlan, D. J. (2006). A suppression subtractive hybridization approach reveals niche-specific genes that may be involved in predator avoidance in marine *Synechococcus* isolates. *Appl Environ Microbiol* **72**, 2730–2737.
- Keim, P. & Smith, K. L. (2002). *Bacillus anthracis* evolution and epidemiology. *Curr Top Microbiol Immunol* **271**, 21–32.
- Keim, P., Kalif, A., Schupp, J., Hill, K., Travis, S. E., Richmond, K., Adair, D. M., Hugh-Jones, M., Kuske, C. R. & other authors (1997). Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. *J Bacteriol* **179**, 818–824.
- Kim, W., Hong, Y. P., Yoo, J. H., Lee, W. B., Choi, C. S. & Chung, S. I. (2002). Genetic relationships of *Bacillus anthracis* and closely related species based on variable-number tandem repeat analysis and BOX-PCR genomic fingerprinting. *FEMS Microbiol Lett* **207**, 21–27.
- Lechner, S., Mayr, R., Francis, K. P., Pruss, B. M., Kaplan, T., Gunkel, E. W., Stewart, G. S. A. B. & Scherer, S. (1998). *Bacillus weihenstephanensis* sp. nov. is a new psychrotolerant species of the *Bacillus cereus* group. *Int J Syst Bacteriol* **48**, 1373–1382.
- Logan, N. A., Carman, J. A., Melling, J. & Berkeley, R. C. (1985). Identification of *Bacillus anthracis* by API tests. *J Med Microbiol* **20**, 75–85.
- Marenda, M. S., Vilei, E. M., Poumarat, F., Frey, J. & Berthelot, X. (2004). Validation of the suppressive subtractive hybridization method in *Mycobacterium agalactiae* species by the comparison of a field strain with the type strain PG2. *Vet Res* **35**, 199–212.
- Marenda, M. S., Sagné, E., Poumarat, F. & Citti, C. (2005). Suppression subtractive hybridization as a basis to assess *Mycoplasma agalactiae* and *Mycoplasma bovis* genome diversity and species-specific sequences. *Microbiology* **151**, 475–489.
- Mohammed, M. J., Marston, C. K., Popovic, T., Weyant, R. S. & Tenover, F. C. (2002). Antimicrobial susceptibility testing of *Bacillus anthracis*: comparison of results obtained using the National Committee for Clinical Laboratory Standards broth microdilution references and Etest agar gradient diffusion methods. *J Clin Microbiol* **40**, 1902–1907.
- Nakamura, L. K. (1998). *Bacillus pseudomycoides* sp. nov. *Int J Syst Bacteriol* **48**, 1031–1035.
- Odendaal, M. W., Pieterse, P. M., de Vros, V. & Botha, A. D. (1991). The biochemical, morphological and virulence profiles of *Bacillus anthracis* isolated in the Kruger National Park. *Onderstepoort J Vet Res* **58**, 21–26.
- Oleastro, M., Monteiro, L., Lehours, P., Mégraud, F. & Ménard, A. (2006). Identification of markers for *Helicobacter pylori* strains isolated from children with peptic ulcer disease by suppressive subtractive hybridization. *Infect Immun* **74**, 4064–4074.
- Priest, F. G., Barker, M., Baillie, L. W., Holmes, E. C. & Maiden, M. C. (2004). Population structure and evolution of the *Bacillus cereus* group. *J Bacteriol* **186**, 7959–7970.
- Radnedge, L., Agron, P. G., Hill, K. K., Jackson, P. J., Ticknor, L. O., Keim, P. & Andersen, G. L. (2003). Genome differences that distinguish *Bacillus anthracis* from *Bacillus cereus* and *Bacillus thuringiensis*. *Appl Environ Microbiol* **69**, 2755–2764.
- Read, T. D., Peterson, S. N., Tourasse, N., Baillie, L. W., Paulsen, I. T., Nelson, K. E., Tettelin, H., Fouts, D. E., Eisen, J. A. & other authors (2003). The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. *Nature* **423**, 81–86.
- Rozen, S. & Skaletsky, H. J. (2000). Primer3 on the WWW for general users and for biologist programmers. In *Bioinformatics Methods and Protocols: Methods in Molecular Biology*, pp. 365–386. Edited by S. Krawetz & S. Misener. Totowa, NJ: Humana Press.
- Sastry, K. S., Tuteja, U., Santhosh, P. K., Lalitha, M. K. & Batra, H. V. (2003). Identification of *Bacillus anthracis* by a simple protective antigen-specific mAb dot-ELISA. *J Med Microbiol* **52**, 47–49.
- Schuch, R., Nelson, D. & Fischetti, V. A. (2002). A bacteriolytic agent that detects and kills *Bacillus anthracis*. *Nature* **418**, 884–889.
- Volokhov, D., Pomerantsev, A., Kivovich, V., Rasooly, A. & Chizhikov, V. (2004). Identification of *Bacillus anthracis* by multiprobe microarray hybridization. *Diagn Microbiol Infect Dis* **49**, 163–171.