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Genetic Diversity Among *Bacillus anthracis*, *Bacillus cereus* and *Bacillus thuringiensis* Strains Using Repetitive Element Polymorphism-PCR

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Abstract

Repetitive element polymorphism-PCR (REP-PCR) is one of the tools that has been used to elucidate genetic diversity of related microorganisms. Using the MB1 primer, REP-PCR fingerprints from 110 *Bacillus* strains within the "*B. cereus* group" have identified eighteen distinct categories, while other more distantly related bacterial species fell within six additional categories. All *Bacillus anthracis* strains tested were found to be monomorphic by f1uorophore-enhanced REP-PCR (FERP) fingerprinting using the MB1 primer. In contrast, other non- *B. anthracis* isolates displayed a high degree of polymorphism. Dendrogramic analysis revealed that the non- *B. anthracis* strains possessing the Ba813 chromosomal marker were divided into two clusters. One of the clusters shared identity with the *B. cereus* strains examined.

Key words: REP-PCR, *B. anthracis*, *B. cereus*, *B. thuringiensis*

Introduction

Bacillus anthracis, *Bacillus cereus* and *Bacillus thuringiensis* are closely related members of the genus *Bacillus*, belonging to the "*B. cereus* group". *B. anthracis* is an extremely virulent pathogen and is the causal agent of anthrax (Dixon *et al*., 1999). The severity of infection by a virulent *B. anthracis* isolate is correlated with the number of microorganisms and the route of entry into the mammalian host (Dixon *et al*., 1999; Hanna 1998). *B. anthracis* is considered to be a very serious biological agent of mass destruction (Higgins *et al*., 1999). *B. cereus* can cause food poisoning and also infects wounds or mucosal membranes, however, it has a much lower incidence of morbidity and mortality (Drobniewski, 1993). *B. thuringiensis* strains are rarely pathogenic in mammals (Hernandez *et al*., 2001), and are routinely used as insecticides (Schnepf *et al*., 1998).

The close phylogenetic relationship between these three species has been demonstrated by sequencing of ribosomal RNA genes (Ash *et al*., 1991; Ash and Collins, 1992) as well as by DNA-DNA hybridization studies (Kaneko *et al*., 1978; Nakamura, 1994; Priest *et al*., 1994; Roloff *et al*., 1996; Seki *et al*., 1978). Multilocus enzyme electrophoresis sequencing nine different genetic loci has lead to the proposal that members of the "*B. cereus* group" should be regarded as a single species (Helgason *et al*., 2000). Analysis of homoduplex and heteroduplex polymorphisms of the 23S–16S intragenic spacer region has also indicated the close genetic relationship of these bacteria (Daffonchio *et al*., 2000). The level of genetic similarity vastly exceeds the 70% criterion that defines a bacterial species (Wayne *et al*., 1987). However, taxonomic arguments continue because of the differences in metabolic capabilities and the distinctively different environmental and host niches which they inhabit (Drobniewski, 1993; Helgason *et al*., 1998; Helgason *et al.*, 2000; Ticknor *et al*., 2001). Recently, 52 strains of Bacilli within the *Bacillus cereus-group* were successfully discriminated using rep-BOX-PCR (Cherif *et al*., 2002). Long-range (LR) repetitive element

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polymorphism-PCR (REP-PCR) has recently been shown to distinguish and differentiate *B. anthracis* strains from other related *Bacillus* species (Brumlik *et al.*, 2001). The LR REP-PCR primer employed in this study was able to unambiguously distinguish 105 *B. anthracis* strains into five distinct groups. The LR REP-PCR system that was used for *B. anthracis* (Brumlik *et al.*, 2001) generated such widely divergent fingerprints that genetic relatedness of non- *B. anthracis Bacillus* species could not be discerned.

Thus, a new primer (MB1) was designed to elucidate the genetic diversity of *B. anthracis*, *B. cereus* and *B. thuringiensis* isolates. In this investigation we describe a REP-PCR system that can be used to examine genetic diversity among closely related *Bacillus* species and strains, as well as fingerprinting of much more distantly related gram-positive and gram-negative bacteria. Fingerprint resolution was accomplished by either conventional agarose gel electrophoresis or by fluorophore-enhanced REP-PCR (FERP) analysis (DelVecchio *et al*., 1995).

Experimental

Materials and Methods

DNA preparation. DNA was isolated from 113 strains *of Bacillus*, including *B. anthracis*, *B. cereus, B. licheniformis*, *B. megaterium*, *B. mycoides*, *B. subtilis* and *B. thuringiensis* (see Table I) using modifications of the method of Schraft and Griffiths (Brumlik *et al*., 2001; Schraft and Griffiths, 1995). High molecular weight DNA suitable for REP-PCR was isolated from *Bacillus* spp. spores using the UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Solana Beach, Calif.). *Bacillus* strains examined in this study were routinely tested for the presence of the *B. anthracis* virulence plasmids pXOl and pXO2, as well as for the presence of the *B. anthracis* chromosomal marker *rpoB* signature (Qi *et al*., 2001). *B. anthracis* virulence plasmids were absent in all non- *B. anthracis* strains, but those strains possessing the Ba813 chromosomal marker are indicated in Table I. DNA isolation from microorganisms such as *Enterococcus faecalis* derived from the modified method of Schraft and Griffiths in that lysozyme was not required. Similarly, DNA isolation from *Escherichia coli* did not require the addition of either lysostaphin or mutanolysin. *Brucella melitensis* DNA was isolated from chloroform-killed cells using the method of Murray and Thompson (1980).

REP-PCR. Each 25 μL MB1 REP-PCR mixture contained 50 mM KC1, 10 mM Tris-HCl (pH 8.3), 2.75 mM $MgC1_2$, 5.0% (vol/vol) dimethyl sulfoxide (DMSO), 100 mM concentration of each dNTP, 25 pmol of 6-carboxyfluorescein-labeled MB 1 primer [5'- (6-carboxyfluorescein, FAM) TGT ACA TAA GAC GAA GCC C -3'], 1.25 U of AmpliTaq (Perkin-Elmer Cetus, Norwalk, Conn.), and 100 ng of template DNA. Once the samples were placed in the PE-9700 thermocycler (Perkin-Elmer Cetus), the template DNA was denatured at 94°C for 5 min. Amplification was accomplished using 35 cycles, with each cycle consisting of denaturation at 94°C for 30 sec, annealing at 62.5°C for 30 sec, and extension at 72°C for 30 sec. Following a final 7 min extension step at 72°C, the samples were stored at –20°C. LR REP-PCR using LL-Rep1LR as a primer was performed as described previously (Brumlik *et al*., 2001).

Agarose gel and Fluorophore-Enhanced REP-PCR (FERP) analysis. Prior to electrophoresis, 8 μL of each FAM-MB1 REP-PCR sample was mixed with 2 μL of 5 X loading buffer (5 X loading buffer contained 0.05% (wt/vol) bromophenol blue, 40.0% (wt/vol) sucrose, 100 mM EDTA (pH 8.0), and 0.5% (wt/vol) sodium dodecyl sulfate). Electrophoresis was then performed in a 1.5% (wt/vol) GTG agarose gel (SeaKem; BioWhittaker, Rockland, Maine) containing 0.5 μg/mL ethidium bromide. Fingerprints were visualized using a ChemiImager™ gel documentation system (AlphaInnotech Inc.; San Leandro, Calif.). Each REP-PCR sample (3.0 μL) was combined with 1.9 μL of deionized formamide, 0.8 μL of 5 X loading buffer, and 0.4 μL of

Table I
Bacterial strains used in this study

MB1 category	Strains	The total number of distinct strains	MB1 category	Strains	The total number of distinct strains
1	*Bacillus* sp. Ba813$^+$ #25		13	*Bacillus* sp. Ba813$^+$ #6681	3
2	*B. anthracis* 7700	26	14	*Bacillus* sp. Ba813$^+$ #3403, #6	
3	*B. thuringiensis* H1	29	15	*B. cereus* UW85	
4	*B. thuringiensis* H8	13	16	*B. cereus* Zimbabwe	5
5	*B. thuringiensis* CT43		17	*B. thuringiensis* H12, H37, H38	
6	*B. thuringiensis* H28, H30		18	*B. mycoides* ATCC 23258	2
7	*B. thuringiensis* H4, H23		19	*B. megaterium* ATCC 33167	
8	*Bacillus* sp. Ba813$^+$ #4, #13		20	*B. subtilis* ATCC 5061	
9	*Bacillus* sp. Ba813$^+$ #11	9	21	*B. licheniformis* ATCC 12139	
10	*Bacillus* sp. Ba813$^+$ #16	5	22	*Enterococcus faecalis* ATCC 51299	
11	*B. thuringiensis* H32		23	*Escherichia coli* ATCC 9637	
12	*Bacillus* sp. Ba813$^+$ #14	3	24	*Brucella melitensis* 16M	

GENESCAN-2500™ ROX standards (Applied Biosystems; Boston, MA). These samples were denatured at 95°C for 2 minutes and were then immediately placed on ice in order to maintain strand-separation prior to being loaded on a 6.5% (wt/vol) polyacrylamide-8 M urea gel. Gels were run for 5 hours using an ABI-377 Automated Sequencer (Applied Biosystems) and the virtual gel images were generated using GENESCAN® 2.5 software (Applied Biosystems). Dendrograms were constructed using DENDRON® 2.3 software (Solltech; Oakdale, lowa) which employed the "absolute difference/total area" method (Solltech Inc., 1992).

Cloning and sequencing of REP-PCR amplicons. REP-PCR was performed as described previously using both the MBl and LL-ReplLR primer, except that in the case of the MBl primer, a non-fluorescently labeled version of the MBl primer was needed in order to facilitate cloning. Unlabeled amplicons were excised from low-melting temperature SeaPlaque agarose gels (Bio Whittaker) and were subsequently purified using a QIA quick Gel Isolation Kit (QIAGEN; Valencia, Calif.). Cloning was carried out using the Original TA Cloning Kit (Invitrogen; Carlsbad, Calif.) in conjunction with competent *Escherichia coli* TOPI0F' cells (Invitrogen). Amplicons were then ligated into the pCR2.1 cloning vector (Invitrogen) prior to sequencing. Sequencing-grade DNA was prepared using a Plasmid Mini Kit (QIAGEN). A minimum of two clones per amplicon were sequenced using an ABI-377 Automated Sequencer (Applied Biosystems). DNA sequences were compared with the *B. anthracis* Ames genome (http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi) and GenBank (http://www.ncbi.nlm.nih.gov:80/BLAST/).

Southern blotting. DNA derived from PCR amplification was either directly electrophoresed in a 1.0% GTG agarose gel or 5 µg of genomic DNA was digested with HindIII prior to being electrophoresed in a 0.7% (wt/vol) GTG agarose gel. DNA was transferred from gels onto Nytran membranes (Amersham; Piscataway, N.J.) according to Sambrook and co-workers (Sambrook *et al*., 1989). *B. thuringiensis* H38 chromosomal DNA served as the template for long-range REP-PCR using the LL-ReplLR primer (Brumlik *et al*., 2001). Digestion of the 3.4 kilobase pair (kb) LL-Rep1LR long-range REP-PCR amplicon with *Eco*RI and HindIII generated a 1.1 kb internal fragment that was subsequently biotinylated using the Random Primer DNA Biotinylation Kit (Kirkegaard & Perry Laboratories, Inc. (KPL, Inc.); Gaithersburg, Maryland). The membranes were probed with the biotinylated 1.1 kb probe for 16 hours at 55°C. Washing and subsequent chemiluminescent detection was carried out according to the instructions of the manufacturer (KPL, Inc.).

Results

REP-PCR using the MB1 primer was able to distinguish *B. anthracis* strains from other "*B. cereus* group" members in all but four cases. Fingerprints from 26 *B. anthracis* strains invariably generated two PCR bands of 525 and 750 base pairs. Fingerprints from other *Bacillus* species produced the 525 bp amplicon in 95% (80/84) of the samples, with four strains showing a profile that would have misidentified them as *B. anthracis* strains (Fig. 1).

Closer examination of the agarose gel indicated that the MB1 primer actually generated a number of different-sized amplicons for most of the non- *B. anthracis* strains (Fig. 1). A fluorescently-labeled MB1 primer (FAM-MB1) was employed to determine whether discriminatory fingerprints could be detected using an ABI-377 Automated Sequencer. FERP fingerprinting analysis was performed on representative samples from each of the twenty-four MB1 categories that were identified in this study. The virtual gel that was generated using GENESCAN® software is shown (Fig. 2, Panel A). Similarities and differences could be observed when the FERP fingerprinting patterns of members of the "*B. cereus* group", which comprise the first eighteen categories, were compared. The same four strains (listed in categories land 17), which would have been misidentified as *B. anthracis* (category 2) when evaluated at the agarose gel level (Fig. 1), also showed a high degree of similarity with respect to each other upon FERP analysis (Fig. 2). FERP fingerprints of distantly related species (categories 19–24) showed a greater number of differences with correspondingly fewer similarities either with respect to each other or to fingerprints from strains belonging to the "*B. cereus* group".

To create a dendrogram using the FERP fingerprinting data, it was necessary to transform the original virtual gel into an inverse image (Fig. 2, Panel B) using DENDRON® software (Solltech, 1992). The bands which were subsequently chosen for dendrogram construction (Fig. 2, Panel C) had to meet certain strict guidelines. First, all bands that were chosen either had to be consistently present or absent from a particular FERP fingerprint. To ensure that this was the case, at least two side-by-side FERP fingerprints were examined for every strain (Table I). Second, all bands that were selected for the construction of the dendrogram were chosen regardless of the observed differences in their f1uorescent intensities. In light of the fact that each fingerprint shown in Figure 2 had a unique profile, it proved unnecessary to impose an extra level of complexity. Third, multiple bands were treated with the same significance as a single band, providing that such bands never appeared individually.

The results obtained from cloning and sequencing the two brightest bands observed in MB1 fingerprints from both categories 1 and 2 (see Figure 1), demonstrated the importance of adhering to the second and third guidelines (Fig. 2, Panel C). The 525 and the 750 bp bands (Fig. 1) were cloned from both *B. anthracis* A19 (category 2) and *Bacillus* sp. Ba813$^+$ #25 (97–27, category 1). The cloned 750 bp amplicon from *Bacillus* sp. Ba813$^+$ #25 generated a distinctive ladder of bands that could be observed on the virtual FERP gel, ranging in apparent size from 725–875 nucleotides (data not shown). In contrast, the corresponding cloned 750 bp

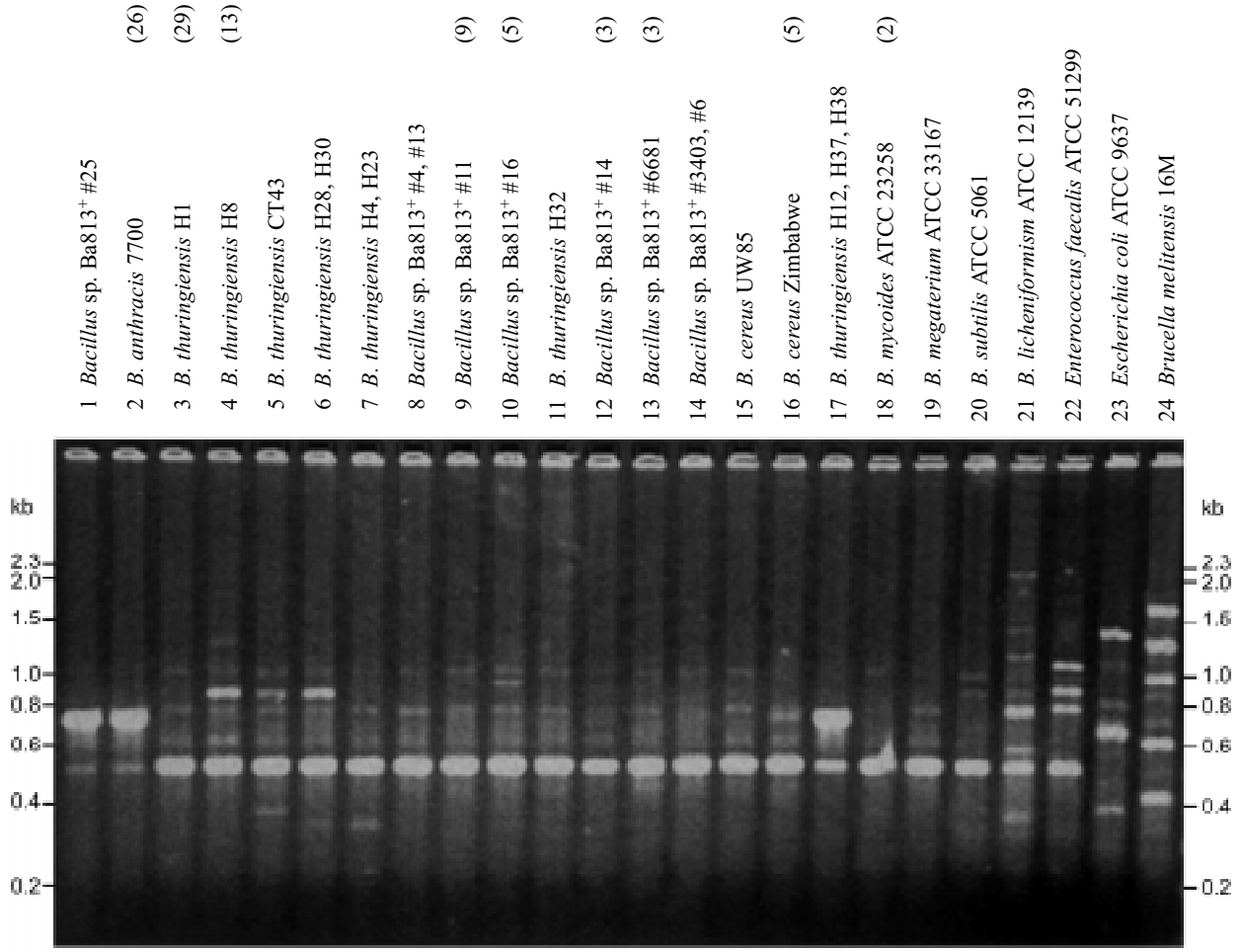

Fig. 1. REP-PCR fingerprinting patterns (generated using the FAM-labeled MB1 primer) detected following electrophoresis in a 1.5% (wt/vol) GTG agarose gel

One representative bacterial strain from each of the twenty-four MBl categories is shown. In cases where more strains were iden tified within a particular MB1 category than could be listed, the total number of distinct strains that belonged within a particular category have been indicated in parentheses (Table I). The sizes of a combined HindIII-digested lambda DNA and 100 base pair ladder is indicated in kilobase pairs (kb) on each side of the gel image.

amplicon from *B. anthracis* A19 generated two predominant bands, having apparent sizes of 725 and 775 nucleotides (data not shown). Identical results were obtained whether genomic DNA (Fig. 2, Panel A) or cloned DNA (data not shown) from these strains served as the template for MB1 REP-PCR fingerprinting.

Portions of the same two house-keeping genes were found to reside on the 750 bp amplicon cloned from either *B. anthracis* A19 (category 2) or *Bacillus* sp. Ba813^{+} #25 (category 1), and these sequences were 95% identical. The 750 bp amplicon comprised the 5' end of an orthologue of the *mmgA* gene from *Bacillus subtilis*, encoding acetyl-CoA acetyltransferase (6; accession no. B69658). This gene was immediately preceded by the 3' end of an orthologue of the *ywjF* gene from *B. subtilis,* encoding fumarate reductase (30; accession no. P45866). The genome data of *B. anthracis* Ames (http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi) and the completed *B. subtilis* genome indicate that these single-copy genes were juxtaposed on the *B. anthracis* chromosome, and were situated 112° apart on the *B. subtilis* chromosome (Kunst *et al.*, 1997).

The cloned 525 bp amplicon comprised an internal portion of the 23S-16S ribosomal rRNA operon. Nine to eleven copies of these genetic loci are distributed around the *Bacillus* chromosome (Patra *et al.*, 2002) which potentially explains the high levels of this amplicon relative to the other MB1 REP-PCR products. A ladder of bands was observed immediately above the 536 nucleotide marker in every case where large amounts of the MB1 525 bp amplicon was generated (Fig. 1 and 2). This may be the result of the potential of single-stranded DNA encoding rRNA to form significant secondary structure. Therefore no bands for dendrogram construction were selected from this region (Fig. 2).

The dendrogram shown in Figure 3, which was based on the FAM-labeled MB1 fingerprinting patterns of 116 strains, showed a similar relationship between these microorganisms as has previously been determined

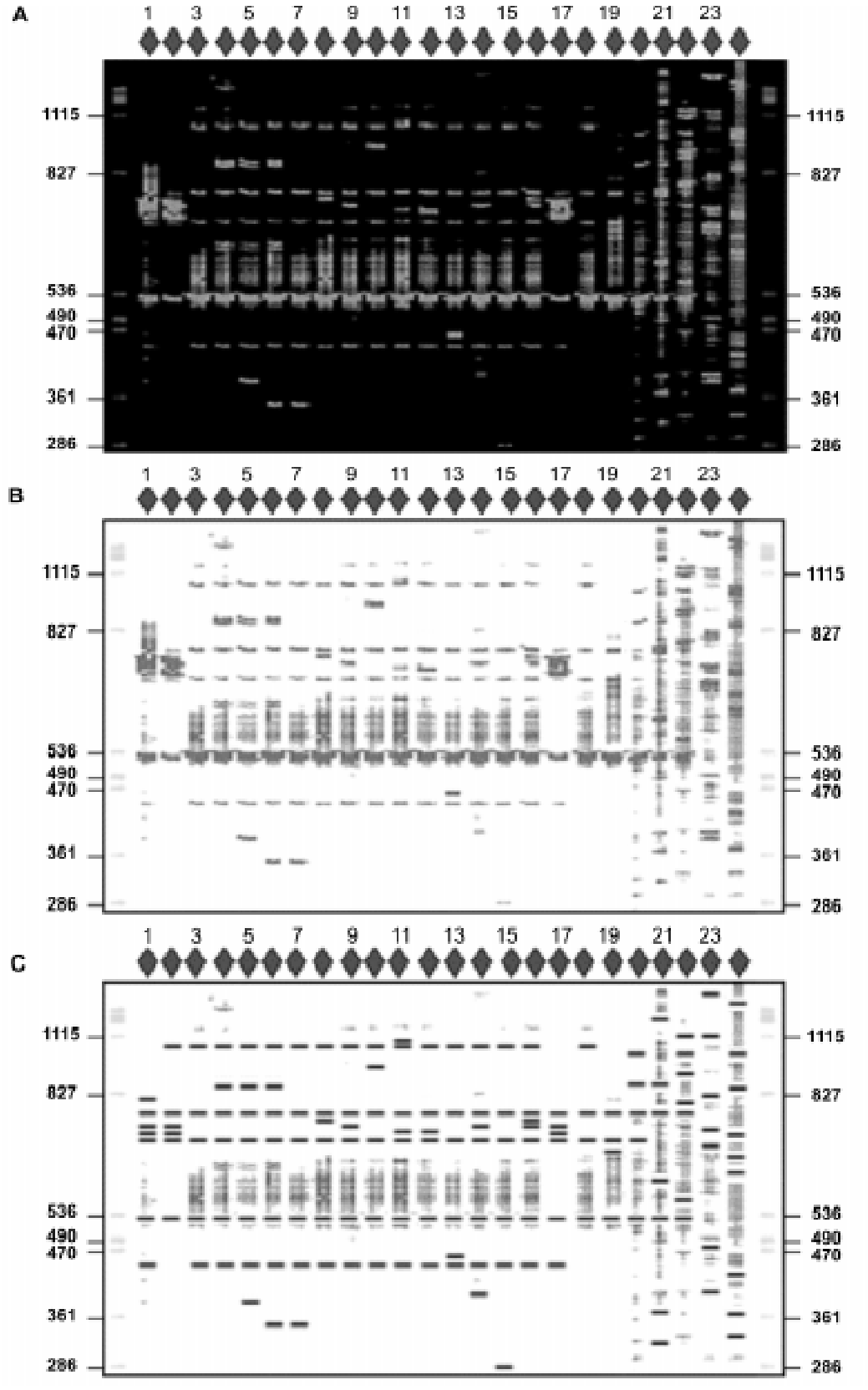

Fig. 2. MB 1 REP-PCR fingerprinting patterns detected following lolyacrylamide gel electrophoresis using an ABI-377 Automated Sequencer

Panel A, shows the virtual gel that was generated by the GENESCANŇ software, with the upper position of the GENESCAN-2500™ ROX standards (red bands) in the outermost lanes of the gel. The sizes of the ROX standards between 286 nucleotides and l, 115 nucleotides in length are shown on each side of the gel images. The lane numbers correspond to the strains shown in Figure l. Panel B shows the inverse image of the virtual gel (generated using the DENDRON® software), which is analogous to the negative that would be used to generate a color photograph. Panel C shows the bands that were used to construct the dendrogram using the DENDRON® software (Solltech Inc., 1992).

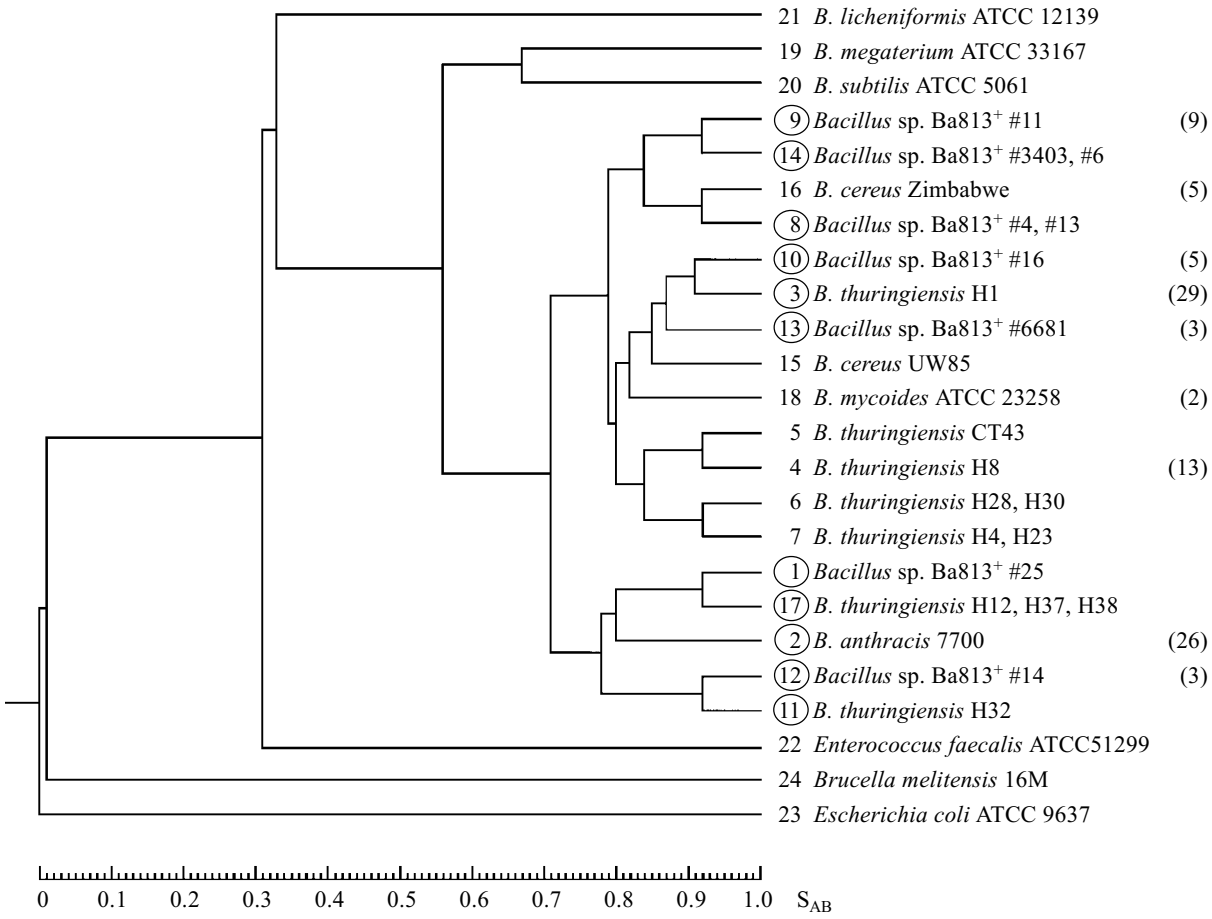

Fig. 3. Dendrogram comparing more than 100 strains within the "*B. cereus* group", along with several more distantly related microorganisms

All twenty-four MBl categories are listed, along with at least one representative species or strain from each category. In cases where more strains were identified than could be listed, the total number of microorganisms that were found in each category is indicated in parentheses. The similarity coefficient (S_{AB}) between MBl categories is indicated along the horizontal axis of the dendrogram, and is a direct reflection of the relatedness between each MB1 category. Category numbers that have been circled indicate that the majority of the strains within that particular category possessed the *B. anthracis* Ba813 chromosomal marker. Categories indicated by plain numbers do not contain any strains possessing the Ba813 chromosomal marker. A broken circle has been used to designate MB1 category 3, since only a minority (24%) of the 29 strains that comprise this category possessed the Ba813 chromosomal marker.

using a variety of other genetic and biochemical tests. Each of the 110 strains that fell within the "*B. cereus* group" were centrally clustered in the dendrogram, having similarity coefficients (S_{AB}) exceeding 0.70 in all cases (Fig. 3).

B. anthracis strains invariably possess the Ba813 chromosomal marker (Ramisse *et al*., 1999). All of the *B. anthracis* strains that were examined in this study were indistinguishable by MB1 fingerprinting. All *B. anthracis* strains were characterized by the absence of a single band that had an apparent size of approximately 450 nucleotides regardless of the plasmid content (Fig. 2 and 3, category 2). However, 32 of the non-*B. anthracis* strains that possessed the Ba813 chromosomal marker (Ramisse *et al*., 1999) were distributed over 10 additional categories. All 11 of the $Ba813^+$ categories fell into two distinct clusters and have been identified by circles in Figure 3. The 13 *B. cereus* strains examined in this study were concentrated in one area of the dendrogram, being classified in categories 3, 9, 10, 15, and 16. The most distant branch-point of *B. cereus*-containing categories had an S_{AB} value of 0.80 (Fig. 3), and overlapped one of two clusters of categories consisting of $Ba813^+$ strains (the upper cluster of circled categories depicted in Figure 3).

The six outlying categories had a lower level of relatedness to other Bacilli based on FAM-labeled MB1 fingerprinting analysis (Fig. 3). These strains were specifically chosen as examples of more distantly related organisms, and their MB1 fingerprinting pattern cannot be considered to be a representative profile for other microorganisms classified within these particular bacterial species (Table I). *Bacillus subtilis* and *Bacillus megaterium* had an equal degree of similarity, having a branch-point S_{AB} value of 0.55 with respect to the remaining categories that fell within "*B. cereus* group". *Bacillus licheniformis* was even more distantly

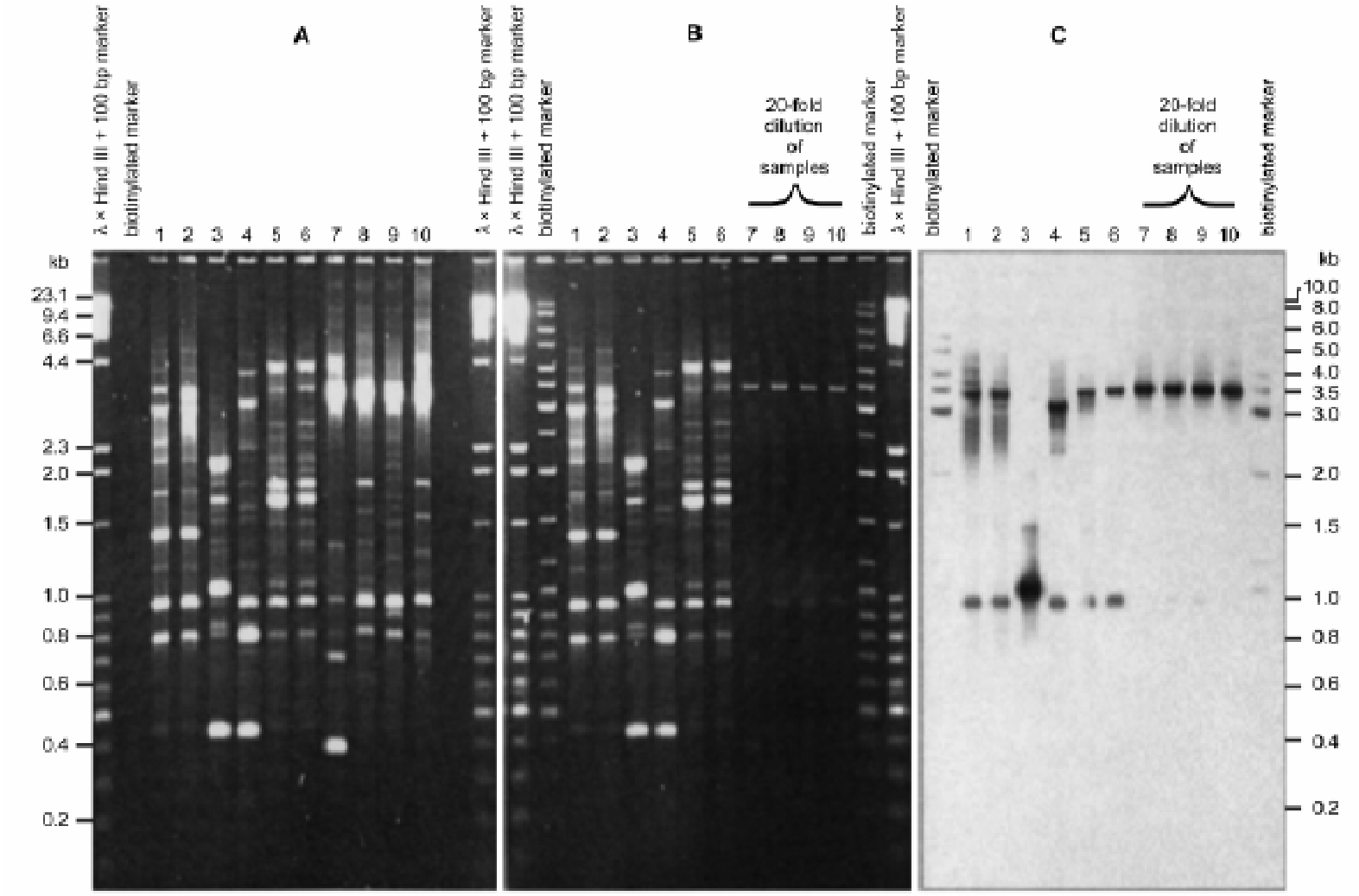

Fig. 4. Southern blot comparing LL-Rep1LR fingerprints among species and strains that are closely related, and those that are distantly related, based on their respective MB1 fingerprinting patterns

Panel A shows the fingerprints generated fol1owing electrophoresis in a 1.0 % (wt/vol) GTG agarose gel. Panel B shows the actual agarose gel that was used for the Southern blot. Twenty-fold dilutions of the REP-PCR samples shown in lanes 7–10 were performed in order to load approximately equal quantities of the 3.4 kb amplicon in every lane (Panel B). Panel C shows the chemiluminescent detection of REP-PCR products using a biotinylated internal fragment comprising the 3.4 kb amplicon from *B. thuringiensis* H38. The lane order is as fol1ows: lane 1, *B. anthracis* 7700 (LL-Rep1LR group B); lane 2, *B. anthracis* A19 (LL-Rep1LR group A); lane 3, *B. thuringiensis* H34; lane 4, *B. thuringiensis* CT43; lane 5, *B. thuringiensis* H12; lane 6, *B. thuringiensis* H37; lane 7, *B. thuringiensis* H38; lane 8, *Bacillus* sp. Ba813^{+} #11 (9594/3); lane 9, *Bacillus* sp. Ba813^{+} #25 (97–27); and lane 10, *B. cereus* Zimbabwe. The sizes of a combined HindIII-digested lambda DNA and 100 base pair DNA ladder is indicated in kilobase pairs (kb) on the left side of the figure, while the sizes of the biotinylated DNA ladders fragments are indicated on the right side of the figure.

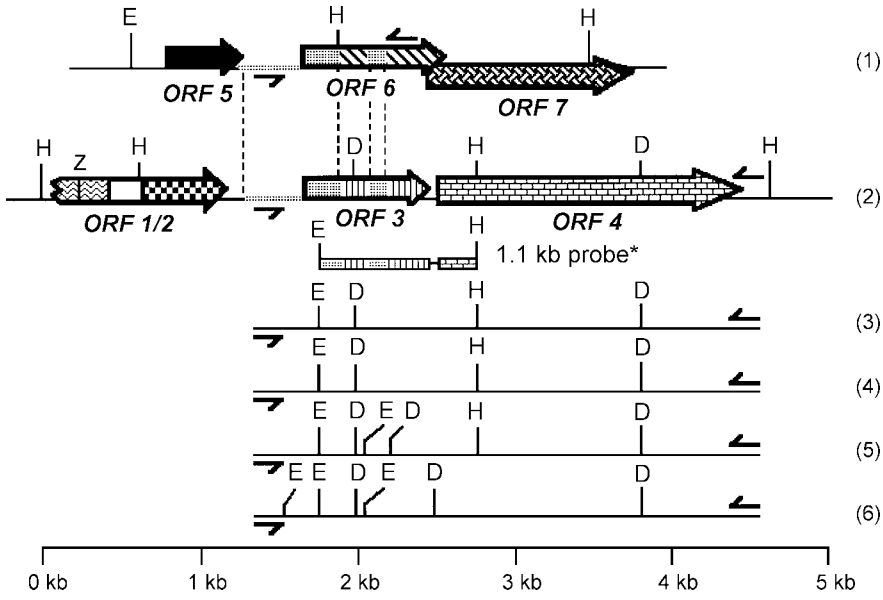

Fig. 5. Organization of the open readings frames (ORFs) that were identified within two distinct loci comprising the *B. anthracis* LL-ReplLR fingerprint, along with the corresponding regions in other *Bacillus* strains

Regions of greater than 70% nucleotide sequence identity are indicated by stippled boxes when they reside within an ORF. A stippled horizontalline has been used to illustrate greater than 70% nucleotide sequence identity between intergenic regions. The 0.95 kb LL-ReplLR amplicon (restriction map l) and the 3.4 kb LL-ReplLR amplicon (restriction map 2) from *B. anthracis* Ames are illustrated at the top of the figure, respectively, with the scale shown in kilobase pairs (kb) at the bottom of the figure. The corresponding regions from the 3.4 kb LL-ReplLR amplicon in the following strains are also shown: *B. thuringiensis* H38 (restriction map 3); *Bacillus* sp. Ba8l3^{+} #25 (97–27; restriction map 4); *Bacillus* sp. Ba8l3^{+} #II (9594/3; restriction map 5); and *B. cereus* Zimbabwe (restriction map 6). The positions of the LL-ReplLR primer-binding sites are shown using smaller arrows. With the exception of ORFl/2, the larger filled arrows with flush 5' ends denote the orientation of each respective ORF listed in Table 2. ORFl/2 is presumed to be a relic, as it lacks an identifiable start codon, as illustrated by the unevenness of its 5' boundary. Moreover, ORFl/2 contains a stop codon (Z) within the reading frame that shares significant homology with the *xlyA* gene from a defective *B. subtilis* prophage (see Table 2). Restriction sites are abbreviated as follows: E, *Eco*RI*;* D, *DraI;* and H, HindIII. The asterisk denotes the internal. l kb biotinylated probe from *B. thuringiensis* H38.

related to other Bacilli, diverging from the previously mentioned branch-point with an S_{AB} value of 0.32. *Enterococcus faecalis* branched even further out, having an S_{AB} value of 0.30. The MB1 fingerprints of the two gram-negative species that were examined, *Brucella melitensis* (category 23) and *Escherichia coli* (category 24), had only one band in comparison to the other MB1 fingerprints. Since the MB1 fingerprints from microorganisms in categories 21–24, generated an extreme number of bands (Fig. 2) compared to the other 20 categories, only the most intense bands were selected for DENDRONN̆ analysis.

Many differences between non- *B. anthracis* LL-Rep1LR fingerprints were commonly observed (Fig. 4, Panel A). The LL-Rep1LR fingerprints *of B. thuringiensis* H12 and H37 (Fig. 4, Panel A, lanes 5 and 6) were the only two fingerprints that were indistinguishable. It was suspected that the very bright 3.4 kb band that was observed in lanes 7–10 might correspond to the same comparatively weak bands that were seen in the *B. thuringiensis* fingerprints (lanes 5 and 6) as well as the *B. anthracis* LL-Rep1LR fingerprints (lanes 1 and 2).

In order to test this hypothesis, the LL-Rep1LR REP-PCR mixtures shown in lanes 7–10 were diluted twenty-fold prior to electrophoresis and Southern blotting. Using an internal 1.1 kb biotinylated portion of the 3.4 kb amplicon from *B. thuringiensis* H38 as the probe, the 3.4 kb amplicon (Fig. 4, Panel C) was shown to be common in all fingerprints for which a corresponding band could be seen in the agarose gel (Fig. 4, Panel B). Although *B. thuringiensis* H34 (lane 3) and CT43 (lane 4) LL-Rep1LR fingerprints did not generate a 3.4 kb band, the biotinylated probe hybridized to a single smaller amplicon in each respective case. The 1.1 kb probe also hybridized to a smaller 0.95 kb band that was present in the *B. anthracis* fingerprints (lanes 1 and 2) as well as in the *B. thuringiensis* fingerprints shown in lanes 4–6 (Fig. 4, Panel C).

The internal 1.1 kb fragment from *B. thuringiensis* H38 that was used as a probe (which originated from the 3.4 kb LL-Rep1LR amplicon) was cloned and its nucleotide sequence determined, as was the 0.95 kb LL-Rep1LR amplicon from *B. anthracis* A19. Comparison of these two nucleotide sequences with the incomplete genome data from *B. anthracis* Ames (httP://www.tigr.org/cgi-bin/BlastSearch/blast.cgi) demonstrated that both of these distinct amplicons had regions of significant nucleotide sequence homology. The open reading frames (ORFs) are described in Fig. 5.

Genomic DNA isolated from alI of the strains examined in Figure 4, with the exception of *B. cereus* Zimbabwe (Fig. 4, lane 10), were digested with HindIII prior to being electrophoresed in a 0.7% agarose gel and transferred to Nytran (Sambrook *et al*., 1989). Following overnight hybridization to the 1.1 kb biotinylated probe, an intense band ranging in size from 2.2–2.3 kb was detected in every lane, with the exception of the lane containing *B. thuringiensis* H34 DNA, in which a larger HindIII fragment was detected (data not shown). A second set of fainter bands, having estimated sizes of 1.7–1.8 kb, were also observed in most lanes (data not shown), which was consistent with the possibility that the 1.1 kb probe had cross-hybridized with chromosomal DNA from which the 0.95 kb LL-Rep1LR amplicon arose (Fig. 5).

Southern blotting of genomic DNA suggested a high degree of conservation of the internal HindllI site within the 3.4 kb LL-Rep1LR amplicon (data not shown). However, as demonstrated in Figure 5, a number of restriction fragment length polymorphisms (RFLPs) could readily be detected by digesting the 3.4 kb amplicon from *B. thuringiensis* H38, *Bacillus* sp. Ba813$^+$ #25, *Bacillus* sp. Ba813$^+$ #11, and *B. cereus* Zimbabwe, with enzymes such as DraI, *Eco*RI, as well as HindIII.

Discussion

Improving strain differentiation within the "*B. cereus* group" of bacteria may eventually lead to a better understanding of the phylogenic and evo1utionary relationship between *B. anthracis*, *B. cereus* and *B. thuringiensis.* Nevertheless the host specificity remains a key characteristic which will separate these three groups of microorganisms without ambiguity. Although it is true that the virulence of *B. anthracis* strains is contingent upon the presence of both of pXO1 and pXO2 (Ramisse *et al*., 1996), the absence of these. plasmids does not reduce the distinctive characteristics of the *B. anthracis* chromosome (Andersen *et al*., 1996; Brumlik *et al*., 2001; Cherif *et al*., 2002; Keim *et al*., 1997; Keim *et al*., 2000; Ramisse *et al*., 1999). Moreover, *B. anthracis* possesses single nucleotide polymorphisms in the *rpoB* gene, a gene that encodes the β-subunit of RNA polymerase, with respect to other species within the "*B. cereus* group". (Qi *et al*., 2001).

MB1 REP-PCR fingerprinting measures the relatedness of microorganisms belonging to the "*B. cereus* group" (Fig. 3) based on colloborating genetic and phenotypic data shown in Table I. More distantly related *Bacillus* species such as *Bacillus licheniformis*, *Bacillus megaterium* and *Bacillus subtilis* could also be compared to strains with the "*B. cereus* group" using the FAM-labeled MB1 primer (Fig. 2). Analysis of the MB1 fingerprinting pattern in agarose gels demonstrated that several unrelated gram-positive and gram-negative bacteria were also capable of generating remarkably complex fingerprints (Fig. 1).

LL-Rep1LR fingerprinting was not found to be particularly useful with respect to examining the genetic relatedness of members of the "*B. cereus* group". This was primarily due to the high degree of complexity that was observed among LL-Rep1LR fingerprints generated by non- *B. anthracis* strains, especially strains possessing the Ba813 chromosomal marker (Brumlik *et al*., 2001). At times this was caused by the dramatic differences in the abundance of common bands such as the 3.4 kb amplicon (Fig. 4). Southern blotting of HindIII-digested chromosomal DNA did not suggest any differences in copy-number when size and chemiluminescent signal intensity of the regions encompassing the common 3.4 kb amplicon were compared (data not shown). The incomplete genome data of *B. anthracis* Ames (http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi) indicates the genes depicted in Figure 5 are found in single copies (Table II). Therefore, the abundance of the 3.4 kb LL-Rep1LR amplicon in a particular fingerprint must be affected by nucleotide sequence differences in at least one of the two LL-Rep1LR primer binding sites and not by differences in the copy number of this genetic locus.

The RFLP studies on particular 3.4 kb amplicons (Fig. 5) suggested that *Bacillus* strains that were more distantly related on the basis of the MB1 dendrogram (Fig. 3), were more likely to show such differences. The RFLP profile (Fig. 5) was identical for *B. thuringiensis* H38 and *Bacillus* sp. Ba813$^+$ #25. These two strains resided within two closely related categories on the MB1 dendrogram (categories 17 and 1, respectively). The next most closely re1ated category, which harbored a11 *B. anthracis* strains that were examined (category 2), showed a distinct RFLP profile for the above Bacilli, since RFLP analysis of the 3.4 kb LL-Rep1LR amplicon from *B. anthracis* strains lacked an EcoRI site (Fig. 5). More distantly related MB 1 categories comprising strains such as *Bacillus* sp. Ba813$^+$ #11 (category 9) and *B. cereus* Zimbabwe (category 16) had a greater number of differences in the RFLP profile of their 3.4 kb LL-Rep1LR amplicons (Fig. 5).

This MB1 fingerprinting study provides a new tool available to investigate the relationships of the microorganisms described in the dendrogram (Fig. 3). The relatedness is reinforced by (*i*) the H-antigen classification of any serotypable *Bacillus* strain that was tested (Table 1), (*ii*) the LL-Rep1LR fingerprint (Fig. 4,

Panel A), (*iii*) the presence and relative abundance of the 3.4 kb LL-Rep1LR amplicon (Fig. 4), and (*iv*) the corresponding RFLP profile of the 3.4 kb amplicon if the band was found to be present (Fig. 5).

As expected, all of the strains belonging to the "*B. cereus* group" resided in MB 1 categories that were centrally located in the dendrogram. This region was flanked by more distantly related *Bacillus* species, and representatives of extremely diverse bacterial species such as *Brucella melitensis*, *Escherichia coli,* and *Enterococcus faecalis.* The fact that *Bacillus* strains possessing the Ba813 chromosomal marker fell into two distinct clusters in the dendrogram, and that the *B. cereus* strains that were examined were found to overlap one of these two clusters, strongly supported the power of the MB1 fingerprinting system to demonstrate genetic relatedness among *Bacillus* species. Closely related MB1 categories tended to share common features such as the appearance of a common 3.4 kb amplicon in their LL-Rep1LR fingerprints notwithstanding the fact that each individual LL-Rep1LR fingerprint often showed significant differences (Fig. 4).

Taken together, these data suggest that the MB1 fingerprinting system is a valid method of measuring the evolutionary relationships between *Bacillus* strains such as *B. anthracis*, *B. cereus* and *B. thuringiensis. B. anthracis* is capable of infecting and killing its mammalian host when it possesses the two virulence plasmids pXO1 and pXO2 (Ramisse *et al*., 1996). *B. thuringiensis* can analogously become insecticidal once *cry* genes have been expressed (Hernandez *et al*., 2001; Lecadet *et al*., 1999; Schnepf *et al*., 1998; Vilas-Boas *et al*., 2002). The *cerA* and *cerB* genes are genetic markers found exclusively in *B. cereus* strains (Drobniewski, 1993). To some extent the ecological niche contributes to the genomic organization of *Bacillus* species and conversely, the presence of certain plasmids or genes establish the environment where each of these species is best suited to survive. These particular genetic markers allow differentiation between these species from a taxonomic point of view. The MB1 fingerprinting method simultaneously examines a number of reference points within the genomes of the microorganisms regardless of their plasmid content. This has facilitated an evaluation of the degree of relatedness between bacterial strains, and is not simply a classification system.

It also appears as though MB1 fingerprinting analysis may be able to show more distant evolutionary relationships between such diverse microorganisms as *Brucella melitensis*, *Enterococcus faecalis*, as well as *Escherichia coli.*

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