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Differentiation of *Bacillus anthracis* from Other *Bacillus cereus* Group Bacteria with the PCR

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Variation among isolates of *Bacillus anthracis* was examined by using restriction fragmentation patterns and the PCR performed with arbitrary and sequence-specific oligonucleotide primers. The patterns were compared with the patterns generated from strains of closely related species belonging to the "*Bacillus cereus* group" of bacteria, including *B. cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides*. All *B. anthracis* profiles were identical for each of 18 restriction enzymes, each of 10 arbitrary PCR primers, and a repetitive extragenic palindrome-specific PCR primer. The PCR profiles generated with a coliphage M13-based primer exhibited slight pattern variation in a 400- to 500-bp band region. The *B. anthracis* profiles were unique compared with the profiles of the other species examined. In these other species, strain-to-strain variations were observed. Our results showed that isolates of *B. anthracis* are almost completely homogeneous, indicating a clonal lineage, and are distinct from other members of the *B. cereus* group and that *B. anthracis*, as a species in its own right, may have evolved only relatively recently.

DNA homology studies (11, 24) have shown that *Bacillus anthracis* is closely related to *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides*. These species have almost identical G+C contents (31 to 34 mol%) (15), and ribosomal DNA sequence data have revealed only minor differences among them (1, 2). In the laboratory, confirmation of suspect isolates as *B. anthracis* is generally accomplished by using tests for gamma phage susceptibility, penicillin susceptibility, motility (*B. anthracis* is not motile), hemolysis (absent or delayed in *B. anthracis*), and the production of capsule and toxin gene products.

Textbook methods for the identification of *B. anthracis* are based on biochemical and serological techniques (reviewed in reference 29), but in practice, the sharing of biochemical and antigenic properties by members of the "*B. cereus* group" is such that these techniques are not reliable for distinguishing one member of the group from another. The main difference between *B. anthracis* and other members of the *B. cereus* group is the presence of two virulence plasmids, pXO1 (which codes for toxin production) and pXO2 (which codes for capsule production). The PCR has been used to detect virulence plasmid genes and is important for the detection of virulent forms of *B. anthracis* in the environment (7, 27). However, the inability of this technique to detect plasmid-free strains that may have arisen from virulent precursors should be kept in mind (29). The basis for distinguishing such plasmid-free isolates from *B. cereus* rests on the gamma phage susceptibility, motility, and hemolysis tests mentioned above.

The ability to distinguish strains of *B. anthracis* is also important for both epidemiological purposes and quality control of vaccine seed cultures ($pXO1^+$ $pXO2^-$). To date, this has not been possible because of the conserved nature of the species and the phenotypic overlap with other members of the *B. cereus* group. Therefore, there is an urgent need for a way to specifically identify all forms, virulent and avirulent, of *B. anthracis* and for a way to distinguish strains within the species.

One technique to determine interrelationships which has been successfully used for gram-positive organisms (19) and in particular for *B. thuringiensis* (18) is DNA fingerprinting using probes that detect restriction fragment length polymorphisms. Differences between organisms, as reflected in the frequency and intersequence heterogeneity of specific repeated sequences distributed throughout the genome, can be revealed by Southern blotting of cleaved DNA with appropriate probes. An alternative way to fingerprint genomes is with the PCR, by using either the randomly amplified polymorphic DNA analysis technique (32) or sequence-specific primers. This method has been used to amplify species-specific sequences for the detection of clinically important food-borne pathogens (16) and in environmental surveillance studies (3). Randomly amplified polymorphic DNA PCR methods have been used to differentiate species and strains with oligonucleotide primers with arbitrary sequences and have been used to differentiate *Lactococcus lactis* (6) and *Clostridium difficile* (17). The principal advantages of this technique are its reproducibility and the lack of a requirement for a detailed knowledge of the genomic organization of the target organism.

The primary focus of this study was to demonstrate differences between isolates of *B. anthracis* at the genetic level for the purposes of (i) reliable and consistent differentiation from other species belonging to the *B. cereus* group, (ii) epidemiological comparison of isolates obtained from outbreaks of anthrax, and (iii) monitoring variations known to occur in vaccine seed strains for quality control purposes.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. All strains were inoculated into 100 ml of L broth and grown with shaking (200 rpm) for 8 h at 37°C.

DNA isolation. The method of Marmur (14) was modified

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TABLE 1. Origins and characterization of the strains used in this study[a]

B. anthracis isolate(s)[b]	Other designation	Origin, year of isolation, and/or other information[c]	Reference(s)
NCTC 8234	Sterne[d]	Cow, South Africa, 1937, pXO1$^+$ pXO2$^-$ human vaccine strain	
ASC 97	Sterne[e]	Cow, South Africa, 1937, pXO1$^+$ pXO2$^-$ human vaccine strain	26
ASC 47		Animal vaccine strain[f]	12
ASC 68	Ames	Cow, United States, 1980	
ASC 162, ASC 167		Guinea pig reisolates of ASC 68	29
NCTC 10340^T	Vollum	Cow, United Kingdom, pre-1939	25
ASC 69	New Hampshire	Human, United States, 1957	22
ASC 182	Pasteur	France, pre-1880s	21
ASC 27, ASC 28, ASC 29, ASC 30, ASC 31		Cattle, United Kingdom, from West Africa, 1977 or 1978	13
ASC 58, ASC 60		Elephant, Namibia, 1983	28
ASC 50, ASC 52, ASC 54, ASC 55, ASC 56, ASC 189		Human epidemic, Zimbabwe, 1982	9, 10
ASC 234		Sewage, 1992	
ASC 122, ASC 126		pXO1$^-$ pXO2$^-$ sewage isolates, United Kingdom, 1989	33
ASC 101, ASC 103		pXO1$^+$ pXO2$^-$ tannery dump isolates, 1988	
ASC 184	Cured Vollum isolate[g]	pXO1$^+$ pXO2$^-$	
ASC 185	Cured Vollum isolates[g]	pXO1$^-$, pXO2$^+$	
NCTC 5444		United Kingdom, 1938	20
ATCC 938		Human, United Kingdom, 1931	8
ASC 65		Cow, Brazil, 1982	5
ASC 112, ASC 114		pXO1$^-$ pXO2$^-$ sewage isolates, United Kingdom, 1988	
ASC 327	Vollum[g]	pXO1$^-$ pXO2$^-$	
ASC 328	Vollum[g]	pXO1$^+$ pXO2$^+$	
ASC 183		pXO1$^-$ pXO2$^+$ penicillin-resistant isolate, 1976[h]	25
ASC 45	Sterne		12

[a] The following other members of the *B. cereus* group were also used in this study: *B. cereus* NCTC 2599^T (T = type strain), NCTC 7464, and NCTC 9939; *B. cereus* F4810/72, F4433/73, F2532/74, and F8035, obtained from the Food Hygiene Laboratory, Central Public Health Laboratory, Colindale, London, England; *B. mycoides* NCTC 09680; *B. thuringiensis* HD37, HD102, and HD225, obtained from H. T. Dulmage, U.S. Department of Agriculture, Brownsville, Tex.; and *B. thuringiensis* F2110/78 (subsp. *kenyae*) and F2113/78 (subsp. *entomocidus*), obtained from the Food Hygiene Laboratory, Central Public Health Laboratory, Colindale, London, England.

[b] ASC, Anthrax Section culture, Division of Biologics, Public Health Laboratory Service Centre for Applied Microbiology and Research, Porton Down, Salisbury, England; ATCC, American Type Culture Collection, Rockville, Md.; NCTC, National Collection of Type Cultures, London, England.

[c] Unless stated otherwise, all *B. anthracis* isolates are pXO1$^+$ pXO2$^+$.

[d] Original Sterne vaccine strain.

[e] Wellcome vaccine strain.

[f] Romanian Stamatin animal vaccine strain (designated strain B1119 by Logan et al. [12]).

[g] Defence Microbiology Division, Chemical and Biological Defense Establishment, Porton Down, Salisbury, England.

[h] ASC 32 cured of the appropriate plasmid by C. P. Quinn, Division of Biologics, Public Health Laboratory Service Centre for Applied Microbiology and Research, Porton Down, Salisbury, England.

to overcome problems with lysis of *B. anthracis* cells. Briefly, cells from 8-h 100-ml cultures (A_{600}, 2.0) were pelleted by centrifugation at 10,000 × *g* and resuspended in 5 ml of 0.1 M potassium phosphate buffer (pH 6.8). After the addition of mutanolysin (final concentration, 100 U/ml; Sigma) and incubation at 37°C for 1 h, lysozyme (Sigma) was added to a concentration of 0.2 mg/ml, and incubation was continued for an additional 1 h, after which the suspension was heated at 60°C for 5 min. The cells were lysed by adding 0.05 volume of a warm (60°C) 25% (wt/vol) sodium dodecyl sulfate solution; after 5 min at 60°C, the cell lysate was allowed to cool to the ambient temperature. Proteins were removed by phenol extraction, and the DNA was collected by spooling with cold absolute ethanol (23). After resuspension in TE buffer (10 mM Tris-Cl [pH 8.0], 1 mM EDTA), RNA was removed by adding DNase-free RNase (final concentration, 1 μg/ml; Boehringer) and incubating the preparation at 37°C for 1 h. The DNA solution was reextracted once with phenol and then three times with chloroform-isoamyl alcohol (24:1, vol/vol). The DNA was collected by spooling with isopropanol, washed for 5 min in increasing concentrations of ethanol (70 to 100%, vol/vol), dried, and finally resuspended in a minimum volume of TE buffer.

Restriction enzyme digestion. The restriction enzymes used to digest total cellular DNA were *Alu*I, *Dra*I, *Dra*II, *Eco*RI, *Eco*RV, *Hae*II, *Hae*III, *Hin*dIII, *Hin*fI, *Hpa*I, *Hpa*II, *Pst*I, *Pvu*II, *Sau*3AI, *Sin*I, *Sty*I, *Taq*I, and *Xba*I. In each case, 5 μg of DNA was digested with the restriction enzyme in accordance with the instructions of the manufacturer (Northumbria Biologicals Ltd., Cramlington, United Kingdom). Digests were heated to 70°C prior to analysis by electrophoresis (23).

Oligonucleotides. Oligonucleotides were synthesized with an Applied Biosystems model 380B DNA synthesizer by using the automated phosphoramidite coupling method or were obtained directly from Alta Biosciences, Edgbaston, United Kingdom. The following arbitrary oligonucleotide primers were used in this study: (i) 5′-TCGAGCTT-3′, (ii) 5′-GAGCTGACCA-3′, (iii) 5′-CGTAACGACGTC-3′, (iv) 5′-CTTCAGATAGACACG-3′, (v) 5′-TGAATGAAGCCAT ACCAA-3′, (vi) 5′-GTATTGTTAACCCAACTG-3′, (vii) 5′-GTATGAGTATTCAACATTC-3′, (viii) 5′-ATAAATAAAA TATATTCAAAAA-3′, (ix) 5′-AAGTTGACAAAATTATT TAAG-3′, and (x) 5′-TGAAGATAAAAATATTGTTTTTA 3′. The following sequence-specific primers were used: (i) 5′-GAGGGTGGCGGCTCT-3′, taken from the sequence of the M13 fingerprinting probe first identified by Vassart et al.

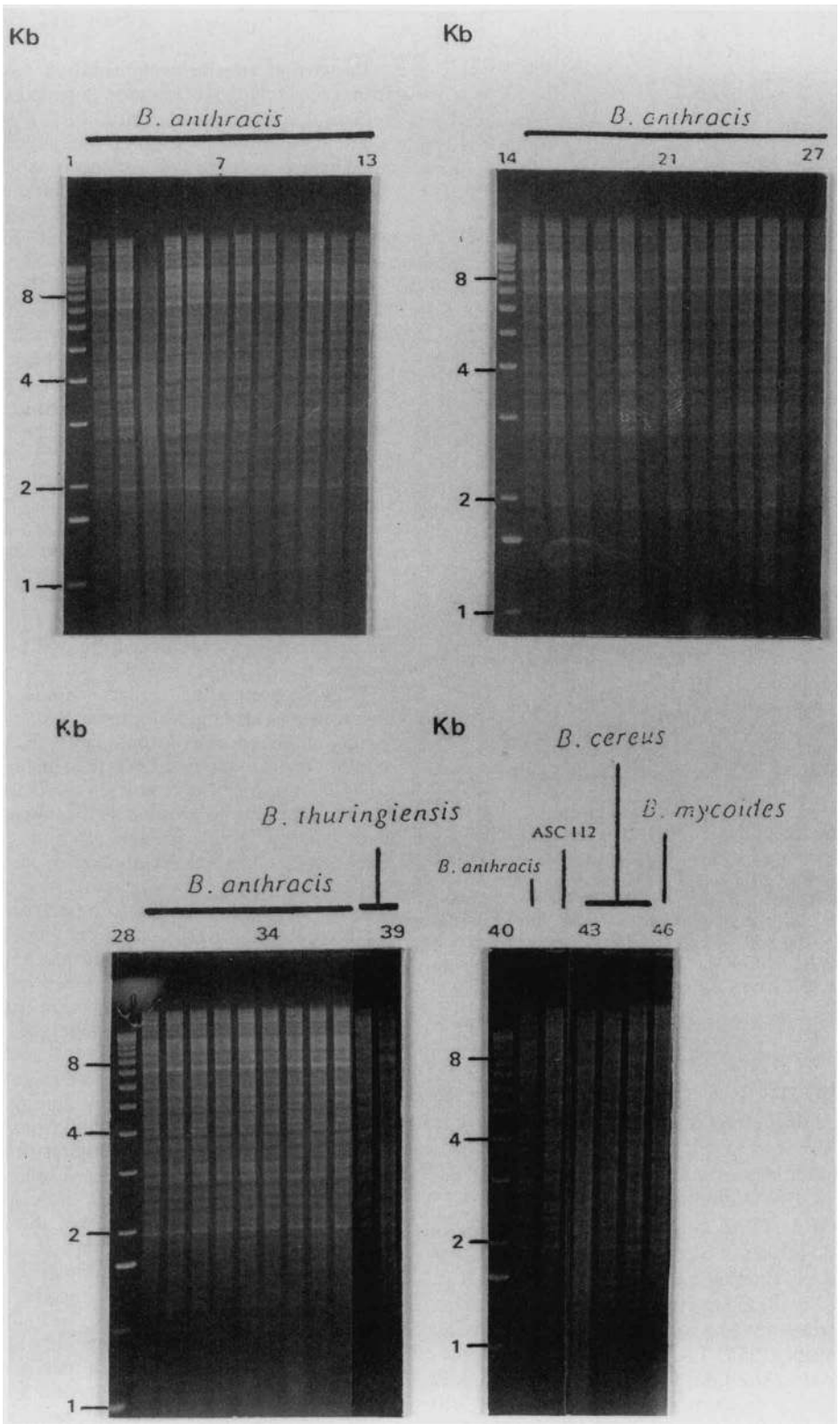

FIG. 1. *Eco*RI digests of total DNAs from members of the *B. cereus* group. DNA was separated by electrophoresis on a 0.7% agarose gel at 2.5 V/cm. Lanes 1, 14, 28, and 40, kilobase ladder; lanes 2 through 13, *B. anthracis* ASC 50, ASC 52, ASC 54, ASC 55, ASC 56, ASC 189, ASC 234, ASC 122, ASC 126, ASC 101, ASC 103, and ASC 327, respectively; lanes 15 through 27, *B. anthracis* ASC 184, ASC 185, ASC 328, ASC 68, ASC 162, ASC 167, NCTC 10340^T, ASC 69, ASC 182, ASC 27, ASC 28, ASC 29, and ASC 30, respectively; lanes 29 through 37, *B. anthracis* ASC 31, ASC 65, ASC 45, ASC 60, NCTC 8234, ASC 97, ASC 47, ASC 58, and ASC 183, respectively; lanes 38 and 39, *B. thuringiensis* HD 37 and F2110/78, respectively; lane 41, *B. anthracis* NCTC 10340^T; lane 42, ASC 112; lanes 43 through 45, *B. cereus* NCTC 2599^T, NCTC 7464, and F4810/72, respectively; lane 46, *B. mycoides* NCTC 09680.

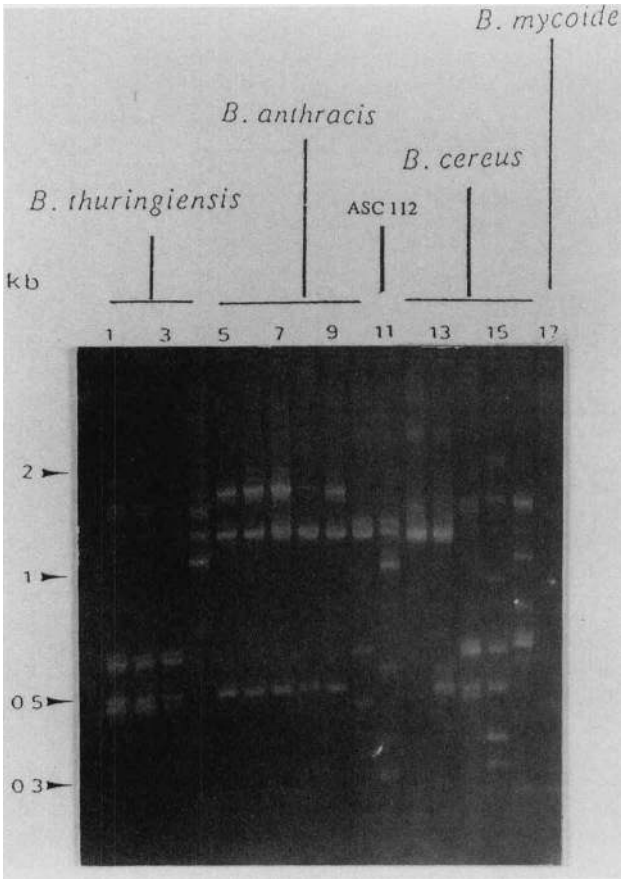

FIG. 2. PCR fingerprinting of *B. cereus* group strains performed with the sequence-specific primer 5′-IIIICGICGICATCIGGC-3′. PCR products were separated by electrophoresis on a 2% agarose gel at 2.5 V/cm. Lane 1, 1-kb ladder; lanes 2 through 5, *B. thuringiensis* HD37, HD102, HD225, and F2113/78, respectively; lanes 6 through 11, *B. anthracis* NCTC 8234, NCTC 10340^T, ASC 68, ASC 69, ASC 182, and ASC 112, respectively; lanes 12 through 16, *B. cereus* F2532/79, F4810/72, F4433/78, NCTC 2599^T, and NCTC 9939, respectively; lane 17, *B. mycoides* NCTC 09680.

(30), and (ii) 5′-IIIICGICGICATCIGGC-3′, taken from the consensus sequence of the repetitive extragenic palindrome described by Versalovic et al. (31).

PCR reaction conditions. Each PCR mixture (100 μl) contained 150 ng of DNA template, 1.25 mM dATP, 1.25 mM dCTP, 1.25 mM dGTP, 1.25 mM dTTP, 3.0 mM Mg^{2+}, each oligonucleotide primer at a concentration of 2 μM, and 2.5 U of *Taq* DNA polymerase. The primers were used (i) singly, (ii) in pairs, or (iii) in several different combinations. The following thermal cycling conditions were used: an initial denaturation step (95°C for 10 min; *Taq* DNA polymerase was added after the first 5 min of this step) and 39 cycles consisting of (i) denaturation at 96°C for 1 s, (ii) annealing at 30°C for 10 s, and primer extension at 72°C for 10 s. Samples were overlaid with 100 μl of mineral oil to minimize evaporation during thermal cycling. When the PCR was completed, the samples were frozen at −20°C to allow removal of the mineral oil without loss of sample; once the samples were thawed, they were analyzed by electrophoresis (23).

RESULTS

Restriction enzyme fragmentation patterns. Total DNAs from the *Bacillus* strains were digested with each enzyme; a typical result (obtained with *Eco*RI) is shown in Fig. 1. For a given restriction enzyme, the patterns obtained for all specifically identified *B. anthracis* isolates were identical. The patterns obtained for other members of the *B. cereus* group were different from the *B. anthracis* pattern and exhibited strain-to-strain differences (Fig. 1, lane 11). One strain included in this study was ASC 112, which was isolated from a site in the United Kingdom that was suspected of being contaminated with anthrax spores. This isolate was morphologically similar to *B. anthracis* but was found to be resistant to gamma phage and penicillin (and hence would normally be regarded as *B. cereus*). The restriction enzyme fragment pattern of the DNA from this isolate was almost indistinguishable from the patterns of the other *B. anthracis* isolates, but a careful examination of certain bands revealed slight differences, indicating that this isolate may represent a naturally occurring hybrid or intermediate between *B. anthracis* and other members of the *B. cereus* group. The inability to specifically identify differences between confirmed *B. anthracis* isolates and the presence of isolates such as ASC 112 indicate that our method is inadequate for specifically and unequivocally differentiating *B. anthracis* from other closely related members of the *B. cereus* group.

PCR fingerprinting. Fingerprinting of *B. cereus* group bacteria was attempted by using sequence-specific and randomly amplified polymorphic DNA PCR methods. The PCR conditions used included various annealing temperatures (30 to 65°C) and Mg^{2+} concentrations (0.5 to 5.0 mM Mg^{2+}) in the PCR mixture to provide the best possible fingerprint with each oligonucleotide primer. No differences were observed between *B. anthracis* isolates when we used arbitrary primers, either singly or in combination with other primers (multiplex), at the annealing temperatures and Mg^{2+} concentrations at which DNA products were formed. Differences were observed between *B. anthracis* and the other members of the *B. cereus* group and between individual strain types, as previously found with restriction fragmentation patterns (Fig. 2). The profiles of PCR-generated DNA fragments for these species and strains did reveal some band sharing with *B. anthracis*, but the profiles were more complex and readily distinguishable. When this technique was used, isolate ASC 112 could be readily distinguished from *B. anthracis* strains, including those *B. anthracis* isolates that lacked one or both of the virulence plasmids. These results demonstrate that the PCR fingerprint obtained for *B. anthracis* is independent of virulence status and distinct from the fingerprints of other members of the group. This method has the advantage that fingerprint profiles are much simpler to read than restriction fragmentation patterns and results can potentially be achieved much more rapidly.

One of the two sequence-specific oligonucleotide primers used, the M13 consensus sequence primer (5′-GAGGGTG GCGGCTCT-3′), yielded fingerprints that revealed minor differences between the *B. anthracis* isolates. These polymorphisms (Fig. 3 and 4) probably represent small differences, either at the primer binding site or in the spaces between putative PCR primer binding sites. On the basis of these differences, *B. anthracis* isolates were divided into three groups (Table 2). These groups are not related to virulence status, infected host species, or geographical origin.

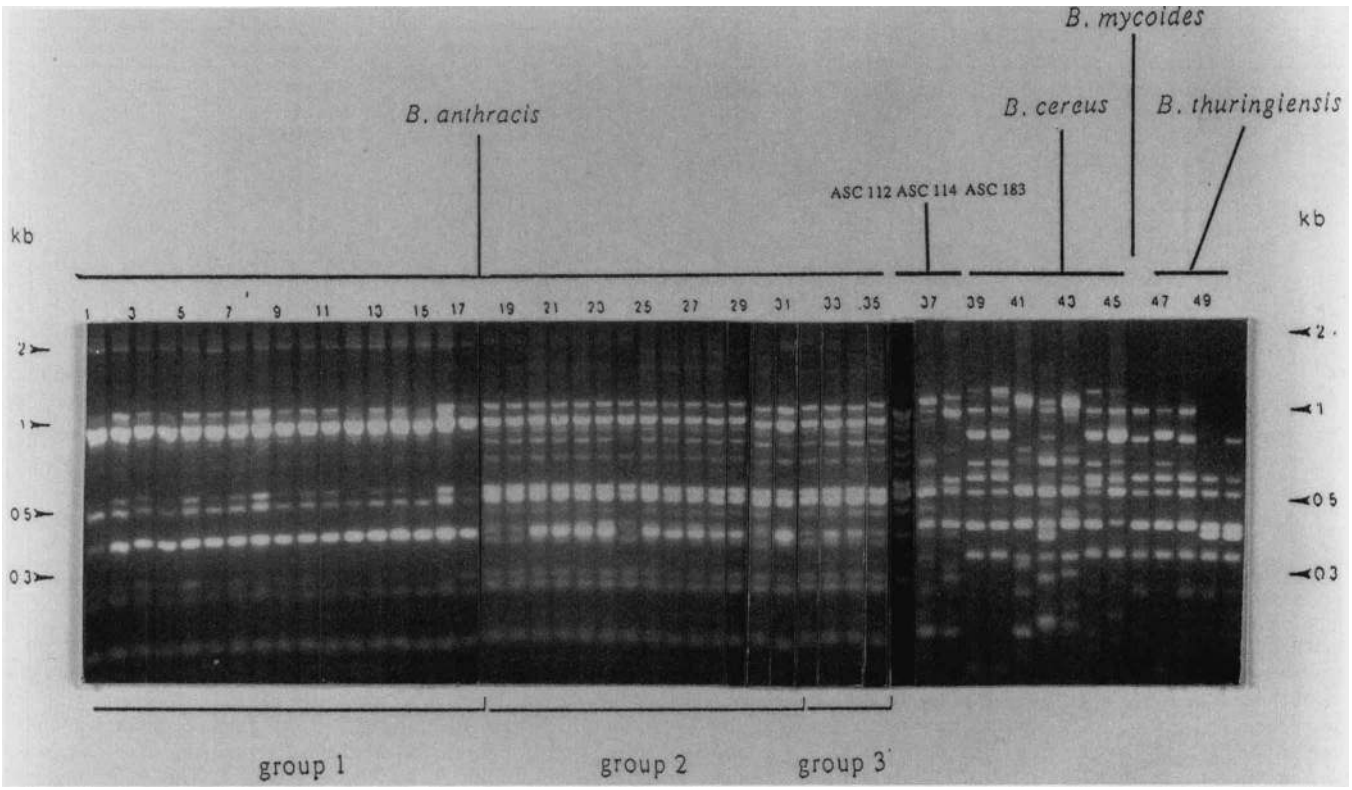

FIG. 3. PCR fingerprinting of *B. cereus* group strains performed with the M13 consensus sequence primer 5′-GAGGGTGGCGGCTCT-3′. PCR products were separated by electrophoresis on a 2% agarose gel at 2.5 V/cm. Lanes 1 through 17, group 1 *B. anthracis* isolates ASC 50, ASC 52, ASC 54, ASC 55, ASC 56, ASC 189, ASC 234, ASC 122, ASC 126, ASC 101, ASC 103, ASC 327, ASC 184, ASC 185, ASC 328, NCTC 5444, and ATCC 938, respectively; lanes 18 through 31, group 2 *B. anthracis* isolates ASC 68, ASC 162, ASC 167, NCTC 10340^T, ASC 69, ASC 182, ASC 27, ASC 28, ASC 29, ASC 30, ASC 31, ASC 65, ASC 45, and ASC 60, respectively; lanes 32 through 35, group 3 *B. anthracis* isolates NCTC 8234, ASC 97, ASC 47, and ASC 58, respectively; lanes 36 through 38, strains ASC 112, ASC 114, and ASC 183, respectively; lanes 39 through 45, *B. cereus* NCTC 2599^T, NCTC 7464, F4810/72, F4433/73, F2532/74, 8035, and NCTC 9939, respectively; lane 46, *B. mycoides* NCTC 09680; lanes 47 through 50, *B. thuringiensis* HD 37, HD 102, F2110/78, and F2113/78, respectively.

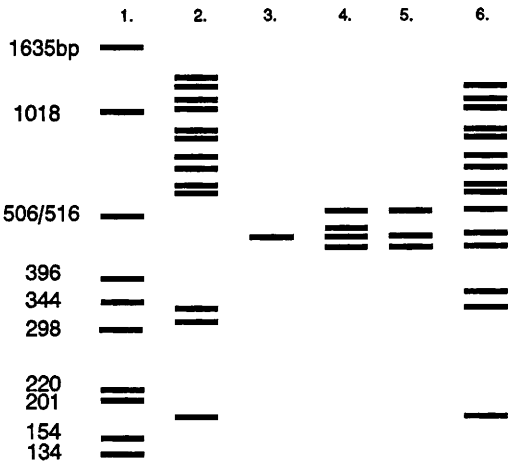

FIG. 4. Schematic representation of the variable regions in the PCR fingerprints of *B. anthracis* strains determined with the M13 consensus sequence primer. Lane 1, 1-kb ladder; lane 2, bands common to all *B. anthracis* isolates; lane 3, bands found in group 1 isolates; lane 4, bands found in group 2 isolates; lane 5, bands found in group 3 isolates; lane 6, fingerprint of ASC 112. The strains in each group are shown in Table 2.

DISCUSSION

The importance of *B. anthracis* as a pathogen requires that a means be sought for both specific identification and differentiation of this taxon from the other members of the closely related *B. cereus* group and subdifferentiation of the species into strains for epidemiological and other purposes. For microbiologists familiar with the *B. cereus* group, members of this group are normally fairly simple to identify in the laboratory. The high degree of sharing of phenotypic characteristics within the group means that further identification of *B. anthracis* and *B. thuringiensis* is based on the detection of virulence factors (i.e., capsule and toxin production in *B. anthracis* and parasporal crystal proteins in *B. thuringiensis*). However, with *B. anthracis*, the existence of avirulent "anthrax-like" organisms in the environment that lack the genes for toxin and/or capsule production can easily defeat such techniques. A more sensitive and revealing approach to identification and differentiation is to assess genome structure. In this study, this approach took the form of an analysis of restriction enzyme digestion patterns of isolated total DNAs; we also examined the more complex aspects of specific sequence distribution that were revealed by DNA fingerprinting with the PCR.

A simple comparison of restriction fragment patterns revealed that at this level of differentiation, *B. anthracis* is homogeneous and that differences between virulent and

TABLE 2. *B. anthracis* groups as determined by M13 PCR fingerprinting

Group	Strain(s)	Geographical origin	Source	Other designation
1	ASC 50, ASC 52, ASC 54, ASC 55, ASC 56, ASC 189	Africa	Human	
	234	United Kingdom	Sewage	
	ASC 122, ASC 126	United Kingdom	Sewage	
	ASC 101, ASC 103	United Kingdom	Tannery	
	ASC 327	United States	Unknown	
	ASC 184	United Kingdom	Cow	Vollum
	ASC 185	United Kingdom	Cow	Vollum
	ASC 328	United States	Unknown	
	NCTC 5444	United Kingdom	Unknown	
	ATCC 938	United Kingdom	Human	
2	ASC 68, ASC 162, ASC 167	United States	Cow	Ames
	NCTC 10340^T	United Kingdom	Cow	Vollum
	ASC 69	United States	Human	New Hampshire
	ASC 182	France	Unknown	Pasteur
	ASC 27, ASC 28, ASC 29, ASC 30, ASC 31	Africa	Cow	
	ASC 65	Brazil	Cow	
	ASC 45	United Kingdom	Cow	Sterne
	ASC 60	Africa	Elephant	
3	NCTC 8234, ASC 97, ASC 47	Africa	Cow	Sterne
	ASC 58	Africa	Elephant	

avirulent forms could not be detected. In contrast, *B. cereus* and *B. thuringiensis* not only produced patterns different from that of *B. anthracis* but also exhibited strain-to-strain heterogeneity. More importantly, this method could not unequivocally identify specific differences between *B. anthracis* and morphologically similar *B. cereus* group isolates that phenotypically appear to be *B. cereus*. DNA homology studies (4) have indicated that the level of similarity between individual isolates of *B. anthracis* is more than 90%, suggesting that the detection of polymorphisms or mutations within restriction enzyme sites, as revealed by DNA digestion, would be almost impossible unless gross rearrangements, deletions, or insertions were present.

When PCR fingerprinting with oligonucleotides having both arbitrary and specific sequences was used and when different annealing temperatures and Mg^{2+} concentrations were used, almost all of the patterns generated from the different *B. anthracis* isolates were identical. These patterns allowed *B. anthracis* to be specifically distinguished from other members of the *B. cereus* group. As observed with the restriction enzyme fragment patterns, PCR fingerprinting revealed a large degree of strain-to-strain heterogeneity in both *B. cereus* and *B. thuringiensis* and also revealed differences between *B. anthracis* and morphologically similar isolates. Such isolates exhibited a high degrees of band sharing with *B. anthracis*, but differences, mainly in the form of bands in addition to the bands in the standard *B. anthracis* profile, were readily distinguished in the fingerprints. These differences were independent of the virulence factor gene content in *B. anthracis*. The similarity of the profiles of certain *B. cereus* group isolates to the general *B. anthracis* profile suggests that these isolates are either (i) *B. cereus* strains that have a similar distribution of the M13 sequence in their genomes or (ii) avirulent *B. anthracis* isolates that represent a more heterogeneous member of the species. To date, heterogeneous isolates of *B. anthracis* have not been isolated from infections.

Penicillin susceptibility is one of the standard criteria used to confirm that an isolate is *B. anthracis*. It is interesting that a penicillin-resistant isolate (Fig. 3, lane 14) produced one of the three standard *B. anthracis* profiles with this fingerprinting primer without altering the profile. Either this organism has acquired the gene(s) for penicillin resistance or an existing gene(s) has in some way become derepressed or activated.

The M13 fingerprinting probe consensus sequence was the only 1 of 12 oligonucleotide primer sequences that revealed differences among *B. anthracis* isolates. The PCR fingerprints obtained with this primer exhibited minor differences in a 400- to 500-bp region which allowed us to assign the isolates to three groups. No correlation between these groups and host or parasite relationship was demonstrated, suggesting that no species-specific strains exist for the initiation of infection. However, the method did distinguish the vaccine strains (NCTC 8234, ASC 47, and ASC 97) from the other cultures; this was independent of plasmid content. Some level of correlation between group and country of origin may be apparent from the data in Table 2, but it must be borne in mind that many cases of anthrax (irrespective of host species) are directly or indirectly acquired from imported animal products, such as animal hides, wool, and hair or feed and fertilizer ingredients. This is exemplified by isolates ASC 27 through ASC 31, which were derived from cows with anthrax at different locations throughout the United Kingdom and were traced to contaminated peanuts imported from West Africa (13).

The lack of great variation in geographically separated isolates could be due to the fact that there are few opportunities for changes to occur because of the lack of chances for cycling of the organism in the environment and poor survival when the organism is in competition with other microorganisms. The opportunity for variation may arise only during the infrequent cycles through host organisms or in the presence of ideal environmental conditions. These limitations may mean that *B. anthracis* has only recently evolved as an independent species.

B. anthracis-like members of the *B. cereus* group (such as ASC 112) produce fingerprints very similar to those of conventional *B. anthracis* strains. These isolates may represent a link between the members of the group and a common ancestor; all of the *B. cereus* group isolates fingerprinted with the M13 primer had several bands in common with *B. anthracis*, suggesting that some conservation of chromosomal organization occurred. This could be investigated

further by investigating the sequences that are amplified between the M13 oligonucleotide primer sequences.

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