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Arbitrary Primer Polymerase Chain Reaction, a Powerful Method To Identify *Bacillus thuringiensis* Serovars and Strains†

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Arbitrary primer polymerase chain reaction technology has been applied to the identification of commercial strains of *Bacillus thuringiensis* by using total DNAs extracted from single bacterial colonies as templates. Characteristic DNA banding patterns can be readily and reproducibly obtained by agarose gel electrophoresis. This method has been used to distinguish commercial products containing *B. thuringiensis* serovar kurstaki (3a3b). When a single primer was used, this method was capable of producing discriminating DNA fingerprints for 33 known serovars. Differentiation from the closely related species *Bacillus cereus* is also readily achieved. This technique should prove to be a powerful tool for identification and discrimination of individual *B. thuringiensis* strains.

The group of bacteria formed by *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus anthracis* is of great interest both economically and medically. One member of this group, *B. thuringiensis*, constitutes by far the most widely used biological insecticide (6) and as such enjoys widespread public acceptance. In contrast, there is increasing concern about the involvement of *B. cereus* in episodes of food poisoning (12), while *B. anthracis* undoubtedly is a serious human pathogen (5). As requirements for environmental impact studies for biological agents become ever more stringent, it becomes a matter of some urgency to develop rapid and reliable methods to distinguish among these three species even though *B. thuringiensis* has been implicated in only a single case of human disease (18, 19).

Differential identification of *B. cereus*, *B. anthracis*, and *B. thuringiensis* is by no means easy. The three species share a great number of phenotypic and biochemical characteristics, such as fatty acid composition (15). The similarities in fatty acid composition are such that Lawrence et al. (15) state that differentiation among *B. thuringiensis*, *B. anthracis*, and *B. cereus* by using fatty acid gas chromatography is not possible if the bacteria are grown on standard complex media. Similarly, in a comparative study of enzyme variation, Zahner et al. (26) obtained results which grouped *B. cereus* and *B. thuringiensis* in a single species. It has also been found by one group of workers (11) that *B. cereus* and commercial strain HD-1 of *B. thuringiensis* produce identical hemolysins.

A characteristic that is often used to distinguish *B. thuringiensis* from *B. cereus* is the presence of parasporal crystals in the former organism. Indeed, in a recent public health study Green et al. (9) considered all *Bacillus* isolates that possessed the endogenous crystals (Cry^+) to be *B. thuringiensis* isolates. However, the fact that the crystal-encoding genes are plasmid borne (7) indicates that the Cry^+ crystal-producing phenotype is susceptible to loss and to transmission to related species. Indeed it has been shown (2, 7) that plasmids can be transferred in vitro among all three species, giving rise to Cry^+ strains of *B. anthracis* and *B. cereus*.

Currently, strains of *B. thuringiensis* are identified and grouped on the basis of their flagellar (H) antigens, a technique which has proven to be useful for species differentiation among the bacilli (4). However, it has been shown that *B. cereus* can cross-react serologically with some of the *B. thuringiensis* flagellar antigens (16) at a frequency close to 30%. Therefore, the distinction between an acrystalliferous *B. thuringiensis* strain and *B. cereus* is tenuous at best when current techniques are used.

The similarities among these species are naturally reflected at the nucleic acid level. Early work by several independent teams of researchers (13, 20, 22) grouped together *B. anthracis*, *B. cereus*, and *B. thuringiensis* on the basis of whole-genome hybridization. In a recent publication, Ash et al. (1) found that the sequences of the 16S rRNAs of the type strains of *B. anthracis* and *B. cereus* were identical, while the sequence differences between *B. cereus* and *B. thuringiensis* were small enough to be accommodated within the concept of intraspecies variation. These differences were also found to be insufficient to allow the design of a species-specific oligonucleotide probe.

In this study, we have applied the newly developed methodology of the arbitrary primer (AP) polymerase chain reaction (PCR) (2, 24, 25) to the problem of distinguishing among commercial *B. thuringiensis* strains and the differentiation of these strains from *B. cereus*. Furthermore, we have examined the utility of this technique for discriminating among the known serovars of *B. thuringiensis*.

MATERIALS AND METHODS

Bacterial strains and media. All of the *B. thuringiensis* strains used in this study and their origins are listed in Table 1. Colonies were first isolated from commercial preparations on PEMBA medium (10). Surface-inoculated plates (inoculated by using a spreader) were incubated at 37°C for 40 h. Colonies were then stained by using the Smirnoff technique (21) in order to detect the presence of parasporal crystals. All of the bacterial colonies used for the AP-PCR were

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TABLE 1. Description of *Bacillus thuringiensis* serovars used in this study

Serovar or biovar	HD or strain no.	Serovar designation
Serovars[a]		
Aizawai	HD-11	7
Alesti	HD-4	3a
Canadensis	HD-554	5a5c
Colmeri	HD-847	20
Darmstadiensis	HD-146	10
Dakota	HD-932	16
Dendrolimus	HD-7	4a4b
Entomocidus	HD-9	6
Finitimus	HD-3	2
Galleriae	HD-29	5a5b
Indiana	HD-521	15
Israelensis	HD-567	14
Kenyae	HD-5	4a4c
Kumamotoensis	HD-867	18
Kurstaki	HD-73	3a3b
Kyushuensis	HD-541	11a11c
Morrisoni	HD-12	8a8b
Ostriniae	HD-501	8a8c
Pakistani	HD-395	13
Pondicheriensis	HD-1011	20a20c
Shandogiensis	HD-1012	22
Sotto	HD-930	4a4b
Subtoxicus	HD-109	6
Thompsoni	HD-542	12
Thuringiensis	HD-2	1
Tochigiensis	HD-868	19
Tohokuensis	HD-866	17
Tolworthi	HD-537	9
Toumanoffi	HD-201	11a11b
Wuhanensis	HD-52	
Yunnaensis	HD-977	20
Biovars		
San diego[b]		8a8b
Tenebrionis[c]		8a8b

[a] Obtained from L. K. Nakamura, U.S. Department of Agriculture, Peoria, Ill.
[b] Obtained from Wendy Gelernter, Mycogen Corp., San Diego, Calif.
[c] Obtained from Abbott Laboratories, North Chicago, Ill.

grown overnight at 30°C on 2YT medium (17). All plasmid curing experiments in which strain HD-1 or HD-73 was used were carried out by growing organisms overnight at 42°C in liquid media. The lack of crystal production was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (17).

AP-PCR primers. A set of 120 random sequence primers that were 9 bases long were synthesized with an Applied Biosystems model 380A DNA synthesizer. The primers were designed in four families having average G+C contents of 90, 60, 45, and 30 mol% by using a program based on a random-number generator (Lotus 123 software run under MS-DOS). Approximately one primer in four gave a pattern, with the primers rich in G+C having a higher frequency of success (25). The three primers used in this study were selected from the full set of 120 primers on the basis of the information content of their patterns. These primers had the following sequences: primer 0910-08, 5'-CCGGCGGCG; primer 0940-12, 5'-ACGCGCCCT; and primer 0955-03, 5'-CCGAGTCCA.

PCR sample preparation. In this work, our primer collection was initially screened against *B. thuringiensis* HD-1 purified genomic DNA by using 1 μg of genomic DNA per reaction mixture (data not shown). However, it has been suggested elsewhere that single bacterial colonies could provide sufficient material for reproducible AP-PCR patterns (24). We found that identical patterns could indeed be obtained directly from bacterial colonies with an absolute minimum of preparative steps. After this discovery primer screening was done directly with isolated colonies. Boiling a harvested colony for 10 min followed by centrifugation gave the best results in terms of ease of preparation, reliability, and reproducibility. After overnight growth at 30°C on 2YT medium plates, one isolated colony was harvested by using a sterile needle and resuspended in 250 μl of sterile distilled water in a 0.5-ml plastic microcentrifuge tube. If the overnight growth plates were stored at 4°C, reproducible patterns could be obtained with colonies harvested after 4 days. Each tube was vortexed for 10 s and then placed in a holder (Altec Laboratories, Bolton, Mass.) (a custom-made device of similar design was also used) to prevent its cap from opening under pressure. The holder was placed in a boiling water bath for 10 min. The tubes were centrifuged at 10,000 × *g* for 30 s at room temperature and then placed on ice. The supernatant was used directly for the PCR.

PCR samples were prepared by using a 10× buffer containing 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 25 mM $MgCl_2$, and 0.1% gelatin. Each PCR tube contained 10× buffer (10 μl), supernatant from a boiled colony (50.5 μl), 2.5 U of *Taq* polymerase (Cetus) (0.5 μl), 400 pmol of primer (1 μl), 200 μM deoxynucleoside triphosphate (16 μl), and water (22 μl) (total volume, 100 μl). Each sample was covered with 2 drops of light mineral oil before it was placed in the thermal cycler. The negative controls were the same as the mixture described above except that the supernatant was replaced with distilled water. One negative control experiment was performed for each set of PCRs.

PCR amplification conditions. A Perkin-Elmer Cetus model TC1 thermal cycler was used for all amplifications. The temperature profile was as follows: denaturation was performed through a 1-min ramp from 72 to 96°C, followed by 1 s at 96°C; for the annealing step we used a 4.75-min ramp from 96 to 30°C, followed by 10 s at 30°C; and for the elongation step we used a 1.75-min ramp from 30 to 72°C, followed by 10 s at 72°C. The steps were repeated for subsequent cycles. A total of 30 cycles of amplification were performed, after which the sample block was kept at 4°C until electrophoresis was performed.

Electrophoresis. A 20-μl portion of the PCR sample was mixed with 2 μl of loading buffer (30% [vol/vol] glycerol, 0.15% bromophenol blue, 0.15% xylene cyanol), and the resulting preparation was loaded onto a 1.5% Tris-acetate–agarose gel containing 200 μg of ethidium bromide per liter. Electrophoresis was performed for 1.5 h at 7 V/cm. The results were visualized with a transilluminator by using 254-nm UV light and were recorded on Polaroid type 57 film.

RESULTS

Fingerprinting of commercial *B. thuringiensis* strains. The major goal of primer screening was to obtain patterns containing several discrete bands between 0.25 and 1.5 kb. This region was chosen because PCR amplification is sometimes inefficient for fragments that are larger than 1.5 kb. Primers which gave satisfactory patterns in this range were then evaluated for their ability to distinguish commercial *B. thuringiensis* strains from each other and also from *B. cereus*. As shown in Fig. 1A, primer 0955-03 produces a

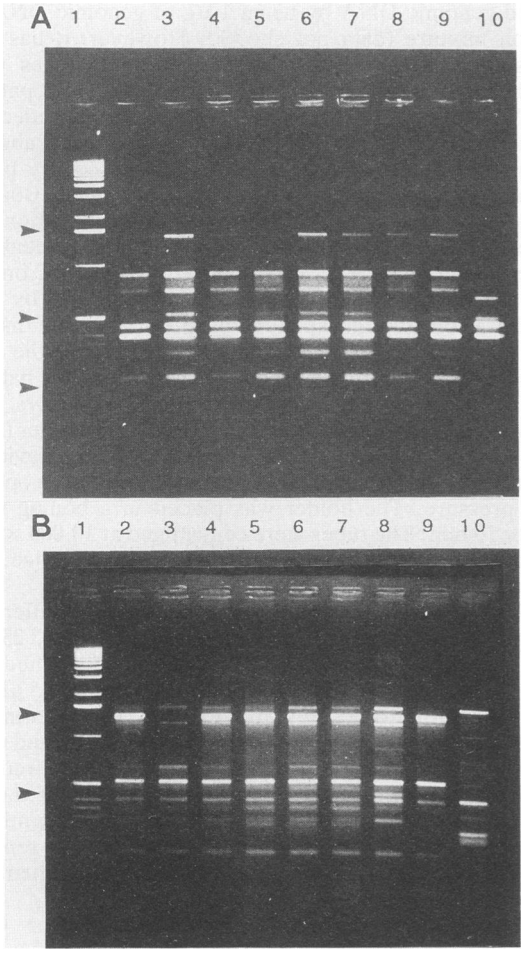

FIG. 1. AP-PCR comparison of different commercial strains of *B. thuringiensis* with *B. cereus*. (A) Primer 0955-03. (B) Primer 0940-12. Lanes 1 contained a 1-kb DNA ladder obtained from Bethesda Research Laboratories, Gaithersburg, Md. The positions of the relevant markers in panel A are indicated by arrowheads at (from top to bottom) 1,635, 516, and 220 bp. Lanes 2, Bernan (strain BC-1); lanes 3, BIODART (strain A-20); lanes 4, Dipel 96F (strain HD-1); lanes 5, Condor (strain EG2348); lanes 6, Foray 48B (strain HD-1); lanes 7, Futura (strain HD-1); lanes 8, Javelin (strain NRD-12); lanes 9, Thuricide (strain HD-1); lanes 10, *B. cereus* QUE. In panel B the upper arrowhead indicates the position of the diagnostic band for strain A-20, and the lower arrowhead indicates the position of the diagnostic band for strain NRD-12. The sources of the commercial strains were as follows: Bernan, Bactec Corporation; Biodart, ICI Forest Products; Condor, Ecogen, Inc.; Dipel 96AF and HD-1-S1980, Abbott Laboratories; Foray 48B, Novo Laboratories, Inc.; Futura, Chemagro Limited; and Javelin and Thuricide, Sandoz.

number of distinct DNA bands between 250 and 1,500 bp. A 1.6-kb band, which is present in each of the *B. thuringiensis* isolates shown in Fig. 1A, varies greatly in intensity. This illustrates the inefficiency of AP-PCR amplification of fragments larger than 1.5 kb. In fact, if a longer photographic exposure of the agarose gel in Fig. 1A is used, the 1.6-kb fragment is clearly identified. Although this primer cannot distinguish among the closely related commercial *B. thuringiensis* strains, it does produce a different DNA banding pattern for *B. cereus* QUE. However, there are a number of

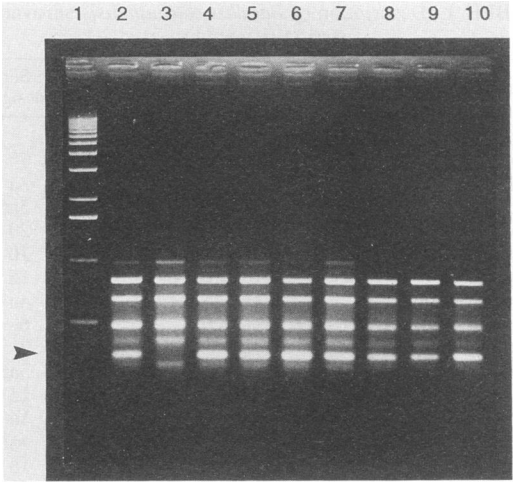

FIG. 2. Differential identification of Condor transconjugant EG2348 by using AP-PCR primer 0910-08. The arrowhead indicates the position of the diagnostic band. Lane 1, 1-kb ladder; lane 2, BIODART (strain A-20); lane 3, Ecogen (strain EG2348); lane 4, Javelin (strain NRD-12); lane 5, strain HD-1-S1980 standard; lane 6, Bernan (strain BC-1); lane 7, Dipel 96AF (strain HD-1); lane 8, Foray 48B (strain HD-1); lane 9, Futura (strain HD-1); lane 10, Thuricide (strain HD-1).

bands of similar sizes, which suggests that these species may be distantly related.

During our screening, one primer (primer 0940-12) was found to be especially useful for distinguishing among three commercial strains, strains HD-1, A-20, and NRD-12 (Fig. 1B). Strain A-20 from the ICI product BIODART can be differentiated from the other strains by the absence of a strong band at around 1.5 kb (Fig. 1B, lane 3) when primer 0940-12 is used. Strain NRD-12 from the Sandoz product Javelin can be distinguished from strain HD-1 by the presence of a characteristic doublet of bands at around 450 bp (Fig. 1B, lane 8). As shown in Fig. 1B, lane 10, *B. cereus* QUE produces a pattern that is drastically different from the patterns produced by the *B. thuringiensis* strains when primer 0940-12 is used.

Of the commercial isolates available to us, only Ecogen's Condor transconjugant EG2348 could not be differentiated with primer 0940-12. Therefore, screening of the random primer band was continued until we found a primer which could distinguish strain EG2348 from the commercial isolates derived from strain HD-1 or NRD-12. The results are shown in Fig. 2. Primer 0910-08 produced identical banding patterns with the nine isolates, with one exception. As shown in Fig. 2, lane 3, the Ecogen strain lacked a characteristic band at 350 bp which was produced by the eight other commercial strains.

Effects of *cry* gene loss on the serovar 3a3b AP-PCR banding pattern. Since the strains belonging to *B. thuringiensis* and *B. cereus* are differentiated essentially on the basis of intracellular crystal production, we examined whether the loss of one or more of the endogenous toxin genes through plasmid curing altered the AP-PCR DNA banding pattern. *B. thuringiensis* serovar 3a3b strains were chosen so that various genotypes ranging from Cry^- to the full complement of cryIA or cryII toxins were represented. As shown in Fig. 3A, primer 0955-03 produced a uniform pattern with all of the serovar 3a3b strains examined regardless of the toxin

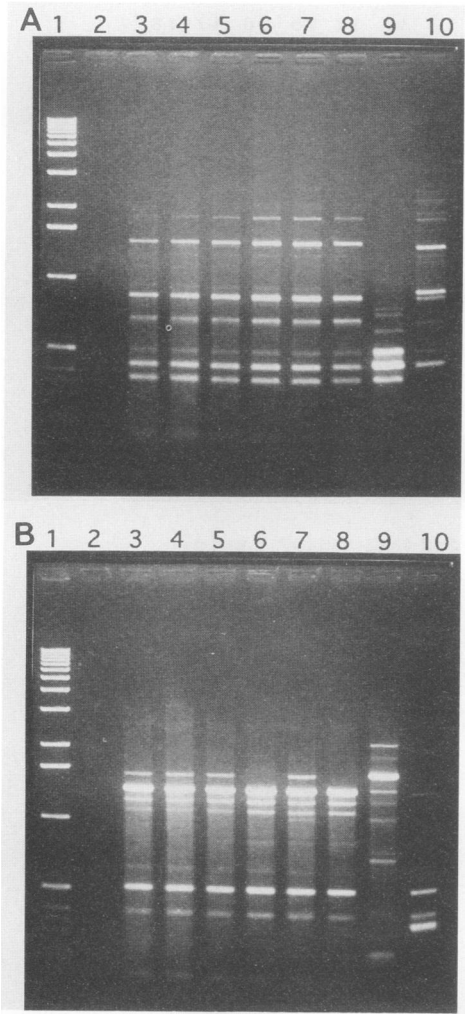

FIG. 3. AP-PCR comparison of *B. thuringiensis* serovar 3a3b strains with *B. cereus*. (A) Primer 0955-03. (B) Primer 0940-12. Lanes 1, 1-kb ladder; lanes 2, negative control; lanes 3, strain HD-1-S1980; lanes 4, strain HD-1 (three genes); lanes 5, strain HD-1 (two genes); lanes 6, strain HD-73; lanes 7, strain HD-1 (Cry^-); lanes 8, strain HD-73 (Cry^-); lanes 9, *B. cereus* QUE; lanes 10, *B. cereus* ATCC 14579. Sources: HD-1 (Cry^-), isolated from HD-1 by W. Moar, Auburn University, Auburn, Ala.; HD-1 (two genes), two-gene [*cryIA*(a) and *cryIA*(c)] HD-1 strain from J. Cabana, Ministère des Forêts du Quebec, Sainte-Foy, Quebec, Canada; HD-1 (three genes), three-gene laboratory strain (22); HD-73 (Cry^-), Cry^- strain of HD-73; *B. cereus* ATCC 14579, American Type Culture Collection; *B. cereus* QUE, P. Lacasse, Ministère de l'Agriculture, des Pêcheries et de l'Alimentation, Sainte-Foy, Quebec, Canada.

gene or crystal content. One important observation is that the two *B. cereus* strains produced patterns that were different from the serovar 3a3b isolate patterns. Furthermore, the banding pattern did not differ among the serovar 3a3b strains even though their plasmid and toxin compositions were variable, suggesting that primer 0955-03 annealed to genomic DNA rather than plasmid DNA. In this case, the loss of crystal production would not result in the reversion of the *B. thuringiensis* Cry^- strain to a *B. cereus* strain. When primer 0955-03 was replaced by primer 0940-12, similar results were observed (Fig. 3B). This primer produced novel banding patterns when total genomic DNAs from the two *B. cereus* strains were used; however, a weak 1.5-kb DNA band was missing from the strain HD-73 isolates when they were compared with the strain HD-1 isolates. It has been shown elsewhere (8) that strain HD-1 possesses approximately nine plasmids, whereas strain HD-73 possesses only six. Therefore, the possibility that the 1.5-kb band produced by the strain HD-1 isolates is plasmid specific cannot be ruled out, although the primer may detect only an inherent chromosomal difference between the two strains.

Applicability of the AP-PCR to the various *B. thuringiensis* serovars. Although strains belonging to the same serovar produce closely related banding patterns, we tried to ascertain whether a given primer that was selected to give several bands with strain HD-1 colonies (serovar kurstaki) would produce distinct patterns with *B. thuringiensis* isolates belonging to different serovars. When primer 0955-03 was used with 33 different serovars of *B. thuringiensis*, it produced a wide range of variable patterns (Fig. 4A and B). In some cases, the primer generated only a few weak bands (e.g., with serovars kumamotoensis, dakota, alesti, and finitimus). Interestingly, serovars aizawa and colmeri appeared to produce similar patterns, as did serovars sotto and thuringiensis. This seems to suggest that the strains are closely related to each other within these pairs; however, these differences must be interpreted with caution since PCR bands of similar size do not necessarily mean that the molecules are identical in sequence.

The two coleopteran-specific biovars (tenebrionis and san diego) also produced patterns that were relatively similar to each other, although there were some striking band differences at sizes larger than 1.6 kb (Fig. 4B, lanes 17 and 18). Once again, care must be exercised in interpreting these results since banding patterns at sizes greater than 1.5 kb can be variable because of inefficient PCR amplification.

DISCUSSION

As described previously, AP-PCR technology can provide fingerprints of complex genomes without the need for prior sequence information. The reproducibility of AP-PCR results can be evaluated from the data in Fig. 1, 2, and 4, in which the different commercial products based on strain HD-1 were expected to produce identical patterns. Some variation in the relative intensities of bands from sample to sample is observed, although this variation usually occurs in very weak bands or when the bands are larger than 1.5 kb. Nevertheless, distinguishing characteristics for certain commercial strains are reproducibly observed, and objective differentiation can be obtained. The AP-PCR DNA patterns which we obtained are surprisingly robust; for example, we found no appreciable effect of Mg^{2+} concentration on the reaction at concentrations between 2.5 and 6.5 mM (data not shown). In agreement with previously published work, the patterns are affected by the amount of DNA present (24). Furthermore, a strong dependence on the type and commercial supplier of the thermostable polymerase used was found. Also in agreement with previously published work, we found that a large amount of primer was required; however, the levels could be reduced from 400 to 200 pmol without a noticeable alteration in yield or DNA banding pattern (3). The number of PCR cycles was reduced from the 45 cycles used by previous workers to 30 cycles without an appreciable sacrifice in yield (25).

The relationship between *B. cereus* and *B. thuringiensis* HD-1 is reflected in some of the AP-PCR patterns in which

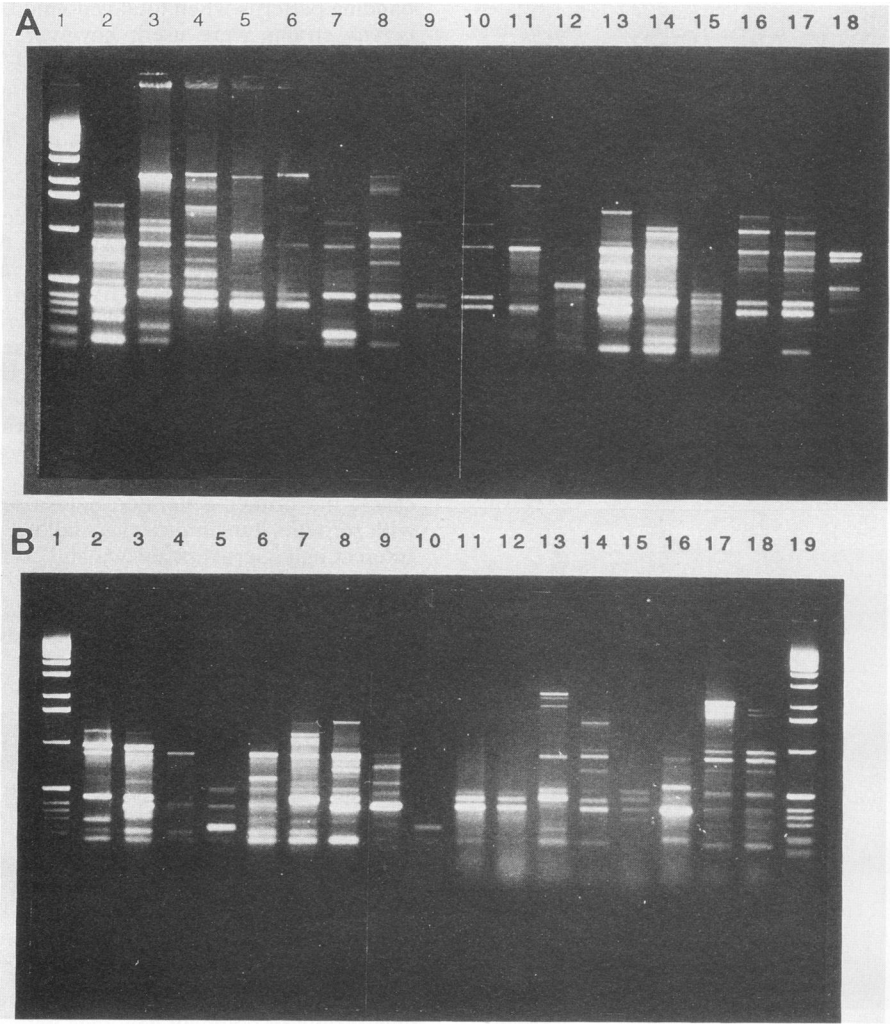

FIG. 4. AP-PCR results obtained with strains of *B. thuringiensis* serovars by using AP-PCR primer 0955-03. (A) Lane 1, 1-kb ladder; lane 2, strain HD-1-S1980; lane 3, serovar pakistani; lane 4, serovar kenyae; lane 5, serovar ostriniae; lane 6, serovar thompsoni; lane 7, serovar canadensis; lane 8, serovar indiana; lane 9, serovar kumamotoensis; lane 10, serovar yunnaensis; lane 11, serovar morrisoni; lane 12, serovar dakota; lane 13, serovar kurstaki; lane 14, serovar subtoxicus; lane 15, serovar toumanoffi; lane 16, serovar aizawai; lane 17, serovar colmeri; lane 18, serovar darmstadiensis. (B) Lanes 1 and 19, 1-kb ladder; lane 2, serovar tolworthi; lane 3, serovar tohokuensis; lane 4, serovar alesti; lane 5, serovar tochigiensis; lane 6, serovar dendrolimus; lane 7, serovar entomocidus; lane 8, serovar galleriae; lane 9, serovar kyushuensis; lane 10, serovar finitimus; lane 11, serovar sotto; lane 12, serovar thuringiensis; lane 13, serovar israelensis; lane 14, serovar wuhanensis; lane 15, serovar pondicheriensis; lane 16, serovar shandogiensis; lane 17, serovar san diego; lane 18, serovar tenebrionis.

these organisms seem to share two or more common bands, although the *B. cereus* pattern is always divergent enough to be distinguishable from the strain HD-1 pattern. If the appropriate primer is chosen, however, the patterns can be drastically different. The results of this study clearly demonstrate that any suspected involvement of *B. thuringiensis* HD-1 in human disease (9) could be verified or disproved through fingerprinting of the bacteria recovered from the patient(s). Positive identification based on an AP-PCR with three or more primers would constitute very strong evidence for the involvement of the commercial strain in question.

Since *B. thuringiensis* is distinguished from *B. cereus* primarily on the basis of crystal production, the relationship of endogenous plasmids (in particular, the toxin gene-bearing plasmids) to the production of amplified PCR DNA bands was also studied. The identical banding patterns produced by the native and heat-cured serovar 3a3b isolates strongly suggest that the DNA PCR fragments were amplified from the bacterial chromosome and not endogenous plasmids. This suggestion is further strengthened by the results obtained with a similarly cured Cry^- serovar 3a3b strain (strain HD-73) possessing an array of plasmids different from the array possessed by strain HD-1. Although no differences were found between the Cry^+ and Cry^- HD-73 isolates, one primer did detect a single band difference between strains HD-73 and HD-1. Whether this band was amplified from plasmid DNA or reflects a natural genomic DNA variation remains to be determined. It is important to note that even though the low-molecular-weight plasmids of strain HD-1 proved to be refractile to heat curing, they formed only a minute amount of the total DNA content. Consequently, the likelihood that a single AP-PCR band was derived from

plasmid DNA is relatively remote, and this likelihood decreases even further when more than one band is considered.

The possibility of studying and assessing the persistence of *B. thuringiensis* in soil and water samples from areas where successive applications of commercial products have been made should be feasible since AP-PCR can rapidly distinguish between indigenous and commercial *B. thuringiensis* strains. Another important application of this technique could be the rapid verification of the presence of particular strains in the different registered *B. thuringiensis* formulations.

Figure 4 shows that a primer that was initially chosen to give a usable pattern with the commercial strain *B. thuringiensis* HD-1 generally produced useful patterns with most of the available *B. thuringiensis* serovars. Of particular interest are biovars san diego and tenebrionis, which gave somewhat different patterns; the san diego pattern produced a strong 2.2-kb band that was absent in the tenebrionis pattern. This result is in contrast with the results of an extensive study (14) in which san diego and tenebrionis strains were found to be indistinguishable on the basis of a biochemical and serological examination. However, since the bands are located at positions above 1 kb, differences between the strains should be confirmed by using a series of different primers.

In conclusion, AP-PCR is a simple and reproducible technique which can be adapted to work reproducibly with boiled bacterial colonies rather than purified genomic DNA. This technology permits rapid identification of closely related commercial strains of *B. thuringiensis*. Furthermore, AP-PCR can distinguish unambiguously between *B. thuringiensis* and the biochemically identical species *B. cereus*. This technique should prove to be a useful tool for environmental assessment and quality control of this important biological insecticide.

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