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Cellular Fatty-Acid Analysis of *Bacillus thuringiensis* Var. *kurstaki* Commercial Preparations

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Cellular fatty acid (CFA) composition of *Bacillus thuringiensis* var. *kurstaki* (Btk) preparations was determined by use of the MIDI Sherlock microbial identification system on a Hewlett-Packard 5890 gas chromatograph. Four commercial preparations—one Btk sample obtained from the U.S. Forest Service, one Btk sample obtained from Dugway Proving Ground, and Btk and *Bacillus thuringiensis* var. *israelensis* (Bti) preparations obtained from American Type Culture Collection (ATCC)—were analyzed and evaluated. This study demonstrated the capability to detect the strain variation in the bacterial species Btk and to clearly differentiate strain variants on the basis of qualitative and quantitative differences in hydrolyzable whole CFA compositions in the preparations examined. We conclude that CFA analysis may be used to identify commercial products but that a more intensive study would be required to evaluate the potential of CFA to provide an inexpensive screening tool applicable to several levels of isolate or product evaluation, including how applied preparations might interact with natural populations over time.

KEYWORDS: Cellular fatty acids; *Bacillus thuringiensis* var. *kurstaki*; *Bacillus thuringiensis* var. *israelensis*; microbial; microorganism fingerprinting

INTRODUCTION

The monitoring of *Bacillus thuringiensis* var. *kurstaki* (Btk) occurrence in the environment is complicated by the increased field use of commercially produced insecticides employing this bacterium. The increased use of commercial preparations also complicates the search for new strains of Btk important in the isolation and study of new strains with enhanced insecticidal properties. In any use of biological materials on a large scale, it is important to develop methods to screen, identify, monitor, and differentiate between applied and native biological materials. Traditionally, varieties of *B. thuringiensis* are differentiated by flagellar agglutination and in the case of Btk are based on the presence of the 3a3b antigen (*1*). However, while flagellar antigens are useful in defining *B. thuringiensis* varieties, they cannot be used to distinguish among strains of Btk.

Several methods are available to identify and differentiate bacterial species at the strain/variant level. However, the applicability of these methods for differentiation between Btk strains/variants has not been demonstrated. The best available methods include ribosomal/plasmid/nucleic acid profiling (*2*−*4*), crystal serology (*5*), and cellular fatty acid (CFA) fingerprinting (*6*−*10*). Of these methods, CFA fingerprinting and automated ribosomal/nucleic acid profiling represent state-of-the-art technology for bacterial identification and differentiation at the species level. Of these two techniques, CFA analysis is less expensive and currently more reliable for subspecies differentiation. CFA analysis on a gas chromatograph (GC) with library construction and numerical analysis software currently represents the best technology for bacterial differentiation at the subspecies level.

In this and other studies, CFA analysis has been used to identify *B. thuringiensis* var. *israelensis* (Bti) and *B. thuringiensis* var. *kurstaki* (Btk) species, subspecies, and strains of bacteria. Instead of relying of antigen detection or a molecular probe, GC is used to determine the CFA composition of vegetative cell walls based on retention time of methyl ester fatty acid derivatives (*11*−*13*). This technique can be used to generate profiles or fingerprints that describe bacterial species or strains and the relationship between the GC profiles can be compared by numerical analysis (*13*, *14*). This technique is used routinely for medical identification of isolates and has been used by others in the comparative characterization of various bacteria at the subspecies level (*15*−*17*). For example, this method was used by Siegel et al. to characterize commercial preparations of Bti and Btk (*8*, *9*) and *Bacillus sphaericus* isolates (*18*) and by Esnard et al. (*6*) for the differentiation of strains of *B. thuringiensis* and *B. thuringiensis* var. *kurstaki*.

The objective of the present study was to determine whether this technique could be used to distinguish between commercially produced Btk preparations and a preparation provided by the U.S. Forest Service. A secondary objective was to determine the requirements for screening various *B. thuringiensis* isolates and various products for similarity. Realizing that different isolates and strains are expected to generate different profiles, a result of continuation of this project would be to

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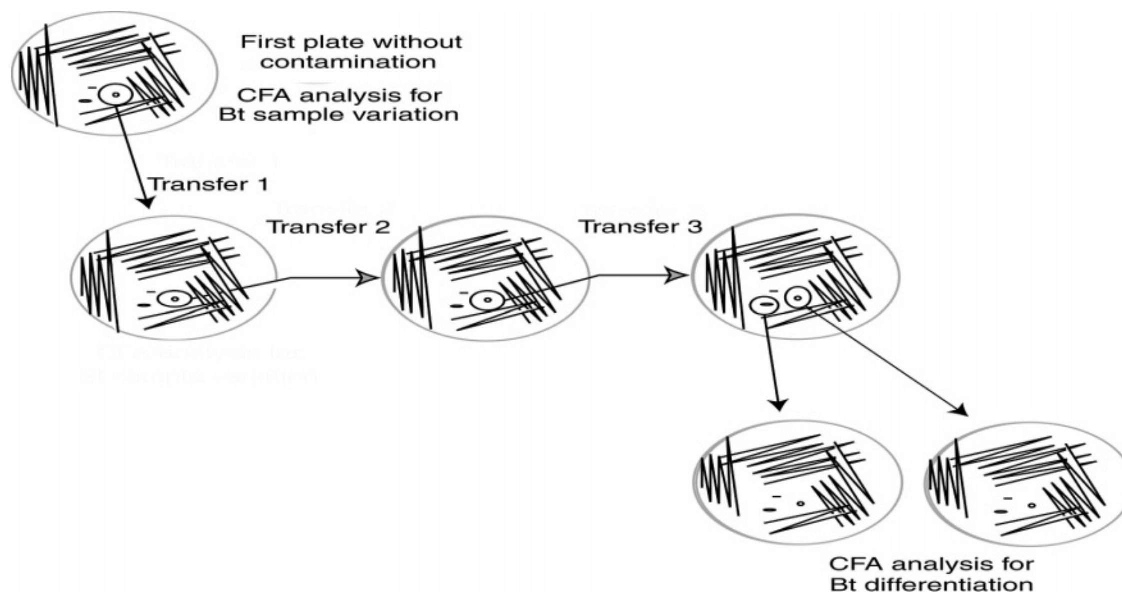

Figure 1. All samples followed the above plating scheme for CFA analysis. Single colonies were transferred as indicated with CFA analysis being completed after transfers 3 from a contaminant-free plate. Approximately 75 mg of bacterial mass, from the fourth and third quadrants, was collected for CFA analysis.

examine how the generated profiles correlated with isolate or product effectiveness. This study was designed to evaluate the potential to provide an inexpensive screening tool applicable to several levels of isolate or product evaluation, including how applied *B. thuringiensis* preparations interact with natural populations over time. This paper presents methods to evaluate these possibilities. Products analyzed included the commercial strains Foray 48B (lot 27-270-PG), Foray 48F (lot 41-987-PG), Dipel 6AF, and Thuricide 48LV. A standard Btk strain (HD-1-S-1980) was provided in the form of a powdered material by the USDA Forest Service (here designated USFS-Bt), and standard strains of Btk and Bti were obtained from American Type Culture Collection (ATCC) as lyophilized materials (ATCC 35866, designated ATCC-Btk, and ATCC 35646, designated ATCC-Bti). Two samples labeled Foray 48B from 4/18/91 were provided as liquid slurries from Dugway Proving Grounds (designated DPG-Bt) from a U.S. Forest Service gypsy moth project on the Wasatch Front.

MATERIALS AND METHODS

Bacterial Strains. Commercial Btk products Foray 48B, Foray 48F, and Dipel 6AF were obtained as 1 L liquid samples. All commercial products were mixed thoroughly, divided into 50 (4) mL aliquots and one bulk sample, and frozen at −85 °C until plated. Two 50 mL samples of one commercial preparation (Foray 48B, 4/18/91), used in a past project along the Utah Wasatch Front, were provided as liquid slurries from Dugway Proving Grounds (DPG-Bt). One DPG sample was divided into 1-mL aliquots and samples were frozen at −85 °C until plated. The USDA Forest Service provided a standard Btk strain (HD-1-S-1980) as a powered material (USFS-Bt). This sample was kept in the refrigerator at 4 °C until plated for fatty acid profiling. Additional standard strains of Btk (ATCC 35866) and Bti (ATCC 35646) were obtained from American Type Culture Collection (ATCC) as lyophilized materials. These samples were grown to 50 mL and stored as aliquots at −85 °C until plated. All products were identified in the Center log by manufacturer and lot number.

Sample Preparation. Standard growth and selection procedures for culturing, isolation, and differentiation of Btk strains/variants were used (*19*). Bacterial streaking and isolation were completed under a class II laminar flow hood. Sample preparation consisted of thawing or hydrating the sample, washing twice in 0.85% saline, and plating on brain heart infusion agar (BHI; Difco Laboratories, Detroit, MI) or trypticase soy agar (TSA; Acumedia Manufacturers, Inc., Baltimore, MD, and BBL Becton-Dickinson Microbiology Systems, Cockeysville, MD). Samples were initially plated on BHI agar to screen for contamination on the basis of colony morphology, and single colonies of *B. thuringiensis* (Bt) were selected and transferred to TSA plates to screen for contamination through a second transfer.

Bt Differentiation. To determine if Bt preparations could be distinguished from one another, single colonies were isolated from each preparation and carried through three transfers, each time transferring a single colony from the previous plate. Cultures of the single-colony isolates were established and used for CFA analysis. For this determination, all samples were collected from the third transfer. Approximately 75 mg (wet cell weight) was removed from each plate, extracted, and methylated according to the MIDI Sherlock microbial identification system protocol.

Bt Sample Variation. To determine the variation in the Bt preparations received, single isolated Bt colonies were transferred from BHI plates to TSA plates and transferred as depicted in **Figure 1**. Single colonies were transferred to TSA and incubated at 28 °C for 24 h for growth of bacterial cell mass used for CFA extraction, methylation, and analysis. Approximately 75 mg (wet cell weight) was removed from each plate, extracted, and methylated according to the MIDI protocol.

Multiple samples of each Bt preparation were extracted over a period of months to maximize heterogeneity. Relationships between the individual isolates were determined by principal component analysis and Euclidean distances by use of algorithms developed by MIDI/Hewlett-Packard and included in the library generation software package.

Instrumentation. A Hewlett-Packard 5890 GC with a 5% phenylmethyl silicone capillary column, flame ionization detector, 7673 auto sampler, 3396 series II integrator, and MIDI/Hewlett-Packard software were used to identify the microbial CFAs and fingerprint the various Btk commercial preparations and Btk and Bti stock cultures.

Sample Analysis. Reproducibility of results was determined by repeating each analysis two times on the above GC equipped with a flame ionization detector (FID). If any sample was questionable, the entire plating, extraction, methylation, and analysis procedure was repeated with new starting materials. With each set of samples processed on the GC, the following controls were included: a MIDI calibration standard repeated every 10th vial and a solvent control. Peak normalization and assignments were made automatically with the MIDI-GC software and compared with a standard mixture of CFAs obtained from MIDI.

Software. MIDI Sherlock 2 software was used to accurately identify microorganisms by fatty acid composition. After sample preparation, the complex mixture was first separated by gas chromatography, and then Sherlock's pattern recognition algorithm was employed to extract quantitative and qualitative information from the raw data. The raw data are compared to Sherlock's extensive databases. The quality of the match as well as the identification made by Sherlock is reported. Sherlock includes programs for operation of the gas chromatograph, automatic calibration and peak naming, data storage, and comparison of the unknown to one or more libraries by use of covariance, principal component analysis, Euclidean distance, and pattern recognition algorithms.

By use of the MIDI software, library entries or profiles were generated from each culture. The dendrogram capability, included with the Sherlock software, was used to determine the relatedness of samples profile sets through Euclidean distance relationships. This cluster type of analysis can indicate sample mixing or species subgroups. Each set included a minimum of two extracted plates. Profiles linking at a distance of ≤2 Euclidean units were considered as belonging to the same strain, on the basis of empirical data collected by MIDI, Inc. Linkage at about 6 Euclidean units would result from analyses of subspecies of biotypes. Species generally link at about 10 Euclidean distances.

Colonies Examined. Additional statistical analysis was conducted on the data in order to determine the correlation among selected fatty acids. One hundred samples were analyzed in this study as follows: 14 Btk, 14 Bti, 14 Foray 48B, 14 Foray 48F, 14 Dipel 6AF, 14 Thuricide 48LV, 8 DPG-Bt, and 8 USFS-Bt.

RESULTS AND DISCUSSION

Bacterial Strains and Sample Preparation. On the basis of colony morphology on BHI and TSA, no bacterial contamination was present in any of the four commercial Btk preparations or Btk and Bti standards from ATCC. However, the Foray 48F and the Dipel 6AF preparations had a bacteriophage present at a relatively high concentration in the preparations received. Therefore, for each CFA analysis, single Bt colonies were selected and screened to ensure that the bacteriophage factor was not carried through the transfers shown in **Figure 1**.

The DPG samples contained at least one bacterial contaminant, bacteria other than *B. thuringiensis,* so the samples were streaked for isolation and the Btk microbes were isolated before proceeding with normal sample preparation and analysis.

Bt Differentiation. Initial screening of Bt samples was performed by conducting CFA analysis of several single-colony isolates from each of the commercial samples received. The variation in the CFA profile dendrograms indicated that there was considerable variation within the different preparations. Therefore, the ability to distinguish between commercial preparations would have to be made by the ability to distinguish between single-colony isolates from each preparation and the degree of variation with in each preparation itself. Representative total CFA profile chromatograms from the eight *B. thuringiensis* preparations were developed from isolates grown, extracted, methylated, and analyzed under identical conditions (**Tables 1** and **2**). For representative total CFA profile determination, sample variability was minimized by carrying single isolates through three transfers to eliminate the variation initially observed in the sample preparations received.

On the basis of software analysis of the representative total CFA profile chromatograms, characterizing the eight Bt preparations examined in this study, CFA was able to distinguish between the commercial preparations tested in each of the 14 analysis comparisons made. If the various Bt preparations examined only contained a single strain, CFA should serve as a fingerprint of the individual Bt preparation. However, this was not the case; differentiation of individual isolates from each preparation produced variable patterns or fingerprints that, while distinguishable from the other samples when tested together, cannot be used to identify a particular Bt preparation. This is because the preparations examined contained multiple Bt strains.

Dendrograms A and B, summarized in **Figure 2**, represent two of the 14 CFA analyses used to characterize each of the eight Bt preparations and to determine the ability of CFA to distinguish between four commercial Bt preparations. As can be seen, CFA analysis did provide a distinction between Btk and Bti as expected and approximately at the same level as found by Siegel et al. (*9*, *20*). On the surface, these figures appear to present somewhat conflicting results with respect to linking patterns. However, because of the plating methods shown in **Figure 1**, they indicate the presence of multiple Bt strains in the preparations examined. These results mirror the relationships observed in the comparison of the CFA profiles analyzed for this project.

Table 1. Cellular Fatty Acids of *B. thuringiensis* var. *kurstaki* (ATCC-Btk-ATCC 35866)[a]

fatty acid	mean percentage ± SD	minimum	maximum
12:0 iso	1.335 ± 0.063 64	1.29	1.38
13:0 iso	10.565 ± 0.190 919	10.43	10.7
13:0 anteiso	2.35 ± 0.0848 53	2.29	2.41
14:0 iso	6.71 ± 0.127 279	6.62	6.8
14:00	3.01 ± 0.028 284	2.99	3.03
15:0 iso	22.395 ± 0.417 193	22.1	22.69
15:0 anteiso	7.09 ± 0.028 284	7.07	7.11
16:1 w/c alcohol	0.875 ± 0.007 071	0.87	0.88
sum in feature 2	4.33 ± 0.028 284	4.31	4.35
16:0 iso	6.595 ± 0.049 497	6.56	6.63
sum in feature 3	12.295 ± 0.106 066	12.22	12.37
16:00	4.165 ± 0.106 066	4.09	4.24
15:0 2OH	1.15 ± 0.183 848	1.02	1.28
Iso 17:1 w10c	2.285 ± 0.091 924	2.22	2.35
Iso 17:1 w5c	5.11 ± 0.014 142	5.1	5.12
17:1 anteiso A	1.72 ± 0.028 284	1.7	1.74
17:0 iso	6.36 ± 0.056 569	6.32	6.4
17:0 anteiso	1.66 ± 0.098 995	1.59	1.73
100% named			

[a] Significant figures given are as provided by the instrument software but could be rounded to two places past the decimal if desired.

Table 2. Cellular Fatty Acids of *B. thuringiensis* var. *israelensis* (ATCC-Bti-ATCC 35646)[a]

fatty acid	mean percentage ± SD	minimum	maximum
12:0 iso	1.15 ± 0.046 583	1.11	1.27
13:0 iso	8.33 ± 0.145 499	8.04	8.42
13:0 anteiso	1.39 ± 0.039 749	1.31	1.41
14:0 iso	5.82 ± 0.107 796	5.73	6.02
14:00	3.44 ± 0.211 187	3.15	3.64
15:0 iso	20.72 ± 0.258 844	20.34	20.98
15:0 anteiso	4.75 ± 0.064 42	4.68	4.85
16:1 w/c alcohol	1.17 ± 0.104 403	1.07	1.39
sum in feature 2	4.48 ± 0.235 202	4.45	5.04
16:0 iso	7.28 ± 0.117 26	7.1	7.42
sum in feature 3	11.75 ± 0.321 823	11.54	12.45
16:00	4.88 ± 0.082 158	4.71	4.92
15:0 2OH	1.69 ± 0.262 011	1.6	2.26
Iso 17:1 w10c	3.86 ± 0.085 849	3.83	4.02
Iso 17:1 w5c	7.09 ± 0.373 47	6.99	7.91
17:1 anteiso A	1.57 ± 0.061 074	1.55	1.7
17:0 iso	9.1 ± 0.728 162	7.46	9.1
17:0 anteiso	1.42 ± 0.164 712	1.12	1.53
100% named			

[a] Significant figures given are as provided by the instrument software but could be rounded to two places past the decimal if desired.

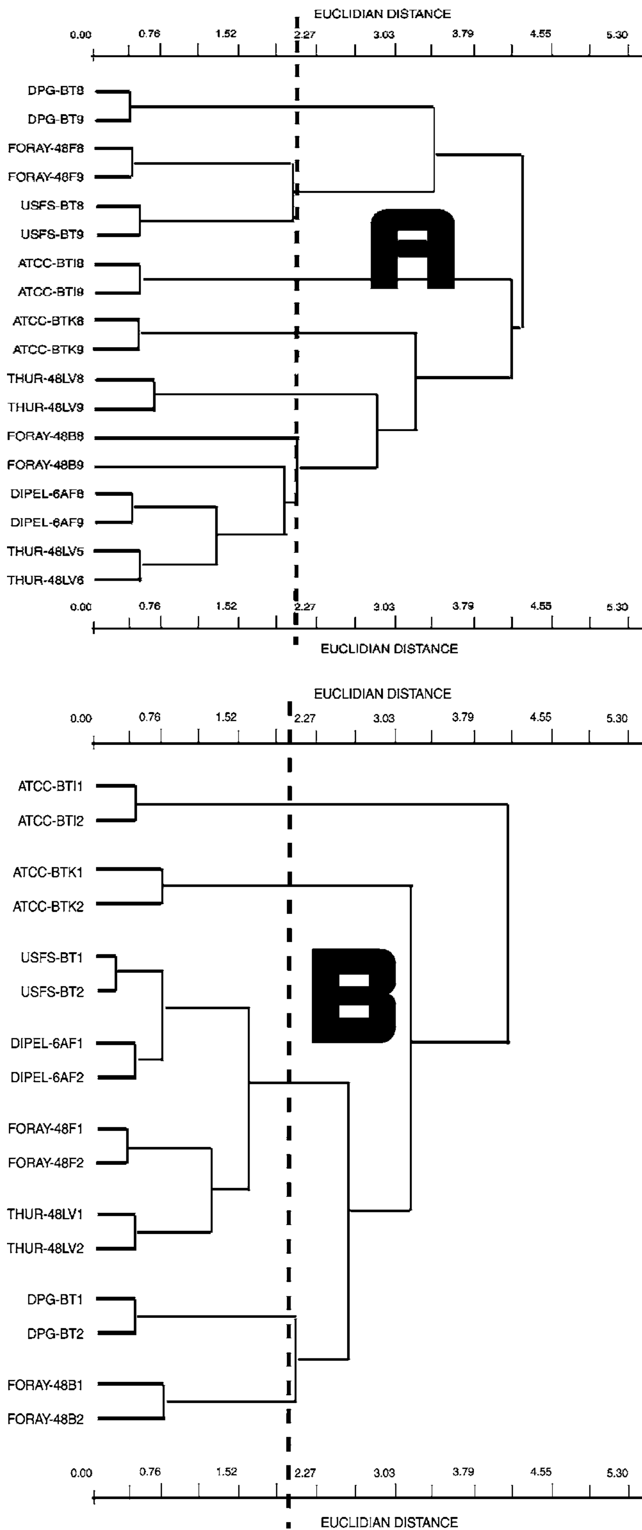

Figure 2. Dendrograms A and B of commercial Bt preparations Dipel 6AF, Foray 48F, Thuricide 48LV, and Foray 48B; ATCC stock preparations of *Bacillus thuringiensis* var. *israelensis* and *Bacillus thuringiensis* var. *kurstaki*; and USFS-Bt and DPG-Bt prepared in pairs from the original preparations as indicated in **Figure 1**.

According to MIDI Inc., as a "rule of thumb" based on empirical experience with thousands of runs of their software, Euclidean distances of 10 or more units distinguish species of bacteria, distances of 6 or more units distinguish subspecies, and distances of 2 or more units distinguish strains. On the basis of this information, dendrogram A indicates that Foray-48B, Thuricide-48LV$_{5+6}$, and Dipel-6AF are the same strain because they link within two Euclidean units. Dendrogram A also indicates that USFS-Bt and Foray-48F are also the same strain. This dendrogram also indicates that DPG-Bt, ATCC-Bti, ATCC-Btk, and Thuricide-48LV$_{8+9}$ are different strains. However, Thuricide-48LV$_{8+9}$ and Thuricide-48LV$_{5+6}$ are just different colonies picked from a TSA plate used to isolate individual microbes from the same Thuricide-48LV preparation.

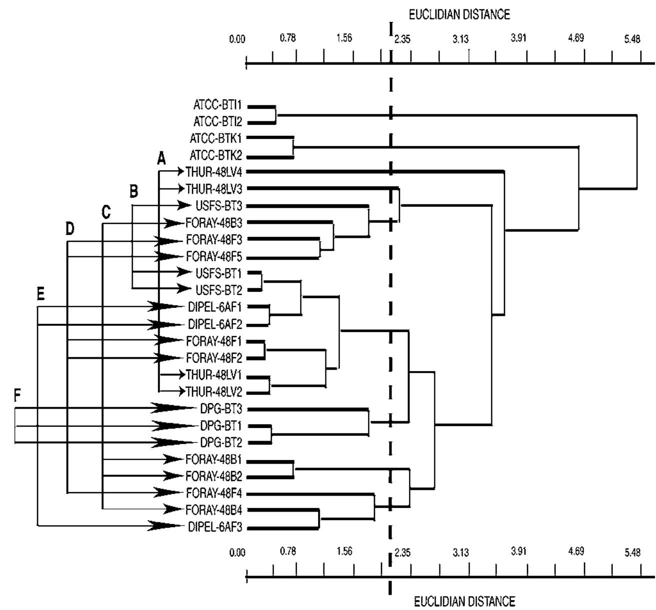

Figure 3. CFA variability observed in individual microbial isolates from the four commercial Bt preparations tested. CFA analysis was performed as indicated in **Figure 1**.

Dendrogram B in **Figure 2** indicates that USFS-Bt, Dipel-6AF, Foray-48F, and Thuricide 48LV are the same strain because they link within 2 Euclidean units. Within this strain are two divisions: USFS-Bt and Dipel-6AF form one division and Foray 48F and Thuricide-48LV form the other division. Dendrogram B indicates that Foray-48B and DPG-Bt are possibly the same strain since they link at just over 2 Euclidean units. It is also indicated that Foray-48B and DPG-Bt are different strains than USFS-Bt, Dipel-6AF, Foray-48F, and Thuricide 48LV and that ATCC-Bti and ATCC-Btk are different strains.

These dendrograms, and the others generated from the CFA analyses performed on individual microbial colonies isolated from the various preparations used in this study, fail to establish a relationship between the commercial samples. As with the chromatograms generated and analyzed for the eight Bt preparations, the dendrograms produced are variable; dendrograms A and B are representative of the variation observed in the 14 CFA analyses performed in this study.

The results summarized in **Figure 3**, and others not shown, indicate considerable variability within the Bt preparations and that not all of the strains present in some preparations have been isolated and examined by CFA. Thuricide-48LV (A) is composed of at least two strains or profiles that link at greater than 2 Euclidean distances, as do the USFS-Bt (B) and Dipel-6AF (E) preparations. Foray 48B (C) has at least three strains or profiles that link at greater than 2 Euclidean distances. The DPG-Bt preparation has two strains or profiles that link within less than 2 Euclidean distances and would therefore be pooled for analysis when the total number of strains in each preparation has been determined. The strains or profiles linking at greater than 2 Euclidean distances would be analyzed separately.

Bt Sample Variability within Individual Preparations. Heterogeneity in commercial Bt preparations was expected, as Siegel et al. (*9*, *20*) had reported up to 11 strains in the preparations of *Bacillus thuringiensis* serovar *kurstaki* and *Bacillus thuringiensis* var. *israelensis* examined. There are several hypotheses that may account for the diversity observed in the preparations examined in this study. However, without knowing the history of each preparation, it can only be speculated whether the strains were introduced on purpose. Without a more comprehensive analysis of each preparation, we cannot determine which strains are present in some preparations and not others, making it difficult to speculate on their origin. For example, if strains were found in commercial preparations but not in stock cultures, then an examination of the presence of the individual strains across all commercial preparations might lead to some insight to whether the strains may have been purposely introduced.

The proposed testing, designed to differentiate between the different commercial preparations, did not include enough individual isolation replicates directly from the parent sample to determine the number of strains (variability) in each commercial preparation. However, the data demonstrated that CFA analysis may be used to identify commercial products but that the more intensive study outlined would be required to evaluate the potential of CFA to provide an inexpensive screening tool applicable to several levels of isolate or product evaluation, including how applied *B. thuringiensis* preparations might interact with natural Bt populations aver time.

With the information gathered in this study, greater numbers of strain profiles are needed to identify all strains within the Btk preparations examined. The increase in precision required to identify all strains present could be obtained through a study structured to isolate approximately 50 individual microbes from each preparation to ensure isolation of all strains present in a given sample. Each strain found should be slanted and catalogued to maximize reproducibility between the initial test and subsequent CFA analysis. A minimum of two CFA analyses should be performed from each slanted isolate to establish variation between replicate samples and to provide an accurate profile of the isolates. Dendrograms need to be developed to establish the relationship between strains within and between samples being tested and to determine the relative number of microbial strains in each preparation. Dendrograms coupled with a coefficient of variation analysis on individual fatty acids both in and between strains found in the different preparations would significantly aid in the determination of relationships between strains and the number of isolates needed for a conclusive determination of population variation within a preparation or in any interaction occurring over time between applied preparations and the natural population at a specific site.

The results of the proposed method not only would allow the identification of commercial Btk strains but also would provide an estimation of the number of samples or analyses needed to correctly identify a commercial preparation in most settings. Once the strains have been identified with the precision indicated, an identification of commercial Btk preparations could be made that would withstand considerable change in the parent culture. This would also provide a relatedness profile of the commercial cultures with a high degree of confidence.

Because of the variability observed with individual bacterial isolates from the eight preparations examined and because of the extensive nature of the study needed to identify all the strains in the commercial preparations, it may be worthwhile to examine CFA analysis performed on whole preparations. While MIDI analysis was developed to distinguish between pure cultures of a single microorganism, the same concepts can be applied to mixtures of microorganisms. A CFA of a mixture of microorganisms should be representative of the relative amounts of different microbial strains present in each preparation. While this technique may yield reproducible results, method development is required to move from single-strain differentiation to whole-preparation CFA analysis and must be supported by intensive CFA analysis as proposed above.

Additionally, it must be remembered that fatty acid analysis is dependent on standardization of methods between tests and laboratories to maintain reproducibility. Prime factors in maintaining this reproducibility are the standardization of media, incubation temperature, and harvesting of culture during the same growth phase. Media type and brand also influence the percent composition of fatty acids that comprise the cell envelope. Other influences include the presence of amino acids such as L-isoleucine and L-valine in the media used for growing bacterial isolates for CFA analysis and the operating conditions of the gas chromatograph, column type, and carrier gas purity. The importance of these factors can be demonstrated with incubation temperature: as incubation temperature increases, the percentage of anteiso fatty acids in the cell envelope decreases. Because anteiso fatty acids have lower melting points than their unbranched and iso counterparts, at higher incubation temperatures bacteria reduced or eliminated them in order to stabilize membrane fluidity. Although our data were replicable, our results are only directly comparable to those of other researchers who use the same growth and extraction protocols as well as the MIDI/Hewlett-Packard microbial identification system.

Peak assignments and area calculations are made automatically by the MIDI/Hewlett-Packard microbial identification instrumentation and software. The two most similar commercial preparations are Thuricide 48LV and Dipel 6AF. These two preparations appear similar enough to warrant further analysis. Additional passages of the preparation will be made on TSA to determine whether the slight differences observed might be due to procedural differences in commercial sample preparation. At this point in sample analysis, they are similar enough to speculate that the products were derived from a common source.

This study has demonstrated the capability to detect strain variation in the bacterial species Btk and to clearly differentiate strain variants on the basis of qualitative and quantitative differences in hydrolyzable whole CFA compositions in the commercial preparations examined. We conclude that CFA analysis may be used to identify commercial products but that a more intensive study would be required to evaluate the potential of CFA to provide an inexpensive screening tool applicable to several levels of isolate or product evaluation, including how applied *B. thuringiensis* preparations might interact with natural Bt populations over time.

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