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USE OF CELLULAR FATTY ACID ANALYSIS TO CHARACTERIZE COMMERCIAL BRANDS OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS*[1]

JOEL P. SIEGEL, A. RAY SMITH,[2] JOSEPH V. MADDOX AND ROBERT J. NOVAK

Illinois Natural History Survey, Center for Economic Entomology, and University of Illinois, Office of Agricultural Entomology, Champaign, IL 61820

ABSTRACT. The cellular fatty acid composition of *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) from 5 commercial brands (Vectobac®, Acrobe®, Skeetal®, Bactimos® and Teknar®), as well as of the current International Standard for *B.t.i.* (IPS 82), was determined using a Hewlett-Packard Microbial Identification System. The original strain of *B.t.i.*, *B.t.* var. *kurstaki*, *B.t.* var. *thuringiensis*, *B.t.* var. *morrisoni* and *Bacillus sphaericus* strain 2362 were used as outgroups. Acrobe, Bactimos, Teknar, Vectobac and IPS 82 consisted of the same strain. Skeetal represented a different strain than the other commercially produced *B.t.i.* Our results indicate that cellular fatty acid analysis can be used to distinguish among the forms of *B.t.i.* produced by various manufacturers.

The search for new strains of *Bacillus thuringiensis* var. *israelensis* (de Barjac) (*B.t.i.*) is complicated by increased field use of commercially produced insecticides employing this bacterium (Brownbridge and Margalit 1986, Asimeng and Mutinga 1992, Orduz et al. 1992). Consequently, when researchers isolate *B.t.i.* from the field, they must first eliminate the possibility that they have recovered a commercially produced *B.t.i.* before they can claim to have discovered a new strain. Currently, varieties of *B. thuringiensis* are differentiated by flagellar agglutination, which is based on the presence of the H antigen (de Barjac and Frachon 1990). All commercially produced *B.t.i.*, the International Standard (IPS 82) and the original isolate (Goldberg and Margalit 1977) possess the H-14 antigen, which defines this variety. Although this character distinguishes *B.t.i.* from the other varieties used in agriculture, such as *B. thuringiensis* var. *kurstaki* (which possesses flagellar antigen 3a3b), it cannot be used to distinguish among strains of *B.t.i.*

Cellular fatty acid (CFA) analysis is a technique that can be used to identify species, subspecies and strains of bacteria. Instead of relying on antigen detection or a molecular probe, gas liquid chromatography (GLC) is used to determine the fatty acid composition of vegetative cell walls based on the retention time of the methyl ester derivatives of the fatty acids (D'Donnell and Norris 1981, Drucker 1981, Frachon et al. 1991, Welch 1991, Schenkel et al. 1992). This technique can be used to generate profiles that describe strains and/or species of bacteria, and the relationship between profiles can be compared by numerical analysis (Manly 1986, Frachon et al. 1991, Welch 1991, Schenkel et al. 1992). Frachon et al. (1991) and Schenkel et al. (1992) have used this technique successfully to describe the relationships between strains of *Bacillus sphaericus*, another species of bacteria with mosquitocidal activity. Stahly and Klein (1992) used this technique to determine that a commercial product used to control *Popillia japonica*, the Japanese beetle, contained *Bacillus polymyxa*, a nonpathogen, instead of the beetle pathogen, *Bacillus popillae*.

The objective of the present study was to determine whether this technique could be used to distinguish commercially produced *B.t.i.* products. The products analyzed were Acrobe® aqueous suspension (produced by American Cyanamid and provided by Clarke Outdoor Spraying, Roselle, IL, lot 1054P3QQ, plus another container with no lot number), Bactimos® technical powder and briquettes (produced by Solvay, technical powder provided by Clarke Outdoor Spraying, and briquettes, provided by Summit Corporation, Baltimore, MD, lot 0502), Skeetal® aqueous suspension (produced by Novo Laboratories, Inc., lot BIN 0015), Teknar® HP-D

[1] Mention of a commercial product does not constitute an endorsement by the Illinois Natural History Survey or University of Illinois.

[2] Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Illinois, Urbana, IL 61801.

Table 1. The cellular fatty acids of *Bacillus thuringiensis* var. *israelensis* (IPS 82).

Fatty acid	Mean percentage ± SD	Min.	Max.
12:0 Iso	0.90 ± 0.45	0.00	1.43
13:0	11.74 ± 1.21	7.93	14.06
13:0 Anteiso	1.36 ± 0.33	0.00	1.79
14:0 Iso	5.60 ± 0.88	3.81	7.01
14:0	3.94 ± 0.37	3.15	4.42
15:0 Iso[1]	30.42 ± 2.19	27.18	34.78
15:0 Anteiso	4.45 ± 0.18	3.96	4.80
16:1 ω7c alcohol	0.65 ± 0.35	0.00	0.98
16:0 Iso	5.23 ± 0.43	4.54	6.33
16:0	3.34 ± 0.48	2.47	4.79
15:0 2OH	0.57 ± 0.30	0.00	0.99
Iso 17:1 ω10c	2.87 ± 0.59	2.33	4.39
Iso 17:1 ω5c	5.50 ± 1.05	4.53	9.25
17:1 Anteiso A	0.79 ± 0.39	0.00	1.08
17:0 Iso	6.21 ± 1.07	4.88	9.28
17:0 Anteiso	0.64 ± 0.35	0.00	1.23
Summed feature 3[2]	3.77 ± 0.53	2.73	4.46
Summed feature 4[3]	11.85 ± 0.59	10.78	13.14

[1] Primary constituent.
[2] Either 16:1 Iso I or 14:0 3OH.
[3] Either 15:0 Iso 2OH or 16:1 ω7t.

aqueous suspension (two containers, Zoecon Corporation, lot 7841040 and Clarke Outdoor Spraying, no lot number), Vectobac® 12AS aqueous suspension (two containers) and Vectobac® technical powder (produced by Abbott Laboratories, lots 61-476-N9, 18-202-BA and 27-801-CD, respectively).

In addition, we compared the commercial strains with the current International Standard for *B.t.i.*, IPS 82 (provided by H. de Barjac, Institute Pasteur, Paris, France, lot 91509). We also compared the commercial strains with the original strain of *B.t.i.* isolated by Goldberg and Margalit (1977), *B. thuringiensis* var. *kurstaki, B. thuringiensis* var. *thuringiensis,* and *B. thuringiensis* var. *morrisoni* (provided in the form of cell suspensions on sterile filter disks by the Bacillus Genetic Stock Center, Department of Biochemistry, The Ohio State University, Columbus, OH). A slant culture of *B. sphaericus* strain 2362 was provided by A. Yousten, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

The bacteria were prepared for analysis as follows. The aqueous suspensions, technical powders, IPS 82 and *B. sphaericus* strain 2362 were streaked onto brain heart infusion (BHI) agar plates (Difco Laboratories, Detroit, MI). The bacteria received from the Bacillus Genetic Stock Center were first revived in BHI broth and then streaked onto BHI plates. The plates were incubated at 30°C for 24 h and then checked for contamination based on colony morphology. Any plate that appeared contaminated was discarded. Single colonies were then transferred to tryptic soy broth (Baltimore Biological Laboratories, Cockeysville, MD) agar (Difco Laboratories) plates and incubated at 28°C for 24 h. All streaking and isolation were done under a laminar flow hood. The cells were then extracted and analyzed according to the protocol of Miller and Berger (1985).

A Hewlett-Packard (Avondale, PA) Microbial Identification System (Model 5890 A) equipped with a 5% phenylmethyl silicone capillary column, flame ionization detector, 7673 automatic sampler, 7673 A controller and 3392 A integrator was used to identify the CFA present in the samples. The GLC settings used were described by Frachon et al. (1991). The fatty acids were identified using a Hewlett-Packard 300 computer equipped with library generation software and with a software library for species identification based on CFA developed by MIDI Corporation (Newark, DE). The GLC was calibrated using a Hewlett-Packard calibration standard kit containing fatty acid methyl esters in 0.8 ml hexane (saturated nC9:0 to nC20:0 plus 2 & 3 hydroxy). These data were then used to generate a profile for each commercial *B.t.i.* isolate and outgroup. The relationships between the profiles were determined by numerical analysis, using algorithms developed by Hewlett-Packard and included in the software package.

Multiple samples were run during a one-year period for each source of bacteria in order to maximize heterogeneity. We ran the most samples for IPS 82 because it is the International Standard. We also ran samples of aqueous suspension and technical powder when possible, in order to determine possible strain differences in these different formulations. We used 32 samples to generate the profile for IPS 82, 23 samples for Teknar, 21 samples for Bactimos, 18 samples for Vectobac, 11 samples for Skeetal, 10 samples for Acrobe, 4 samples for the original isolate of *B.t.i.*, 4 samples for *B. thuringiensis* var. *kurstaki,* 4 samples for *B. thuringiensis* var. *thuringiensis,* 8 samples for *B. thuringiensis* var. *morrisoni* and 8 samples of *B. sphaericus* strain 2362.

The profile for IPS 82 is summarized in Table 1. The predominant fatty acid is 15:0 iso (13-methyltetradecanoic acid), composing 30.42% of the total CFA. This is the principal fatty acid in all the bacteria studied. The range of carbon chain length is 12–17. The GLC could not resolve every individual CFA present, and this lack of resolution is indicated in the table by Summed feature 3 and Summed feature 4. This designation

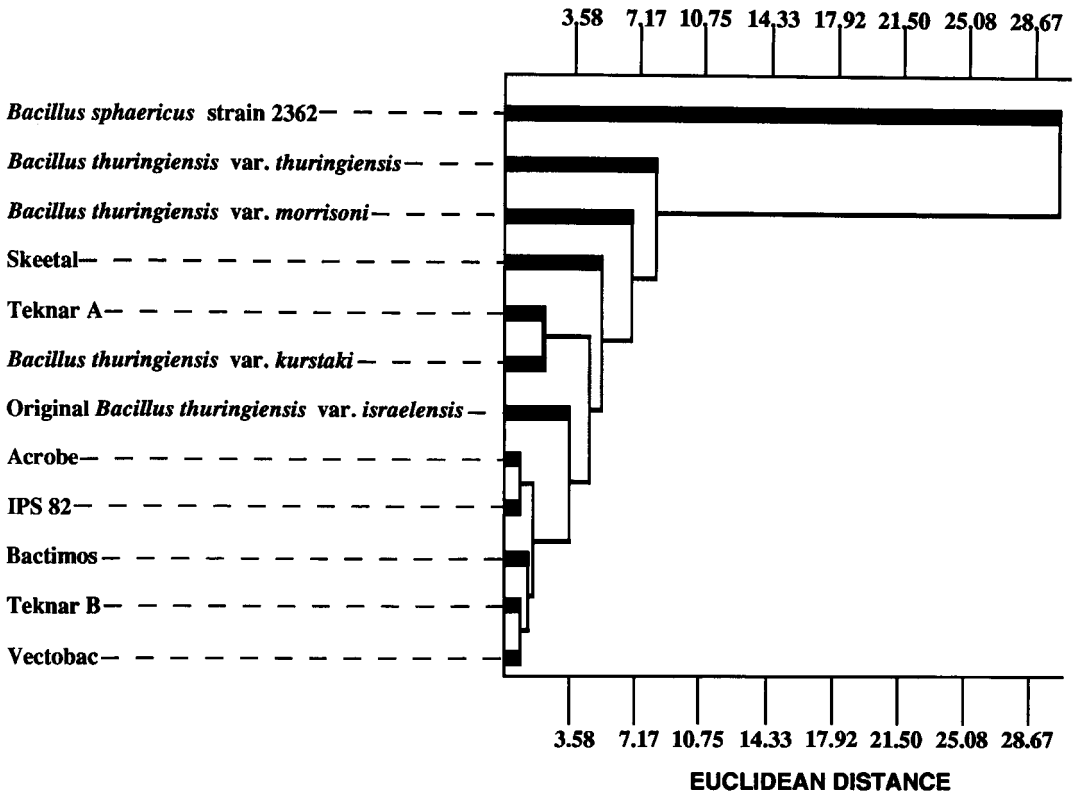

Fig. 1. Dendrogram of 5 commercially produced isolates of *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*), the international standard for *B.t.i.* and 5 outgroups, consisting of the original isolate of *B.t.i., B. thuringiensis* var. *kurstaki, B. thuringiensis* var. *morrisoni, B. thuringiensis* var. *thuringiensis* and *B. sphaericus* strain 2362.

indicated that there were 2 overlapping peaks. Our profile was validated by comparing the profile of our samples to profiles in the MIDI Corporation software library for *B. thuringiensis*. All the *B.t.i.* isolates were correctly identified as belonging to the species *B. thuringiensis* and the *B. sphaericus* strain 2362 was correctly identified to species using this software library.

The data for the different formulations produced by a manufacturer were pooled, with the exception of Teknar, for the following reasons. There were no differences between the samples from the 2 containers of aqueous Vectobac and the Vectobac technical powder. Likewise, there was no significant difference among samples from Bactimos briquettes, Bactimos granules (on corncob) and Bactimos technical powder. Three vials of IPS 82 powder were compared and there was no significant difference between vials; thus the data were pooled, resulting in a single profile. Similarly, a single profile was created for *B. thuringiensis* var. *kurstaki, B. thuringiensis* var. *thuringiensis, B. thuringiensis* var. *morrisoni* and *B. sphaericus* strain 2362. Two profiles were necessary for Teknar because the samples from lot 7841040 (received in 1990) differed from the samples from the aqueous suspension provided by Clarke Outdoor Spraying (received in 1992). The profile Teknar A (15 samples) refers to lot 7841040, and the profile Teknar B (8 samples) refers to the material received from Clarke Outdoor Spraying.

The relationships among the *B.t.i.* in the 5 commercial products, IPS 82 and the outgroups are summarized in Fig. 1. According to MIDI Corporation, 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, 6 or more units distinguish subspecies and 2 or more units distinguish strains. The dendrogram in Fig. 1 correctly indicates that *B. sphaericus* strain 2362 is a different species of bacteria because it links up beyond 10 Euclidean units. Acrobe, Bactimos, Teknar B, Vectobac and IPS 82 link within 2 Euclidean units, indicating that they belong to the same strain. Within this strain there are 2 divisions; Acrobe and IPS 82 form one division,

and Teknar B, Vectobac and Bactimos form the other division. We interpret this to mean that Acrobe is the most similar to IPS 82 but reiterate that all belong to the same strain. These commercial strains, with the exception of Teknar A and Skeetal, link up with the original isolate of *B.t.i.* before linking up with the other serotypes of *B. thuringiensis* that we tested. This may indicate a correlation between cellular fatty acid composition and flagellar antigen type, but further research is needed to determine whether a correlation does in fact exist.

The relationships among the commercially produced *B.t.i.* isolates summarized in Fig. 1 were validated by preparing 2 unmarked samples each of Acrobe, Bactimos, IPS 82 and Skeetal for GLC analysis. Acrobe, Bactimos and IPS 82 were chosen to represent the strain comprising the majority of the commercial *B.t.i.*, and Skeetal was chosen because it belonged to a different strain or perhaps a different serotype of *B. thuringiensis*. The samples were run blindly through the Hewlett-Packard Microbial Identification System, and all samples were correctly identified to strain of *B.t.i.*

The division of Teknar into 2 strains, as well as the separation of Skeetal from the other commercially produced *B.t.i.*, was unexpected. It is unlikely that our data simply reflect variation in production conditions and/or differences in growth media during production, rather than true strain differences, for the following reasons. First, the Vectobac used in our study was from several lots, as was the Bactimos, but the individual samples did not differ from one another. Acrobe was sampled from several containers and also did not differ. Therefore, if the samples from other manufacturers were uniform, it is reasonable to assume that the same conditions should hold for Teknar and Skeetal as well. Second, we controlled for possible production differences between manufacturers by growing all of the *B.t.i.* analyzed on the same medium and incubating all plates for the same time period. We therefore conclude that the difference between the 2 containers of Teknar reflected an actual strain difference. The similarity between Teknar A and *B. thuringiensis* var. *kurstaki* may indicate contamination of Teknar A with *B. thuringiensis* var. *kurstaki* somewhere in the manufacturing and packaging process. This hypothesis is plausible because *B. thuringiensis* var. *kurstaki* is produced for control of agricultural pests. However, since we did not determine the serotype of the bacteria tested, we cannot prove this hypothesis. We note that the more recently produced Teknar B belongs to the same strain as IPS 82. Similarly, Skeetal was so different from the other products that we suspect that the colonies we analyzed belong to a different serotype of *B. thuringiensis*. It would be very interesting to determine the serotype of Skeetal as well. We were unable to determine whether or not more recently produced Skeetal also belongs to the same strain as IPS 82 because we only had one container of Skeetal.

In conclusion, our data indicate that Acrobe, Bactimos, Teknar B, Vectobac and IPS 82 consist of the same strain of *B.t.i.* At the very least, Skeetal and Teknar A belong to other strains of *B.t.i.* and may in fact belong to other serotypes of *B. thuringiensis*. Our results suggest that it is possible to generate a common profile for currently produced commercial *B.t.i.* and that by using the profile, we could identify commercially produced *B.t.i.* to strain in unmarked samples in the laboratory. Further research is necessary to determine whether or not this technique will identify commercially produced *B.t.i.* recovered from the field.

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