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CELLULAR FATTY ACID ANALYSIS OF *BACILLUS THURINGIENSIS* SSP. *ISRAELENSIS* (ONR-60A)[1]

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ABSTRACT. The cellular fatty acid composition of *Bacillus thuringiensis* ssp. *israelensis* (*B.t.i.*) ONR-60A was determined by gas–liquid chromatography (GLC). Isolates of ONR-60A were obtained from the *Bacillus* Genetic Stock Center, Pasteur Institute, and USDA culture collections. These isolates were compared to one another and the culture collections differed in the strains that they contained. Eleven separate strains of ONR-60A were identified, based on fatty acid composition of the cell envelope. The current Pasteur Institute international standard for *B.t.i.*, IPS 82, was also analyzed and it consisted of 3 strains using these same criteria. Profiles were created for each strain found in ONR-60A and IPS 82, and validated against profiles of commercially produced *B.t.i.* from 2 time periods, 1983–84, and 1990–94. Approximately 84% of the commercially produced *B.t.i.* (134 out of 160 agar plates) was identified as belonging to the strains found in ONR-60A and in IPS 82. The most prevalent strain found in IPS 82 is also the most prevalent strain in the recently produced commercial material, 40.8% of the commercial plates analyzed, but commercially produced *B.t.i.* also retained a strong ONR-60A ancestry (35% of the plates analyzed).

INTRODUCTION

In 1977, the discovery of *Bacillus thuringiensis* spp. *israelensis* de Barjac (*B.t.i.*) was reported (Goldberg and Margalit 1977). This subspecies of *Bacillus thuringiensis* produced a new class of toxins known as Cry IV, borne on a 72-Md plasmid (Margalit and Dean 1985, Höfte and Whiteley 1989). Margalit and Dean (1985) and Margalit (1990) reported that all known cultures in use were derived from a single colony designated ONR-60A. They further stated that 12 agar plates were made from this single colony, and each in turn was pooled and lyophilized in 12 glass vials. Over a period of time ONR-60A was sent to the World Health Organization (WHO) (Geneva, Switzerland), the Pasteur Institute Reference Laboratory (Paris, France), J. Briggs of the WHO Collaborating Center for the Biological Control of Vectors of Human Diseases at Ohio State University (Columbus, OH), D. Dean at the *Bacillus* Genetic Stock Center (Columbus, OH) and S. Singer at Western Illinois University (Macomb, IL). *Bacillus thuringiensis* spp. *israelensis* originating from material received by the WHO was given the accession numbers WHO 1884 and WHO 1897. The first primary powder of *B.t.i.* was R153-78, fermented by R. Bellon Laboratories, France, from material provided by the Pasteur Institute, and this was used both in the initial field and mammalian safety testing (Shadduck 1980). The original standard for *B.t.i.* was created by the Pasteur Institute, designated IPS-78, and was subsequently replaced by the more potent current standard, IPS 82 (McLaughlin et al. 1984, Dulmage et al. 1985). Siegel et al. (1993) reported that Acrobe®, Bactimos®, Teknar®, and Vectobac® belonged to the same strain as IPS 82, based on analysis of the fatty acid composition of the cell envelope, and that these commercial products and IPS 82 differed from ONR-60A received from the Pasteur Institute (accession number T14 001).

Siegel et al. (1993), utilizing gas–liquid chromatography (GLC) reported that a 15:0 iso (chain length 15 carbon) fatty acid, 13-methyl tetradecanoic acid, was the most common fatty acid in the cell envelope, followed by a 13:0 iso fatty acid. The coefficient of variation (CV) for the 13:0 iso fatty acid in IPS 82 was 10.3. The CVs for this fatty acid in Bactimos and Vectobac were even higher, 13 and 16, respectively. Values this high may simply reflect that this fatty acid is quite variable, or it may mean that we had created composite profiles consisting of several strains. In order to resolve this issue we decided to analyze additional colonies of both IPS 82 and commercially produced *B.t.i.*, as well as the original ONR-60A isolates deposited in 3 culture collections. In this paper we identify and compare the fatty acid composition of isolates listed as ONR-60A in the culture catalogs of the *Bacillus* Genetic Stock Center, Pasteur Institute, and USDA collections. Comparative data on the relationship of these ONR-60A isolates to commercially produced *B.t.i.* and IPS 82 are also presented.

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

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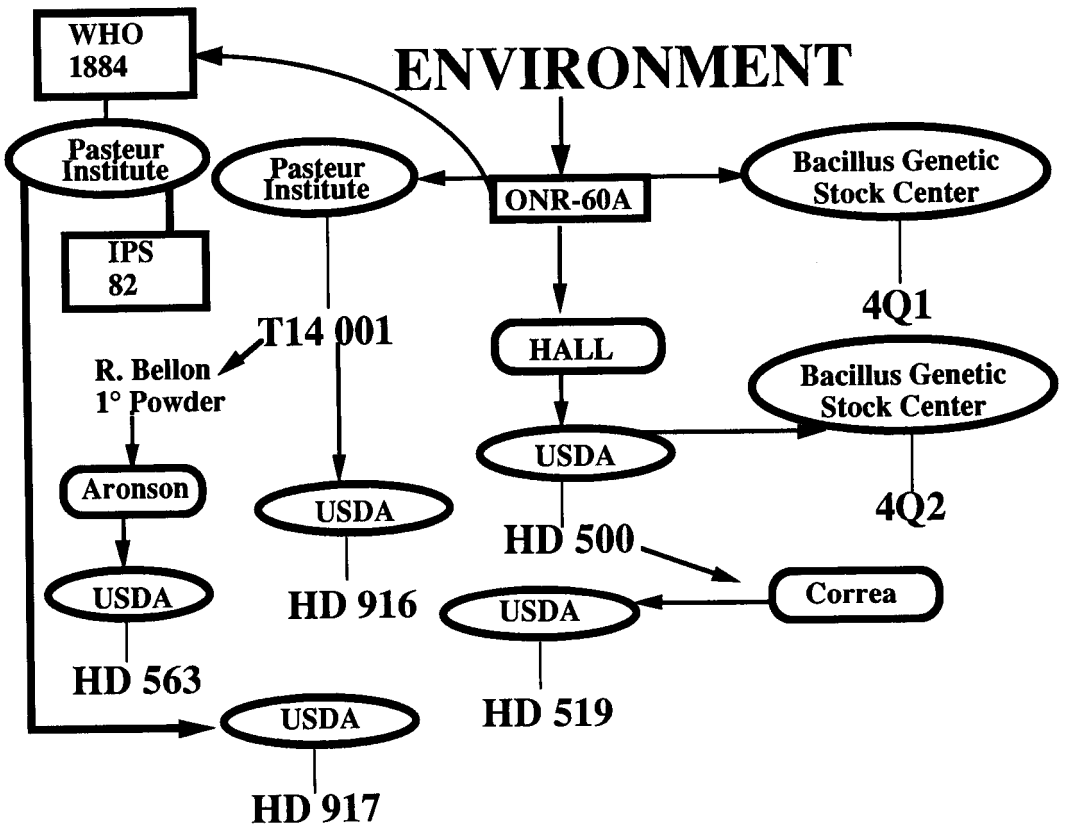

Fig. 1. Source and relationship of the *Bacillus thuringiensis* ssp. *israelensis* strains ONR-60A, WHO 1884, and IPS 82 used in this study.

MATERIALS AND METHODS

Source and identity of cultures and commercial material: Daniel R. Zeigler, curator of the *Bacillus* Genetic Stock Center, The Ohio State University, Columbus, OH, provided 4Q1 (ONR-60A) and 4Q2 (derived from HD 500). Lawrence K. Nakamura, curator of the USDA *Bacillus thuringiensis* culture collection, Peoria, IL, provided HD 500 (ONR-60A donated to the USDA collection by I. M. Hall), HD 519 (a pigmented variety isolated from HD 500 by J. A. Correa), HD 563 (derived from R153-78 and donated by J. N. Aronson), HD 916 (ONR-60A donated by H. de Barjac), and HD 917 (WHO 1884 donated by H. de Barjac). Isabelle Thiery of the Pasteur Institute, Paris, France, provided T14 001 (ONR-60A received from J. Margalit) from the *Bacillus thuringiensis* and *Bacillus sphaericus* Collection and IPS 82 lot 91509. The relationship between these isolates is summarized in Fig. 1. The cultures from the *Bacillus* Genetic Stock center and T14 001 were received as spore-impregnated filter disks and strips, the cultures from the USDA collection were received as lyophilized pellets, and IPS 82 was received as a technical powder. The filter strips/disks were placed onto brain heart infusion (BHI) agar plates (Difco Laboratories, Detroit, MI) and rehydrated with BHI broth. IPS 82 was sprinkled onto BHI agar plates, and the lyophilized pellets were suspended in BHI broth and incubated at 30°C for 24 h, and then 0.3 ml of this suspension was streaked onto BHI agar plates. Acrobe granules formulated by Clarke Outdoor Spraying, Roselle, IL, and aqueous suspension produced by American Cyanamid, lot 1054P3Q; Bactimos technical powder provided by Clarke Outdoor Spraying, Roselle, IL, and briquettes provided by Summit Corporation, Baltimore, MD, lot 0502; Teknar HP-D aqueous suspension, Zoecon Corporation, lot 7841040; and Vectobac 12AS aqueous suspension (2 containers) and technical powder, Abbott Laboratories, lots 61-476-N9, 18-202-BA, and 27-801-CD, were all streaked onto BHI agar plates for this study. Three vials of commercially produced technical powder dating back to the study of McLaughlin et al. (1984) marked standard II,

standard III, and standard IV, were also used and these technical powders were sprinkled onto BHI plates. All BHI plates were incubated at 30°C.

Sample preparation and analysis: Single isolated colonies were picked from the BHI agar plates and transferred, one colony per plate, to tryptic soy broth (TSB) (Baltimore Biological Laboratories, Cockeysville, MD), agar plates (Difco Laboratories) and incubated at 28°C for 24 h. All streaking and isolation were done under a laminar flow hood. Approximately 40–50 mg wet weight of cells were removed from each plate and the fatty acids in these cells were extracted and methylated according to the protocol of Miller and Berger (1985). The fatty acid methyl esters were identified as described by Siegel et al. (1993). The GLC was calibrated every 10th vial with a Hewlett-Packard calibration standard kit containing fatty acid methyl esters in 0.8 ml hexane (saturated nC9:0 to nC20:0 plus 2 and 3 hydroxy). A reagent control was also included with every run.

The relationships between the individual plates were determined by principal component analysis, Euclidean distance, and hierarchical unpaired group method using arithmetic averages (UPGMA) clustering techniques using algorithms developed by Hewlett-Packard and included in the library generation (LG) software package. Using LG software, library entries were generated from each culture, and we refer to these library entries as profiles. The relationship between profiles was determined by dendrogram analysis. Each library entry contained a minimum of 2 extracted plates. Profiles that linked at a distance ≤ 2 Euclidean units were considered as belonging to the same strain, based on empirical data collected by MIDI, Inc. When 2 profiles contained ≤ 4 colonies each and belonged to the same strain they were combined. Strains that were identical were given the same letter of the alphabet and strains that were dissimilar were designated by unique letters of the alphabet. Additional statistical analysis was conducted on the data for 4Q2 using PROC PRINCOMP on mainframe SAS (version 6, SAS Institute Inc., Cary, NC) in order to determine the correlation among the fatty acids and the loading values of the principal components.

Numbers of colonies examined: Three hundred fifty-five plates were analyzed in this study as follows: 15 plates 4Q1, 16 plates 4Q2, 15 plates HD 500, 8 plates HD 519, 15 plates HD 563, 12 plates HD 916, 16 plates HD 917, 20 plates T14 001, 78 plates IPS 82, 40 plates from standards II, III, and IV, and 120 plates from Acrobe, Bactimos, Teknar, and Vectobac. Multiple samples were run over a 6-month period to maximize heterogeneity. If there was any question concerning a particular run, it was repeated with new material.

RESULTS

Descriptive statistics for ONR-60A, WHO 1884, and IPS 82: Twenty fatty acids were used in the analysis of all isolates, of which 7 consistently accounted for $\geq 5\%$ of the fatty acids identified. These 7 fatty acids and their CVs are summarized in Table 1. All were iso fatty acids, indicating that they had an L-leucine amino acid primer. The CV for the primary constituent, 13-methyl tetradecanoic acid (15:0 iso), ranged from 1 to 8, and the CV for the 13:0 iso fatty acid had a similar range. The 17:0 iso fatty acid appeared most variable, with the CV ranging from 1 to 10. In isolate 4Q2, 3 fatty acids were positively correlated with the 15:0 iso primary constituent, iso 17:1 ω5C (0.76), the minor fatty acid 17:1 anteiso A (0.34), and 17:0 iso (0.34). These were the 5th, 13th, and 4th most abundant constituents, respectively. The 3 fatty acids most negatively correlated with the 15:0 iso fatty acid in 4Q2 were the 14:0 iso (-0.89), 16:0 iso (-0.74), and 15:0 2OH (-0.72). These fatty acids were the 6th, 7th, and 8th most abundant constituents. Nine fatty acids were positively correlated with the 13:0 iso in 4Q2, and the 2 strongest positive correlations were for the 14:0 (0.72) and the 13:0 anteiso (0.69). These are the 9th and 15th most abundant constituents. The strongest negative correlation was with the 17:0 iso (-0.52). These strong correlations are indicative of a highly ordered data set and 3 principal components typically accounted for $>90\%$ of the variation in all profiles. In 4Q2, the first principal component was a contrast of the primary constituent (15:0 iso) plus the 3 fatty acids that were positively correlated to it versus the remainder of the fatty acids. The first principal component accounted for $>60\%$ of the variance. The 2nd and 3rd principal components were more difficult to interpret but appeared to contrast the shorter and longer chain fatty acids.

Profiles ONR-60A: The ONR-60A analyzed consisted of 11 separate strains. Six initial profiles for T14 001 and HD 916 were combined into 3 composite profiles because they contained fewer than 4 plates each and joined at a distance less than 2 Euclidean units (Fig. 2). Four profiles could not be combined and consisted of the minimum of 2 plates each. The remaining profiles ranged from 4 to 48 plates. The 3 composite profiles shown in Fig. 2 are indicated by brackets, and the strain boundary of 2 Euclidean units is denoted by a dotted line. Six profiles joined before this boundary; 9 joined after the boundary

Table 1. Mean percent composition and coefficient of variation of cellular fatty acids comprising ≥5% of the cell envelope of ONR-60A, WHO 1884, and IPS 82.

Strain	13:0 iso	14:0 iso	15:0 iso	16:0 iso	iso 17:1 ω5c	17:0 iso	Sum feature 4[1]	n
HD 500-1	9.2/4	5.1/3	33.8/2	5.4/5	5.5/3	7.8/5	11.5/2	10
HD 500-2	9.5/5	5.6/1	31.4/1	5.6/4	5.7/3	7.8/3	11.8/1	5
HD 519	9.9/7	—	30.9/3	—	8.0/3	8.2/7	11.1/2	8
HD 563-1	8.9/6	5.0/8	31.7/2	5.4/9	6.7/9	7.5/6	12.2/4	7
HD 563-2	9.2/2	—	34.5/2	—	6.7/3	7.4/2	11.6/3	4
HD 563-3	11.4/8	—	35.6/2	—	6.7/6	7.4/9	12.2/9	2
HD 563-4	10.4/3	—	37.8/3	—	6.6/13	7.1/7	10.9/2	2
HD 916-4	8.9/1	5.2/4	32.0/2	6.0/3	6.0/4	9.2/4	12.3/4	6
HD 917-1	11.1/4	6.5/7	27.7/3	5.8/5	5.5/8	6.2/10	11.9/2	7
HD 917-2	12.6/1	6.2/3	29.3/2	5.1/3	5.7/2	5.9/2	12.1/1	5
4Q1	10.2/3	—	32.3/2	—	6.7/3	7.3/4	12.1/2	15
4Q2	10.3/4	5.0/4	32.0/2	—	6.7/3	7.3/5	12.2/2	16
T14 001-1	12.9/4	5.8/7	31.3/5	5.2/7	—	6.4/9	11.4/4	7
T14 001-2 + HD 916-1	10.5/3	6.2/11	30.6/8	6.1/8	5.0/15	6.8/10	12.1/5	7
T14 001-3 + HD 916-2	9.0/6	5.4/5	33.1/2	6.1/9	5.5/6	8.4/4	11.2/6	6
T14 001-4 + HD 916-3	9.0/8	5.1/3	31.6/1	6.7/10	5.3/1	9.6/1	10.5/1	2
T14 001-5	8.4/4	5.9/5	29.7/2	7.2/1	—	8.6/6	11.4/6	4
IPS 82-1	12.1/6	6.4/6	29.7/3	5.2/6	5.2/6	5.7/10	12.0/5	48
IPS 82-2	10.0/6	5.7/8	31.0/3	5.4/6	6.0/5	7.0/8	12.0/2	28
IPS 82-3	8.3/6	—	29.4/1	6.3/1	5.9/7	9.1/3	11.4/4	2

[1] Either 15:0 iso 2OH or 16:1 ω7t.

but before the subspecies boundary of 6 Euclidean units. Four profiles, 4Q1, 4Q2, and a profile from HD 500 and HD 563 belonged to one strain (F). The composite profile formed by a strain from T14 001 and HD 916, and a strain in HD 500, joined before 2 Euclidean units, indicating that they all belonged to a single strain as well (C). The remaining profiles represent separate strains. These strains were not equally distributed among the 3 collections, and the most common strain, F, occurred in isolates from the USDA and the *Bacillus* Genetic Stock Center collections.

Profiles IPS 82 and WHO 1884: IPS 82 consisted of 3 strains and the relationship between these strains and the ONR-60A strains is shown in Fig. 3. There were 3 strains in IPS 82, 2 of which comprised 76 of the 78 plates analyzed and one minor strain that comprised 2 plates. The 2 major strains are designated L and F in Fig. 3. Strain L accounted for 62.5% (48/78) of the plates and strain F accounted for 35.9% (28/78). The profile for IPS 82 strain F joined with profiles from 4Q1, 4Q2, HD 500, and HD 563 before 2 Euclidean distance units, indicating that they belong the same strain.

The relationship between IPS 82 and WHO 1884 is shown in Fig. 4. There were 2 strains in HD 917, and their profiles are given the letters L and M in the dendrogram. The profiles marked L in IPS 82 and HD 917 join before 2 Euclidean distance units, indicating that they belong to the same strain. The 2nd strain in HD 917 joins just outside 2 units, indicating that it is not identical but very similar.

Validation of ONR-60A and IPS 82 profiles: Thirty-six out of the 40 plates originating from standards II–IV belonged to strain F (90%), and 5% of the plates (2/40) belonged to strain G (Table 2). Five percent of the plates did not fit any of our profiles, resulting in a successful identification level of 95%. The predominant strain in the new commercially produced *B.t.i.* was strain L, 40.8% (49/120); 35% of the plates (42/120) belonged to ONR-60A strains A, B, D, F, and H, and 4.2% (5/120) of the plates belonged to strain N, which was the minor strain in IPS 82. We could not identify 20% of the plates (24/120) using our profiles. These unidentified plates fell within the range for subspecies, 6 Euclidean distance units. Our identification level for the new commercially produced *B.t.i.* was 80%. The material from standards II–IV contained only 2 ONR-60A strains, with strain F predominant at 90%; the new commercially produced material was heterogeneous. No single strain accounted for 50% of the plates analyzed, and 5 different ONR-60A strains were observed. The percentage of strain L in the commercially produced *B.t.i.* was lower than in IPS 82, and the per-

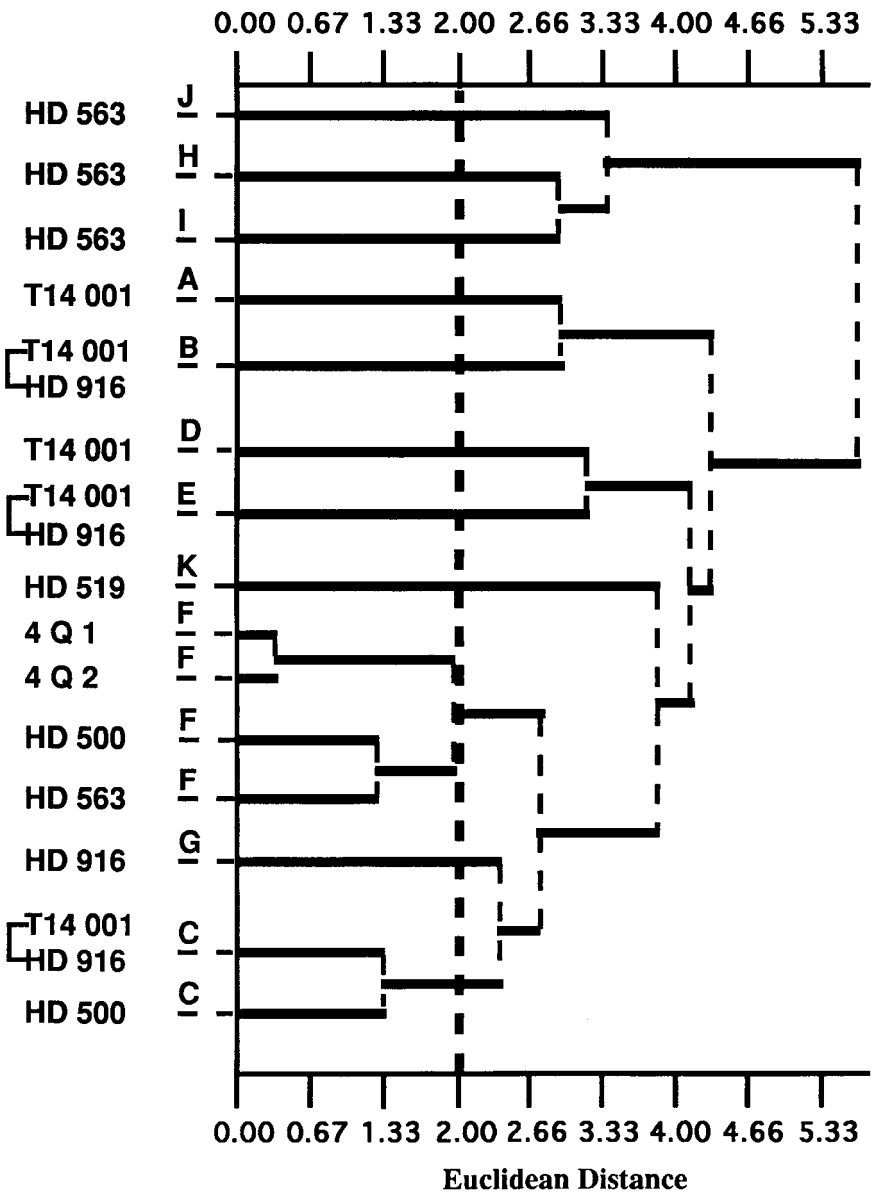

Fig. 2. Dendrogram of the relationship among 15 profiles created from *Bacillus thuringiensis* ssp. *israelensis* ONR-60A. Identical profiles, which link before 2 Euclidean distance units, are marked by the same letter. These unique letters correspond to strain designations in the text.

centage of strain F was lower in the new commercially produced *B.t.i.* compared to IPS 82.

DISCUSSION

ONR-60A strains: We were surprised by the heterogeneity of ONR-60A. Initially we had expected to find perhaps 2 or 3 strains in ONR-60A, because of the pigmented variant HD 519, and perhaps 2 strains in IPS 82, because of the heterogeneity of the 13:0 iso fatty acid. Instead, we found that ONR-60A obtained from culture collections consisted of 11 strains and IPS 82 consisted of 3 strains. There are several hypotheses that may account for this diversity. The first hypothesis is that the majority of the strains that we identified were contaminants introduced over the past 18 years. We do not know the complete

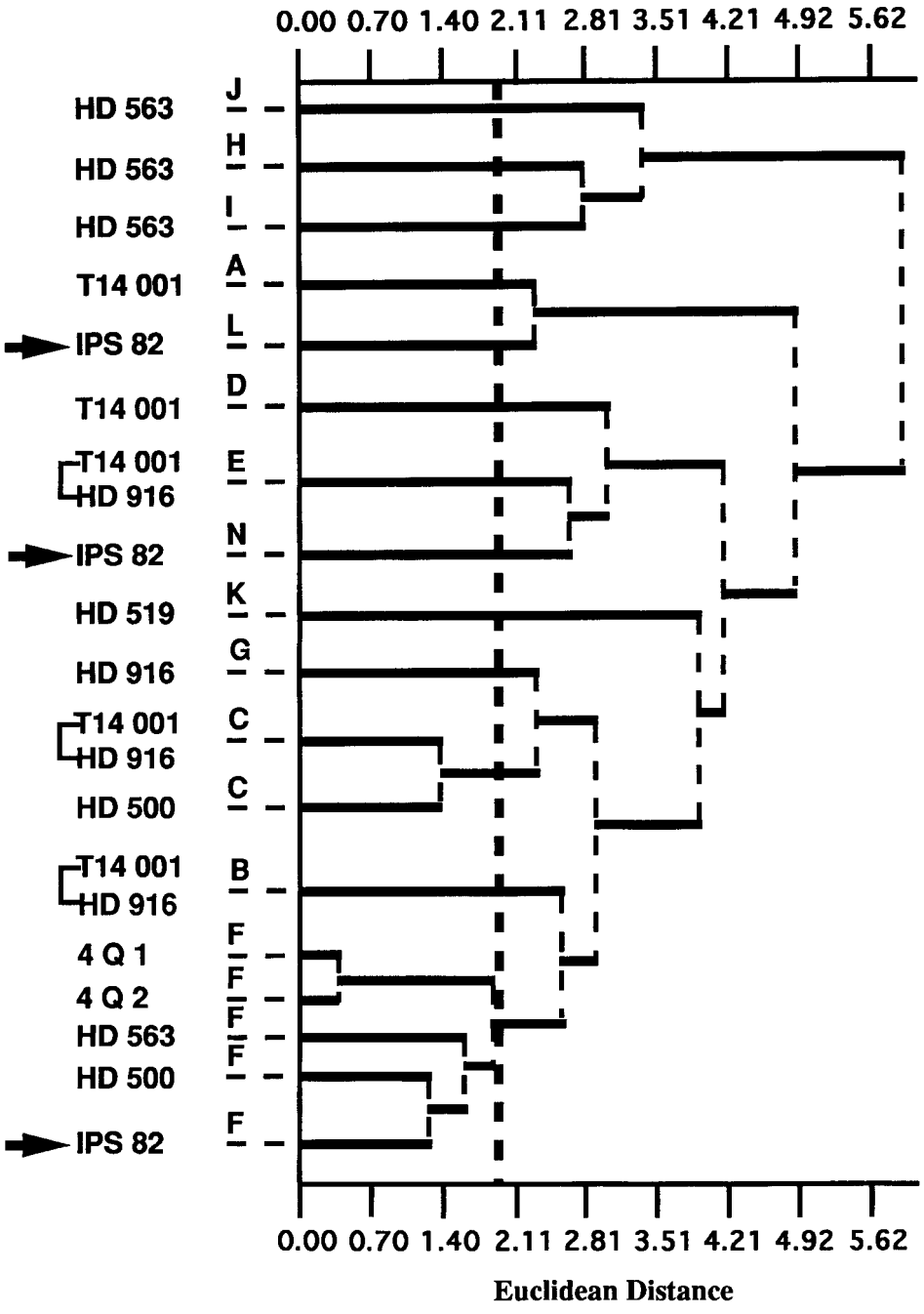

Fig. 3. Dendrogram of the relationship between *Bacillus thuringiensis* ssp. *israelensis* IPS 82 and ONR-60A. The IPS 82 profiles are marked by arrows, and identical profiles, which link before 2 Euclidean distance units, are marked by the same letter. These unique letters correspond to strain designations in the text.

history of some of the isolates deposited in the strain collections, and contaminants may have been deposited along with ONR-60A into these collections. If one were to choose strains likely to be contaminants, our candidates would be strain K in HD 519 and strains I and J in HD 563, because they do not occur in other cultures or commercially produced *B.t.i.* However, we do

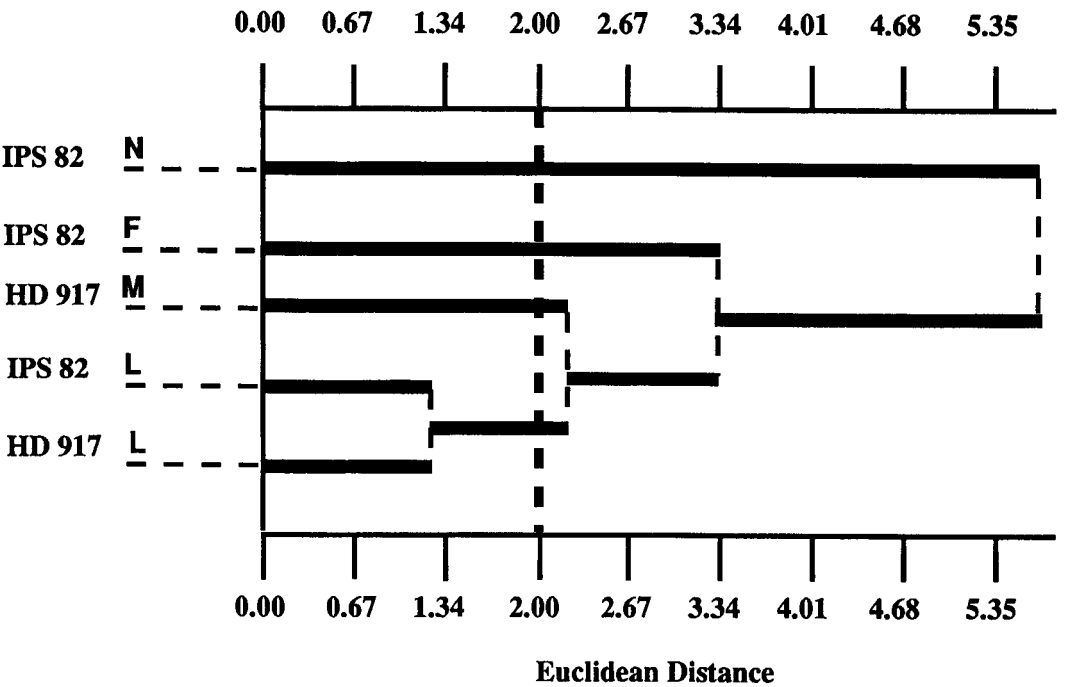

Fig. 4. Dendrogram of the relationship between *Bacillus thuringiensis* ssp. *israelensis* IPS 82 and WHO 1884. Identical profiles, which link before 2 Euclidean distance units, are marked by the same letter. These unique letters correspond to strain designations in the text.

not think that this hypothesis accounts for all of the diversity observed, because 4 strains occur in several collections as well as in commercial products. The second hypothesis is that the original isolate of ONR-60A contained multiple strains of *B.t.i.* Subsequently, inadvertent selection by the curators of the strain collections for strains that grew best in their culture system and both purposeful and inadvertent selection by industry for the most toxic strains combined with the ability to grow under industrial conditions, eliminated some strains and conserved others. This hypothesis explains the unequal distribution of these strains among the culture collections and commercially produced material. For example, the ONR-60A from the Pasteur Institute, T14 001, contained the most strains, 5, but did not contain the strain that we designated F, which was the most common. However, strain F was present in HD 563, which was derived from R153-78, which in turn originated from ONR-60A sent to R. Bellon laboratories by the Pasteur Institute. Strain F may have been lost if the curators of the Pasteur Institute collection did not recognize that they were dealing with a mixture, or if media and culture conditions changed at some point so that the growth of other strains was favored. The final hypothesis is that ONR-60A originated from the single colony described by Margalit and Dean (1985) and Margalit (1990). During the process of subculturing by the numerous laboratories that investigated this subspecies over the years, spontaneous mutants were selected and these lines continued under favorable cultural conditions. If these mutants had superior growth or toxicity properties under industrial conditions, the mutants may have been purposefully retained by industry. Likewise, if these mutants retained mosquitocidal activity and had a similar colony morphology, they may have been retained by the curators of the strain collections.

We cannot disprove the second or third hypotheses, 18 years after the fact, and both hypotheses share the same mechanism after the initial isolation of ONR-60A, which is a combination of purposeful and inadvertent selection resulting in the diversity that we observed. There is some evidence that the protocols differed between culture collections because the 2 isolates from the *Bacillus* Genetic Stock Center only contained a single strain whereas the isolates from the USDA and Pasteur Institute contained several strains. This issue has a practical application, because it suggests that if one researcher uses material from the Pasteur Institute, and another

Table 2. Number of colonies of commercially produced *Bacillus thuringiensis* ssp. *israelensis* matching strain profiles created for ONR-60A, WHO 1884, and IPS 82. The strains are represented by letters and IPS 82 is included for comparison.

Source	A	B	C	D	E	F	G	H	I	J	K	L	M	N	?[1]	*n*
Old *B.t.i.*[2]	0	0	0	0	0	36	2	0	0	0	0	0	0	0	2	40
IPS 82	0	0	0	0	0	28	0	0	0	0	0	48	0	2	0	78
New *B.t.i.*[3]	15	14	0	5	0	6	0	2	0	0	0	49	0	5	24	120

[1] Plates that did not match the strain profiles.
[2] Material from the study of McLaughlin et al. (1984).
[3] Acrobe®, Bactimos®, Teknar®, and Vectobac® obtained between 1990 and 1994.

researcher uses material from the *Bacillus* Genetic Stock Center, even though both are labeled ONR-60A, they are not identical. Our findings have additional ramifications because there are multiple deposits of ONR-60A in the collections of the USDA and Pasteur Institute, so that even within a culture collection, different isolates may not be comparable. Finally, the hypothesis that some current strains are contaminants is also compatible with either the second or third hypothesis.

IPS 82: Although we did not evaluate toxicity, it is possible that the 15-fold increase in the toxicity of IPS 82 compared to the original IPS 78 standard (McLaughlin et al. 1984, Dulmage et al. 1985) resulted from the addition of strain L. Researchers at the Pasteur Institute created the new standards, IPS 82, from WHO 1884 stock, because this isolate produced larger insecticidal crystal proteins (I. Thiery, personal communication). We hypothesize that these researchers increased the percentage of strain L in the mixture that was WHO 1884. We do not know if strain L does indeed produce larger crystals than does strain F, or even if insecticidal crystal protein (ICP) size is necessarily correlated with potency. If IPS 82 originated solely from WHO 1884, then this isolate was a mixture of L, F, M, and N, based on the composition of IPS 82 and HD 917. We plan to obtain additional cultures of WHO 1884 as well as WHO 1897, and determine their relationship to our 3 strains of IPS 82.

Commercially produced B.t.i.: The findings in this paper do not contradict our previous report that Acrobe, Bactimos, Teknar, and Vectobac belong to the same strain as IPS 82. In retrospect, our previous profiles indicated that these commercial products and IPS 82 contained strain L. We have now increased the precision of our profiles so that we can identify ONR-60A strains, and our data suggest that IPS 82 was blended into the commercial seed cultures at some point after 1983–84, probably no later than 1987. As early as 1988 (Siegel, unpublished data) strain L appeared in commercially produced material.

The data for commercially produced *B.t.i.* validated our ONR-60A profiles, and indicated that several ONR-60A strains are present in commercial products (Table 2). As expected, standards II–IV contained the most prevalent strain, F. However, we were surprised by the dominance of strain F, given its unequal distribution in the isolates from the culture collections. For example, HD 500 consisted of only 33% F, whereas standards II–IV were 90% F, based on the agar plates that we analyzed. It is possible that industrial production techniques favored this strain, or that our sample size was not representative of the overall population. We do not think that this last point is likely, because we sampled the technical powder on several occasions and picked our colonies at random. In contrast to the old material, recently produced commercial *B.t.i.* was quite heterogeneous, although strain L was the most prevalent. The prevalence of ONR-60A strains A and B, 12.5% and 11.7%, respectively, in the new material, in contrast to their absence from standards II–IV, may reflect selection due to changes in submerged culture technology over the past decade.

In our previous paper (Siegel et al. 1993), we noted that a now discontinued commercial product, Skeetal®, belonged to a different strain than did IPS 82. We analyzed additional colonies of Skeetal and found that this product contained 5 strains. We then compared these Skeetal strains to every profile reported in this paper and still could not link them to any other strain at a distance of 2 Euclidean units. It is still possible that Skeetal may have originated from WHO 1884 or WHO 1897, and until we create additional profiles from these isolates, the origin of Skeetal remains unclear.

Culture conditions: One shortcoming associated with fatty acid analysis is the lack of standardization between research laboratories. Media and temperature influence the percent composition of fatty acids that comprise the cell envelope. For instance, as incubation temperature increases the percentage of anteiso fatty acids in the cell envelope decreases. These anteiso fatty

acids have a substantially lower melting point than their unbranched and iso counterparts so they are eliminated in order to stabilize membrane fluidity. Additionally, because the iso-odd fatty acids have an L-leucine amino acid primer, anteiso-odd fatty acids have an L-isoleucine amino acid primer, and iso-even fatty acids have an L-valine primer, the lack of these amino acids in the medium will also affect the percent composition of the fatty acids in the cell envelope (Suzuki et al. 1993). There are additional permutations associated with the conditions that the gas–liquid chromatograph (GLC) is operated under, such as temperature, rate of temperature increase, column type, and gas carrier. Although our data were replicable and our variation was low (as measured by CV), our results are only directly comparable to those of other researchers who use the same growth and extraction protocols as well as the Hewlett-Packard Microbial Identification system. These other research institutes include the Pasteur Institute (Frachon et al. 1991), and commercial diagnostic laboratories (Stahly and Klein 1992). To emphasize this point, there have been 2 recently published papers analyzing fatty acid composition of nematicidal *Bacillus thuringiensis* and *Bacillus thuringiensis* ssp. *kurstaki* (HD-1) during fermentation (Esnard et al. 1994, Rowe and Margaritis 1994). However, we cannot relate our data to these findings because of the different media, temperature, and GLC settings used. As a final comment concerning technique, given our experience with ONR-60A, we suggest that summaries of fatty acids unaccompanied by either the CV or standard deviation should be interpreted with caution.

In summary, instead of a single strain, ONR-60A currently consists of 11 strains, based on differences in fatty acid composition of the cell envelope. This diversity may have been present in the original isolate of ONR-60A, or may have resulted from the cumulative effects of spontaneous mutations combined with the introduction of contaminants over the past 18 years. These strains were unequally distributed among the 3 culture collections. There was a single strain in the *Bacillus* Genetic Stock Center isolates, whereas the USDA collection contained 6 strains. Three of these 11 strains may have been contaminants, because they were unique to a single isolate and did not occur in commercially produced material. One strain, F, occurred in 2 culture collections, and this same strain was found in 90% of the plates derived from material used in the study of McLaughlin et al. (1984). The current standard, IPS 82, consisted of 3 strains, one of which was identical to strain F in our dendrogram analysis. IPS 82 contained 2 other strains as well, designated L and N, and we suggest that it is the presence of strain L that gives IPS 82 at its improved properties. However, we did not test the toxicity or host spectrum of our strains. This same strain L is the most prevalent strain in commercially produced *B.t.i.* We acknowledge that we have examined only a single isolate of WHO 1884 (HD 917) and until we have examined other isolates, we cannot rule out the possibility that other strains, including F, were in WHO 1884 from the beginning. We intended to analyze additional isolates of WHO 1884 and WHO 1897 from the collections of the USDA and Pasteur Institute to resolve this issue. Finally, we repeat our caution that at this point in time, there is no single strain of ONR-60A and suggest that studies using isolates from different culture collections, or even isolates corresponding to different accession numbers within a culture collection, may not be comparable.

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