Differentiation of Six Strains of *Bacillus thuringiensis* by Hydrolyzable Fatty Acid Composition

Joseph Esnard,† Thomas L. Potter,‡ and Bert M. Zuckerman*,†

Department of Plant Pathology and Mass Spectrometry Facility, College of Food and Natural Resources, University of Massachusetts, Amherst, Massachusetts 01003

Six potential biocontrol strains of *Bacillus thuringiensis* grown under identical conditions on fatty-acid-free King's B medium were differentiated on the basis of their hydrolyzable fatty acid content. Thirty-four compounds were identified as their methyl esters in hydrolysates, among which the major fatty acids were i-15:0 (13.7-23.2%), i-13:0 (6.8–10.7%), i-14:0 (5.0–7.7%), 14:0 (3.0–4.1%), a-15:0 (3.9–9.9%), i-16:0 (3.6–7.2%), 16:0 (3.2–11.6%), i-17:0 (3.2–9.8%), 18:0 (tr-13.5%), a monounsaturated C_{16} acid (4.5–9.9%), a monounsaturated branched C_{17} acid (2.8–5.8%), a diunsaturated C_{18} acid (0–5.8%), and a monounsaturated C_{18} acid (0.3–4.8%). A cyclopropanoid fatty acid, cy17:0(9), was detected at low concentration in three strains, including the anthelmintic strain CR-371. The presence or absence and the relative ratios of certain chromatographic peaks in combination with a Wilcoxon-signed-rank-test analysis of pairwise differences in the total chromatographic profile distribution were used to differentiate the six bacterial strains.

INTRODUCTION

Bacillus thuringiensis (**Bt**) is the most widely used bacterial species for biological control of insects. Tanada and Kaya (1993) recently reviewed the importance of the Bacillaceae in biocontrol of insects. The potential of **Bt** products as alternatives to synthetic pesticides broadened with the discovery of **Bt** strains toxic to pathogenic protozoans, parasitic liver flukes, and mites (Feitelson et al., 1992). Recently, Zuckerman et al. (1993) demonstrated, for the first time in a field trial, the effective control of plant-parasitic nematodes by a novel strain of **Bt** (designated CR-371). Biocontrol methods using **Bt** are being applied in many countries throughout the world (Powell et al., 1990).

Increasingly, new **Bt** strains specific for protozoan, liver fluke, mite, nematode, and insect pest management are being discovered. Rapid, accurate, and economically attractive methods for their differentiation from previously isolated **Bt** strains are needed. Among other considerations, definitive differentiation is needed when organisms are patented. In this study, gas chromatographic profile analysis of the hydrolyzable fatty acids from several biocontrol strains including CR-371 was explored. Because fatty acids are suitable for instrumental analysis and are synthesized by highly regulated enzyme systems, they have proved to be extremely useful in bacterial systematics (Komagata and Suzuki, 1987; Sasser, 1990). Fatty-acid-based groupings agree with DNA homology (Roy, 1988).

To our knowledge, all studies reported thus far that have used fatty acid composition for characterization within the genus *Bacillus* demonstrate successful differentiation of species but not strains. This study was undertaken to demonstrate the capability to differentiate **Bt** strains on the basis of comparisons of their hydrolyzable fatty acid compositions. It is essentially the differentiation of our novel antinematode strain (CR-371) from five of Mycogen Corp.'s already described **Bt** strains that are purportedly active against plant-parasitic nematodes (Edwards et al., 1992) but which, to our knowledge, have not been demonstrated to be efficacious in authentic field trials.

MATERIALS AND METHODS

Bacterial Strains. Six strains of *B. thuringiensis* were analyzed. **Bt** strain CR-371 was a novel organism, while NRRL B-18243, NRRL B-18244, NRRL B-18245, NRRL B-18246, and NRRL B-18247 were five different strains patented by Mycogen Corp. (Edwards et al., 1992) obtained from the American Type Culture Collection. CR-371's potential to reduce population levels of several plant-parasitic and/or free-living nematodes has been demonstrated (Zuckerman et al., 1993). All strains had been previously stored at −80 °C in cryopreservation buffer (per 200 mL, 20 mL of 1 M NaCl, 10 mL of 1 M phosphate buffer, 60 mL of glycerol; after autoclaving, 0.6 mL of 0.1 M $MgSO_4$). Each **Bt** strain was quadrant streaked onto solid fatty-acid-free King's B medium (*Pseudomonas* agar F) in triplicate 6-cm Petri plates and grown in the dark for 48 h at 28 ± 0.1 °C.

Extraction and Derivatization of Fatty Acids. The fatty acid extraction procedure was a modification of that of Vannieuwenhuyze and Sandra (1987) and Sasser (1990). It involved saponification of bacterial lipids with KOH and their methylation with methanolic HCl. Fifty milligrams wet weight of cells was carefully scraped from the fourth quadrant of three plates of each **Bt** strain. King's B medium (50 mg in triplicate) was used as the control. Each sample was placed in a 5-mL borosilicate serum bottle, and 1 mL of 5% KOH in 50% aqueous methanol was added. The serum bottles were crimp-sealed with Teflon-faced silicon rubber septa with one-piece tear-away aluminum caps, shaken rapidly for 2–3 s, and then placed in a heating module at 100 °C for 30 min with occasional shaking. The contents were allowed to cool to room temperature, after which the aluminum caps were removed. One and a half milliliters of 25% hydrochloric acid (12.1 N) in methanol was added to each bottle, which was sealed, agitated, and then returned to the heating module for 10 min at 80 °C. After cooling, methylated fatty acids were extracted by adding 1 mL of hexane to each serum bottle which was then shaken vigorously (with the Teflon septum held in place by hand) for 15 s. The aqueous (bottom) phase was discarded, and the hexane fraction was washed with 1 mL of phosphate buffer (0.025 M KH_2PO_4 plus 0.025 M Na_2HPO_4, pH 7) by agitating for 15 s. Each fatty acid solution was transferred to a small vial and assayed immediately by capillary gas chromatography or stored at −10 °C.

Gas Chromatography–Mass Spectrometry. A Hewlett-Packard Model 5989A GC–MS system (Avondale, PA) was used.

† Department of Plant Pathology.
‡ Mass Spectrometry Facility.

0021-8561/94/1442-1251$04.50/0

The chromatograph was equipped with a Hewlett-Packard polyimide coated fused silica HP-5 capillary column (30 m × 0.25 mm i.d.) coated with a 0.25-μm film of cross-linked bonded phenyl (5%)–dimethylsiloxane (95%) gum. Helium served as the carrier gas (column head pressure of 70 kPa). Initial column temperature was held at 60 °C for 1 min and then increased 4 °C/min to 250 °C and held for 10 min. Injection was at 250 °C in the splitless mode. The capillary column was directly coupled to the ion source through a heated transfer line. The mass spectrometer was operated in the electron impact (70 eV) and ammonia chemical ionization modes (CI). Under CI conditions, the ammonia gas pressure was set at 0.9 Torr and the source temperature at 150 °C.

Gas Chromatography with Flame Ionization. To test the reproducibility of the results, analyses were repeated three times on a Varian 3700 gas chromatograph equipped with a flame ionization detector (FID, 250 °C) at the end of a 50-m BP-5 column (SGE, i.d. 0.22 mm with 0.25-μm film). On-column injection techniques were used (Grob and Grob, 1978). The helium carrier gas head pressure was 141 kPa. The same temperature program in GC–MS analyses described above was followed. Peak areas were normalized on the largest peak, i-15:0. The coefficient of variation ranged from 0.64 to 1.01% (n = 3).

Peak Assignments. Peak assignments were made by comparing retention times and mass spectra to data obtained with a standard mixture of bacterial fatty acid methyl esters (Matreya, catalog no. 1114). Relative retention times were determined by calculations relative to an internal standard ($C_{22}H_{46}$). Saturated branched-chain fatty acids were identified by cochromatographic comparisons of their relative retention times to those of the standards (Suutari and Laakso, 1993). **Bt** fatty acids not represented in the standard fatty acid mixture were identified by searching several mass spectral data bases (McLafferty and Stauffer, 1989; Ausloos et al., 1992; Mass Spectrometry Data Centre, 1993) and comparisons of their relative retention times with published results (Suutari and Laakso, 1993).

Statistical Analyses. A simple algebraic formula was introduced for pairwise differentiation of the **Bt** strains based on F, the proportion of hydrolyzable fatty acid types in the elution profile common to both strains (s_1 and s_2)

$$F = n(s_1 \cap s_2)/n(s_1 \cup s_2) \quad (1)$$

where $n(s_1 \cap s_2)$ is the number of common fatty acids shared by both sets (strain profiles) and $n(s_1 \cup s_2)$ is the total number of distinct fatty acids between both strain profiles (shared fatty acids counted only once). When F was less than one, the two **Bt** strains being compared were considered dissimilar. Theoretically, $0 \leq F \leq 1$. Brown et al. (1979) introduced a similar formula for DNA divergence studies

$$F = n_{12}/(n_1 + n_2 - n_{12}) \quad (2)$$

where n_1 and n_2 are the sizes of populations 1 and 2, respectively, and n_{12} is the number of restriction fragments common to both populations, for estimating the proportion of ancestral restriction sites that remain unchanged in the two populations. The shared presence or absence of certain bacterial fatty acids (Lambert et al., 1983) and the relative ratios (Roy, 1988; Chase et al., 1992) of certain fatty acid methyl esters have been used to differentiate bacteria.

In addition, distributions of hydrolyzable fatty acids from the **Bt** strains were compared using Wilcoxon's signed rank test (Wilcoxon, 1945, 1947), which took into account both the direction and the size of the differences between two sets of paired measurements. Before Wilcoxon's test was applied, the area counts in the profiles from each **Bt** strain were first "normalized" by dividing all peaks by the area corresponding to the same large peak (i-15:0) in each chromatogram. Statistical analyses were performed using MSTAT-C software (Freed and Eisensmith, 1990). Our motivation for using the Wilcoxon signed rank test rather than more complex procedures was that it provided a means of analysis without making assumptions regarding the distribution underlying the population of measurements. An assumption of the existence of a normal distribution in chromatographic measurements, for example, may not be acceptable (Massart and Kaufman, 1983). In commonly used statistical linear discriminant analysis or multivariate analysis of variance procedures, hundreds of samples are needed to describe a multivariate normal population adequately (Han et al., 1990). The number is dependent on the number of variables used in the comparison (Lavine et al., 1988). Other limitations are that values must be assigned for each observation in a sample's data set. This requires use of estimation techniques when observations for a given compound are below the analytical detection limit in some but not all data sets. The alternative is to reject the "non-detect" data and in the process lose information that may very effectively discriminate target populations.

RESULTS AND DISCUSSION

For patenting purposes it became necessary to differentiate our nematicidal **Bt** from five already differentiated **Bt** strains patented by Mycogen and nematicidal to plant-parasitic nematodes. GC–MS and GC-FID analyses of hydrolyzable fatty acids showed clear differences in all combinations of the six strains taken a pair at a time. It was possible to separate the six strains on the basis of (i) the absence or presence of certain fatty acids, (ii) ratios of certain diagnostic peaks within a chromatogram, and (iii) results of the Wilcoxon matched-pairs signed rank test.

GC Profiles. Panels A–F of Figure 1 show the total ion chromatograms of the fatty acids from six **Bt** strains grown under identical conditions. These profiles appear to be similar and would probably lead to tight clustering if it were appropriate to use parametric statistical analysis (where a type II error could be committed). Table 1 provides peak assignments for 34 compounds identified as their methyl esters. Thirteen fatty acids were found in high amounts in the six strains: i-15:0 (13.7–23.2%), i-13:0 (6.8–10.7%), i-14:0 (5.0–7.7%), 14:0 (3.0–4.1%), a-15:0 (3.9–9.9%), i-16:0 (3.6-7.2%), 16:0 (3.2–11.6%), i-17:0 (3.2–9.8%), 18:0 (tr-13.5%), a monounsaturated C_{16} acid (4.5–9.9%), a monounsaturated branched C_{17} acid (2.8–5.8%), a diunsaturated C_{18} acid (0–5.8%), and a monounsaturated C_{18} acid (0.3–4.8%). The relatively high content of stearic acid (13.5%) in strain NRRL B-18247 was remarkable. To our knowledge, this is the first report of such a high amount of stearic acid (18:0) in *B. thuringiensis*. Small amounts of the C_{17} analogue of lactobacillic acid, *cis*-9,10-methylenehexadecanoic acid [cy17:0(9)], occurred in three strains including the antinematodal CR-371.

On the basis of the types of fatty acids present or absent (Table 1), the nematicidal **Bt** (CR-371) was determined to be closest to Mycogen's strain NRRL B-18243, and NRRL B-18244 most resembles NRRL B-18245. These similarities (F = 1 and 0.97, respectively) are reflected in Table 2, which lists the proportion (F) of fatty acids common to a pair of **Bt** strains.

Peak Ratios. Despite this close resemblance, it was not difficult to differentiate these strains since the ratios of their fatty acid contents for certain peaks were very different (Table 3). The ratio of peak 22 (branched 17:1) to peak 17 (i-16:0) in strain CR-371 clearly differentiated it from strain NRRL B-18243 (Table 3; Figure 1). The ratio of these two peaks and that of peak 25 to peak 12 separate strain NRRL B-18244 from NRRL B-18245. All other pairwise comparisons based on peak ratios showed strong differences between all **Bt** strains (Table 3). Quick and clear-cut separation of strains can be achieved, therefore, by using both the magnitude and direction (<1 vs >1) of the differences in peak ratios.

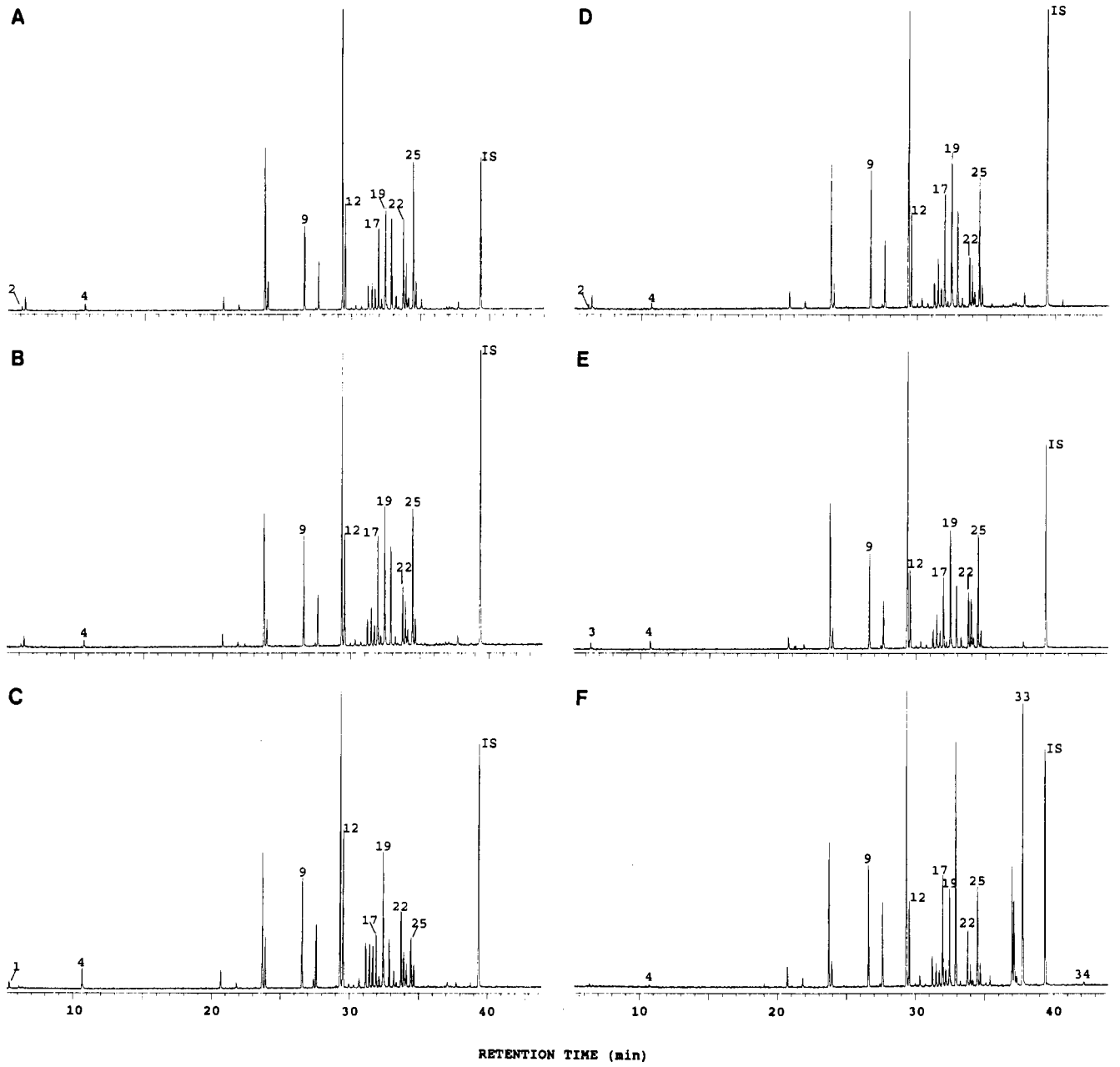

Figure 1. Total ion chromatograms of fatty acid methyl esters for **Bt** strains CR-371 (A), NRRL B-18243 (B), NRRL B-18234 (C), NRRL B-18245 (D), NRRL B-18246 (E) and NRRL B-18247 (F). Peak identities are given in Table 1. IS, internal standard.

Statistical Analyses. Nonparametric statistical analyses were used to rigorously test the significance of the differences between the six **Bt** strains. The test was selected on the basis of the rationale of Han et al. (1990), Massart and Kaufman (1983), and Steel and Torrie (1980) that distribution-free statistics apply when the assumption of normality is unacceptable. Accordingly, both MS and FID data were analyzed by the Wilcoxon signed rank test. A test criterion and normal deviate were generated and tested for each comparison. All paired comparisons showed very highly significant differences ($p < 0.0001$; $n = 3$). These results corroborate previous conclusions from biochemical and genetic analyses that the Mycogen strains are different.

The detection of saturated iso- and anteiso-branched- and straight-chain fatty acids between C_{12} and C_{17} with i-15:0 most abundant in the six **Bt** strains agreed with the results of previous analyses on **Bt** (Kaneda, 1968). We report in addition C_4, C_6, C_8, C_{18}, and C_{20} hydrolyzable acids (Table 1) that were very useful for separation of the strains. Stearic acid (peak 33, Figure 1F) in particular was detected at an appreciable concentration in strain NRRL B-18247. To our knowledge this is the first study on the differentiation of **Bt** strains that utilized all components detected in each profile. Though fatty acid analyses are known to be extremely useful in bacterial systematics (O'Donnell, 1985; Saddler et al., 1987; Komagata and Suzuki, 1987; Sasser, 1990), a majority of reports on species novae or strains present only a few (rarely >10) fatty acids with percentage composition. Bacterial fatty acid chromatograms usually contain more than 30 detectable peaks, the patterns of which have not been fully utilized in the differentiation of strains and species.

Our study aimed to demonstrate that our nematicidal **Bt** strain (CR-371) is different from Mycogen's five **Bt** strains. In all cases claiming strain novelty, evidence of a difference is necessary for patenting purposes. Many studies on fatty acid content investigate the extent of clustering (or similarity) among species or strains. It is expected that most bacterial strains of the same species

Table 1. Fatty Acid Composition of Six *B. thuringiensis* Strains

no.[a]	t_R[b]	compd	MW[c]	ID[d]	%I[e]	%II	%III	%IV	%V	%VI
1	5.41	i-6:0	130	*	0.0	0.0	0.5	0.0	0.0	0.0
2	6.13	MTP[f]	134	*, #	0.4	0.2	0.2	0.3	0.0	0.0
3	6.37	BA[g]	146	*, #	0.9	0.5	0.0	0.9	0.4	0.2
4	10.68	BAA[h]	150	*, #	0.8	0.6	1.5	0.6	0.9	0.1
5	20.71	i-12:0	214	+, *	1.0	1.0	1.3	1.1	1.1	1.0
6	21.84	12:0	214	+, *	0.4	0.4	0.6	0.6	0.3	0.5
7	23.73	i-13:0	228	*, #	9.9	7.9	8.9	8.1	10.7	6.8
8	23.94	a-13:0	228	*, #	1.9	1.8	3.4	1.5	1.6	1.4
9	26.61	i-14:0	242	+, *	5.0	6.6	6.9	7.7	6.3	5.5
10	27.63	14:0	242	+, *	3.0	3.4	4.1	4.1	3.4	3.8
11	29.36	i-15:0	256	+, *	21.3	19.3	19.3	18.3	23.2	13.7
12	29.56	a-15:0	256	+, *	6.5	6.9	9.9	5.7	5.6	3.9
13	30.33	15:0	256	+, *	0.4	0.6	0.2	0.7	0.5	0.6
14	31.22	16:1	268	*	1.6	1.7	2.9	1.6	1.5	1.5
15	31.50	16:1	268	*	1.7	2.6	3.0	3.1	2.5	1.2
16	31.73	16:2	266	*	1.4	0.1	2.9	1.5	1.4	0.9
17	31.93	i-16:0	270	+, *	5.3	7.2	3.6	7.0	5.1	5.2
18	32.19	16:1	268	*	0.8	0.8	0.9	0.6	0.5	1.1
19	32.47	16:1	268	*	6.7	9.3	9.3	9.9	8.5	4.5
20	32.92	16:0	270	+, *	5.9	6.3	3.2	6.2	4.8	11.6
21	33.29	17:2	280	*	1.1	0.8	1.3	0.8	0.9	0.3
22	33.77	17:1	282	*	5.8	3.9	5.3	3.4	4.3	2.8
23	33.97	br17:1	282	*	2.9	3.1	2.6	2.8	3.7	1.1
24	34.14	br17:1	282	*	1.1	1.3	1.7	1.2	1.2	0.4
25	34.48	i-17:0	284	+, *	9.8	9.2	3.2	7.6	8.4	4.9
26	34.68	17:0	284	+, *	2.0	2.2	1.7	1.6	1.4	1.2
27	35.08	cy17:0(9)	282	+, *	0.8	0.2	0.0	0.0	0.0	0.3
28	35.38	br18:0	282	*	0.2	0.3	0.1	0.5	0.3	0.6
29	36.88	i-18:0	298	*, #	0.2	0.4	0.2	0.3	0.2	0.0
30	36.97	18:2	294	*	0.0	0.0	0.1	0.4	0.0	5.8
31	37.12	18:1	296	*	0.3	0.5	0.5	0.5	0.3	4.8
32	37.30	br19:0		*	0.2	0.1	0.3	0.3	0.2	0.8
33	37.75	18:0	298	+, *	0.6	0.8	0.3	1.3	0.5	13.5
34	42.21	20:0		*	0.0	0.0	0.0	0.0	0.2	0.2

[a] Peak numbers correspond to Figure 1. [b] Relative retention time in minutes. [c] Molecular weights of methyl esters based on chemical ionization mass spectra. [d] Identification based on (+) comparison with authentic standards, (*) mass spectral interpretation and data base search, or (#) GC retention time. [e] Relative percentage fatty acid composition in Bt strains CR-371 (I), NRRL B-18243 (II), NRRL B-18244 (III), NRRL B-18245 (IV), NRRL B-18246 (V) and NRRL B-18247 (VI). [f] 2-(Methylthio)propanoic acid. [g] Butanedioic acid. [h] Benzylacetic acid.

Table 2. *F* Values[a] from the Pairwise Comparisons of the Six Strains of *B. thuringiensis*

strain	CR-371	NRRL B-18243	NRRL B-18244	NRRL B-18245	NRRL B-18246	NRRL B-18247
CR-371	1	1	0.91	0.94	0.93	0.88
NRRL B-18243		1	0.91	0.94	0.93	0.88
NRRL B-18244			1	0.97	0.91	0.85
NRRL B-18245				1	0.93	0.88
NRRL B-18246					1	0.88
NRRL B-18247						1

[a] *F* value is the proportion of fatty acids common to any pair of strains.

would cluster to some extent. However, in the absence of PCR-based methods (Brousseau et al., 1993) or DNA sequencing techniques which would give the definitive qualitative difference between strains, detection of the qualitative and quantitative differences in the total chromatographic pattern of bacterial fatty acids can be a useful tool for separation of strains.

The Massachusetts **Bt** isolate (CR-371) described herein originated from nematode-suppressive soils in Costa Rica (Zuckerman et al., 1989) and was extensively tested for efficacy in controlling root-knot nematode (*Meloidogyne incognita*) and lesion nematode (*Pratylenchus penetrans*) in greenhouse trials in Massachusetts. The results of these studies and those of 2-year large-scale field trials in Puerto Rico showing significant reduction in root-knot nematode

Table 3. Ratios of Certain Diagnostic Peaks in the Chromatogram of Hydrolyzable Fatty Acids from Six *B. thuringiensis* Strains

peak ratios[a]	CR-371	NRRL B-18243	NRRL B-18244	NRRL B-18245	NRRL B-18246	NRRL B-18247
P12/P9	>1	>1	>1	<1	<1	<1
P17/P12	<1	<1	<1	>1	<1	>1
P22/P17	>1	<1	>1	<1	<1	<1
P25/P12	>1	>1	<1	>1	>1	>1
P25/P19	>1	<1	<1	<1	<1	>1
P25/P22	>1	>1	<1	>1	>1	>1
P33/P12	<1	<1	<1	<1	<1	>1

[a] Identities of peaks are listed in Table 1. P, peak.

injury and significant yield increases were reported by Zuckerman et al. (1993).

The claim for nematicidal activity for the five Mycogen **Bt** strains, as described by the 1992 Mycogen patent (Edwards et al., 1992), is based on laboratory trials showing nematicidal activity on the free-living nematode *Caenorhabditis elegans*. Those knowledgeable in testing for anthelmintic activity utilize *Caenorhabditis* as a preliminary laboratory screen, indicating potential candidates for *in vivo* testing. In our Massachusetts laboratory, fewer than 1% of candidate anthelmintics that give positive results against *Caenorhabditis* reach the field-testing stage. In contrast, CR-371 is presented as different and having valid activity against plant nematodes on the basis of positive results in extensive greenhouse and field experiments.

We demonstrated significant differences among the six **Bt** strains by a combination of mere absence/presence and relative peak ratios with a nonparametric statistical test of significance comparable in purpose to the *t*-test. Parametric statistical techniques were deemed inappropriate for the separation of the six strains mainly because of an otherwise inappropriate assumption of normality in each data set.

Conclusion. This study has demonstrated the capability to detect the intraspecific variation in the bacterial species *B. thuringiensis* and to clearly differentiate strains on the basis of qualitative and quantitative differences in hydrolyzable whole cell fatty acid compositions. Moreover, our antinematode strain CR-371 is clearly differentiated from Mycogen's five nematicidal **Bt** strains. A nonparametric statistical analysis for paired comparisons was suitable for testing the significance of the differences between chromatographic profiles of the bacterial fatty acids and hence strain separation.

ACKNOWLEDGMENT

This work was supported by a grant to B. M. Z. from the Corporation for the Technological Development of Tropical Resources, Commonwealth of Puerto Rico.

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Received for review November 1, 1993. Accepted February 18, 1994.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts*, April 1, 1994.