APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1997, p. 1006–1010 Vol. 63, No. 3
0099-2240/97/$04.00+0

Comparison of the Cellular Fatty Acid Composition of a Bacterium Isolated from a Human and Alleged To Be *Bacillus sphaericus* with That of *Bacillus sphaericus* Isolated from a Mosquito Larvicide

J. P. SIEGEL,[1,2]* A. R. SMITH,[3] AND R. J. NOVAK[1,2]

Illinois Natural History Survey, Center for Economic Entomology, Champaign, Illinois 61820,[1] and Department of Natural Resources and Environmental Sciences[2] and Department of Veterinary Pathobiology, College of Veterinary Medicine,[3] University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Received 29 July 1996/Accepted 22 December 1996

The cellular fatty acid (CFA) composition of the cytoplasmic membrane of a bacillus isolated from a human lung and deposited in the National Collection of Type Cultures as *Bacillus sphaericus* NCTC 11025 was determined by gas-liquid chromatography. The CFA composition of *B. sphaericus* 2362, isolated from a microbial larvicide, and those of *B. sphaericus* reference strains obtained from public collections were also determined. Samples were grouped by hierarchical cluster analysis based on the unpaired-group method using arithmetic averages. Samples that linked at a Euclidean distance of ≤2.0 U were considered to belong to the same strain. NCTC 11025 and the type strain of *B. sphaericus*, ATCC 14577, were mixed; all other isolates were monotypic. The predominant fatty acid in NCTC 11025 was 12-methyltetradecanoic acid, while the predominant fatty acid in the remaining isolates was 13-methyltetradecanoic acid. NCTC 11025 linked to the other isolates at a Euclidean distance of 83.8 U, and we concluded that it belongs to a different species that we could not identify. We could distinguish among six DNA homology groups of *B. sphaericus* by using fatty acids. Within DNA homology group IIA, strain 2362 could be distinguished from other strains belonging to serotype H5a, 5b. We concluded that CFA analysis is a useful technique to determine if future human isolates identified as *B. sphaericus* in fact belong to other species of bacteria or whether the isolates originated from commercial products.

Bacillus sphaericus (Meyer and Neide) is a heterogeneous species based on DNA homology (14) containing strains that have commercial potential as mosquito larvicides. In 1980, *B. sphaericus* was divided into six groups (I, IIA, IIB, III, IV, and V) based on DNA homology; all larvicidal strains belong to DNA homology group IIA. This entomopathogenic group is also distinct from the other DNA homology groups based on numerical analysis of auxanograms, rRNA gene restriction patterns, random amplified polymorphic DNA fingerprinting, and cellular fatty acid (CFA) analysis (1, 5, 9, 14, 17, 18, 23, 28). DNA homology group IIA can be divided into subgroups by serotyping and phage typing (5). Larvicidal activity of the most toxic strains is due to the production of two proteins of 41.9 and 52.4 kDa which act as a binary toxin. Many of the most toxic strains of *B. sphaericus* are members of serotype H5a, 5b, and one strain belonging to this serotype, strain 2362 (isolated from blackflies in Nigeria), is fermented commercially. The sole *B. sphaericus* larvicide registered in the United States is Vectolex (strain 2362; Abbott Laboratories, North Chicago, Ill.).

Concerns about the human safety of larvicides containing *B. sphaericus* have been raised by Dixon (6) and Drobniewski (7), based on several reports of fatal human infections (2, 3, 7, 10). However, the role played by *B. sphaericus* in these fatalities is questionable. First, in all cases, the DNA homology group(s) that these human isolates belonged to is unknown. Second, with the exception of NCTC 11025, recovered post mortem from a pseudotumor of a lung (10), these isolates were not deposited in any collection; consequently, their mammalian pathogenicity cannot be confirmed. The importance of this last point is underscored in a case reported by Allen and Wilkinson (2). Although bacilli identified as *B. sphaericus* were isolated from antemortem cultures of blood, spinal fluid, and urine, these isolates were not pathogenic when inoculated intravenously and intraperitoneally into young rabbits.

In contrast to the case reports mentioned above, the pathogenicity of several isolates belonging to DNA homology group IIA (serotypes H2a, 2b; H5a, 5b; and H25) has been tested by the World Health Organization, as well as by industry. Rats, euthymic and athymic mice, and rabbits were exposed by oral administration; inhalation; ocular irritation; and intraocular, subcutaneous, intraperitoneal, and intracerebral injection. Some of these studies also included the lung isolate NCTC 11025. None of the isolates tested, including NCTC 11025, were pathogenic (4, 20, 21). Despite these safety studies, it seems likely that with increased use of microbial insecticides and concomitant human exposure to these products, further concerns will be raised.

The purpose of this study was to answer two questions. First, what is the relationship between NCTC 11025 and *B. sphaericus* 2362? Second, can we create a unique profile or "fingerprint" for *B. sphaericus* 2362, so that it can be distinguished from other strains within serotype H5a, 5b, as well as other DNA homology groups? Here we report the utility of CFA analysis for answering these questions.

MATERIALS AND METHODS

Sources of commercial and collection larvicidal cultures of serotype H5a, 5b. Vectolex CG (Abbott Laboratories), formulated on corncob granules, was received in 1990 and 1995 (see Table 1, strain 2362, lots 29-087-K1 and 08-640-N8). Samples were taken from these two time periods to confirm that there was no

* Corresponding author. Mailing address: 172 Natural History Survey, 607 East Peabody Drive, Champaign, IL 61820. Phone: (217) 244-3427. Fax: (217) 333-2359. E-mail: jsiegel@mail.inhs.uiuc.edu.

change in Vectolex due to either inadvertent or purposeful selection for clones that grow best under industrial fermentation conditions. *B. sphaericus* was isolated from this larvicide by shaking the granules onto brain heart infusion (BHI) agar plates (Micro Diagnostics, Addison, Ill.) and then incubating the plates at 28°C for 24 h. The following cultures were obtained from the collection of the Pasteur Institute (Paris, France): SPH 88 (current International Potency Standard for strain 2362, lot BSP 247A), SO5 010 (reference strain for strain 2362), SO5 214 (isolated from a European larvicide, Spherix, strain 2362), SO5 001 (reference strain for serotype H5a, 5b, strain 1593), and SO5 002 (strain 1593-4). SPH 88 was received as a powder, and this powder was shaken onto BHI agar plates, which were incubated as described above. The other cultures from the Pasteur Institute were received as spore-impregnated filter strips. The filter strips were placed onto BHI agar plates and rehydrated with BHI broth (Micro Diagnostics). The plates were incubated as described above.

Sources of nonlarvicidal *B. sphaericus* cultures. The type strain for *B. sphaericus*, ATCC 14577, was received as a lyophilized pellet from the American Type Culture Collection (Rockville, Md.). The culture was revived by suspending the pellet in BHI broth at 28°C for 24 h, and then 0.1 ml was streaked onto BHI agar plates, which were incubated at 28°C for 24 h. The following DNA homology group reference strains (agar slants) were received from A. A. Yousten, Virginia Polytechnic Institute and State University, Blacksburg: ATCC 7055 (reference strain for DNA homology group IIB), NRS 592 (reference strain for DNA homology group III), NRS 400 (reference strain for DNA homology group IV), and NRS 1198 (reference strain for DNA homology group V). A loopful of material from each slant was transferred to BHI agar plates and incubated as described above.

Source of human isolate. Two cultures of NCTC 11025 (isolated from a human lung) were received as lyophilized pellets from the National Collection of Type Cultures (Central Public Health Laboratory, London, United Kingdom). The cultures were revived by suspending each lyophilized pellet in BHI broth and incubating the suspension at 25°C for 4 days. After this incubation period, 0.1 ml of the BHI broth was streaked either onto tryptic soy broth agar (TSA) plates or TSA plates supplemented with 5% sheep blood (TSAB plates; Remel, Lenexa, Kans.) that were then incubated at 28°C. NCTC 11025 was not streaked onto BHI agar plates because of the prolonged incubation in BHI broth.

Other *Bacillus* species used in this study. The type strain of *B. globisporus*, ATCC 23301, was received from the American Type Culture Collection as a lyophilized pellet. The culture was revived by suspending the pellet in BHI broth at 28°C for 24 h, and then 0.1 ml was streaked onto TSAB plates, which were incubated at 28°C.

CFA sample preparation and analysis of data. Single-colony isolates of *B. sphaericus* NCTC 11025 and *B. globisporus* were obtained by using a four-quadrant streak pattern and then transferring single colonies to either TSA or TSAB plates. These plates were incubated at 28°C for 24 or 48 h in accordance with the standard TSA protocol of MIDI Corporation (Newark, Del.). Initially, all of the *B. sphaericus* samples were incubated for 24 h on TSA plates, and colonies of NCTC 11025 derived from a single lyophilized pellet were incubated for 48 h because of slow growth. In a second experiment, NCTC 11025, derived from a second lyophilized pellet, and *B. globisporus* were incubated on TSAB plates for 48 h. Colonies obtained from this second pellet of NCTC 11025 were also incubated on TSA plates for 48 h to compare their fatty acid values to the profiles created from the first pellet.

Approximately 40 to 50 mg (wet weight) of cells in the early stationary phase was then harvested from each plate, and the fatty acids were extracted and methylated (9, 16, 19, 24). The fatty acid methyl esters were identified with a Hewlett-Packard (Avondale, Pa.) Microbial Identification System HP 5898A, consisting of a 5890A gas-liquid chromatograph equipped with a 5% phenylmethyl silicone capillary column, a flame ionization detector, a 7673 automatic sampler, a 7673A controller, a 3392A integrator, and a Hewlett-Packard 300 computer. The gas-liquid chromatograph was calibrated every 11th vial with a Hewlett-Packard calibration standard kit containing fatty acid methyl esters in 0.8 ml of hexane (saturated nC9:0 to nC20:0 plus 2 and 3 hydroxy). A reagent control was included with every run. The species determination for each plate was based on a software library (TSBA version 3.8) developed by MIDI Corporation that compared the fatty acid composition of the extracted plates to library values. A separate computer record was generated for each plate, and we refer to these records as entries (9, 22, 24, 27).

Profile creation. The phenetic relationship among isolated colonies of each strain was determined by using Euclidean distance. Euclidean distance is a multivariate measure of the distance between variables and is calculated by using the Pythagorean theorem (15). We did not standardize the values used in our calculations because they were all in the same scale, percentage. Dendrograms were generated by using the library generation software (version 1) developed by MIDI Corporation, as well as the cluster analysis platform in JMP (SAS Institute Inc., Cary, N.C.). The hierarchical clustering method employed was the unpaired-group method using arithmetic averages (UPGMA). We began our analysis by clustering entries from a single accession number or single lot of *B. sphaericus*. Entries that linked at a Euclidean distance of ≤2.0 U were considered to belong to the same strain, based on empirical data from MIDI Corporation, as well as our own experience (22, 24). Subsequently, these individual entries were grouped into a new file that we refer to as a profile. Each profile contains the average value for every CFA identified in at least two plates. The next stage of our analysis examined the relationship among profiles by using UPGMA analysis. Profiles that linked at a Euclidean distance of ≤2.0 U were considered to belong to the same strain, and profiles that linked at a Euclidean distance of >10.0 U were considered to belong to different species, based on empirical data collected by MIDI Corporation (22, 24). We then validated our profiles by examining histograms of the primary and second and third most common CFAs obtained from the individual colonies that comprised our profiles, as well as the coefficient of variation (CV) of the principal fatty acid. The CV is the quotient of the standard deviation divided by the mean. Profiles were considered valid if the CV of the principal fatty acid was ≤2% (22, 24).

Reproducibility. The reproducibility of our method was tested by analyzing the data for 200 separate injections of the calibrator mixture with the cluster option in JMP and determining the Euclidean distance at which all of the calibrator entries linked together. We also evaluated the reproducibility of this method by comparing 10 plates of NCTC 11025 obtained from the second lyophilized pellet (incubated at 28°C for 48 h on TSA) to our profiles for NCTC 11025 that were created from the first lyophilized pellet.

Comparison of profiles for NCTC 11025 and existing literature values. The CFA values for our NCTC 11025 profiles were compared to values reported by Kämpfer (11) for the genus *Bacillus*. A set of data containing both our values and those of Kämpfer was created. The phenetic relationships within this set of data between NCTC 11025 and other species of bacilli were calculated by using the cluster analysis program in JMP. The type strain of the species in this database closest to NCTC 11025 was then obtained from the American Type Culture Collection. Single colonies of this type strain were isolated and incubated, and their CFAs were extracted as described above. Entries for the colonies were grouped into profiles as described above. The Euclidean distance between NCTC 11025 and this new *Bacillus* species was calculated to determine if NCTC 11025 belongs to this other species.

Numbers of plates analyzed. Two hundred sixty-seven plates were used in this study to create profiles as follows: 59 plates of NCTC 11025, 13 plates of SO5 010, 13 plates of Vectolex 1995, 16 plates of Vectolex 1990, 19 plates of SO5 214, 29 plates of SPH 88, 9 plates of SO5 001, 14 plates of SO5 002, 15 plates of ATCC 14577, 15 plates of NRS 592, 16 plates of NRS 400, 14 plates of ATCC 7055, 16 plates of NRS 1198, and 29 plates of *B. globisporus*. Multiple samples were run on several dates to maximize heterogeneity.

Growth of NCTC 11025 for morphological description. NCTC 11025 was streaked on TSAB plates and incubated aerobically at 25°C for 9 days. Slides were made, Gram stained, and then examined with a compound microscope.

RESULTS

Descriptive statistics. NCTC 11025 was gram positive and had primarily straight rods, with occasional serpentine forms. Spores were spherical and terminal with bulging of the sporangia. Colonies were soft, up to 7 mm in diameter.

The identities of the fatty acids present in the profiles of *B. sphaericus* and NCTC 11025 incubated on TSA plates and their mean percent composition and CV are reported in Table 1. Fifteen fatty acids were used in this analysis. Four fatty acids (i15:0, a15:0, 16:1 ω7 alcohol, and i16:0) accounted for approximately 80% of the total fatty acid composition of the cytoplasmic membrane. One fatty acid (i15:1 at 5) appeared at trace levels, ≤0.32%, or was lacking in the plates analyzed. Another fatty acid could not be resolved and is either an i17:1 I or an a17:1 B fatty acid; this fatty acid was identified as summed feature 5 in our printout. The only isolate that could not be identified by the MIDI Corporation TSA database was NCTC 11025.

NCTC 11025 differed from all of the other *B. sphaericus* strains examined that were grown on TSA in both the identity of its primary fatty acid (a15:0 rather than i15:0) and its lack of two secondary fatty acids common to all of the other strains, i17:1 ω10c and i17:0. The percent composition of the 16:1 ω7 alcohol fatty acid in NCTC 11025 was also noticeably lower.

When NCTC 11025 was incubated on TSAB, an additional three fatty acids were present in the cytoplasmic membrane (Table 2). These fatty acids were 14:0, i17:1 ω5c, and 18:0 and together comprised 2.73% of the fatty acids identified. The fatty acid identified as summed feature 5 was missing.

Profile creation. NCTC 11025 and ATCC 14577 were polytypic and contained two strains each based on our analysis. The profile for the predominant strain in each isolate was designated A and the profile for the secondary strain was designated

TABLE 1. Mean percent composition and CV of CFAs for the profiles of *B. sphaericus* and NCTC 11025 incubated at 28°C on TSA plates

Strain	Mean % composition (CV) of:														
	i14:0	14:0	i15:1 at 5	i15:0	a15:0	15:0	i14:0 30H	16:1 ω7 alcohol	i16:0	16:1 ω11c	16:0	i17:1 ω10c	i17:0	a17:0	SF 5[a]
NCTC 11025-A	8.32 (7.0)			5.13 (7.0)	66.74 (1.0)	0.81 (14.0)		3.55 (10.0)	4.66 (6.0)	1.11 (9.0)	0.97 (17.0)			6.36 (8.0)	2.01 (35.0)
NCTC 11025-B	7.81 (3.0)			3.76 (5.0)	68.49 (1.0)	0.62 (16.0)		2.97 (5.0)	5.08 (3.0)	1.08 (13.0)	1.01 (13.0)			6.75 (3.0)	1.79 (7.0)
SO5 010	2.34 (4.0)	0.54 (12.0)		66.40 (1.0)	4.17 (7.0)	0.51 (71.0)		9.50 (1.0)	5.05 (3.0)	2.37 (7.0)	0.65 (34.0)	2.34 (3.0)	4.09 (4.0)	0.77 (10.0)	0.69 (14.0)
SPH 88	2.36 (5.0)	0.55 (6.0)		66.93 (1.0)	4.46 (5.0)	0.42 (57.0)	0.50 (9.0)	9.01 (5.0)	4.96 (5.0)	2.35 (5.0)	0.54 (22.0)	2.11 (7.0)	3.86 (5.0)	0.77 (9.0)	0.58 (21.0)
Vectolex 1990	2.30 (7.0)	0.56 (12.0)		65.62 (1.0)	4.41 (6.0)	0.49 (67.0)	0.35 (60.0)	9.48 (3.0)	5.39 (7.0)	2.47 (5.0)	0.62 (20.0)	2.35 (6.0)	4.05 (5.0)	0.84 (33.0)	0.75 (11.0)
Vectolex 1995	2.16 (4.0)	0.42 (3.0)		66.52 (1.0)	4.07 (4.0)	0.74 (10.0)	0.50 (33.0)	9.34 (2.0)	5.18 (5.0)	2.18 (4.0)	0.52 (10.0)	2.38 (4.0)	4.39 (4.0)	0.77 (7.0)	0.64 (25.0)
SO5 214	2.22 (7.0)	0.51 (7.0)	0.32 (5.0)	66.50 (1.0)	4.07 (4.0)	0.66 (17.0)	0.48 (4.0)	9.02 (3.0)	4.90 (4.0)	2.47 (5.0)	0.49 (10.0)	2.61 (6.0)	3.89 (5.0)	0.68 (11.0)	0.64 (25.0)
S05 001	2.26 (5.0)	0.56 (4.0)	0.28 (15.0)	66.96 (1.0)	5.58 (6.0)		0.73 (6.0)	8.26 (4.0)	4.20 (2.0)	2.46 (4.0)	0.52 (35.0)	2.19 (7.0)	3.36 (8.0)	0.96 (11.0)	0.72 (38.0)
S05 002	1.91 (4.0)	0.68 (4.0)		63.75 (2.0)	8.07 (5.0)		0.51 (8.0)	8.25 (2.0)	4.14 (5.0)	3.08 (4.0)	0.54 (14.0)	1.91 (4.0)	4.09 (7.0)	1.56 (12.0)	1.02 (5.0)
ATCC 14577-A	3.44 (6.0)			51.03 (1.0)	3.27 (4.0)			15.84 (5.0)	11.51 (5.0)	2.82 (6.0)	0.55 (35.0)	4.64 (7.0)	4.88 (4.0)	0.62 (31.0)	1.02 (6.0)
ATCC 14577-B	4.11 (6.0)	0.32 (4.0)	0.31 (0)	48.13 (1.0)	3.04 (6.0)			17.43 (4.0)	13.59 (3.0)	2.58 (3.0)	0.46 (1.0)	4.00 (4.0)	4.58 (2.0)	0.56 (6.0)	0.89 (9.0)
ATCC 7055	2.51 (5.0)	0.51 (7.0)		51.67 (1.0)	10.14 (4.0)	0.72 (16.0)	0.33 (10.0)	11.16 (2.0)	9.07 (2.0)	2.77 (5.0)	0.72 (5.0)	1.36 (4.0)	4.64 (3.0)	2.70 (7.0)	1.26 (4.0)
NRS 592	5.62 (2.0)	0.60 (4.0)		54.78 (0)	7.81 (5.0)	1.00 (9.0)		12.65 (1.0)	8.92 (3.0)	1.98 (2.0)	0.44 (6.0)	1.03 (3.0)	2.31 (3.0)	1.16 (6.0)	1.00 (4.0)
NRS 400	5.32 (3.0)	0.45 (5.0)		51.63 (1.0)	8.35 (2.0)	0.85 (13.0)	0.49 (4.0)	12.79 (1.0)	11.57 (3.0)	1.82 (4.0)	0.68 (8.0)	0.91 (3.0)	2.67 (3.0)	1.52 (4.0)	0.72 (3.0)
NRS 1198	2.08 (9.0)	0.31 (9.0)		38.72 (2.0)	6.28 (7.0)	0.82 (6.0)		20.36 (4.0)	17.14 (2.0)	2.73 (9.0)	0.61 (14.0)	1.79 (4.0)	4.56 (9.0)	2.18 (16.0)	2.27 (9.0)

[a] Summed feature (SF) 5 is either 17:1 iso I or 17:1 anteiso B.

TABLE 2. Mean percent composition and CV of CFAs for the profiles of NCTC 11025 and *B. globisporus* incubated at 28°C for 48 h on TSAB plates

Strain	Mean % composition (CV) of:																
	i14:0	14:0	Unk[a] 14.258	i15:0	a15:0	15:0	16:1 ω7 alcohol	16:0 N alcohol	i16:0	16:1 ω11c	16:0	i17:1 ω5c	i17:0	a17:0	18:0	19:0 methyl	SF 5[b]
NCTC 11025	5.64 (4.0)	0.35 (41.0)		4.79 (8.0)	67.22 (1.0)	0.54 (28.0)	2.42 (6.0)		3.57 (5.0)	2.54 (9.0)	2.72 (7.0)	1.12 (81.0)		6.63 (4.0)	1.26 (12.0)		
ATCC 23301-A	3.00 (5.0)	0.71 (12.0)	0.39 (66.0)	4.65 (4.0)	53.90 (1.0)	0.56 (12.0)	2.32 (9.0)	0.46 (48.0)	2.56 (9.0)	6.42 (8.0)	5.92 (11.0)	3.90 (10.0)	0.77 (9.0)	9.46 (6.0)	3.9 (16.0)	0.55 (35.0)	
ATCC 23301-B	3.04 (2.0)	0.98 (0.0)		4.85 (7.0)	53.18 (2.0)	0.60 (4.0)	1.90 (3.0)	1.90 (3.0)	2.54 (4.0)	7.03 (4.0)	7.16 (3.0)		0.84 (2.0)	9.04 (1.0)	4.28 (9.0)		3.15 (9.0)
ATCC 23301-C	3.21 (10.0)	0.97 (12.0)		4.13 (6.0)	51.89 (1.0)	0.61 (13.0)	1.58 (2.0)	0.63 (3.0)	2.21 (5.0)	7.30 (1.0)	8.36 (5.0)	2.57 (7.0)	0.85 (9.0)	8.11 (1.0)	6.28 (12.0)	0.69 (17.0)	
ATCC 23301-D	2.81 (3.0)	0.93 (10.0)		4.37 (7.0)	50.97 (0.0)	0.72 (32.0)	1.65 (2.0)		2.35 (1.0)	6.48 (17.0)	7.57 (5.0)	2.74 (10.0)	0.79 (0.65)	8.99 (11.0)	4.53 (7.0)	0.78 (3.0)	
ATCC 23301-E	3.46 (5.0)	0.74 (24.0)		5.03 (11.0)	56.27 (0.0)		2.76 (1.0)		2.62 (11.0)	5.64 (8.0)	4.92 (13.0)	4.28 (9.0)	0.69 (17.0)	9.10 (2.0)	3.21 (0.0)	0.39 (0.0)	

[a] Unnamed fatty acid with equivalent chain length of 14.258.
[b] Summed feature (SF) 5 is either 17:1 iso I or 17:1 anteiso B.

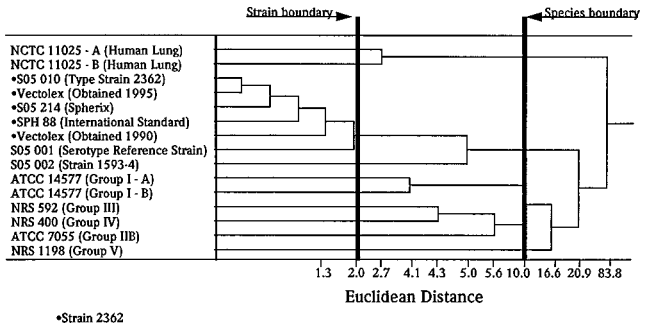

FIG. 1. UPGMA cluster dendrogram depicting the relationship between profiles of *B. sphaericus* derived from public culture collections, laboratory collections, and a commercially produced mosquito larvicide, Vectolex. This dendrogram was created with the cluster platform of JMP (SAS Institute).

B. NCTC 11025-A accounted for 72.7% (34 of 43) of the plates analyzed, and ATCC 14577-A accounted for 86.7% (13 of 15) of the plates analyzed. Both strains in NCTC 11025 formed a cluster at a Euclidean distance of 2.7 U, and both strains in ATCC 14577 formed a cluster at a Euclidean distance of 4.1 U.

A dendrogram depicting the relationship between all of our profiles derived from TSA is shown in Fig. 1. The profiles fell into two groups. The major group contained all isolates except NCTC 11025. These profiles linked together inside a Euclidean distance of 20.9 U. The minor group consisted of the two profiles of NCTC 11025. The NCTC 11025 profiles linked together at a Euclidean distance of 2.7 U and linked to the other profiles at a Euclidean distance of 83.8 U. These distances indicate that NCTC 11025 is not *B. sphaericus.*

Comparison between NCTC 11025 and *B. globisporus*. *B. globisporus* was polytypic when incubated on TSAB for 48 h. The profile for the predominant strain was designated A, and the profiles for the secondary strains were designated B, C, D, and E. *B. globisporus* A accounted for 72.4% (21 of 29) of the plates, and *B. globisporus* B through E accounted for 2 plates each. NCTC 11025 was monotypic under these conditions (16 plates) (Table 2). When the *B. globisporus* profiles were compared to that of NCTC 11025, the *B. globisporus* profiles linked together at a Euclidean distance of 5.9 U and linked to NCTC 11025 at a distance of 16.3 U. These distances indicate that although NCTC 11025 is not *B. globisporus*, it is more similar to *B. globisporus* than to *B. sphaericus.*

Reproducibility of the method. Of the 200 calibrator entries, 180 linked together inside a Euclidean distance of 0.1 U. All of the entries linked together inside a Euclidean distance of 0.5 U. The 10 plates obtained from the second lyophilized pellet of NCTC 11025 were identified by our NCTC 11025-A profile with similarity values of >0.8. These samples were then incorporated into the NCTC 11025-A profile.

DISCUSSION

Kaneda (12, 13) was the first to state that it is possible to create fingerprints of the genus *Bacillus* by using CFA analysis. The genus could be divided into major and minor groups based on the ratio of two fatty acids, i15:0 and a15:0. The a15:0 fatty acid, 12-methyltetradecanoic acid, is the predominant fatty acid in the major group, and the i15:0 fatty acid, 13-methyltetradecanoic acid, is the predominant fatty acid in the minor group. Other researchers have confirmed this finding (8, 9, 19, 22, 24, 25). More recently, Kämpfer (11) studied the fatty acid patterns of 313 *Bacillus* strains and Frachon et al. (9) examined 114 strains of *B. sphaericus* by using this technique. These researchers reported that in every strain analyzed, the i15:0 fatty acid predominated in *B. sphaericus*. The samples we analyzed (Table 1) are in agreement with these findings, except for NCTC 11025. The predominance of the a15:0 fatty acid in NCTC 11025 indicates that it was incorrectly identified as *B. sphaericus* by Isaacson et al. (10).

We evaluated the possibility that the predominance of the a15:0 fatty acid in NCTC 11025 was an artifact due to a longer incubation time by analyzing additional plates of *B. sphaericus* 2362 (obtained from Vectolex) incubated on TSA plates for 192 h at 28°C. There was no shift toward production of the a15:0 fatty acid; in fact, there was a 5% increase in the percentage of the i15:0 fatty. We conclude that the predominance of the a15:0 fatty acid in NCTC 11025 was not due to prolonged incubation. We also considered the possibility that the distant linkage of NCTC 11025 to the other profiles was dependent on the clustering algorithm used. We also calculated the Euclidean distance between profiles by using Centroid, Complete Linkage, Single Linkage, and Ward's clustering algorithms. In every instance, the NCTC 11025 profiles linked to the other profiles at Euclidean distances of >72 U.

When we incorporated the values of Kämpfer (11) into our set of data, *B. globisporus* linked to NCTC 11025 at a Euclidean distance of 10.3 U. Since this linkage occurred at the species boundary empirically derived by MIDI Corporation, we decided to examine the relationship between NCTC 11025 and *B. globisporus* by using our own profiles. This was necessary to standardize the growth medium and incubation time, both of which affect the identity and percentage composition of the fatty acids in the cytoplasmic membrane (13, 15, 21, 23). In addition, we were interested in determining if NCTC 11025 would still differ from *B. sphaericus* when grown on a medium commonly used in clinical diagnostic laboratories. NCTC 11025 was distinct from *B. globisporus* and remained unidentified; the a15:0 fatty acid still predominated.

We do not believe that our findings of heterogeneity in NCTC 11025, ATCC 14574, and ATCC 23301 indicate a problem with the reproducibility of CFA analysis. Our data for 200 separate injections of the calibrator (17 fatty acids) linked inside our empirically derived strain boundary of 2.0 Euclidean distance units. Our CV for the fatty acids in the calibrator ranged from 0.2 to 1.8. We conclude that the observed heterogeneity was not an artifact. Perhaps the samples selected for culture were mixed from the beginning and several clonal populations were then maintained in culture. Alternatively, the isolates selected for ATCC 14574, ATCC 23301, and NCTC 11025 may have initially been single colonies that subsequently mutated. It is difficult to reconcile our findings for these three samples with the standard definition of a strain, i.e., "the descendants of a single isolation in pure culture and usually made up of a succession of cultures ultimately derived from an initial single colony" (26). Our data suggest that caution is warranted when analyzing material obtained from culture collections, but these findings do not affect our conclusion that NCTC 11025 is not *B. sphaericus*.

There has been an evolution in the use of CFA analysis, as technological advances have enabled large sets of data to be analyzed. Kaneda (12, 13) concentrated on the i15:0/a15:0 fatty acid ratio in the genus *Bacillus* and ignored all of the other fatty acids. Esnard et al. (8) utilized all of the fatty acids identified but did not use the statistical technique of cluster analysis. Kämpfer (11) utilized all of the fatty acids identified and employed UPGMA clustering techniques but created profiles with unacceptable variation (as high as 49%) for the predominant fatty acid. Frachon et al. (9) and Schenkel et al. (18) used methods that approximate ours but did not specify

whether their fatty acid extracts came from single-colony isolates and also did not report the CV for the primary fatty acid. We believe that our approach to profile creation is unique and that the technique of CFA analysis has the greatest power of discrimination when a single colony is streaked per plate and the data are clustered by using hierarchical UPGMA analysis.

In summary, NCTC 11025 was misidentified as *B. sphaericus*. If the concerns raised by Dixon (6) and Drobniewski (7) are based on the case report of Isaacson et al. (10), then they are unwarranted. We note that NCTC 11025 underwent mammalian safety testing and was not pathogenic (20), yet neither author cited the relevant report. Assessment of the significance of the other case reports of *B. sphaericus* infections in humans is complicated by our inability to obtain these clinical isolates.

CFA analysis was a useful tool that successfully distinguished among *B. sphaericus* DNA homology groups, as well as strains within a serotype. If *B. sphaericus* is isolated from humans in the future, it is possible, by using CFA characterization, to compare the new isolate(s) to our existing profiles for strain 2362. We believe that this technique is also applicable to other species of bacteria that are used as insecticides or fungicides.

ACKNOWLEDGMENTS

We are grateful to I. Thiery of the Pasteur Institute, A. A. Yousten of Virginia Polytechnic Institute and State University, and B. Melin of Abbott Laboratories for providing cultures and larvicide. We thank M. H. Vodkin, J. V. Maddox, and four anonymous reviewers for reading the manuscript.

This research was supported in part by the Illinois Waste Tire Act, grant SENR TM-2, and in part by funds received from Abbott Laboratories.

REFERENCES

1. **Alexander, B., and F. G. Priest.** 1990. Numerical classification and identification of *Bacillus sphaericus* including some strains pathogenic for mosquito larvae. J. Gen. Microbiol. **136:**367–376.
2. **Allen, B. T., and H. A. Wilkinson III.** 1968. A case of meningitis and generalized Shwartzman reaction caused by *Bacillus sphaericus*. Hopkins Med. J. **125:**8–13.
3. **Banerjee, C., C. I. Bustamente, R. Wharton, E. Talley, and J. C. Wade.** 1988. *Bacillus* infections in patients with cancer. Arch. Intern. Med. **148:**1769–1774.
4. **de Barjac, H., V. Cosmao Dumanoir, S. Hamon, and I. Thiery.** 1987. Safety tests of mice with *Bacillus sphaericus* serotype H-5a, 5b, strain 2362. WHO mimeograph document WHO/VBC/87.948. World Health Organization, Geneva, Switzerland.
5. **de Barjac, H.** 1990. Classification of *Bacillus sphaericus* strains and comparative toxicity to mosquito larvae, p. 228–236. *In* H. de Barjac and D. J. Sutherland (ed.), Bacterial control of mosquitoes & blackflies: biochemistry, genetics & applications of *Bacillus thuringiensis israelensis* and *Bacillus sphaericus*. Rutgers University Press, New Brunswick, N.J.
6. **Dixon, B.** 1994. Keeping an eye on *B. thuringiensis*. Bio/Technology **12:**435.
7. **Drobniewski, F. A.** 1994. The safety of *Bacillus* species as insect vector control agents. J. Appl. Bacteriol. **76:**101–109.
8. **Esnard, J., T. L. Potter, and B. M. Zuckerman.** 1994. Differentiation of six strains of *Bacillus thuringiensis* by hydrolyzable fatty acid composition. J. Agric. Food Chem. **42:**1251–1255.
9. **Frachon, E., S. Hamon, L. Nicolas, and H. de Barjac.** 1991. Cellular fatty acid analysis as a potential tool for predicting mosquitocidal activity of *Bacillus sphaericus* strains. Appl. Environ. Microbiol. **57:**3394–3398.
10. **Isaacson, P., P. H. Jacobs, A. M. R. Mackenzie, and A. W. Mathews.** 1976. Pseudotumour of the lung caused by infection with *Bacillus sphaericus*. J. Clin. Pathol. **29:**806–811.
11. **Kämpfer, P.** 1994. Limits and possibilities of total fatty acid analysis for classification and identification of *Bacillus* species. Syst. Appl. Microbiol. **17:**86–98.
12. **Kaneda, T.** 1968. Fatty acids in the genus *Bacillus*. J. Bacteriol. **95:**2210–2216.
13. **Kaneda, T.** 1977. Fatty acids of the genus *Bacillus*: an example of branched-chain preference. Bacteriol. Rev. **41:**391–418.
14. **Krych, V. K., J. L. Johnson, and A. A. Yousten.** 1980. Deoxyribonucleic acid homologies among strains of *Bacillus sphaericus*. Int. J. Syst. Bacteriol. **30:**476–484.
15. **Manly, B. F. J.** 1986. Multivariate statistical methods: a primer. Chapman & Hall, London, England.
16. **Miller, L., and T. Berger.** 1985. Gas chromatography. Application note 228-41. Hewlett-Packard Co., Avondale, Pa.
17. **Muro, M. A., W. J. Mitchell, and F. G. Priest.** 1992. Differentiation of mosquito-pathogenic strains of *Bacillus sphaericus* from nontoxic varieties by ribosomal RNA gene restriction patterns. J. Gen. Microbiol. **138:**1159–1166.
18. **Priest, F. G., M. Goodfellow, and C. Todd.** 1988. A numerical classification of the genus *Bacillus*. J. Gen. Microbiol. **134:**1847–1882.
19. **Schenkel, R. G. M., L. Nicolas, E. Frachon, and S. Hamon.** 1992. Characterization and toxicity of mosquito larvae of four *Bacillus sphaericus* strains isolated from Brazilian soils. J. Invertebr. Pathol. **60:**10–14.
20. **Siegel, J. P., and J. A. Shadduck.** 1990. Mammalian safety of *Bacillus sphaericus*, p. 321–331. *In* H. de Barjac and D. J. Sutherland (ed.), Bacterial control of mosquitoes & blackflies: Biochemistry, genetics & applications of *Bacillus thuringiensis israelensis* and *Bacillus sphaericus*. Rutgers University Press, New Brunswick, N.J.
21. **Siegel, J. P., and J. A. Shadduck.** 1990. Clearance of *Bacillus sphaericus* and *Bacillus thuringiensis* spp. *israelensis* from mammals. J. Econ. Entomol. **83:**347–355.
22. **Siegel, J. P., A. R. Smith, J. V. Maddox, and R. J. Novak.** 1993. Use of cellular fatty acid analysis to characterize commercial brands of *Bacillus thuringiensis* var. *israelensis*. J. Am. Mosq. Control Assoc. **9:**330–334.
23. **Singer, S.** 1990. Introduction to the study of *Bacillus sphaericus* as a mosquito control agent, p. 221–227. *In* H. de Barjac and D. J. Sutherland (ed.), Bacterial control of mosquitoes & blackflies: biochemistry, genetics & applications of *Bacillus thuringiensis israelensis* and *Bacillus sphaericus*. Rutgers University Press, New Brunswick, N.J.
24. **Smith, A. R., and J. P. Siegel.** 1996. Cellular fatty acid analysis for the classification and identification of bacteria, p. 179–222. *In* W. P. Olson (ed.), Automated microbial identification and quantitation. Interpharm Press, Inc., Buffalo Grove, Ill.
25. **Stahly, D. P., and M. G. Klein.** 1992. Problems with *in vitro* production of spores of *Bacillus popillae* for use in biological control of the Japanese beetle. J. Invertebr. Pathol. **60:**283–291.
26. **Staley, J. T., and N. R. Krieg.** 1984. Classification of procaryotic organisms: an overview, p. 1–7. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore, Md.
27. **Welch, D. F.** 1991. Applications of cellular fatty acid analysis. Clin. Microbiol. Rev. **4:**422–438.
28. **Woodburn, M. A., A. A. Yousten, and K. H. Hilu.** 1995. Random amplified polymorphic DNA fingerprinting of mosquito-pathogenic and nonpathogenic strains of *Bacillus sphaericus*. Int. J. Syst. Bacteriol. **45:**212–217.