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RECOVERY OF COMMERCIALLY PRODUCED *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* AND *BACILLUS SPHAERICUS* FROM TIRES AND PREVALENCE OF BACILLI IN ARTIFICIAL AND NATURAL CONTAINERS[1]

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ABSTRACT. We conducted surveys to identify the species of spore-forming bacteria present in natural and artificial containers. Most of our samples came from Illinois. Identification was based on the cellular fatty acid composition of the bacterial cell wall. In addition, we utilized a custom database for commercially produced strains of *Bacillus thuringiensis* var. *israelensis* (*Bti*) and *B. sphaericus,* to differentiate between larvicidal isolates with commercial or native origin. Native *Bti* was present at low levels in almost all habitats but was not recovered from bromeliads and metal containers. In temporary woodland pools, 27.9% of the colonies recovered were native *Bti.* We did not recover larvicidal *B. sphaericus* in untreated habitats. VectoBac® and VectoLex® were applied to tires containing water and the tires were sampled 3 months and 9 months after treatment. Isolates of *Bti* and *B. sphaericus* with commercial origin were recovered as long as 9 months after application. We noticed numerous cadavers of *Aedes triseriatus* in several tires 9 months after treatment with VectoBac. We could not determine if this mortality resulted from recycling of *Bti* in these tires or whether insecticidal crystal proteins from the original treatment were resuspended. *Bacillus thuringiensis* var. *israelensis* isolates with commercial ancestry were recovered from untreated tires 9 months after application. Isolates of larvicidal *B. sphaericus* that differed from the bacteria in VectoLex were also recovered from untreated tires.

KEY WORDS Fatty acid analysis, *Bacillus thuringiensis* var. *israelensis, Bacillus sphaericus,* persistence, container

INTRODUCTION

Numerous surveys have been conducted worldwide for novel isolates of the entomopathogen *Bacillus thuringiensis* Berliner. This bacterium is primarily considered a soil microorganism (Ohba and Aizawa 1986, Martin and Travers 1989), although Smith and Couche (1991) presented evidence that it can be isolated from the phylloplane (leaf surfaces) of conifers and deciduous trees. Currently, 82 serovars of *B. thuringiensis* are known and the insect host spectrum of *B. thuringiensis* includes coleopteran, dipteran, and lepidopteran pest species (Lecadet et al. 1999). The serovar *B. thuringiensis israelensis* de Barjac (*Bti*), initially isolated in 1976 from cadavers of *Culex pipiens* L., is used in vector control (Margalit 1990). This serovar produces insecticidal crystal proteins (ICPs) that, when ingested and activated in the gut, are toxic to larvae belonging to the genera *Aedes, Anopheles, Culex,* and *Psorophora.* These proteins are also toxic to black fly larvae belonging to the genera *Austrosimulium* and *Simulium* (Molloy 1990, Mulla 1990). Subsequent surveys have demonstrated that *Bti* is not always associated with mosquitoes because it has been recovered from leaf surfaces (2% of 81 isolates [Smith and Couche 1991] and 6% of 96 isolates [Damgaard et al. 1997]) and residue samples from an animal feed mill (5% of 477 *B. thuringiensis* isolates [Meadows et al. 1992]).

Bacillus sphaericus Meyer and Neide is the other member of the genus *Bacillus* used in vector control. *Bacillus sphaericus* is a heterogeneous species and only isolates belonging to DNA homology group IIA are toxic to mosquito larvae (Krych et al. 1980, Rippere et al. 1997). The 1st strain with mosquito activity (K) was isolated from moribund *Culiseta incidens* Thomson, and strain 2362 (used in VectoLex®, Valent BioSciences Corp., Libertyville, IL) was isolated from a nonmosquito source (*Simulium* adults collected in Nigeria). Other mosquito-active strains of *B. sphaericus* have been isolated from Lepidoptera and Orthoptera (Singer 1990). As of 1994, the collection of the Pasteur Institute contained 9 serotypes of mosquito-active *B. sphaericus* (Anonymous 1994). In contrast to *B. thuringiensis,* the overwhelming majority of these isolates came from insects, although mosquitocidal *B. sphaericus* has been isolated from soil and water (Schenkel et al. 1992). The only commercial *B. sphaericus* larvicide registered in the United States is VectoLex.

One obstacle to surveying larval habitat for bacteria is rapidly identifying the species recovered. Automated microbial identification systems are now available and enable rapid identification (Sackin and Jones 1993). One system, based on gas–liquid chromatography and marketed by MIDI, Inc. (Newark, DE), utilizes cellular fatty acid (CFA) analysis to identify species, subspecies, and strains of bacteria. The MIDI database contains informa-

[1] Use of a commercial product does not constitute an endorsement by the authors.

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tion on 500 species of bacteria. Numerous genera of aerobic and anaerobic bacteria have been analyzed using CFA analysis and many of these studies utilized the MIDI system (Welch 1991, Wauthoz et al. 1995, Smith and Siegel 1996). The genus *Bacillus* has been extensively characterized using CFA analysis, with particular emphasis on entomopathogenic species (Kaneda 1968; Frachon et al. 1991; Schenkel et al. 1992; Stahly and Klein 1992; Siegel et al. 1993, 1995, 1997, 2000; Esnard et al. 1994; Kämpfer 1994). The MIDI system also enables users to create custom databases and the authors have made entries for commercially produced *B. thuringiensis* var. *kurstaki, Bti,* and *B. sphaericus* (Siegel et al. 1993, 1995, 1997, 2000).

This study had 2 objectives. Our 1st objective was to determine the prevalence of *Bti* and larvicidal *B. sphaericus,* as well as other species belonging to the genus *Bacillus,* in permanent pools of water and both artificial and natural containers, using CFA analysis and the MIDI system. Our 2nd objective was to apply the larvicides VectoBac® (Valent BioSciences) and VectoLex to tires and to determine, by CFA analysis and the use of a custom database, the persistence of *Bti* and *B. sphaericus* originating from these formulations, as well as the ability of these bacteria to spread to untreated tires after application.

MATERIALS AND METHODS

Sample collection and preparation: Water samples (5–10 ml) were collected from 6 bromeliads, 6 metal containers, 2 ponds, 2 roadside ditches, 2 salt marshes, 10 tree holes, 3 temporary woodland pools, and 30 waste tires using sterile disposable 5-ml pipettes. The bromeliads sampled were on the grounds of the Florida Medical Entomology Laboratory (FMEL), Vero Beach, FL, and the drainage ditches sampled were just outside the grounds of FMEL. The salt marsh samples came from the Anahuac National Wildlife Refuge and J. D. Murphree Wildlife Management Area in Texas. The tire samples came from Kankakee County, IL, and the metal containers, ponds, and tree holes were sampled in central and southern Illinois. The woodland pools were located in Busey Woods (Urbana, IL) and were not treated with larvicide. Sampling occurred from late spring through midsummer. All samples were placed in marked sterile 15-ml centrifuge tubes. When sediment was present in the container, the sediment was stirred so that it was included in the sample. Soil and leaf litter (3 samples) was scooped using an autoclaved metal spatula. These samples were placed in sterile 15-ml centrifuge tubes containing 10 ml of sterile distilled water. All samples were pasteurized in a water bath at 65°C for 30 min in order to kill non–spore-forming bacteria. After pasteurization, the centrifuge tubes were agitated and 0.3 ml of the pasteurized sample was streaked onto brain–heart infusion (BHI) agar plates (Micro Diagnostics, Addison, IL). Streaking and colony isolation were performed in a class II biological safety cabinet (The Baker Co., Sanford, ME). The plates were then incubated at 28°C for 48 h and the visible colonies were transferred to fresh BHI plates, 1 colony per plate, and incubated for 24 h. Samples from these BHI plates were then transferred to tryptic soy broth agar (TSA) plates (Remel, Lenexa, KS), 1 colony per plate, and these plates were incubated for 24 h at 28°C in accordance with the standard TSA protocol of MIDI (Miller and Berger 1985). All of the samples were pooled by habitat type in our analysis.

Sample identification: Approximately 40–50 mg (wet weight) of cells in the early stationary phase were harvested from each TSA plate and the fatty acids were extracted and methylated (Miller and Berger 1985). The resulting 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 MIDI calibration standard kit containing fatty acid methyl esters in 0.8 ml of hexane (saturated C9:0 to nC20:0 plus 2 and 3 hydroxy). A reagent control was included with each run. A separate computer record was generated for each sample and this record included the fatty acid composition of the sample and the species identification expressed as a similarity index (similarity values were given for the standard MIDI database and our custom database and were calculated by the database software). According to the criteria of MIDI, a sample is considered identified by the database when its similarity index is ≥ 0.5. In our study isolates with an index below this threshold were classified as unidentified.

As noted in the introduction, 82 serovars of *B. thuringiensis* currently are known and we do not have custom database entries for all serovars. Taxonomically, *B. thuringiensis* is considered part of the *B. cereus* group and can only be distinguished from *B. cereus* by the presence of ICPs (Sneath 1986). The MIDI database has entries for both *B. cereus* and *B. thuringiensis,* and our custom database includes entries for *B. thuringiensis* serovars *aizawai, kurstaki, and morrisoni,* and the type strain of *B. cereus.* However, in this study we did not examine each isolate identified as *B. thuringiensis* or *B. cereus* for the presence of ICPs. Therefore, except for serovar *israelensis,* isolates belonging to all serovars of *B. thuringiensis,* regardless of our database identification, were grouped with the isolates identified as *B. cereus.* We also grouped isolates of *B. sphaericus* that did not belong to DNA homology group IIA.

Sources of commercially produced Bti *and* B.

sphaericus: VectoBac and VectoLex formulated on corncob granules (8–10 mesh and 10–14 mesh, respectively) were received from Abbott Laboratories, North Chicago, IL (VectoBac and VectoLex are now marketed by Valent BioSciences, but are fermented by Abbott Laboratories). The sources of other commercially produced *B. thuringiensis, Bti,* and larvicidal *B. sphaericus* used to create our custom database and the methodology used to validate entries are described by Siegel et al. (1995, 1997, 2000). In this study, isolates of *Bti* and larvicidal *B. sphaericus* that were indistinguishable from the strains in our commercial database are classified as having commercial ancestry, because we cannot distinguish between isolates that were produced commercially and isolates that were descended from commercially produced bacteria. Isolates that differed from our commercial database are classified as native.

Validation of the MIDI database identification of nonentomopathogenic bacilli: Water and soil samples collected throughout Illinois (1991–93) were prepared as described above and the bacilli isolated were analyzed. The type strains of the most commonly identified species belonging to the genus *Bacillus* (based on the MIDI database) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Samples of *B. amyloliquefasciens* (ATCC 23350), *B. cereus* (ATCC 14579), *B. megaterium* (ATCC 14581), *B. mycoides* (ATCC 6462), and *B. pumilus* (ATCC 7061) were then grown, extracted, and analyzed using CFA analysis and the MIDI database to confirm that the database values were correct.

Bioassay of isolates identified as Bti *or larvicidal* B. sphaericus *from tires: Culex restuans* Theobald larvae were exposed to samples identified as mosquitocidal bacilli as follows. Second- and 3rd-stage larvae were placed in wax cups (20 larvae per cup) containing 100 ml of water and a slurry of ground TetraMin® fish food (Tetra, Morris Plains, NJ). A single loopful (approximately 40–50 mg wet weight) of the isolate was added to the water by immersing the loop and then agitating it. The wax cup was then gently swirled and covered with a disposable petri dish cover. Cups were then covered with a paper towel (to eliminate aggregation in a single shaded area) and incubated at 28°C. Mortality was evaluated at 1, 6, 24, 48, and 168 h after exposure. When all of the larvae died within 48 h (no incidence occurred of no mortality at 48 h and 100% mortality at 168 h), the isolate was classified as larvicidal; in many instances larvae became moribund within the 1st hour. Mortality was confirmed by prodding the larvae and swirling the cup.

Determination of diagnostic test parameters of our database: The diagnostic test parameters sensitivity, specificity, accuracy, false-positive rate, and the predictive values of a positive and negative identification were calculated using a 2 × 2 table (Kelsey et al. 1986). Our baseline standard (referred to in diagnostic test literature as a gold standard) for the validity of our identification of an isolate as *Bti* or larvicidal *B. sphaericus* was the bioassay. If an isolate that was identified as *Bti* or larvicidal *B. sphaericus* did not kill larvae, the identification was counted as a false positive. In the case of *Bti,* a false positive was placed in the *B. cereus/B. thuringiensis* category, whereas in the case of *B. sphaericus,* a false positive was placed in the nonlarvicidal *B. sphaericus* category.

Forty-five isolates initially identified as *Bti* (5 of the tire isolates identified as *Bti* were not bioassayed) and 12 isolates initially identified as *B. cereus/B. thuringiensis* were bioassayed. The latter isolates were tested to determine if non-*Bti* isolates were larvicidal. Twenty-seven isolates identified as larvicidal *B. sphaericus* and 17 isolates identified as *B. sphaericus* belonging to other DNA homology groups were bioassayed. The latter *B. sphaericus* isolates were included to evaluate our determination, based on our database, that they were nonlarvicidal.

Tire treatment: The site for the waste tire persistence study was an experimental tire dump located in Trelease Woods, a mixed deciduous woodlot located in Champaign County, IL. The trees were predominantly oak and maple. In August 1995, 15 granules of VectoBac (255 mg) or 150 granules of VectoLex (375 mg) were added to 12 marked tires containing water (6 tires received VectoBac and 6 tires received VectoLex). One half of these tires were in direct sunlight whereas the rest were shaded. All tires contained leaves and other debris and had been in the dump for at least 6 years. The tires were sampled 3 months after application in November 1995, and 9 months after application in May 1996, by stirring the water with the disposable pipette and then collecting the sample. Additionally, in May 1996, 6 tires containing water adjacent to the treated tires and 6 tires containing water located >20 m were also sampled.

RESULTS

Identification of *Bacillus* species found in larval habitat

The species of spore-forming bacteria isolated from artificial and natural containers are summarized in Table 1. Colonies identified as native *Bti* were obtained from waste tires and tree holes, but they only comprised 9.5% and 2.7% of the bacilli recovered from these habitats, respectively. *Bacillus thuringiensis* var. *israelensis* was not recovered from metal containers or bromeliads. The predominant group recovered was *B. cereus/B. thuringiensis.* Nonlarvicidal *B. sphaericus* (DNA homology groups other than IIA) was recovered from waste tires, metal containers, and tree holes. Among the artificial and natural containers sampled, bromeli-

Table 1. Spore-forming bacilli recovered from artificial and natural containers (151 colonies analyzed). These containers were not treated with microbial insecticides.

Species of *Bacillus* based on cellular fatty acid analysis	Percentage of sample
Waste tires (n = 74 colonies)	
B. cereus/B. thuringiensis[1]	44.6
Unidentified	23.0
B. sphaericus (nonlarvicidal)	14.9
B. thuringiensis var. *israelensis* (native)	9.5
B. mycoides	4.1
B. macerans	1.4
B. megaterium	1.4
B. pumilus	1.4
Metal containers (n = 12 colonies)	
B. cereus/B. thuringiensis[1]	33.0
Unidentified	33.0
B. sphericus (nonlarvicidal)	25.0
B. mycoides	9.0
Deciduous tree holes (n = 37 colonies)	
B. cereus/B. thuringiensis[1]	73.0
Unidentified	10.8
B. sphaericus (nonlarvicidal)	5.4
B. laterosporus	5.4
B. thuringiensis var. *israelensis* (native)	2.7
B. mycoides	2.7
Bromeliads (n = 28 colonies)	
B. cereus/B. thuringiensis[1]	42.9
Unidentified	39.3
B. megaterium	14.3
B. mycoides	3.6

[1] This category pools all other serovars of *B. thuringiensis* with *B. cereus.*

Table 2. Spore-forming bacilli recovered from soil, leaf litter, and pools of water (140 colonies analyzed). These areas were not treated with microbial insecticides.

Species of *Bacillus* based on cellular fatty acid analysis	Percentage of sample
Soil and leaf litter (n = 18 colonies)	
B. cereus/B. thuringiensis[1]	61.1
B. megaterium	16.7
B. sphaericus (nonlarvicidal)	11.1
Unidentified	11.1
Woodland pools and roadside ditches (n = 43 colonies)	
B. cereus/B. thuringiensis[1]	44.2
B. thuringiensis var. *israelensis* (native)	27.9
Unidentified	14.0
B. sphaericus (nonlarvicidal)	9.3
B. amyloliquefasciens	4.7
Ponds and salt marsh (n = 79 colonies)	
Unidentified	49.4
B. cereus/B. thuringiensis[1]	29.1
B. brevis	11.4
B. laterosporus	2.6
B. megaterium	2.6
B. thuringiensis var. *israelensis* (native)	1.3
B. sphaericus (nonlarvicidal)	1.3
B. marinus	1.3
B. mycoides	1.3

[1] This category pools all other serovars of *B. thuringiensis* with *B. cereus.*

ads contained the greatest percentage of bacilli unidentified by our system (39.3%).

The species of spore-forming bacteria isolated from soil, leaf litter, woodland pools, roadside ditches, ponds, and salt marshes and their prevalence in these habitats are summarized in Table 2. The most common species recovered in these habitats was *B. cereus/B. thuringiensis* and this species predominated in soil and leaf litter. Native *Bti* was prevalent in shaded temporary woodland pools, where it comprised 27.9% of the colonies analyzed. The isolates from the woodland pools could be grouped into 4 strains using CFA analysis, all of which had larvicidal activity. *Bacillus thuringiensis* var. *israelensis* was not recovered from soil and leaf litter, roadside ditches, and salt marshes. Nonlarvicidal *B. sphaericus* was recovered from all habitats listed in Table 2. Ponds and salt marsh contained the greatest percentage of bacilli unidentified by our system (49.4%).

We can combine the other species of bacillus recovered into 3 groups, based on Sneath (1986). *Bacillus amyloliquefasciens, B. brevis, B. megaterium, B. mycoides,* and *B. pumilus* are species commonly isolated from soil and plant material. *Bacillus macerans* and *B. laterosporus* are species relatively scarce in soil or rarely isolated from soil and water and *B. laterosporus* has been isolated from dead honeybee larvae (*Apis mellifera* L.). Finally, *B. marinus* has been isolated from marine sediments. When we combine nonlarvicidal *B. sphaericus* with the other nonpathogenic bacilli listed above, 18.2% (53/291) of the isolates are listed by Sneath (1986) as commonly collected from soil and plant material. If the *B. cereus/B. thuringiensis* group is included, soil bacteria accounted for 62.5% of the colonies analyzed (182/291).

Recovery of *Bti* and larvicidal *B. sphaericus* from treated tires

Isolates with commercial ancestry were more common than native larvicidal bacteria in treated tires. Three months after application, isolates with commercial ancestry were the only larvicidal bacteria recovered (Table 3). Almost one half of the samples (47%) were unidentified. Nine months after application, the majority of the *Bti* collected (90%) had commercial ancestry. In contrast, 53% of the larvicidal *B. sphaericus* collected had commercial ancestry. Nonlarvicidal *B. sphaericus* was as prevalent as larvicidal *B. sphaericus.* In addition, 2 species of bacteria commonly encountered in soil (*B. megaterium* and *B. mycoides*) were collected.

In our samples from untreated adjacent tires, both native *Bti* and *Bti* isolates with commercial ancestry were recovered 9 months after treatment.

Table 3. Recovery of *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* from tires.

Species of *Bacillus* based on cellular fatty acid analysis	Percentage of sample
3 months after treatment (n = 34 colonies)	
Unidentified	47.1
B. thuringiensis var. *israelensis* (commercial ancestry)	20.6
Larvicidal *B. sphaericus* (commercial ancestry)	20.6
B. cereus/*B. thuringiensis*[1]	5.9
B. sphaericus (nonlarvicidal)	5.9
9 months after treatment (n = 142 colonies)	
Unidentified	26.4
B. cereus/*B. thuringiensis*[1]	24.3
B. thuringiensis var. *israelensis* (commercial ancestry)	18.6
B. sphaericus (nonlarvicidal)	11.4
Larvicidal *B. sphaericus* (commercial ancestry)	5.7
Larvicidal *B. sphaericus* (native)	5.0
B. megaterium	5.0
B. thuringiensis var. *israelensis* (native)	2.1
B. mycoides	1.4
Adjacent untreated tires, 9 months later (n = 48 colonies)	
B. cereus/*B. thuringiensis*[1]	25.0
Unidentified	22.9
B. megaterium	21.0
B. thuringiensis var. *israelensis* (commercial ancestry)	14.5
B. sphaericus (nonlarvicidal)	10.5
B. thuringiensis var. *israelensis* (native)	2.1
Untreated tires > 20 m away, 9 months later (n = 37 colonies)	
B. sphaericus (native)	29.7
B. cereus/*B. thuringiensis*[1]	29.7
Unidentified	22.9
B. thuringiensis var. *israelensis* (native)	16.2
B. mycoides	5.4

[1] This category pools all other serovars of *B. thuringiensis* with *B. cereus*.

Table 4. Bioassay results of the custom database identification of *Bacillus thuringiensis* var. *israelensis*.[1]

Database identification	Cups of dead larvae[2]	Cups of live larvae
Positive	24	21
Negative	0	12

[1] Diagnostic test parameters for the custom database identification are as follows. Sensitivity (24/24) = 100%. Specificity (12/33) = 36.4%. Accuracy (36/57) = 63.2%. Predictive value of a positive identification (24/45) = 53.3%. Predictive value of a negative identification (12/12) = 100%. False positive rate (21/33) = 63.6%.

[2] Baseline standard was toxicity to 2nd- to 3rd-instar *Culex restuans* within 48 h.

Table 5. Bioassay results of the custom database identification of larvicidal *Bacillus sphaericus*.[1]

Database identification	Cups of dead larvae[2]	Cups of live larvae
Positive	26	1
Negative	0	17

[1] Diagnostic test parameters for the custom database identification are as follows. Sensitivity (26/26) = 100%. Specificity (17/18) = 94.4%. Accuracy (43/44) = 97.7%. Predictive value of a positive identification (26/27) = 96.3%. Predictive value of a negative identification (17/17) = 100%. False positive rate (1/18) = 5.6%.

[2] Baseline standard was toxicity to 2nd to 3rd-instar *Culex restuans* within 48 h.

Bacillus megaterium was also recovered and was as prevalent as larvicidal bacilli. The samples from the untreated tires >20 m away contained both native *Bti* and larvicidal *B. sphaericus*. *Bacillus mycoides* was recovered from these tires as well.

Nine months after application, we noticed numerous cadavers of *Aedes triseriatus* (Say) floating on the surface of the water in several tires treated with VectoBac. Several cadavers and water samples were collected, and *Bti* was recovered. We could not determine if this mortality resulted from recycling of *Bti* in these tires or whether ICPs from the original treatment had been resuspended and ingested by the larvae.

Diagnostic test parameter values of the *Bti* and *B. sphaericus* database

The sensitivity (ability to detect an isolate with larvicidal activity) of our *Bti* database was 100%, because all isolates with larvicidal activity were identified as *Bti* (Table 4). Specificity (ability to correctly identify an isolate with no larvicidal activity) was 36.4%, because only 12 isolates were negative according to both the database and the bioassay. The false-positive rate was 63.6% and the overall accuracy of the database was 63.2%. The greatest confidence should be placed in a negative identification by our database, because its predictive value was 100%. The prevalence of *Bti* in all of the samples assayed, based on the bioassay, was 53.3%.

The sensitivity of our larvicidal *B. sphaericus* database was 100%, because all isolates with larvicidal activity were identified (Table 5). In contrast to the results of our *Bti* database, our *B. sphaericus* database had a high specificity (94.4%). Only a single isolate that was identified as larvicidal failed to kill larvae in our bioassay. The false-positive rate was 5.6% and the overall accuracy was 97.7%. The greatest confidence should be placed in a negative identification by our database, because its predictive value was 100%, but a positive identification also had a high predictive value (96.3%). The prevalence of larvicidal *B. sphaericus* in the samples assayed, based on the bioassay, was 59.1%.

DISCUSSION

We stress that the serovar name *israelensis* does not preclude recovery of native *Bti* from North America, and its prevalence in natural and artificial containers (2.7–9.5%) is consistent with the findings of Smith and Couche (1991) and Meadows et al. (1992). The frequency of recovery of *Bti* (27.9%) in Busey Woods was an exception. Recovery of *Bti* from this habitat is interesting, because as noted in the introduction, *Bti* was originally isolated from larvae and cadavers collected from a pool in Israel. Unlike the circumstances surrounding the discovery of *Bti,* no mosquitoes were present when the samples were collected. *Bacillus thuringiensis* var. *israelensis* is not always associated with mosquitoes (Meadows et al. 1992) and has even been isolated from a saturniid moth, *Hylesia metabus* (Vassal et al. 1993). *Bacillus thuringiensis* var. *israelensis* might be introduced into temporary pools when nondipteran insects or other arthropods or leaves fall into the water. Nondipteran reservoirs could also introduce native *Bti* into waste tires and tree holes.

Initially, given the low overall prevalence of *Bti* in the habitats sampled, *Bti* seems unlikely to be an important natural mortality factor for mosquitoes in the United States. However, if our data are in fact representative, when the results are stratified by habitat, *Bti* could play a role in regulating populations of *Aedes vexans* Meigen or other species that utilize temporary pools as larval habitat, provided that sporulation occurs and ICPs are produced. Furthermore, if nondipteran arthropod hosts are present for native *Bti*, fluctuations in the populations of the hosts may also influence the temporal distribution of this bacterium. At this point in time, researchers have identified 17 crystal proteins produced by various serovars of *B. thuringiensis* that have dipteran activity (the current listing of Cry proteins can be viewed at *http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html* and the host spectrum of these Cry proteins can be viewed at *http://www.glfc.forestry.ca/english/res/Bt_HomePage/netintro.html*). Because *B. cereus/B. thuringiensis* was recovered from all habitats including salt marsh, if some of these isolates produced mosquito-toxic ICPs, they may also be a source of larval mortality.

When we shift our focus from *Bti* to *B. sphaericus,* with the exception of Trelease Woods, which will be discussed in depth below, larvicidal *B. sphaericus* was not recovered. Nonlarvicidal *B. sphaericus* was most prevalent in waste tires (14.9%), but isolates were collected from all habitats except bromeliads. Our data on the distribution of nonlarvicidal *B. sphaericus* are similar to those reported by Dias et al. (1999). In their study, nonlarvicidal *B. sphaericus* comprised 2.1% of the bacilli isolated from Argentine soil. Sneath (1986) lists *B. sphaericus* as occurring in soil and freshwater and marine sediments, which concurs with our data for nonlarvicidal *B. sphaericus.*

Assessing the significance of our recovery of what we designated as native larvicidal *B. sphaericus* from Trelease Woods is difficult. Because these isolates were only recovered at 1 location, they may have descended from the bacteria in VectoLex that no longer matched our commercial database. They were not present in the samples taken from the treated tires 3 months after application nor were they recovered outside of Trelease Woods; therefore, one could argue that their ancestry was commercial. Their introduction into untreated adjacent tires could have occurred when the treated tires overflowed or when splashing occurred. However, overflow and splashing do not explain the recovery of these isolates >20 m from the application site. The distant isolates probably were deposited by wind, but wind could deposit either native larvicidal *B. sphaericus* or larvicidal *B. sphaericus* with a commercial ancestry. Native larvicidal *B. sphaericus* possibly was present in low numbers in all habitats sampled but was missed because of the small sample size used in our study. Ultimately, the origin of these isolates cannot be determined for 2 reasons. First, we did not sample all of the tires in the Trelease Woods tire dumps immediately before treatment, so we do not know if the larvicidal *B. sphaericus* isolates were present. Second, even if we had, covering the tires after sampling also would have been necessary to eliminate the deposition of native larvicidal *B. sphaericus* by the wind or falling leaves at a later date. This was not done because our focus was on persistence under field conditions. What seems irrefutable is that in samples obtained from numerous larval habitats, larvicidal *B. sphaericus* was rarely encountered.

The question raised above concerning the ancestry of the native larvicidal *B. sphaericus* can also be raised about the native *Bti* isolates collected in Trelease Woods, and is inextricably linked to the issue of recycling. If multiplication did not occur in tires, no opportunity existed for either species of larvicidal bacillus to produce variants that did not fit our CFA database. In that case, the larvicidal bacteria collected came from the formulated product. If we assume that the larvicidal isolates that did not match our commercial database were mutants, the predominance of isolates with commercial ancestry in treated tires (78% of the bacilli identified) 3 months after treatment may be evidence that recycling did not occur. This coincides with previous reports of the duration of control of commercial formulations in tires, 75 days and 63 days for *Bti* and *B. sphaericus,* respectively (Novak et al. 1985, Siegel and Novak 1999). Our observation of cadavers of *Ae. triseriatus* in tires 9 months after treatment with VectoBac is unusual, but this finding still does not prove that multiplication occurred. A fraction of the original treatment possibly was resuspended (we recovered both native *Bti* and com-

mercial ancestry *Bti* from these tires). This raises an additional issue, which is the stability of our CFA profiles. Even if recycling did occur, it would still not necessarily lead to a change in the CFA composition of the cell wall. In the laboratory, colonies of *Bti* were serially transferred to new BHI plates every other day for a 3-week period and their CFA values did not change. However, changes in these colonies may have occurred if the experiment was carried out longer. When we compared isolates from commercially produced *B. thuringiensis* var. *kurstaki* separated by 20 years, we found them to be indistinguishable (Siegel et al. 2000). We conducted similar studies on 2 samples of commercially produced larvicidal *B. sphaericus* separated by 5 years and found them to be indistinguishable as well (Siegel et al. 1997). Although our laboratory data indicate that our CFA entries are stable, we cannot rule out the possibility that variants may emerge under field conditions. Further study is necessary to address this issue.

We regard the recovery from untreated tires of isolates of *Bti* with commercial ancestry as one of the more intriguing findings in this study. It is easy to envision how heavy rains might transport either carrier corncob granules or water containing larvicide into adjacent tires when stacked treated tires overflow, but this does not necessarily mean that the ICPs are transported as well. We must distinguish between the recovery of bacteria from a tire and a tire having a larvicidal concentration of ICPs in the feeding zone. What is puzzling is that the mechanisms mentioned above should also transport larvicidal *B. sphaericus* with a commercial ancestry, yet we did not recover any. Our calculations may be distorted by the small sample size (48 colonies analyzed), but we are still left with the question of why only *Bti* with a commercial ancestry was recovered.

If our isolates of native larvicidal *B. sphaericus* actually descended from the strain used in VectoLex, then the data become even more intriguing. How did larvicidal *B. sphaericus* get transported >20 m from the treated tires? We noticed considerable deposition of soil into tires over time, and it is reasonable to assume that a portion of the contents of treated tires may become airborne in strong winds and be deposited in new sites, especially during a drought. From the perspective of control, it would be ideal if a single application of a microbial larvicide succeeded in not only killing the existing larvae in treated tires but also colonized untreated tires. However, in order to be effective after colonization, recycling would be necessary to ensure the concentration of larvicidal toxins necessary for control. Although analysis of our data does not provide clearcut evidence that effective colonization or recycling occurred, we did establish that strains of both *Bti* and *B. sphaericus* with commercial ancestry were recovered months after application. Furthermore, in several tires 9 months after application, larvicidal ICPs were present in a sufficient concentration to kill mosquito larvae.

Our database identification that isolates were *Bti* did not necessarily mean that these isolates were larvicidal. As reported in Table 4, 46.7% (21/45) of the isolates identified as *Bti* were not toxic to larvae, presumably because they lost the plasmid that contained the genes necessary for the production of ICPs (Sekar 1990). Although our estimate of the diagnostic test parameters for our CFA database is based on a small sample size and high frequency of *Bti* isolates analyzed, if this is a process that frequently happens in the field over time, this may be a mechanism that limits efficacy of *Bti* despite recycling. In contrast, only 1 isolate identified as larvicidal *B. sphaericus* did not kill mosquitoes (Table 5). The genes for the larvicidal toxins of *B. sphaericus* lie on the chromosome (Davidson and Yousten 1990) and are less likely to be lost than toxin genes that are located on a plasmid. This suggests that if recycling does occur, *B. sphaericus* may be effective for a longer period than *Bti,* but further studies are needed confirm this. With regard to using CFA analysis to identify native *Bti,* analysis of our data on the predictive value of our database suggests that an additional test such as bioassay or confirmation that ICPs are present is necessary to document toxicity.

Overall, waste tires seem to be excellent collectors of bacteria commonly encountered in soil and our Trelease Woods data underscore the dynamic nature of the bacterial populations in waste tires. *Bacillus megaterium* and *B. mycoides* were absent at 3 months but were present in the 9-month samples. The prevalence of nonlarvicidal *B. sphaericus* doubled during this time period. Abiotic factors such as water temperature, water level, and the decay rate of the organic content inside a tire combined with deposition of both new species and strains of bacteria undoubtedly influence bacterial populations. Our data on the spore-forming bacteria collected from the tires in Trelease Woods, with the exception of larvicidal *B. sphaericus,* show marked similarities to the data from the other habitats surveyed. If the larvicidal *B. sphaericus* collected in Trelease Woods was native, then it was the only bacterial species identified that was unique to tires.

In summary, we found that the majority of bacilli collected in artificial containers and tree holes were common soil bacteria. Native *Bti* was present at low levels in almost all habitats. In a temporary woodland pool, *Bti* comprised 27.9% of our samples and we have no explanation for this high prevalence. When *Bti* and *B. sphaericus* were applied to tires, both species were recovered from treated tires as long as 9 months after application. We could not distinguish between multiplication in tires and resuspension of the original treatment. Both species survived the Illinois winter and *Bti* isolates indistinguishable from the strains present in VectoBac

were recovered from adjacent untreated tires. Our CFA database was more likely to correctly predict larvicidal activity for *B. sphaericus*. We suggest that this discrepancy is the result of the location of the *Bti* ICP genes on a plasmid, which is easier to lose than a chromosome. Our findings on the persistence of these larvicidal bacilli and their movement may prove helpful when public health and vector control programs consider various larvicides for mosquito control. Further research is necessary to assess the stability of fatty acid profiles over numerous generations in the field.

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