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Article *in* Journal of Invertebrate Pathology · May 2011

DOI: 10.1016/j.jip.2011.01.003 · Source: PubMed

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Journal of Invertebrate Pathology 107 (2011) 11–15

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Investigation of biota associated and natural infection by sporulated bacteria in Culicidae larvae from São Paulo state, Brazil

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ARTICLE INFO

Article history:
Received 9 April 2010
Accepted 9 January 2011
Available online 15 January 2011

Keywords:
Bacillus thuringiensis
Bacillus cereus
Lysinibacillus sphaericus
Culicidae larvae and bacterial biodiversity

ABSTRACT

The aim of this study was to investigate the naturally occurring bacteria from Culicidae larvae found in São Paulo state between 2006 and 2008 from the Tiete Ecological Park in the municipality of Caraguatatuba. Bacterial strains were obtained after surface sterilization of larvae followed by thermal treatment and incubation in bacteriological media under laboratory conditions. Identification was determined using cytomorphology, biochemical and physiological tests. Strains were characterized by qualitative evaluation of biological activity against Culicidae larvae, comparing protein profiles obtained by electrophoresis of crystal protoxins, electrophoresis of *Lysinibacillus sphaericus* isoenzymes, detection of the L_2 fraction of Hemolytic BL enterotoxin and amplification of DNA using Multiplex-PCR to detect HBL, NHE, CytK, BceT and EntFM enterotoxins. The results obtained in this study demonstrated that the endosymbiotic bacterial diversity belonging to *Bacillus* and related genera associated with Culicidae larvae is limited to a number of species that does not vary throughout the seasons.

1. Introduction

Microbial control of insect vector populations can be highly effective and generally has advantages over chemical control because many are host specific and safe for non-target organisms. Bacteria used successfully in microbial control programs to suppress mosquito larval populations are *Bacillus thuringiensis* and *Lysinibacillus sphaericus* (WHO, 1999). However, new microbial agents are being investigated due to the rapid appearance of resistance to *L. sphaericus* (Rao et al., 1995). In 1989, a biological control program against black flies in Brazil was initiated using *B. thuringiensis* serovar *israelensis* and resulted in excellent control of the target species (Araújo-Coutinho, 1995). This successful program initiated a search for other entomopathogenic agents, mainly Brazilian strains of *B. thuringiensis* serovar *israelensis* and other bacteria capable of controlling diptera that are disease vectors (Cavados et al., 2001).

During the 1990s, a worldwide program for isolation of entomopathogenic *Bacillus* was encouraged by the World Health Organization (WHO). In Brazil, studies which were made in the Southeast region and focused on Simuliidae (black fly) and Culicidae (mosquito) larvae from which 18 strains of *B. thuringiensis* and one of *L. sphaericus* were isolated (Cavados et al., 2001). Among the isolates, at least one that had high toxicity against *Simulium pertinax* and *Aedes aegypti* larvae, was shown to be more effective than the strains used in the commercial production of bio-insecticides (Cavados et al., 2005). As part of this research program, the histopathological effects of these toxins in the posterior midgut cells of *S. pertinax* larvae provided additional knowledge on the mechanisms of δ-endotoxins in larvae of dipteral (Cavados et al., 2004).

The larvicidal activity of *B. thuringiensis* is due to the toxins found in the parasporal inclusions that are produced at the time of sporulation. Collectively referred to as δ-endotoxins they comprise a diverse group of proteinaceous toxins that have highly specific activity against larvae, especially those of the Lepidoptera, Diptera and Coleoptera (Feitelson et al., 1992). The toxins with high larval activity for diptera (Cry4A, Cry4B, Cry11A and Cyt1A) are found in *Bti* and in some other serovarieties that produce the same or similar toxins (Lacey et al., 1982).

Invertebrates provide a rich habitat for micro-organisms. The interactions between invertebrate hosts and the microbes they harbor are numerous, and they play a key role both in host physiology and whole ecosystem processes (Reeson et al., 2003). The intestinal microbiota of hosts can protect against infection or invasion by

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0022-2011/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2011.01.003

pathogens (Moal and Servin, 2006). Members of the *Bacillus cereus sensu lato* group have also been identified as commensals in insect intestines (Jung and Kim, 2006). Given the importance of these microbial associations to both invertebrates and the environment as a whole, is remarkable how little is known about them.

The success of the program to isolate new entomopathogenic bacterial strains led to a search for other autochthonous pathogens that infect Brazilian Diptera to identify new microbial control agents. The aim of this study was to investigate sporulated biota associated and natural infection by these bacteria in Culicidae larvae in São Paulo state between 2006 and 2008.

2. Material and methods

2.1. Collection of entomological material

Culicidae larvae were collected every 15 days over a 3-year period at two locations; one in the Tiete Ecological Park and other in the municipality of Caraguatatuba, both in São Paulo state. The larvae were returned to the laboratory in the water in which they were collected to allow some of them to be examined while still alive. Larvae went through a process of sorting for signs of disease and those that exhibited symptoms of bacterial infection were submitted to a sterilization process with sequential baths, during five minutes, of 70% ethanol, 10% sodium thiosulfate and 5% sodium hypochlorite.

2.2. Bacteria isolation

A vortex mixer was used to prepare a homogeneous solution in sterile water of the infected macerated larvae. Two milliliters were taken from the solution and submitted to thermal screening by exposure to 80 °C for 20 min to kill non-sporulated bacteria. After heat treatment, 100 μL were plated on BHI agar and on a specific medium based on egg-yolk lecithin for isolation of *Bacillus* (Vasconcellos and Rabinovitch, 1994) and then incubated for 24 h at 30 °C according to Cavados et al. (2004). After incubation, fresh smears were made for cytomorphological studies under an optical microscope at 1000× magnification.

2.3. Biochemical and physiological test

Biochemical and physiological tests were performed according to Gordon et al. (1973) and Claus and Berkeley (1986) to taxonomically identify the species of the isolated bacterial strains. These tests used culture mediums that contained specific organic compounds. The biochemical tests applied were: Voges-Proskauer Reaction (VP); production of nitrate to nitrite; formation of acids from sugars (glucose, arabinose, xylose, manitol); production of gas from glucose; hydrolysis of starch, casein and gelatin; use of citrate and tyrosine; production of the enzymes lecithinase, catalase and oxidase; and production of indole. The physiological factors analyzed were: growth temperature range (40 °C, 45 °C, 50 °C, 55 °C, 60 °C and 65 °C); growth under anaerobic conditions; growth in pH 5.7 and in the presence of sodium chloride (NaCl) at different concentrations (5%, 7% and 10%). All biochemical and physiological tests were evaluated by comparing the results of the isolates, with the results of the positive and negative control strains used for each test. This information was used to classify the isolated species using the *Bacillus* species dichotomic key according to Gordon et al. (1973).

2.4. Qualitative evaluation of biological activity

The lineages of *B. thuringiensis* and *L. sphaericus* isolates were grown in Agar Nutrient and Agar NYSM, respectively, for 48 h at 30 °C ± 1 °C. Afterwards the cultures were suspended in sterile saline solution (0.85% NaCl) and aliquots of 1 mL of the bacterial suspension, with an optical density equal to 0.1 and wavelength of 600 nm were added to 50 mL of distilled water in disposable plastic cups containing 15 3rd instar larvae of *Ae. aegypti* and *Culex quinquefasciatus* followed by incubation at 25 °C ± 1 for 24 and 48 h, respectively. As a toxicity reference for the *Cx. quinquefasciatus* larvae, lineages of *L. sphaericus* SSII-1 (LFB-FIOCRUZ 847) and *L. sphaericus* 2362 (LFB-FIOCRUZ 736) were used; as a toxicity reference for *Ae. aegypti* larvae, lineages of *B. thuringiensis* serovar *israelensis* (LFB-FIOCRUZ 584) and *B. thuringiensis* serovar *kurstaki* (LFB-FIOCRUZ 263) were used. They were also used as a standard in this study. All the lineages were tested in duplicate and larvae without bacterial suspensions were used as negative controls. The biological activity for *Ae. aegypti* and *Cx. quinquefasciatus* larvae was recorded 24 and 48 h post-treatment, respectively. The biological activity was evaluated according to Oliveira et al. (1998).

2.5. Electrophoresis of crystal protoxins

For this characterization, a protein profile analysis of the crystals produced by the new lineages of *B. thuringiensis* and *L. sphaericus* was used. This analysis was carried out through electrophoresis in a 12% gel of Polyacrylamide-Dodecyl Sodium Sulfate/SDS–PAGE as described by Laemmli and Favre (1973).

2.6. Electrophoresis of isoenzymes (multilocus enzyme electrophoresis – MLEE)

Agarose at 1% (p/v) in an appropriate buffer for the enzyme was prepared and used for conducting electrophoresis. The strains of *L. sphaericus* submitted to electrophoresis were cultivated in NYSM, pH 7.2 at 33 °C. After 18 h, when the cells were still in the vegetative phase of growth, the bacterial strains were suspended in 400 mL of lysis buffer (Triton X-100 1%; 0.1 M Buffer Tris pH 8.0; EDTA 0.001 M; DDT 0.001 M and e-p-amino-n-caproic acid 13%). Approximately 30 mg of glass beads with a 0.5 mm diameter were added to each sample, and then homogenized in a magnetic agitator for one minute. In this way, a chemical and physical lysing of the bacterial cells was made. The resulting material was then conditioned at 0 °C. The methodology for these analyses followed the well-known method by Zahner et al. (1989).

2.7. Detection of the L_2 fraction of Hemolytic BL enterotoxin

The strains of *B. thuringiensis* isolated were grown in 5 mL of BHI broth with 0.1% of glucose and placed in an incubator at 33 °C for 18–24 h. After this time, 100 μL from each culture was inoculated in 25 mL of BHI broth with 0.1% of glucose and then incubated in a "shaker" at 33 °C at 250 rpm for 13–18 h. They were then pelleted at 4 °C using 2200 rpm for 20 min. Enterotoxin detection followed the methodology described in the BCET-RPLA TD950 kit from OXOID.

2.8. Extraction, dosage and amplification of DNA using Multiplex-PCR technique

The extraction of DNA followed the method used by Harwood and Cutting (1990) cited in Santos (2000). After extraction, DNA concentration was analyzed according to Yang et al. (2005) by using Multiplex-PCR techniques to amplify specific regions of DNA through the use of specific primers of genes that code for the enterotoxins (NHE, HBL, CytK, EntFM and BceT), thus generating a characteristic profile of each lineage.

Table 1
Number of collected larvae in Tiete Ecological Park and Caraguatatuba, São Paulo state between the years of 2006 and 2008.

Larvae	Tiete Ecological Park Years		Caraguatatuba	
	2006	2007	2007	2008
Normal	779	1510	997	7035
Symptom A1	9	60	21	19
Symptom B2	23	286	17	431
Symptom C3	0	259	64	482
Total	811	2115	1099	7967

Number of larvae collected of each symptom among 3 years at Tiete Ecological Park and Caraguatatuba, São Paulo state. A1 – larvae with whitish tegument and milky appearance; B2 – larvae with purple stains on the tegument and darkening of the gastric caeca; C3 – larvae with white stains along the intestine and bioluminescence of the intestine.

3. Results

Between 2006 and 2008, 11,992 of *Culex* spp. and *Aedes* spp. larvae were collected, of which 10,321 larvae did not exhibit any apparent symptoms and 1671 exhibited some type of symptom that could not be attributed to a particular pathogen group (microsporidia, viruses, fungi) (Table 1). Three morphological manifestations were identified. In Symptom A, the larva exhibited a whitish integument with a milky appearance; in Symptom B, darkening of the gastric caeca occurred and the presence of purple stains on the integument; and in Symptom C, there was bioluminescence of the intestine and the appearance of white regions along the intestine (Fig. 1). In Tiete Ecological Park, 1.10% of the larvae had Symptom A in 2006 and 2.84% in 2007. For Symptom B, the prevalence was 2.84% in 2006 and 13.5 2% in 2007. For Symptom C, in 2006 no larvae were identified, but in 2007 12.24% of the larva had this symptom. In Caraguatatuba, 1.91% in 2007 and 0.24% in 2008 of the larva had Symptom A. For the larvae with Symptom B, the rate was 1.55% in 2007 and 5.40% in 2008 and those with Symptom C the rate was 5.82% in 2007 and 6.05% in 2008.

3.1. Isolation and identification of bacteria

One hundred and sixteen strains of bacteria belonging to the genus *Bacillus*, *Lysinibacillus* and *Brevibacillus*, were isolated and identified by biochemical and physiological tests. They were the following: 19 *B. thuringiensis*, 23 *B. cereus*, 22 *L. sphaericus*, 10 *B. amyloliquefaciens*, 22 *B. megaterium*, 4 *Br. brevis*, 5 *B. licheniformis*, 6 *B. pumilus*, 2 *B. circulans*, 1 *B. globisporus*, 1 *B. badius* and 1 *B. mycoides*.

3.2. Qualitative analysis of biological activity

Seven strains of *B. thuringiensis* and three strains of *L. sphaericus* resulted in larval mortality greater than 50% against *Ae. aegypti.* and *Cx. quinquefasciatus*, respectively. The other strains tested showed low larval toxicity with mortality rates between 0% and 20% for *Ae. aegypti* and *Cx. quinquefasciatus*.

3.3. Electrophoresis of crystal protoxins

Electrophoresis analysis in 12% gel polyacrylamide-dodecyl sodium sulfate/ SDS–PAGE for *B. thuringiensis* identified seven samples that exhibited possible profiles of *B. thuringiensis* serovar israelensis IPS-82 serotype H-14 (LFB-FIOCRUZ 584) (Fig. 2), with proteins of 125, 135, 68 and 28 kDa that are known to be responsible for toxicity to larvae of Diptera. The other samples tested exhibited similar profiles to serovars *brasiliensis*, *yunnanensis*, *nigeriensis* or unknown profiles. For *L. sphaericus*, all strains have been tested and three of them contained a binary toxin with the molecular weights of 42 and 51 kDa which is similar to the reference strain 2362 that is toxic for Diptera.

3.4. Electrophoreses of isoenzymes

The three strains of *L. sphaericus* analyzed showed similar enzymatic profiles to the entomopathogenic strain 2362 and allele 2 in the genetic locus for ALDH (Alanine Dehydrogenase), an important

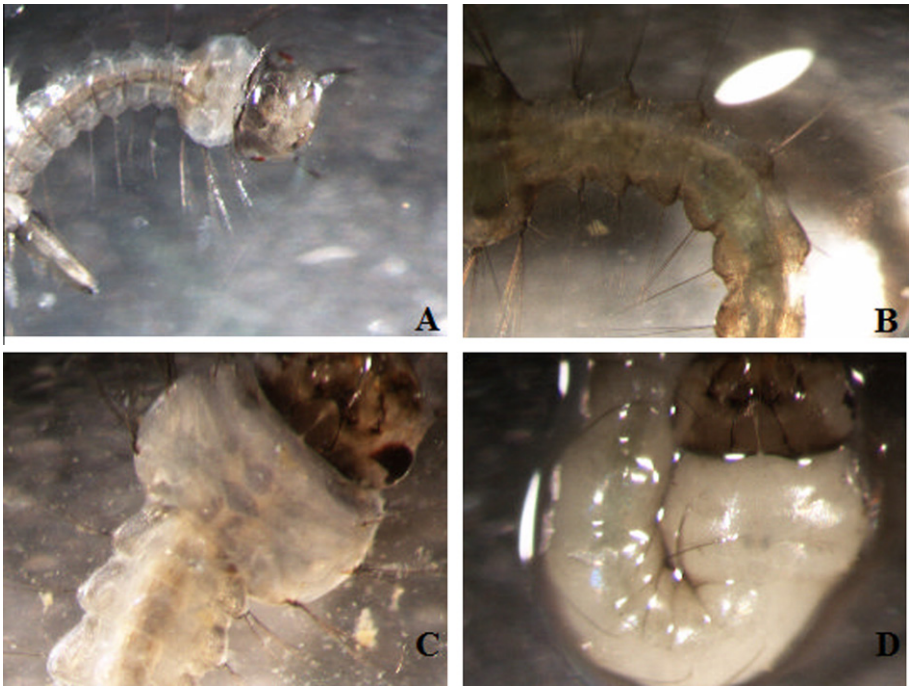

Fig. 1. Symptoms found in the *Culex quinquefasciatus* larvae. (A) Normal larvae; (B) larvae with purple stains on the tegument and darkening of the gastric caeca, symptom B; (C) larvae with white stains along the intestine and bioluminescence of the intestine, symptom C; (D) larvae with whitish tegument and milky appearance, symptom A.

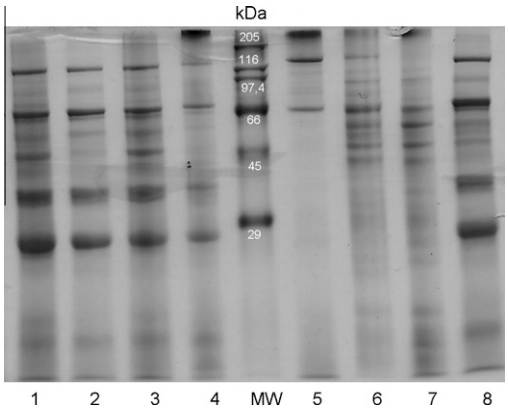

Fig. 2. SDS–PAGE to *B. thuringiensis* crystal proteins profile analyses. Kilo Dalton (kDa); Molecular weight (MW); Lane 1-*Bt israelensis* LFB-FIOCRUZ 584; Lanes 2–4 and 8 – Analyzed samples showing similar profile of serovar *israelensis;* Lane 5 – *Bt kurstaki* LFB-FIOCRUZ 263; Lane 6 – *Bt morrisoni* LFB-FIOCRUZ 781; Lane 7 – Analyzed sample showing similar profile of serovar *morrisoni*.

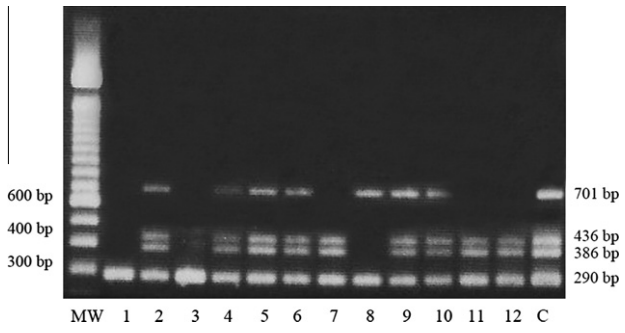

Fig. 3. Agaroses Gel Electrophoresis showing fragments corresponding to enterotoxins genes detected by Multiplex-PCR. Legend: Molecular weight (MW); Base pair (bp) – Enterotoxin T (BceT) (701 pb); Enterotoxin Hemolytic BL (HBL D) (436 pb) and (HBL C) (386 pb); and Enterotoxin FM (EntFM) (290 pb). Lanes 1–12 – tested samples; C – Positive control (*B. cereus* LFB-FIOCRUZ 406).

characteristic for the indication of entomopathogenic strains of *L. sphaericus*. This confirms the data obtained in the protein profile and in the qualitative bioassays of biological activity that were conducted.

3.5. Detection of the L_2 fraction of the Hemolytic BL enterotoxin

Among the *B. thuringiensis* strains isolated, 19 samples were tested, 15 of which were positive for L_2 fraction of the Hemolytic BL enterotoxin. The Hemolytic BL enterotoxin is composed of three fractions. The presence of fraction L_2 does not assure the presence of toxic action. However, it is an indication that more tests should be performed because the use of this lineage could be unsuitable for insect control because this toxin has known activity in mammals.

3.6. Extraction, dosage and amplification of DNA using the Multiplex-PCR technique

B. thuringiensisstrains were analyzed for genes that code for the three subunits of enterotoxin non-hemolytic (NHE) and the three subunits of Hemolytic BL (HBL) enterotoxin, and to the presence of genes for the enterotoxins FM, CytK and BceT. Electrophoresis was carried out in 1.5% agarose gel (Fig. 3). The results obtained were: genes *nheABC* were found in 16, 17 and 17 strains, respectively. For *hblACD* complex 15 strains was positive, *cytK* gene was found in 12 strains. Seventeen strains were positive for *entFM* and *bceT* was detected in 7 strains. Also, it should be pointed out that the data obtained showed that only one strain was negative for all the genes of the enterotoxins tested.

4. Discussion

Strains were identified by biochemical and physiological tests following the initial characterization based on colony morphology and cytomorphology. Strains of *B. thuringiensis* and *L. sphaericus* are frequently isolated from larvae of Diptera (Cavados et al., 2001, 2004). However, the isolation of other species of the genus *Bacillus* and close relatives (*B. amyloliquefaciens, B. subtilis, B. pumilus, B. licheniformis, B. cereus, Br. brevis, B. megaterium, B. mycoides, B. badius, B. globisporus and B. circulans*) is less common.

B. amyloliquefaciens is considered to be similar to *B. subtilis*, although this species is different from the latter in terms of C + G content. With all the characteristics of the genus *Bacillus*, *B. amyloliquefaciens* is commonly found in soil (Claus and Berkeley, 1986). Prior to this report, there is no evidence that this species possesses any entomopathogenic properties. However, there are *B. subtilis* lineages that produce entomopathogenic substances (Das and Mukherjee, 2006).

Selinger et al. (2008) showed the capacity of genetically modified *B. pumilus* to express endotoxins of *B. thuringiensis* that confer entomopathogenicity. However, the natural production of these toxins by *B. pumilus* has not been described. Likewise, *B. licheniformis*, which is used in production of bacterial antibiotics (Veith et al., 2004), has also not been associated with pathogenicity in insects. Despite the isolation of these species from dead or sick larvae, it is not possible to confirm that these species caused that infection.

B. megaterium is a Gram-positive soil bacterium that is important in the food industry as it causes foodborne illnesses. Osborn et al. (2002) reported the isolation of these micro-organisms from larvae of a lepidopteran host and demonstrated that they were toxic to the insect but with very low toxicity (10% mortality).

Hu et al. (2005) reported entomopathogenicity caused by some strains of *B. cereus* against larvae of Diptera possibly due to the capacity of this species for natural recombination, in this case with toxic genes from *B. thuringiensis*. Various studies have shown that these two species are members of the same group within the genus *Bacillus*, the *B. cereus sensu lato* group. Some authors consider *B. thuringiensis* and *B. cereus sensu stricto* as one species, making it even more likely that characteristics of one species could be found in the other (Luthy and Wolfersberger, 2000).

There is no evidence that *B. mycoides* isolated from larvae in this study is entomopathogenic. Its presence is possibly opportunistic or perhaps represents a symbiotic relationship with this mosquito host (Villas-Bôas et al., 2007). Also, *Br. brevis*, a species normally isolated from soil and food has previously been isolated from dead larvae of Diptera. However, it has not demonstrated any pathogenic activity under laboratory conditions (Mohsen et al., 1989).

According to Darriet and Hougard (2002), *B. circulans* has biological activity against *Ae. aegypti, Cx. quinquefasciatus* and *Anopheles gambiae*, but is less toxic than *L. sphaericus*. However, the factor responsible for this biological activity has not yet been identified but the production of chitinases in some strains is known which could have some entomopathogenic activity (Wiwat et al., 1999).

We could find no report in the literature concerning the isolation of *B. badius* and *B. globisporus* from insect larvae. This study is the first to report that these species are associated with mosquito larvae as part of their natural microbiota.

There are reports of insect larvae with external symptomology similar to those found in the present study. A bacterial species belonging to *Paenibacillus* genus, *Pae. popilliae*, previously known

as *B. popilliae*, causes an illness known as Milky Disease in coleopteran larvae, *Popillia japonica*. This name is due to the fact that bacterial spores germinate and multiply in insect hemolymph causing a milky appearance (Claus and Berkeley, 1986). There are no reports of diptera infection caused by this bacterium, which has specials growth necessities and is host specific.

The results from the qualitative bioassays of the *B. thuringiensis* strains against *Ae. aegypti* larvae were in agreement with those from SDS–PAGE. The strains that exhibited a protein profile similar to the serovar *israelensis* had high biological activity. Also, the results from qualitative bioassays with *L. sphaericus* strains against *Culex* sp. larvae were in agreement with those obtained in the MLEE, where a similar enzymatic profile to entomotoxic 2362 lineage with allele 2 in the genetic locus for ALDH (Alanine Dehydrogenase) was confirmed.

The characterization using Multiplex-PCR of the *B. cereus* enterotoxingenes in strains of *B. thuringiensis* is a necessary precaution, especially when dealing with potential entomopathogenic strains since they could become the main active ingredient of bioinsecticides. Strains of *B. cereus* that produce enterotoxins have been associated with outbreaks of gastroenteritis in humans (Mantynen and Lindstrom, 1998). The fact that *B. cereus* and *B. thuringiensis* are species with high genetic similarity within the genus *Bacillus*, makes it possible that characteristics present in one of the species can also appear in the other. For this reason it is necessary to verify the presence of enterotoxins in *B. thuringiensis* strains that are under consideration as bio-insecticides.

The results obtained in this study demonstrated that the endosymbiotic bacterial diversity within the genus *Bacillus* and associated with *Cx. quinquefasciatus* larvae, is dominated by a limited number of species that does not vary throughout the seasons. This is likely due to the lifestyle of these species and the fact that 99% of the bacterial community cannot be cultured.

Acknowledgments

We are grateful to Dr. James J. Becnel (ARS – USDA) for the review of manuscripts. This research was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (2008/05700-2).

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