THE DETECTION OF *BACILLUS THURINGIENSIS* IN MASS REARING OF *CACTOBLASTIS CACTORUM* (LEPIDOPTERA: PYRALIDAE)

VERENA-ULRIKE LIETZE[1], GEORGE SCHNEIDER[2], PANNIPA PROMPIBOON[1] AND DRION G. BOUCIAS[1]
[1]Entomology and Nematology Department, University of Florida, 970 Natural Area Drive, Gainesville, FL 32611, USA

[2]Florida Department of Agriculture and Consumer Services, Division of Plant Industry, 1911 S.W. 34th Street, Gainesville, FL 32608, USA

ABSTRACT

A colony of the cactus moth, *Cactoblastis cactorum* Berg, suffered a die-off that involved 100% larval mortality in selected rearing containers. Preliminary microscope examination of wet mounts prepared from dead larvae revealed the presence of numerous uniform, highly refractive particles reminiscent of bacterial spores. Utilizing a combination of bacteriological, molecular, and chemical methods the causal agent responsible for this die-off was found to be a strain of the insecticidal *Bacillus thuringiensis* var. *kurstaki*. Significantly, larvae that were killed supported bacterial growth and sporulation. The gregarious feeding habit of this insect combined with the ability of this bacterium to amplify in dead larvae explains in part the observed rapid spread of sepsis in the rearing containers. Screening the various diet ingredients demonstrated that the cannellini bean flour harbored a variety of heat resistant bacilli including both *Bacillus cereus* and *B. thuringiensis* implicating it as the likely source of toxicity.

Key Words: cactus moth, Bt, insect rearing, entomopathogen

RESUMEN

Una colonia de la polilla del cactus, *Cactoblastis cactorum* Berg, sufrió una mortalidad de 100% de las larvas en recipientes de cria seleccionadas. Una examinación microscópica preliminaria de montajes humedos preparados de larvas muertas, revelaron la presencia de numeros particulares altamente refractivos semejantes de esporas de bacteria. Utilizando una combinación de métodos bacteriologicos, moleculares y químicos, encontramos que el agente causante responsable para el muerte fue una cepa insecticida de *Bacillus thuringiensis* var. *kurstaki*. Significativamente, las larvas que fueron eliminadas soportaron el crecimiento y esporulación bacterial. El comportamiento de este insecto de alimentarse gregariamente combinado con la abilidad de esta bacteria para amplificarse en larvas muertas, explica en parte, el esparcimiento rapido observado de sepsis en los recipientes de cria. Al filtrar los varios ingredientes de dieta, demonstramos que la harina de frijol cannellini albergó una variedad de bacilos resistentes al calor incluyendo ambas *Bacillus cereus* y *B. thuringiensis* que implica que probablemente sea la fuente de toxicidad.

Cactoblastis cactorum Berg (Lepidoptera: Pyralidae), or cactus moth, is recognized for its beneficial role as a biological control agent of invasive prickly pear cactus, *Opuntia* spp. (Caryophyllales: Cactaceae), in Australia. This insect, a native of Argentina, has recently invaded the Caribbean, Central America, and coastal areas of the southeastern U.S. and threatens to destroy the diversity of native *Opuntia* species (Zimmermann et al. 2001). In response to its U.S. presence, USDA-APHIS in collaboration with other federal and state agencies outlined a plan to contain and prevent the western expansion of its geographical range by a combination of control tactics. One component of the management plan included the implementation of the sterile insect technique (Carpenter et al. 2001). In order to provide the insects for sterilization, a large-scale rearing program of this moth was initiated in 2006 by the Florida Department of Agriculture, Division of Plant Industry (DPI) in Gainesville, Florida in cooperation with the USDA-ARS, Crop Protection and Management Research Laboratory in Tifton, Georgia.

To date, the establishment of large-scale colonies for mass rearing of this insect on an artificial diet has been hindered by the presence of entomopathogens. On several occasions, these colonies have become chronically infected with microsporidia. The responsible pathogen replicating in the Malpighian tubules spreads via fecal deposits to healthy conspecifics and is gradually amplified within containers. The presence of microsporidia causes retardation in larval development and often death at larval-pupal transition. Whether or not the *Nosema*-like microsporida de-

tected in recent years in these colonies is the same as that collected by Pemberton & Cordo (2001) is unknown. However, implementing both an increased level of sanitation and destruction of larvae in rearing containers with detectable levels of microsporidia has decreased the impact of this disease on the colony to tolerable levels. Other issues including unexplained aberrations in larval development have been observed in limited numbers of rearing containers.

Recently, rearing containers were found to contain large numbers of heavily melanized, mixed-aged dead larvae. Unlike the situation observed with microsporidia infection, all larvae in these containers were killed within days suggesting the presence of a highly virulent virus or toxin-producing bacterial pathogen. Microscope examination of wet mounts from these insects revealed that all of the dead larvae contained numerous uniform, highly refractive particles reminiscent of bacterial spores. In this study, we have isolated and identified the causal agent of disease to be a *Bacillus thuringiensis* strain that is lethal and that replicates within *C. cactorum*.

Materials and Methods

Insect Rearing

Egg sticks were obtained from a *C. cactorum* colony located at the USDA-ARS laboratory in Tifton, Georgia in order to reduce the incidence of *Nosema* and other entomopathogens. Egg sticks were held at 27°C and 70% relative humidity (RH) for 21 d or until egg sticks showed characteristic darkening prior to hatch. Egg sticks, totaling 300 eggs, were then transferred to a diet block and sealed in 4-liter Rubbermaid® containers. These containers were incubated at 26-27°C and 50-55% RH under a photoperiod of 8.5 h:15.5 h (L:D). At approximately 3 weeks post-transfer the solid container lid was exchanged for one with a single 5-cm diameter screened vent to allow for moisture exchange and reduce the likelihood of mold development on the diet block or frass. A new diet block was added at 4 weeks post-transfer and then weekly thereafter until no more pupae were formed. Collection of pupae was performed weekly once initiated. The containers were continually monitored for excess moisture and the venting adjusted accordingly with different screened lid configurations to limit mold development. To enhance sanitation, spent diet blocks, silk, and frass were removed during container servicing for food addition or collection of pupae. The diet was composed of the same basic ingredients previously developed for *C. cactorum* by Marti & Carpenter (2008). The main constituents were as follows: 2.5 L of boiling water, 630 g of cannellini bean flour, 186 g of Brewer's yeast, 100 g of sucrose, 45 g of agar, 9.6 g of ascorbic acid, 6 g of methyl paraben, and mold inhibitor (15 mL of a solution consisting of 418 mL propionic acid, 42 mL phosphoric acid, and 540 mL water). The diet was prepared in 8-L batches in a Hobart HCM450 Cutter mixer and then poured to a depth of 2 cm in cookie sheets and left to harden. Once firm it was cut into 4 × 6 cm blocks which were then dipped into beeswax to provide a thin waxy outer layer to simulate a cactus cladode and help retain moisture within the food block.

Detection and Isolation of Bacteria

Individual dead larvae were randomly selected from containers, transferred to sterile microcentrifuge tubes containing 500 µL of 0.85% NaCl and homogenized with a sterile pestle. The homogenates were incubated at 70°C for 30 min, subsequently cooled on ice for several minutes, and then filtered through Miracloth™ (22-25 µm pore size, Calbiochem Inc., Gibbstown, NJ) to remove insect debris. The filtrates were streaked (100 µl/plate) on nutrient agar (NA) and incubated at 28°C. The growth development of the bacterial colony phenotype was monitored daily. After 3 d, single colonies were randomly selected and isolated to new NA plates. Plates were incubated at 28°C to produce colonies for bioassays. Selected colonies developing on these plates were Giemsa-stained and examined with a light microscope.

Biochemical and Molecular Characterization of a *Bacillus thuringiensis*

Colonies displaying the typical phenotype described in the results section were selected from the NA plates and propagated on trypticase soy broth agar (TSBA) plates at 28°C for 2-3 d. Cells were harvested and treated chemically to extract and convert the fatty acids present in the cell wall or cell membrane fractions to fatty acid methyl esters (FAMEs) following the methods described by Botha & Kock (1993). The total cellular FAMEs were analyzed by GC and the resulting profiles matched with those of yeasts available in the Microbial Identification system (MIDI) database in Sherlock Version 4.5 software (Microbial ID, 1993).

Selected bacterial clones were subjected to the polymerase chain reaction (PCR) based identification reaction outlined by Vidal-Quist et al. (2009). DNA was obtained by subjecting 24-h-old cultures to thermal shock (Bravo et al., 1998). The primer pair, Un1F 5'-CATGATTCATGCGGCAGATAAAC and Un1R 5'-TTGTGACACTTCTGCTTCCCATT, was used to amplify a 277-bp region of the *cry1* gene (Vidal-Quist et al. 2009). Resulting PCR products were sequenced with an ABI Prism DNA Sequencer at the Interdisciplinary Center for Biotechnology Research Core Facility at the University of Florida, Gainesville, and the DNA sequences were compared to those deposited in GenBank with BLAST (blastn).

Bioassays

The initial tier of assays was conducted to determine if the particles observed in the killed larvae were infectious. Approximately 100 mg of dead larvae were homogenized in 1 mL of sterile water and filtered through Miracloth™. The filtrate was applied onto the outer surface of a block of diet. These blocks were then infested with 25-50 mixed age larvae and incubated at 25°C. These treated blocks were inspected daily.

A second series of bioassays were conducted on bacteria isolated from heat-treated homogenates from dead insects. A culture of *B. thuringiensis* var. *kurstaki* HD-1 was incorporated as a positive control for the bioassays. Bacteria from both sources were grown on NA for 5 d and sporulating cultures transferred and suspended into 1 mL of sterile 0.85% NaCl solution. These suspensions were mixed vigorously and bacterial/spore concentrations were estimated by measuring optical density at 600 nm. In addition, serial dilutions were prepared and spot-plated onto NA to determine colony forming units (CFUs). A range of dilutions were applied to small blocks of diet (10^1 to 10^6 CFUs/cm^2 of diet surface) that lacked the wax coating. A total of 10 third instars, sampled from containers deemed clean of any detectable disease, were placed on each block in individual 2.5-oz plastic cups. Appropriate controls were established on diet treated either with saline (blank) or with dilutions of a nonpathogenic *Bacillus cereus* spore preparation (10^6 CFUs/cm^2 of diet surface). Insects were incubated at 28°C under a photoperiod of 12 h:12 h (L:D) and observed daily. Tissue samples from dead larvae were sampled postmortem and examined under a phase contrast microscope.

Larvae from each treatment that succumbed to sepsis as well as insects fed the *B. cereus* preparations were assayed to estimate bacteria growth and sporulation. Insects collected 24 h postmortem and living larvae from *B. cereus* treatments were weighed, homogenized in sterile saline (1 mg of insect tissue/100 µL) and incubated at 70°C for 30 min to select for heat resistant bacterial spores. Heat-treated homogenates were serially diluted and 2-µL aliquots of each dilution were spot-plated onto NA to estimate the number of *in vivo* produced endospores. Statistical analysis was conducted with the Statistical Analysis System (SAS) for Windows (SAS, 2004). To obtain normal distribution, CFU counts were $\log_{10}$ transformed. Transformed data were subjected to ANOVA with the mixed procedure of SAS and means were separated by the least-square (lsmeans) statement. Untransformed data were expressed as average CFU/mg insect tissue ± standard error.

Results and Discussion

The onset of disease symptoms in the colony were dramatic, and within days after initial detection all larvae within an affected container succumbed to sepsis. None of these individuals contained detectable *Nosema*-like spores that were found previously to infest this colony (Fig. 1). Microscopic examination of the tissue smears from freshly killed larvae revealed the presence of numerous Gram-positive rod shaped bacteria (Fig. 2A). Within 24-48 h postmortem, the rod shaped bacteria in the dead larvae sporulated producing oval endospores and associated inclusions (Fig. 2B). The initial tier of assays demonstrated that crude insect homogenates were highly virulent to second through fourth instars. Within 24 h of exposure 100% of the larvae exposed to homogenate-treated diet were dead and displayed external symptoms identical to those observed in the colony.

Plating of heat-treated insect homogenates produced a uniform pattern containing thousands of bacterial colonies. These colonies were cream-colored and opaque with undulating margins and had the general phenotype of bacilli. Examination of either wet mounts or Giemsa-stained smears of the 1-d-old cultures revealed the presence of numerous rod shaped bacteria measuring 4-6 µm long by 1 µm in diameter. In addition to these rods, a wide range of longer rods extending greater than 25 µm in length were observed in these cultures (Fig. 3A). After 3 d of incubation the cultures produced an abundance of highly refractive oval spores. Microscopic examination of Giemsa-stained preparations revealed that these cultures also produced numerous parasporal crystals (Fig. 3B) suggesting that the causal agent was *B. thuringiensis* (Bt).

The MIDI analysis of spore-forming bacteria isolated from heat-treated dead larvae best fit (0.649) to the *B. cereus* subgroup A. The second best fit (0.440) was *B. thuringiensis* var. *kurstaki*. The inability of this method to place this isolate close to known *B. thuringiensis* may reflect the wide variation among different Bt isolates and/or the fact that the precision of MIDI is reliant on a high stringency of method standardization (Adams et al. 2005). The association of Bt isolates with *B. cereus* subgroup A is in agreement with the results of both MIDI and sequence analyses that have been conducted on this bacterial group (Wintzingerode et al. 1997; Bavykin et al. 2004). In light of the presence of parasporal crystals in cultures derived from dead *C. cactorum*, a series of PCRs were conducted using universal primers designed to amplify a fragment of the *cry* toxin. All of the tested bacteria including the control *B. thuringiensis* HD-1 strain produced identical 245-bp trimmed amplicons that had 100% homology to the *B. thuringiensis cry1Ab* gene. In light of

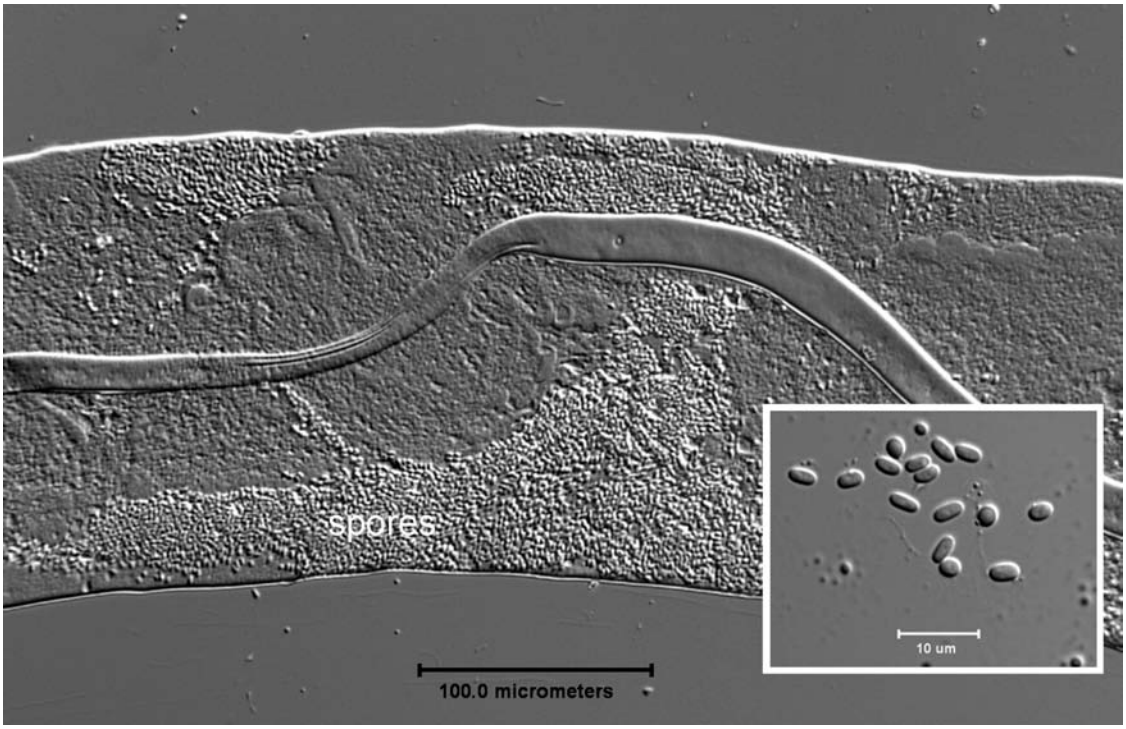


Fig. 1. Differential interference contrast micrograph of Malpighian tubules dissected from *Nosema*-infected *Cactoblastis cactorum* larvae. Numerous spores are produced throughout the length of tubules. As the infection develops, infected cells lyse and release numerous oval-shaped spores (see insert) into the hemocoel.

detection of the δ-endotoxin gene the bacterial isolates derived from *C. cactorum* have been denoted as BtCc.

The source of the BtCc was likely the ground bean flour used in the artificial diet. Incubation of the cannellini beans in nutrient broth after heat treatment produced an array of sporulating Gram-positive colonies. The majority of clones (19 out of 20) were Gram-positive bacilli that produced distinct centrally located endospores. These clones lacked detectable parasporal crystals, produced no PCR-generated amplicons with

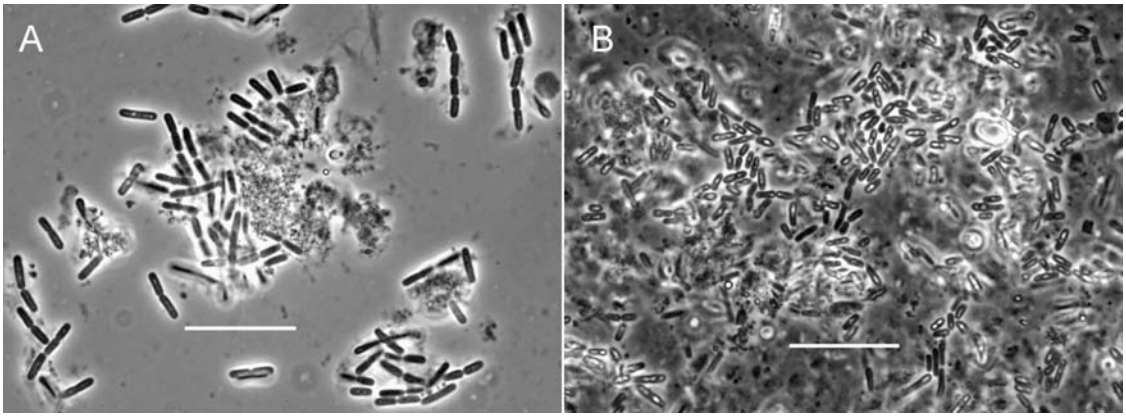


Fig. 2. Phase contrast micrograph of tissue smears dissected from *Cactoblastis cactorum* fed homogenates of dead larvae sampled from infected rearing containers. Note the initial production of rod-shaped vegetative cells in the tissues sampled several h *postmortem* (A). After 24 h *postmortem* (B) these vegetative cells in the hemolymph underwent sporulation producing numerous highly refractive endospores and associated crystals (bars equal 20 μm).

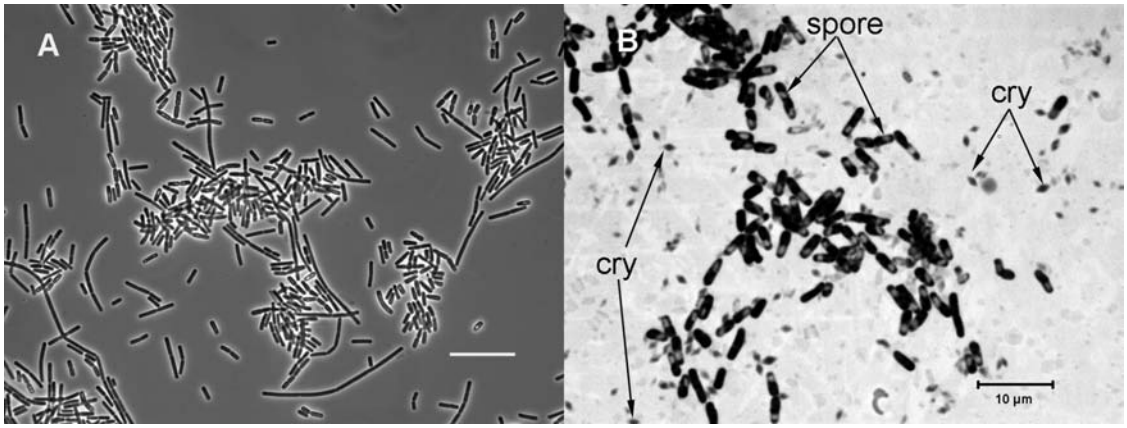


Fig. 3. Micrographs of bacterial cells from nutrient broth cultures derived from heat-treated tissue homogenates of dead *Cactoblastis cactorum* larvae. (A) Phase contrast micrograph of the exponentially growing vegetative cells (bar equals 20 µm). (B) Light micrograph of Giemsa-stained cells harvested during the stationary growth phase depicting sporulating cells with unstained refractive spores and numerous parasporal shaped crystals (cry) presumed to be composed of the highly insecticidal δ-endotoxin.

the *cry* primers, and were nonpathogenic to *C. cactorum*. These clones were presumed to be a nonpathogenic *B. cereus*. However, one clone originating from the heat-treated bean flour produced spores and associated parasporal crystals that were identical to those in Fig. 1B. This clone subjected to PCR diagnostic testing produced an amplicon with a sequence identical to that observed with isolates derived from the dead larvae.

The bioassays with spore crystal preparations demonstrated that *C. cactorum* was highly sensitive to *B. thuringiensis*. Exposure to diet treated with high dosages (10^4-10^6 spores /cm^2 of diet) of either BtCc or HD-1 strains killed 100% of the test larvae within 48 h post-exposure. At these higher dosages there was no evidence of feeding as reflected by the lack of frass production. It is assumed that the ingestion of Bt-produced δ-endotoxins induced an immediate disruption of gut tissue that preceded larval death. At a lower dosage of 10^3 spores/cm^2 of diet, the two Bt preparations killed approximately 50% of the test larvae. Treatments below this dosage level, including control treatments, caused no larval mortality after 1 week of incubation. The overall sensitivity of *C. cactorum* to the HD-1 is in agreement with the data of Bloem et al. (2005). In their report, dipping individual cladodes of *Opuntia stricta* in the 1X Dipel rate (Valent USA Corp., Walnut Creek, CA) resulted in 100% neonate mortality.

Larvae that succumbed from the BtCc and HD-1 treatments supported both bacterial growth and sporulation. The BtCc isolate appeared to be better adapted to replicate/sporulate in *C. cactorum* than the HD-1 isolate. Plating homogenates of dead larvae taken from treatments with 10^4 or 10^5 spores/cm^2 of diet demonstrated that the BtCc strain produced $4.1 \times 10^5 \pm 1.4 \times 10^5$ heat resistant spores/mg larval tissue which was significantly greater ($t = 2.54$, $P = 0.016$, $df = 1$) than $2.0 \times 10^5 \pm 1.1 \times 10^5$ heat resistant spores/mg larval tissue produced in HD-1 killed larvae. Insects fed diet treated with nonpathogenic *B. cereus* (high dosage) harbored $2.5 \times 10^2 \pm 1.0 \times 10^2$ heat resistant spores/mg larval tissue. These spore concentrations may be considered as background levels present in the food bolus. The ability of Bt to develop in this insect may explain the rapid spread of this pathogen in the rearing containers. The response of an insect to *B. thuringiensis* treatment depends upon the level and the type of bacterial toxins produced by the bacterial strain, the presence and levels of endospores, and/or the intrinsic properties of the hosts (Tanada & Kaya 1993). Many insects are highly susceptible to exposure to the δ-endotoxins and undergo immediate gut paralysis followed by death within 1d post-exposure without subsequent growth of the ingested *B. thuringiensis*. In the present study, sporulating cultures of both HD-1 and BtCc presumably produced δ-endotoxins that caused a rapid toxemia in treated larvae. Significantly, the BtCc and to a lesser extent the HD-1 strain were able to invade, replicate, and sporulate in *C. cactorum*. These findings conflict with the earlier bioassay results of Huang & Tamashiro (1966). In this previous report, *C. cactorum* was found to be highly sensitive to the toxic effects of *B. thuringiensis*, but dead larvae did not support bacterial sporulation. Additional experiments conducted by Huang & Tamashiro (1966) demonstrated that the homogenates of larvae killed by *B. thuringiensis*, although containing vegetative cells, had no impact on healthy conspecifics. It should be noted that Huang & Tamashiro (1966) conducted their assays with a *B. thuringiensis* var. *thuringiensis*.

Their results suggested that application of this *B. thuringiensis* isolate, although producing short-term pest suppression, would not cycle in the insect population.

In general, the response of *C. cactorum* to the BtCc strain is similar to that found with stored product insects and silkworm species that are highly susceptible to the δ-endotoxins and also serve as a substrate for bacterial development (Tanada & Kaya 1993). The ability of the highly toxic BtCc isolate to replicate and sporulate in *C. cactorum* is a key requisite for its long-term survival and dissemination in the population. *Cactoblastis cactorum* feeds in aggregates within the cladodes of the cactus. Under this scenario, a single larva succumbing to BtCc would produce enough spores/δ-endotoxins to kill all of the associated healthy conspecifics feeding within a cactus or in containers with semisynthetic diet.

ACKNOWLEDGMENTS

The authors gratefully acknowledge James Carpenter and Susan Drawdy for providing the colony egg sticks utilized to rear *C. cactorum* and Michael Banaszek for providing the larvae and diet ingredients for analysis.

REFERENCES CITED

ADAMS, D. J., GURR, S., AND HOGGE, J. 2005. Cellular fatty-acid analysis of *Bacillus thuringiensis* var. *kurstaki* commercial preparations. J. Agric. Food Chem. 53: 512-517.

BAVYKIN, S. G., LYSOV, Y. P., ZAKHARIEV, V., KELLY, J. J., JACKMAN, J., STAHL, D. A., AND CHERNI, A. 2004. Use of 16S rRNA, 23S rRNA, and gyrB gene sequence analysis to determine phylogenetic relationships of *Bacillus cereus* group microorganisms. J. Clin. Microbiol. 42: 3711-3730.

BLOEM, S., MIZELL III, R. F., BLOEM, K. A., HIGHT, S. D., AND CARPENTER, J. E. 2005. Laboratory evaluation of insecticides for control of the invasive *Cactoblastis cactorum* (Lepidoptera: Pyralidae). Florida Entomol. 88: 395-400.

BOTHA, A., AND KOCK, J. L. 1993. Application of fatty acid profiles in the identification of yeasts. Intl. J. Food Microbiol. 19: 39-51.

BRAVO, A., SARABIA, S., LOPEZ, L., ONTIVEROS, H., ABARCA, C., ORTIZ, A., ORTIZ, M., LINA, L., VILLALOBOS, F. J., PENA, G., NUNEZ-VALDEZ, M. E., SOBERON, M., AND QUINTERO, R. 1998. Characterization of *cry* genes in Mexican *B. thuringiensis* strain collection. Appl. Environ. Microbiol. 64: 4965-4972.

CARPENTER, J. E., BLOEM, K. A., AND BLOEM, S. 2001. Applications of F_1 sterility for research and management of *Cactoblastis cactorum* (Lepidoptera: Pyralidae). Florida Entomol. 84: 531-536.

HUANG, S. S., AND TAMASHIRO, M. 1966. The susceptibility of *Cactoblastis cactorum* (Berg) to *Bacillus thuringiensis* var. *thuringiensis.* Proc. Hawaiian Entomol. Soc. 14: 213-221.

MARTI, O. G., AND CARPENTER, J. E. 2008. Rearing *Cactoblastis cactorum* (Lepidoptera: Pyralidae) on a factitious meridic diet at different temperatures and larval densities. Florida Entomol. 91: 679-685.

MICROBIAL ID INC. (1993) Microbial Identification System Operating Manual, Version 4. Microbial ID Inc. (Newark, Del.).

PEMBERTON, R. W., AND CORDO, H. A. 2001. *Nosema* (Microsporida: Nosematidae) species as potential biological control agents of *Cactoblastis cactorum* (Lepidoptera: Pyralidae): surveys for the microsporidia in Argentina and South Africa. Florida Entomol. 81: 527-529.

SAS. 2004. User's Guide, version 9.1. Cary, NC: SAS Institute.

TANADA, Y., AND KAYA, H. K. 1993. Insect Pathology. San Diego: Academic Press.

VIDAL-QUIST, J. C., CASTAÑERA, P., AND CABRERA, J. G. 2009. Simple and rapid method for PCR characterization of large *Bacillus thuringiensis* strain collections. Curr. Microbiol. 59: 421-425.

WINTZINGERODE, F., RAINEY, F. A., KROPPENSTEDT, R. M., AND STACKEBRANDT, E. 1997. Identification of environmental strains of *Bacillus mycoides* by fatty acid analysis and species-specific 16S rDNA oligonucleotide probe. FEMS Microbiol. Ecol. 24: 201-209.

ZIMMERMANN, H. G., MORAN, V. C., AND HOFFMANN, J. H. 2001. The renowned cactus moth, *Cactoblastis cactorum* (Lepidoptera: Pyralidae): Its natural history and threat to native *Opuntia* floras in Mexico and the United States of America. Florida Entomol. 84: 543-551.