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Short Communication

Identification of two mosquitocidal *Bacillus cereus* strains showing different host rangesHyun-Woo Park^{a,*}, Sabrina R. Hayes^a, Gail M. Stout^b, Ginny Day-Hall^c, Mark D. Latham^b, John P. Hunter^c^aJohn A. Mulrennan, Sr., Public Health Entomology Research & Education Center College of Engineering Sciences, Technology & Agriculture, Florida A&M University, Panama City, FL 32405, USA^bManatee County Mosquito Control District, Palmetto, FL 34221, USA^cCharlotte County Environmental & Extension Services, Port Charlotte, FL 33980, USA

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ABSTRACT

Mosquitocidal bacteria, M413 and C32 have been isolated from sediment samples collected from woodland and ditch, respectively. Gas chromatographic analysis of fatty acids methyl esters (GC-FAME) and 16S rRNA gene sequence alignment results showed these isolates belong to *Bacillus cereus*. The SDS-PAGE analysis of sporulated cultures of both isolates showed two major bands very similar in size. Interestingly, however, M413 is mainly toxic to 4th instars of *Ochlerotatus taeniorhynchus* whereas C32 is to those of *Culex quinquefasciatus*.

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1. Introduction

Mosquitocidal bacteria are environmentally friendly alternatives to chemical insecticides for controlling vector mosquitoes and therefore there have been tremendous world-wide efforts to isolate novel mosquitocidal bacteria with improved efficacy (Federici et al., 2006; Park et al., 2007). Despite these efforts, however, only *Bacillus thuringiensis*, *Bacillus sphaericus*, *Brevibacillus laterosporus* and *Clostridium bifermentans* are known to be mosquitocidal (Thiéry et al., 1992; Orlova et al., 1998; Federici et al., 2006; Park et al., 2006). All of these bacteria produce parasporal endotoxin crystals during sporulation and these crystal proteins are believed to be responsible for their mosquitocidal activity. Among mosquitocidal bacteria known, *B. thuringiensis* subsp. *israelensis* is the most potent and effective, and produces 4 major mosquitocidal toxins of Cry4A, Cry4B, Cry11A and Cyt1A in a parasporal body (Federici et al., 2006).

Two spore-forming bacteria, M413 and C32, were isolated from sediment samples collected from a woodland in Manatee County, Florida and a ditch in Charlotte County, Florida, respectively, using the method previously described (Park et al., 2007). For identification of M413 and C32, gas chromatographic analysis of fatty acids methyl esters (GC-FAME) (Osterhout et al., 1991; Kaufman et al., 1999) and 16S rRNA gene sequence alignment (Lane, 1991; Kolbert and Pershing, 1999) were performed. For GC-FAME, recovered

bacteria were grown on nutrient agar plates at 30 °C overnight and a 4 mm loop was used to harvest about 40 mg of bacterial cells of the streaked plates. The cells were placed in a clean 13 × 100 mm culture tubes. One ml of 15% NaOH in 50% aqueous methanol was added to each tube containing cells. The tubes were securely sealed with Teflon lined caps, vortexed briefly and heated in a boiling water bath for 5 min, at which time the tubes were vigorously vortexed for 10 s and returned to the water bath to complete the 30 min heating. The cooled tubes were uncapped; 2 ml of methanolic HCl was added. The tubes were capped and briefly vortexed. After vortexing, the tubes were heated for 10 min at 80 °C. Addition of 1.25 ml of hexane-methyl-*tert*-butyl ether (1:1, v/v) to the cooled tubes was followed by recapping and gentle tumbling on a rotary shaker for 10 min. The tubes were uncapped and the aqueous phase was pipetted out and discarded. About 3 ml of diluted NaOH was added to the organic phase remaining in the tubes; the tubes were recapped and tumbled for 5 min. Following uncapping, about 2/3 of the organic phase was pipetted into a gas chromatograph vial. Samples were analyzed with the 6850 Gas Chromatograph (Agilent technologies, Santa Clara, CA) and the Sherlock MIS software (MIDI Inc., Newark, DE). For 16S rRNA gene sequence alignment, the 16S rRNA genes from both isolates were amplified from genomic DNA by PCR. Primers used were universal 16S primers that correspond to positions 0005F and 0531R (Lane, 1991). Cycle sequencing of the 16S rRNA amplification products was carried out using DNA polymerase and dye terminator chemistry. Excess dye-labeled terminators were removed from the sequencing reactions, collected by centrifugation, dried under vacuum and frozen at –20 °C until ready to load.

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Table 1
Mosquitocidal activity of the *B. cereus* isolates in comparison with *B. thuringiensis* subsp. *israelensis* IPs-82

Bacterial strain	LC ₅₀ (fiducial limits) ^a			
	<i>Ae. aegypti</i>	<i>An. quadrimaculatus</i>	<i>Cx. quinquefasciatus</i>	<i>Oc. taeniorhynchus</i>
<i>B. cereus</i> C32	N/T ^b	N/T	82.2 (50.6–132.9)	739.2 (491.8–1459.3)
<i>B. cereus</i> M413	N/T	1155 (759.8–2654.7)	N/T	89.2 (66.0–119.9)
<i>B. thuringiensis</i> subsp. <i>israelensis</i> IPS-82	11.6 (9.7–13.9)	50.7 (37.8–70.1)	5.9 (2.9–8.7)	18.7 (13.4–24.9)

^a 24-h mortality, in nanograms per milliliter.

^b Not able to determine LC₅₀ using 1 µg/ml as the highest concentration.

Samples were resuspended in a formamide solution and denatured prior to loading. The samples were electrophoresed on the 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) and analyzed using the DNA Library D16S2 (MIDI Inc., Newark, DE). The results showed that M413 has 0.74% genetic difference and the Similarity Index of 0.846 with *Bacillus cereus*. C32 also has 0.74% genetic difference and the Similarity Index of 0.845 with *B. cereus*.

B. cereus forms the *B. cereus* group with two other closely related *Bacillus* species, *Bacillus anthracis* and *B. thuringiensis* (Vilas-Bôas et al., 2007). *B. cereus* has been extensively studied mainly because it is considered a pathogen of mammals causing food poisoning. However, Khyami-Horani et al. (1999) reported that *B. cereus* isolated from various habitats in Jordan showed activity against *Culiseta longiareolata* without providing the median lethal concentrations (LC₅₀s). Furthermore, it has been found to be the dominant bacterial species in the guts of *Aedes aegypti* and *Culex quinquefasciatus* (Luxananil et al., 2001). When *B. cereus* strains isolated from mosquito larval guts were transformed with a recombinant plasmid containing the *bin* toxin genes from *B. sphaericus* 2297, recombinant *B. cereus* strains showed significantly higher toxicity than the wild type *B. sphaericus* 2297 against *Cx. quinquefasciatus* larvae (Luxananil et al., 2003). However, significant mosquitocidal activity of the wild type *B. cereus* has never been reported against major species such as *Aedes*, *Anopheles*, *Culex* and *Ochlerotatus*.

For preliminary mosquito bioassay, both isolates were grown in 50 ml of GYS medium for 3 days at 30 °C, and *Ochlerotatus taeniorhynchus* and *Cx. quinquefasciatus* 4th instars maintained at John A. Mulrennan, Sr., Public Health Entomology Research & Education Center, Florida A & M University were used. Ten ml of undiluted bacterial culture was transferred to a 15 ml conical tube, five mosquito larvae were added and tubes were placed at 28 °C. Ten ml of tap water with five larvae was used as a control. Both isolates showed 100% mortality against these mosquito species after 24 h whereas control did not show any mortality. Therefore, advanced bioassay was performed to calculate LC₅₀s and to determine the host range of these isolates using the method previously established (Park et al., 2003, 2005). Lyophilized powder of sporulated cultures of M413, C32 and *B. thuringiensis* subsp. *israelensis* IPS-82 (Institut Pasteur, Paris, France) was tested against *Ae. aegypti*, *Cx. quinquefasciatus* and *Oc. taeniorhynchus* early 4th instars and *Anopheles quadrimaculatus* 3rd instars. *Ae. aegypti* and *An. quadrimaculatus* eggs were purchased from Benzon Research (Carlisle, PA). The results showed that M413 is mainly toxic to *Oc. taeniorhynchus* (LC₅₀ = 89.2 ng/ml) whereas C32 is to *Cx. quinquefasciatus* (LC₅₀ = 82.2 ng/ml) (Table 1). M413 was not active against *Ae. aegypti* and *Cx. quinquefasciatus*, and showed low toxicity against *An. quadrimaculatus* (LC₅₀ = 1155 ng/ml). Interestingly, it was not active against *Ae. aegypti*, a closely related species of *Oc. taeniorhynchus*. C32 did not show any toxicity against *Ae. aegypti* or *An. quadrimaculatus*, but showed low toxicity against *Oc. taeniorhynchus* (LC₅₀ = 739.2 ng/ml). None of the isolates showed higher toxicity against any mosquito species tested than *B. thuringiensis* subsp. *israelensis* IPS-82.

SDS–polyacrylamide gel electrophoresis (PAGE) was performed using the lysed GYS cultures of M413 and C32 according to estab-

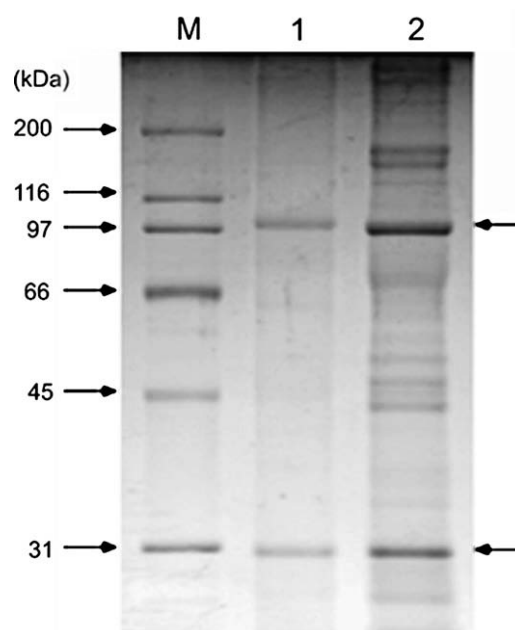


Fig. 1. Analysis of whole cell culture of the mosquitocidal *B. cereus* isolates. Sedimented spores and cellular debris obtained from equal volumes of culture medium at the end of sporulation were loaded into each lane. M, molecular size marker; lane 1, C32; lane 2, M413. Arrows indicate ~97- and 30-kDa protein bands.

lished methods (Laemmli, 1970; Park et al., 2003, 2005). During sporulation, both M413 and C32 produced two distinct major proteins of ca. 97 and 30 kDa, although M413 produced additional minor proteins with various sizes (Fig. 1). In most cases, when insecticidal bacterial cultures are analyzed using SDS–PAGE, major bands appearing on the gel are from crystal proteins (Park et al., 2003, 2005; Federici et al., 2006). However, crystals or inclusions were not observed from either isolate by both phase contrast and transmission electron microscopy (data not shown). Therefore, proteins produced from C32 and M413 during sporulation could be soluble or other non-crystal proteins such as surface layer (Pena et al., 2006; Hu et al., 2008) or spore coat (Bailey-Smith et al., 2005; Johnson et al., 2006) proteins. Identification of these two major proteins along with other minor ones is under evaluation using high-performance liquid chromatography with tandem mass spectrometry detection (LC–MS/MS) (Golovko and Murphy, 2008). Based on the LC–MS/MS results, individual proteins will be overproduced and their mosquitocidal activities will be determined. If the two major proteins of C32 and M413 detected are responsible for mosquitocidal activity, their sequences may be highly conserved with some variable regions that make difference in the toxicity spectrum.

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