

Submitted to: **Journal of the American Mosquito Control Association**

Scientific Note

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Isolation of *Bacillus sphaericus* with improved efficacy against *Culex quinquefasciatus*

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ABSTRACT. A *Bacillus sphaericus* highly toxic to fourth instar *Culex quinquefasciatus* was isolated from sediment samples collected at Winter Beach marsh in Indian River County, Florida. This isolate, named WBM 1-1-13, showed significantly higher toxicity compared with strain 2297. WBM 1-1-13 is equivalent, though better initially, to the toxicity of strain 2362, the active ingredient of VectoLex[®]. Furthermore, the Winter Beach marsh isolate produced more Bin per unit medium than strain 2362 suggesting that this new isolate could be useful for *Culex* control.

KEY WORDS *Bacillus sphaericus*, *Culex quinquefasciatus*, Bin toxin

Mosquitocidal *Bacillus sphaericus* produces two different types of toxins – Bin (for binary toxin) and Mtx (for mosquitocidal toxin) (Charles et al. 1996, Charles and Nielsen-LeRoux 2000, Federici et al. 2003). The former is composed of a 42-kDa toxin domain (BinA) and a 51-kDa binding domain (BinB). These two proteins are co-crystallized during sporulation. Three different types are known for Mtx specifically – Mtx1 (100 kDa), Mtx2 (31 kDa), and Mtx3 (36 kDa). Unlike the Bin toxin, they do not form crystals and, therefore, are degraded quickly upon synthesis during the vegetative stage. Highly mosquitocidal strains of *B. sphaericus* such as 2297 and 2362 produce both types of toxins whereas other strains produce only either Bin or Mtx.

Because of its longer persistence in polluted water and relatively high toxicity against certain species of mosquitoes, *B. sphaericus* 2362 (Weiser 1984) has been marketed under the product name VectoLex[®] (Valent BioSciences). This bacterial strain is

used for mosquito control operations along with another microbial insecticide, *B. thuringiensis* subsp. *israelensis* (Goldberg and Margalit 1977). For years, researchers have been unable to discover more potent *B. sphaericus* strains than 2362. Here we report the isolation of *B. sphaericus* (named here as WBM 1-1-13) with improved efficacy compared with 2362. WBM 1-1-13 was isolated from a sediment sample collected at the Winter Beach marsh in Indian River County, Florida using a procedure modified from Dhindsa et al. (2002). This isolate was identified with the taxonomic key of Thiéry and Frachon (1997).

Genomic DNA of *B. sphaericus* WBM 1-1-13 was analyzed along with those of control strains, 2297 and 2362, by polymerase chain reaction (PCR) using *Taq* DNA polymerase (New England Biolabs) and primers specific to known *B. sphaericus* toxin genes – *bin*, *mtx1*, *mtx2* and *mtx3*. We found that WBM 1-1-13 contained all four toxin genes that control strains had. Because the Bin toxin is the major source for mosquitocidal activity of *B. sphaericus*, the 3.5-kb DNA fragment of WBM 1-1-13 containing the *bin* operon and its promoter was amplified using Vent[™] DNA polymerase (New England Biolabs), 5'-AACTGCAGCTTGTC AACATGTGAAGATTA AAGGTAACTTTC-3' as a forward primer and 5'-AACTGCAGCTTCGCAGCTTTTTTATAAACGTCGTGACTTTA-3' as a reverse primer. The amplified fragment was cleaned, digested with *Pst*I and cloned into the same site in pUC19. Then, the cloned original fragment was sub-cloned into pUC19 using restriction enzymes – *Pst*I, *Kpn*I, *Eco*RI and *Xba*I, and sequenced at the Core Instrumentation Facility, Institute for Integrative Genome Biology, University of California, Riverside (GenBank accession number: DQ875600). The deduced amino acid sequence of WBM 1-1-13 was then aligned with those of control

strains using Vector NTI version 10.3.0 (Invitrogen). Five amino acids of WBM 1-1-13 were different from those of 2297 for BinA (at positions 99, 105, 125, 135 and 267), and 2 from those of 2362 (at positions 48 and 124). Three amino acids of WBM 1-1-13 were different from those of 2297 for BinB (at positions 314, 317 and 389) and 2 from those of 2362 (at positions 370 and 372).

For further characterization, *B. sphaericus* WBM 1-1-13 and two control strains were grown in 25 ml of sporulation medium, MBS (Kalfon et al. 1983) in 250 ml flasks at 300 rpm for 3 days at 30°C. Equal amounts of each sporulated culture were compared using SDS-polyacrylamide gel electrophoresis (PAGE). The Bin yield of WBM 1-1-13 per unit medium was less than 2297 but twice of 2362 (Fig. 1). To determine whether differences in yield were due to the number of bacterial cells, numbers of spores per unit medium produced by three *B. sphaericus* strains were counted. The results analyzed using the Super ANOVA program (Abacus Concepts) showed numbers of spores produced by WBM 1-1-13 ($4.3 \times 10^7/\text{ml}$) and 2362 ($4.7 \times 10^7/\text{ml}$) were not statistically different whereas 2297 produced significantly more spores ($7.9 \times 10^7/\text{ml}$) than the others. This suggested that the increase in Bin production of WBM 1-1-13 was possibly due to greater Bin crystal synthesis.

Laboratory-reared fourth instar *Ochlerotatus taeniorhynchus* (Wiedemann) and *Culex quinquefasciatus* (Say) obtained from the John A. Mulrennan, Sr., Public Health Entomology Research & Education Center were used to determine the toxicity of WBM 1-1-13 along with control strains 2297 and 2362 as described previously (Park et al. 2005) (Table 1). None of the strains showed significant toxicity against larvae of *Oc. taeniorhynchus*. However, initial activity of WBM 1-1-13 (LC_{50} at 24 h = 19.6 ng/ml)

was significantly greater than that of 2297 (LC₅₀ at 24 h = 106.2 ng/ml) against fourth instars of *Cx. quinquefasciatus* and slightly elevated, but not significant, for 2362 (LC₅₀ at 24 h = 27.1 ng/ml). Interestingly, toxicity of WBM 1-1-13 (LC₅₀ at 48 h = 16.1 ng/ml) was not significantly improved after 48 h compared with the two control strains.

In conclusion, as a new isolate, *B. sphaericus* WBM 1-1-13 produced significantly more Bin toxin per unit medium, and showed equivalent to, but better initial, toxicity to *Cx. quinquefasciatus* than 2362. It could be useful for controlling *Culex* vectors that transmit many medically important diseases.

We thank Brian A. Federici and Dennis K. Bideshi for their help for sequencing the *bin* gene, and Michael Hudon for his assistance for collecting sediment samples. We are also grateful to Michael Greer, Kenneth Shaffer, and Jamie Coughlin for providing larval mosquitoes during the study. This study was supported by the mosquito research grant from the Florida Department of Agriculture and Consumer Services (award number: 012014).

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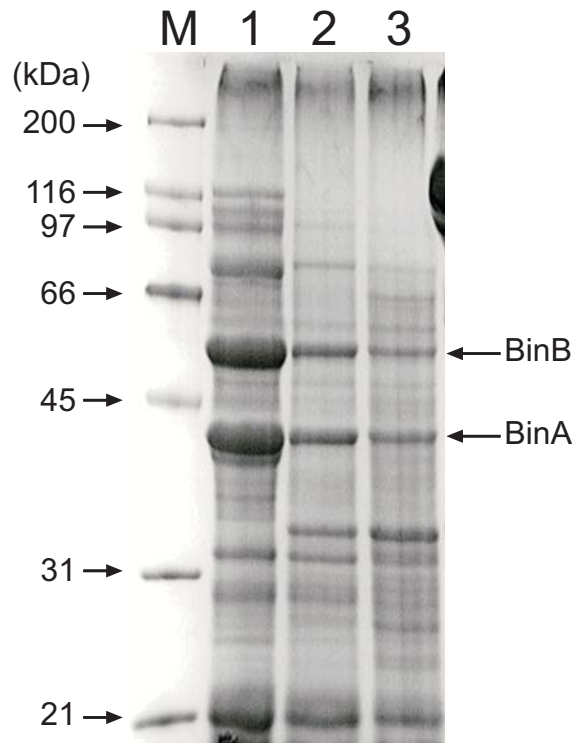


Fig. 1. Comparative Bin toxin yields produced per unit medium by *Bacillus sphaericus*.

Lane M, molecular size marker; lane 1, 2297; lane 2, WBM 1-1-13; lane 3, 2362.

BinA (42 kDa) and BinB (51 kDa) are indicated using arrows.

Table 1. Mosquitocidal activity of existing and newly isolated *Bacillus sphaericus* strains to fourth instars of *Culex quinquefasciatus*.

Strain	LC (ng/ml) of fourth instars (range)		
	50%	95%	Slope
24 hr			
2297	106.2 (83.8 – 134.7)	529.8 (369.5 – 895.9)	2.4 ± 0.3
2362	27.1 (20.5 – 35.7)	199.9 (128.3 – 390.4)	1.9 ± 0.2
WBM1-1-13	19.6 (14.6 – 25.8)	152.3 (98.2 – 294.1)	1.9 ± 0.2
48 hr			
2297	35.0 (25.1 – 46.6)	284.5 (180.3 – 589.8)	1.8 ± 0.3
2362	13.5 (9.6 – 18.4)	148.8 (91.1 – 310.6)	1.6 ± 0.2
WBM 1-1-13	16.1 (12.1 – 21.1)	111.1 (73.6 – 205.5)	2.0 ± 0.2