

Grant Title:

Isolation of Mosquitocidal Bacteria with Improved Efficacy from Various Mosquito Habitats in Florida

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Initially, soil/mud samples were collected from salt marshes and ditches at Panama City and Panama City Beach (with assistance of Beach Mosquito Control District) in Bay County and at Vero Beach (with assistance of Dr. Harry Zhong and Indian River Mosquito Control District) in Indian River County. Total 19 samples were brought to the PI's laboratory and screened using the procedure described in the proposal. In addition, letters for asking soil/mud sample collection for the project were sent out to 55 mosquito control programs in Florida. Among them, 20 mosquito control programs had responded the letter and agreed to send samples. During the first quarter of the project, 8 mosquito control programs – Anastasia Mosquito Control District (53 samples), Charlotte County Environmental & Extension Service (7 samples), Collier Mosquito Control District (24 samples), East Flagler Mosquito Control District (6 samples), Escambia County Mosquito and Rodent Management Division (39 samples), Gulf County Mosquito Control (23 samples), Manatee County Mosquito Control District (12 samples) and Saint Lucie County Mosquito Control District (16 samples) – sent us samples from various mosquito habitats including ditch, salt marsh, puddle, swamp, woodland, pastureland, pond and basin. Total number of samples received from these mosquito control programs was 180. During this report period, total 390 isolates out of 15 samples were screened, and 238 out of 390 isolates were identified as spore-formers using phase contrast microscopy. When the sporulated cultures of these spore-formers were used to test mosquitocidal activity (preliminary bioassay), 23 and 15 isolates showed more than 70% mortality against 4<sup>th</sup> instars of *Ochlerotatus taeniorhynchus* and *Culex quinquefasciatus*, respectively. When the advanced bioassay using lyophilized powders of bacterial cultures that showed 100% mortality in the preliminary bioassay, one Bs isolate (WBM-1-13) showed high toxicity against *Cx. quinquefasciatus* 4<sup>th</sup> instars. Total 18 Bs-like and 45 Bt-like isolates were observed in this period.

During the second quarter, only 5 more samples were received from Lake County Mosquito/Aquatic Plant Management. Total 403 isolates out of 20 samples were screened, and 247 out

of 403 isolates were identified as spore-formers using phase contrast microscopy. When the sporulated cultures of these spore-formers were used to test mosquitocidal activity (preliminary bioassay), 22 and 10 isolates showed more than 70% mortality against fourth instars of *Ochlerotatus taeniorhynchus* and *Culex quinquefasciatus*, respectively. However, none of these isolates showed significant toxicity when the advanced bioassay using lyophilized powder of bacterial culture was performed.

To characterize WBM 1-1-13, *B. sphaericus* isolate showed promising results in the previous report period, *Oc. taeniorhynchus* and *Cx. quinquefasciatus* fourth instars were used to determine the LC values of WBM 1-1-13 along with two control strains, 2297 and 2362. The latter is an active ingredient of VectoLex<sup>®</sup>. None of them showed significant toxicity against fourth instars of *Oc. taeniorhynchus*. Against fourth instars of *Cx. quinquefasciatus*, initial activity of WBM 1-1-13 (LC<sub>50</sub> at 24 h = 19.6 ng/ml) was significantly higher than that of 2297 (LC<sub>50</sub> at 24 h = 106.2 ng/ml) and was a little higher, but not significant, to that of 2362 (LC<sub>50</sub> at 24 h = 27.1 ng/ml). Interestingly, toxicity of WBM 1-1-13 (LC<sub>50</sub> at 48 h = 16.1 ng/ml) did not improved significantly after 48 h due to its high initial activity whereas those of two control strains did [LC<sub>50</sub>s at 48 h = 35.0 (for 2297) and 13.5 (for 2362)] (Table 1).

To determine a protein profile, *B. sphaericus* WBM 1-1-13 and two control strains were grown in 25 ml of sporulation medium, MBS in 250 ml flasks at 300 rpm, for 3 days at 30°C. When the same amount of each sporulated culture was compared using SDS-polyacrylamide gel electrophoresis (PAGE), the Bin yield of WBM 1-1-13 per unit medium was less than that of 2297 but at least twice as much as that of 2362 (Figure 1). To determine whether these differences in yield were due to the differences in number of bacterial cells, numbers of spores per unit medium produced by three *B. sphaericus* strains were counted. The results showed numbers of spores produced by WBM 1-1-13 ( $4.3 \times 10^7$ /ml) and 2362 ( $4.7 \times 10^7$ /ml) were not statistically different whereas 2297 produced significantly more spores ( $7.9 \times 10^7$ /ml) than the others. This suggested that more Bin production of WBM 1-1-13 than 2362 was not because of more cells per unit medium but possibly because of larger Bin crystal synthesis.

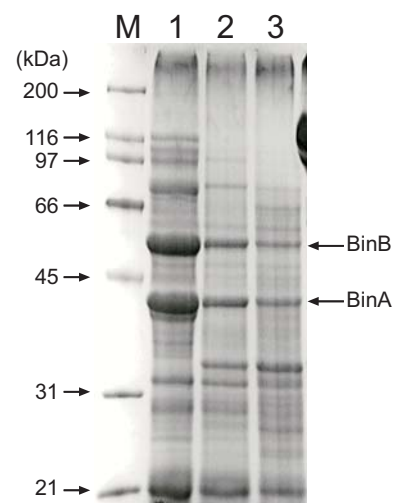
A 3.4-kb DNA fragment containing the *bin* toxin gene of WBM 1-1-13 was amplified using 5'-A ACTGCAGCTTGTCAACATGTGAAGATTAAGGTAACCTTTC-3' as a forward primer and 5'-AAC TGCAGCTTCGCAGCTTTTTTATAAACG TCGTGACTTTA-3' as a reverse primer. The amplified fragment was cleaned, digested with *Pst*I and cloned into 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 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 aligned with those of control strains using Vector NTI version 10.3.0 (Invitrogen). For BinA, 5 amino acids of WBM 1-1-13 were different from those of 2297 (at positions 99, 105, 125, 135 and 267), and 2 from those of 2362 (at positions 48 and 124). For BinB, 3 amino acids of WBM 1-1-13 were different from those of 2297 (at positions 314, 317 and 389) and 2 from those of 2362 (at positions 370 and 372).

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

Figure 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.



We received 400 samples from Orange County Mosquito Control during the third quarter. Total 406 isolates out of 19 samples were screened, and 249 out of 406 isolates were identified as spore-formers using phase contrast microscopy. When the sporulated cultures of these spore-formers were used to test mosquitocidal activity (preliminary bioassay), 28 and 23 isolates showed more than 70% mortality against fourth instars of *Ochlerotatus taeniorhynchus* and *Culex quinquefasciatus*, respectively. However, none of these isolates showed significant toxicity when the advanced bioassay using lyophilized powder of bacterial culture was performed.

During the final quarter, we have screened 25 samples from 9 different counties. Total 1,200 isolates were recovered and 252 of them identified as ‘of interest.’ Out of 252 isolates, 158 were Bt-type and 94 were Bs-type. One hundred thirty two isolates were screened further after excluding isolates that look similar by phase contrast microscopy. Preliminary mosquito bioassay using whole culture was performed against early 4<sup>th</sup> instars of *Culex quinquefasciatus* and *Ochlerotatus taeniorhynchus* (Table 1).

Table 1. Mosquitocidal activity of bacterial isolates against *Cx. quinquefasciatus* and *Oc. taeniorhynchus* 4<sup>th</sup> instars using whole cell culture

Mortality (%) <sup>a</sup>	No. of isolates	
	<i>Cx. quinquefasciatus</i>	<i>Oc. taeniorhynchus</i>
100 – 70	42	37
69 – 30	16	12
29 – 0	54	83

<sup>a</sup> Mortality was recorded after 24 hr of incubation.

Within 24 hr, 30 and 26 isolates showed 100% mortality against *Cx. quinquefasciatus* and *Oc. taeniorhynchus*, respectively. Among them, 22 isolates showed 100% mortality against both mosquito species. Using isolates showing 100% mortality, advanced mosquito bioassay was performed. Sporulated bacterial culture was lyophilized and 6 different concentrations of the lyophilized powder ranging from 5 to 200 ng/ml were used. Unfortunately, none of them showed either equivalent or higher toxicity compared with the control strains, *Bacillus thuringiensis* subsp. *israelensis* and *B. sphaericus* 2362.

Although we could not find any bacterial isolate with high toxicity during this period, we have some isolates that have not been completely analyzed but showed potentials. These isolates will be

continuously analyzed. In addition, because bacterial pellets were used for protein gel analysis, strains that did not have major protein bands in SDS-PAGE but showed 100% mortality within 5 hr in preliminary bioassay may secrete mosquitocidal proteins rather than forming crystals. Therefore, supernatant of these bacterial cultures will be treated with NaOH and ammonium sulfate to precipitate secreted proteins. Then the precipitated proteins will be concentrated and dialyzed for bioassay.

The *B. sphaericus* WBM 1-1-13 isolated from Indian River County has been filed for the US patent (Park, H.-W., C. M. Mangum and H. Zhong. 2007. A novel mosquitocidal bacterium and its toxin. US Patent Application No. 11/770,203), and a manuscript describing this isolate has been submitted for publication to the Journal of the American Mosquito Control Association (Manuscript No. 5663; Please see the attachment). Lastly, an undergraduate student to assist this project during this summer (July – August) was hired.