Insecticidal Activity of *Chromobacterium vaccinii*[1]

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Abstract New *Chromobacterium* sp. strains were isolated from water collected from several localities in the eastern United States and were subsequently identified as the species *Chromobacterium vaccinii* Soby et al. Bacteria were cultured in a liquid medium and applied to artificial insect diets in the laboratory. All of the recently discovered *C. vaccinii* isolates tested were toxic to larvae of the seedcorn maggot, *Delia platura* (Meigen), but had little activity against larvae of the diamondback moth, *Plutella xylostella* (L.). None of the new isolates of *C. vaccinii* were toxic to adults of the red flour beetle, *Tribolium castaneum* (Herbst).

Key words: *Chromobacterium vaccinii*, *Delia*, *Plutella*, *Tribolium*

The genus *Chromobacterium* is comprised of Gram-negative bacteria that typically occur in soil and water. Many isolates produce the purple pigments violacein and deoxyviolacein. Until recently, the genus *Chromobacterium* was represented by a single species, *Chromobacterium violaceum* Bergonzini. Then, Martin et al. (2007) reported the discovery of a second species, *Chromobacterium subtsugae* Martin et al., which possessed insecticidal activity against the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) as well as against several other insect pests. Since that time, an additional seven species of *Chromobacterium* have been described. A preparation of *C. subtsugae* is now commercially available as an organic insecticide under the trade name Grandevo™ (Marrone BioInnovations, Davis, CA, USA). Asolkar et al. (2014) reported that *C. subtsugae* produces three insecticidal factors including "chromamide A," violacein, and one unidentified compound.

Chromobacterium vaccinii Soby et al. was originally isolated from cranberry, *Vaccinium macrocarpon* Ait., bogs in Massachusetts, USA (Soby et al. 2013), and a patent was granted for this species claiming insecticidal activity against dipterous and lepidopterous insects (Martin and Soby 2016). However, to date no studies demonstrating insecticidal activity in *C. vaccinii* have been published in scholarly journals. Vöing et al. (2015) reported a draft genome sequence for this species and refer to it as a potential biocontrol agent against mosquitoes, but give no data on

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Table 1. Origins of *Chromobacterium* isolates included in the present study.

Isolate	Species	Locality	Habitat
PRAA4-1^T	*C. subtsugae*	Frederick Co., Maryland	Forest soil
MWU205^T	*C. vaccinii*	Cape Cod National Seashore, Massachusetts	Wild cranberry bog
IIBBL 21-1	*C. vaccinii*	Beltsville, Prince Georges Co., Maryland	Pond
IIBBL 163-2	*C. vaccinii*	Kent Co., Maryland	Pond
IIBBL 178-1	*C. vaccinii*	Montgomery Co., Maryland	Puddle
IIBBL 192-1	*C. vaccinii*	Laurel, Prince Georges Co., Maryland	Pond
IIBBL 194-1	*C. vaccinii*	Poland, Androscoggin Co., Maine	Sphagnum bog
IIBBL 199-1	*C. vaccinii*	Poland, Androscoggin Co., Maine	Pond
IIBBL 211-1	*C. vaccinii*	Camden Co., New Jersey	White cedar swamp
IIBBL 224-1	*C. vaccinii*	Charles Co., Maryland	Tidal marsh

insecticidal activity. Herein, we report new isolates of *C. vaccinii* with insecticidal activity along with similar results for the type strain of *C. vaccinii*.

Materials and Methods

Isolation and culture of bacteria. Water samples were collected from aquatic habitats at several localities in the eastern United States (Table 1). *Chromobacterium* isolates were obtained as described by Blackburn et al. (2017). Bacteria were cultured on a solid medium modified from Keeble and Cross (1977) consisting of 1 g yeast extract (Bacto; Becton, Dickinson; Sparks, MD, USA), 3 g nutrient broth (Bacto), 10 g glucose (Sigma-Aldrich, St. Louis, MO, USA), and 18 g agar (Bacto), per liter of water. The pH of the medium was adjusted to 7.3 by the addition of sodium hydroxide. After this mixture was autoclaved and allowed to cool to 55°C, sterile-filtered solutions of the antibiotics neomycin (Bio-Serv, Flemington, NJ, USA) and cycloheximide (Sigma-Aldrich) were added to make a final concentration of 50 mg/Lof each antibiotic. For bioassays, isolates were cultured in a liquid medium with the same components listed above but without agar or antibiotics. Liquid cultures were shaken on an orbital shaker (Model C25KC, New Brunswick Scientific, Edison, NJ, USA) at 200 rpm and 24°C for 96 h. Positive controls were isolate PRAA4-1^T, the type strain of *C. subtsugae* (Martin et al. 2007), and the type strain of *C. vaccinii*, MWU205^T (Soby et al. 2013).

Identification of bacteria. Isolates were identified based on their 16S rRNA gene sequences. The 16S rRNA genes were amplified from genomic DNA using

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primers universal to prokaryotes, fD1 (AGAGTTTGATCCTGGCTCAG; Weisburg et al. 1991) and R16R0 (GATACCTTGTTACGACTTAACCCC; Lee et al. 1993) and sequenced with the same primers using BigDye® terminator chemistry on an ABI Genetic Analyzer 3130XL (PE Applied Biosystems, Grand Island, NY, USA).

Seedcorn maggot bioassays. Larvae of the seedcorn maggot, *Delia platura* (Meigen), were obtained from a colony maintained on whole lima beans with meat and bone meal (Baker Commodities, Rochester, NY, USA) at the Invasive Insect Biocontrol and Behavior Laboratory (USDA/ARS, Beltsville, MD, USA). Bioassays were conducted with freeze-dried diet pellets after Martin (2004). An artificial diet consisting of 50 g ground lima beans (Bio-Serv), 1.35 g meat and bone meal, and 10 g agar in 500 ml water was used. All dry materials were added to boiling water and blended. Hot diet was pipetted into 96-well enzyme-linked immunosorbent assay (ELISA) plates with a volume of 300 μl per well, allowed to cool, and frozen. Diet was then freeze-dried and resulting pellets were removed from the ELISA plates. Plastic bioassay trays (Bio-BA 128®, Bio-Serv) were used. Approximately 1 g dry quartz sand, passed through a 850 um-mesh screen, was placed in each cell of the bioassay tray and moistened with 150 μl of deionized water. Two diet pellets were placed on top of the moist sand and rehydrated with 300 μl of liquid each (whole culture or water). Two 6-d-old larvae were placed in each cell. Cells were covered with vented plastic covers. Trays were held at 20% relative humidity (RH) and 27°C in an incubator (Model I36VLC8, Percival, Perry, IA, USA) equipped with a desiccant drier (Model IAT-75RE, Innovative Air Technologies, Covington, GA, USA) for 21 d. Puparia were then counted. Proportion pupation was calculated, normalized by arcsine $\sqrt{}$ transformation, and analyzed by analysis of variance (ANOVA) (PROC GLM, SAS Institute 2010). When significant treatment effects were found, means were separated by the least significant difference (LSD) test.

Two bioassays of the seedcorn maggot were conducted. The first bioassay included isolates PRAA4-1^T, MWU205^T, IIBBL 21-1, and a negative control (water only). The second bioassay included PRAA4-1^T, IIBBL 199-1, IIBBL 224-1, and a control. In all bioassays, 24 larvae per treatment were included. Both bioassays were replicated four times.

Diamondback moth bioassays. Larvae of the diamondback moth, *Plutella xylostella* (L.), were obtained from a colony maintained on artificial diet (Shelton et al. 1991) at the Invasive Insect Biocontrol and Behavior Laboratory (USDA/ARS, Beltsville, MD, USA). Freeze-dried pellets of gypsy moth, *Lymantria dispar* (L.), diet (Bell et al. 1981) were used because they absorb and hold liquid better than do pellets of diamondback moth diet (Shelton et al. 1991), and the larvae develop to pupation normally on both diets. One diet pellet was placed in each cell of a bioassay tray, without sand. Pellets were rehydrated as before. Two early second instars were placed on each pellet. Trays were held at 27°C for 14 d. Proportion pupation was calculated and analyzed as above.

Three bioassays were conducted with the diamondback moth. The first bioassay included PRAA4-1^T, IIBBL 21-1, MWU205^T, and a control. The second bioassay included PRAA4-1^T, IIBBL 194-1, IIBBL 211-1, IIBBL 224-1, and a control. The third bioassay included PRAA4-1^T, IIBBL 163-2, IIBBL 178-1, IIBBL 192-1, IIBBL 199-1, and a control. Twenty-four larvae per treatment were included in all bioassays. The first bioassay was replicated five times; the second and third bioassays were replicated four times each.

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Red flour beetle bioassay. Adults of the red flour beetle, *Tribolium castaneum*, were obtained from Carolina Biological Supply, Burlington, NC, USA. The method of Milutinović et al. (2013) was used to test this insect. Briefly, 10 ml of liquid culture or water were mixed with 1.25 g of a dry medium consisting of 95% organic white flour (Arrowhead Mills, Boulder, CO, USA) and 5% brewer's yeast (Bio-Serv). Forty microliters of this mixture was pipetted into each well of a transparent 96-well ELISA plate (Costar® 3369, Corning, Inc., Corning, NY, USA) and dried for 24 h at 50°C. One adult beetle was placed in each well and the plate was covered with an adhesive transparent plastic cover (SealPlate®, Sigma-Aldrich, St. Louis, MO, USA.) An insect pin was used to punch three holes in the cover over each cell for ventilation. Plates were held at 27°C. Beetles were scored at 6 wk. Proportion mortality was calculated and analyzed as above.

Two bioassays were conducted with the red flour beetle. Isolates PRAA4-1^T, IIBBL 21-1, MWU205^T, and a control were included in the first bioassay. The second bioassay included PRAA4-1^T, IIBBL194-1, IIBBL 211-1, IIBBL 224-1, and a control. Twenty-four beetles per treatment were included and each bioassay was replicated four times.

Results

Identification of bacteria. The 1,180 nucleotide sequences obtained for the 16S gene from isolates IIBBL 163-2, IIBBL 178-1, IIBBL 192-1, IIBBL 194-1, IIBBL 199-1, and IIBBL 211-1 were found to be identical to the corresponding sequence of the *C. vaccinii* MWU205^T 16S rRNA gene available in GenBank (National Center for Biotechnology Information [NCBI] Reference Sequence: NZ_JZJL01000120.1) while IIBBL 224-1 differed by four nucleotides. The four substitutions found in the IIBBL 224-1 gene were restricted to two pairs of residues 37 nucleotides apart and probably represent two (or fewer) events. Based on their 16S sequence homology, these isolates were *C. vaccinii*. The eight 16S rRNA gene sequences encoded in IIBBL 21-1 were obtained from full genomic sequence data (GenBank assembly: ASM185527v1) and revealed that five of eight total copies of the 16S rRNA gene encoded in the genome were identical to that of *C. vaccinii* type strain MWU205^T while three 16S gene copies differed by a single nucleotide. The identity of isolate IIBBL 21-1 as *C. vaccinii* was further confirmed by alignment of its entire 5-Mb genome sequence with that of MWU205^T (GenBank assembly: ASM97133v1). Blast-based average nucleotide identity (ANIb) was calculated for the aligned genome sequences using the application JSpecies and found to be ~98.2% identical, well above the 95% threshold, indicating that the two strains are the same species (Richter and Rosselló-Móra 2009).

Bioassays. In both bioassays against the seedcorn maggot, rates of pupation were significantly affected by treatment (first bioassay: $F = 28.95$; df = 3, 9; $P = 0.0001$; second bioassay: $F = 36.07$; df = 3, 9; $P = 0.0001$) (Table 2). Pupation on all isolates of *C. vaccinii* was lower than those on the control. Rates of pupation on the new isolates of *C. vaccinii* were lower than those on PRAA4-1^T. Rates of pupation on PRAA4-1^T were lower than those on the control in both bioassays.

Rates of pupation by larvae of the diamondback moth were significantly affected by treatment in all tests (first bioassay: $F = 40.15$; df = 3, 12; $P = 0.0001$; second

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Table 2. Bioassays of *Chromobacterium* spp. against the seedcorn maggot.

Bioassay	Isolate	% Mean Pupation ± SE*	*F*	df	*P*
1	Control	74.0 ± 13.65 A	28.95	3, 9	0.0001
	PRAA4-1^T	15.6 ± 10.12 B			
	MWU205^T	7.3 ± 5.98 BC			
	IIBBL 21-1	3.1 ± 3.13 C			
2	Control	87.5 ± 2.41 A	36.07	3, 9	0.0001
	PRAA4-1^T	69.8 ± 8.22 B			
	IIBBL 224-1	31.3 ± 10.42 C			
	IIBBL 199-1	28.1 ± 7.49 C			

* Within a bioassay, means with the same uppercase letter are not significantly different by LSD ($P > 0.05$).

bioassay: $F = 32.75$; df = 4, 12; $P = 0.0001$; third bioassay: $F = 7.89$; df = 5, 15; $P = 0.0001$) (Table 3). Rates of pupation by the diamondback moth were slightly lower on the *C. vaccinii* isolates than on the control in the second bioassay but did not differ from the control in the other bioassays. Pupation on PRAA4-1^T was lower than those on all other treatments in all tests.

Mortality of the red flour beetle was significantly affected by *Chromobacterium* isolate in both bioassays (first bioassay: $F = 161.08$; df = 3, 9; $P = 0.0001$; second bioassay: $F = 69.68$; df = 4, 12; $P = 0.0001$) (Table 4). High rates of mortality of this insect on PRAA4-1^T were seen in both bioassays, but no other isolate differed from the water control.

Discussion

All new isolates of *C. vaccinii* isolated in the eastern United States and tested against the seedcorn maggot exhibited insecticidal activity, as did the type strain of *C. vaccinii*, MWU205^T. The spectrum of activity of *C. vaccinii* clearly differed from that of the well-characterized biopesticide strain *C. subtsugae* PRAA4-1^T. While PRAA4-1^T was toxic to all insect species assayed in the present study, *C. vaccinii* was highly toxic only to the dipteran seedcorn maggot. The type strain of *C. vaccinii*, MWU205^T, did not differ in activity from the control against the diamondback moth. Some other isolates of *C. vaccinii* showed very limited activity against the diamondback moth. No isolate of *C. vaccinii* was toxic to the red flour beetle.

Even though they were collected from geographically and ecologically diverse locations, no substantial differences in insecticidal activity were noted among the new isolates of *C. vaccinii*. Isolates included in the present study represented collections from nine sites in four states. In no bioassay were significant differences among isolates of *C. vaccinii* found. These results suggest there is low variability in insecticidal activity among *C. vaccinii* isolates from a relatively wide geographic area.

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Table 3. Bioassays of *Chromobacterium* spp. against the diamondback moth.

Bioassay	Isolate	% Mean Pupation ± SE*	*F*	df	*P*
1	Control	72.5 ± 8.80 A	40.15	3, 12	0.0001
	MWU205^T	65.8 ± 6.37 A			
	IIBBL 21-1	60.0 ± 9.37 A			
	PRAA4-1^T	9.2 ± 5.17 B			
2	Control	96.9 ± 1.04 A	32.75	4, 12	0.0001
	IIBBL 194-1	84.9 ± 3.90 B			
	IIBBL 224-1	82.3 ± 5.48 B			
	IIBBL 211-1	80.2 ± 4.29 B			
	PRAA4-1^T	31.3 ± 5.51 C			
3	Control	87.5 ± 5.38 A	7.89	5, 15	0.0008
	IIBBL 192-1	85.4 ± 7.70 A			
	IIBBL 199-1	81.3 ± 3.61 A			
	IIBBL 163-2	78.1 ± 14.27 A			
	IIBBL 178-1	77.1 ± 4.96 A			
	PRAA4-1^T	36.5 ± 1.04 B			

* Within a bioassay, means with the same uppercase letter are not significantly different by LSD ($P > 0.05$).

Table 4. Bioassays of *Chromobacterium* spp. against the red flour beetle.

Bioassay	Isolate	% Mean Mortality ± SE*	*F*	df	*P*
1	PRAA4-1^T	95.8 ± 1.70 A	161.08	3, 9	0.0001
	Control	9.4 ± 1.99 B			
	IIBBL 21-1	8.6 ± 1.49 B			
	MWU205^T	7.6 ± 1.87 B			
2	PRAA4-1^T	87.5 ± 7.41 A	69.68	4,12	0.0001
	IIBBL 211-1	12.5 ± 2.95 B			
	Control	9.4 ± 1.95 B			
	IIBBL 194-1	8.3 ± 2.95 B			
	IIBBL 224-1	5.2 ± 1.99 B			

* Within a bioassay, means with the same uppercase letter are not significantly different by LSD ($P > 0.05$).

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The genetic determinants accounting for the differences in activity among species of *Chromobacterium* have not yet been characterized, but suggest multiple insecticidal factors with different activity spectra. The present results would be consistent with the presence of a factor or factors in cultures of PRAA4-1^T that are toxic to the diamondback moth and red flour beetle and that are not present, or are present at lower levels, in cultures of *C. vaccinii*.

Soby et al. (2013) reported that *C. vaccinii* could be isolated from roots of cranberry, but not from several other plants growing in the same bogs. However, cranberry was absent from all sampling sites in the current study. Thus, while *C. vaccinii* may have a special relationship with cranberry where this plant occurs, cranberry is not required for the presence of *C. vaccinii*. In our experience sampling wetlands and bogs, *C. vaccinii* appears to be the most abundant and broadly distributed species of *Chromobacterium* in the eastern United States.

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