Insecticidal Activity of a Recently Described Bacterium, *Chromobacterium sphagni*[1]

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Abstract Several isolates of the recently described bacterial species *Chromobacterium sphagni* Blackburn et al. were obtained from water collected from *Sphagnum* bogs in West Virginia and Maine. Bacterial isolates were cultured in a liquid medium and applied to artificial insect diets in the laboratory. The new isolates were toxic to larvae of the gypsy moth, *Lymantria dispar* (L.), and the diamondback moth, *Plutella xylostella* (L.), but were not toxic to larvae of the seedcorn maggot, *Delia platura* (Meigen), or adults of the red flour beetle, *Tribolium castaneum* (Herbst).

Key Words *Chromobacterium*, *Lymantria*, *Plutella*, *Delia*, *Tribolium*

The genus *Chromobacterium* is comprised of Gram-negative bacteria that typically occur in soil and water. Many isolates produce purple pigments, violacein, and deoxyviolacein. Martin et al. (2007a) reported the discovery of a new species, *C. subtsugae* Martin et al., that possesses insecticidal activity against the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), as well as several other insect pests. Additionally, activity of this species against the southern green stink bug, *Nezara viridula* L., and corn rootworms, *Diabrotica* spp., was reported by Martin et al. (2007b). A commercial preparation of *C. subtsugae* is now available for use as an organic insecticide under the trade name Grandevo™ (Marrone BioInnovations, Davis, CA). Asolkar et al. (2014) reported that *C. subtsugae* produced three insecticidal factors, including chromamide A, violacein, and one unidentified compound. No other data on the insecticidal activity of any *Chromobacterium* species have been published in scholarly journals.

Chromobacterium subtsugae was described from a single isolate collected in the Catoctin Mountains of Maryland, USA. Attempts to find additional isolates of the same species in the same area were unsuccessful. Efforts are ongoing to find additional isolates of *C. subtsugae* and other species of *Chromobacterium* with insecticidal activity. Recently, a new species, *C. sphagni* Blackburn et al., was described from *Sphagnum* bogs in West Virginia and Maine (Blackburn et al. 2017).

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Herein, we report results of bioassays of the new species against representative insects and compare its activity with that of *C. subtsugae*.

Materials and Methods

Bacteria. Water samples were collected from *Sphagnum* bogs in Red Creek Plains near Laneville, WV on 20 August 2012 and near Poland, ME on 21 June 2013. The isolation of the bacteria and their determination as a new species are described by Blackburn et al. (2017). Isolates were maintained on a solid medium modified from Keeble and Cross (1977), consisting of 1 g yeast extract (Bacto; Becton Dickinson, Sparks, MD), 3 g nutrient broth (Bacto), 10 g glucose (Sigma-Aldrich, St. Louis, MO), and 18 g agar (Bacto) per liter. 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) and cycloheximide (Sigma-Aldrich) were added to make a final concentration of 50 mg/liter of each antibiotic. Isolates were subcultured weekly and maintained at 24°C. 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.

Four isolates of *C. sphagni* were obtained from the West Virginia samples and were designated 14B-1 (the type strain), 14B-4, 14B-5, and 14B-6. Twelve isolates were obtained from the Maine samples. These isolates were designated 36-1 through 36-6 and 37-1 through 37-6.

Gypsy moth bioassay. Gypsy moth, *Lymantria dispar* (L.), eggs were obtained from USDA/APHIS, Otis Air National Guard Base, Buzzards Bay, MA. Larvae were reared to the early second instar on artificial diet (Bell et al. 1981). Bioassays were conducted with freeze-dried diet pellets according to Martin (2004). Briefly, hot diet (Bell et al. 1981) was poured into 96-well enzyme-linked immunosorbent assay (ELISA) plates with a volume of 300 μl per well, were allowed to cool, and were frozen. Diet was then freeze dried, and resulting pellets were removed from the ELISA plates. One pellet was placed in each cell of a plastic bioassay tray (Bio-BA 128®; Bio-Serv). Each pellet was rehydrated with 300 μl of either undiluted liquid culture or, for a control, water only. Two early second instars were placed in each cell, and the cells were covered with vented transparent plastic covers (Bio-CV 16®; Bio-Serv). Trays were held at 27°C for 6 d. Larvae were then scored as alive or dead. Proportion mortality was calculated, normalized by arcsine $\sqrt{}$ transformation, and analyzed by analysis of variance (ANOVA; PROC GLM) (SAS Institute 2010). When significant treatment effects ($P < 0.05$) were found, means were separated by the least significant difference test.

Included in the gypsy moth bioassay were West Virginia isolates 14B-1, 14B-4, 14B-5, and 14B-6. Also included were *C. subtsugae* isolate PRAA4-1^T, as a positive control, the *Bacillus thuringiensis* Berliner var. *israelensis* isolate IBL-163 as a negative control, and water only. Twenty-four larvae per treatment were included, and the bioassay was replicated five times.

Diamondback moth bioassays. Larvae of the diamondback moth, *Plutella xylostella* (L.), were obtained from a colony maintained on artificial diet (Shelton et

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al. 1991) at the Invasive Insect Biocontrol and Behavior Laboratory (USDA/ARS, Beltsville, MD). The procedure was similar to that used for the gypsy moth, except that the insects were reared to pupation before being scored. Pellets of gypsy moth diet (Bell et al. 1981) were again used because they absorb and hold liquid better than do pellets of diamondback moth diet, and the larvae develop to pupation normally on both diets. Proportion pupation was calculated and analyzed as above.

Two experiments were conducted on diamondback moth. The first experiment included the same treatments that the gypsy moth bioassay included. The second experiment included Maine isolates 37-2 and 37-5 (selected from among 12 isolates based on preliminary tests), West Virginia isolate 14B-1, PRAA4-1^T, and a water-only control. Twenty-four larvae per treatment were included, and both experiments were replicated five times.

Seedcorn maggot bioassays. Larvae of the seedcorn maggot, *Delia platura* (Meigen), were obtained from a colony maintained on whole lima beans with meat and bonemeal (Baker Commodities, Rochester, NY) at the Invasive Insect Biocontrol and Behavior Laboratory (USDA/ARS, Beltsville, MD). For the bioassays, a medium consisting of 50-g ground lima beans (Bio-Serv), 1.35-g meat and bonemeal, and 10-g agar in 500 ml water was used. All dry materials were added to boiling water and blended. Freeze-dried pellets were then made as before. Approximately 1-g dry quartz sand, passed through a 850 μm screen, was placed in each cell of the bioassay trays. The sand in each cell was moistened with 260 μl of deionized water. Two diet pellets were placed on top of the moist sand and rehydrated with 300 μl of liquid each. Two 6-d-old larvae were placed in each cell. Cells were covered with vented plastic covers. Trays were held at 27°C, 60% relative humidity for 7 d and then at 24°C, 40% relative humidity for 14 additional days. Puparia were then counted. Proportion pupation was calculated and analyzed as above.

Two experiments were conducted with the seedcorn maggot. In the first experiment, isolates 14B-1, PRAA4-1^T, and IBL 163 were included, along with a water-only control. In the second experiment, isolates 14B-1, 37-2, 37-5, and PRAA4-1^T and control were included. In both experiments, 24 larvae per treatment were included, and the experiments were each replicated five times.

Red flour beetle bioassay. Adults of the red flour beetle, *Tribolium castaneum* (Herbst), were obtained from Carolina Biological Supply, Burlington, NC. The method of Milutinovic' et al. (2013) was used to test this insect. Briefly, 10 ml of liquid culture or water was mixed with 1.25 g of a dry medium consisting of 95% organic white flour (Arrowhead Mills, Boulder, CO) 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) 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). An insect pin was used to punch three holes in the cover over each well for ventilation. Plates were held at 27°C. Beetles were scored at 6 weeks. Proportion mortality was calculated and analyzed as above.

A single experiment was conducted with the red flour beetle. Isolates 14B-1, 37-2, and PRAA4-1^T were included. As negative controls in this experiment, the nontoxic *B. thuringiensis* var. *finitimus* isolate IBL-717 and water-only control were

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Table 1. Bioassays of West Virginia *Chromobacterium* isolates against the gypsy moth, diamondback moth, and seedcorn maggot (means ± standard errors).

Treatment/ Isolate	Gypsy Moth Mortality (%)*	Diamondback Moth Pupation (%)*	Seedcorn Maggot Pupation (%)*
Water only	1.7 ± 1.67 A	68.6 ± 3.29 A	**
IBL-163	0.8 ± 0.83 A	71.1 ± 4.69 A	85.8 ± 4.68 A
PRAA4-1 ^{T}	29.2 ± 9.03 B	28.3 ± 6.64 B	11.07 ± 4.64 B
14B-1	29.2 ± 10.37 B	5.0 ± 2.43 D	85.8 ± 4.64 A
14B-4	29.2 ± 10.46 B	9.2 ± 3.33 CD	**
14B-5	30.5 ± 10.43 B	20.8 ± 6.04 BC	**
14B-6	28.3 ± 7.84 B	15.8 ± 4.25 BC	**

* Means with the same letter are not significantly different by least significant difference ($P > 0.05$).
** Not tested.

also included. Twenty-four beetles per treatment were included, and the experiment was replicated four times.

Results

Gypsy moth bioassay. The mortality of the gypsy moth larvae was significantly affected by isolate ($F = 13.22$; df = 6, 24; $P = 0.0001$). Mortality was higher on all West Virginia isolates than that on the control (Table 1). None of the West Virginia isolates differed from PRAA4-1^{T}. The mortality of larvae on *B. thuringiensis* IBL-163 did not differ from that on the water control.

Diamondback moth bioassays. In the first experiment, percent pupation was significantly affected by the *Chromobacterium* isolate ($F = 23.40$; df = 6, 24; $P = 0.0001$). Rates of pupation were lower on all *Chromobacterium* treatments than that on the control (Table 1). Pupation was lower on West Virginia isolate 14B-1 than on PRAA4-1^{T} or isolates 14B-5 or 14B-6. Pupation on IBL-163 did not differ from that on the water control.

In the second experiment, pupation was again significantly affected by isolate ($F = 14.77$; df = 4, 16; $P = 0.0001$). Pupation on the Maine isolates 37-2 and 37-5 was intermediate between that of the control and that of the other *Chromobacterium* isolates (Table 2). In this experiment, 14B-1 did not differ from PRAA4-1^{T}.

Seedcorn maggot bioassays. In the first experiment, percent pupation was significantly affected by treatment ($F = 108.14$; df = 2, 8; $P = 0.0001$). Percent pupation on 14B-1 did not differ from that of IBL-163 (Table 1), but pupation of PRAA4-1^{T} was lower than that of the other two treatments.

In the second experiment, pupation was again significantly affected by *Chromobacterium* isolate ($F = 9.62$; df = 4, 16; $P = 0.0001$). Pupation was lower

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Table 2. Bioassays of Maine and West Virginia *Chromobacterium* isolates against the diamondback moth, seedcorn maggot, and red flour beetle (means ± standard errors).

Treatment/ Isolate	Diamondback Moth Pupation (%)*	Seedcorn Maggot Pupation (%)*	Red Flour Beetle Mortality (%)*
Water only	72.5 ± 8.80 A	45.0 ± 5.17 A	9.4 ± 1.99 A
IBL-717	**	**	11.5 ± 4.92 A
PRAA4-1^T	9.2 ± 5.17 C	20.0 ± 2.04 B	95.8 ± 1.70 B
14B-1	13.3 ± 5.50 C	51.7 ± 8.29 A	16.7 ± 7.61 A
37-2	45.0 ± 9.72 B	51.7 ± 6.54 A	24.1 ± 8.13 A
37-5	43.3 ± 12.19 B	44.2 ± 4.49 A	**

* Means with the same letter are not significantly different by least significant difference ($P > 0.05$).
** Not tested.

on PRAA4-1^T than that on the other treatments, but no other treatments differed from the water control in this experiment (Table 2).

Red flour beetle bioassay. Mortality of the red flour beetle was significantly affected by treatment ($F = 39.37$; df = 4, 12; $P = 0.0001$). Mortality was higher on PRAA4-1^T than that on any other treatment (Table 2), but no other treatments differed from the water control.

Discussion

Isolates of *C. sphagni* from both West Virginia and Maine exhibited insecticidal activity. However, the spectrum of activity of the new isolates clearly differed from that of *C. subtsugae* PRAA4-1^T. Although PRAA4-1^T was toxic to all insect species included in the present study, the new isolates were only toxic to the lepidopterans, the gypsy moth and diamondback moth. The reasons for this difference in activity are unknown at the present time but suggest that multiple insecticidal factors with different activity spectra are present in PRAA4-1^T. Our results are consistent with the presence of a factor or factors in cultures of PRAA4-1^T that are toxic to the seedcorn maggot and red flour beetle and that are not present, or are present at lower levels, in cultures of *C. sphagni*.

In the first experiment with the diamondback moth, results indicated that West Virginia isolate 14B-1 might have greater activity than *C. subtsugae* PRAA4-1^T and, therefore, could have the potential to be a superior insecticide. However, in the second experiment, no significant difference between these isolates was seen. No indication that any of the Maine isolates would be superior insecticides was seen.

In no experiment did the *B. thuringiensis* isolates IBL-163 and IBL-717 cause appreciable mortality. These results indicate that the presence of bacterial culture alone in the media does not cause mortality in the insects tested. Positive results

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with *Chromobacterium* cultures are thus consistent with the presence of one or more toxic factors in these bacterial cultures.

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