Insecticidal Activity of *Chromobacterium phragmitis*, a Recently Described Bacterium from Tidal Marshes[1]

Robert R. Farrar, Jr., Dawn E. Gundersen-Rindal, Daniel Kuhar, and Michael B. Blackburn[2]

USDA-ARS, Invasive Insect Biocontrol and Behavior Lab, 10300 Baltimore Ave., Bldg. 007, Rm. 301, Beltsville, Maryland 20705 USA

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Abstract Several isolates of the recently described bacterial species *Chromobacterium phragmitis* Blackburn et al. were obtained from water collected from low-salinity tidal marshes in Maryland and Virginia. Bacteria were cultured in a liquid medium and applied to artificial diets in the laboratory. One of two Maryland isolates, IIBBL 113-1, was highly toxic to larvae of the cabbage looper *Trichoplusia ni* (Hübner) and the diamondback moth, *Plutella xylostella* (L.). The other Maryland isolate, IIBBL 112-1^T, and the Virginia isolates were less toxic to these species. Maryland isolates were toxic to larvae of the seedcorn maggot *Delia platura* (Meigen), while a Virginia isolate, IIBBL 274-1, was of intermediate toxicity against *D. platura*. The *C. phragmitis* isolate IIBBL 113-1 was toxic to adults of the red flour beetle *Tribolium castaneum* (Herbst), while other *C. phragmitis* isolates had no activity against this species.

Key Words *Chromobacterium phragmitis*, *Trichoplusia*, *Plutella*, *Delia*, *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 was represented by a single species, *Chromobacterium violaceum* Bergonzini. Martin et al. (2007) reported the discovery of a new species, *Chromobacterium subtsugae* Martin et al., that possesses insecticidal activity against the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), as well as several other insect pests. An extract of *C. subtsugae* is now commercially available as an organic insecticide under the trade name Grandevo^{TM} (Marrone BioInnovations, Davis, CA). Asolkar et al. (2014) reported that *C. subtsugae* produces three insecticidal factors, including "chromamide A", violacein, and one unidentified compound. *Chromobacterium subtsugae* was described from a single isolate collected in the Catoctin Mountains of Maryland and attempts to find additional isolates of the same species in the same area have been unsuccessful.

Efforts are ongoing to find additional isolates of *C. subtsugae* and other *Chromobacterium* spp. with insecticidal activity. Blackburn et al. (2017) reported the discovery of *Chromobacterium sphagni* Blackburn et al. in *Sphagnum* bogs in West Virginia and Maine. It was found to be toxic to lepidopterous insects but not toxic to

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[2]Corresponding author (email: Mike.Blackburn@ars.usda.gov).

dipterous or coleopterous insects (Farrar et al. 2018a). Recently, an additional new species, *Chromobacterium phragmitis* Blackburn et al., was described from low-salinity tidal marshes in Maryland and Virginia (Blackburn et al. 2019). Herein, we report results of bioassays of the new species against representative insects.

Materials and Methods

Bacteria. Bacteria were isolated from water samples collected from low-salinity tidal marshes in Maryland and Virginia and were determined as a new species, *C. phragmitis*, as described by Blackburn et al. (2019). Two isolates of *C. phragmitis* were obtained from the Maryland samples and were designated IIBBL 112-1^T and IIBBL 113-1. Eleven isolates were obtained from the Virginia samples. These isolates were designated IIBBL 269-1, IIBBL 269-2, IIBBL 273-1, IIBBL 273-2, IIBBL 274-1 through IIBBL 274-4, and IIBBL 277-1 through IIBBL 277-3. From among the Virginia isolates, IIBBL 274-1 was selected for bioassays based on preliminary tests. For positive controls, all bioassays included a *C. subtsugae* isolate, either PRAA4-1^T, isolated from forest soil in Maryland (Martin et al. 2007); IIBBL 205-1, isolated from pond water in New Jersey (R.R. Farrar, Jr. and M.B. Blackburn, unpublished); or both of these isolates.

All 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 and cycloheximide (both 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 in an orbital shaker (model C25KC; New Brunswick Scientific, Edison, NJ) at 200 rpm and 24°C for 96 h.

Cabbage looper bioassay. Eggs of the cabbage looper *Trichoplusia ni* (Hübner) were obtained from Benzon Research (Carlisle, PA). Larvae were reared to early second instars on artificial diet (King and Hartley 1985). Bioassays were conducted with freeze-dried diet pellets after Martin (2004). Briefly, hot diet was poured 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. One pellet was placed in each cell of a plastic bioassay tray (Bio-BA 128®; Bio-Serv, Flemington, NJ). Each pellet was rehydrated with 300 μl of either undiluted liquid culture or, for a negative control, deionized water only. One larva was placed in each cell, and the cells were covered with vented transparent plastic covers (Bio-CV 16®; Bio-Serv). Larvae were held at 27°C and scored as alive or dead at 3 d and 6 d. Proportion mortality was calculated, normalized by arcsine $\sqrt{}$ transformation, and analyzed by analysis of variance (PROC GLM, SAS Institute 2010). When significant treatment effects ($P < 0.05$) were found, means were separated by the least significant difference (LSD) test.

Against the cabbage looper, treatments included IIBBL 205-1, Virginia isolate IIBBL 274-1, Maryland isolates IIBBL 112-1^T and IIBBL 113-1, and a control of water only. Twenty-four larvae per treatment were included, and the bioassay was replicated four times.

Diamondback moth bioassay. 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 (IIBBL; USDA/ARS, Beltsville, MD). 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. Pellets were rehydrated as above. Two early second instars were placed on each pellet. Trays were held at 27°C until all insects died or pupated. Insects were scored at 3 d, 6 d, and pupation. Proportion mortality was calculated and analyzed as above.

The same treatments included in the cabbage looper bioassay were included in the diamondback moth bioassay. Twenty-four larvae per treatment were included, and the bioassay was replicated four 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 bone meal (Baker Commodities, Rochester, NY) at IIBBL. Bioassays were conducted with freeze-dried diet pellets made as described above. 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. Approximately 1 g dry quartz sand, passed through an 850-μm screen, was placed in each cell of a bioassay tray and moistened with 150 μl of deionized water. Two diet pellets were placed on top of the moist sand and rehydrated as above. Two 6-d-old larvae were placed in each cell. Cells were covered with vented plastic covers. Trays were held at 20% relative humidity and 27°C in an incubator (model I36VLC8; Percival, Perry, IA) equipped with a desiccant drier (model IAT-75RE; Innovative Air Technologies, Covington, GA) for 21 d. Puparia were then counted. Proportion pupation was calculated and analyzed as above.

Two bioassays were conducted with the seedcorn maggot. The first bioassay included IIBBL 112-1^T, IIBL 113-1, PRAA4-1^T, and a control of water only. The second bioassay included IIBBL 274-1, IIBBL 112-1^T, IIBBL 205-1, and a control. Twenty-four larvae per treatment were included, and each bioassay was replicated four times.

Red flour beetle bioassays. 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

Table 1. Mortality of cabbage looper larvae on *Chromobacterium* isolates (means ± standard errors).

Treatment	Mortality, 3 d (%)*	Mortality, 6 d (%)*
Water only	7.3 ± 4.29 A	10.4 ± 6.25 A
IIBBL 112-1^T	13.5 ± 3.13 AB	15.6 ± 2.62 AB
IIBBL 205-1	21.9 ± 6.45 B	28.1 ± 8.05 B
IIBBL 274-1	25.0 ± 6.13 B	35.4 ± 7.70 B
IIBBL 113-1	100.0 ± 0.00 C	100.0 ± 0.00 C

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

27°C. Beetles were scored at 5 weeks. Proportion mortality was calculated and analyzed as above.

Two bioassays were conducted with the red flour beetle. The first bioassay included IIBBL 112-1^T, IIBBL 274-1, IIBBL 205-1, and a control. The second bioassay included IIBBL 113-1, IIBBL 205-1, PRAA4-1^T, and a control. Twenty-four beetles per treatment were included. The first bioassay was replicated four times, whereas the second bioassay was replicated five times.

Results

Cabbage looper bioassay. Significant differences among isolates of *C. phragmitis* against the cabbage looper were found at both 3 d ($F = 59.43$; df = 4, 12; $P = 0.0001$) and 6 d ($F = 47.08$; df = 4, 12; $P = 0.0001$) (Table 1). Mortality on isolate IIBBL 113-1 was 100% within 3 d. Mortality on isolate IIBBL 112-1^T did not differ from that on water only at 3 d or 6 d. Mortality on isolates IIBBL 205-1 and IIBBL 274-1 was intermediate between that on water only and that on isolate IIBBL 113-1.

Diamondback moth bioassay. Significant differences among isolates of *C. phragmitis* were also found against the diamondback moth at 3 d ($F = 16.86$; df = 4, 12; $P = 0.0001$), 6 d ($F = 40.49$; df = 4, 12; $P = 0.0001$), and pupation ($F = 127.79$; df = 4, 12; $P = 0.0001$) (Table 2). Mortality on isolate IIBBL 113-1 reached 100% by pupation. Mortality on IIBBL 112-1^T and IIBBL 274-1 was significantly higher than that on the control only at pupation.

Seedcorn maggot bioassays. In the first bioassay, mortality on *C. phragmitis* was significantly affected by bacterial isolate ($F = 43.03$; df = 3, 11; $P = 0.0001$). Mortality was higher on *C. phragmitis* than that on *C. subtsugae*, although this difference was statistically significant ($P < 0.05$) only for isolate IIBBL 112-1^T (Table 3). In the second bioassay, mortality was again significantly affected ($F = 30.20$; df = 3, 9; $P = 0.0001$) by isolate. In this test, mortality on isolate IIBBL 274-1 was intermediate between that on water only and those on isolate IIBBL 112-1^T or *C. subtsugae*, whereas the latter isolates did not differ (Table 3). In both bioassays, mortality on all *Chromobacterium* isolates was significantly higher ($P < 0.05$) than that on water only.

Table 2. Mortality of diamondback moth larvae on *Chromobacterium* isolates (means ± standard errors).

Treatment	Mortality, 3 d (%)*	Mortality, 6 d (%)*	Mortality at Pupation (%)*
Water only	7.3 ± 3.56 A	12.5 ± 4.50 A	12.5 ± 4.50 A
IIBBL 112-1^T	15.6 ± 4.92 AB	21.9 ± 6.88 A	26.0 ± 5.98 B
IIBBL 274-1	13.5 ± 4.29 AB	25.0 ± 4.50 A	26.0 ± 4.62 B
IIBBL 205-1	28.1 ± 4.29 B	57.3 ± 7.09 B	81.3 ± 4.96 C
IIBBL 113-1	61.5 ± 6.67 C	91.7 ± 2.95 C	100.0 ± 0.00 D

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

Red flour beetle bioassays. Mortality of red flour beetles was significantly affected in both the first bioassay ($F = 31.58$; df = 3, 9; $P = 0.0001$) and the second ($F = 22.45$; df = 3, 12; $P = 0.0001$). In the first bioassay, neither *C. phragmitis* isolate, IIBBL 112-1^T or IIBBL 274-1, differed significantly ($P > 0.05$) from water only (Table 4). In contrast, in the second bioassay, mortality on *C. phragmitis* isolate IIBBL 113-1 was significantly ($P < 0.05$) higher than that on any other treatment. Mortality on *C. subtsugae* isolates was significantly ($P < 0.05$) higher than that on water only in both bioassays.

Table 3. Mortality of seedcorn maggot larvae on *Chromobacterium* isolates (means ± standard errors).

Treatment	Mortality at Pupation (%)*
Bioassay 1	
Water only	36.5 ± 9.06 A
PRAA4-1^T	90.6 ± 5.48 B
IIBBL 113-1	97.9 ± 1.20 BC
IIBBL 112-1^T	99.0 ± 1.04 C
Bioassay 2	
Water only	10.5 ± 5.51 A
IIBBL 274-1	37.5 ± 4.50 B
IIBBL 112-1^T	87.5 ± 5.38 C
IIBBL 205-1	88.5 ± 3.13 C

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

Table 4. Mortality of red flour beetle adults on *Chromobacterium* isolates (means ± standard errors).

Treatment	Mortality (%)*
Bioassay 1	
Water only	11.5 ± 4.29 A
IIBBL 274-1	14.4 ± 3.70 A
IIBBL 112-1^T	9.4 ± 5.98 A
IIBBL 205-1	88.5 ± 3.56 B
Bioassay 2	
Water only	9.2 ± 4.25 A
PRAA4-1^T	38.3 ± 9.45 B
IIBBL 205-1	39.2 ± 8.19 B
IIBBL 113-1	54.2 ± 9.03 C

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

Discussion

Insecticidal factors appear to vary greatly among isolates of *C. phragmitis*, even between isolates collected in the same marsh at the same time (IIBBL 112-1^T and IIBBL 113-1). Isolate IIBBL 113-1 was toxic to both lepidopterans but particularly toxic to the cabbage looper. All cabbage looper larvae on IIBBL 113-1 died within 3 d. These larvae apparently fed very little and produced little or no frass. Some feeding by diamondback moth larvae on this isolate was evident, but all of these larvae died before pupation. The other *C. phragmitis* isolates were less toxic to the lepidopterans, with IIBBL 112-1^T not differing from the control against the cabbage looper. Isolate IIBBL 113-1 was also toxic to the red flour beetle, whereas isolates IIBBL 112-1^T and IIBBL 274-1 showed no activity against this insect. In contrast, both Maryland isolates of *C. phragmitis* were highly toxic to the seedcorn maggot, whereas toxicity of the Virginia isolate was intermediate between those isolates and the control.

Chromobacterium phragmitis isolate IIBBL 113-1 was more toxic than *C. subtsugae* in all bioassays, although this difference was not statistically significant ($P > 0.05$) against the seedcorn maggot. Its range of activity was broader than that found for *C. sphagni* (Farrar et al. 2018a). These results indicate that IIBBL 113-1 may be useful as the basis for insecticidal products.

The findings of great variability among *C. phragmitis* isolates contrast with those of Farrar et al. (2018b) who found that the insecticidal activity of isolates of *Chromobacterium vaccinii* collected from nine sites in four states (United States) varied very little. Farrar et al. (2018a) found that isolates of *C. sphagni* from two sites in two states (United States) also varied but not to the extent seen with *C. phragmitis*.

Asolkar et al. (2014) reported that *C. subtsugae* produces three insecticidal factors, including chromamide A, violacein, and one unidentified compound. The insecticidal factors produced by *C. phragmitis* are unknown at present. Results of the present study are consistent with the presence of a factor or factors in isolate IIBBL 113-1 that are toxic to the lepidopterans, as well as the red flour beetle, that are absent, or present at lower levels in the other isolates.

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