# Two New Bacterial Pathogens of Colorado Potato Beetle (Coleoptera: Chrysomelidae)

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**ABSTRACT** Other than *Bacillus thuringiensis* Berliner, few bacteria are lethal to the Colorado potato beetle (*Leptinotarsa decemlineata* [Say]), a major pest of potatoes and eggplant. Expanded use of biologicals for the control of Colorado potato beetle will improve resistance management, reduce pesticide use, and produce novel compounds for potential use in transgenic plants. Using freeze-dried, rehydrated artificial diet in pellet form to screen bacteria lethal to other insects, we determined that strains of *Photorhabdus luminescens* killed Colorado potato beetle larvae. The $LC_{50}$ for second instar larvae of strain HM5-1 was $6.4 \pm 1.87 \times 10^7$ cells per diet pellet. In an attempt to find additional naturally occurring *P. luminescens* strains toxic to Colorado potato beetle larvae, we recovered, from soil, bacteria that produced a purple pigment. This bacterial strain, identified as *Chromobacterium* sp. by 16S ribosomal DNA sequencing, was also toxic to Colorado potato beetle larvae within 3 d. The $LC_{50}$ for second instar larvae for these bacteria was $2.0 \pm 0.79 \times 10^8$ cells per diet pellet, while the $LC_{50}$ was approximately 1 log lower for third instar larvae. *P. luminescens* appeared to kill by means of a protein toxin that may be similar to the described lepidopteran protein toxins. Based on the heat and acid stability, the toxin or toxins that *Chromobacterium* sp. produces, while not fully characterized, do not appear to be typical proteins. In both bacteria, the toxins are made after exponential growth ceases.

**KEY WORDS** insect biocontrol, insect pathogen, *Leptinotarsa decemlineata*, *Chromobacterium violaceum*, *Photorhabdus luminescens*

THE COLORADO POTATO BEETLE *Leptinotarsa decemlineata* (Say) is a major insect pest of potatoes, tomatoes, and eggplant. Microbial control can be an effective alternative to chemical control. *Bacillus thuringiensis* Berliner can successfully control this pest as a foliar spray (Ferro et al. 1997) or in transgenic plants (Perlak et al. 1993). Other successful biocontrol agents for this pest include fungi, of which *Beauveria bassiana* (Balsamo) Vuillemin has been the most successful in some areas such as Europe and the northern United States, and under certain conditions such as early season applications in Virginia requiring high humidity and low temperatures (Groden and Lockwood 1991, Poprawski et al. 1997, Martin et al. 1999).

*Bacillus popilliae* Dutky was the first bacterial pesticide (milky spore) to successfully control beetles (Japanese beetles) (Dutky 1940). Other bacteria have offered promise of beetle control, but have not been commercial successes. Various strains of *B. thuringiensis* are toxic to scarab beetles (Ohba et al. 1992) and *Diabrotica* spp. (Tailor et al. 1992). *Serratia entomophila* Grimont et al. and *Serratia proteamaculans* (Paine and Stansfield) (Jackson et al. 1993) cause amber disease in grass grubs and are being developed in New Zealand. *Spiroplasma leptinotarsae* Hackett et al. has been implicated as a disease-causing agent in Colorado potato beetle (Hackett et al. 1996), but few other bacteria have been used to effectively control Colorado potato beetles (Onstad 2001).

*Photorhabdus luminescens* Thomas and Poinar, with nematodes as a vector, has been described as pathogenic to Lepidoptera (Forst and Nealson 1996). High molecular weight protein complexes isolated from *P. luminescens* are toxic to lepidopteran and coleopteran insects (Bowen et al. 1998, Guo et al. 1999). One of these complexes, Tca, has been shown to disrupt the midgut epithelium of tobacco hornworm larvae (Blackburn et al. 1998).

Purple bacteria such as *Chromobacterium violaceum* Bergonzini have infrequently been isolated from insects, and have not been previously considered as insect pathogens (Bucher 1981). This species of bacteria has been isolated from the digestive tract of the larger grain borer (*Prostephanus truncatus* [Horn]), where it may be involved in cellulose digestion (Vazquez-Arista et al. 1997) forming a symbiotic rather than a pathogenic association. However, *C. vio-*

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*laceum* is mainly known for its production of a purple pigment, violacein, which has antimicrobial activity against Gram-positive and Gram-negative bacteria (Duran et al. 1983) and *Trypanosoma cruzi* Chagas (Duran et al. 1994).

In the present work, we describe two strains of *Chromobacterium* sp. and *P. luminescens* that kill Colorado potato beetle larvae. We determine conditions for optimal insect toxicity and begin to characterize the toxic activity.

## Materials and Methods

**Bacterial Strains and Media.** *P. luminescens* strains HM5-1, W5-14, and NC5-1 were subcultures of strains Hm, W14, and Nc (obtained from Dr. D. Bowen, University of Wisconsin, Madison, WI), which have been described as orally toxic to caterpillars (Bowen and Ensign 1998). Purple colonies of bacterial strain PRAA4-1 were isolated from Maryland forest soil by S. Stone (Eleanor Roosevelt High School student, Greenbelt, MD) by plating on L-agar. The purple bacteria were Gram-negative rods, and preliminarily identified as *C. violaceum* by comparison with descriptions in Bergey's manual (Sneath 1984). When the first 500 bp of the 16S ribosomal DNA were sequenced, the identification was only to the genus *Chromobacterium* (Accugenix, Newark, DE). *B. thuringiensis* variety *tenebrionis* NTEN3-2 was obtained from Novodor FC (Abbott Labs, Chicago, IL) and used for comparisons of toxicity.

Bacteria were cultured in L-broth (Atlas 1997) or on L-agar. We also used recovery medium (RM) (Martin et al. 1998), which contained half the nutrients of L, for bacterial recovery from insects.

**Preliminary Toxin Characterization.** Bacteria were grown on L-agar plates at 25°C. At preselected time intervals, bacteria were harvested from plates. Cells were titered and used directly or diluted in sterile water for insect bioassays.

For further toxin characterization, bacteria were grown in L-broth or RM-broth and shaken at 125 rpm for appropriate times at 25°C. Cells were titered and used directly or diluted in sterile water. Cell-free supernatants were obtained from liquid cultures that had been grown at 25°C for 24 h shaking at 150 rpm, or from plates whose cells had been harvested in sterile water. Cells were removed by centrifugation, and the resulting supernatant was sterilized by passage through a 0.45-$\mu$m nylon filter or a 0.45-$\mu$m polyethersulfone (PES) filter (Millipore, Bedford, MA).

The filtered supernatants of both *Chromobacterium* sp. PRAA4-1 and *P. luminescens* HM5-1 were exposed to heat (65°C for 10 min) and protease XIV (30°C for 30 min) (Sigma-Aldrich, St. Louis, MO) to test for toxin stability. To further characterize the *Chromobacterium* sp. PRAA 4-1 toxin, we filtered the supernatant through 100K and 10K polysulphone filters (Vecta-Spin 3; Whatman International, Maidstone, United Kingdom) and evaluated their toxicity against second instar Colorado potato beetle.

**Koch's Postulates.** To fulfill Koch's postulates, second instar larvae that had died after treatment with bacteria were surface sterilized and placed in a 1.5-ml microcentrifuge tube; 0.5 ml of water was added; and the cadaver was ground with a motorized pestle (Sigma-Aldrich). Insect parts were allowed to settle, and dilutions were plated and incubated at 25°C for 48 h in order for pigmented microbes to be distinguished. Using Biolog system of identification (Biolog, Hayward, CA; version 3.5), substrate utilization profiles of the recovered microbes were compared with the profiles of the bacteria originally fed.

**Recovery from Diet Pellets.** Because we could not recover the fed bacteria from all insects that had died after having consumed diet rehydrated with the bacteria, we determined the recovery of bacteria from diet pellets. Approximately $10^7$ bacteria were added to each diet pellet in triplicate. Pellets were harvested immediately and at 1–7 d to determine survival over time. Pellets were suspended in 5 ml of sterile water and blended in a Stomacher blender (Techmar, Cincinnati, OH) on high for 90 s. Cells were titered and compared with original titer.

**Insects.** The Colorado potato beetle colony originated from eggs sent from the New Jersey Department of Agriculture in 1996. The colony has been maintained on potato foliage, and field-collected insects are introduced yearly to maintain genetic diversity. Insects were reared from eggs for bioassays on Insect Biocontrol Lab (IBL) diet (Gelman et al. 2001) made with defined ingredients as well as potato leaf powder and tomato fruit powder.

**Bioassays.** For bioassays, IBL diet was used as rehydrated freeze-dried pellets (Martin 2004). The diet was made, as described (Gelman et al. 2001), without neomycin, poured into 96-well polypropylene plates (GreinerBioOne, Longwood, FL), frozen overnight (−20°C), and then dried in a Virtis Advantage Freeze Drier (Virtis, Gardiner, NY). The dry IBL diet pellets without neomycin were removed from the molds, placed in sterile plastic bags, and stored at 4°C before use.

Colorado potato beetle adults were fed potato foliage. The eggs laid on potato foliage were harvested and hatched on diet. Larvae for bioassays were reared on diet. Incubation was initially in the dark so that the larvae would feed on the diet, and then on a light-dark regime of 16:8 (L:D) with 46% RH at 24°C. Diet was changed every 4 d.

Thirty-two diet pellets were used for each treatment in bioassays. Each diet pellet was placed in a well (1.6 cm diameter × 1.6 cm deep) in white plastic bioassay trays (C-D International, Ocean City, NJ) and rehydrated with 0.3 ml of water (controls) or suspensions containing dilutions of the pathogen (treatments). One Colorado potato beetle larva was added to each pellet. Wells were sealed with film and holes made in the film with insect pins. Insects were incubated as described for rearing, and mortality recorded at 24, 48, 72, 96, and 120 h. $LC_{50}$ were determined using PROBIT procedure from SAS, using cell counts as the dose (SAS Institute 1999). Cell counts

were used because the specific cause of mortality was unknown. Bioassays with control mortality above 5% were discarded. We tested first, second, and third instars and adults for toxicity. Fourth instar test results are not reported because of consistently high control mortality. Test results for second and third instar larvae were the mean of at least three separate trials.

## Results

**Initial Toxicity.** Three strains of *P. luminescens* were tested against second instar Colorado potato beetle larvae. At 96 h after treatment, mortality for undiluted 48-h-old whole cultures of NC5-1 was 6.2%, W5-14 was 40.6%, and HM5-1 was 100% at $\approx 10^8$ cells of each strain per pellet. We continued using HM5-1 for additional experiments.

From one Maryland forest soil sample rich in decaying hemlock leaves, we recovered purple bacterial colonies on L-agar. The pH of the soil was 4.7, and the moisture content was 46.6%. The original sample had a total aerobic microbial cell count of $1.75 \times 10^6$ colony-forming units (cfu)/g of soil, about half of which were purple colonies. Because some pigmented bacteria such as *Serratia marcescens* Bizo are toxic to insects (Grimont and Grimont 1978), we fed these bacteria to Colorado potato beetle larvae. In the first bioassay, over 78% of the second instar beetle larvae died within 3 d. The age of the culture was not recorded. The larvae also appeared to stop feeding, no diet was consumed, and little to no frass was produced even by the survivors.

**Koch's Postulates.** *P. luminescens* gold-pigmented colonies were recovered from surface-sterilized larvae (30) that died after feeding on *P. luminescens* HM5-1. Recovery was highest in larvae that had been dead for >48 h after exposure to HM5-1, indicating growth in the insect cadaver after death. The golden colonies comprised 0.001–0.1% of the total colonies recovered. When the bacteria grown from these colonies were fed to larvae after growth on L-agar for 48 h, there was 100% mortality from an undiluted culture ($1.08 \times 10^8$ cfu/diet pellet) in 72 h. The colonies recovered matched the previous profiles using the Biolog system for substrate utilization.

*Chromobacterium* sp. purple-pigmented colonies were recovered from only 12 of 30 insects that died after exposure to PRAA4-1 whole cultures. The total number of bacteria recovered per larvae averaged $1.15 \pm 0.95 \times 10^7$, while the amount of *Chromobacterium* sp. PRAA4-1 recovered ranged from $2.5 \times 10^3$ from a larvae that died at 24 h to a high of $6.5 \times 10^5$ from a larvae that died at 72 h. An undiluted culture of purple bacteria grown from colonies recovered from these insects was also toxic (100% mortality in 72 h) when fed to Colorado potato beetle larvae ($2.5 \times 10^8$ cfu/diet pellet) after growth on L-agar for 48 h.

**Recovery from Diet Pellets.** We were able to recover *P. luminescens* HM5-1 from diet pellets at 1 wk after initially adding $10^7$ cfu/diet pellet. Recovery at 24 h without insect feeding was $\approx 10^5$ cfu/diet pellet. Initial attempts to recover *Chromobacterium* sp. PRAA4-1 from diet pellets after 1 wk failed. We were able to recover <0.2% ($9.18 \times 10^5$) of the PRAA4-1 cells ($5.49 \times 10^8$) initially added to diet pellets, and by 24 h the numbers of viable cells recovered had dropped to <500 cfu (level of detection). Growth of *Chromobacterium* sp. PRAA4-1 was found to be slightly inhibited by some components of the diet when tested separately (methyl-para-hydroxybenzoate, sorbic acid, and tomato fruit powder) (Martin 2003).

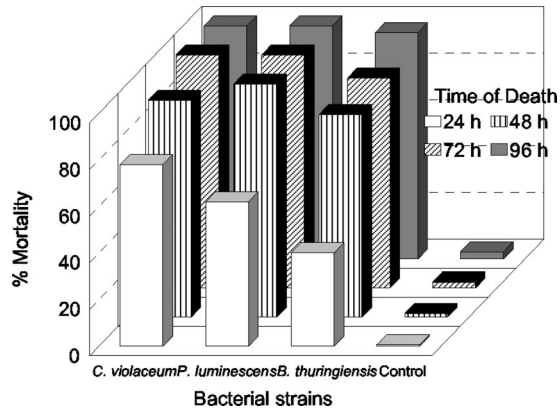


**Fig. 1.** Comparison of mortality of bacterial cultures. Single plates of each bacteria were harvested identically. The number of bacteria applied per diet pellet was $2.54 \times 10^8$ for PRAA4-1 (*Chromobacterium* sp.), $5.1 \times 10^7$ for HM5-1 (*P. luminescens*), and $2.3 \times 10^8$ for NTEN3-2 (*B. thuringiensis*). Shaded bars are the time posttreatment.

**Toxicity of the *P. luminescens* and *Chromobacterium* sp.** In an initial comparison of strains, it was found that the toxicity of both *P. luminescens* HM5-1 and *Chromobacterium* sp. PRAA4-1 was similar to *B. thuringiensis* NTEN3-2 toxicity fed to second instar Colorado potato beetle larvae at the same dilution (Fig. 1). This comparison was made when the bacteria were grown on the same media under identical conditions with stationary-phase (48-h) cultures. In this preliminary experiment, both *P. luminescens* HM5-1 and *Chromobacterium* sp. PRAA4-1 killed the larvae faster than the *B. thuringiensis* NTEN3-2. $LC_{50}$ could not be compared as *B. thuringiensis* toxicity is not correlated with the number of viable cells (Angus 1956), and the toxic factor of *Chromobacterium* sp. PRAA 4-1 is not yet known.

Many *B. thuringiensis* strains produce toxins only upon sporulation after growth is finished (Schnepf et al. 1998); therefore, we determined the time of maximum lethality. For *P. luminescens* HM5-1, there was high mortality among larvae exposed to cultures grown for 24 h (Fig. 2A). This mortality changed little with how long the *P. luminescens* HM5-1 was grown. Although dropping slightly at 72 h, *P. luminescens* HM5-1 cultures had reached stationary phase by 24 h, with a doubling time of 45 min.

The age of the *Chromobacterium* sp. PRAA4-1 made more of a difference in initial mortality, but not total mortality (Fig. 2B). At 25°C, the doubling time was 40

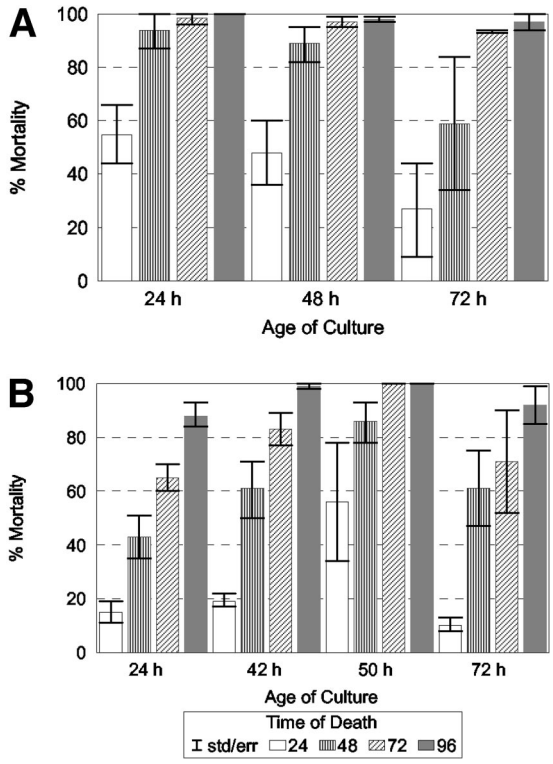


**Fig. 2.** (A) Effect of age of culture of HM5-1 (*P. luminescens*) on mortality of second instar Colorado potato beetle larvae. The number of viable bacteria applied per diet pellet was 24 h, $1.62 \times 10^7$; 48 h, $1.18 \times 10^8$; 72 h, $1.80 \times 10^8$. Shaded bars are the time posttreatment. (B) Effect of age of culture of PRAA4-1 (*Chromobacterium* sp.) on mortality of second instar Colorado potato beetle larvae. The number of viable bacteria applied per diet pellet was 24 h, $1.2 \times 10^8$; 40 h, $2.6 \times 10^8$; 50 h, $8.6 \times 10^8$; 72 h, $5.6 \times 10^8$. Shaded bars are the time posttreatment.

min, and stationary phase was reached within 24 h. The toxic activity of *Chromobacterium* sp. PRAA4-1 appeared later, with maximum toxicity of cultures grown on solid media occurring at 50 h (100% mortality within 72 h). As PRAA4-1 cultures aged to 10 d, the titer dropped to <1,000 viable cells, but the toxicity remained high (84% mortality), even though larvae did not start to die until 48 h after being placed on the diet (data not shown). Further stability studies conducted on a 28-d liquid whole culture had 87.4% mortality.

In liquid culture (50 ml in 250-ml baffled flasks), *Chromobacterium* sp. PRAA4-1 had high toxicity (75% mortality) when incubated at 25°C, 125 rpm for 24 h. The toxicity in liquid culture grown for 24 h decreased (25% mortality) with increased shaking speed (150 rpm) and increased temperature (27°C). In one experiment, when the culture temperature was inadvertently raised to 35°C, no measurable toxicity was noted in the bioassay, and no viable cells were recovered.

First instar larvae were the most susceptible to both *Chromobacterium* sp. PRAA4-1 and *P. luminescens* HM5-1. Starving the larvae before placing them on the diet did not change the total mortality, but did affect how quickly they died. In an experiment comparing fed and starved late first instar larvae, 81.3% of the starved larvae died within 24 h after being exposed to pellets to which $7.2 \times 10^8$ *Chromobacterium* sp. PRAA4-1 cells had been added. Only 56.3% of the fed first instar larvae were dead at 24 h after exposure to the same dose. Second instar fed larvae exposed to the same dose, at the same time, showed 91% survival at 24 h. The $LC_{50}$ for *Chromobacterium* sp. PRAA4-1 unexpectedly decreased from second to third instar larvae (Table 1).

**Table 1. $LC_{50}$ of Colorado potato beetle by instar after 96 h**

| Stage | *P. luminescens* HM5-1 | *Chromobacterium sp.* PRAA4-1 |
|---|---|---|
| 1st instar | $1.0 \times 10^7$ | $8.0 \times 10^6$ |
| 2nd instar | $6.4 \pm 1.87 \times 10^7$[a] | $2.0 \pm 0.79 \times 10^8$ |
| 3rd instar | $3.41 \pm 2.8 \times 10^8$ | $3.24 \pm 1.18 \times 10^7$ |
| Adult | None[b] | None[b] |

[a] Mean ± standard error of number of bacteria applied per pellet based on at least three bioassays with five concentrations per bioassay and $LC_{50}$ calculated by PROBIT analysis. First instar assay was not replicated.
[b] Adult beetles stopped eating.

**Preliminary Characterization of Toxic Activity.** For *P. luminescens* HM5-1, the mortality of larvae treated with filter-sterilized supernatants from 24- and 48-h cultures averaged ≈60% of the whole culture toxicity. This mortality caused by the filter-sterilized supernatant dropped to 33% when the culture was grown for 72 h. There was no mortality when the supernatant was heated or treated with protease.

When filter-sterilized supernatants of *Chromobacterium* sp. PRAA4-1 were tested against second instar Colorado potato beetle larvae, the mortality was dependent on the age of the culture. Cell-free supernatants from cultures up to 48 h old had only 9–20% the toxicity of the whole culture, while the toxicity of supernatants from cultures older than 72 h was 55–86% of whole cultures (Table 2). This confirmed previous observations that cultures older than 7 d with few viable cell counts were also toxic; however, peak toxicity occurred when cell number was maximal. Filtered supernatants were clear or only slightly purple compared with whole cultures when filtered through nylon filters. Supernatants filtered through PES membranes retained their purple color. Purified violacein

**Table 2. Comparison of mortality of whole cultures and sterile supernatants of PRAA4-1**

| Culture type | % Mortality | | | |
|---|---|---|---|---|
| | 24 h | 48 h | 72 h | 96 h |
| 48-h total culture[a] | 15.2 | 36.4 | 63.6 | 84.3 |
| 48-h filtered supernatant | 3.0 | 9.1 | 9.1 | 15.6 |
| 96-h total culture[b] | 0 | 28.1 | 53.1 | 100 |
| 96-h filtered supernatant | 0 | 13.3 | 60.0 | 86.0 |

[a] A total of $8.15 \times 10^8$ viable cells applied per pellet.
[b] Fewer than 10,000 viable cells applied per pellet.

(the purple pigment) tested against larvae was not toxic.

For *Chromobacterium* sp. PRAA4-1, there were no differences in toxicity of filtrates that were heated (60% filtrate, 61.5% heat-treated filtrate). Furthermore, heat treatments of whole cultures including autoclaving did not reduce toxicity. There was a slight decrease in toxicity when filtrates were treated with protease type XIV (51.7% protease-treated filtrate) compared with those filtrates that were not treated. When a supernatant that caused 55.2% mortality in second instar larvae was passed through a 100K filter, the mortality dropped to 16.1%, and to 11.1% when passed through a 10K filter.

## Discussion

New pathogens against Colorado potato beetle will give growers alternatives to *B. thuringiensis*, as well as chemicals, for control of this pest. Because *Chromobacterium* sp. and *P. luminescens* are Gram-negative bacteria, they are unlikely to have mechanisms similar to *B. thuringiensis* for killing, and could be employed in resistance management. Although these bacteria may not ultimately be practical as pathogens, the toxic factors they possess may be of use in new formulations or when deployed in transgenic plants. For *P. luminescens* HM5-1, we expect that the toxic factors will resemble those proteins described from *P. luminescens* W14 (Bowen et al. 1998, Guo et al. 1999) and *S. entomophila* (Hurst et al. 2000). But for *Chromobacterium* sp. PRAA4-1, these toxins are likely to be novel, as the properties of heat stability and protease resistance differ from both *P. luminescens* and *B. thuringiensis.*

The *P. luminescens* strain HM5-1 and the *Chromobacterium* sp. strain PRAA4-1 have approximately the same toxicity on a per cell basis. While first instar Colorado potato beetle larvae were most susceptible to both bacteria, it is just not practical for field applications to test against first instar larvae, as this larval stage is the shortest and causes the least damage. The time to kill second instar larvae can be somewhat variable depending on whether the larvae were starved or fed, but at high doses usually all larvae are dead by 120 h. The decrease in the $LC_{50}$ from second to third instar larvae for *Chromobacterium* sp. PRAA4-1 may not be because of increased sensitivity to toxin, but because of increased consumption of the toxin.

As mentioned above, it is highly probable that *P. luminescens* HM5-1 kills by means of one or more protein toxins. Cell-free filtrates of *P. luminescens* HM5-1 kill beetle larvae and are susceptible to heat inactivation, which is consistent with a protein toxin or toxins. Although the toxins from *P. luminescens* HM5-1 may prove to be similar in primary structure to those described from *P. luminescens* W14, there is the possibility that toxins from *P. luminescens* HM5-1 will prove to be more potent against the Colorado potato beetle than these previously described toxins. Essentially nothing is known about quantitative differences in the specific activity of these toxins from different strains of these bacteria. Characterization of toxins from *P. luminescens* HM5-1 is currently underway.

There are many lines of evidence that indicate that *Chromobacterium* sp. PRAA4-1 produces factors that are toxic to Colorado potato beetle larvae. The bacteria survive <24 h in the diet pellet, yet remain lethal to larvae. Cultures with few viable cells are comparable in toxicity to cultures with $>10^8$ viable cells. *Chromobacterium* sp. could not be recovered consistently from dead larvae. Most directly, cell-free supernatants can retain about half the toxicity of the whole cultures, and autoclaved cultures also retain activity. Even culture fluid that has passed through a 10K filter retains some toxicity (10%). Initial experiments have been contradictory in regard to the nature of the *Chromobacterium* sp. PRAA4-1 toxin. The best evidence suggests that most of the toxin is a high molecular weight compound or aggregate, and has a high degree of heat stability. But there is also the possibility that some of the toxicity may be caused by a smaller molecule. In fact, there may be multiple toxins, as treatment with protease or heat seems to affect some of the toxicity, but not all. It appears that some of the toxins are cell associated and are released from or are less associated with the cells as the culture ages.

The 16S ribosomal DNA sequencing indicated that PRAA4-1, while belonging to the genus *Chromobacterium,* is different enough to be considered another species. Another important deviation from the description of the *C. violaceum* species (Sneath 1984) is that the optimal temperature for growth of this isolate is 25°C.

In view of Bucher's (1981) claim that *C. violaceum* was not pathogenic to insects, it was surprising that these purple bacteria killed Colorado potato beetle larvae. A previously described isolate of *C. violaceum* had no toxic effect on greater grain borer and was implicated in cellulose digestion in the insect gut (Vazquez-Arista et al. 1997). The *C. violaceum* type strain (ATCC 12472) was not toxic to Colorado potato beetle under bioassay conditions identical to those used for *Chromobacterium* sp. PRAA4-1 (data not shown).

Although bacteria were applied at a high concentration to each pellet, it was apparent that the larvae consumed very little diet, which would indicate a much lower lethal dose than the concentration applied to the diet pellet. In previous diet studies, one to five larvae per treatment do not eat, as measured by weight gain (Martin 2004). The most noticeable observation compared with the control was the lack of frass production, implying that the *Chromobacterium* sp. PRAA4-1 cultures had antifeeding activity. Alternatively, the toxic factor(s) may act on the insect gut; decreased frass production is a commonly observed symptom of toxins that destroy the midgut, such as the δ-endotoxins of *B. thuringiensis* or the Tc proteins of *P. luminescens.*

With these new pathogens there is a great deal of work to be done, including maximizing toxin produc-

tion. Some of the variables, such as age of culture and temperature, have been determined, but others, such as media and conditions for growth in liquid culture and scale up, have not. More work is necessary to determine economic feasibility. With the experience of *B. thuringiensis* protein toxin, the handling of the *P. luminescens* protein toxin has some precedence. We expect the development to be similar. For *Chromobacterium* sp. toxin, it is still too early to speculate, but the laboratory stability of the toxin after formation suggests that it would also be stable in the environment. We are continuing work to further characterize the toxic activity from *Chromobacterium* sp. PRAA4-1, as well as isolating additional strains of *P. luminescens* from the environment.

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