

# Remarkable susceptibility of the diamondback moth (*Plutella xylostella*) to ingestion of Pir toxins from *Photorhabdus luminescens*

Michael B. Blackburn\*, Robert R. Farrar, Nicole G. Novak & Susan D. Lawrence

United States Department of Agriculture, Agricultural Research Service, Insect Biocontrol Laboratory, Building 011A, Room 214, Henry A. Wallace Beltsville Agricultural Research Center, Beltsville, MD 20705, USA

Accepted: 18 May 2006

**Key words:** *Leptinotarsa decemlineata*, *Lymantria dispar*, *Manduca sexta*, *Heliothis virescens*, juvenile hormone esterase, leptinotarsin, Lepidoptera, Plutellidae, Enterobacteriaceae

## Abstract

Genes encoding Pir toxins were cloned and sequenced from *Photorhabdus luminescens* (Enterobacteriaceae) strain Hm. Cultures of *Escherichia coli* expressing the Pir A and B proteins were highly toxic when fed to larvae of *Plutella xylostella* L. (Lepidoptera: Plutellidae), as had been reported previously. Histological examination of *P. xylostella* larvae fed with recombinant *E. coli* revealed gross abnormalities of the midgut epithelium, with profound swelling and shedding of the apical membranes. However, the recombinant *E. coli* had no effect on the growth or mortality of larval *Heliothis virescens* F. (Lepidoptera: Noctuidae), *Manduca sexta* L. (Lepidoptera: Sphingidae), *Lymantria dispar* L. (Lepidoptera: Lymantriidae), or *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae). Based on these results, *P. xylostella* is at least 300-fold more susceptible to Pir toxins than other insect species tested, suggesting that they may not be broadly useful as insecticidal proteins. Because Pir B has sequence similarities with N-terminal portions of Cry proteins from *Bacillus thuringiensis*, we also tested the recombinant *E. coli* against a strain of *P. xylostella* that is resistant to the Cry 1A toxin, but found no difference in mortality between resistant and susceptible strains.

## Introduction

The entomopathogenic bacterium *Photorhabdus luminescens* (Enterobacteriaceae) (Thomas & Poinar, 1979), an enteric symbiont of heterorhabditid nematodes, has been found to produce several toxins that are active against insects. Two similar types of high molecular weight protein complexes, Toxin complexes a and d (Tca and Tcd), have been shown to be orally active against a number of different pest species (Bowen et al., 1998; Guo et al., 1999; Blackburn et al., 2005). Two other large proteins that are distinct from the Tcs, Mcf1 and Mcf2, induce apoptosis in hemocytes and midgut epithelium of insects upon injection (Daborn et al., 2002; Waterfield et al., 2003).

More recently, yet another class of toxins has been described from *P. luminescens*. Duchaud et al. (2003) described two genetic loci, each comprising a pair of genes (*plu4093-plu4092* and *plu4437-plu4436*), which produced insecticidal products when expressed in *Escherichia coli*. Both loci

produced toxins with oral activity against *Plutella xylostella* L. (Lepidoptera: Plutellidae), while *plu4093-plu4092* was also shown to be active against the mosquitoes *Aedes aegypti* L., *Culex pipiens* L., and *Anopheles gambiae* Giles (Diptera: Culicidae). Disruption of *plu4092* was found to abolish toxicity against both mosquitoes and *P. xylostella*. Interestingly, the predicted products of both *plu4092* and *plu4436* share significant sequence similarity with a putative juvenile hormone esterase of the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) (Vermunt et al., 1997). In addition, both the predicted *P. luminescens* proteins and the beetle protein share N-terminal sequence similarities with the N-termini of *Bacillus thuringiensis*  $\delta$ -endotoxins. Waterfield et al. (2005) demonstrated that each of the genes in the *P. luminescens* loci were required for toxicity when injected into larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae), but the combination was not sufficient to cause mortality in *Manduca sexta* L. (Lepidoptera: Sphingidae) by either injection or oral administration. These authors also found no juvenile hormone esterase activity associated with any

\*Correspondence: E-mail: blackbm@ba.ars.usda.gov

of the *P. luminescens* genes, and questioned whether the beetle protein was actually a juvenile hormone esterase, pointing out that juvenile hormone esterase activity of the beetle protein had never been demonstrated, and that the identification of the protein was based largely on developmental profiles. In the absence of firm information as to the functional identity of the beetle protein, Waterfield et al. (2005) suggested the nomenclature 'Photorhabdus insect related' proteins (Pir) A and B to refer to products of the *P. luminescens* genes, with Pir A referring to products of *plu4093/4437* homologs, and Pir B to products of *plu4092/4436* homologs.

Although the role Pir B homologs play in both *L. decemlineata* and *P. luminescens* remains unclear, evidence presented by Crosland et al. (2005) suggests these proteins are related to leptinotarsin, a neurotoxic protein present in the hemolymph of several *Leptinotarsa* species (Hsiao & Fraenkel, 1969). Two of three sequences of proteolytically generated peptides from purified  $\beta$ -leptinotarsin-h, isolated from *Leptinotarsa haldemani* Rogers, were nearly identical to internal sequences of the *L. decemlineata* 'juvenile hormone esterase' (Crosland et al., 2005).  $\beta$ -Leptinotarsin-h is a potent neurotoxin to both invertebrates and vertebrates that stimulates  $\text{Ca}^{2+}$  influx and neurotransmitter release (Crosland et al., 2005).

In order to determine the potential of these proteins for insect control we have cloned and sequenced a Pir A/B locus from *P. luminescens* strain Hm, and tested cultures of *E. coli* expressing this locus against selected lepidopteran pests [*P. xylostella*, *Heliothis virescens* F. (Lepidoptera: Noctuidae), *M. sexta*, and *Lymantria dispar* L. (Lepidoptera: Lymantriidae)], as well as the beetle, *L. decemlineata*. Sequence similarities of Pir B with  $\delta$ -endotoxins of *B. thuringiensis* compelled us to compare a Cry 1A resistant strain of *P. xylostella* with our susceptible laboratory strain. Finally, using histological techniques, we surveyed the midgut of *P. xylostella* larvae fed PirA/B expressed in *E. coli* for evidence of pathology.

## Materials and methods

### Pir gene cloning, confirmation, and toxin preparation

Pir A and Pir B were amplified from *P. luminescens* strain Hm genomic DNA using primers 5'-TAG TGT CTT GGT GAT TTA TTG TGG TAT-3' and 5'-CTT TGT TGG CTG CTT TCA CTC ACC C-3'. PCR products were ligated to pGEM-T (Promega, Madison, WI, USA) using methods described in the instruction manual. Pir A and B were amplified from pGEM using the primers 5'-ATT CAT GAG GAA AAT AAA TAT GTC TAG AAT AAC CA-3' and 5'-AAG GAT CCT ATT TTT CAT CAT CAT TTA TCA ACT G-3'. PCR products were digested with *Bsp*HI/*Bam*HI

and ligated to pET24-d (EMD Biosciences, Madison, WI, USA) at the *Nco*I/*Bam*HI sites. The resulting construct allows for inducible expression of Pir A and B genes from the T7 promoter following instructions in the manufacturer's manual. This allowed isolation of protein bands from SDS-PAGE gels that were unique to the Pir A and B construct. Mass spectrometry of tryptic digests of these bands confirmed their identities as Pir A and B (Protein Sequence Core Facility, University of Florida, Gainesville, FL, USA). Although the pET24 vector allowed for much greater expression of Pir A and B, the expressed proteins formed inclusion bodies, and bioassay of induced cultures indicated that the toxicity was not proportionally higher. These results suggested a higher specific activity for Pir A/B expressed from the pGEM construct, and a single active pGEM clone, pGEM-Pir A/B, was selected for feeding bioassays. To produce a uniform stock of toxin for bioassay, pGEM-Pir A/B was grown in 200 ml Luria broth containing ampicillin at 30 °C until turbid. Cells were pelleted by centrifugation and resuspended in 20 ml of the culture supernatant to which an equal volume of glycerol was added. This stock of bacterial suspension was then stored at -15 °C. A similar preparation of *E. coli* transformed with the empty pGEM vector was prepared and used as a negative control in bioassays.

### Insects

We obtained eggs of five species of insects from stock cultures: *P. xylostella* and *L. decemlineata* (Insect Biocontrol Laboratory USDA-ARS, Beltsville, MD, USA), *H. virescens* (Benzon Research, Carlisle, PA, USA), *L. dispar* (Otis Methods Development Center, Otis ANGB, MA, USA), and *M. sexta* (University of Arizona, Tucson, AZ, USA). *Plutella xylostella* and *H. virescens* were reared through the first stadium on artificial diet (King & Hartley, 1985 and Shelton et al., 1991, respectively) and tested as second instar larvae. The other species were tested as neonates.

We also obtained a strain of *P. xylostella* (Benzon Research) that is resistant to the Cry1A  $\delta$ -endotoxin of *B. thuringiensis* (Bt-resistant strain). This strain has been shown to be >1000-fold less susceptible to *B. thuringiensis* ssp. *kurstaki*, strain HD-1, than is the strain maintained at the Insect Biocontrol Laboratory (IBL strain) (HG Goh & DB Gelman, unpubl.).

### Plants

Host plants included turnip (*Brassica napus* L. cv. Seven Top) for *P. xylostella*, lima bean (*Phaseolus lunatus* L. cv. Baby Fordhook) for *H. virescens*, potato (*Solanum tuberosum* L. cv. Kennebec) for *L. decemlineata*, lettuce (*Lactuca sativa* L. cv. Black Seeded Simpson) for *L. dispar*, and tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) for *M. sexta*. All

plants were grown in a greenhouse under a regime of  $24 \pm 3$  °C, with the photoperiod supplemented to L16:D8 h by sodium vapor lamps. A commercial potting medium (Pro Mix BX®, Premier Brands, Red Hill, PA, USA) was used. Plants were 6–8 weeks old when used.

#### Bioassays

Bioassays were conducted to test the toxicity of pGEM-Pir A/B to the five species listed above. To account for possible variation in the concentration of active proteins in the culture stock, *P. xylostella* was used as a standard in all bioassays. When each trial of one of the other insects was conducted, a duplicate trial of *P. xylostella* (IBL strain) was also conducted. Each of the other insects was then compared with *P. xylostella*. A similar test in which Bt-resistant *P. xylostella* was compared with the IBL strain was also conducted.

For each trial, serial dilutions of pGEM-Pir A/B were prepared with concentrations varying in half-log increments. The highest concentration in all tests was a 10-fold dilution of the stock (a 50-fold dilution of the original cultures). Also prepared were controls of water only and of a 10-fold dilution of the suspension of the empty pGEM vector. A wetting agent, Triton X-155® (Union Carbide, Danbury, CT, USA), was included in all suspensions at a concentration of 0.05%. A cork borer was used to cut disks (1 cm diameter) from leaves. Disks were dipped in the suspensions, laid on moist paper, and allowed to dry. For each trial, the same suspensions were used to treat disks for both *P. xylostella* and the other species. Disks were placed individually with larvae on moist filter paper in cells of plastic bioassay trays (Bio-BA-128, CD International, Pitman, NJ, USA). The cells were closed with ventilated clear plastic covers. Larvae were held in an incubator at 27 °C for 48 h. Twenty-four larvae of each species/dilution were included in each trial, and the test was replicated four times for each species, except for the test of the Bt-resistant strain of *P. xylostella*, which was replicated five times.

Because preliminary observations indicated that most mortality of *P. xylostella* larvae occurred between 24 and 48 h, the trials were scored at 48 h. Mortality was recorded on all treatments. In addition, the first 10 surviving insects of species other than *P. xylostella* on the highest concentration of the pGEM-Pir A/B suspension and on the suspension of empty pGEM vector were weighed. *Plutella xylostella* larvae were not weighed because very few survived on the highest concentration of toxin (see Results).

#### Statistical analyses

For each trial of each species, mortality data were adjusted for mortality on the water control by Abbott's (1925) formula. Percentage mortality was then calculated and

normalized by arcsine  $\sqrt{\%}$  transformation. Concentrations of culture were transformed logarithmically. Data were then analyzed by analysis of variance (ANOVA) for effects of species (*P. xylostella* vs. each other species), concentration of culture suspensions, and the interaction of concentration by species (PROC GLM; SAS Institute, 1999). Data were also analyzed separately for the effects of concentration on each species. Also compared by ANOVA were the water control and the empty pGEM vector control for each species. Weights of surviving insects were analyzed by ANOVA for effects of treatment on each species. Because species other than *P. xylostella* were tested at different times, no other statistical comparisons among species were made.

Tests on the same stock suspension of pGEM-Pir A/B were conducted over a period of 120 days. To evaluate storage stability of the toxin suspension at  $-15$  °C, data on *P. xylostella* only at the three highest concentrations of toxin from all bioassays were analyzed by ANOVA for effect of storage time (time from the start of the study to the start of each bioassay) and concentration on mortality. Twenty-one trials of *P. xylostella* were included in this analysis.

#### Histology

*Plutella xylostella* larvae fed a 10-fold dilution of pGEM-Pir A/B or empty pGEM vector suspensions for 24 h were fixed in Carnoy's B for 3 h, put through three changes of absolute ethanol, three changes of xylene, and embedded overnight in Paraplast-Xtra® (Oxford Labware, St. Louis, MO, USA). Embedded larvae were sectioned on a rotary microtome at 5  $\mu$ m. Sections were relaxed on water at 40 °C, mounted on gelatin-coated slides, dried, and placed horizontally in a drying oven at 40 °C overnight. Mounted sections were deparaffinized in three changes of xylene, transferred through three changes of absolute ethanol, and rehydrated through a series of aqueous ethanol solutions. Sections were stained with Carazzi's hematoxylin (Carazzi, 1911) followed by Casson's trichrome (Kiernan, 1990) and examined under a compound microscope.

#### Results

The proteins encoded by the genes cloned from strain Hm (GenBank accession number DQ459368) were nearly identical to those predicted from the *plu4092-plu4093* genes of strain TT01 (Duchaud et al., 2003) with 95% and 92% sequence identity, respectively. The *plu4092* homolog of strain Hm was 34% identical to the putative juvenile hormone esterase of *L. decemlineata*.

In all tests comparing *P. xylostella* with other species, the interaction of species by pGEM-Pir A/B concentration was significant (Table 1). When species were analyzed

**Table 1** Results of statistical analyses of tests comparing the effects of pGEM-Pir A/B against the IBL strain of *Plutella xylostella* and four other species of phytophagous insects

Test species <sup>1</sup>	Figure <sup>2</sup>	Effect on	Effect of	F	d.f.	P
<i>L. decemlineata</i>	1A	Both species	Species	148.69	1,33	0.0001
			Concentration	64.61	1,33	0.0001
		<i>L. decemlineata</i>	Concentration*species	69.38	1,33	0.0001
			Concentration	0.12	1,15	0.7313
			Concentration	147.14	1,15	0.0001
<i>L. dispar</i>	1B	Both species	Species	132.16	1,33	0.0001
			Concentration	174.61	1,33	0.0001
		<i>L. dispar</i>	Concentration	88.35	1,33	0.0001
			Concentration	11.20	1,15	0.0044
			Concentration	185.83	1,15	0.0001
<i>H. virescens</i>	1C	Both species	Species	104.51	1,33	0.0001
			Concentration	76.18	1,33	0.0001
		<i>H. virescens</i>	Concentration*species	96.18	1,33	0.0001
			Concentration	1.00	1,15	0.3321
			Concentration	124.14	1,15	0.0001
<i>M. sexta</i>	1D	Both species	Species	142.73	1,33	0.0001
			Concentration	79.95	1,33	0.0001
		<i>M. sexta</i>	Concentration	59.96	1,33	0.0001
			Concentration	0.72	1,15	0.4098
			Concentration	148.28	1,15	0.0001

<sup>1</sup>Species being compared with *P. xylostella*.<sup>2</sup>Figure showing corresponding means.

separately, mortality of *P. xylostella* larvae increased with increasing concentrations of pGEM-Pir A/B in all tests (Figure 1, Table 1). Of the other species, only *L. dispar* showed a small, although statistically significant, increase in mortality with increasing concentration of pGEM-Pir A/B (Figure 1B, Table 1). Mortality of insects fed the suspension of the empty pGEM vector did not differ from that of insects fed the water control in any species (Table 2). Weights of surviving insects on the highest concentration of pGEM-Pir A/B did not differ from those of insects on

the empty pGEM vector, except in the case of *L. decemlineata*, in which those on the empty vector were slightly smaller than those on the pGEM-Pir A/B (Table 3).

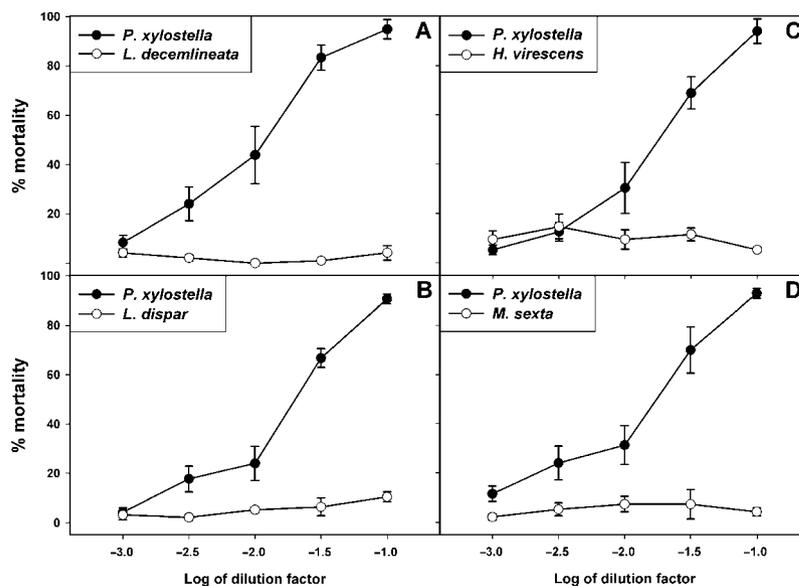
Mortality of both the Bt-resistant and the IBL strain of *P. xylostella* increased with increasing concentrations of pGEM-Pir A/B (Figure 2;  $F_{1,42} = 271.28$ ,  $P = 0.0001$ ). The two strains did not differ significantly in their response to pGEM-Pir A/B ( $F_{1,42} = 3.23$ ,  $P = 0.0794$  for effects of strain, and  $F_{1,42} = 0.06$ ,  $P = 0.8076$  for the strain\*pGEM-Pir A/B concentration interaction). There was no difference

**Table 2** Mortality of *Plutella xylostella* and four other insect species fed suspensions of empty pGEM vector or water control

Test <sup>1</sup>	Species	% mortality ( $\pm$ SE) on		F	d.f.	P
		pGEM	Water			
<i>L. decemlineata</i>	<i>L. decemlineata</i>	2.1 (1.20)	2.1 (1.20)	0.00	1,3	0.9999
	<i>P. xylostella</i>	6.3 (4.96)	5.2 (2.62)	0.03	1,3	0.8688
<i>L. dispar</i>	<i>L. dispar</i>	1.0 (1.04)	0.0 (0.00)	1.00	1,3	0.3910
	<i>P. xylostella</i>	6.3 (4.96)	5.2 (2.62)	0.04	1,3	0.8543
<i>H. virescens</i>	<i>H. virescens</i>	9.4 (3.13)	11.5 (3.13)	0.49	1,3	0.5360
	<i>P. xylostella</i>	3.1 (1.04)	6.3 (2.69)	2.59	1,3	0.2060
<i>M. sexta</i>	<i>M. sexta</i>	7.3 (2.62)	1.0 (1.04)	4.77	1,3	0.1170
	<i>P. xylostella</i>	4.2 (1.70)	5.2 (3.13)	0.02	1,3	0.9077

<sup>1</sup>Species being compared with *P. xylostella*.

**Figure 1** Mortality of *Plutella xylostella* larvae compared with mortality of four other species of phytophagous insects fed leaf disks treated with suspensions of pGEM-Pir A/B.



in mortality between the water control and the empty pGEM vector control for the IBL strain ( $F_{1,4} = 4.04$ ,  $P = 0.1148$ ) or for the Bt-resistant strain ( $F_{1,4} = 1.50$ ,  $P = 0.2884$ ). The potency of the pGEM-Pir A/B suspension, as measured by its effect on the IBL strain of *P. xylostella*, did not change significantly in storage at  $-15^{\circ}\text{C}$  over the 120-day course of the study ( $F_{1,59} = 0.98$ ,  $P = 0.3262$ ).

Histological examination of sectioned *P. xylostella* larvae fed leaf disks dipped in pGEM-Pir A/B suspension revealed gross abnormalities of the midgut when compared with sections of larvae fed leaf disks dipped in the empty pGEM vector controls. The columnar cells showed swelling and shedding of their apical membranes into the gut lumen. Although all regions of the midgut were affected, swelling of the epithelium in the posterior midgut was generally more profound (Figure 3).

## Discussion

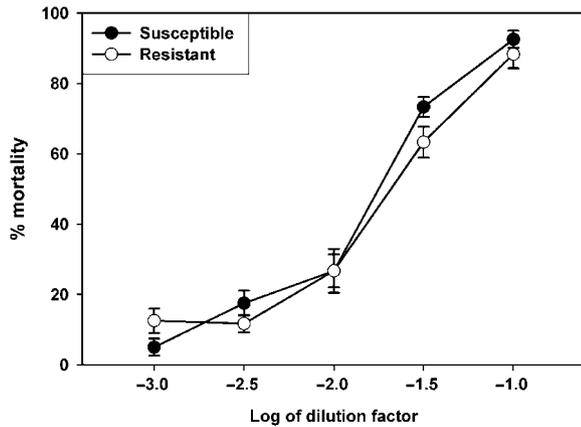
Although *P. xylostella* seem quite susceptible to PirA/B, we were unable to detect any effect on any other species tested.

In our assays, there was some slight increase in mortality of *L. dispar* larvae fed the highest concentration, but we found no difference in the weights of surviving *L. dispar* larvae and the control group. This last point leads us to believe that the slight increase in mortality is a statistical aberration, but does not preclude the possibility that a small proportion of *L. dispar* larvae are highly susceptible. Our results suggest that *P. xylostella* is at least 300-fold more susceptible than the other species tested. The marked susceptibility of *P. xylostella* larvae could be due either to differences in gut protease profiles, or to differences in toxin interaction with specific gut receptors. The very specific response of *P. xylostella* relative to the other lepidopterans we tested is in contrast to the uniform response of the three mosquito species tested by Duchaud et al. (2003).

Although the Pir B protein shares some sequence similarities with the *B. thuringiensis*  $\delta$ -endotoxins, we found no difference between *P. xylostella* larvae from our susceptible laboratory colony and a commercially available Cry 1A-resistant strain. The susceptibility of the resistant strain is

**Table 3** Weights (mg) of surviving insects of four species fed suspensions of pGEM-Pir A/B or an empty pGEM vector control

Species	Weight ( $\pm$ SE) on		F	d.f.	P
	pGEM-Pir A/B	Empty pGEM			
<i>L. decemlineata</i>	3.77 (0.156)	3.16 (0.152)	8.32	1,75	0.0051
<i>L. dispar</i>	1.04 (0.068)	1.03 (0.072)	0.00	1,75	0.9839
<i>H. virescens</i>	1.05 (0.075)	1.00 (0.075)	0.25	1,75	0.6178
<i>M. sexta</i>	4.35 (0.113)	4.46 (0.100)	0.66	1,75	0.4182



**Figure 2** Mortality of Bt-resistant and susceptible *Plutella xylostella* larvae fed leaf disks treated with suspensions of pGEM-Pir A/B.

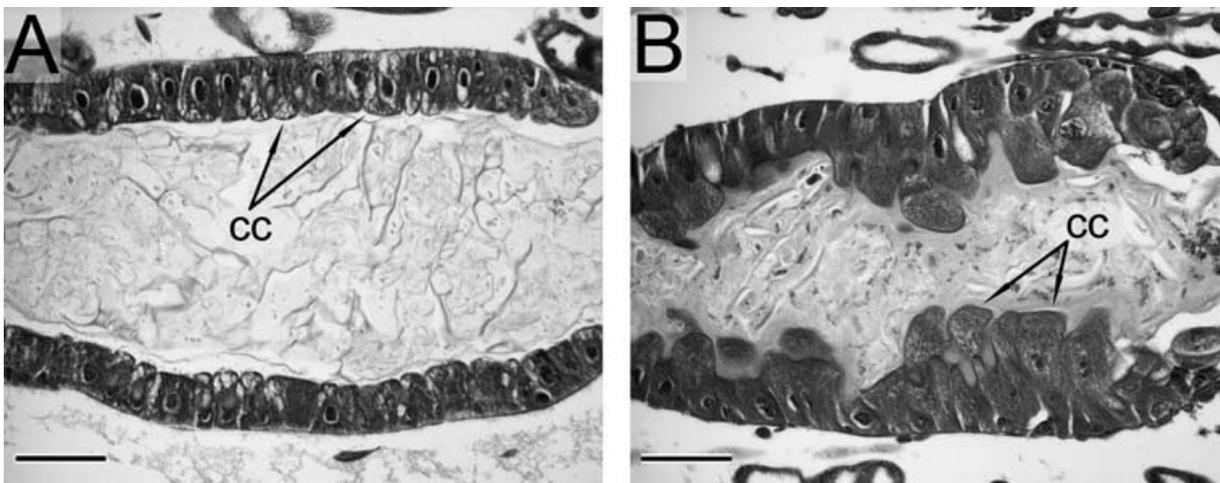
probably due to the fact that similarities between Pir B and the  $\delta$ -endotoxins reside in the N-terminal domain of the endotoxins, which is associated with membrane insertion, and not with domains associated with specific interactions with midgut receptors.

In previous reports, no attempt was made to identify the target organ affected by Pir toxins. In *P. xylostella*, the only lepidopteran affected by oral administration of Pir toxins, we have demonstrated that the midgut is the primary site of action. The pathology produced by the toxin is similar to that seen in other gut active toxins, that is, swelling and shedding of the apical gut membranes. Although the entire gut typically showed abnormalities, damage seemed to be most consistent in the posterior midgut, near the junction

with the hindgut. This contrasts with the effect of *P. luminescens* Tca on the midgut of *M. sexta*, in which the most profound effects were noted in the anterior midgut (Blackburn et al., 1998).

The possibility that the developmentally regulated *L. decemlineata* protein described by Vermunt et al. (1997) is actually a leptinotarsin or a related protein (Crosland et al., 2005) may shed some light on the mode of action of the Pir toxins. The leptinotarsins, originally described by Hsiao & Fraenkel (1969) and Hsiao (1978) from the hemolymph of *L. decemlineata* and other *Leptinotarsa* species, are toxic to both insects and vertebrates. They are neurotoxins that cause rapid influx of  $Ca^{2+}$  through presynaptic  $Ca^{2+}$  channels and trigger neurotransmitter release (Crosland et al., 2005). It would be worthwhile to determine if this mode of action has any relevance to the observed effects of the Pir toxins on the midgut epithelium of *P. xylostella*, or if the Pir toxins possess leptinotarsin-like neurotoxicity.

The actual biological roles that the Pir toxins of *P. luminescens* and their beetle counterparts play in their respective organisms remain a mystery. Ironically, Armer et al. (2004) have speculated that the leptinotarsin of *L. decemlineata* may provide the beetle some protection against the nematode *Heterorhabditis marelatus* which, of course, carries the bacterium *P. luminescens* as an enteric symbiont. These authors document a heat labile factor in the beetle's hemolymph, which causes a phenotypic shift in *P. luminescens* to a secondary form that does not support nematode growth. Heat denaturation at 60 °C caused the disappearance of a protein very similar in size to leptinotarsin from beetle hemolymph, and abolished the ability of the hemolymph to cause the phenotypic shift in *P. luminescens*.



**Figure 3** Histological sections of *Plutella xylostella* larvae fed a suspension of *Escherichia coli* containing either (A) an empty pGEM vector, or (B) pGEM-Pir A/B. Note the dramatic apical swelling of the columnar cells (cc) in larvae feeding on *E. coli* containing the Pir genes (B). Both A and B show the most posterior region of the midgut, adjacent to the hindgut (right is posterior). Scale bars = 50  $\mu$ m.

The results of the current study suggest that the application of the Pir toxins in insect control may be limited. The oral activity against *P. xylostella* appears to be an anomaly among the Lepidoptera, although we cannot preclude the possibility that the toxins are active against the other species tested at higher concentrations. In addition, the possible relation of the Pir toxins to the leptinotarsins may indicate problems with vertebrate toxicity.

### Acknowledgements

The authors wish to thank John M. Domek for his technical contributions to early phases of this study, and David J. Bowen for supplying *P. luminescens* strain Hm. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

### References

- Abbott WS (1925) A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology* 18: 265–267.
- Armer CA, Rao S & Berry RE (2004) Insect cellular and chemical limitations to pathogen development: the Colorado potato beetle, the nematode *Heterorhabditis marelatus*, and its symbiotic bacteria. *Journal of Invertebrate Pathology* 87: 114–122.
- Blackburn MB, Domek JM, Gelman DB & Hu JS (2005) The broadly insecticidal *Photorhabdus luminescens* Toxin complex a (Tca): activity against Colorado potato beetle and sweet potato whitefly. *Journal of Insect Science* 5: 32.
- Blackburn M, Golubeva E, Bowen D & ffrench-Constant RH (1998) A novel insecticidal toxin from *Photorhabdus luminescens*: histopathological effects of toxin complex A (Tca) on the midgut of *Manduca sexta*. *Applied and Environmental Microbiology* 64: 3036–3041.
- Bowen D, Rocheleau TA, Blackburn M, Andreev O, Golubeva E et al. (1998) Insecticidal toxins from the bacterium *Photorhabdus luminescens*. *Science* 280: 2129–2132.
- Carazzi D (1911) Eine neue Haematoxylinloesung. *Zeitschrift für Wissenschaftliche Mikroskopie und für Mikroskopische Technik* 28: 273.
- Crosland RD, Fitch RW & Hines HB (2005) Characterization of  $\beta$ -leptinotarsin-h and the effects of calcium flux agonists on its activity. *Toxicon* 45: 829–841.
- Daborn PJ, Waterfield N, Silva CP, Au CPY, Sharma S & ffrench-Constant RH (2002) A single *Photorhabdus* gene, *makes caterpillars floppy* (Mcf), allows *Escherichia coli* to persist within and kill insects. *Proceedings of the National Academy of Sciences of the United States of America* 99: 10742–10747.
- Duchaud E, Rusniok C, Frangeul L, Buchrieser C, Givaudan A et al. (2003) The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. *Nature Biotechnology* 21: 1307–1313.
- Guo L, Fatig RO III, Orr GL, Schafer BW, Strickland JA et al. (1999) *Photorhabdus luminescens* W-14 insecticidal activity consists of at least two similar but distinct proteins: purification and characterization of toxin A and toxin B. *The Journal of Biological Chemistry* 274: 9836–9842.
- Hsiao TM (1978) Comparative studies on hemolymph protein toxins of Leptinotarsa beetles. In: Rosenberg P (Ed), *Toxins: Animal, Plant, and Microbial*. Pergamon Press, Oxford, pp. 675–688.
- Hsiao TH & Fraenkel G (1969) Properties of Leptinotarsin, a toxic hemolymph protein from the Colorado potato beetle. *Toxicon* 7: 119–130.
- Kiernan JA (1990) *Histological and Histochemical Methods: Theory and Practice*. Pergamon Press, New York, NY, USA.
- King EG & Hartley GG (1985) *Heliothis virescens*. *Handbook of Insect Rearing*, Vol. II (ed. by P Singh & RF Moore), pp. 232–328. Elsevier, New York, NY, USA.
- SAS Institute (1999) SAS Online Doc®, Version 8. SAS Institute Inc., Cary, NC, USA.
- Shelton AM, Cooley RJ, Kroening MK, Wilsey WT & Eigenbrode SD (1991) Comparative analysis of two rearing procedures for diamondback moth (Lepidoptera: Plutellidae). *Journal of Entomological Science* 26: 17–26.
- Thomas GM & Poinar GO (1979) *Xenorhabdus* gen. nov. a genus of entomopathogenic bacteria of the family Enterobacteriaceae. *International Journal of Systematic Bacteriology* 29: 352–360.
- Vermunt AM, Koopmanschap AB, Vlaskovits JM & de Kort CA (1997) Cloning and sequence analysis of cDNA encoding a putative juvenile hormone esterase from the Colorado potato beetle. *Insect Biochemistry and Molecular Biology* 27: 919–928.
- Waterfield NR, Daborn PJ, Dowling AJ, Yang G, Hares M & ffrench-Constant RH (2003) The insecticidal toxin *Makes caterpillars floppy 2* (Mcf2) shows similarity to HrmA, an avirulence protein from a plant pathogen. *FEMS Microbiology Letters* 229: 265–270.
- Waterfield N, Kamita SG, Hammock BD & ffrench-Constant RH (2005) The *Photorhabdus* Pir toxins are similar to a developmentally regulated insect protein but show no juvenile hormone esterase activity. *FEMS Microbiology Letters* 245: 47–52.