Photorhabdus Virulence Cassettes Confer Injectable Insecticidal Activity against the Wax Moth

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Two recently sequenced genomes of the insect-pathogenic bacterium Photorhabdus and a large Serratia entomophila plasmid, pADAP, have phage-related loci containing putative toxin effector genes, designated the “Photorhabdus virulence cassettes” (PVCs). In S. entomophila, the single plasmid PVC confers antifeeding activity on larvae of a beetle. Here, we show that recombinant Escherichia coli expressing PVC-containing cosmid from Photorhabdus has injectable insecticidal activity against larvae of the wax moth. Electron microscopy showed that the structure of the PVC products is similar to the structure of the antibacterial R-type pyocins. However, unlike these bacteriocins, the PVC products of Photorhabdus have no demonstrable antibacterial activity. Instead, injection of Photorhabdus PVC products destroys insect hemocytes, which undergo dramatic actin cytoskeleton condensation. Comparison of the genomic organizations of several PVCs showed that they have a conserved phage-like structure with a variable number of putative anti-insect effectors encoded at one end. Expression of these putative effectors directly inside cultured cells showed that they are capable of rearranging the actin cytoskeleton. Together, these data show that the PVCs are functional homologs of the S. entomophila antifeeding genes and encode physical structures that resemble bacteriocins. This raises the interesting hypothesis that the PVC products are bacteriocin-like but that they have been modified to attack eukaryotic host cells.

The recent sequencing of the Photorhabdus genome confirmed that this entomopathogenic bacterium produces an astonishing array of putative insecticidal toxins (7). Three classes of toxins have now been characterized. The members of the first class, the toxin complexes, are orally toxic to caterpillar pests (2) and have recently been used to create insect-resistant transgenic plants (14). The members of the second class, the “makes caterpillars floppy” toxins (Mcf1 and Mcf2), are potent toxins that are active upon injection (4, 18). Mcf1 mimics BH3 domain-only proteins, which are proapoptotic proteins found in mitochondria, and promotes apoptosis both in the insect gut and in mammalian tissue culture cells (6). Third and most recently, the “Photorhabdus insect-related” proteins (PirAB) have been shown to be binary toxins with both injectable (17) and oral activities in some insects (7). Given the requirement that Photorhabdus bacteria should kill the insect host into which they are released by their nematode vector and the numerous predicted toxins encoded in the Photorhabdus luminescens TT01 genome (7), it seems likely that other classes of insecticidal toxin remain to be discovered. In this light, here we investigated homologs of an antifeeding locus of the free-living entomopathogenic bacterium Serratia entomophila (11). We speculated that the antifeeding effect of the prophage-like locus in S. entomophila may reflect low antigut toxicity and therefore the possibility that the homologs of this locus in Photorhabdus are also toxic to insects.

In S. entomophila, the 120-kb pADAP plasmid confers “amber disease,” a disease associated with “clearing” of midgut of the New Zealand grass grub (12). The pADAP plasmid contains genes encoding both homologs of the insecticidal toxin complex (tc genes) (8), termed sepA, sepB, and sepC (12), which are responsible for insect gut clearance, and also a prophage-like locus responsible for a separate “antifeeding” effect (11). This pADAP prophage-like locus contains 18 putative open reading frames for predicted proteins with high levels of similarity to phage tail and base plate proteins, as well as a putative effector protein which is putatively responsible for the antifeeding activity against the grass grub (11). Similarly, the genomes of two recently sequenced strains of Photorhabdus, P. luminescens strain TT01 (11, 19) and Photorhabdus asymbiotica strain ATCC 43949 (this study), contain numerous copies of these prophage-like loci, each encoding a different putative effector protein. Some of the putative effectors exhibit predicted amino acid similarity to parts of known multidomain toxins, such as the Mcf cytotoxin from P. luminescens (4), toxin A from Clostridium difficile (5), YopT from Yersinia enterocolitica (21), and the active site of cytotoxic necrosis factor (CNF1) from Escherichia coli (13). Others exhibit no predicted similarity to known effectors and so may represent novel effectors with novel modes of action.

In order to determine if the products of Photorhabdus virulence cassettes (PVCs) have insecticidal activity, as suggested by the antifeeding activity of the product of the pADAP PVC locus in S. entomophila, here we investigated their injectable activity against larvae of the wax moth Galleria mellonella. We found not only that E. coli strains with PVC-containing cosmids are toxic to larvae after injection but also that the same E. coli strains produce structures similar to one type of bacteriocins, the R-type pyocins. Unexpectedly, the PVC products have no antibacterial activity but trigger rapid destruction of insect phagocytes. The hemocyte destruction is associated with actin
condensation, an effect that can be recapitulated by expressing putative effector proteins from the PVCs directly in tissue culture cells. Transposon mutagenesis of a single PVC showed that injectable toxicity is disrupted by insertion within a central subset of open reading frames. These results support the hypothesis that the PVCs are functional homologs of the anti-feeding genes of \textit{S. entomophila} but leave the precise mechanism of toxicity associated with the bacteriocin-like structure unclear.

**MATERIALS AND METHODS**

**2D electrophoresis and protein identification.** Supernatant proteins from \textit{E. coli} EC100 carrying either the e4DF10 cosmid (which encodes PaPVCpfn) or the control vector pWB2 were phenol precipitated, and the protein was resuspended in 150 µl CDU, which contained 9 M urea, 4% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), 130 mM dithiothreitol (DTT), and 1× HALT protease inhibitor cocktail mixture (Pierce), and was incubated for 2 h at room temperature. Samples were then centrifuged for 30 min at 88,760 x g. For protein quantification, a RedPlate protein quantitation kit (Molecular Probes) was used. For two-dimensional (2D) gel electrophoresis, the Multichip II system (GE Healthcare) was used for isoelectric focusing and horizontal sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis with Immobiline DryStrip gels and precast 12.5% homogeneous SDS gels (GE Healthcare). Briefly, Immobiline Dry Strip gels were rehydrated overnight in rehydration buffer, which consisted of 8 M urea, 2% CHAPS, 0.002% bromophenol blue, 18 mM DTT, and 0.5% IPG buffer, containing 15 µg of the protein sample. Isoelectric focusing was performed at 20°C. For the IPG 3–10 strip the upper limits for current and power were set at 2 mA and 2 W, respectively. In phase 1 the voltage was kept at 300 V for 1 h, in phase 2 the voltage was increased to 3,500 V over 2,900 V h, and in phase 3 the voltage was kept at 3,500 V for 7,100 V h. The resulting strips were then frozen at −80°C for subsequent analysis. Before SDS gel electrophoresis, strips were equilibrated for 15 min in 50 mM Tris (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, and 65 mM DTT. In a second equilibration step, the 65 mM DTT was replaced by 135 mM iodoacetamide. The Immobiline DryStrip gel was then transferred onto the SDS polyacrylamide gel and electrophoresed. Before staining, gels were washed overnight in a fixation solution containing 40% ethanol and 10% acetic acid. Silver staining was performed according to the protocol of a PlusOne silver staining kit (GE Healthcare), omitting gelatine-free gels from the sensitizing solution and formaldehyde from the silver nitrate solution. Protein spots from silver-stained gels were cut from the acrylamide and sent to the protein sequencing facility at the University of the West of England. Matrix-assisted laser desorption ionization—time of flight (MALDI-TOF) analysis of trypsin-digested protein samples was used to generate a spectrum, which was compared to all proteins in the protein database.

**Cosmid libraries and insect bioassay.** Cosmid libraries of \textit{P. asymbiotica} ATCC 43949, \textit{P. luminescens} strain T010, and \textit{Photorhabdus temperata} strain K122 were prepared in \textit{E. coli} EC100 and arrayed into 96-well microtiter plates with MWG Biotech, Munich, Germany, as described previously (4). Insect bioassays were performed as described elsewhere (17). Briefly, 10-µl portions of a whole bacterial culture or a cell-free supernatant were injected into Gelattella larvae that had been cooled at 4°C overnight. The animals were then held at 25°C and scored for “blackening,” morbidity, and mortality at 1 and 24 h postinjection. “Heated” bacterial supernatants were heated to 90°C for 20 min to inactivate PTC toxicity.

**Recombinant expression of putative effectors.** The \textit{logT}-like and \textit{prlP}-like genes from PaPVCpfn and PaPVCpfn were PCR amplified from \textit{P. asymbiotica} strain ATCC 43949 genomic DNA using the following primers: \textit{logT}F4 (TAAGAG CCAAATTTAATACGAAAGTGA), \textit{paLogT-R} (TATGAACTTTAGG GTAATGGTTGGA), \textit{paPPLF} (AATTGAACCTCAACCATACGAGA), and \textit{paPPLF} (GGTCAGGTATTAAACACGCTT). The PCR products were purified and cloned into the arabinose-inducible expression plasmid pBAD30. The resulting clones were sequenced to confirm their identities, and the optimal expression conditions were determined. Briefly, −80°C glycerol stocks were used to inoculate 5 ml of fresh LB medium supplemented with 0.2% (wt/vol) glucose and the appropriate antibiotic for selection. Bacteria were grown overnight at 30°C with shaking, and 1 ml of the culture was then harvested, resuspended in 100 ml of the same medium, and incubated in an orbital incubator at 37°C until the optical density at 600 nm was 0.7 to 0.9. Cells were then harvested at room temperature by centrifugation at 4,000 rpm for 10 min. The pellet was resuspended in 100 ml of fresh LB medium supplemented with the appropriate antibiotic and the inducer l-arabinose at a concentration of 0.2% (wt/vol). Optimized times for induction were determined experimentally, and cells were then harvested. The bacterial cell pellet was resuspended in 10 ml of 1× phosphate-buffered saline (PBS) and sonicated (four 20-s sonications at 45 mA using a Branson 450 digital Sonifier) fitted with a tapered probe. The freshly sonicated samples were then diluted in 1× PBS for injection into \textit{Galleria} larvae and for SDS-polyacrylamide gel electrophoresis analysis to confirm expression of the target protein.

**Homocyste counts and actin staining.** Injected \textit{Galleria} larvae were bleed onto microscopic slides by piercing the larval cuticle. Four animals were bled per treatment, and hemocytes were counted in four random microscopic fields of view on each slide. This provided an estimate of the number of recoverable (circulating) hemocytes. A tetramethyl rhodamine isocyanate (TRITC)-phalloloid conjugate was used to stain the homocyste cytoskeleton (1). Briefly, preinjected \textit{Galleria} larvae were bled onto coverslips, and the hemocytes were allowed to attach to the glass surface by incubation at room temperature for 20 min, after which they were fixed and stained with TRITC-phalloloid.

**PVC purification and electron microscopy.** Overnight cultures of \textit{E. coli} with PVC-containing cosmids were grown overnight in LB medium at 30°C with aeration. The cultures were centrifuged at 8,000 rpm in a GS3 rotor for 30 min at 4°C. The supernatant was decanted to remove each cell pellet, and the centrifugation procedure was repeated to remove any remaining cells. Each cell-free supernatant was then centrifuged at 150,000 × g for 90 min at 4°C in a Sorval ultracentrifuge to harvest particular material. The supernatants were then discarded, and the pellets were washed by gentle resuspension in 1× PBS (5 × 150,000 × g for 90 min at 4°C) to pellet the particulate material. Each pellet was finally resuspended in 500 µl of ice-cold PBS and stored at 4°C. As a second step in the PVC purification procedure the particulate preparations were further separated by DEAE-Sepharose chromatography. For DEAE-Sepharose chromatography, the particular material recovered after the first high-speed centrifugation described above was resuspended in 10 ml of ice-cold PBS, and an equivalent volume of DEAE-Sepharose affinity matrix was added. The preparations were incubated at 4°C until the supernatant was harvested. The bacterial cell pellet was resuspended in 10 ml of ice-cold PBS and again harvested by centrifugation. This washing step was repeated another three times, and the final resuspended in 10 ml of elution buffer (0.5 M NaCl, 50 mM phosphate buffer [pH 7.4]). The resin was removed by centrifugation, and the supernatant containing the PVCs was again centrifuged at 150,000 × g for 90 min at 4°C to pellet the particulate material. The particular material was finally resuspended in 600 µl of ice-cold PBS and stored at 4°C.

For transmission electron microscopy (TEM) pioloform-covered 300-mesh copper grids were coated with a fine layer of carbon were used as substrates for the protein fractions. The four following aqueous negative stains were tested with the protein samples: 1% uranyl acetate, 3% ammonium acetate, 1% phosphotungstic acid, 2% sodium silicotungstate. The preferred stain, 3% methylamine tungstate, produced acceptable contrast and minimum artifacts and was subsequently used for all samples viewed by TEM. The coated grids were exposed to UV light for 16 h immediately prior to use to ensure adequate wetting of the substrate. A 10-µl drop was applied to the TEM grid, and the protein was allowed to settle for 5 min. Liquid was absorbed with filter paper from the edge of the grid and replaced immediately with 10 µl of filtered negative stain. The drop was partially removed with filter paper, and the grids were allowed to air dry thoroughly before they were viewed with a JEOL 1200EX transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV.

**Effector expression in mammalian cells.** The \textit{prlP}-like and \textit{sepC}-like genes of \textit{PaPVCpfn} were PCR amplified from \textit{P. asymbiotica} genomic DNA using primers (5′ to 3′) \textit{paprskR} \textit{pbfamH1} (TTAGAGCATTCTTTAATATAGCTAATCC; BamHI site), \textit{paprskR} \textit{pbfamH1} (GTCGTCATATCGAGATAATCA; Psfl site), \textit{paprskR} \textit{pbfamH1} (GGGGAACGTATTACACACCCT; Psfl site), and the \textit{pbfamH1} \textit{PVCpfn} gene was PCR amplified using primers \textit{pbfamH1} \textit{pbfamH1} (TTAGAGCATTCTTTAATATAGCTAATCC; BamHI site) and \textit{paprskR} \textit{pbfamH1} (TAGTCGACGATAATCA; Psfl site) (the underlining indicates mismatched nucleotides incorporated to generate the restriction site described; all PCR products were purified using standard techniques and were cloned as \textit{MYC}-tagged fusions into the relevant restriction sites of the mammalian expression plasmid pRK5myc. Clones were sequenced to confirm their identities. These constructs were cotransfected into NIH-3T3 cells along with pEGFPActin as previously described (16). Briefly, the \textit{MYC}-tagged fusion allowed us to detect, visualize, and
localize transient expression of the target protein (Pnf, SepC, or Plu1690), while the pEGFPactin revealed the condition of the cytoskeleton in the same cells.

RESULTS AND DISCUSSION

Genomic organization. To determine the genomic organization of the PVCs in different Photobacterium species, we identified and compared PVCs from *P. luminescens* strain TT01 (7), *P. temperata* strain K122, and *P. asymbiotica* ATCC 43949. PVC loci were found to be distributed around the genome in both *P. asymbiotica* ATCC 43949 and *P. luminescens*. However in the case of *P. luminescens*, four PVC elements were also clustered in a tandem array between a type IV DNA conjugation pilus operon and a mukB replicon partitioning gene. In *P. asymbiotica* only one of these PVC elements was present at this locus, while in *P. temperata* this PVC cassette was replaced by a Mu-like phage, suggesting that there is a common insertion site for mobile elements. A detailed comparison of the different predicted open reading frames in the different PVCs revealed their phage-like structure. Thus, each PVC unit was predicted to encode 15 to 20 proteins, each with predicted similarity to phage tail, phage base plate assembly, fimbrial usher, and putative effector proteins from other pathogenic bacteria (Fig. 1, top panel). We noted that each PVC unit had different putative effector sequences, but they were always in the equivalent position (Fig. 1, top panel), as documented for other prophages (3). The different PVC effectors were also often flanked by transposons, suggesting a possible mechanism for their insertion into the PVC or movement between different PVCs. Finally, we noted that one PVC element was consistently inserted adjacent to the mukB locus, a locus involved in plasmid maintenance and stability (20). This suggests either that mukB may be a communal insertion point for these phage-like elements or that it may regulate the insertion of plasmids containing these elements. Interestingly, a type IV DNA con-

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![FIG. 1. PVC loci of *P. asymbiotica*. (Top panel) Conserved PVC unit consisting of 13 to 15 open reading frames encoding predicted proteins with high levels of similarity to different phage components. Note the variable number of putative effector proteins encoded at one end of each PVC locus. These effectors are either unnamed or bear the name of the protein to which they exhibit the highest level of similarity in a BlastX search (see text). (Bottom panel) 2D gels of *E. coli* proteins prepared from recombinant bacteria with (left gel) and without (right gel) the PaPVCpnf cosmid. The circled proteins are unique to PaPVCpnf-expressing *E. coli*, and three of the most abundant of these proteins were confirmed to be PVC-encoded proteins by picking the spots from the gel, analyzing them by MALDI-TOF analysis, and subsequently matching predicted tryptic digests of all PVC-encoded proteins. Orf 1, Orf 2, and Orf 3 correspond to the first three open reading frames in the PaPVCpnf unit.](http://jb.asm.org/)

#### GENOMIC ORGANIZATION

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jugation pilus operon was found immediately 5’ of this PVC element insertion site. A similar operon was also found on the pADAP plasmid, where it was responsible for conjugative transfer of the plasmid. It is therefore tempting to speculate that this operon in *Photorhabdus* is involved in horizontal movement of the PVC elements. We selected two model elements from *P. asymbiotica*, designated PaPVCpnf and PaPV ClopT, for further experimental investigation.

**Expression and structure.** We examined the supernatant of PaPVCpnf cosmid-containing *E. coli* via 2D gel electrophoresis and subsequent MALDI-TOF analysis of expressed *Photorhabdus* proteins (Fig. 1, bottom panel). Visual examination of 2D gels of *E. coli* with and without PVC-carrying cosmids revealed several differences (Fig. 1, bottom panel). Subsequent MALDI-TOF analysis of trypsin digests of proteins present only in the PVC-expressing *E. coli* and subsequent interrogation of a database of predicted PVC-encoded proteins confirmed that three of the most abundant differentially expressed proteins had a PVC origin and corresponded to the first three predicted open reading frames in the PVC locus (Fig. 1, top panel). To determine the structure of the final exported PVC, we purified preparations of PaPVCpnf-expressing recombinant *E. coli* by using TEM. Electron microscopy revealed the presence of structures highly reminiscent of R-type pyocins (15). These structures were ~30 nm wide and had variable lengths; some examples were more than 800 nm long (Fig. 2). They

![FIG. 2. Transmission electron micrographs of PVC products, showing their similarity to R-type pyocins. (A) Relaxed PVC product, showing only the outer sheath (arrow). (B) Contracted PVC product, showing extrusion of the inner needle (white arrow) from outer sheath (black arrow).](http://jb.asm.org/)

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comprised a rigid contractile outer sheath and a thin hollow inner “needle” structure (approximately 6 nm wide), analogous to R-type pyocins. Examples of both “contracted” and “relaxed” forms were seen (Fig. 2). Although the precise mode of action of R-type pyocins themselves is not known, it has been suggested that their antibacterial activity is achieved by injection of the needle into bacterial cells via contraction of the sheath (15). However, investigation of this potential mode of action for the PVC products was beyond the scope of the current study.

**Insecticidal activity.** As R-type pyocins are bacteriocins with well-documented antibacterial activity (15), we tested purified PVC products with a range of different bacteria. We were unable to demonstrate any activity against either *E. coli* or *Micrococcus luteus*. We therefore also tested insecticidal activity; we injected *E. coli* K-12 strain EC100 carrying individual PVC-containing cosmids into larvae of the wax moth. Following injection of whole cultures of PVC-expressing *E. coli*, larvae died within 24 h (Fig. 3A). For *E. coli* expressing PaPVCpfn, larvae into which cell-free supernatant alone was injected became black and moribund 15 min after injection (Fig. 3B). This injectable activity in the supernatant from PaPVCpfn-expressing *E. coli* was eliminated by heat treatment prior to injection (Fig. 3C). Surprisingly, a comparison of the mortality data (Fig. 3A) resulting from injection of *E. coli* carrying PVC products from different *Photobacterium* species suggested that PVC products from the human pathogen *P. asymbiotica* (PaPVC) caused blackening in 80 to 100% of the larvae, whereas those from *P. luminescens* (PlPVC) exhibited a phenotype in only 10 to 40% of the larvae. (B) Larvae into which only supernatants from *E. coli* expressing PaPVCpfn was injected showed rapid blackening and death within 30 min. (C) Control larvae into which a heated supernatant from *E. coli* expressing PaPVCpfn was injected showed no effects.

**In vivo effect on hemocytes.** Following injection of *E. coli* K-12 strain EC100 not expressing any bacterial virulence factors, the insect immune system normally clears the infecting *E. coli* via a combination of antibacterial peptide production and the phagocytosis and nodulation reactions associated with insect hemocytes (10). Therefore, in order to understand why PVC-expressing *E. coli* is not successfully neutralized by the insect immune system, we bled *Galleria* larvae 30 min after injection with PaPVCpfn supernatants and examined their hemocytes. Simple counting of recoverable hemocytes revealed a dramatic decrease in the number of intact hemocytes 30 min after injection (Fig. 4A). Further staining of the few remaining hemocytes with TRITC-phalloidin revealed that their actin cytoskeletons were compressed in the residual cell bodies (Fig. 4B). The dramatic decrease in the number of recoverable hemocytes from infected insects as little as 30 min after injection demonstrated the rapid onset of the insecticidal effects mediated by PVC products from *P. asymbiotica*.

**FIG. 3.** Mortality of *Galleria* larvae into which *E. coli* expressing individual PVC-containing cosmids was injected. (A) Percentage of larvae showing blackening at 24 h after injection of either whole PVC-expressing *E. coli* cultures (left) or culture supernatant of PaPVCpfnalone (right). Note that all the PVC-expressing cosmids from *P. asymbiotica* (PaPVC) caused blackening in 80 to 100% of the larvae, whereas those from *P. luminescens* (PlPVC) exhibited a phenotype in only 10 to 40% of the larvae. (B) Larvae into which only supernatants from *E. coli* expressing PaPVCpfn was injected showed rapid blackening and death within 30 min. (C) Control larvae into which a heated supernatant from *E. coli* expressing PaPVCpfn was injected showed no effects.
(Fig. 4A) helps explain how PVC-expressing *E. coli* can persist in the presence of insect phagocytes or hemocytes.

To confirm that the putative effectors within *Photorhabdus* PVCs do indeed represent novel antieukaryotic effectors, we expressed several effectors directly in transfected mammalian cells and looked for rearrangements of the actin cytoskeleton and changes in nuclear morphology. Putative PVC effectors were transfected into NIH-3T3 cells using the mammalian expression vector pRK5myc (6). This vector expresses each effector as a fusion protein with Myc, which allows detection of it in transfected cells using an anti-Myc antibody (6). To assess the subsequent integrity of the actin cytoskeleton, we cotransfected the same cells with a construct expressing EGFP actin (Fig. 5). Consistent with its dramatic effect on insect hemocytes, the effector that exhibited similarity to Pnf, encoded by PaPVCpnf, dramatically reduced the cell bodies of transfected cells, leaving only a bundle of condensed actin and a disintegrating nucleus (Fig. 5C). Interestingly, a second putative effector also encoded on the PaPVCpnf element, which exhibited similarity to the C-terminal region of the SepC toxin, produced long arms of actin-rich filaments in transfected cells (Fig. 5D). Finally, a third effector, encoded by *plt1690*, which exhibited similarity to part of the *P. luminescens* cytotoxin Mcf (4), also reduced the cell bodies of transfected cells, leaving an intact nucleus surrounded by several actin-rich bundles (Fig. 5B). Together, these data suggest that the PVC-encoded effectors are capable of rearranging the actin cytoskeleton of host cells. However, it is not clear if the antihemocytic effects of PVC effector injection are related to this phenotype in mammalian tissue culture cells.

**Transposon mutagenesis.** In order to determine which open reading frames in an individual PVC are required for injectable insecticidal activity, we performed transposon mutagenesis of the PaPVCpnf cosmid and then examined the toxicity of the resulting mutants containing single insertions in single open reading frames. The results of the mutagenesis (Fig. 6) showed that a small central section of open reading frames, including the putative effector gene encoding a protein with similarity to the known effector Lop-T, are required for insecticidal activity.

**Conclusions.** Here we describe the first functional studies of phage-like PVC structures found as repetitive cassettes in two
different genera of entomopathogenic bacteria, *Photorhabdus* (19) and *Serratia* (11). These prophage-like loci were described both as an antifeeding locus on the pADAP plasmid of *S. entomophila* (11) and as repetitive loci in the genomes of *P. luminescens* TT01 (19) and *P. asymbiotica* ATCC 43949 (19).

We found that these loci encode particles whose structure is similar to the structure of R-type pycocins. However, unlike R-type pyocins, the PVC products have no demonstrable antibiotic activity, but they do have injectable activity against larvae of the wax moth. Together, these data suggest that the PVC products, together with the product of the antifeeding locus on pADAP of *S. entomophila* (11), may be bacteriocin-like structures modified to have activity against insect phagocytes. However, many central questions related to the biology of both the PVCs and the *S. entomophila* antifeeding genes remain unanswered. First, what is the relative role of phage-like genes and the putative effectors in PVC toxicity? Second, are the hemocytes the primary target of the PVC products, or

![Figure 5](image-url)  
**FIG. 5.** Expression of PVC effectors in mammalian tissue culture cells. (A) NIH-3T3 cells transfected with the pRK5myc (red) vector alone and cotransfected with a second construct expressing actin-EGFP (green) had a normal actin cytoskeleton. Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI) (blue). (B) Cells transfected with pRK5myc expressing the Mcf-like effector Plu1690 contained only fragments of successfully transfected cells which showed actin condensation. (C) Cells transfected with pRK5myc expressing the Pnf-like effector had contracted cell bodies and condensed actin. (D) Cells transfected with pRK5myc expressing the SepC-like effector had remarkable actin-rich cabling, a phenotype never seen in cells from the pRK5myc transfected controls.

![Figure 6](image-url)  
**FIG. 6.** Transposon mutagenesis of PaPVCpnf. The positions of insertions in the PVC that either disrupt toxicity (solid triangles) or maintain toxicity (open triangles) are indicated. Note that insertions that disrupt toxicity are all clustered in the center of the PVC unit in a region that contains the gene encoding the putative Lop-T-like effector.
is their effect more widespread? Third, does the mode of action of these novel structures involve the needle-like delivery mechanism inferred for R-type pyocins? Finally, what is the role of these prophage-like elements in the ecology of different *P. luminescens* species? All of these questions about these interesting new structures remain to be investigated. However, the PVCs may well be important in conferring novel virulence either against different types of insects or, in the case of the *P. asymbiotica* strains isolated from human wounds (9), potentially against humans.

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