

Insecticidal Toxic Proteins Produced by *Photorhabdus luminescens akhurstii*, a Symbiont of *Heterorhabditis indica*

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Abstract: We describe the isolation and characterization of an insect pathogenic bacterium from the entomopathogenic nematode *Heterorhabditis indica* (Karnataka strain), an isolate from the southern regions of India. The strain has been identified and characterized by phenotypic, biochemical tests and PCR-RFLP analysis of the 16S rRNA gene as *Photorhabdus luminescens* subsp. *akhurstii*. The insecticidal toxin complex produced by this bacterium has been purified through a series of steps including ultrafiltration, anion exchange chromatography, and gel filtration chromatography. The toxin consists of two protein complexes of approximately 1,000 kD and was active against the larvae of *Spodoptera litura* and *Galleria mellonella*.

Key words: bacteria, entomopathogenic, *Galleria mellonella*, *Heterorhabditis*, insect, insecticidal, nematode, *Photorhabdus*, *Spodoptera litura*, toxin.

Entomopathogenic nematodes of the genus *Heterorhabditis* and *Steinernema* are effective parasites against insect pests. Their distribution has been reported from various regions of the world. Virulence, ease of culturing, and a high reproductive efficiency has led to successful integration of these nematodes into pest management programs intended to control soilborne pests (Kaya and Gaugler, 1993).

Members of the genus *Heterorhabditis* have a mutualistic association with bacteria *Photorhabdus* spp. that reside in their intestinal tract (Boemare et al., 1993). This bacterium was first described by Khan and Brooks (1977) and was placed as a species of the genus *Xenorhabdus*. Later, Boemare et al. (1993), on the basis of DNA relatedness studies, placed it in a new genus, *Photorhabdus*, and until recently only one species, *P. luminescens*, had been assigned.

Heterorhabditis has been isolated from several climatic regions, and a number of different species have been described (Liu et al., 2000). The bacterium *Photorhabdus* has typically been isolated from the nematode *Heterorhabditis*. Free-living forms from soil and water have not been isolated. Species of *Photorhabdus* have been delineated recently employing PCR-RFLP analysis of the 16S rRNA genes (Brunel et al., 1997; Fischer-Le Saux et al., 1998). Several strains isolated from different geographical locations were characterized and grouped into different species and sub-species (Fischer-Le Saux et al., 1999). However, the nematode *H. indica* (Poinar et al., 1992) was first described from the Indian subcontinent, but isolates from Asia were not included in this analysis.

On entering the insect, *Photorhabdus* is released into

the host haemocoel where they multiply rapidly and kill the insect. Several extracellular macromolecules, such as proteases, lipases, and broad-spectrum antibiotics, are produced by the bacteria. These agents degrade the insect cadaver upon which the nematode feeds and multiply (Forst and Neilson, 1996). Optimal multiplication of the nematodes requires a domination of the microflora by *P. luminescens* as it synthesizes some food signals (Strauch and Ehlers, 1998).

Insecticidal toxic proteins from *P. luminescens* strain W-14 consist of high molecular weight complexes that include toxin A and toxin B. These toxin complexes have been well-characterized both at a biochemical level and by identifying their genes (Bowen et al., 1998; Guo et al., 1999). Though nematodes have been reported from varied geographical locations, insecticidal proteins from different isolates of *P. luminescens* have not been characterized in strains other than W-14.

In this study we report the isolation and identification of *P. luminescens* from an Indian (Karnataka) strain of *H. indica* and the purification of its insecticidal toxins.

MATERIALS AND METHODS

Isolation of bacteria and identification: *Heterorhabditis indica* (Karnataka strain) was obtained from the Sugarcane Breeding Institute, Coimbatore, India, and maintained on fifth instar *Galleria mellonella* and *Spodoptera litura* larvae. The infective juveniles (IJ) of *H. indica* were surface-sterilized for 3 hours with 0.1% merthiolate solution (w/v) containing streptomycin (5,000 units/ml), followed by three rinses in sterile Ringers' solution. Fifty IJ were applied onto a moist filter paper (5.5 cm) placed in a 60 × 15-mm petri dish. Alternatively, 40 to 50 IJs were microinjected (in 5–10 µl Ringers' solution) into the insect using a syringe fitted with a 26-G needle (Kaya and Stock, 1997). After 36 hours, insects were surface-sterilized with 1.0% sodium hypochlorite for 2 minutes and rinsed three times with sterile water. After drying, the cadaver was opened with a sterile scissors, avoiding rupture of the midgut. A drop of haemolymph from the exposed cadaver was streaked into MacConkey agar. The cultures were incubated for 2 days at 28 °C, and the red-colored luminescent colo-

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nies were picked and subcultured in 5 ml LB broth for 2 days at 28 °C with constant shaking.

The bacterial strain was identified employing PCR-RFLP of the 16S rRNA gene. Total genomic DNA was extracted from cultured isolates (Sambrook et al., 1989). The PCR amplification of the 16S rRNA gene employed the following primers

5'-GCA GAG TTA GAT CTT GGC TC-3'

5'-AAG GAG GTG ATC CAG CCG CA-3'

PCR cycling conditions were as follows: denaturation at 94 °C for 30 seconds, reannealing at 55 °C for 30 seconds, followed by extension at 72 °C for 90 seconds. The reaction products were resolved in 1% agarose gels containing 2% of ethidium bromide and viewed with ultraviolet light.

PCR products were eluted from agarose gels using QIA quick-spin columns (Qiagen Corp., Hilden, Germany) according to the manufacturer's instructions. The eluted DNA (2 to 2.5 µg) was digested individually with Msp I, Dde I, Alu I, Hae III, and Hinf I (USB Corp., Cleveland, OH), per the manufacturer's recommended protocol. The restricted DNA was resolved in a 3% agarose gel and the restriction profile documented.

Bacterial cultures and growth conditions: *Photobacterium luminescens* (Strain K-1) was isolated, as described above, from *H. indica*, Karnataka strain. Primary colonies were selected on NBTA agar based on phenotypic criteria of bioluminescence and colony morphology (Akhurst and Boemare, 1990; Boemare and Akhurst, 1988). Individual colonies were grown on LB broth to an OD₆₀₀ of 0.5 and stored in 20% Glycerol at -70 °C.

Preparation of culture supernatant: Following growth on LB media, cultures were centrifuged at 5,000 rpm for 30 minutes at 4 °C. The supernatant was vacuum filtered through GF/C and GF/D (Whatman International Ltd., Maidstone, England) glass microfiber filters. The filtrate was concentrated by ultrafiltration through an Amicon (Millipore Corp., Bedford, MA) 8000 series stir-cell fitted with a 100-kDa membrane. Proteins were concentrated approximately 20-fold using the procedure.

Ion-exchange column chromatography: The ultrafiltration retentate was centrifuged at 12,000 rpm for 20 minutes and the supernatant fractionated (100 mg crude protein as determined by the Bradford method) through a DE 52 column (4 × 20-cm) equilibrated with 20 mM phosphate buffer pH 7.0 (buffer A). The column was washed with one bed volume of equilibration buffer (buffer A) and bound proteins eluted with a linear gradient of 0 to 0.7M KCl (50 ml each) prepared in equilibration buffer; 2-ml fractions were collected.

Insect rearing: *Spodoptera litura* larvae were reared from eggs (supplied by A. V. N. Paul and G. T. Gujjar of the Department of Entomology, IARI, New Delhi India). The eggs were hatched at 28 °C and 60 ± 5° RH.

A day prior to hatching (at the black head stage) the eggs were surface-sterilised with 0.002% bleach solution for 2 minutes and washed repeatedly by Milli Q-purified water. Immediately after hatching, the larvae were provided with fresh, clean castor leaves that were exchanged daily. Upon emergence, the adult moths were held in 20 × 15-cm jars, and 20% honey solution in cotton was provided as food. Folded papers were kept in the jar to serve as substrate for resting and egg laying. Alternatively, the larvae on hatching were feed on artificial diet as described by Paul (1998). *Galleria mellonella* was reared according to the procedure outlined by Woodring and Kaya (1988) with minor modifications.

Toxin assay: Fractions resolved by ion exchange chromatography (10 µl) were injected aseptically, using a 1-ml syringe with a-26-G needle, into the hemocoel of 5th-6th instar *S. litura* and *Galleria mellonella* larvae. Five insects were used to assay each fraction, and care was exercised to eliminate cross contamination of proteins from different fractions. Following injection, insects were observed for 48 hours for viability and other morphological changes. If at least four of the five insects injected with the fraction died in 48 hours, the fraction was considered insecticidal.

Protein determinations: Protein concentrations were determined according to the method of Bradford (1976). Native and SDS-PAGE analysis was carried out on 5% and 10% gels, respectively (Laemelli, 1970). Molecular weights of the protein were calculated according to the method of Bryan (1977), with slight modifications using the nondenatured protein molecular weight marker kit (Sigma Chemical Co., St. Louis, MO, Technical Bulletin No. MKR-137).

Purification of insecticidal proteins by gel filtration: Fractions that eluted off the ion exchange chromatography column and showed insecticidal activity were pooled and concentrated to 1.5 mg/ml on a Centricon-100 (Amicon, Millipore Corp., Bedford, MA), with a 100-kD cut-off membrane. The concentrate was resuspended in 5 ml of 50 mM phosphate buffer, pH 7.0, containing 50 mM KCl (Buffer B). A 1.5-ml aliquot of the sample was resolved on FPLC (Pharmacia, Uppsala, Sweden) through a TSK gel G 3000 SW gel filtration column, and 1-ml fractions were collected. All fractions were assayed for biological activity as described previously.

Second anion exchange chromatography: Fractions from the gel filtration column containing insecticidal proteins were pooled and refractionated on a DE52 anion exchange column. The column (1 × 4 cm) was equilibrated with 20 mM buffer A and fractions eluted with a linear gradient of 0 to 0.5 M KCl prepared in buffer A. These fractions were assayed for insecticidal activity against the larvae of *S. litura*.

RESULTS AND DISCUSSION

Isolation of *Photobacterium luminescens*: Infection of 5th to 6th instar *G. mellonella* and *S. litura* larvae with *H. indica*

resulted in the recovery and isolation of bright-pink to red-colored, convex, opaque colonies on MacConkey agar. Preliminary investigation with Gram-staining and oxidative reactions confirmed that the bacterial colonies were Gram-negative and catalase-positive. The bacterial culture grown on broth for 48 hours was highly luminescent in the dark. On NBTA agar, the bacteria formed greenish colonies with a clear zone (halo) around them. A 1.5-kb PCR product was amplified using 16S rRNA gene-specific primers. The restriction pattern of amplified DNA with different restriction enzymes (Fig. 1) displayed near identity with the pattern for *P. luminescens* subsp. *akhurstii* isolated from another *H. indica* isolate (Table 1) (Brunel et al., 1997; Fischer-Le Saux et al., 1998).

Taxonomic studies of entomopathogenic nematodes and their symbiotic bacteria have revealed the specificity that nematodes possess to their symbiont, *Photorhabdus* with *Heterorhabditis* and *Xenorhabdus* with *Steinernema* (Boemare et al., 1997). Using the technique of PCR-based ribotyping of 16S rRNA gene, a number of *Photorhabdus* and *Xenorhabdus* isolates from various locales in the Caribbean region have been identified (Brunel et al., 1997; Fischer-Le Saux et al., 1998). Recently, Fischer-Le Saux et al. (1999) proposed a polyphasic classification of *P. luminescens* derived from various geographical regions. They reported a correlation between the bacterial samples from different ecological regions and their maximum growth temperatures. The *Photorhabdus* symbionts from *Heterorhabditis bacteriophora* and *H. megidis* originating from different parts of the world are grouped into different species and subspecies; those from *H. indica* fall into *P. luminescens* subsp. *akhurstii*.

TABLE 1. Comparison of PCR-RFLP product molecular weight^a between *P. luminescens akhurstii* (type strain CIP 105564) and *P. luminescens akhurstii* (strain K-1).

Dde I		Hinf I		Hae III		Alu I		Msp I	
T. strain	K-1								
510	510	680	680	320	320	530	530	470	470
320	320	340	340	290	290	340	340	440	440
280	280	330	330	180	180	240	240	160	160
250	250	180	180	170	170	210	210	130	130
154	154	170	170	155	155	100	100	120	120
120	120	155	155	100	100			115	115
		100	100	70	70			70	70
		70	70						

^a All values denote molecular weights (in base pairs) of RFLP bands. Molecular weights of the type strain are as in Fischer-Le Saux et al. (1998).

The RFLP profile of our isolate (K-1) also displayed near identity with the pattern for the *P. luminescens* subsp. *akhurstii* (Fig. 1; Table 1), confirming the isolate as a member of this group. The result that *Photorhabdus* symbionts of *H. indica* from different regions share close identity indicates that the bacteria nematode symbiosis is very specific in this case—unlike *H. bacteriophora* and *H. megidis*. Also, as *H. indica* was first reported from India (Poinar et al., 1992), this result would give more credence to nematode identification through the identification of symbiotic bacteria.

Purification of insecticidal toxin: Upon ribotyping this strain, we extended the study of K-1 by analyzing the insecticidal toxin complex produced by it. The insecticidal toxins from *P. luminescens* recently have been reported (Bowen and Ensign, 1998) and their genes cloned and characterized (Bowen et al., 1998) from the strain W-14. However, W-14 has not been characterized or typed with respect to the various *Photorhabdus* species and subspecies. In the absence of these critical data, other workers in this field are left without a reference on which to base their further study.

A preliminary analysis for this *P. luminescens* (K-1) isolate established that the injection of 100 cells was lethal for *G. mellonella* in 48 hours. The toxin was secreted into the growth medium as the culture supernatant also killed the insect in 48 hours.

The bacteria were cultured on LB broth for 48 hours. Following centrifugation and removal of fine granules through GF/C and D filters, the supernatant was concentrated. The retentate of ultrafiltration was lethal to 5th instar *S. litura*, while the filtrate was not active. The retentate was resolved on a DE-52 anion exchange column. Bioassay of different fractions revealed the elution of insecticidal proteins corresponding to the gradient range of 0.15 and 0.3M KCl (Fig. 2A). These active fractions were pooled and concentrated through a Centricon-100 filter. The retentate was resolved through a TSK gel 3000 SW-gel filtration FPLC column, and fractions were bioassayed for insecticidal activity (Fig. 2B). Analysis of active fractions by native PAGE

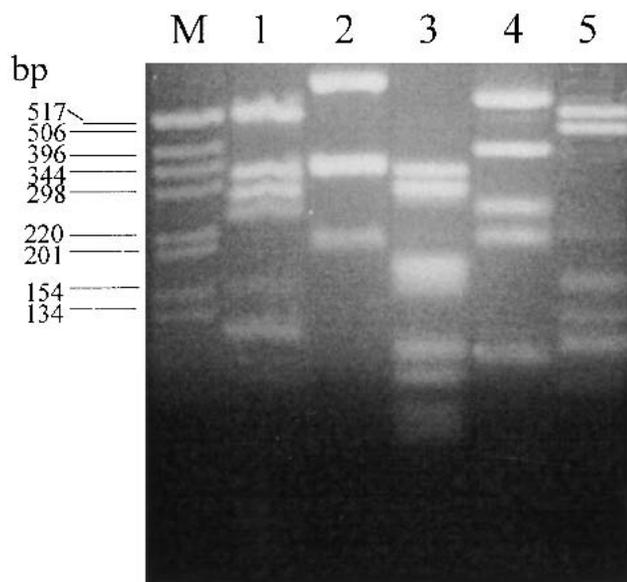


FIG. 1. Restriction pattern of PCR-amplified product of 16S rRNA gene digested with Dde I (lane 1), Hinf I (lane 2), Hae III (lane 3), Alu I (lane 4), and Msp I (lane 5). Molecular weight marker (1-kb) was co-electrophoresed in the marker lane.

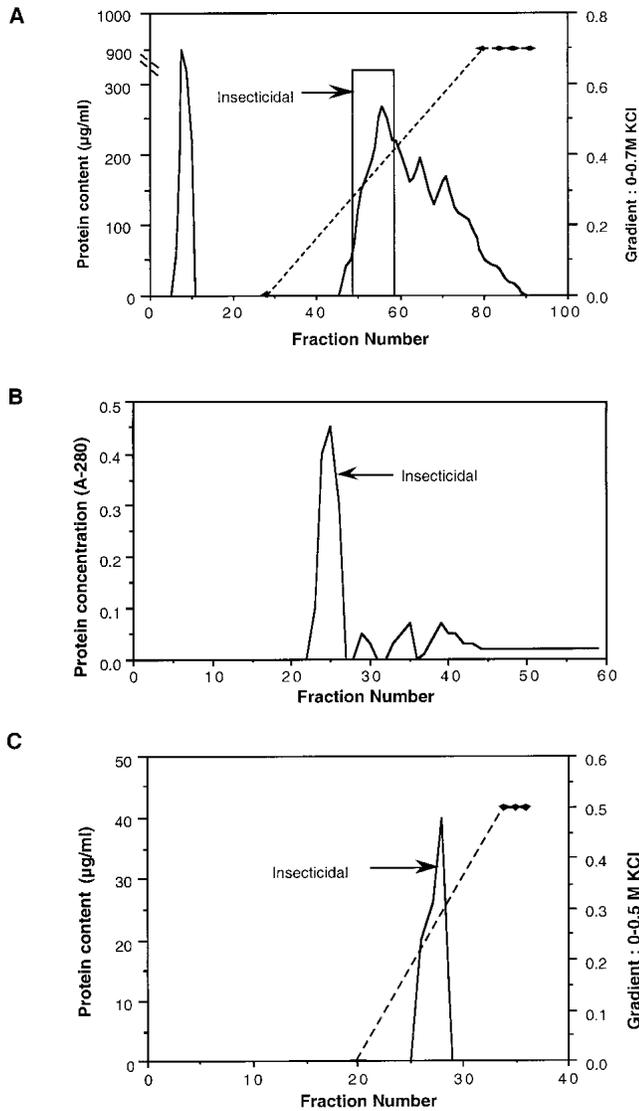


FIG. 2. Purification of *P. luminescens* (K-1) insecticidal protein. A) Separation through a DE-52 anion exchange chromatography. Dotted line, 0 to 0.7 M KCl gradient. The active fractions (denoted by a box) were pooled and loaded on B) a TSK Gel G 3000 SW gel filtration column. Pooled active fractions were further purified through C) a mini column of DE 52 anion exchange chromatography. Dotted line, 0 to 0.5 M KCl gradient.

revealed three bands—one corresponding to the molecular mass of 850 and the other two to 1,000 kD (Fig. 3A). The fractions with insecticidal activity were pooled, concentrated, and resolved on a second DE-52 column and eluted with a linear gradient of 0 to 0.5M KCl. The insecticidal fractions eluted between 0.15 to 0.25 M KCl (Fig. 2C). On analysis of the bioactive fractions with native PAGE, only two bands of approximately 1,000 kD mass were observed (Fig. 3A).

A preliminary characterization of the insecticidal toxin revealed that it is sensitive to boiling, does not display antimicrobial activity against *E. coli* K-12, and can be stored without any loss of activity at 4 °C for 10 days (data not shown). This toxin complex is active against larvae of *S. litura* and *G. mellonella* at a concen-

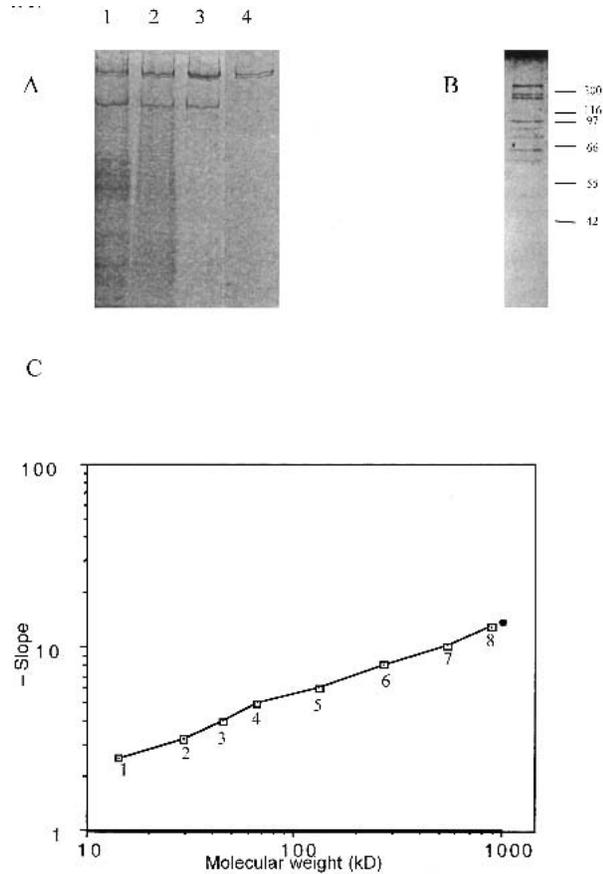


FIG. 3. A) Resolution of the toxic protein fractions by native poly acrylamide gel electrophoresis (PAGE), 5% gel. Native sample (lane 1), first ion exchange column (lane 2), gel filtration column (lane 3), second anion exchange column (lane 4). B) Resolution of toxic protein purified through second anion exchange column by SDS-PAGE, 10% gel. The gels were stained with coomassie brilliant blue. C) Molecular weight determination of purified toxin (protein from Fig. 3A, lane 4); the standard proteins are 1. lactalbumin (14 kD), 2. carbonic anhydrase (29 kD), 3. chicken egg albumin (45 kD), 4. bovine serum albumin (monomer 66 kD), 5. bovine serum albumin (dimer 132 kD), 6. urease (trimer 272 kD), 7. urease (hexamer 545 kD), and 8. apoferritin (dimer 900 kD). *, *Photorhabdus* toxin.

tration of 0.4 to 0.5 µg upon delivery into haemocoel (data not shown). Resolution of the toxin on 10% SDS-PAGE revealed that the toxin consists of seven subunits of approximately 280, 170, 150, 97, 90, 60, and 50 kD masses (Fig. 3B).

The high molecular weight of the active toxin fraction (Fig. 3C) is a property similar to the toxin complex reported by Bowen and Ensign (1998). In the absence of information about the structure and sequence of the toxin protein, it is difficult to make a realistic comparison of the two complexes isolated from different strains of *P. luminescens*.

With the reclassification of the genus *Photorhabdus* into three species and four subspecies, it is essential to characterize the toxin profile from each of the subspecies. This is the first report on the insecticidal proteins from *P. luminescens* subsp. *akhurstii*. Further studies on this toxin would help in increasing our arsenal to com-

bat insect pests through the development of transgenic plants and serve as an alternative to *Bt* transgenic, wherein the development of resistance in insects has been widely reported (Ramachandran et al., 1998).

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