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PirAB protein from *Xenorhabdus nematophila* HB310 exhibits a binary toxin with insecticidal activity and cytotoxicity in *Galleria mellonella*

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ABSTRACT

PirAB (*Photorhabdus* insect-related proteins, PirAB) toxin was initially found in the *Photorhabdus luminescens* TT01 strain and has been shown to be a binary toxin with high insecticidal activity. Based on GenBank data, this gene was also found in the *Xenorhabdus nematophila* genome sequence. The predicted amino acid sequence of *pirA* and *pirB* in the genome of *X. nematophila* showed 51% and 50% identity with those gene sequences from *P. luminescens*. The purpose of this experiment is to identify the relevant information for this toxin gene in *X. nematophila*. The *pirA*, *pirB* and *pirAB* genes of *X. nematophila* HB310 were cloned and expressed in *Escherichia coli* BL21 (DE3) using the pET-28a vector. A PirAB-fusion protein (PirAB-F) was constructed by linking the *pirA* and *pirB* genes with the flexible linker $(Gly)_4$ DNA encoding sequence and then efficiently expressed in *E. coli*. The hemocoel and oral insecticidal activities of the recombinant proteins were analyzed against the larvae of *Galleria mellonella*. The results show that PirA/B alone, PirA/B mixture, co-expressed PirAB protein, and PirAB-F all had no oral insecticidal activity against the second-instar larvae of *G. mellonella*. Only PirA/B mixture and co-expressed PirAB protein had hemocoel insecticidal activity against *G. mellonella* fifth-instar larvae, with an LD_{50} of 2.718 μg/larva or 1.566 μg/larva, respectively. Therefore, we confirmed that PirAB protein of *X. nematophila* HB310 is a binary insecticidal toxin. The successful expression and purification of PirAB laid a foundation for further studies on the function, insecticidal mechanism and expression regulation of the binary toxin.

1. Introduction

Bacteria of the genera *Xenorhabdus* and *Photorhabdus* have a mutual symbiotic relationship with entomopathogenic nematodes (EPN) of the genus *Steinernema* and *Heterorhabditis*, respectively. The bacteria that symbiotically associate with EPN are released into the insect hemocoel upon nematode invasion and kill their insect host by producing many toxins. The toxins produced by these two bacteria have oral insecticidal activity and hemocoel insecticidal activity. The insect is then killed mainly by these toxins, the bacteria continue to replicate within the insect cadaver and the nematodes feed on the bacterial-insect medium (Hinchliffe et al., 2010). For *Photorhabdus*, several classes of toxins have been characterized such as Tcs (Waterfield et al., 2005a,b), Mcf (Daborn et al., 2002), PVC (Yang et al., 2006) and PirAB (Ffrench-Constanta et al., 2007). For *Xenorhabdus*, Xpt (Morgan et al., 2001), XnGroEL (Shi et al., 2012), Txp40 (Brown et al., 2004) and XaxAB toxins (Vigneux et al., 2007) have been shown to be toxic for the insect host.

The PirAB toxin was initially found in the *Photorhabdus luminescens* TT01 strain and is encoded at two distinct loci in the *P. luminescens* TT01 genome, *plu4093/plu4092* and *plu4437/plu4436*, respectively (Duchaud et al., 2003). PirAB has been shown to be a binary toxin that is host-specific with both injectable and oral activities against a range of insects. Waterfield et al. (2005a,b) suggested that PirA and PirB may refer to the products of *plu4093/4437* and *plu4092/4436* homologs, respectively. Moreover, PirA and PirB components are necessary for the injectable activity against *Galleria mellonella*, but not for oral activity against *Manduca sexta* (Waterfield et al., 2005a,b). Both loci produced toxins with oral activity against *Plutella xylostella*, and a histological examination revealed serious abnormalities of the midgut epithelium (Blackburn et al., 2006). The PirAB toxin was also shown to be active against the mosquitoes *Aedes aegypti*, *Culex pipiens*, and *Anopheles gambiae* (Duchaud et al., 2003; Ahantarig et al., 2009). A recent report on the abundance of the *pirAB* toxin transcripts (*plu4093-2* and *plu4437-6*) of the *P. luminescens laumondii* TT01 strain suggested that the deployment of the toxin transcripts occurred during the early stages of the infection process (Castagnola et al., 2016). All original bacteria strains in the above studies came from *P. luminescens*, and

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PirAB toxins had high insecticidal activity against insect pests in the simultaneous presence of the two proteins.

Follow-up studies demonstrated that *pirAB* is a binary toxin gene that exists in other bacteria, such as *P. asymbiotica* (Wilkinson et al., 2009), *X. nematophila* (GenBank data), *Yersina intermedia* (Hurst et al., 2016), and *Vibrio parahemolyticus* (Lee et al., 2015). We identified *pirA* and *pirB* genes (408 bp and 1290 bp, respectively) in the whole gene sequence of the *X. nematophila* ATCC19061 genome (GenBank access No.: FN667742). Both the toxicity and relationship to virulence of PirAB from the above bacteria have been investigated, but the characterization of the PirAB toxin gene of *Xenorhabdus* has not yet been assessed, although the gene sequence information is known. To determine the characteristics and potential of this protein for insect control, we cloned and sequenced *pir*A, *pir*B and *pir*AB genes from *X. nematophila* HB310. Moreover, we heterologously expressed these locus in *Escherichia coli* and tested the recombinant proteins against *G. mellonella*. Meanwhile, a PirAB-fusion protein (PirAB-F) was constructed and its insecticidal activity was compared with a PirA/PirB mixture and co-expressed PirAB. The possible mechanism for the insecticidal activity of PirAB is also discussed.

2. Materials and methods

2.1. Insects and bacteria

G. mellonella larvae were obtained from the Pest Biocontrol Laboratory (PBL), Hebei Agricultural University, China. Larvae were fed an artificial diet (22% maize meal, 22% wheat germ, 11% dried milk, 5.5% dry yeast, 17.5% bee wax, 11% honey, and 11% glycerin) and reared at 29 °C and 70% RH under a 14 h light: 10 h dark photoperiod.

The *X. nematophila* HB310 strain used in this study was isolated and stored in the Pest Biocontrol Laboratory (PBL), Hebei Agricultural University, China. Broth cultures were grown from a single primary phase colony in an LB medium at 28 °C on a shaker at 200 rpm. *E. coli* DH5a and *E. coli* BL21(DE3) (Novagen, USA) were cultured at 37 °C for sequencing, cloning and expression.

2.2. Cloning and bioinformatics analysis of the pirA and pirB genes

Total DNA extracted from *X. nematophila* HB310 was used as a template for amplification of the whole ORF of *pirA* and *pirB*. Oligos designed based on the sequence of *X. nematophila* ATCC19061 (GenBank access no: FN667742) are listed in Table 1. Oligo pairs PirA-F/PirA-R and PirB-F/PirB-R were used to amplify *pirA* and *pirB*, respectively. The resultant PCR products were ligated into the pMD19-T vector (Takara, Japan). The resultant plasmids were named pMDT-*pirA* and pMDT-*pirB*, respectively. The resulting plasmids were used to transform *E. coli* DH5α. Then, the recombinant plasmids were subjected to sequencing.

To obtain information on the proteins, a bioinformatic analysis of the genes *pirA* and *pirB* was performed. NCBI BLAST was used to determine the homologous sequence to the *pirA* and *pirB* genes (http://www.ncbi.nlm.nih.gov/BLAST/). The amino acid sequences predicted by the nucleotide sequences were used for the molecular phylogenetic analysis. Multiple amino acid sequence alignments were analyzed with DNAMAN 7.0 of Lynnon Biosoft Co. The secondary structure was predicted using PSIpred (http://bioinf.cs.ucl.ac.uk/psipred/). Phylogenetic trees were constructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987) with MEGA Version 5 (Tamura et al., 2011). The reliability of the clustering patterns was assessed by bootstrapping (Felsenstein, 1985). All the sequences used for comparison in this paper were obtained from GenBank.

2.3. Construction of expression plasmids

Plasmid pMDT-*pirA* and pMDT-*pirB* were double-digested with *BamH*I and *Sal*I (Takara, Japan). The digestion products were then ligated into a pET-28a expression vector double-digested by *BamH*I and *Sal*I and transformed into *E. coli* BL21 (DE3) competent cells. For co-expression of *pirAB* in BL21 (DE3), Co PirAB-F/Co PirAB-R was used to amplify *pirAB*. The PCR products of *pirAB* were double-digested by *BamH*I and *Sal*I, and cloned into pET-28a sequentially to generate co-expression plasmid pET-Co*pirAB*. For PirAB fusion expression, oligo pairs FPirAB-AF/AR and FPirAB-BF/BR were used to amplify *pirA* and *pirB*, respectively; and, PCR products of *pirA* and *pirB* were used as DNA template by FPirAB-AF/BR. The encoding sequence of flexible linker (Gly4) was incorporated into the oligo FPirAB-BF. The PCR products were double-digested by *BamH*I/*Sal*I and then *BamH*I/*Sal*I fragment containing *pirA*-linker-*pirB* chimeric gene was cloned into pET-28a to generate PirAB-fusion expression plasmid pET-F*pirAB*. DNA sequencing confirmed all the constructs.

2.4. Heterologous expression and purification of the recombinant proteins

The inserted DNA fragments were sequenced again to verify the correctness. Recombinant strains were grown in LB medium supplemented with kanamycin (100 μg mL^{-1}) at 37 °C to an OD_{600} nm of 0.6–0.8. Expression of genes was induced by isopropyl-beta-Dthiogalactopyranoside (IPTG) to a final concentration of 1 mM for 3 h. Recombinant *E. coli* cells were centrifuged (10,000*g*, 10 min), washed three times with distilled water, and suspended in 10 mL of sterile phosphate buffered saline (PBS). The suspension was lysed by sonication and centrifuged at 4 °C, 10,000*g* for 10 min. The soluble fusion protein was purified using His-Tagged Protein Purification Kit (CWBIO, China) as described by the manufacturer. Solutions were then

Table 1
Primers used in this study.

Toxin detected	Primer	Sequence	Tm (°C)	GC (%)
PirA	PirA-F	5′-CGC***GGATCC***ATGATTACAATCAATATC-3′	58	41
	PirA-R	5′-CGC***GTCGAC***TTATAGATTGTAGCCGG-3′	62	54
PirB	PirB-F	5′-CGC***GGATCC***ATGAATAATGAACCG-3′	59	50
	PirB-R	5′-CGC***GTCGAC***CTATGATTTTTTATC-3′	55	42
Co-expressed PirAB	Co-PirAB-F	5′-CGC***GGATCC***ATGATTACAATCAATATC-3′	58	41
	Co-PirAB-R	5′-CGC***GTCGAC***TGATTTTTTATCAGCTATCATATGG-3′	64	41
PirAB-fusion	F-PirAB-AF	5′-CGC***GGATCC***ATGATTACAATCAATATC-3′	58	41
	F-PirAB-AR	5′-GATCCTGATCCAGAACCTAGATTGTAGCCGGT AACAAAAGCAC-3′	71	47
	F-PirAB-BF	5′-CTACAATCTAGGTTCTGGATCAGGATCAGGTTCAATG AATAATGAACCGATGAATACTAATGAATCACAAG-3′	73	37
	F-PirAB-BR	5′-CGC***GTCGAC***CTATGATTTTTTATC-3′	55	42

Note: The Italic/Bold letter nucleotides are the *BamH*I and *Sal*I restriction sites, respectively.