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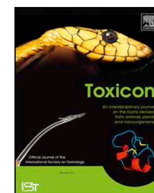
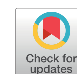


Short communication

A 37 kDa Txp40 protein characterized from *Photorhabdus luminescens* sub sp. *akhurstii* conferred injectable and oral toxicity to greater wax moth, *Galleria mellonella*



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ABSTRACT

Photorhabdus luminescens is a gram-negative bacterium that symbiotically associates with insect-parasitic nematode, *Heterorhabditis indica*. Herein, we have characterized an insecticidal gene, *Txp40* (1008 bp) from the indigenous isolates of *P. luminescens*, and tested its bioefficacy against *Galleria mellonella* via injectable and oral bioassay. The recombinant protein characterized from *P. luminescens* strain H3 exhibited comparatively greater insect toxicity than strain H1 in terms of LD_{50} and LT_{50} values. Txp40 holds great potential to replace Bt toxins in global agriculture.

Xenorhabdus and *Photorhabdus* are two genera of bacteria (gram negative, Enterobacteriaceae family) that symbiotically associate with the nematode genera, *Steinernema* and *Heterorhabditis*, respectively (Akhurst and Boemare, 1990). The nematode-bacterium pair is capable of invading and killing the larval stages of numerous insects, including Coleopteran, Lepidopteran, Dipteran, Dictyopteran and Orthopteran pests (Georgis, 2002).

The symbiotic bacteria are carried in the gut of their associated nematode infective juveniles, which search for insect hosts in the soil and penetrate into host hemocoel via cuticle or natural openings. In most cases bacteria alone are highly virulent upon being released into the insect hemocoel by nematodes. Bacteria produce a range of toxins that kill insects. When insect cadaver is depleted on nutrients due to colonization of nematode and bacteria, they reassociate and emerge from cadaver to find new hosts (Forst and Clarke, 2002).

Since the direct use of *Xenorhabdus* and *Photorhabdus* as a biopesticide is severely limited due to their incapability to survive in water or soil for long, it is important that the insecticidal toxicity of these bacteria can be reproduced in other bacteria, microorganisms or plants for their exploitation. To enable this, several novel toxin genes were identified from the *Xenorhabdus* and *Photorhabdus* spp. and their insecticidal activity was confirmed by expressing them in heterologous host, *Escherichia coli* followed by bioassay against different insects (Bowen et al., 1998; Morgan et al., 2001; Waterfield et al., 2001, 2005; Daborn et al., 2002; Duchaud et al., 2003; Yang et al., 2006). In addition, full scale sequencing of *P. luminescens* strain TT01 (Duchaud et al., 2003) and sample sequencing of *P. luminescens* strain W14 (Ffrench-Constant et al., 2000) revealed that they contain a wide range of putative virulence factors including high molecular weight toxin complexes (Tc), lipopolysaccharides (LPS), proteases, lipases and a range of different antibiotics.

However, in country like India which contains diverse agro-climatic zones the according insect pest problem varies substantially, exploration of a diversity of toxins from the native isolates of *Photorhabdus* sp. can enrich the repository for pest management practices. Incidentally, Bt cotton variety Bollgard II (released by Monsanto India Ltd.) that expresses bacterial Cry toxins has failed to manage pink bollworm (*Pectinophora gossypiella*) which affected 50% of cotton crop in last two to three years in Maharashtra state of India (The Hindu news report, 2017; http://www.thehindu.com/news/cities/mumbai/pink-bollworm-may-eat-up-half-of-states-cotton-crop/article20493492.ece). Earlier, in an isolated study, a truncated Tc toxin of *P. luminescens* was expressed in *Arabidopsis thaliana* and the isolated toxin from transgenic plants strongly suppressed the growth of southern corn rootworm (*Manduca sexta*) *in vitro* (Liu et al., 2003).

Tc toxins of *Photorhabdus* consists of large protein subunits which are intrinsically difficult to express transgenically in crop plants. Their full potential can't be exploited unless the protein can be trimmed down to smaller active domains. Earlier, a ubiquitous toxin protein, Txp40 (corresponds to 2415 bp nucleotides) was isolated from *P. luminescens* strain V16 that conferred injectable toxicity to *Helicoverpa armigera* (Brown et al., 2006). A homologue of Txp40, i.e. A24tox (42 kDa

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secreted protein) characterized from *X. nematophila* also showed insect toxicity (Brown et al., 2004). In the present study, the longest open reading frame (ORF) of Txp40 (1008 bp) was cloned from the native Indian strains of *P. luminescens* subsp. *akhurstii*, expressed in *E. coli* and the recombinant proteins were tested for their bioefficacy against the larvae of greater wax moth, *Galleria mellonella*.

Firstly, four strains of *P. luminescens* subsp. *akhurstii* such as IARI-SGMG3 or H1 (16S rRNA accession no. JX221722), IARI- SGGJ2 or H2 (KJ995729), IARI-SGHR2 or H3 (HQ637411) and IARI-SGMS1 or H5 (HQ637414) were isolated from the infective juveniles (IJs) of *Heterorhabditis indica* which were collected from various agro-climatic zones of India (nematode strain details are documented in Kumar et al., 2015 and Kushwah et al., 2017). Nematode strains were maintained in our laboratory on fourth instar larvae of *G. mellonella* by following the standard procedure (McMullen and Stock, 2014). Symbiont bacterial strains were isolated from freshly hatched surface sterilized IJs by following the methodology described in Kumar et al. (2016) and Kushwah et al. (2017). Pure green colonies were isolated from nutrient bromothymol blue agar (NBTA) plates and strain identity was confirmed via sequencing of 16S rDNA.

2415 bp nucleotide sequence (Genbank accession no. DQ242625) corresponding to full-length *Txp40* gene was translated to amino acid sequences to identify the potential ORF in Expasy server (https://www.expasy.org/). The longest ORF of 1008 bp was identified an primers were designed as 5′GGATCCATGGTTATACAATTAACACCTGATGA3′(sense oligonucleotide) and 5′AAGCTTTCATATATTTTTATAATGAGTTCCAACACT3′(antisense oligonucleotide). The *Bam*HI and *Hind*III restriction enzyme sites are underlined. Genomic DNA was isolated from different strains of *P. luminescens* by using PureLink genomic DNA mini kit (Thermo Fisher Scientific). *Txp40* gene was PCR amplified from those strains using standard protocol with high-fidelity Phusion DNA polymerase (Invitrogen). PCR products were cloned in pGEM-T (Promega) and transformed into *E. coli* DH5α competent cells (New England Biolabs). Recombinant plasmids were isolated from the positive clones (QIAGEN Plasmid Miniprep kit) and sequenced via Sanger sequencing method.

Subsequently, *Txp40* insert in pGEM-T backbone was double digested with *Bam*HI and *Hind*III, gel purified and ligated (using T4 DNA ligase, Promega) into pET29a expression vector at *Bam*HI and *Hind*III restriction sites to produce Txp40-His tag fusion protein. Transformed pET29a colonies were screened via restriction digestion. *Txp40*-pET29a plasmids were transformed into *E. coli* BL21 (DE3) cells via electroporation. Positive colonies were selected by screening against the antibiotic kanamycin (50 μg/ml) in Luria-Bertani (LB) medium. 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was used for recombinant protein induction at 37 °C for 4 h (at 0.6 OD) and checked on 12% SDS-PAGE against uninduced cell culture. The pellet of *E. coli* cells harbouring Txp40 protein was suspended in 50 mM Tris buffer and lysed via sonication. Recombinant protein was purified from *E. coli* lysate via affinity purification using Ni-NTA column (Qiagen) and checked on 12% SDS-PAGE. Concentration of protein was estimated using Bradford's method with BSA as standard. The similarity search, motif and fold recognition of protein was performed using standard bioinformatics analyses. The identity of the protein was confirmed by mass spectroscopy. Trypsin-digested protein samples were analysed via MALDI-TOF assay by outsourcing to Sandor Life Sciences Pvt. Ltd., Hyderabad. The peak lists of peptides captured by MassLynx software (v 4.0) were searched against MASCOT (http://www.matrixscience.com/) and NCBI non-redundant protein database using standard parameters.

For insect bioassays, *G. mellonella* was reared at 28 °C on artificial diet containing 20 parts wheat flour, 20 parts corn flour, 1 part yeast, 2 parts each of milk powder, glycerol and honey. Ampicillin (20 mg per kg body weight) was co-administered to prevent infection of other contaminating bacteria. Larvae were surface sterilized with cotton swabs dipped in 70% ethanol. The bioassays were conducted *in vitro* in 6 well sterile polystyrene tissue culture plates. For injectable toxicity assay, 5–10 μl of purified protein (at10, 50, 100 and 500 ng of protein per larva; average larval mass was approximately 500 mg) was injected through intersegmental membrane (below the prolegs) into the abdomen of fourth instar larvae via hypodermic syringe (26s gauge, 10 μl capacity Hamilton syringe, Sigma-Aldrich), incubated at 28 °C and observations on insect mortality was recorded at 12 h time interval. For oral toxicity assay, different concentrations of toxin protein (200, 500, 1000 and 1500 ng/g of insect diet) was fed to the first instar larvae, incubated at 28 °C and observations on insect mortality was recorded at 7 days post inoculation. *Photorhabdus luminescens* culture was used as positive control and LB broth as negative control. Additionally, the weight of insect diet was recorded after 7 days in order to calculate the average diet consumed by each larva. Each treatment consisted of 15 replicates and was repeated at least thrice. Mortality data were used to calculate LD_{50} (median lethal dose) values using SPSS v.21 (IBM Corp., NY, USA) software. LT_{50} (median lethal time) values were calculated using GraphPad Prism 5.0 (GraphPad Software Inc., CA, USA) software.

The longest ORF of *Txp40* gene (1008 bp) was PCR-amplified successfully from the genomic DNA of *P. luminescens* strain H1, H2, H3 and H5. Genbank accession numbers obtained for these genes are MH052595, MH052596, MH255892 and MH052597, respectively. *Txp40* gene of all the four strains exhibited 92–94% sequence similarity (Query coverage – 100%, E value – 0) at the nucleotide level to the corresponding gene of *P. luminescens* strain NLK-1, V16, *P. luminescens* subsp. *laumondii* strain TT01 and *X. bovienii* strain T363 using BLAST search query in NCBI non-redundant database (data not shown). The secondary structure prediction indicated that Txp40 protein (335 amino acids long) is predominantly alpha-helical containing 45% helix and 9% beta strands (Supplementary Fig. 1). Txp40 is possibly located in bacterial inner membrane and secreted by typeIII secretion system as no signal peptide cleavage site was predicted at the N-terminus of protein. InterProScan (EMBL-EBI) results revealed that Txp40 has no detectable homologues in the present database.

The Txp40 protein was characterized via bacterial expression system for *P. luminescens* strains H1 and H3 in the present study. However, Txp40 cloned from strains H2 and H5 could not be expressed after repeated attempts. Premature translation stoppage due to codon degeneracy (although there were no apparent frame shift, sequence of Txp40 differed among these four strains by several amino acids; Supplementary Fig. 2) during the process of protein expression cannot be ruled out in this case. The cloning of PCR amplified *Txp40* gene in pET29a was confirmed via restriction digestion (Supplementary Fig. 3). IPTG-induced Txp40 protein (of *P. luminescens* strain H1 and H3) was purified and loaded on SDS-PAGE for analysis. Post purification and concentration, the yield of Txp40 (of both strains) was approximately 2.5 mg per litre of *E. coli* culture. The identity of the protein was confirmed by mass spectroscopy. An analysis of the tryptic digest of Txp40 of strain H1 and H3 identified peptides that covered 70.4 and 74.2% of the predicted ORFs, respectively, including peptides from both the N and C termini (Supplementary Fig. 4). As evident from Fig. 1A, the molecular weight of the expressed protein is approximately 37 kDa which is in line with the predicted weight of the translated protein (analysed in Expasy server: https://www.expasy.org/).

Upon intra-hemocoel injection, toxin protein corresponding to strain H1 and H3 killed majority of the *G. mellonella* larvae at 12 h (at 500 ng/larva dose) compared to zero mortality in negative control. Dead insects turned brick red coloured as observed with the positive control. Orally administered Txp40 toxin caused greatest insect mortality at 1500 ng/g diet at 7 days post feeding. In case of positive control, all the larvae were dead during their first instar stage while in negative control larvae were developed to third or fourth instar stages. Qualitative data are depicted in Fig. 1 (B,C). LD_{50} values for strain H1-derived toxin were reduced from 67.25 ng/larva at 12 h to 20.36 ng/larva at 72 h. Whereas, LD_{50} values for strain H3-derived toxin were reduced from 20.76 ng/larva at 12 h to 17.68 ng/larva at 72 h

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