

Original Research Article

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## Isolation, Cloning and Expression of Insecticidal-Protein-Encoding Gene *tcdA* from *Photorhabdus luminescens* in *Escherichia coli*

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### ABSTRACT

#### Keywords

*tcdA*, *Photorhabdus luminescens*, Cloning and expression.

#### Article Info

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In this study, the *tcdA* coding gene of *Photorhabdus luminescens* was inserted in pET32a (+) plasmid with extra His-tag sequence. *Photorhabdus luminescens* strain was isolated from Western Ghats of Karnataka, which is symbiotically associated with entomopathogenic nematode *Heterorhabditis* spp. The DNA sequence of cloned toxin gene (7551 bp) has an open reading frame encoding 2506 amino acids with a predicted molecular mass of 283kDa. The integrity of the constructed plasmid was confirmed using restriction digestion and PCR. The *tcdA* gene was expressed after induction with IPTG in *Escherichia coli* BL21. Recombinant *tcdA* was purified by Ni-NTA Affinity Chromatography.

### Introduction

Entomopathogenic nematodes can provide effective biological control of several important soil insect pests that occur in cryptic habitats (Georgis and Manweiler, 1994; Koppenhofer, 2000). Favorable characteristics include formation of a durable infective stage that can be stored for long periods and applied by conventional methods, and persistence in the environment following application. In addition, these beneficial nematodes are specific to insects, safe to non-target organisms including humans other vertebrates and plants, they do not pollute the environment (Poinar, 1990; Ehlers, 1991). Accordingly, there is an intense interest to isolate these nematodes from different regions

of the world that are climatically adapted and have the potential for biological control of pests in that area.

Bacteria of the genera *Photorhabdus* and *Xenorhabdus* form a mutually beneficial symbiotic complex with the entomopathogenic nematodes (EPNs) in the families' Heterorhabditidae and Steinernematidae respectively which are able to infect kill and reproduce in many insect species (Boemare *et al.*, 1993; Thomas and Poinar, 1983). In this mutualistic relationship the bacteria are necessary to kill insect hosts and for nematode development within the insect (Boemare *et al.*, 1993; Boemare, 2002).

The bacteria are transported by the free-living infective juvenile (IJ) stage of the EPN being located in the anterior part of the *Heterorhabditis* intestine or in a special intestinal vesicle in most of the *Steinernema* spp. (Bird and Akhurst, 1983; Martens and Goodrich-Blair, 2005). Although these bacteria might survive in soil for 1 week in the absence of the nematode (Morgan *et al.*, 2001) in natural conditions the IJs act as active vectors that survive in soil until they find a suitable host.

The toxin complex (*tc*) genes of *Photorhabdus* encode insecticidal high molecular weight Tc toxins. These toxins have been suggested as useful supplement or offer an alternative to those derived from *Bacillus thuringiensis* for expression in insect-resistant transgenic plants. However, *tca* and *tcc* loci encode for several open reading frames (ORFs) (Ffrench-Constant *et al.*, 2003), thus producing multiple components per locus; *tcb* and *tcd* are comprised of a single long ORF. The toxin complexes (Tcs) are encoded by the PAI I (pathogenicity island I) (Waterfield *et al.*, 2004) and have been identified as high molecular weight insecticidal toxins comprised of multiple subunits (Ffrench-Constant *et al.*, 2007). There are four such complexes, namely *tca*, *tcb*, *tcc* and *tcd*, found in different loci.

The three complexes show significant similarity to one another; therefore three basic types of genetic elements have been identified: the *tcdA*-like element, equivalent to the combination of *tcaA* and *tcaB*, the *tcdB*-like element, equivalent to the *tcaC* and the *tccC*-like element. *tcdA*-like elements are responsible for establishing primary toxicity, while the *tcdB*/*tccC*-like elements are potentially toxic (Pinheiro and Ellar, 2007), *tcbA* and *tcdA* also share 50 percent identity overall, as well as a similar predicted pattern of both carboxy- and amino-terminal

cleavage. It was postulated that these proteins might thus be homologs (to some degree) of one another. Furthermore, the similar, large size of *tcbA* and *tcdA*, and also the fact that both toxins appear to act on the gut of the insect, may suggest similar modes of action (Ffrench-Constant and Bowen, 1999). Against sensitive insects, the potency of toxin A compares favorably with published values for *Bt* toxins (Liu *et al.*, 2003) and therefore, we chose to study toxin A, looking at its importance in the field of agriculture. In this study, the *Photorhabdus luminescens* *tcdA* gene has been cloned, expressed in *Escherichia coli* (*E. coli*) BL21 and purified.

## Materials and Methods

### Bacterial strains

*Photorhabdus luminescens* strain was isolated from western ghat of Karnataka, which is symbiotically associated with entomophagous nematode *Heterorhabditis*. Standard culture of *Photorhabdus luminescens* was obtained from ICgeb. *Escherichia coli* strain was used for cloning, and maintenance of different DNA fragments. A prokaryotic expression vector pET32a (+) (Novagene) was used for recombinant protein production.

The recombinant plasmid was transformed into *E. coli*, BL21 as host strain. LB broth or LB agar was supplemented when required with 100 µg/mL ampicillin. All chemicals were purchased from Himedia.

DNA extraction protocol was followed according to Sambrook *et al.*, (1989). Total DNA isolated was quantified by following the ethidium bromide spotting method as given by Sambrook and Russel (2001).

### Designing of primers

Primer pairs were designed to amplify the full-length region of *tcdA* gene and primers

were also designed to amplify the internal regions of the *tcdA* gene. From the sequence information available in NCBI database (accession number AF188483). Primers were designed using Fast-PCR software and were synthesized from Sigma Genset, Germany

### Full length primer for *tcdA*

Forward primer with *Bam*H I site for cloning into pET32a (+)  
5'ctcacgcggatccttattaatggtgtagcgaatatgc (38mer) Reverse primer with *Xho* I site for cloning into pET32a (+) 5'-gtgcagctcgagttattaatggtgtagcgaatatgc (37mer).

Internal primers for *tcdA*: (1) *tcdAi* gene F: C gatcggatccatgaacgagctgtgaaaag (*Bam*HI) R: Tacttagctagcaccgaggacgcttttcg (*Nhe* I) (2) *tcdAii* gene F: Cggtgctagcggcatttgaagctaactcgt (*Nhe* I) R: Gtgagggccccaccaggtaccatctgat (*Apa* I) and (3) *tcdAiii* gene: F: ggtggggccctcactttgtagagatgataaag (*Apa* I) R: gtgcagctcgagttattaatggtgtagcgaatatgc (*Xho* I)

### Polymerase chain reaction

Different concentrations of primers 2.5, 5 and 10 pM were used to optimize amplification using total DNA of *Photothabdus luminescens*. Primers at 5 pM concentration were found optimum and used in all further studies. Taq DNA polymerase, 10x assay buffer and mixed dNTPs were obtained from M/s Bangalore Genei Private Ltd., Bangalore. Eppendorf Master Cycler (5331) was used to run the PCR programme (Table 1).

### Gel elution of the PCR amplicon

The Ferment as gel extraction kit was used to elute the different predicted size of 2.4kb, 2.7kb and 2.4kb in *tcdAi*, *tcdAii* and *tcdAiii* respectively from the agarose block as described in user's manual.

### Ligation of three fragments of *tcdA* gene

A total reaction volume of 25µl was set up for ligation by mixing the following components in a 0.5 ml tube: Purified PCR fragments of *tcdAi*, *tcdAii* and *tcdAiii* 5.0µl each, 10X ligation buffer 3.0µl, PEG4000 solution 3.0µl, BSA 0.5µl, Deionised water upto 3.0µl and T4 DNA ligase 0.5µl Ligation was carried out at 22°C for 16 hrs.

The ligated fragment was used for cloning in expression vector pET-32a (+). The ligated fragment and the vector pET-32a were simultaneously subjected to digestion with *Bam*HI and *Xho*I restriction enzyme in separate reaction. 2.0 µl of both insert and vector were run on 1.2 Percent agarose gel and the remaining sample was purified using Fermentas purification Kit. Ligation reaction was set at 1: 3 (v/v) vector to insert ratio and ligation was carried out according to manufacturer instructions using T4 DNA ligase, for overnight at 4°C. Approximately 50 ng of the insert was ligated in pET-32a (+) (Novagen) digested with *Bam*HI and *Xho*I restriction enzymes and the recombinant vector was named as pET-32a (+) *tcdA*. construct pET-32a(+) –*tcdA* was transformed by heat shock into *E. coli* competent cells BL21 DE2. Standard techniques for these steps such as plasmid DNA preparation, ligation, competent cell preparation and transformation were followed, as described previously (Sambrook and Russell, 2001). Ampicillin-resistant colonies were grown until OD 600 = 0.4 to 0.6 in LB medium containing 100 µg/ml of Ampicillin, at 37°C with agitation.

The plasmids were purified and analyzed for restriction enzyme digestion with *Bam* HI and *Nhe*-1, *Nhe*-1 and *Apa*-1, *Apa*-1 and *Xho*-1 to confirm the presence of *tcdA*. PCR amplification using *tcdA* full length primer was also carried out to further confirm the

clone. Confirmed clones were used further to optimize the expression of *tcdA* in bacterial system

### **Expression and purification of *tcdA***

For expression of *tcdA*, the purified pET32a (+) *tcdA* construct was transformed in *E. coli* BL21 (DE3) cells (Novagen, USA) by heat shock method. The selected *E. coli* clone showing expression of *tcdA* was inoculated into 100 ml LB broth containing ampicillin (100µg/ml) and grown at 37°C for 10-12 hrs as pre-inoculum. 50 ml of the pre-inoculum was inoculated to 500ml of LB medium containing ampicillin (100µg/ml) and incubated on a shaker at 37°C until OD reached a value of 0.6 at 600nm. When the bacterial suspension reached an O.D value of 0.6 at 600nm, culture was induced with 0.5mM IPTG (iso propyl β-thiogalactopyranoside) to a final concentration and incubation was continued at 30°C for overnight in a shaker-incubator. The induced culture was harvested by centrifugation at 6000 rpm for 12 min at 4°C.

The cell pellet was suspended in 30 ml of lysis buffer and was disrupted by sonication on ice bath for 15 min, the cell debris was pelleted by centrifugation at 12000 rpm for 10 min at 4°C and both the supernatant and the cell debris pellet were stored at -80°C until tested.

Expressed protein was purified by chromatography through Ni-agarose (Invitrogen), from the insoluble phase of lysate using Guanidine hydrochloride 6 M to dissolve the pellet, according to the manufacturer's protocol. Briefly, two ml of Ni-NTA resin was packed into a syringe, washed and equilibrated in 10 column volumes of deionized water, followed by 10 column volumes of binding buffer (pH = 7.8, K3(PO4) 50 mM, NaCl 400 mM, KCl

100mM, 10 mM Imidazole, 10% Glycerol, 0.5% Triton X-100). The filtered supernatant of lysate insoluble phase through a 0.45 µm membrane was loaded onto a Ni-NTA column and then washed with 10 column volumes washing buffer containing 20 mM Imidazole. Target protein was eluted using an Imidazole gradient (100-500 mM) in the binding buffer. Subsequently, the eluted solution containing protein was collected. The concentration of purified protein was determined by Bradford method. The purified *tcdA* was analyzed by SDS-PAGE gel and Coomassie brilliant blue staining.

### **Results and Discussion**

#### **Amplification and cloning of DNA coding for *tcdA***

Amplification of 2.4kb, 2.7kb and 2.4kb in *tcdAi*, *tcdAii* and *tcdAiii* respectively, followed by ligation of these fragment and ligated fragment was cloned successfully in the pET32a(+) expression vector. The integrity of the constructed vector pET32a (+) *tcdA* was confirmed by restriction digestion analysis. The sequencing of constructed plasmid with full length primer *tcdA* were performed (Plate 1 and 2).

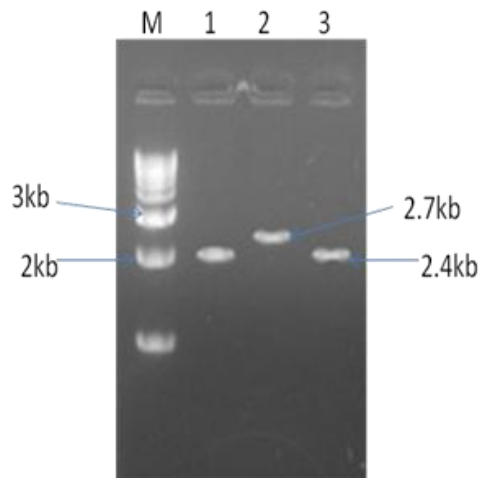
#### **Expression and purification of *tcdA***

The *tcdA* protein was expressed immediately after induction with IPTG. The expected recombinant fusion protein of 283 kDa was detected after induction of the culture with IPTG. Most of it was found to be localized inside the inclusion bodies (pellet of cell lysate) in the cells. The maximum amount of *tcdA* was obtained overnight after induction by IPTG at 30°C with agitation (Plate-3). After purification of *tcdA* by Ni-NTA Affinity Chromatography, the yield of the purified solution was estimated by Bradford method about 0.350 µg/ml of culture

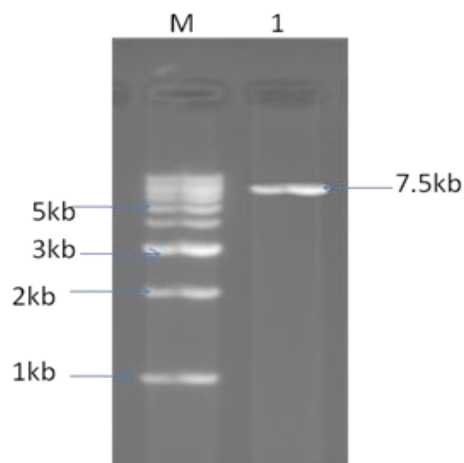
**Table.1** PCR amplification conditions employed for amplification of *tcdA*, *tcdAi*, *tcdAii* and *tcdAiii* genes

Stage	Step	Temperature (°C)				Duration				No. of cycles
1	Initial Denaturation	94				4min				1
2	Denaturation	94				30sec				}30
3	Annealing	<i>tcdAi</i>	<i>tcdAii</i>	<i>tcdAiii</i>	<i>tcdA</i>	30sec				
		55	60	58	58					
4	Extension	72				<i>tcdAi</i>	<i>tcdAii</i>	<i>tcdAiii</i>	<i>tcdA</i>	
						2.5min	2.5min	2.5min	8min	
5	Final Extension	72				15min				1
6	Hold	4				infinity				

**Plate.1** PCR amplification of (1) *tcdAi*, (2) *tcdAii* and (3) *tcdAiii*, gene from genomic DNA using specific primer (M) marker

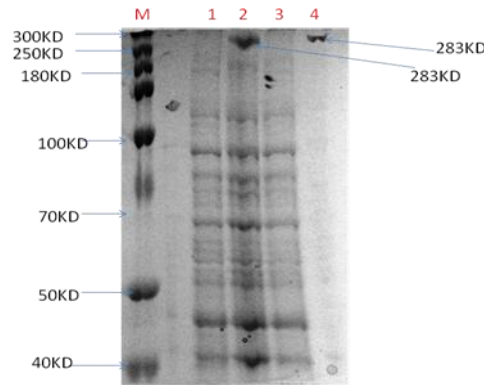


**Plate.2** PCR amplification of (1) *tcdA* gene using full length primer (M) marker





**Plate.3 SDS PAGE analysis for expression of *tcdA* gene**



In this study the expression vector pET32a (+) was used to construct expressing vector, pET32a (+) *tcdA*. The pET System is the most powerful system yet developed for the cloning and expression of recombinant proteins in *E. coli* (Novagen's pET System Manual and Novagen, Technical Bulletin 009). Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cells. T7 RNA polymerase is so active that, when fully induced, almost all of the cell's resources are converted to target gene expression (Novagen's pET System Manual and Novagen, Technical Bulletin 009).

By transferring the plasmid pET32a (+) *tcdA* into *E. coli* BL21 DE3, expression was induced by the addition of IPTG. This vector encodes the His•Tag sequences that allow easy purification, quantification and detection of target proteins (Meng *et al.*, 2009). His•Tag domain follows the cloning sites, allowing C-terminal fusions by cloning in the appropriate continuous reading frame. The His•Tag sequence is very useful as a fusion partner for protein purification. His•Tag fusion proteins can be affinity purified under fully denaturing conditions (Novagen's pET System Manual and Novagen, Technical Bulletin 009).

In this study, *Bam*HI and *Xho*-I cloning sites were chosen for insertion. Therefore, His•Tags sequences were incorporated in the expressed protein. These additional tags increased the size

of the expressed protein. The restriction digestion and PCR results confirmed the integrity of the cloning. The *tcdA* was purified successfully and this can be use for further studies. One of the most important toxic protein against different insect pest was made available by production of a recombinant *E. coli* expressing the *tcdA* gene as a fusion protein to easy purification.

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