

# Insilico Analysis of Novel *hipAB*, *ccdBA*, and *yoeB-yefM* Toxin-Antitoxin Homolog's from the Genome of *Xenorhabdus nematophila*

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**Abstract** *Xenorhabdus nematophila* is a motile gram-negative bacteria belonging to the family *Enterobacteriaceae* and is a natural symbiont of a soil nematode of family *Steinernematidae*. The bacterium is essential for effective killing of the insect host and is required by the nematode to complete its life cycle. *X. nematophila* can be grown under standard laboratory conditions. As the bacterium enters the stationary phase of growth cycle it secretes several extracellular products, which include lipase(s), phospholipase(s), protease(s), and several broad spectrum antibiotics in the insect hemolymph. Recently, the genome of *X. nematophila* has been completely sequenced and annotated version is available in the NCBI database. In this study the genome of *X. nematophila* was extensively analyzed bioinformatically with NCBI server ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Our results showed the presence of *hipAB*, *ccdBA*, *yoeB-yefM* toxin-antitoxin homologues at different loci in genome. Later, phylogenetic as well as physiochemical analysis of each toxin-antitoxin pair was done. Extensive promoter analysis of each toxin-antitoxin module was performed with BPROM ([www.softberry.com](http://www.softberry.com)) to dissect the various transcription factors which may control the transcription of such novel identified putative TA modules in *X. nematophila*. Existence of all the three operons has been confirmed by polymerase chain reaction (PCR) amplification using operon specific primers.

**Keywords** Toxin-antitoxin System, Putative *hipAB*, *ccdBA* and *yoeB-yefM* Operon, Genome, Phylogenetic Analysis, Promoter Analysis, *X. Nematophila*

## 1. Introduction

The term “toxin-antitoxin system” usually abbreviated as “TA system” comprises a functional element consisting of a biologically active protein molecule and a corresponding inhibitor, whose nature and inhibitory mechanism depend on the system's class affiliation. Components of such systems are encoded within polycistronic operons, often with partially overlapping open reading frames. These systems are wide spread among bacteria as well as archaea[1,2,3,4] and evolved to carry out diverse functions. However, their common feature is an enzymatic activity detrimental for the cell metabolism. Such toxic activity has been demonstrated to switch bacterial cells over to a dormant state, leading to cell death during prolonged exposure. In most cases various stress stimuli are responsible for TA system activation.

The signaling pathway in such instances is often related to other stress-induced response pathways. Moreover, it is well documented that in some cases the activity of TA systems

stabilizes mobile genetic elements, therefore comprising an important mechanism of plasmids maintenance.

The biological activity of a toxin comprising a component of a TA system is usually (but not always) that of an endoribonuclease. The classification of TA systems is based on the mechanism of inhibition of the toxin as well as on operon auto regulatory functions. Initially two classes of TA systems were identified[5], but subsequent discoveries extended the classification to three classes[6]. Class I includes systems in which the antitoxin is an antisense RNA forming duplexes with the toxin mRNA. This leads to inhibition of translation in a process known as RNA interference. Examples of such systems are chromosomally located operons found in *Escherichia coli* namely *tisAB*[7] and *symER*[8], as well as plasmid loci *parB*[9] of *E. coli* and *par* of *Enterococcus faecalis*[10][11] and a homologous plasmid operon of *Staphylococcus aureus*[12]. Class II encompasses a wide range of TA systems; Antitoxins of this class are proteins. The biological activities exhibited by the toxins include transcription inhibition by targeting gyrase function and interference with translation through an mRNA interferase activity, which may or may not rely on ribosome binding. The endoribonucleolytic activity of mRNA interferases is often sequence specific gives a short overview

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of the class II TA systems and their characteristics. Class III comprises a single member only. This system is encoded in the *toxIN* operon of *Erwinia carotovora*, a plant pathogen. In this case inhibition of ToxN toxin activity is driven by RNA molecules directly interacting with the toxin molecules [13][14]. On the basis of different TA Modules, bacteria have developed multiple complex mechanisms ensuring an adequate response to environmental changes. In this context, bacterial cell division and growth are subject to strict control to ensure metabolic balance and cell survival.

*X. nematophila* is a motile gram-negative bacteria belonging to the family *Enterobacteriaceae* [15]. It forms symbiotic association in the gut of a soil nematode of family *Steinemmatidae* [16]. The *X. nematophila* isolated, studied so far have been obtained from nematodes harvested from soil samples. Free-living forms of the bacterium have not yet been isolated from soil or water sources, which suggest that the symbiotic association may be essential for the survival of the bacteria in the environment. The bacteria in turn, are essential for effective killing of the insect host and are required by the nematode to complete its life cycle [17][18]. As the bacteria enter the stationary phase of their growth cycle, they secrete several extracellular products, which include lipase(s), phospholipase(s), protease(s), and several broad spectrum antibiotics [19][20]. These products are believed to be secreted in the insects hemolymph when the bacteria enter stationary phase conditions.

Since TA modules are involved in the survival of bacterium under stress conditions. Therefore, it could be possible that similar kind of survival mechanism may exist in *X. nematophila* which protects bacterium during its stress full life cycle inside insect hemolymph or in insect carcass (due to exponential growth of bacterium which leads to nutrient deprivation). In our earlier study we have identified three putative TA modules includes *relB*, *relE* and *mazF* [21] from the genome of *X. nematophila*. In this study we have identified three additional putative TA modules such as *hip-AB*, *ccd-BA* and *yoe-yef-BM*. These operons located in the genome of *X. nematophila*, molecular weights as well as isoelectric point of each toxin and antitoxin proteins have been identified with the help of Expasy server ([www.expasy.org](http://www.expasy.org)) which suggests that all three novel identified TA modules in this study belongs to Type II TA category. Extensive promoter analysis were done with BPROM ([www.softberry.com](http://www.softberry.com)) to dissect the various transcription factors which control the transcription of such novel identified putative TA module in *X. nematophila*.

## 2. Methods

### 2.1. Identification and Genetic Organization of Putative TA Modules

Complete genome sequence of *X. nematophila* ATCC 19061 was available in NCBI server ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) under RefSeq database with accession number NC\_014228.1.

The entire genome sequence of *X. nematophila* was analyzed bioinformatically for the presence of *hip-AB*, *ccd-BA*, and *yoeB-yefM* homolog's. Operons coding these TA modules were downloaded and analyzed with ORF (Open Reading Frame) finder Software from NCBI server.

### 2.2. Protein-Protein Blast

Protein databank was searched by protein-protein blast performed with putative toxin-antitoxins encoded by the various operon from the genome of *X. nematophila* using NCBI server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

### 2.3. Phylogenetic Analysis

Different annotated protein sequences were multiple aligned and phylogenetic trees were constructed by using CLC Genomics Workbench (version 4.9) software.

### 2.4. Physiochemical Properties

All the physio-chemical properties of putative toxin-antitoxin proteins have been determined by Expasy server ([www.expasy.org](http://www.expasy.org)).

### 2.5. Promoter Analysis

Identification of putative pro-motors associated with ORFs was determined by software BPROMO ([www.softberry.com](http://www.softberry.com)).

### 2.6. Genomic DNA Isolation

*X. nematophila* culture was inoculated from glycerol stock in 50 ml and grown overnight at 28°C, 200 rpm. Overnight grown culture was pelleted down by centrifugation at 5000 rpm for 10 minutes at 4°C. The pellet was resuspended in 4 ml TE buffer pH8 (10 mM Tris HCl, 1mM EDTA) and 0.5 ml of 10% SDS was added. 30 µl of proteinase K (20mg/ml) was added to the resuspended culture and incubated at 37°C for one hour. After complete lysis of the cells, 1 ml of 5 M NaCl was added and mixed gently. 750 µl CTAB NaCl mixture was added to the lysate and incubated at 65°C for 20 minutes. Later equal volume of chloroform: Isoamyl alcohol mix (approx 7.5ml) was added and mixed gently. It was centrifuged at 12,000 rpm at 4°C for 30 minutes. To the aqueous phase containing genomic DNA, 12.5 µl of RNase (2mg/ml) was added. The supernatant was incubated at 37°C for one and half hours; equal volume of phenol: chloroform: isoamyl alcohol mixture was added and mixed properly. The tubes were centrifuged at 12,000 rpm, for 30 minutes at 4°C. The supernatant was again extracted with equal volume of phenol: chloroform: isoamyl alcohol mixture. The supernatant (aqueous phase) was collected in korex tube and 0.6 volume of isopropanol was added and mixed properly. korex tube was centrifuged at 10,000 rpm for 20 minutes at 4°C. The pellet was washed with 70 % ethanol and kept for drying in room temperature. Finally the pellet was dissolved in 0.5 ml autoclaved water and run on 0.8% agarose gel.

### 2.7. Amplification of Putative *HipAB*, *ccdAB* and *yoeB-yefM* Operon by Polymerase Chain reaction (PCR) from Genome

The DNA sequence encoding putative *hipA* toxin and its antitoxin *hipB* respectively were obtained by PCR amplification using genomic DNA as template (Denaturation at 94°C for 40 sec, annealing at 60°C for 40 sec and extension at 68°C for 1 min 30 sec, total 30 cycles) for cloning with primer pair JSR25 and JSR26. Sequence encoding putative *ccdB* toxin and its antitoxin *ccdA* respectively were obtained by PCR amplification using genomic DNA as template (Denaturation at 94°C for 40 sec, annealing at 60°C for 40 sec and extension at 68°C for 50 sec, total 30 cycles) for cloning with primer pair JSR30 and JSR31. Sequence encoding putative *yoeB* toxin and its antitoxin *yefM* respectively were obtained by PCR amplification using genomic DNA as template (Denaturation at 94°C for 40 sec, annealing at 60°C for 40 sec and extension at 68°C for 50 sec, total 30 cycles) for cloning with primer pair JSR20 and JSR21.

**Table 1.** List of primers used in this study

Primer	Sequences
JSR25	5'CGC GGA TCC ATG AAG ATC ACATCA CCA CAA AC 3'
JSR26	5'CCC AAG CTT TTA CCC TGC ACA AAG CCT GCC 3'
JSR30	5'CGC GGA TCC ATG ACA CGT GTG CTT ACA CG 3'
JSR31	5'CCC AAG CTT TCA AAA CCC GTC AAA GAG TAA ATC 3'
JSR20	5'CGC GGA TCC ATG AAT GTT ATG AGC TAT TCA GC 3'
JSR21	5' CCC AAG CTT TTA GTA GTG ATA GCG ACA TTG 3'

## 3. Result and Discussion

### 3.1. Genetic Organization of *hip-AB*, *ccd-BA*, and *yoeB-yefM* Homolog's

*X. nematophila* genome was analyzed bioinformatically and in this study we have identified three loci corresponding to three different TA modules. HipA toxin homolog encoded by 1299 bp and its antitoxin homolog HipB encoded by 251 bp was located in an operon designated as "XNC1\_operon 0810" which lies between 4076692-4077990 base pair of *X. nematophila* genome in complementary orientation as shown in Figure1 (a).

In the second identified putative TA module, putative CcdB toxin encoded by 305 bp whereas its antitoxin homolog CcdA encoded by 278bp. Both genes were located in locus designated as operon "XNC1\_operon0014" which lies between 82145-83430 base pair as shown Figure1 (b).

Third identified putative TA module is homolog of *yoeB-yefM* TA module. Putative YoeB toxin encoded by 254bp whereas its putative antitoxin YefM homolog encoded by 254 bp which is located in operon designated as

operon "XNC1\_operon0711". This operon lies between 3607111-3607616 base pair in the genome of *X. nematophila* as shown in Figure 1(c).

### 3.2. Analysis by ORF Finder (Open Reading Frame Finder) and Identification of Protein Families

All the corresponding protein sequences encoded by different TA modules were deduced by ORF finder. First putative HipA toxin composed of 432 amino acids and its putative HipB antitoxin composed of 83 amino acids. The Protein-protein blast with HipA protein showed a conserved domain belongs to *couple\_hipA* super family, *hipA\_N* super family, and *hipA\_C* super family as shown in Figure 2 (a). Blast results showed its similarity with *hipA* domain containing protein of *Enterobacter sp.638* with 69% identity (accession no. YP\_001176760.1). Similarly, protein-protein blast with HipB protein showed a conserved domain belongs to *HTX\_XRE* super family as shown in Figure 2 (b). The blast results showed its 59% identity with *XRE* super family transcriptional regulators of *Enterobacter sp.638* (accession no. YP\_00117676.1).

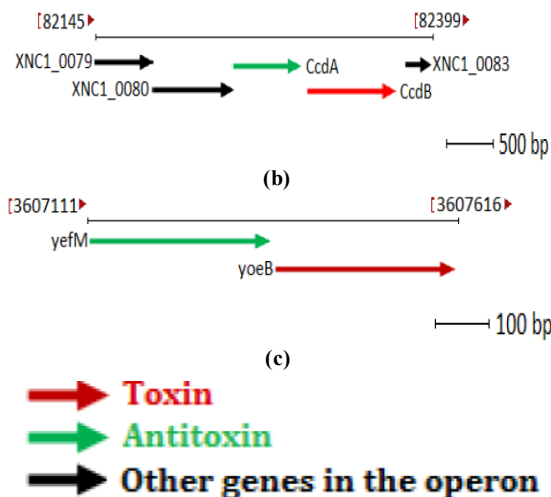
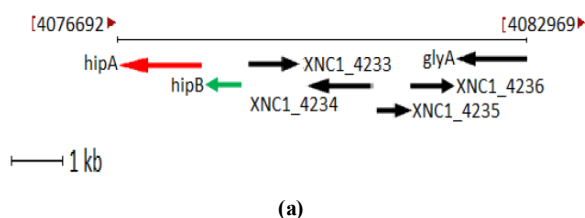
Putative CcdA antitoxin composed of 92 amino acids and its putative toxin CcdB composed of 113 amino acids. Protein-protein blast with putative CcdA protein showed conserved domain belongs to CcdA super family as shown in Figure 3 (a). It showed 68% identity with CcdA protein from *Photobacterium luminescens subsp. laumondii TTO1* (Accession no. NP\_929535.1). Protein-Protein blast with putative CcdB protein showed a conserved domain belongs to CcdB super family as shown in Figure 3 (b). Blast result with putative CcdB showed that CcdB toxin protein from *X. nematophila* was 67% identical with CcdB cytotoxic from *Photobacterium luminescens subsp. laumondii TTO1* (Accession no. NP\_929536.1).

Putative YoeB toxin composed of 84 amino acids and its putative YefM antitoxin composed of 84 amino acids. Protein-protein blast with putative YefM antitoxin protein showed a conserved domain belongs to *PhdYefM* antitoxin super family as shown in Figure 4 (a). Blast results showed its similarity with *yefM* antitoxin protein from *Prevent host death protein from Delta proteobacterium MLMS-1*. (Accession no. ZP\_01288746.1) with 77% identity. Blast results showed that putative YoeB toxin has a conserved domain at N-terminal which belongs to plasmid stability super family as shown in Figure 4 (b). Blast results showed that putative YoeB toxin showed 81% identity with hypothetical protein from *Legionella drancourtii LLAP12 LDG\_6480* (Accession no. ZP\_09620091.1). Although putative toxin was encoded by genome but belong to plasmid stability family, this could be possible only due to the horizontal gene transfer commonly present in the prokaryotic system.

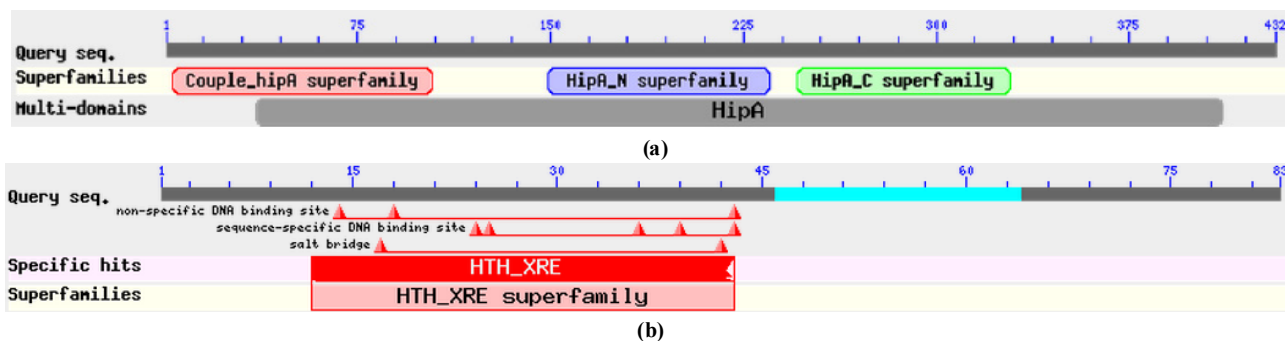
### 3.3. Physiochemical Properties

The physiochemical properties of putative toxin-antitoxin proteins have been determined by ExPASy server

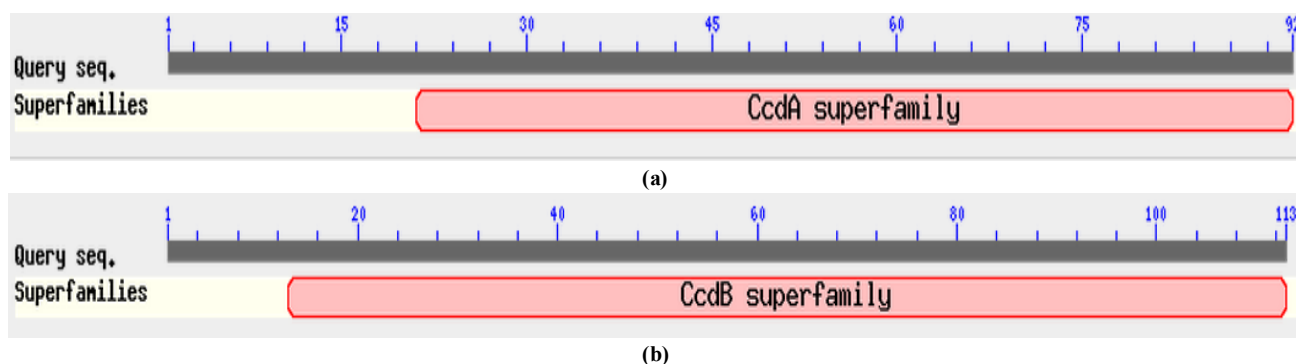
(www.expasy.org) which showed that HipA toxin had pI of 6.57 with molecular weight of 48,446 Da whereas its putative antitoxin HipB had pI 10.16 with molecular weight 9480 Da. CcdA antitoxin had pI of 8.89 with molecular weight of 10,546 Da where as its putative antitoxin CcdB had pI 5.09 with molecular weight of 11,543 Da. YoeB toxin had pI of 8.76 with molecular weight of 10,246 Da whereas its putative antitoxin YefM had pI 5.25 with molecular weight of 9512 Da.



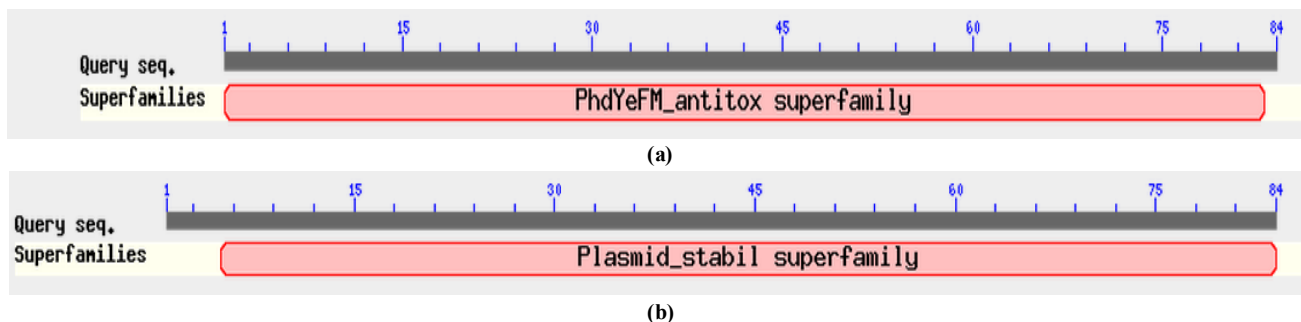
**Figure 1.** Genetic organization of TA homolog in the genome of *X. nematophila*. (a) *hipAB* TA homolog (b) *ccdBA* TA homolog (c) *yoeB-yefM* homolog



**Figure 2.** (a). Conserved domain of putative HipA toxin protein. (b). Conserved domain of putative HipB antitoxin protein



**Figure 3.** (a). Conserved domain of putative CcdA antitoxin protein. (b) Conserved domain of putative CcdB toxin protein



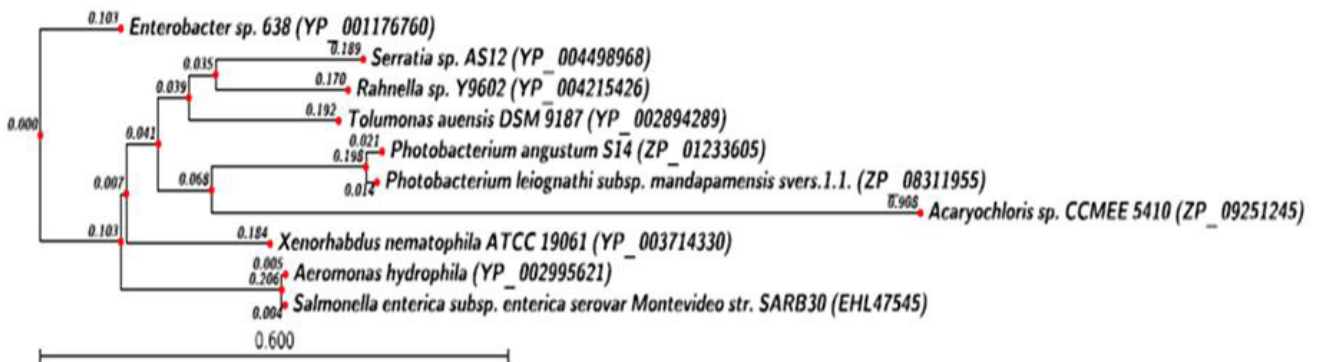
**Figure 4.** (a). Conserved domain of YefM antitoxin protein (b) Conserved domain of YoeB toxin protein

### 3.4. Phylogenetic Analysis of HipAB, CcdBA, and YoeB-YefM

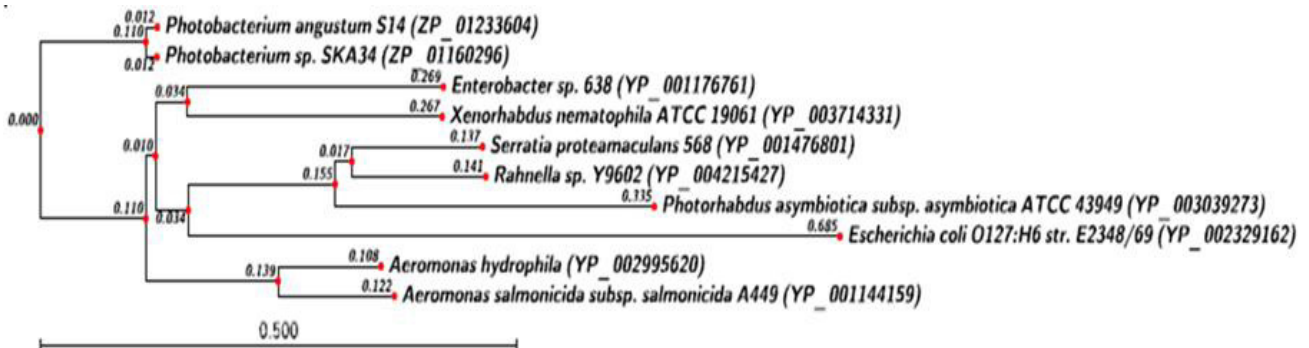
Phylogenetic analysis of HipA toxin from *X. nematophila* revealed that it forms a distinct branch of toxin from other bacteria as shown in Figure 5. Toxins from other bacteria such as *Salmonella enterica* subsp. *enterica* serovar Montevideo str. SARB30 (EHL 47545.1) and *Aeromonas hydrophila* (YP\_0029 95621.1), which are closed to the *X. nematophila* and located at proximal site in the phylogenetic tree. Whereas toxins from *Photobacterium leiognathi* subsp. *mandapamensis* svers.1.1. (ZP\_08311955.1), *Photobacterium angustum* S14 (ZP\_01233605.1), formed a separate cluster. *Rahnella* sp. Y9602 (YP\_00421 5426.1), *Serratia* sp. AS12 (YP\_0044 98968.1), forms the third cluster located at distal from *X. nematophila*. Phylogenetic analysis of HipB antitoxin from *X. nematophila* revealed that it forms very close related toxin with *Enterobacter* sp. 638 (YP\_001176761.1), and both formed a unique cluster

separated from the other two clusters formed by toxins from other bacteria as shown in Figure 6.

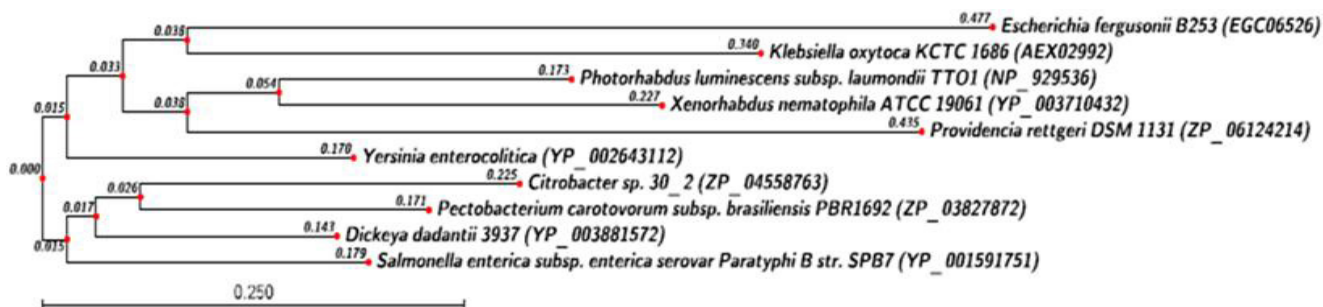
Phylogenetic analysis of the CcdB toxin revealed that there were three distinct clusters formed by toxins from different bacteria however, toxin from *X. nematophila* ATCC 19061 is very close *Photobacterium luminescens* subsp. *laumondii* T101 as shown in Figure 7. Second cluster of toxin has formed from *Pectobacterium carotovorum* subsp. *Brasiliensis* PBR1692 (ZP\_03827872.1), *Citrobacter* sp.30\_2 (ZP\_04558763.1). Third cluster of toxin has formed *Klebsiella oxytoca* KCTC 1686 (AEX\_029 92.1), *Escherichia fergusonii* B253 (EGC\_06526.1). In between second cluster and *X. nematophila* ATCC 19061, the antitoxin of *Yersinia enterocolitica* (YP\_002643112.1) has separated out evolutionary. CcdA antitoxins from other bacteria such as *Yersinia enterocolitica* (YP\_002643111.1), was separated in very beginning from the putative antitoxin from *X. nematophila* ATCC 19061 as shown in Figure 8. Similarly another antitoxin from other bacteria such as *Citrobacter* sp. 30\_2 (ZP\_04558764.1) separated apart later.



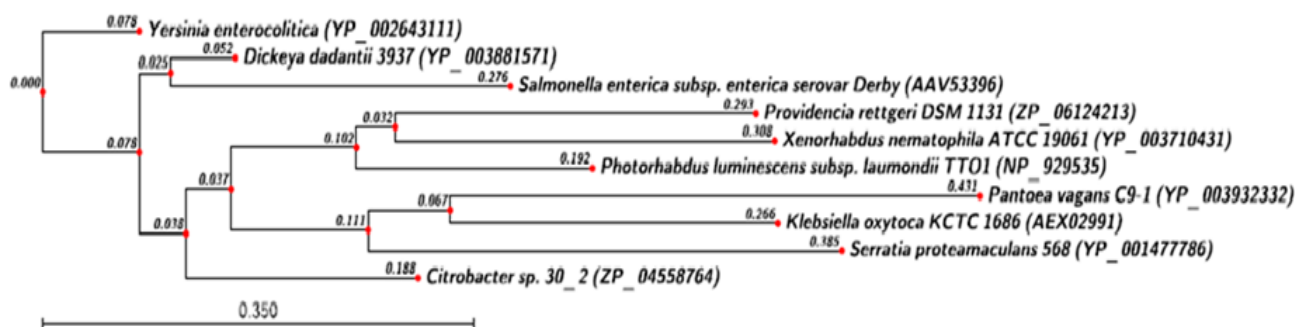
**Figure 5.** Phylogenetic analysis of Toxin *hip-A* protein. Phylogenetic tree constructed based on amino acid sequence of toxin from other bacteria sp. Sequences were aligned by using CLC Genomics workbench (VERSION 4.9) software. Gene accession number for the various protein were as follows: *X. nematophila* ATCC 19061 (YP\_003714330.1), *Enterobacter* sp. 638 (YP\_001176760.1), *Salmonella enterica* subsp. *enterica* serovar Montevideo str. SARB30 (EHL47545.1), *Aeromonas hydrophila* (YP\_002995621.1), *Tolumonas auensis* DSM 9187 (YP\_002894289.1), *Rahnella* sp. Y9602 (YP\_004215426.1), *Serratia* sp. AS12 (YP\_004498968.1), *Photobacterium leiognathi* subsp. *Mandapamensis* svers.1.1. (ZP\_08311955.1), *Photobacterium angustum* S14 (ZP\_01233605.1), *Acaryochloris* sp. CCME 5410 (ZP\_09251245.1)



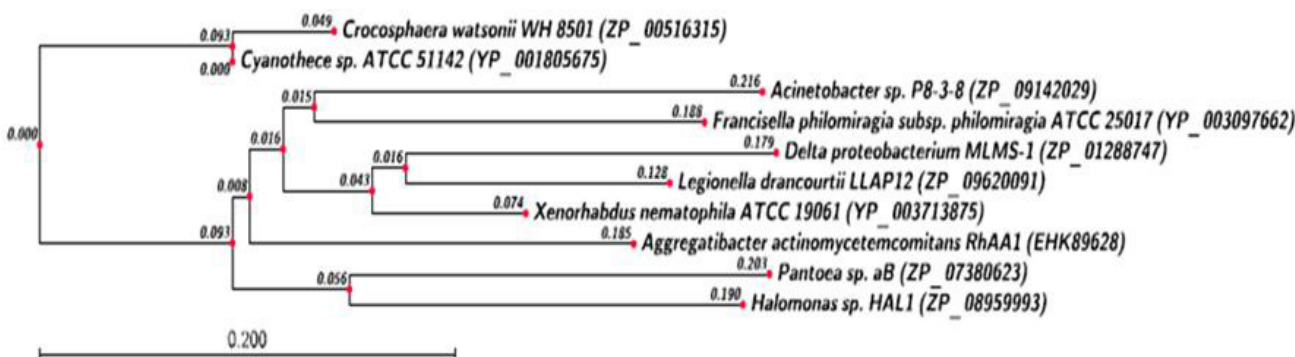
**Figure 6.** Phylogenetic analysis of Antitoxin in *hip-B* protein. Phylogenetic tree constructed based on amino acid sequence of antitoxin from other bacterial sp. Sequences were aligned by using CLC Genomics workbench (version 4.9) software. Gene accession number for the various protein were as follows: *X. nematophila* ATCC (YP\_003714331.1), *Aeromonas hydrophila* (YP\_002995620), *Aeromonas salmonicida* subsp. *salmonicida* A449 (YP\_001144159.1), *Enterobacter* sp. 638 (YP\_001176761.1), *Photobacterium* sp. SKA34 (ZP\_01160296.1), *Photobacterium angustum* S14 (ZP\_01233604), *Serratia proteamaculans* 568 (YP\_001476801.1), *Rahnella* sp. Y9602 (YP\_004215427), *Photobacterium leiognathi* subsp. *mandapamensis* svers.1.1. (ZP\_08311955.1), *Escherichia coli* O127:H6 str. E2348/69 (YP\_002329162)



**Figure 7.** Phylogenetic analysis of Toxin *ccd-B* protein. Phylogenetic tree constructed based on amino acid sequence of toxin from other bacterial sp. Sequence were aligned by using CLC Genomics workbench (version 4.9) software. Gene accession number for the various protein were as follows *X. nematophila* ATCC 19061 (YP\_003710432.1), *Photorhabdus luminescens* subsp. *laumondii* TTO1 (NP\_929536.1), *Salmonella enterica* subsp. *enterica* serovar *Paratyphi B* str. *SPB7* (YP\_001591751.1), *Yersinia enterocolitica* (YP\_002643112.1), *Dickeya dadantii* 3937 (YP\_003881572.1), *Pectobacterium carotovorum* subsp. *brasiliensis* PBR1692 (ZP\_03827872.1), *Klebsiella oxytoca* KCTC1686 (AEX\_02992.1), *Citrobacter* sp. 30\_2 (ZP\_04558763.1), *Providencia rettgeri* DSM 1131 (ZP\_06124214.1), *Escherichia fergusonii* B253 (EGC\_06526.1)



**Figure 8.** Phylogenetic analysis of *ccd-A* Antitoxin protein. Phylogenetic tree constructed based on amino acid sequence of antitoxin from other bacterial sp. Sequence were aligned by using CLC Genomics workbench (version 4.9) software. Gene accession number for the various protein were as follows *X. nematophila* ATCC 19061 (YP\_003710431.1), *Photorhabdus luminescens* subsp. *laumondii* TTO1 (NP\_929535), *Salmonella enterica* subsp. *enterica* serovar *Derby* (AAV53396.1), *Citrobacter* sp. 30\_2 (ZP\_04558764.1), *Providencia rettgeri* DSM 1131 (ZP\_06124213.1), *Dickeya dadantii* 3937 (YP\_003881571.1), *Yersinia enterocolitica* (YP\_002643111.1), *Klebsiella oxytoca* KCTC 1686 (AEX02991.1), *Serratia proteamaculans* 568 (YP\_001477786.1), *Pantoea vagans* C9-1 (YP\_003932332)

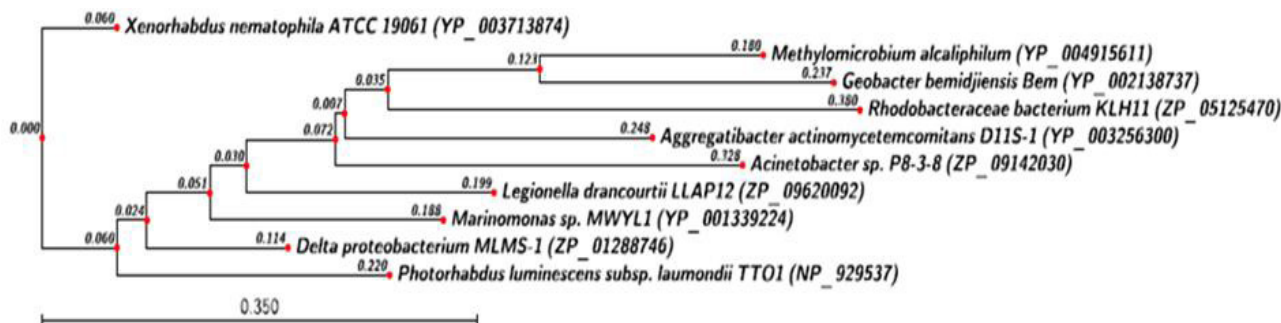


**Figure 9.** Phylogenetic analysis of *Yoe-B* Toxin protein. Phylogenetic tree constructed based on amino acid sequence of toxin from other bacterial sp. Sequence were aligned by using CLC Genomics workbench (version 4.9) software. Gene accession number for the various protein were as follows *X. nematophila* ATCC 19061 (YP\_003713875.1), *Legionella drancourtii* LLAP12 (ZP\_09620091), *Delta proteobacterium* MLMS-1 (ZP\_01288747.1), *Francisella philomiragia* subsp. *philomiragia* ATCC 25017 (YP\_003097662.1), *Aggregatibacter actinomycetemcomitans* RhAA1 (EHK89628.1), *Cyanobacter* sp. ATCC 51142 (YP\_001805675.1), *Acinetobacter* sp. P8-3-8 (ZP\_09142029.1), *Crocosphaera watsonii* WH 8501 (ZP\_00516315.1), *Pantoea* sp. aB (ZP\_07380623.1), *Halomonas* sp. HAL1 (ZP\_08959993.1)

Phylogenetic analysis of the YoeB toxin from *X. nematophila* ATCC 19061 revealed that toxin of *Legionella drancourtii* LLAP12 (ZP\_09620091) was the most closed to it as shown in Figure 9. The toxin from another bacterium such as *Delta proteobacterium* MLMS-1 (ZP\_01288747.1) form a cluster with toxin of *Legionella drancourtii* LLAP12 (ZP\_09620091). There was another cluster of toxin from bacteria such as *Halomonas* sp. HAL1 (ZP\_08959993.1) and *Pantoea* sp. aB (ZP\_07380623.1) located at distal from *X. nematophila*. The toxin from *Cyanobacter* sp. ATCC 51142 (YP\_001805675.1) and *Crocosphaera watsonii* WH 8501 (ZP\_00516315.1) was separated from the beginning. Analysis of



the YefM antitoxin from *X. nematophila* ATCC 19061 revealed that it form distinct clusters formed by antitoxins from other bacteria as shown in Figure 10.



**Figure 10.** Phylogenetic analysis of YefM antitoxin protein. Phylogenetic tree constructed based on amino acid sequence of antitoxin from other bacterial sp. Sequence were aligned by using CLC Genomics workbench (version 4.9) software. Gene accession number for the various protein were as follows *X. nematophila* ATCC 19061 (YP\_003713874), *Delta proteobacterium* MLMS-1 (ZP\_01288746.1) *Photorhabdus luminescens* subsp. *laumondii* TTO1 (NP\_929537), *Legionella drancourtii* LLAP12 (ZP\_09620092.1), *Marinomonas* sp. MWYL1 (YP\_001339224.1), *Aggregatibacter actinomycetemcomitans* D11S-1 (YP\_003256300.1), *Acinetobacter* sp. P8-3-8 (ZP\_09142030.1), *Geobacter bemidjensis* Bem (YP\_002138737.1), *Methylobacterium alcaliphilum* (YP\_004915611.1), *Rhodobacteraceae bacterium* KLH11 (ZP\_05125470.1)

### 3.5. Promoter Analysis

148 base pairs upstream region to a putative *hipAB* module contained -35 and -10 promoter like elements along with RpoD17 sigma-D (sigma70) binding sequences as shown in Figure 11. The binding sequence of RpoD17 present in upstream region of putative -35 box and some binding sequences of RpoD17 were overlap with it. RpoD17 is the heat shock sigma factor and is transcribed from two promoters PC and PHS. Synthesis of RpoD mRNA from PC is constitutive under both steady-state and heat-shock growth conditions, while that of PHS is transiently induced upon heat-shock[22]. The putative promoter consensus sequences in the -35/-10 region of the RpoD transcriptional initiation site are highly similar to those of *E. coli* sigma70. Many bacterial species exhibit a general stress response that can be induced by numerous very different stress conditions and, phenotypically renders the cells broadly stress resistant.

taagtatccctccaaaaggataaaagtaacttatcccttttaag  
ggataatttcaacctattcctacaagctatccaatgagctatcac  
aaccaactgcaatggcgccgggaaagggaagctcgtctgttga  
cgccccctcaggaggcATG

**Figure 11.** 148bp nucleotide sequence upstream region of *hip-AB* operon showing putative -10 and -35 promoter like elements along with RpoD17 sigma D (sigma 70) binding sequences

152 base pairs upstream region to putative *ccdBA* TA module contained -35 and -10 promoter like elements, along with the three transcription factor binding site like OxyR, ArgR and ArcA. In *ccdBA* putative promoter the binding sequences of OxyR and ArgR is overlap with each other, similarly the binding sequences of ArgR and ArcA also overlap with each other. OxyR is the oxidative stress regulator which acts as the transcriptional dual regulator for the expression of antioxidant genes in response to oxidative stress in particular, elevated levels of hydrogen peroxide or stress full chemical environment. The OxyR regulon includes genes involved in peroxide metabolism. In addition,

expression of OxyR is positively regulated by cAMP activated Crp protein during exponential growth and negatively regulated by RpoS when cells enter stationary phase[23]. The transcription factor of ArgR transcription factor or protein is a homohexameric protein that, when complexed with arginine, represses arginine biosynthetic genes by binding at sites overlapping their promoters. ArgR complexes with L-arginine represses the transcription of several genes involved in biosynthesis and transport of arginine, transport of histidine and its own synthesis[24][25][26] and activates genes for arginine catabolism[27]. ArgR is also essential for a site-specific recombination reaction that resolves plasmid ColE1 multimers to monomers and is necessary for plasmid stability[28]. The ArcA is a dual transcriptional regulator for anoxic redox control it acts primarily as a negative transcriptional regulator under anaerobic conditions. ArcA represses operons involved in respiratory metabolism, encoding products such as tricarboxylic acid cycle enzymes, enzymes of the glyoxylate shunt, and enzymes of the pathway for fatty acid degradation[29]. It has been suggested that the most significant role of ArcA is under micro aerobic conditions.

143bp upstream region of *yoeB-yefM* module was analyzed. It contained putative -35 and -10 promoters like elements. In the upstream of -35 box it has binding region for transcription factors RpoD19 [30], and at the upstream of -10 box it has binding site for another transcription factor called as Tyr. Both the binding sequences of the putative promoter like elements involve in transcription at sigma70 dependent promoters. The general RpoS is a sigma subunit of RNA polymerase, is a very close relative of the vegetative sigma70 RpoD. The homologous of sigma70 are known to activate specific regulons associated with heat-shock or morphological developmental stages such as bacterial sporulation or flagellar biogenesis. TyrR can acts as dual character as a repressor and as an activator. This involves in transcription at sigma70 dependent promoters. So, on the basis of all above information it is quite possible that

*yoeB-yefM* operon might be activated during the heat shock and oxidative stress conditions.

tcagatgaattccccggcaGATTAAATTCATAataCTGCCAaa  
 -10 Box oxyR argR -35 Box  
 ccttatcaggtTATTAACCTgatcacacgAATTAATAAAA  
 argR arcA  
 cgcaaagcaccgaggatttttctgcagaaggtgtgtagataAg  
 caatgtgagtggaggatcaccaATG

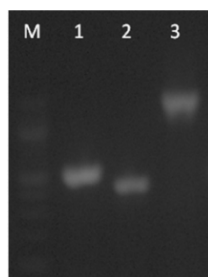
**Figure 12.** 152bp nucleotide sequence upstream region of *ccdBA* were analyzed. It contained putative -35 and -10 promoter like elements, upstream of -35 box and downstream of -10 box and has oxyR, argR and arcA binding sequences respectively

tcttctggataatttttctcctcagatcttataaaatctggg  
 rpo-D19 -35 BOX  
 gaaacaatctgattatggctaaagccataaaACAGATTGAGT  
 tyr -10 box  
 acttaacATTGTACAAGTACAATgttaAgtactcaaattctgtg  
 aggtaatgaccATG

**Figure 13.** 143bp nucleotide sequence upstream region of *yoeB-yefM* were analyzed. It contained putative -35 and -10 promoter like elements, upstream of -35 box and downstream of -10 box and has rpo-D19 and tyr binding sequences respectively

#### PCR amplification

Genomic DNA was isolated from the *X. nematophila* as protocol described in method. 585 bp DNA fragment encoding *ccdBA* module obtained by PCR amplification using genomic DNA as template as shown in lane 1 Figure 14 and 505 bp DNA fragment encoding *yoeB-yefM* module as shown Figure 14, lane 2. 1550 bp DNA fragment encoding *hipAB* module obtained by PCR amplification using genomic DNA as template as shown in Figure 14, lane 3.



**Figure 14.** PCR amplification of *ccdBA*, *yoeB-yefM* B and *hipAB* operon using genomic DNA as template. Lane M, 100bp ladder; lane 1, 585 bp *ccdBA* operon; lane 2, 505 bp *yoeB-yefM* operon; and lane 3, 1550 bp *hipAB* operon

## 4. Conclusions

TA modules have been associated with bacterial programmed cell death and programmed cell survival or persistence[31][32] under various unfavorable conditions which could be physical, chemical or nutrient depleted conditions. *X. nematophila* encounters competition with soil microflora in insect cadaver as well as with antibacterial protein from the insect hemolymph in its life cycle. Therefore under these conditions, the role of TA modules in

*X. nematophila* cannot be ruled out. In this study, sequenced and annotated genome of *X. nematophila* has been studied bioinformatically for the identification of putative TA modules in its genome. Three TA module homolog's *hipBA*, *ccdAB* and *yoeB-yefM* has been identified. Genomic organization revealed that *hipAB* module is located in "operon="XNC1\_operon 0810". Protein-protein blast of *hipA* showed its similarity with *hipA* toxin from *Enterobacter sp.638* and *hipB* showed its similarity with XRE super family respectively. Therefore, we can presume that in *X. nematophila* *hipA* and *hipB* will form a putative *hipAB* TA module. Difference in their individual pI's (HipA toxin: pI 6.57 and putative HipB antitoxin: pI 10.16) will tend them to form complex in physiological conditions. From the predicted promoter analysis due to the presence of RpoD17 we inferred that transcription of *hipA* and putative *hipB* will occur either under different environmental conditions such as high temperature or stress.

Similarly, second putative *ccdBA* TA module is located in "XCN\_loperon0014". This TA module encodes for *ccdA* antitoxin which showed similarity with *ccdA* antitoxin protein from *Photorhabdus luminescens subs laumondii* TT 01, where as its putative CcdB toxin showed similarity with CcdB toxin protein from *Photorhabdus luminescens subs. laumondii*. In this case also difference in individual pI (Putative CcdB toxin: pI 5.09 and CcdA antitoxin: pI 8.89) will tend them to form complex in physiological conditions. From predicted promoter analysis, presence of oxyR, argR, and arcA indicates that transcription of putative *ccdBA* module will occur either under SOS conditions and might be tightly regulated. Therefore, we can presume that in *X. nematophila* gene encoding *ccdAB* will form a novel TA module.

In the third putative *yoeB-yefM* TA module is located at operon "XCN\_loperon0711". Protein-protein blast with YefM antitoxin protein showed its similarity to the prevent host death protein *phd* (Antitoxin) from *Delta proteobacterium mlms-1* where as YoeB toxin from *X. nematophila* showed its identity with hypothetical protein from *Legionell adranccourtillap* 12 LDG\_6480. Therefore, putative YefM antitoxin will form a pair with YoeB to toxin. In this case due the differences in individual pI's (Putative YoeB toxin: pI 8.76 and putative antitoxin YefM pI 5.27) they will form complex in physiological conditions. Promoter predicted revealed the presence of signature sequences for the binding of rpo-D19 and tyr, which indicate transcription from this promoter under heat shock conditions. In silico identification of putative *hipBA*, *ccdBA* and *yoeB-yefM* toxin-antitoxin modules has been confirmed by PCR amplification using genomic DNA as template.

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