**Insilico Analysis of Novel Relb, Rele and Mazf Toxin-Antitoxin Homolog’s from the Genome of Xenorhabdus Nematophila**

Jitendra Singh*, Ravi Kumar Chaudhary, Pradeep Gautam

School of Biotechnology, Gautam Buddha University, Greater Noida, Uttar Pradesh, 201308, India

**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 and known to secrete several extracellular products, which include lipase(s), phospholipase(s), protease(s), and several broad spectrum antibiotics as the bacteria enter the stationary phase of their growth cycle and are believed to be secreted 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 we have extensively analyzed bioinformatically the genome of *X. nematophila* with NCBI server (www.ncbi.nlm.nih.gov). Our results showed the presence of *relB, relE*, and *mazF* toxin-antitoxin homolog’s at different loci in the genome. Later, various genes present in these loci were studied for phylogenetic as well as physiochemical analysis. Promoter analysis of each module has been done to know the various transcription factors involved in their transcription.

**Keywords**  
Toxin-antitoxin system, Putative *relB, relE, mazF*, genome, phylogenetic analysis, promoter, *X. nematophila*

1. Introduction

*Xenorhabdus nematophila* is a motile gram-negative bacteria belonging to the family *Enterobacteriaceae*[1]. It forms symbiotic association in the gut of a soil nematode of family *Steinernematidae*[2]. All the *Xenorhabdus* isolates, 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[3,4]. *X. nematophila* can be grown under standard laboratory conditions. Growth *in vitro* is probably supported by the rich nutrient supply of the laboratory growth media and lack of competition that normally exists in the soil environment. 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[5,6] that can be assayed in the culture media.

These products are believed to be secreted in the insect hemolymph when the bacteria enter stationary phase conditions. The degradative enzymes break down macromolecules of the insect cadaver to provide the developing nematode with nutrient supply, while the antibiotics suppress contamination of the cadaver by other soil microorganisms. Cytoplasmic inclusion bodies, composed of highly expressed crystalline proteins, are also produced by the bacterium during stationary-phase growth[7]. In general, bacteria exposed to a plethora of environments possess molecular responses that regulate the degradation of defective or unnecessary proteins and mRNA molecules. These defective unnecessary proteins or mRNA molecules proves burden during nutritional stress which decrease cell survival rate. To get rid of these faulty molecules produced during transcription or translation and to increase cell survival rate during nutritional or antibiotic stress a unique control mechanism is operated that help prokaryotes to cope with these unfavourable conditions. This control mechanism consist of two components together known as toxin-antitoxin (TA) modules and these modules form a non toxic complex in a favourable condition[8,9] while on the other hand modulate the global levels of transcription and replication during exposure of nutritional stress or antibiotic stress due to over expression of toxic component.

Generally in prokaryote TA modules codes for two components and they are of three types: in type I TA module,
the antitoxin are small antisense RNAs that repress translation of the toxin genes[10,11] whereas in type II the antitoxin are proteins in nature and combine with and neutralize the toxin[12]. Type III encodes a small RNA antitoxin that combines with and neutralise toxin protein[13]. Toxin-antitoxin (TA) systems or modules are broadly distributed in prokaryotes in multiple copies[14,15] and all TA operons are auto -regulated at the level of transcription by the antitoxins in which antitoxin component neutralizes its cognate toxin[16]. For example, the relBE and mazEF TA systems are global inhibitors of translation and cleave mRNA during amino acid starvation that leads to the reduction of post starvation rate of translation[17-19]. Moreover, these systems also enhance relative competitiveness of alternative sigma factors (σ) or transcription factors to prioritize transcription of stress related genes[20,21]. Since E. coli TA modules are involved in the survival of E. coli following stress conditions therefore, it could be possible that similar kind of survival mechanism exist in X. nematophila which protects bacterium during its stressful life cycle inside the insect hemolymph or in insect carcass (due to exponential growth of bacterium which leads to nutrient deprivation). Therefore, it is necessary to study toxin-antitoxin (TA) modules of X. nematophila in detail. Recently, the genome of X. nematophila has been sequenced, annotated and available in NCBI database. Moreover, so far not a single toxin-antitoxin (TA) module from X. nematophila has been reported. Therefore, this gave us opportunity to extensively search X. nematophila genome bioinformatically with NCBI server (www.ncbi.nlm.nih.gov) for the identification of such toxin-antitoxin modules. In this study we have identified three putative TA modules such as relB, relE and mazEF, in the genome of X. nematophila. Interestingly, in our case relBE module is divided into two separate modules relB and relE, and they are located very far from each other in genome, each having their own toxin and antitoxin components respectively. Molecular weights as well as isoelectric point of each toxin and antitoxin proteins have been identified with Expasy server (www.expasy.ch) which suggests that all the three novel identified TA modules have their own toxin and antitoxin components respectively. Molecular weights as well as isoelectric point of each toxin and antitoxin proteins have been identified with Expasy server (www.expasy.ch) which suggests that all the three novel identified TA modules have their own toxin and antitoxin components respectively. Molecular weights as well as isoelectric point of each toxin and antitoxin proteins have been identified with Expasy server (www.expasy.ch) which suggests that all the three novel identified TA modules have their own toxin and antitoxin components respectively. Molecular weights as well as isoelectric point of each toxin and antitoxin proteins have been identified with Expasy server (www.expasy.ch) which suggests that all the three novel identified TA modules have their own toxin and antitoxin components respectively.

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) in two different databases; INSDC database with accession number FN667742.1 and RefSeq database with accession number NC_014228.1. The entire genome sequence of X. nematophila was downloaded from the Refseq database due to its nonredundant nature and was analyzed bioinformatically for the presence of relB, relE and mazF homolog’s. Identified operons coding these toxin-antitoxin modules were downloaded and analyzed with ORF (Open Reading Frame finder) finder software from NCBI server (http://www.ncbi.nlm.nih.gov/gorf/gorf.html)

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 physiochemical properties of putative toxin-antitoxin proteins have been determined by Expasy server (www.expasy.ch)

2.5. Promoter Analysis

Identification of putative promoter associated with ORFs were determined by software BPROMO (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 pelletized from 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:i soamyl 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:i soamyl 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 mazF, relB and relE operon by polymerase chain reaction (PCR) from genome

The DNA sequence encoding mazF and its putative antitoxin mazE respectively were obtained by PCR amplification using genomic DNA as template (Denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 68°C for 50 sec, total 30 cycles) for cloning with primer pair JSR1 and JSR2. Sequence encoding relB antitoxin and its putative toxin respectively were obtained by PCR amplification using genomic DNA as template (Denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 68°C for 50 sec, total 30 cycles) for cloning with primer pair JSR6 and JSR7. Sequence encoding relE and its putative antitoxin respectively were obtained by PCR amplification using genomic DNA as template (Denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 68°C for 50 sec, total 30 cycles) for cloning with primer pair JSR11 and JSR12.

Table 1. Primer list with sequence used in polymerase chain reaction

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>JSR1</td>
<td>5’ CGCGGATCC ATG TGT TTA TTC TCT ATT GAG 3’</td>
</tr>
<tr>
<td>JSR2</td>
<td>5’ CCGCGATCC TCA TCA GTC TTT TAC GGG 3’</td>
</tr>
<tr>
<td>JSR6</td>
<td>5’ CGCGGATCC ATG ACC ACC AAT CAA ATA AGG 3’</td>
</tr>
<tr>
<td>JSR7</td>
<td>5’ CCGCGATCC TTA AAA AGA AAA ATT CCT CAG 3’</td>
</tr>
<tr>
<td>JSR11</td>
<td>5’ CGCGGATCC ATG TCT ATT GAG 3’</td>
</tr>
<tr>
<td>JSR12</td>
<td>5’ CGCGGATCC ATG TCT ATT GAG 3’</td>
</tr>
</tbody>
</table>

3. Result and Discussion

3.1. Genetic Organization of relB, relE, and mazF Homolog’s

Complete genome of X. nematophila was analyzed bioinformatics for the identification for toxin-antitoxin homologs. We have identified three loci corresponding to three different toxin-antitoxin modules. mazF toxin homolog along with gene encoding a hypothetical protein was located in operon consist of 530 bp, annotated as “XCN_1operon0855” which lies between 4269050-4269581 bp of X. nematophila genome as shown in Figure 1 (a). Whereas, relB antitoxin homolog along with part of Qin prophage system was located as a part of operon “XCN_1operon0367” consist of 3278 bp which lies between 1841962-1845241 bp of X. nematophila genome in the complement orientation as shown in Figure 1(b). Interestingly, relE toxin homolog along with stability protein gene stbd was located in separate operon consist of 526 bp, annotated as “XCN_1operon0557” which lies between 2939581-2940108 bp of X. nematophila genome as shown in Figure 1(c). Therefore, in our case relB and relE homologs were located separately in genome rather than forming usual single relBE toxin-antitoxin module as present in other prokaryotic system[17]. All operons were further analyzed with ORF (Open Reading Frame Finder).

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

All three identified operons were analyzed by ORF finder. First operon “XCN_1operon0855” encoded two different genes, first gene correspond to a hypothetical protein of 52 amino acids, where as second gene corresponds to MazF toxin protein of 125 amino acids. Their corresponding protein sequences were deduced by ORF finder. Protein-protein blast with hypothetical protein encoded by first gene of operon did not show any conserved domain. Interestingly, it showed 58% identity with MazE antitoxin protein from vibrio cholerae 1587 (Accession no. ZP_01950671.1). Therefore, we have designated this hypothetical protein from X. nematophila as putative MazE antitoxin. Similarly, protein-protein blast with MazF toxin protein encoded by the second gene showed a conserved domain in its N’ terminal belonging to PemK superfamily as shown in Figure 2. Blast results showed that MazF toxin protein from X. nematophila was 75% identical with MazF toxin protein from Photobacterium profundum SS9 (Accession no. YP_130852.1).

Second operon “XCN_1operon0367” which encoded four different genes but they are in a complement orientation. First gene of an operon corresponds to a RelB antitoxin protein of 84 amino acids, where as second gene corresponds to Qin prophage system coding for 57 amino acids. Third and fourth gene codes for uhpB and uhpC gene respectively. uhpC codes for sensory histidine kinase which is part of the two-component regulatory system with UphA whereas, uhpB codes for a membrane protein. All the corresponding protein sequences encoded by four genes were deduced by ORF finder. Protein-protein blast with RelB antitoxin protein showed a conserved domain belonged to RelB superfamily as shown in Figure 3 (a). Blast results showed its similarity with RelB antitoxin protein from Photorhabdus luminescent subs laumondii TT 01 (Accession no. NP_927617.1) with 89% identity. Similarly, protein-protein blast with Qin prophage system protein encoded by the second gene showed 81% identity with hypothetical protein YE0510A from Yersinia enterocolitica subsp enterocolitica 8081 (Accession no YP_001004871.1) and 71% identity with RelE toxin protein from Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949. We have designated this protein as putative toxin. Blast results showed that putative toxin encoded by second gene of operon have a conserved domain at N’ terminal which belonged to plasmid stability super family as showed in Figure 3 (b). Although putative toxin was encoded by genome but it belonged to plasmid stability family, this could be possible only due to the horizontal gene transfer, commonly present in the prokaryotic system. Third operon “XCN_1operon0557” encoded two different genes in complement orientation, first gene corresponding to a stability protein Stbd of 83 amino acids, where as second gene corresponds to RelE protein of 94 amino acids. Their corre-
sponding protein sequences were deduced by ORF finder. Protein-protein blast with stability protein StbD encoded by first gene of operon showed a conserved domain at its N’ terminal belonged to PhdYefM antitoxin superfamily as shown in Figure 4 (a). Blast result showed that stability protein StbD from *X. nematophila* is 84% identical with Stability protein StbD (Antitoxin) from *Escherichia coli* ED1a (Accession no. YP_002418779.1). Therefore, we have designated this protein as putative antitoxin. However, protein-protein blast with RelE protein showed a conserved domain at its N’ terminal belonged to plasmid stability superfamily as shown in Figure 4 (b). Blast result showed that RelE protein from *X. nematophila* is 73% identical with stability protein StbE from *Edwardsiella ictaluri* 93-146 (YP_002933420.1) and 71% identical with toxin component of toxin-antitoxin sytem of RelE family from *Edwardsiella tarda* ATCC 23685 (Accession no ZP_06714851.1). In this case also RelE was encoded by genome but it belonged to a plasmid superfamily, again it is an example of horizontal gene transfer.

Figure 5.(a) Phylogenetic analysis of MazF toxin protein from X. nematophila. Phylogenetic tree constructed based on the amino acid sequence of similar toxins from other bacteria. Sequences were multiple aligned by using CLC Genomics Workbench (version 4.9) software. Gene accession numbers for the various proteins were as follows: Xenorhabdus nematophila ATCC 19061 (YP_003714544.1), Photobacterium profundum SS9 (YP_130852.1), Vibrio cholerae MZO-3 (ZP_01955077.1), Marinobacter aquaeolei VT8 (YP_0569901.1), V. vulnificus ZP_01950607.1), Vibrio vulnificus (YP_001393092.1), Photobacterium profundum SS9 (YP_130851.1). CLC Genomics Workbench (version 4.9) software was used to construct the phylogenetic tree.

Figure 5.(b) Phylogenetic analysis of putative MazE antitoxin protein from X. nematophila. Phylogenetic tree constructed based on the amino acid sequence of similar antitoxins from other bacteria. Sequences were multiple aligned by using CLC Genomics Workbench (version 4.9) software. Gene accession numbers for the various proteins were as follows: Xenorhabdus nematophila ATCC 19061 (YP_003714543.1), Vibrio cholerae 1587(ZP_01950607.1), Vibrio vulnificus (YP_00379092.1), Photobacterium profundum SS9 (YP_130851.1). CLC Genomics Workbench (version 4.9) software was used to construct the phylogenetic tree.

Figure 6.(a) Phylogenetic analysis of relB antitoxin protein from X. nematophila. Phylogenetic tree constructed based on the amino acid sequence of antitoxins from other bacteria. Sequences were multiple aligned by using CLC Genomics Workbench (version 4.9) software. Gene accession numbers for the various proteins were as follows: Xenorhabdus nematophila ATCC 19061 (YP_003712183.1), Photobacterium phosphoreum subsp. laumondii TTO1 (NP_927617.1), Versinia enterococcal subs. enterococcal 8081 (YP_001004870.1), Candidatus Regiella insecticola LSR1 (ZP_07396152.1), Escherichia coli E22 (ZP_03046989.1),Providencia rustigianii DSM 4541 (ZP_05972866.2), Providencia rustigianii DSM 4541 (ZP_05972866.2), Commensalibacter intestini A911 (ZP_09013002.1), Salmohaella enterica subs. arizonae serovar 62:z4,z23:--, str. RSK2980 (YP_001572182.1), Pantoaea sp. SL1_M5 (ZP_09512769.1), Aggregatibacter actinomycetemcomitans RhAA1 (EHK91012.1) CLC Genomics Workbench (version 4.9) software was used to construct the phylogenetic tree.

Figure 6.(b) Phylogenetic analysis of putative toxin protein from X. nematophila. Phylogenetic tree constructed based on the amino acid sequence of antitoxins from other bacteria. Sequences were multiple aligned by using CLC Genomics Workbench (version 4.9) software. Gene accession numbers for the various proteins were as follows: Xenorhabdus nematophila ATCC 19061 (YP_003712183.1), Photobacterium phosphoreum subsp. laumondii TTO1 (NP_927617.1), Versinia enterococcal subs. enterococcal 8081 (YP_001004870.1), Candidatus Regiella insecticola LSR1 (ZP_07396152.1), Escherichia coli E22 (ZP_03046989.1),Providencia rustigianii DSM 4541 (ZP_05972866.2), Providencia rustigianii DSM 4541 (ZP_05972866.2), Commensalibacter intestini A911 (ZP_09013002.1), Salmohaella enterica subs. arizonae serovar 62:z4,z23:--, str. RSK2980 (YP_001572182.1), Pantoaea sp. SL1_M5 (ZP_09512769.1), Aggregatibacter actinomycetemcomitans RhAA1 (EHK91012.1) CLC Genomics Workbench (version 4.9) software was used to construct the phylogenetic tree.
3.3. Phylogenetic Analysis of MazF, RelB and RelE

Protein-protein blast with MazF, RelB and RelE showed similar proteins from other bacteria. All such similar protein sequences were downloaded, multiple aligned and phylogenetically analyzed with CLC Genomics Workbench (version 4.9) software. Phylogenetic analysis of MazF toxin of X. nematophila revealed that it formed a distinct branch from the toxins of other bacteria as shown in Figure 5 (a). Although, toxin of Photobacterium profundum S99 which showed maximum identity with MazF of X. nematophila was located phylogenetically very far from X. nematophila, it also formed a first distinct cluster with toxin of Vibrio cholerae MZO-3. Toxins from other bacteria such as Rahnella sp. Y9602 and Yersinia mollaretii ATCC 43969 formed their own second cluster where as toxins from Escherichia coli B185, Aggregatibacter actinomycetemcomitans RhAA1 and Thioalkalivibrio thiocyanoxidans ARH 4 lies between first cluster and X. nematophila ATCC 19061. Toxin of Marinobacter aquaeolei VT8 was separated from these two clusters in the very beginning however, toxin of Beggiatoa sp. PS was separated from second cluster just before toxin of X. nematophila ATCC 19061. As far as putative antitoxin MazE from X. nematophila ATCC 19061 is concerned, it was separated phylogenetically far from the antitoxin of Vibrio cholerae 1587 in very beginning as shown in figure 5 (b). However, antitoxin from two Photobacterium profundum S99 species formed a distinct cluster from the putative antitoxin MazE of X. nematophila whereas, antitoxin of Vibrio vulnificus was located between these two.

Phylogenetic analysis of RelB antitoxin from X. nematophila ATCC 19061 revealed that there were two distinct clusters formed by similar antitoxins from other bacteria. Although X. nematophila ATCC 19061 is very close to Photobacterus, its RelB antitoxin forms a unique evolutionary branch. Antitoxin of Escherichia coli H120 was separated in the very beginning from two main clusters however; antitoxin of Photobacterus asymbiotica subsp. asymbiotica ATCC 43949 formed a separate first cluster along with antitoxins from Yersinia enterocolitica subsp. enterocolitica 8081 and Candidatus Regiella insecticola LSR1 which was far apart from antitoxin of X. nematophila ATCC 19061. Antitoxin of Commensalibacter intestini A911 formed a separate branch which was phylogenetically close to antitoxins of X. nematophila ATCC 19061 as well as of Providencia rustigianii DSM 4541. Antitoxin of Erwinia pyrifoliae Ep1/96 lies between antitoxin of Providencia rustigianii DSM 4541 and second cluster formed by antitoxins from Pseudomonas fluorescens WH6, and Shigella dysenteriae 1012 as shown in figure 6 (a). Phylogenetic studies with putative toxin of X. nematophila ATCC 19061 revealed that
it was very close to toxin of *Photobacterium luminescens* subsp. *laumondii* TTO1 and both formed a unique cluster separated from the other two clusters formed by toxins from other bacteria. Toxin of *Candidatus Regiella insecticola* LSR1 was separated in very beginning from the putative toxin of *X. nematophila* ATCC 19061 whereas, cluster formed by toxins from *Escherichia coli* E22 and *Yersinia enterocolitica* subsp. *enterocolitica* 8081 lies at distal end however, branch formed by the toxins from *Providencia rustigianii* DSM 4541 and *Commmensalibacter intestini* A911 located at the proximal to putative toxin of *X. nematophila* ATCC 19061. Toxin of *Pantoea* sp. SL1_M5 was located between toxin of *Commensalibacter intestini* A911 and third cluster formed by toxins from *Salmonella enterica* subsp. *arizonae* serovar 62:z4,z23-- str. RSK2980 and *Aggregatibacter actinomycetemcomitans* RhAA1 as shown in Figure 6 (b).

Phylogenetic analysis of RelE toxin of *Xenorhabdus nematophila* ATCC 19061 revealed that there were three distinct clusters formed by toxins from other bacteria as shown in figure 7 (a). Toxin of *Nitrosomonas europaea* ATCC 19718 was separated in the very beginning from toxin of *Xenorhabdus nematophila* ATCC 19061. Although *Xenorhabdus nematophila* ATCC 19061 is very close to *Photobacteriud* but its toxin formed unique evolutionary branch which was separated from distinct cluster formed by toxins from *Photobacterium luminescens* subsp. *laumondii* TTO1 and *Edwardsiella ictaluri* 93-146. Second cluster formed by toxins from *Erwinia pyrifoliae* and *Pectobacterium atrosepticum* SCR11043 lies proximal to *Xenorhabdus nematophila* ATCC 19061 whereas, cluster formed by toxins from *Escherichia coli* ED1a, *Salmonella enterica* subsp. enterica serovar Enteritidis and *Shigella dysenteriae* 1012 located at distal end whereas, toxin of *Tolumonas auensis* DSM 9187 was located between second and third cluster as unique branch.

Phylogenetic studies with putative antitoxin of *Xenorhabdus nematophila* ATCC 19061 revealed that it was very close to antitoxin of *Escherichia coli* ED1a but were separated from each other as distinct branches of phylogenetic tree. Antitoxin of *Aeromonas caviae* Ae398 was separated in very beginning from putative antitoxin of *Xenorhabdus nematophila* ATCC 19061. Antitoxins from closely related bacterium *Photobacterium luminescens* subsp. *laumondii* TTO1 (NP_929364.1) and *Edwardsiella tarda* ATCC 23685 formed separate cluster which was located proximal to putative antitoxin of *X. nematophila* ATCC 19061, whereas cluster formed by antitoxins from *Erwinia pyrifoliae* DSM 12163 along with *Pectobacterium atrosepticum* SCR11043 formed a cluster which was located at distal position to a branch formed by putative antitoxin of *X. nematophila* ATCC 19061. Antitoxins from *Rahnella* sp. Y9602, *Hae-mophilus influenzae* PittGG and *Tolumonas auensis* DSM 9187 had their own separate branches between antitoxin of *Escherichia coli* ED1a and a cluster formed by antitoxins from *Erwinia pyrifoliae* DSM 12163 and *Pectobacterium atrosepticum* SCR11043 as shown in Figure 7 (b).
arginine, transport of histidine, and its own synthesis[41-43]. These information’s, we inferred that putative maEF toxin-antitoxin module could be transcribed under unfavourable conditions such as physical or chemical stress.

3.5.2. relB Promoter Analysis

294 bp upstream region of relB were analyzed. It contained putative -10 and -35 promoter elements. In upstream region of relB, overlapping RpoD16, LexA, and ArgR binding sequences were observed above -35 regions whereas, RpoD18 binding sequence was overlapping with -10 region. Fis binding sequence was also observed below -10 region as shown in Figure 9. rpoD transcription factor was named as sigma factor-70 (σ^70).

This sigma factor helps RNA polymerase to bind to its specific binding site of gene promoters. Also known as "housekeeping" sigma factor or primary sigma factor, which transcribes the majority of genes in cells and make those proteins that are essential to keep the cell alive. LexA represses the transcription of several genes involved in the cellular response to DNA damage or inhibition of DNA replication[28,29] as well as its own synthesis[30]. This regulation is known as the SOS response[28]. When DNA is damaged, the RecA co-protease binds to single-stranded DNA in the damaged region to form a filament[31,32]. This filament interacts with the LexA dimer to activate its self-cleavage activity by an allosteric mechanism, causing the dissociation of LexA from its DNA targets and the induction of the SOS regulon for repair of the broken DNA[33,34]. The conformational flexibility of unbound LexA is the key element in establishing a coordinated SOS response[35]. LexA is widely distributed in bacteria, and it appears that it emerged from gram-positive group[36]. It shows two domains, the N-terminal domain involved in DNA binding via a helix-turn-helix[37] and the C-terminal domain involved in dimerization and in cleavage activity[38,39]. To repress transcription, LexA blocks the access of RNA polymerase to target promoters[40]. 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 complexed with L-arginine represses the transcription of several genes involved in biosynthesis and transport of arginine, transport of histidine, and its own synthesis[41-43] and activates genes for arginine catabolism[44]. ArgR is also essential for a site-specific recombination reaction that resolves plasmid CoIE1 multimers to monomers and is necessary for plasmid stability[45]. The role of ArgR in this latter reaction may be structural[45]. Fis, "factor for inversion stimulation", is a small DNA-binding and bending protein whose main role appears to be the organization and maintenance of nucleoid structure through direct DNA binding and by modulating gyrase[46,47] and topoisomerase I production[48] as well as regulation of other proteins that modulate the nucleoid structure, such as CRP, HNS, and HU. Fis directly modulates several cellular processes, such as transcription, chromosomal replication, DNA inversion, phage integration/excision, and DNA transposition[49,50]. As a transcriptional regulator, Fis regulates the expression of many genes involved in translation (rRNA and tRNA genes), virulence, biofilm formation, energy metabolism, stress response, central intermediary metabolism, amino acid biosynthesis, transport, cell structure, carbon compound metabolism, amino acid metabolism, nucleotide metabolism, motility and chemotaxis[49-51]. A transcriptome analysis has shown that transcription of approximately 21% of genes is modulated directly or indirectly by Fis[46]. All the above information direct that transcription of RelB operon in X. nematophila is tightly regulated under normal conditions, where transcription occurs only and only under various stress conditions.

3.5.3. relE Promoter Analysis

80 bp upstream region of relE operon were also analysed. It contained putative -10 and -35 promoter elements. In upstream region of relE, RpoH2 overlapping with -35 towards -10 region where as FUR overlaps RpoH2 towards -10 region as shown in Figure 10. RpoH2 is an alternative sigma factor. All organisms react to heat stress with the expression of heat-shock genes in order to cope with accumulating unfolded proteins. In Escherichia coli, heat-shock response is initiated at the transcriptional level by an alternative sigma factor s32 (RpoH). During heat stress, RpoH associates with RNA polymerase (RNAP) and initiates transcription of the heat-shock regulon, coding mainly for molecular chaperones and proteases. The transient induction of heat-shock response is tightly controlled by a regulatory feedback loop.
coupling the amount of heat-shock proteins to the cellular protein folding state. The transcriptional activator FUR, for "Ferric Uptake Regulation," is capable of controlling its own synthesis[52-55] and controls the transcription of genes involved in iron homeostasis[52,54,56-64] and a minor sigma factor that initiates transcription of ferric citrate transport genes in response to the presence of periplasmic iron(III) dicitrate[52,59,65]. This regulator also participates in the regulation of transcription of many other genes involved in different cellular functions: flagellum chemotaxis[66], methionine biosynthesis[66], acid and oxidative stresses[57,67] metal ion stress[66,68-70] resistance to cobalt and nickel[71], the tricarboxylic acid cycle[72,73], glycolysis and gluconeogenesis[57], respiration[66,72,73], porins[73], purine metabolism[66], 2,3-dihydroxybenzoate biosynthesis phage DNA packaging[57], etc. Therefore, it is quite possible that relE operon could be activating during the heat, oxidative and metal ion stress conditions.

Figure 10. 80 bp nucleotide sequence of upstream region of relE operon containing -10, and -30 promoter like elements along with rpoH2 and Fur binding sequences

3.6 Genomic DNA and PCR amplification

Genomic DNA was isolated from the X. nematophila as protocol described in method and shown in figure 10 (A). 530 bp DNA fragment encoding mazF and its putative antitoxin mazE respectively were obtained by PCR amplification using genomic DNA as template as shown in lane 1 figure 11 (B). Whereas, 417 bp DNA fragment encoding relB and its putative toxin were obtained by PCR amplification using genomic DNA as template as shown in lane 2 figure 11 (B) and 526 bp DNA fragment encoding relE and its putative antitoxin were obtained by PCR amplification as shown in lane 3 figure 11 (B).

Figure 11. (A) Genomic DNA isolation from X. nematophila. Lane 1, 2 and 3 genomic DNA at 3µg, 4 µg and 5 µg concentration. (B) PCR amplification of mazF, relB and relE operon using genomic DNA as template. Lane M, 100bp ladder; lane 1, 506 bp mazF operon; lane 2, 416bp relB operon; and lane 3, relE 526bp operon

4. Conclusions

In general, bacteria exposed to various unfavourable conditions possess molecular responses that regulate the degradation of defective or unnecessary proteins and mRNA molecules. One well-described quality control mechanism of Escherichia coli involves toxin-antitoxin (TA) modules. TA modules have been associated with bacterial programmed cell death (PCD) and programmed cell survival (PCS) or persistence[74-76] under various unfavourable conditions which could be physical, chemical or nutrient depleted condition. Since, X. nematophila which protects itself during stressed life cycle inside insect hemolymph or in insect carcass (due to exponential growth of bacterium which leads to nutrient deprivation) therefore, role of toxin-antitoxin (TA) modules in X. nematophila cannot be ruled out. With this background recently sequenced and annotated genome of X. nematophila has been studied bioinformatically for the identification of putative toxin-antitoxin modules in its genome. Three such modules mazF, relB and relE has been identified and studied in detail. Genomic organization revealed that mazF toxin module is located in "XCN_1operon0855" operon in a downstream position to a hypothetical protein. Protein-protein blast of MazF and hypothetical protein showed their similarity with MazF toxin from Photobacterium profundum SS9 (Accession no. YP_130852.1) and MazE antitoxin protein from vibrio cholerae 1587 (Accession no. ZP_01950607.1) respectively. Therefore, hypothetical protein from X. nematophila was designated as putative MazE antitoxin. Difference in their individual pI’s (MazF toxin: pI 8.73 and putative MazE antitoxin: pI 4.41) will tend them to form complex in physiological conditions. Therefore, we can presume that in X. nematophila mazF and mazE will form a putative mazEF toxin-antitoxin module. From the predicted promoter analysis, due to the presence of RpoH3 and CpxrR, we inferred that transcription of mazF and putative mazE will occur either under different environmental conditions such as high temperature or envelope stress. Similarly, second operon “XCN_1operon0367” encodes four genes in complement orientation. First gene of this operon encodes for RelB antitoxin which showed similarity with RelB antitoxin protein from Photorhabdus luminescense subs laumondii TT 01 (Accession no. NP_927617.1) where as second gene encode putative toxin part of Qin prophase toxin-antitoxin component system. Protein-protein blast with toxin protein showed its 81% identity with hypothetical protein YE0510A from Yersinia enterocolitica subsp enterocolitica 8081 (Accession no YP_001004871.1) and 71% identity with RelE toxin protein from Photorhabdus asymbiota subsp. asymbiota ATCC 43949. In this case also difference in individual pI (Putative toxin: pI 9.52 and RelB antitoxin: pI 4.41) will tend them to form complex in physiological conditions. From predicted promoter analysis, presence of RpoD3, LexA, ArgR and Fis indicates that transcription of relB and its putative toxin will occur either under SOS conditions and might be tightly regulated. Therefore, we can presume that in
X. nematophila gene encoding putative toxin and relB will form a novel toxin-antitoxin module. However, in other bacteria toxin component of this kind of module is denoted as relE[17,19]. Interestingly, in X. nematophila genome relE gene was located separately at operon “XCN_10operon0557” which encode two different genes in complement orientation. Protein-protein blast with putative stability protein StbD, encoded by first gene showed a conserved domain at its N’ terminal belonged to PhdYeFM antitoxin superfamily. Therefore, putative StbD from X. nematophila is similar to Stability protein StbD (Antitoxin) from Escherichia coli ED1a (Accession no. YP_002418779.1). Protein-protein blast with protein encoded by second gene relE from X. nematophila showed 73% identity with stability protein StbE from Edwardsiella ictaluri 93-146 (YP_002933420.1) and 71% identity with toxin component of toxin-antitoxin system of RelE family from Edwardsiella tarda ATCC 23685 (Accession no ZP_06714851.1). Therefore, putative StbD antitoxin will form a pair with RelB toxin, which could be a novel toxin-antitoxin module in X. nematophila. In this case due to the differences in individual p’l’s Putative RelE toxin: pI 9.71 and putative antitoxin StbD: pI 4.17 they will form complex in physiological conditions. Predicted promoter revealed the presence of signature sequences for the binding of rpoH, which indicate transcription from this promoter under heat shock conditions. Apart from rpoH presence of FUR signature sequences revealed its transcription under iron regulated conditions. Therefore, in the end we conclude that in X. nematophila we have identified three novel toxin-antitoxin modules from its genome and their existence is proved with the assistance of wet lab experiments.

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