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Homology Modeling and *in silico*Structural Annotation of the Toxin-Antitoxin Systems in *Pseudomonas aeruginosa*

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Abstract

***Pseudomonas aeruginosa* is a common Gram-negative, rod-shaped bacterium that can cause disease in humans & animals. A species of considerable medical importance, *P. aeruginosa* is a prototypical "multidrug resistant (MDR) pathogen" recognised for its ubiquity, its intrinsically advanced antibiotic tolerance mechanisms. *P. aeruginosa* is associated with serious illnesses – especially hospital acquired infections, cystic fibrosis and traumatic burns in human. Recent studies suggested that toxin-antitoxin systems play a key role in the tolerance.Inthe present study, we found five toxin-antitoxin systems in the genome of *P. aeruginosa*, out of which two are located on plasmid (pNOR-200)& three are located on chromosome.We applied homology modeling techniques to study the sequence & structure relationships of toxin-antitoxin systems. The study reveals the presence of toxin-antitoxin system in *P. aeruginosa* that interact with RNA and proteins in the cellular environment and halt normal cellular process by inhibiting the molecules involve in translation, transcription and other important metabolic pathways that leads to multidrug tolerance.**

Keywords:*P. aeruginosa,* genome, multidrug tolerance,toxin-antitoxin system, homology modelling, structural annotation.

Introduction

P. aeruginosa is a multidrug resistant pathogen recognized for its ubiquity, its intrinsically advanced antibiotic resistance mechanisms, and its association with serious illnesses in hospital acquired infections such as ventilator-associated pneumonia and various sepsis syndromes [1]. The organism is considered opportunistic bacterial pathogen and cause cystic fibrosis and traumatic burns. It is also found generally in the immune-compromised host but can also infect the immune-competent host [2]. The treatment of *P. aeruginosa* infections can be difficult due to its natural resistance to antibiotics [3]. In general, bacteria exposed to a plethora of environments possess molecular responses that regulate the degradation of unnecessary proteins and mRNA molecules [4]. These unnecessary proteins and 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 [5]. This control mechanism consists of two components together known as toxin-antitoxin (TA) modules [6,7].These TA systems form a non toxic complex in a favourable condition[8,9] while on the other hand, it's modulate the global levels of transcription and translation during exposure of antibiotic stress due to over expression of toxic component.Generally in prokaryote TA system 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].In addition, Type III encodes a small RNA antitoxin that combines

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with and neutralise toxin protein[13]. Toxin-antitoxin (TA) systems are broadly distributed in prokaryotes in multiple copies[14,15]and all TA operons are auto-regulated at the level of transcription by the antitoxinsin which antitoxin component neutralizes its cognate toxin[16]. For example, the *relBE* and *higBA* 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].Since *E. coli* TA systems are involved in the survival of *E.coli* under stress conditions therefore, it could be possible that similar kind of survival mechanism exist in *P. aeruginosa.*However, in *E. coli* these systems are well studied but in *P. aeruginosa* they are not well studied, a very less information is available of sequence-structure-function relationships of TA systems in *P. aeruginosa*. Therefore, it is necessary to study toxin-antitoxin (TA) systems of *P. aeruginosa*in detail. In this study we have studied three putative TA systems such as *relBE, higBA* and*parDE* in the genome of *P. aeruginosa.*

Materials and methods

1. ***Genetic organization, Sequence retrieval & Data sets***

Initially full lengths of five pairs of toxin antitoxin amino acid sequences for the *P. aeruginosa* proteins were retrieved from the GenPept, a protein sequence database, of National Centre for Biotechnology Information (NCBI). All toxin-antitoxin proteins were inspected with NCBI (http://www.ncbi.nlm.nih.gov/)protein database for proteins [21]. Thenthe all toxin-antitoxin sequences werestoredasanFASTAformatfor furtheranalysis.

2. ***Analysis of physicochemical properties***

The physiologicaland chemical properties ofthetargetedproteinsequences were analyzed by the Expasy tool ProtParam(http://web.expasy.org/protparam/) [22]. The propertiesincludingaliphaticindex(AI),GRAVY(grandaverage ofhydropathy), extinction coefficients,isoelectricpoint (pI),and molecularweightwereanalyzedusingthistool.

3. ***Sub cellular localization prediction***

Determinationofthesub cellular localization iscrucialfor understanding proteinfunctionandisalsovitalforthe genome analysis. Predictionofsub cellularlocalizationof*P. aeruginosa*was carried out by CELLO v.2.5 which is a multiclass support vector machine classification system [23,24].

4. ***Comparative proteomics***

TheBLASTPprogramofNCBIdatabase(http://www.ncbi.nlm.nih.gov/)wasused forsearchingthe similaritywithtoxin-antitoxinproteins of *P. aeruginosa*againstthenon-redundantdatabasewithdefaultparameter[25].Then theTAproteins wereanalyzedfor thepresence ofconserved domains basedon sequencesimilaritysearch with closeorthologousfamilymembers.Forthispurposethreedifferent bioinformaticstoolsanddatabases includingProteinsFamilies Database(Pfam),NCBIConservedDomainsDatabase(NCBI-CDD) andSUPERFAMILY wereused[26-28].TheSUPERFAMILYannotationisbasedon acollectionofhidden Markovmodels,whichrepresentstructural protein domainsattheSCOPsuperfamilylevel.Theannotation isproduced byscanningprotein sequencesfromovercompletely sequencedgenomesagainstthehiddenMarkovmodels. Finally, the phylogeny analysis was obtained by CLC Sequence Viewer v7.0.2 (http://www.clcbio.com/) for better understanding about their comparative evolution.

5. ***Multiplesequencealignmentandsecondary structure analysis***

The amino acid sequence of *P. aeruginosa*toxin-antitoxinproteins and their all identified homologs were subjected to multiple sequence alignment (MSA) for recognizing the conserved residues and patterns using CLASTAL-W program implemented in MEGA-7 program. The conserved residues were noted down for further analysis. Toget

structuralandfunctionalinsightsthroughthesequence comparison,acombinedapproachwas implemented.Wefetched several annotatedantitoxin protein sequences of*P. aeruginosa*speciesfromtheNCBIProteindatabase andtheirmultiplesequence alignment(MSA)withthetargetedproteinwereobtainedthrough theBioEditbiologicalsequencealignmenteditor [29].Afterthat, thesealignedsequenceswereusedfortheprediction ofthesecondary structure byusingEsPript3.0[30].

6. ***Homology prediction & Homologymodelling***

Homology prediction technique was used to identify the existence of similar regions in the sequences of different species. The homology prediction program, BLASTp, was used to predict the bacterial homologs of *P. aeruginosa*proteins. The amino acid sequences of all the identified homologs were downloaded in FASTA format and used for further analysis in comparison with the amino acid sequence of *P. aeruginosa*toxin-antitoxinproteins. Homologymodellingwas usedtodeterminethethreedimensionalstructureof *P. aeruginosa*.A BLASTpsearchwith defaultparameterswas performedagainsttheBrookhavenProtein DataBank(PDB)tofindsuitable templatesforhomologymodeling.Thetertiarystructurewas predictedbyMODELLER throughHHpredtoolsoftheMaxPlanckInstituteforDevelopment Biology[31-34].

Results and discussion

The physiological and chemical properties of the toxin-antitoxin proteins were assessed by ProtParam tool (Table 1). These are including aliphatic index (AI), instability index (II), pI, extinction coefficient and average hydropathicity. All of these calculations are related to the stability of the protein and that are correlates with proper function [35]. The subcellular localization of all toxin-antitoxin proteins was predicted by CELLO v.2.5. All proteinswere predicted to have their localization in cytoplasm except ParD protein which was predictedin periplasm.

Table 1: PROTPARAM tool analysis results for the targeted TA proteins

S. No.	A/T Protein	No. of amino acids	Mw	Pi	(asp+glu)/ (arg+lys)	Ext. Coefficient	Aliphatic Index (AI)	Instability Index (II)	GRAVY*	Sub cellular Localization Prediction
1.	TOX1	68	7559.08	4.14	17/6	13980	57.50	61.52	-1.052	Cytoplasmic
	TOX2	57	5900.73	5.12	8/6	5500	104.91	28.39	0.284	Cytoplasmic
2.	T/AT1	83	8767.15	9.69	8/11	5500	100.00	27.57	-0.060	Cytoplasmic
	T/AT2	131	13700.78	4.33	16/9	14105	121.45	15.98	0.616	Cytoplasmic
3.	RelB	93	10462.00	9.45	11/14	13980	103.76	49.74	-0.217	Cytoplasmic
	RelE	75	8568.64	5.48	12/9	13980	100.40	59.02	-0.397	Cytoplasmic
4.	HigA	101	11184.65	4.86	14/8	9970	91.98	45.36	-0.175	Cytoplasmic
	HigB	92	10646.19	7.87	12/13	18115	86.96	60.19	-0.421	Cytoplasmic
5.	parD	83	9103.27	4.96	13/10	6990	88.31	34.65	-0.512	Periplasmic
	parE	103	11715.73	9.13	13/16	11585	94.95	36.48	-0.098	Cytoplasmic

* Grand average of hydropathicity

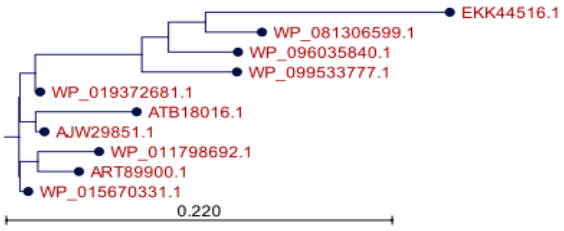
The BLASTp search against non-redundant database showed a higher homology with toxin-antitoxin proteins from different species (table 2). Phylogenetic analysis was depicted in the Figure 1-2, by using the same data. The output of the tree with the true distance guided us about the evolutionary similarity of different toxin-antitoxin genes as well as proteins.Numerous web tools were used to search the conserved domains and potential function of TA proteins. Based on consensus predictions made by Pfam, NCBI-CDD and

SUPERFAMILY suggested that the protein belong to different superfamilies superfamily domains and are currently classified as type II toxin- antitoxin systems. Pfam server predicted the e-values of all TA type II toxin-antitoxin systems (table-2).

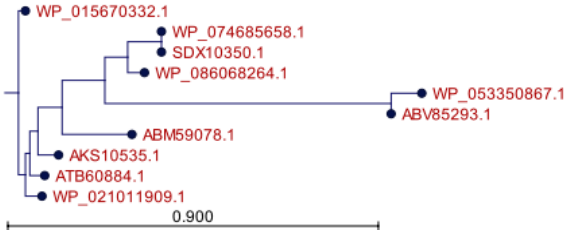
Table 2: Similar toxin-antitoxin proteins obtained from non-redundant database.

TA system	**Plasmid (pNOR-200) toxin-antitoxin systems**					
	Organism	**Protein name**	**Identity**	**Score**	**E-value**	**Entry name**
Tox-1 Antitoxin	*Achromobacter*sp.	antitoxin	99%	137	4e-41	AJW29851.1
	Xanthomonasfuscans	hypothetical	86%	119	4e-34	WP_096035840.1
	Escherichia coli 8.0569	transcriptional regulator	71%	73.9	4e-15	EKK44516.1
Tox-2 toxin	*Klebsiellapneumoniae*	hypothetical protein	88%	80.9	5e-19	AKS10535.1
	Nitrosomonaseutropha	DNA-damage-protein J	67%	71.2	3e-15	SDX10350.1
	Rickettsia montanensis	type II TA system	42%	35.0	0.99	WP_014409734.1
T-AT1 Antitoxin	*Bordetellabronchiseptica*	antitoxin	100%	166	6e-52	KCV24973.1
	Xanthomonascitri	transcriptional regulator	99%	165	1e-51	WP_033483272.1
	*Thiomonas*sp. FB-Cd		95%	161	3e-50	WP_031406255.1
T-AT2 toxin	*Xanthomonasgardneri*	PIN domain-containing protein	99%	253	3e-85	WP_046934155.1
	*Thauera*sp. K11		97%	249	9e-84	WP_096453044.1
	Klebsiellapneumoniae		98%	249	2e-83	WP_085903587.1
	Chromosomal toxin-antitoxin systems					
relE antitoxin	*Pseudomonas* sp.	ribbon-helix-helix (RHH)*****	99%	150	7e-46	KJJ21186.1
	PA0125		99%	149	1e-45	AAT50665.1
	P. jinjuensis		86%	132	5e-39	WP_084314647.1
RelB (c) toxin	PA0124	toxin	99%	187	4e-60	AAT49350.1
	P. delhiensis		71%	135	8e-40	WP_089392789.1
	P. knackmussii		68%	135	2e-39	WP_043248044.1
higB antitoxin	*P. xanthomarina*	plasmid maintenance system killer protein	88%	175	2e-55	WP_065983611.1
	Pseudomonas sp.		86%	173	9e-55	WP_015478854.1
	P. kunmingensis		88%	173	1e-54	WP_090522284.1
higA toxin	PA4674	addiction module	96%	202	8e-66	AAT49451.1
	Pseudomonas borbori		87%	186	1e-59	WP_090505068.1
	P. xanthomarina		81%	176	1e-55	WP_073303647.1
ParD antitoxin	uncultured bacterium	partitioning protein	100%	167	2e-52	AFR44061.1
	Klebsiellapneumoniae	antitoxin	99%	166	4e-52	WP_087638928.1
	*Photorhabdus*sp.		93%	160	8e-50	WP_088374051.1
ParE toxin	*Klebsiellapneumoniae*	toxin	95%	201	2e-65	WP_087638929.1
	Neorhizobiumgalegae		83%	181	2e-57	WP_046627180.1
	*Roseibium*sp. TrichSKD4		83%	181	3e-57	WP_009469534.1

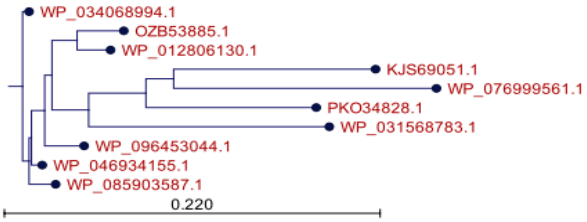
*Predicted transcriptional regulator



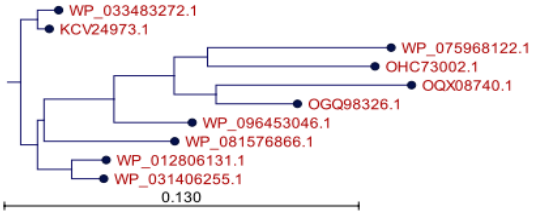
(1a) Tox1 antitoxin



(1b) Tox2 toxin



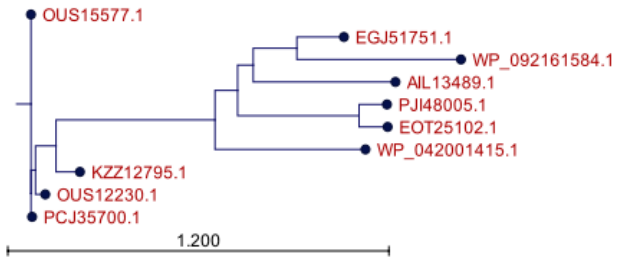
(2a) T-AT1 antitoxin



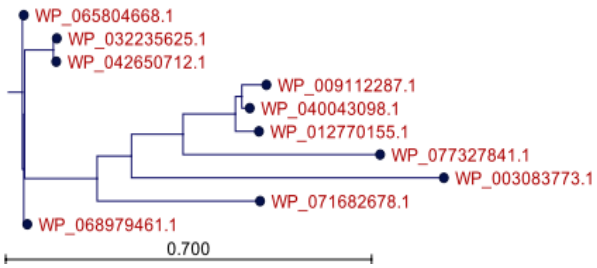
(2b) T-AT2 antitoxin/ toxin

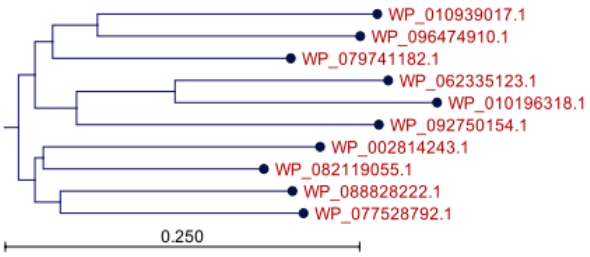
Fig. 1: Phylogenetic analysis of pNOR-2000 plasmid based Tox1/Tox2 and T-AT1/T-AT2 antitoxin/ toxin proteins. Phylogenetic tree constructed based on the amino acid sequence of toxins from other bacteria Sequence were aligned by using CLC Genomics Workbench (version 7.6.2) software.

(3a) RelE

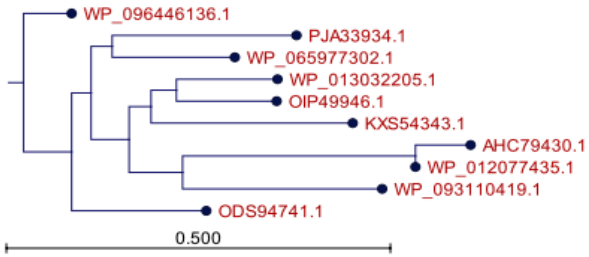


(3b) RelB

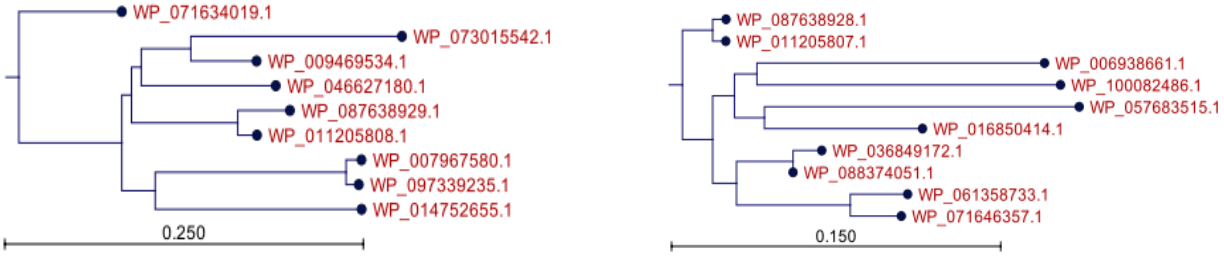




(4a)HigB



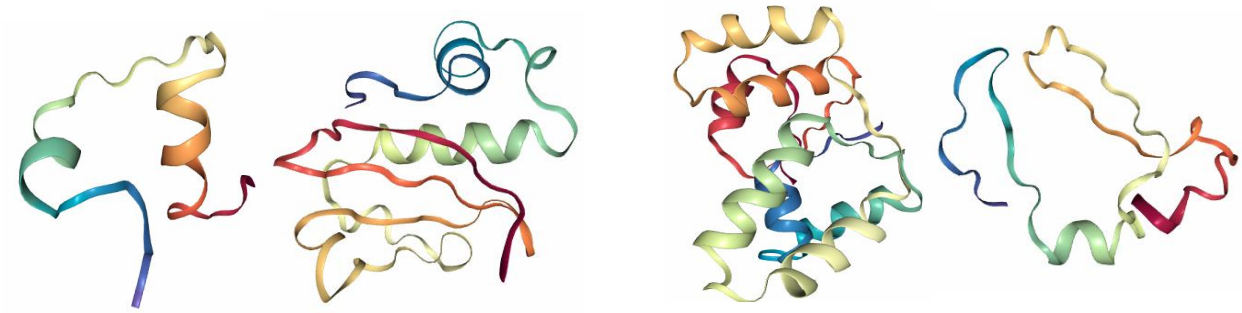
(4b)HigA



(5a) ParD **(5b)ParE**

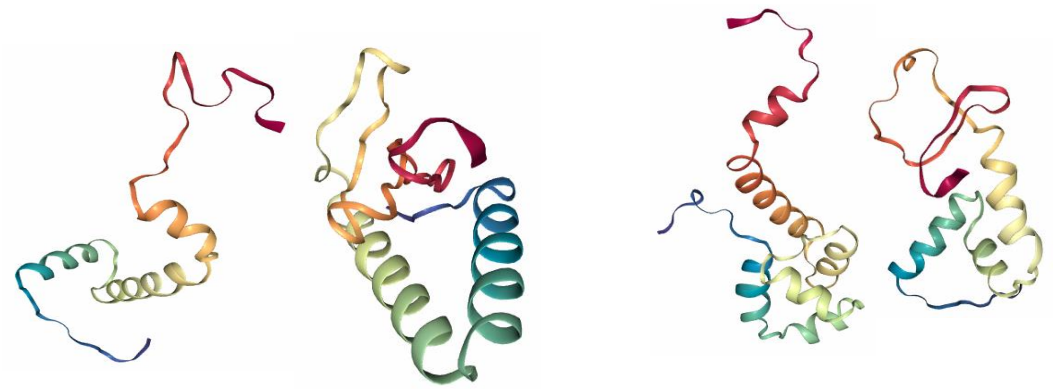
Fig. 2: Phylogenetic analysis of chromosomalRelBE, HigBA andParDE toxin/antitoxin proteins in *P. aeruginosa*. Phylogenetic tree constructed based on the amino acid sequence of toxins from other bacteria Sequence were aligned by using CLC Genomics Workbench (version 7.6.2) software.

MSAofdifferent toxin-antitoxin proteins of*P. aeruginosa*aredepicted in the figure6-7.The secondarystructureoftheproteinsare alsoincludedinthisfigure and showedthattheyaremostlyconservedthroughoutt healignment alongwiththedifferent toxin-antitoxin protein of other species.

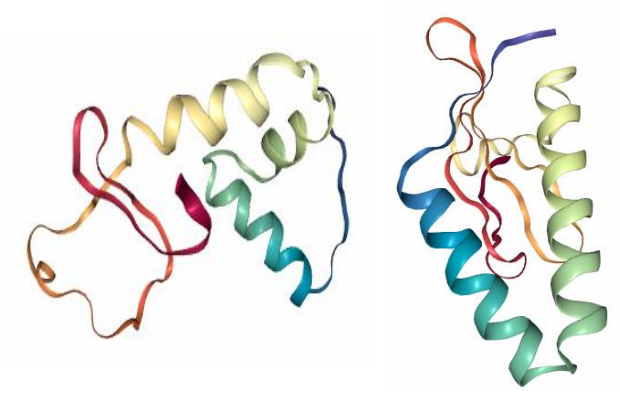


(6a)Tox1/Tox2 **(6b) T-AT1/T-AT2**

Fig3. Predicted three-dimensional model structure of pNOR-2000 plasmid based **Tox1/Tox2 & T-AT1/T-AT2 antitoxin/toxin proteins. The N-terminal end started with β sheet (Blue) and the C-terminal end is coiled structure (Red).**



(7a)RelB/RelE **(7b)HigB/HigA**



(7c)ParD/ParE

Fig 4. Predicted three-dimensional model structure of chromosomal based RelBE, HigBE&ParDE antitoxin/toxin proteins. The N-terminal end started with β sheet (Blue) and the C-terminal end is coiled structure (Red).

Homologymodellingis anindispensablepartof thestructural genomicsintherecentpastforthecomparativemodellingofvarious unknownstructurewithenormoustools[36,37].Int hisstudy we predicted& constructedthe comparativethree-dimensional modelofTA proteinsthroughhomologymodelling.Thatisdepicted inthefigure3-4.

Discussion & Conclusion

Homologymodelingandcomparativeproteomics approaches were usedtopredictthethree-dimensional structureforthe toxin-antitoxin system in *P. aeruginosa*. Five toxin-antitoxin systems (Tox1/Tox2, T-AT1/T-AT2, RelBE, HigBA and ParDE) were analyzed; out of which two (Tox1/Tox2 and T-AT1/T-AT2) were located on pNOR-200 plasmid and three (RelBE, HigBA and ParDE) were located on chromosome of *P. aeruginosa*.The physiological and chemical properties suggested that all toxin-antitoxin proteins are cytoplasmic except ParD antitoxin protein which is periplasmic. The BLASTp search resulted that these proteins have higher homology with toxin-antitoxin proteins from different species. Phylogenetic analysis also showed evolutionary similar with different toxin-antitoxin genes and proteins. MSAofdifferent toxin-antitoxin proteins of*P. aeruginosa*aredepicted the secondarystructureoftheproteinsaremostlyconservedwiththedifferent toxin-antitoxin protein of other species. All the above findings suggested that the all toxin-antitoxin proteins are type II TA system. Hopefully, this comprehensive study on this track mightproduce some breakthrough leads for impending research. The further functional annotation of these toxin-antitoxin proteins is required to give clear picture of structure-function relationship.

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