Computational identification and evolutionary analysis of toxins in Mosquitocidal *Bacillus thuringiensis* strain S2160-1

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Abstract

Mosquitocidal *Bacillus thuringiensis* (*Bt*) strain S2160-1 was proposed to be an alternative to *Bacillus thuringiensis* subsp. *israelensis* (*Bti*). Discovering and validating a toxic gene by experimentation was a complex and time-consuming task, which can benefit from high-throughput sequencing analysis. In this research, we predicted and identified toxic proteins in the strain S2160-1 based on the draft whole genome sequence data. Through a local BLASP, 46 putative toxins were identified in S2160-1 genome, by searching against a customized *B. thuringiensis* toxin proteins database containing 653 protein or peptide sequences retrieved from public accessible resources and PCR/clone results in our laboratory (e value = $1e-5$). These putative toxins consist of 42 to 1216 amino acids. The molecular weights are ranged from 4.86 to 137.28 kDa. The isoelectric point of these candidate toxins varied from 4.3 to 10.06, and 16 out of which had a pH greater than 7.0. The analysis of tertiary structure and PFAM domain showed that 12 potential plasmid toxins may share higher similarity (9/12 QMEAN4 score > 0.3) with known *Bt* toxins. In addition, functional annotation indicated that these 12 potential toxins were involved in “sporulation resulting in formation of a cellular spore” and “toxin activity”. Moreover, multiple alignment and phylogenetic analysis were carried out to elucidate the evolutionary relationship among 101 known crystal or toxin proteins from public database and them with MEGA 6.0. It indicated that PS2160P2_1 and PS2160P2_153 may be potential Cry4-like toxins in *Bt* S2160-1. This research may lay the foundation for future functional analysis of *Bt* S2160-1 toxin proteins to reveal their biological roles.

Keywords *Bacillus thuringiensis* · Crystal proteins · Phylogenetic analysis · Evolution · Genome

Abbreviations

CTGs Candidate toxin genes

TER-CTGs Candidate toxin genes with tertiary structure

CD-CTGs Candidate toxin genes with conserved domain

GO-CTGs Candidate toxin genes with GO annotations

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Introduction

Bacillus thuringiensis is an aerobic Gram-positive spore-forming entomopathogenic bacterium. It can be isolated from a wide variety of environmental sources such as soil,

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water, dead insects, plant surfaces and insect feces (Federici 1999). In the unfavorable growth conditions, *B. thuringiensis* might generate spores, and most of the bacteria are accompanied by the production of a crystalline protein, which was called parasporal crystal or δ -endotoxin. To different insect larvae, the parasporal crystals have different pathogenicity, and some of them such as parasporins were reported to be able to kill cancer cells (Nagata et al. 2009; Palma et al. 2014; Poornima et al. 2010; Schnepf et al. 1998). In the families of the delta endotoxins produced by *B. thuringiensis*, Cry toxins and Cyt toxins were the most widely studied families (Jouzani et al. 2017). In past few decades, more than 700 genes encoding Cry crystal proteins were identified and characterized in *Bt* Toxin Nomenclature (http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/) (Crickmore and Feitelson 2016). As the insecticidal crystal protein had specific insecticidal activity to target insects but safe to non-target organisms, it was widely used for controlling insect pests in agriculture and forestry as a biological control agent. However, long term use of the same *Bt* insecticide or single cropping of insect-resistant transgenic crop will lead to insect resistance to *Bt* toxin protein, therefore, it is very urgent to constantly explore new *Bt* strains and novel toxins for the pest control (Bellile and Vonesh 2016; Tabashnik et al. 2013). Cry1Ab, which was identified and cloned by molecular biological approaches from *B. thuringiensis* strain SY49-1, was reported with insecticidal activity, and the insecticidal activity had been improved by the technique of site-directed mutagenesis. Moreover, a new *B. thuringiensis* with multiple insecticidal effects showed previously was constructed by means of genetic engineering, binding transfer, etc. (Azizoglu et al. 2016; Zhang et al. 2012). However, these methods of driftlessly mining and modifying new toxin molecules need doing a lot of experiments and consuming a large number of manpower and material

resources, which is time-consuming, costly and inefficient. In an improvement over conventional experiment screening, next generation sequencing (NGS) has been used for identification of new toxins from *Bt* strains. To narrow the scope of candidate toxins by screening test and improve the identification efficiency of toxins, here, a bioinformatics method was proposed to computationally identify toxins in whole genome based on sequence similarity and conserved functional domains of toxins.

Bt S2160-1 with acute mosquitocidal activity was isolated from soil samples from Dawangling Forest Nature Reserve (Guangxi, China) and proposed as an alternative to *Bti* for mosquitocidal products (Zhang et al. 2012). In 2013, the whole genome sequencing of *Bt* S2160-1 have been done and its draft genome was assembled that contains three replicas, a nucleoid genome in length of 5.46 Mb and two plasmid genomes with the lengths of 544 and 309 kb (Zhong et al. 2013). Cry4Cb3 with mosquitocidal activity against *Culex quinquefasciatus* was identified with mass spectrometry (Zhang et al. 2014). Nevertheless, there have been no systematic studies of the toxic protein of the *Bt* strain S2160-1. With the completion of whole genome sequencing of *Bt* S2160-1, we had opportunity to investigate a genome-wide analysis of crystal proteins.

In this study, an in silico search of *Bt* 2160-1 genome database was conducted to identify crystal proteins. We applied multiple tools and programs to conduct in-depth analysis of each toxin proteins, including the physiological and biochemical properties, sequence structures, functional domains. Then, the GO function annotation was proceeded to study the potential functions of crystal toxins. Furthermore, a thorough comparative analysis of *Bt* 2160-1 toxin proteins to those from other *Bt* strains was performed (Fig. 1). Our results presented here may provide a subset of potential candidate toxins for

Fig. 1 The workflow of computational identification and evolution analysis for toxic proteins in *Bt* S2160-1

future engineering modifications of mosquitocidal activity characteristics in *Bt* S2160-1.

Materials and methods

Sequence retrieval and construction of the local protein database for toxins

Bacillus thuringiensis Toxin Nomenclature (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/intro.html) (Crickmore and Feitelson 2016): The *Bacillus thuringiensis* delta endotoxin nomenclature committee was set up in 1993 to update the nomenclature originally devised in 1989 by Hofte and Whitele. The corresponding protein sequence of delta endotoxins within nomenclature was downloaded from NCBI according to the GI and accession numbers (up to July, 2016). In addition, sequences cloned by our lab that have not submitted to public database were also included. Finally, 653 toxin proteins were selected to create a local toxin protein database. To preliminarily filter out the potential toxins, the peptide sequences of *Bt* S2160-1 were used as query sequences to search against the local database via local BLASTP (e value = $1e-5$).

Sequence analysis of potential toxins in *Bt* strain S2160-1

To make a preliminary judgment on whether a candidate protein meets the physicochemical parameters of toxic proteins, the theoretical isoelectric point (pI), molecular weight (MW) and amino acid composition of these candidate toxins in *Bt* strain S2160-1 were calculated by ProtParam (<http://web.expasy.org/protparam/>) (Wilkins et al. 1999). SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) was used to predict the secondary protein structure of these candidate toxins in S2160-1 with default parameters: Window width: 17, Similarity threshold: 8, Number of states: 4. Then, protein sequences of these candidate toxins were uploaded to SWISS-MODEL (<http://swissmodel.expasy.org/>) (Biasini et al. 2014; Kiefer et al. 2009) to build the protein tertiary structure homology modelling. To explore the domain organization of these candidate toxins, PFAM (<http://pfam.xfam.org/>) (Finn et al. 2016) was performed. Moreover, the functional annotation of these potential toxins was performed using Blast2GO program (Conesa and Gotz 2008), including three parts: biological process, molecular function and cellular component.

Phylogenetic and sequence alignment analysis of hypothetical toxins in *Bt* S2160-1

Multiple sequence alignments of hypothetical toxin protein sequences of *Bt* S2160-1 and other known *Bt* toxins

from UniprotKB were performed using ClustalW tool of MEGA 6.0 (Molecular Evolutionary Genetics Analysis) with default parameters. Phylogenetic tree was constructed using maximum likelihood method with 100 replicates of bootstrap analysis in MEGA 6.0 (Tamura et al. 2013). The phylogenetic tree visualization and later landscaping is made with iTOL (Interactive Tree of Life) online tool (Letunic and Bork 2016).

Results

Computational identification of *Bt* strain S2160-1 candidate toxins

A customized local protein database was constructed with 621 sequences manually maintained by Crickmore et al., and 32 genes cloned by our research team but not registered in public databases (up to 2016-07). BLASTP (version 2.2.28) was performed locally to identify potential toxic proteins (e value = 0.00001) in *Bt* strain S2160-1. Each blast hit was assigned to the corresponding group incorporating four ranks as described by Crickmore et al., in which the boundaries represent approximately 95, 78, and 45% sequence identity (Crickmore et al. 1998). Finally, we identified 46 candidate toxin genes (marked as CTGs), consisting of 6 potential vip, 37 cry and 3 cry/cyt genes (Supplementary Materials Table S1). For the sake of convenience, each gene was named as following: If genes located on circular chromosome, they were named like *ns2160_gene_id_2* and the corresponding encoding protein was named like NS2160_2. Otherwise, genes mapped on plasmids were named as *pS2160p1_gene_id_2* and the corresponding proteins were named as PS2160P1_2.

Computation of physical and chemical parameters for the candidate toxic proteins

As described previously, we collected 653 toxins, which were currently known or kept only in our lab. Results of ProtParam indicated that the conserved *Bt* toxin proteins held the following physical and chemical properties: (1) count of amino acids ranged from 78 to 1522, (2) MW ranged from 8.59 to 170.47 kDa, and (3) pI ranged from 4.07 to 9.79. To determine whether these 46 candidate toxic proteins had the similar features as the conserved *Bt* toxin proteins, we computed the physical and chemical parameters for all of them. It showed that protein sequences of these CTGs contained 42 (NS2160_814) ~ 1216 (PS2160P2_1) amino acids (aa) (Supplementary Materials Table S2). The MW of them ranged from 4.86 to 137.28 kDa, and the pI value ranged from 4.3 (PS2160P1_40) to 10.06 (PS2160P2_241).

It seemed that the physical and chemical properties of these CTGs were similar to that of the conserved *Bt* toxin proteins.

Structure and conserved motif analysis of the candidate toxic proteins

To investigate whether proteins coded by these 46 CTGs in *Bt* S2160-1 had homological model with known *Bt* insecticidal crystal protein, SWISS-MODEL (<https://www.swissmodel.expasy.org/>) (Biasini et al. 2014; Kiefer et al. 2009) was used to predict the protein tertiary structures. It showed that 14 of 46 candidate proteins had at least one matching result with the three-tier models of known *Bt* insecticidal crystal proteins (Supplementary Materials Table S3). The genes coding these 14 proteins were designed as candidate toxin genes with tertiary structure (marked as TER-CTGs).

Typically, the active conformation of *Bt* δ -endotoxins was composed of three distinct structural domains: Endotoxin_N, Endotoxin_M and Delta_endotoxin_C (Bravo et al. 2007; Galitsky et al. 2001; Palma et al. 2014), which play important roles in entomopathogenicity, membrane insertion and pore formation, receptor binding, etc. Some crystal proteins do not have typical endotoxin domains, but contain RICIN or Toxin_10 domain (Itsko et al. 2005; Kelker et al. 2014). The distributions of typical endotoxin domains in these 46 candidate toxin proteins were investigated with PFAM (<http://pfam.xfam.org/>) (Finn et al. 2016). Fortunately, we found that (1) NS2160_5341, PS2160P1_21, PS2160P1_40, PS2160P1_49 and PS2160P2_146 had δ _endotoxin_C domain, (2) PS2160P1_2, PS2160P1_20, PS2160P1_39, PS2160P1_50, PS2160P1_54, PS2160P2_1, PS2160P2_134, PS2160P2_147, PS2160P2_157, and PS2160P2_246 had Endotoxin_N, Endotoxin_M and Delta_endotoxin_C domains (Supplementary Materials Table S3). As far as is known, most toxic genes if not all Cry and Cyt protein-coding genes were mapped on plasmids present in *Bt*, not on the chromosome. In our results, all of them except NS2160_5341 were located on plasmids. So, we marked them as candidate toxin genes with conserved domains (CD-CTGs).

GO functional annotation for CTGs

The sequence diversity of *Bt* strain S2160-1 toxins indicated that these toxin proteins may be involved in multiple biological processes related to pathogen. To understand the biological processes associated with *Bt* strain S2160-1 toxins, we performed GO annotation of the candidate toxins. Finally, 31 of 46 CTGs were annotated to GO terms (marked as GO-CTGs). The results are shown in Supplementary Materials Table S4. The majority of toxic protein-coding CTGs located on plasmids were involved in binding (25 CTGs), cellular process (19 CTGs), multi-organism process (17 CTGs),

interspecies interaction between organisms (17 CTGs), sporulation (14 CTGs), development process (14 CTGs) and signaling receptor binding (12 CTGs). Meanwhile, nine plasmid proteins and four chromosome proteins participated in metabolic process, organic substance metabolic process, nitrogen substance metabolic process, cellular metabolic process and primary metabolic process. From which, it was reasonable to speculate that these 13 proteins may not true toxins in *Bt* strain S2160-1. In addition, three proteins (NS2160_3788, NS2160_3497, NS2160-3703) located on S2160-1 chromosome functioned in biosynthetic process, small molecule metabolic process and oxidation–reduction process. Based on the GO functional annotation analysis, we found that CTGs of *Bt* strain S2160-1 could function in the development process of sporulation, signaling receptor binding and interspecies interaction between organisms.

Summarily, we identified 46 potential toxins in *Bt* strain S2160-1 via BLASTP. Combining the analysis of tertiary structure, conserved domains and GO annotation of these candidate toxins, 12 plasmid genes of 46 CTGs from S2160-1 were more likely to be a *Bt* toxin (Fig. 2a). From the functional analysis of these CTGs, the intersection of CDS-CTGs, TER-CTGs and GO-CTGs was assigned into GO:0090729 (MF: toxin activity), GO:005102 (MF: signaling receptor binding), GO:0009405 (BP: pathogenesis) and GO:030435 (BP: sporulation resulting in formation of a cellular spore) (Fig. 2b). Moreover, PS2160P1_49 and PS2160P1_50 were annotated as mosquitocidal toxin-like protein, which was consistent with mosquitocidal activity of *Bt* S2160-1 (Fang and Zhang 2012) (Table 1).

Phylogenetic analysis of the toxin genes in 12 *Bt* strains

To investigate the phylogenetic relationships of toxins between *Bt* S2160-1 and that of other *Bt* strains, a phylogenetic tree was constructed with maximum likelihood (ML) method for 100 bootstraps in MEGA 6.0 (Tamura et al. 2013). The phylogenetic tree included the above 12 computational predicted toxic proteins from *Bt* S2160-1 and 101 known toxins from UniprotKB (Fig. 3). From the evolution tree, most of the computational predicted toxic proteins (9/12) from S2160-1 had higher similarity (bootstrap value > 0.68) with toxins from *Bacillus thuringiensis* subsp. *Israelensis* (BACTI), which was known as mosquitocidal model *Bt* strain that could produce specific insecticidal toxins during sporulation and had been widely used in the larval mosquito controls (Guan et al. 2012, 2014). On the basis of phylogenetic analysis, we found that PS2160P2_1, PS2160P2_153 and PS2160P2_147 had similarity with Cry4 class crystal proteins. In detail, PS2160P2_1 showed high identities of less than 45% to the best hit Cry4Ba_BACTI, PS2160P2_147 had high

Fig. 2 The Venn diagrams of CTGs, TER-CTGs, CD-CGTs and GO-CTGs and the network of genes and GO terms. **a** CTGs represent the 46 candidate toxin genes identified with BLAST. TER-CTGs indicate those 14 toxin gene-coding proteins with tertiary structure. CD-CTGs represented the 14 toxin gene-coding proteins with conserved endotoxin domains. GO-CTGs indicated 31 genes with GO annotation. **b** The network of genes and GO terms. The circle nodes in the network represent CTGs, and the square nodes represent GO terms. The color of square nodes was colored with the corresponding category

Table 1 The general features of 12 toxin genes in *Bt* strain S2160-1

Protein name	Number of aa	Genome location	Description with GO
PS2160P1_2	699	p01:2258-4357	Cry-like protein
PS2160P1_20	525	p01:23917-25491	Cry30-like protein
PS2160P1_21	69	p01:25575-25784	Cry30-like protein
PS2160P1_39	601	p01:42105-43907	Pesticidal crystal protein
PS2160P1_40	96	p01:43985-44275	Cry53Ab1-like protein
PS2160P1_49	117	p01:50832-51185	Mosquitocidal toxin-like protein
PS2160P1_50	575	p01:51263-52987	Mosquitocidal toxin-like protein
PS2160P1_54	688	p01:55475-57541	Pesticidal crystal protein
PS2160P2_1	1216	p02:216-3866	Cry-like protein
PS2160P2_134	802	p02:114830-117238	Cry-like protein
PS2160P2_147	552	p02:129794-131449	Cry50-like protein
PS2160P2_153	1157	p02:136654-140127	Pesticidal crystal protein

Fig. 3 The maximum likelihood phylogenetic tree of toxin genes in *Bt* strains. 12 CTGs of *Bt* S2160-1 and 101 known toxins from UniProtKB database were used to construct the tree with bootstrap

repeated 100 times, and the bootstrap value larger than 0.50 was shown in the figure. Proteins from S2160-1 were marked with red circle. Each known toxin was marked with gray circle

identities of 53% to its best hit Cry4Ba_BACTI and PS2160-1_153 had high identities of 48% with its best hit Cry4Aa_BACTI. Meanwhile, PS2160P1_2, PS2160P1_20, PS2160P1_21, PS2160P1_54 had closed evolutionary relationship with Cry10 (bootstrap value > 50). In summary, the construction of phylogenetic tree could better reflect the relationship among the toxin genes in *Bt* strains that could be helpful to classify and name the toxin genes as well as to infer the function of toxins in *Bt* S2160-1 strain.

Discussion

B. thuringiensis (*Bt*) is a natural typical insecticidal bacterium. Using *Bt* bacteria as biological pesticides or developing transgenic *Bt* crops is becoming the main directions of research and development in agrochemical and seed industry. *Bt* always performed substantial variations in insecticidal toxicity. Therefore, how to quickly identify new *Bt* strains and how to discover new *Bt* toxic proteins are the basis for establishing competitive advantages in these industries.

The development of genome sequencing technology, especially the next generation sequencing, provides new means for discovering *Bt* toxins in large scale. In this study, we employed the data of next generation sequencing (NGS) data and bioinformatics approaches to computationally discover and identify *Bt* toxin proteins in *Bt* strain S2160-1, which demonstrated that it was an efficient strategy comparing with the previous traditional PCR method.

In theory, NGS could produce a large number of sequences quickly and indiscriminately. On the other hand, the standard computational process could identify all types of *B. thuringiensis* toxin proteins, including Cry proteins, Cyt proteins and Vip proteins. Thus, it was expected that computational method was more efficient and time-saving in identifying *Bt* toxins than traditional PCR-based systems. In fact, we identified 46 candidate toxins for *Bt* strain S2160-1. The tertiary structure analysis with SWISS-MODEL showed that 15 of 46 proteins (TER-CTGs) encoded by the CTGs had a matching result with the three-tier models of known *Bt* insecticidal crystal protein. Meanwhile, 12 proteins (CD-CTGs) among these 15 TER-CTG-encoded proteins were located on circular plasmids, which contained at least one of delta-endotoxin domains. The functional annotation showed that they might be involved in the biological processes or molecular function related to *Bt* pathogen (Fig. 2b). The good news was that four toxins of the insecticide genes Cry30Ea1 (NCBI Acc No. ACC95445), Cry30Ga2 (NCBI Acc No. HQ638217), Cry50Ba1 (NCBI Acc No. GU446675) and Cry54Ba1 (NCBI Acc No. GU446677) previously cloned in *Bt* S2160-1 were confirmed by our results (Zhang et al. 2012) (PS2160P1_54 corresponded to Cry30Ea1, PS2160P1_20 corresponded to Cry30Ga2, PS2160P2_147 corresponded to Cry50Ba1, and PS2160P1_2 corresponded to Cry54Ba1). Additionally, PCR approach had identified three distinguishing bands of parasporin proteins with 140, 130 and 30 kDa in size but remained unknown, which was perfectly matched with this study that PS2160P2_1 was predicted to be with 137.28 kDa and PS2160P2_153 was predicted to be with 130.57 kDa. Combining the results of BLASTP and phylogenetic tree, it was reasonable to infer that PS2160P2_1 and PS2160P2_153 may be potential Cry4-like toxins in *Bt* S2160-1. It was obvious that our research might facilitate to detect those Cry toxins with three-domain through bioinformatics methods.

Remarkably, our research also identified those candidate toxins without typical three domains (Kirouac et al. 2002; Vachon et al. 2012). For example, the protein coded by *pS2160p1_gene_id_8* did not contain typical delta endotoxin domains, whereas contained RICIN domain as similar as Cry35 class crystal proteins and Cyt1Ca, and with an active tertiary structure prediction result. Previous researches reported that the *Bt* toxins Cyt1Ca contained RICIN domain, and had 50% similarity to the β -trefoil modules which was

found in various toxins containing RICIN domains, such as the mosquitocidal toxin protein Mtx1 from *Bacillus sphaericus* (Berry et al. 2002; Manasherob et al. 2006). Therefore, it was reasonable to speculate that the protein coded by *pS2160p1_gene_id_8* may be a member of two-domain fusion toxin as Cyt1Ca.

In conclusion, this computational research based on NGS was proposed to mine cry genes from *B. thuringiensis*. The method was able to mine toxins in an efficient and fast way. As a result, 46 candidate toxins were identified from S2160-1. The GO function annotation revealed that 23 of them were annotated to known pesticidal crystal proteins. Among them, 12 toxins with typical three domains can be identified accurately. In the end, 12 proteins with all features were chosen to investigate their evolutionary relationship with known toxins. The above results proved the efficiency of this method in mining *toxin* genes. In fact, the mining of novel sequences is associated with the selection of known reference sequence. It was important to note that the quality of sequence and assembled genome affects the final prediction results, so a choice would need to be made between the cost and efficiency of sequencing. And this method could mine all classes of toxin proteins based on sequence similarity. This research might make key contributes to further study on the generic features of *B. thuringiensis* S2160-1 toxins.

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Compliance with ethical standards

Conflict of interest No conflict of interest declared.

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