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ORIGINAL PAPER

Genomic–proteomic analysis of a novel *Bacillus thuringiensis* strain: toxicity against two lepidopteran pests, abundance of Cry1Ac5 toxin, and presence of InhA1 virulence factor

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Abstract

Bacillus thuringiensis (*Bt*) is a biological alternative to the indiscriminate use of chemical insecticides in agriculture. Due to resistance development on insect pests to *Bt* crops, isolating novel *Bt* strains is a strategy for screening new pesticidal proteins or strains containing toxin profile variety that can delay resistance. Besides, the combined genomic and proteomic approaches allow identifying pesticidal proteins and virulence factors accurately. Here, the genome of a novel *Bt* strain (*Bt* TOL651) was sequenced, and the proteins from the spore–crystal mixture were identified by proteomic analysis. Toxicity bioassays with the spore–crystal mixture against larvae of *Diatraea saccharalis* and *Anticarsia gemmatalis*, key pests of sugarcane and soybean, respectively, were performed. The toxicity of *Bt* TOL651 varies with the insect; *A. gemmatalis* (LC_{50} = 1.45 ng cm^{-2}) is more susceptible than *D. saccharalis* (LC_{50} = 73.77 ng cm^{-2}). Phylogenetic analysis of the *gyrB* gene indicates that TOL651 is related to *Bt kenyae* strains. The genomic analysis revealed the presence of *cry1Aa18*, *cry1Ac5*, *cry1Ia44*, and *cry2Aa9* pesticidal genes. Virulence factor genes such as phospholipases (*plcA*, *piplc*), metalloproteases (*inhA*), hemolysins (*cytK*, *hlyIII*, *hblA*, *hblC*, *hblD*), and enterotoxins (*nheA*, *nheB*, *nheC*) were also identified. The combined use of the genomic and proteomic data indicated the expression of Cry1Aa18, Cry1Ac5, and Cry2Aa9 proteins, with Cry1Ac5 being the most abundant. InhA1 also was expressed and may contribute to *Bt* TOL651 pathogenicity. These results provide *Bt* TOL651 as a new tool for the biocontrol of lepidopteran pests.

Keywords Biopesticide · *Bacillus thuringiensis* · Cry proteins · Genome sequence · Proteomic · Toxicity · Lepidopteran pest

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Introduction

Sugarcane (*Saccharum officinarum*), corn (*Zea mays*), and soybean (*Glycine max* L) are high-value crops and applied to different purposes such as food and biofuel production (de Matos et al. 2020; Heinrichs et al. 2017). The sugarcane borer, *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae), is a key pest of sugarcane and corn. The velvetbean caterpillar, *Anticarsia gemmatalis* (Hübner 1818) (Lepidoptera: Noctuidae), is a key pest of soybean (Horikoshi et al. 2022; Mendonça et al. 2020). These insects are among Brazil's most relevant Lepidoptera pests causing damage to the raw material used in food and biofuel processing (Silva 1995; Praça et al. 2004; Moscardi et al. 2012; Dinardo-Miranda et al. 2013).

Biocontrol is a safe alternative to reduce the use of chemical insecticides in crop pest management. Entomopathogenic

microorganisms have been considered important agents for this purpose with relevant importance for the bacteria *Bacillus thuringiensis* (*Bt*) (Bel et al. 2017; Daquila et al. 2019; Sanahuja et al. 2011). Biological control products based on *Bt* are being used as biopesticides for decades and currently have the majority of the biological control market share in the world (Arthur and Dara 2019; Lacey et al. 2015; Sena da Silva et al. 2022). *Bt* is a Gram-positive, spore-forming bacterium that can produce parasporal crystal proteins during the sporulation phase (Cry and Cyt proteins) (Pohare et al. 2021). These proteins when ingested by a susceptible insect are solubilized in the alkaline midgut environment, acquiring an oligomeric form, binding to the midgut cell membrane, leading to the destruction of these cells and their insecticidal proprieties (Frankenhuyzen 2009; Bravo et al. 2007, 2011; Pinheiro and Valicente 2021). *Bt* can produce pesticidal proteins during the vegetative phase (Vip proteins) (Pohare et al. 2021). Virulence factors such as metalloproteases, chitinases, hemolysins, and enterotoxins also represent *Bt* pathogenicity (Malovichko et al. 2019; Palma et al. 2014).

Bt is an important biopesticide used against lepidopteran pests as spray formulations and *Bt* crops (transgenic plants that express Cry and/or Vip3 proteins) (Castro et al. 2019; Daquila et al. 2019; Horikoshi et al. 2022; Srikanth et al. 2011). However, the evolution of resistance to *Bt* crops in lepidopteran pests has been reported, including in *D. saccharalis* (de Oliveira et al. 2022; Huang et al. 2015) and *A. gemmatalis* (Pezenti et al. 2021). Therefore, the isolation of novel *Bt* strains is an important strategy for the discovery of new pesticidal proteins or strains containing a range of toxin profiles that can delay the target insect's resistance.

Characterizing novel *Bt* strains and studying the genome and proteome is important to understand their pathogenicity. Genome sequencing technology has accelerated the discovery of novel pesticidal proteins, secondary metabolites, and virulence factors in *Bt* (Zghal et al. 2018; Liu et al. 2017; Cardoso et al. 2020; Day et al. 2014; Jeong et al. 2017; Jia et al. 2016; Liu et al. 2015). However, considering not all of the coding regions predicted from the annotated genome sequence are expressed, there are cryptic pesticidal proteins (Quan et al. 2016; Rang et al. 2015). So, the pesticidal proteins expression profile could be explored using proteomic analysis. Thus, in combination with genomic studies, proteomic analysis allows the accurate identification of pesticidal proteins and virulence factors in different *Bt* strains (Baragamaarachchi et al. 2019; Gomis-Cebolla et al. 2018; Khorramnejad et al. 2020; Wu et al. 2011). Furthermore, from the genomic–proteomic analysis it is also possible to estimate the protein abundance in purified parasporal crystals and spore–crystal mixtures (Baragamaarachchi et al. 2019; Huang et al. 2012; Khorramnejad et al. 2020).

This study sequenced the genome of a novel strain *Bt* TOL651, toxic against *A. gemmatalis* and *D. saccharalis*, and the entomopathogenic characteristics were explored. Additionally, an LC–MS/MS analysis of the spore–crystal mixture was performed to determine the expressed proteins.

Materials and methods

Culturing of TOL651 strain

Bacillus-like colonies were isolated from soil samples collected in Tocantins State (Brazil) (11º43′45″S; 49º04′07″W) according to the previously described protocol (Monnerat et al. 2001). To screen for *Bt* strains, Petri dishes with a selective NYSM medium (nutrient yeast extract salt medium) [8 g/l of nutrient broth (Difco, USA), 0.103 g/l of $CaCl_2{\cdot}2H_2O$, 0.01 g/l of $MnCl_2{\cdot}4H2O$, 0.203 g/l of $MgCl2{\cdot}6H_2O$] (Kalfon et al. 1983) containing 100 mg/l penicillin G were used and the culture grown for 24 h at 30 ± 0.5 °C at 180 rpm. Then, each colony was individually analyzed and identified by phase-contrast microscopy (×1000) to verify the presence of inclusion bodies and crystals (Frankland and Frankland 1887). The *Bt* TOL651 was selected among 87 crystal-forming *Bt* strains (87 strains from 2.445 *Bacillus*-like colonies), due to being the most toxic among different isolated *Bt* strains and tested simultaneously against *Diatrea saccharalis* and *Anticarsia gemmatalis* in the selective bioassays, according to Monnerat et al. (2007). *Bt* HD-1was isolated from the commercial sample (Dipel WP 32 g/kg, Sumitomo Chemical do Brasil Representações Ltda., SP) and used as a reference strain (Cerqueira et al. 2017; Lazart et al. 2021; Sathyan et al. 2022). *Bt* HD-1 has been designated as the primary US reference standard strain for toxicity against lepidopteran insects (Dulmage 1973). *Bt* TOL651 strain was cultured at 28 °C for 12 h on solid Luria–Bertani medium (LB) (10 gL^{-1} tryptone, 5 gL^{-1} yeast extract, 10 gL^{-1} NaCl, and 20 gL^{-1} agar). A single bacterial colony was inoculated in the LB liquid medium at 28 °C, 200 rpm for 16 h, which was used as a starter culture for spore–crystal mixture production and in the genomic DNA extraction step.

SDS-PAGE analysis of cry proteins

For sporulation and crystal production, a starter culture was transferred to the CCY medium (30 ml) (13 mM KH_2P04, 26 mM K_2HP04, 0.002% [w/v] L-glutamine, 0.1% [w/v] casein hydrolysate, 0.1% [w/v] Bacto Casitone, 0.04% Bacto Yeast extract, 0.6% [w/v] glycerol, 0.05 M $ZnCl_2$, 0.5 M $MgCl_2$, 0.01 M $MnCI_2$, 0.2 M $CaCl_2$, 0.05 M $FeCl_3$) and incubated at 28 °C, 200 rpm for 72 h. For SDS-PAGE analysis, the crystals were purified using hexane and low-speed

centrifugation, according to Mounsef et al. (2014). The spores and crystals were collected for centrifugation at 6000 rpm, 4 °C for 10 min, and the pellet was washed twice by suspending it in saline solution (1 M NaCl containing 0.01% Triton X-100) by centrifugation (6000 rpm, 4 °C for 10 min). Then, the pellet was suspended in a 50 ml centrifuge tube with 27 ml of saline solution and sonicated at 100 W of potency for 10 min. Then, 3 ml of hexane was added to the suspension following the centrifugation at 6000 rpm, 4 °C for 10 min. This procedure was repeated three times. The pellet was washed twice with cold distilled water by centrifuging. Then, the crystals were solubilized using 50 mM NaOH buffer at 30 °C and quantified using Bradford reagent (Bio-Rad protein assay). Following this, 7 µg of solubilized crystals was analyzed by 12% sodium dodecyl sulfate-–polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue (R250, 0.4%).

Microscopy

The spore–crystal mixture of TOL651 was collected and diluted in sterile water. Then, 100 µL of this dilution was deposited over metallic supports to be dried for 24 h at 37 °C, covered with gold for 180 s using an Emitech apparatus (model K550), and observed in a Zeiss scanning electron microscope (model DSM 962) at 10 or 20 kV.

Toxicity against *Diatraea saccharalis* and *Anticarsia gemmatalis*

Eggs from *D. saccharalis* and *A. gemmatalis* were obtained from Biocontrole (biocontrole.com.br) and Embrapa Recursos Genéticos e Biotecnologia—CERNAGEN (Brasília, DF, Brazil), respectively. The insect's eggs were maintained under ideal rearing conditions (i.e., 26 °C ± 1 °C and 70 ± 10% RH and 10:14 h (L: D) photoperiod) (Schmidt et al. 2001), on specific artificial diets prepared for *D. saccharalis* (Hensley and Hammond 1968) and *A. gemmatalis* (Greene et al. 1976).

Bioassays against *D. saccharalis* were performed using 24-well cell culture plate (TPP, Techno Plastic Products AG). 1.5 ml of diet was poured into each well and, after solidification, aliquots 35 µL of ten spore–crystals dilutions (from 0.1 to 1000 ng/cm^2) were spread on the diet surface. Subsequently, 1-day-old second instar larvae were placed in each plate. Trays were closed with acrylic lids, keeping them under controlled conditions (26 °C ± 1 °C and 70 ± 10% RH and 10:14 [L: D] h). After 48 h, the surviving larvae were individually transferred to six-well cell culture plates containing a rearing diet. Larvae mortality was evaluated again on day 7 (Praça et al. 2004). Larvae were considered dead when stimulated and no movement was detected.

In the *A. gemmatalis* bioassays, a total volume of 3 ml artificial diet was poured into 30 ml plastic cups. After solidification, aliquots (150 µl) of ten spore–crystal concentrations (from 0.1 to 1000 ng/cm^2) were applied on the diet surface and dried at room temperature. Then, ten 1-day-old second instar larvae of *A. gemmatalis* were added to each cup. The cups were covered with plastic lids and kept under the same conditions described above. After 48 h, the surviving larvae were placed in 30 ml cups containing a rearing diet and the mortality was assessed. Larval mortality was evaluated again on day 5 as described (da Silva et al. 2004).

All the bioassays were performed in triplicate. The commercial strain HD-1 was used as a reference and sterile water, pH 7.0, was added as the control group. The lethal concentrations (LC_{50} and LC_{95}) were determined by Probit analysis (Finney 1971) using the PoloPlus 1.0 (LeOra Software Berkeley, CA, USA).

Genome sequencing, data assembly, and annotation

Total DNA from the *Bt* TOL651 strain was extracted and purified by the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. The DNA concentration and purity were checked by the NanoDrop™ 8000 apparatus (ThermoFisher Scientific, Waltham, MA, USA) and then stored at − 20 °C until further use. Genome sequencing was performed using Illumina MiSeq technologies (paired-end application, 2x ~ 80 bp, average insert size of 200 bp), and the coverage of 137 X. FastQC v.0.11.9 (Andrews 2015) was used for the reads libraries quality analyses, with reads being trimmed using Geneious v.10.2.6 (Kearse et al. 2012) (Workflow Trim and Filter, Error Probability: 0.05). The de novo assembly was performed using SPAdes v.3.10.0, using default parameters (Bankevich et al. 2012). The assembled contigs (= > 500 bp) were run through MeDuSa v.1.6 (Bosi et al. 2015) for scaffolding, using complete genomes *Bt* YBT-1520 and *Bt* HD-1 (NCBI RefSeq NZ_CP004858 and CP004870 respectively) strains as a reference dataset. The quality of the final assembly was assessed using Quast v.5.0.2 (Gurevich et al. 2013) in standard mode, and completeness percentage, and N50 and L50 values were obtained. The annotation and CDS prediction of scaffolds were performed using RASTtk 2.0 (Brettin et al. 2015).

Average nucleotide identity (ANI) and phylogenetic relationships

The genome similarity was assessed through ANI using JSpeciesWS (Richter et al. 2015) on Tetra Correlation Search (TCS) function for selecting related genomes. The Heatmap dendrogram was created using the Morpheus tool (https://software.broadinstitute.org/morpheus). The

phylogenetic relationship analysis was performed using the *gyrB* gene (DNA gyrase subunit B) including other genes of the closely related *Bacillus* spp. strains retrieved from GenBank. The sequences were aligned using ClustalW, and a phylogenetic tree was created using MEGA 11 (Kumar et al. 2018), using the neighbor-joining method and bootstrap percentages based on 1000 replications.

Gene identification of pesticidal proteins

Putative pesticidal proteins were determined using Blastx, through the Btoxin_Digger tool (scaffolds as a query) (Liu et al. 2021) and a customized database (CDS predicted as a query). The customized database was created through Geneious, using Add/Remove Database tool, from the *Bt* pesticidal proteins list available at the *Bt* nomenclaturewebsite (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/toxins2.html). CDS with homology to the *Bt* pesticidal proteins was filtered using E-value 0.001 and word size 6 parameters. To check the presence of novel putative Cry sequences in TOL651, the sequences obtained from the non-redundant protein database NCBI were used (Lazart et al. 2021).

Identification of virulence factors and antibiotic resistance genes

The potential virulence factors were predicted using the bacterial virulence factor database (VFDB) (Liu et al. 2019). The TOL651 genome was screened for antibiotic resistance factors, using the Resistance Gene Identifier (RGI), within Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al. 2023), according to the parameters: perfect, strict, complete genes only and 95% identity nudge.

Proteomic analysis

The proteins in the spore–crystal mixture of the *Bt* TOL651 strain were identified by LC–MS/MS at the Biotech Company Veritas /Life Sciences at the University of São Paulo (USP, Ribeirão Preto, SP, Brazil). The sample of the spore–crystal mixture was washed three times in 1× PBS (phosphate-buffered saline) and resuspended in 750 µL of solubilization buffer (8 M urea, 0.5% Octyl-glucopiranoside (OG) and 0.05 M Tris–HCL, pH 8.8). Then, the sample was sonicated (three cycles of 60 s, 30% amplitude, and shut off for 2 s) and maintained on ice. The solubilized proteins were quantified through the Bradford method using Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories), according to the manufacturer's instructions.

The preparation of the sample for advanced mass spectrometry consisted of three main steps: (i) reduction and alkylation of proteins, (ii) enzymatic digestion using trypsin, and (iii) cleanup/desalting. Briefly, 50 µg of the sample was subjected to disulfide bridge reduction using 50 µg of DTT (dithiothreitol) and 60 min of incubation at 37 °C. Then, the process was followed by alkylation using 250 µg of I.A (iodoacetamide) and 60 min at room temperature in the dark. Finally, the sample was diluted five times in Tris–hydrochloride (0.05 M Tris–HCl, pH 8.8) and incubated with 2 µg of trypsin (Promega, V511A) at 37 °C overnight.

Previously to the mass spectrometry application, the cleanup/desalting of the sample was performed using C18 resin (Supleco). The column was calibrated using 2% acetonitrile containing 0.1% formic acid, and the elution was performed with 50% acetonitrile. The sample was then dried in a speed vac and applied in a mass spectrometer (Termo Fisher Orbitrap Eclipse), coupled to a nanoflow nano LC–MS/MS chromatography system (Dionex Ultimate 3000 RLSCnano System, Thermofisher). Peptides were separated in nanoEase MZ peptide BEH C18 column (130A, 1.7 µm, 75 µm × 250 mm, Waters) 300 nL/min using a 4–50% acetonitrile gradient for 90 min. The data were obtained on MS1 in the range of M/Z 375–1500 (120,000 resolution, AGC target 1E6, maximum time injection of 100 ms). The most abundant ions were submitted to MS/MS (30% collision energy, 1.2 m/z, AGC target 1E5, 15000 resolution).

The raw data were converted to mzXML format and processed using PatternLabV (Santos et al. 2022). The data were analyzed against the database created using CDS translated to the TOL651 genome (Generate Search DB option). The contaminant library content of common MS contaminant sequences (e.g., trypsin, keratins, and albumin) was included in the database. The modifications selected in the search were carbamidomethyl (C), deamination (NQ, variable), and oxidation (M, variable). Enzyme trypsin (fully specific), two maximum missed cleavages, and initial precursor mass tolerance of 10 ppm were set as Comet parameters. The acceptable FDR (false discovery rate) estimates of 3% at spectral, 2% at peptide, and 1% at protein levels and MS and MS/MS tolerance errors of 10 ppm were added as parameters in the Filtering (SEPro) options.

The functional annotation of the identified proteins was performed using UniProtKB/Swiss-Prot database, and the graphical summary of functional classification was created using GO terms through the WEGO 2.0 tool (Web Gene Ontology Annotation Plot) (Ye et al. 2018).

Data availability

This whole genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JANVFA000000000. The version described in this paper is JANVFA010000000.

Results

Protein profile and morphological and toxicity analysis

Cry protein profile of *Bt* TOL651 revealed the presence of two major protein bands of approximately 130 and 65 kDa size, indicating the presence of both Cry1 and Cry2 proteins, also observed in the reference strain (Fig. 1A). The morphological analysis revealed the presence of spores and bipyramidal and cuboidal crystals in this strain, also indicating the expression of Cry1 (bipyramidal crystals) and Cry2 (cuboidal crystals) proteins (Fig. 1B).

The insect bioassays using spore–crystal mixtures of *Bt* TOL651 and the reference *Bt* HD-1 strain showed that both strains were toxic to *D. saccharalis* and *A. gemmatalis* (Table 1). However, the *Bt* TOL651 showed significantly higher toxicity to *D. saccharalis* and *A. gemmatalis* when compared to the *Bt* HD-1 strain (Table 1). The RT_{50} estimate showed that the *Bt* TOL651 strain was 1.97- and 1.75-fold more toxic than the *Bt* HD-1 strain against *D. sacharalis* and *A. gemmatalis*, respectively (Table 1).

Genomic characterization

The draft genome of *Bt* TOL651 was obtained and consists of ~ 6.17 Mb with 35.3% GC content (Table 2). A total of 7003 coding sequences (CDS) were found, out of which 4812 proteins had functional assignments and 2191 were considered hypothetical proteins. Sixty-three tRNA and 5 rRNA genes were also annotated (Table 2). In the subsystem class distribution, most of the genes were involved, in decreasing order: amino acids and derivatives metabolism (387); carbohydrate (265); cofactors, vitamins, prosthetic groups, pigments metabolism subsystems (161); protein metabolism (155); and nucleosides/nucleotides metabolism (118) (Fig. 2).

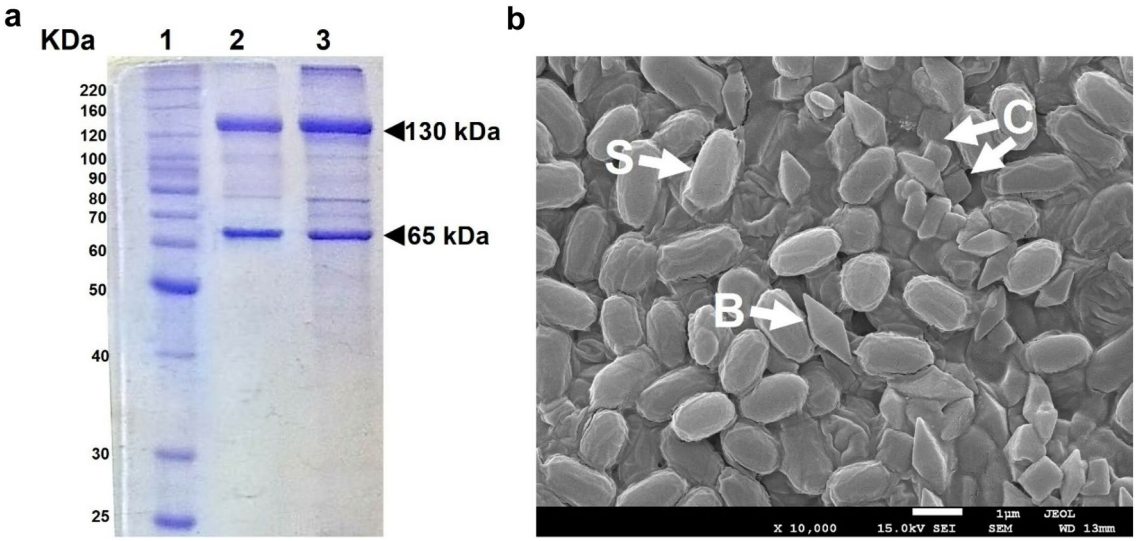

Fig. 1 SDS-PAGE analysis of Cry proteins and scanning electron microscopic of spore–crystal mixture of *Bt* TOL651. **a** Protein profile: Lane 1—molecular mass markers; Lane 2—HD-1; Lane 3—TOL651. Arrows indicate likely protein band size. **b** The view of spores (S) and bipyramidal (B) and cuboidal (C) crystals enlarged 2,000 (×) times and approximated 10,000 times (×)

Table 1 Lethal concentration of spore–crystal mixture from *Bt* TOL651 against larvae of *A. gemmatalis* and *D. saccharalis*

	Strain	Slope ± SE	LC_{50}* ng.cm^{-2} (IC)^c	LC_{95}** ng.cm^{-2} (IC)^c	χ^2	*P*	RT_{50}
D. saccharalis	TOL651	2.69 ± 0.26	73.77 (63.23–87.56)	300.37 (222.34–461.86)	0.62	0.94	1.97
	HD-1	2.78 ± 0.30	145.11 (125.10–164.81)	566.51 (499.94–794.44)	5.28	0.15	
A. gemmatalis	TOL651	4.08 ± 0.48	1.45 (1.33–1.56)	3.66 (3.06–4.84)	0.51	0.91	1.75
	HD-1	3.99 ± 0.56	2.55 (2.30–2.79)	6.58 (5.40–9.18)	3.21	0.20	

*Lethal concentration

**Confidence interval, 95% probability

RT_{50} = toxicity ratio = LC_{50} HD-1/LC_{50}TOL651 (Robertson et al. 2017)

Table 2 Genome features of the *Bt* TOL651 strain

General features	Value
Scaffolds (no.)	256
N50 (bp)	819,816
L50 (no.)	3
N bases (%)	1.5
Completeness (%)	98
Genome Length (bp)	6,176,245
GC Content (%)	35.34
Coding sequences (CDS) (no.)	7003
tRNA (no.)	63
rRNA (no.)	9
Proteins with functional assignments (no.)	4812
Hypothetical proteins (no.)	2191

Bt TOL651 relationships with other *Bt* strains

In the search for *Bt* genomes highly correlated to *Bt* TOL651, different *Bt* strains' genomes were compared with *Bt* TOL651. ANI values were obtained and showed that *Bt* TOL651 was highly similar to other *Bt* strains (ANI ≥ 94%). High ANI values were observed for *Bt* TOL651 and other *Bt* strains such as *Bt* kurstaki T03a001 (99.06%), *Bt* kurstaki HD73 (99.00%), *Bacillus* sp. G3 (98.88%), Bt YC-10 (98.77%), *Bt mexicanensis* 27 (98.63%), *Bt* NBIN-66 (98.41%), *Bt galleriae* HD-29 (98.28%), and *Bt aizawai* Leap01 (98.04%). However, *Bacillus cereus* (*Bc*) B4158 genome also was highly correlated with *Bt* TOL651 (ANI = 98.16%) (Fig. 3). The phylogenetic analysis using gyr*B* gene indicated that *Bt* TOL651 was clustered with *Bt kenyae* BGSC-4F2 and IEBC-T04B001 strains (Fig. 4).

Genes related to pesticidal proteins, virulence factors, and antibiotic resistance

Based on the *Bt* database and Btoxin_Digger, a total of four *cry1* and *cry2*-type genes were found in two scaffolds (18 and 21) in the genome of *Bt* TOL651. The CDS regions peg.5608, peg.5616, and peg.6113 showed high homology to the *cry2Aa9, cry1Ia44*, *and cryAc5* genes, respectively. On the other hand, the peg.5617 CDS region showed high homology to the *cry1Aa18* gene in the custom *Bt* database, but was not found in the Btoxin_Digger (Table 3, Fig. 5). Finally, the peg.3270 CDS region was retrieved using Btoxin_Digger and custom *Bt* database, demonstrating high homology to the *spp1Aa1* gene (~ 80%).

Virulence factor genes from different classes (adherence, enzyme, immune evasion, iron acquisition, regulation, secretion system, toxins, acid resistance, magnesium uptake, surface protein anchoring, and others) were also found in the genome sequence of *Bt* TOL651 (Table S1). Among these genes, we can point out enzymes such as phospholipases (*plcA*, *piplc*) and metalloproteases (*inhA*), and other toxins such as hemolysins (*cytK*, *hlyIII*, *hblA*, *hblC*, *hblD*) and enterotoxins (*nheA*, *nheB*, *nheC*). (Table S1). Four putative antibiotic resistance genes were identified in the

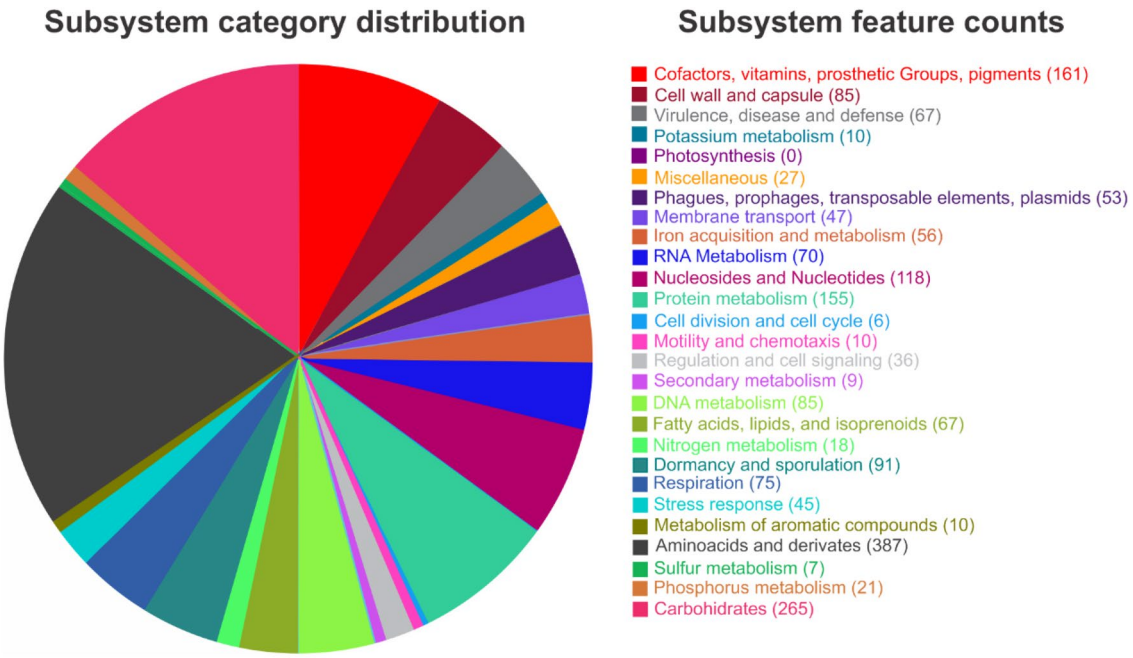

Fig. 2 Subsystem category distribution of genes in the genome of *Bt* TOL651 based on RAST annotation server

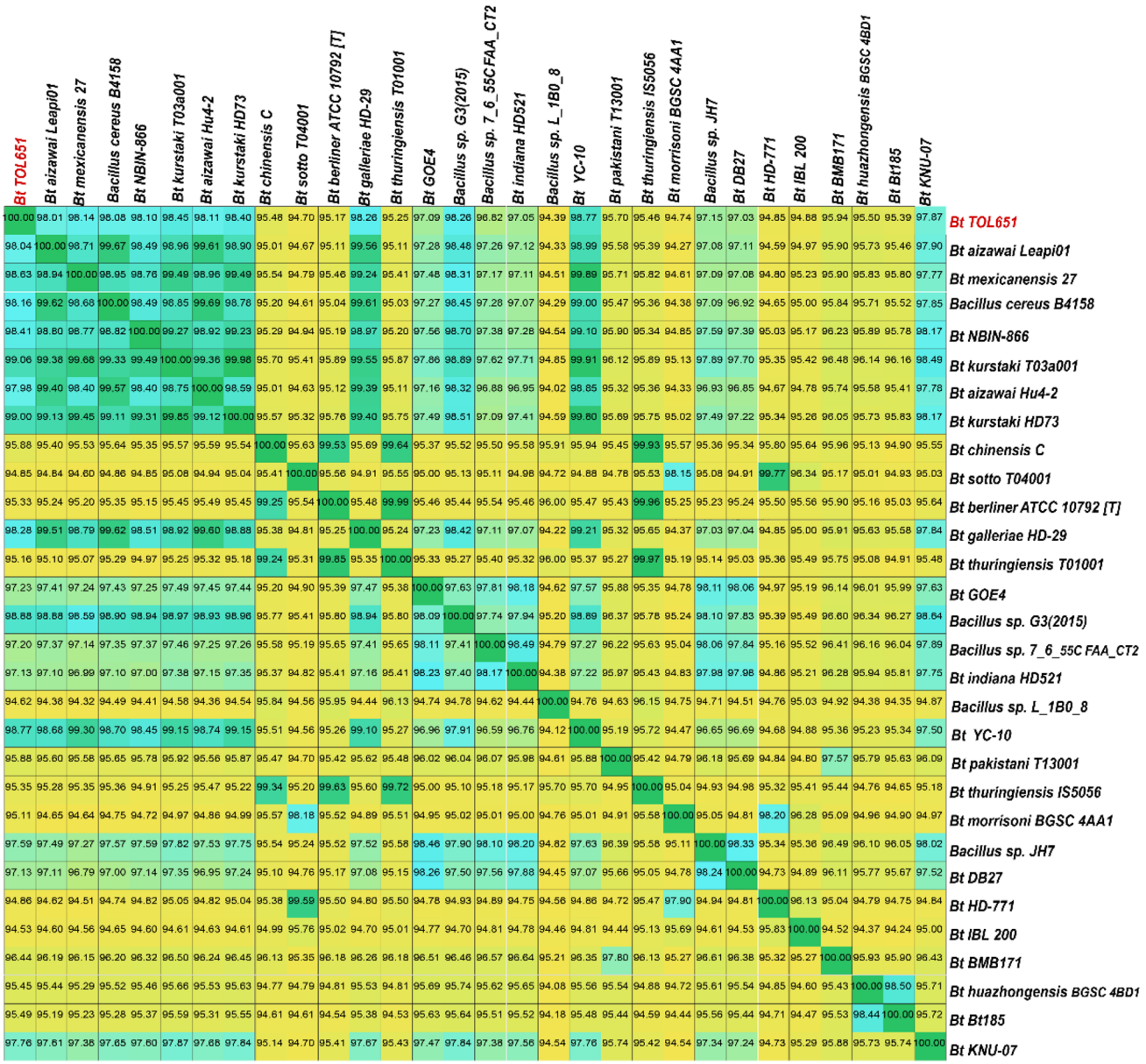

Fig. 3 Heat map of average nucleotide identity (ANI) based on genomic comparison *Bt* TOL651 and other 29 related strains. The percentage identities are listed on the map

genome of *Bt* TOL651, including *BcII* gene (subclass B1 beta-lactamase) (% identity: 90.12; % length of reference sequence: 122.66), *BcI*, and two *BcIII* genes (class A beta-lactamase) (% identities: 95.42, 86.83 and 76.58, respectively, % length of reference sequences: 100.65, 100.32 and 100, respectively).

Proteomic of spores–crystal mixture

The general functional classification carried out by LC–MS/MS analysis of the spore–crystal mixture of *Bt* TOL651 revealed that detected proteins sequences were involved in 11 GO terms related to cellular components, 10 GO terms related to molecular functions, and 19 terms related to biological processes (Fig. 6). In the cellular component groups, most proteins were related mainly to cell and membrane components. Furthermore, the molecular function classification was represented by proteins with catalytic and binding activities; next, in the biological process category, most proteins belonged to metabolic and cellular processes.

The comparison among genomic and proteomic data indicated that 24 coding sequences were identified in the proteins' sequences and, based on unique peptide count, the most abundant proteins were: Cry1Ac5, Cry2Aa9,

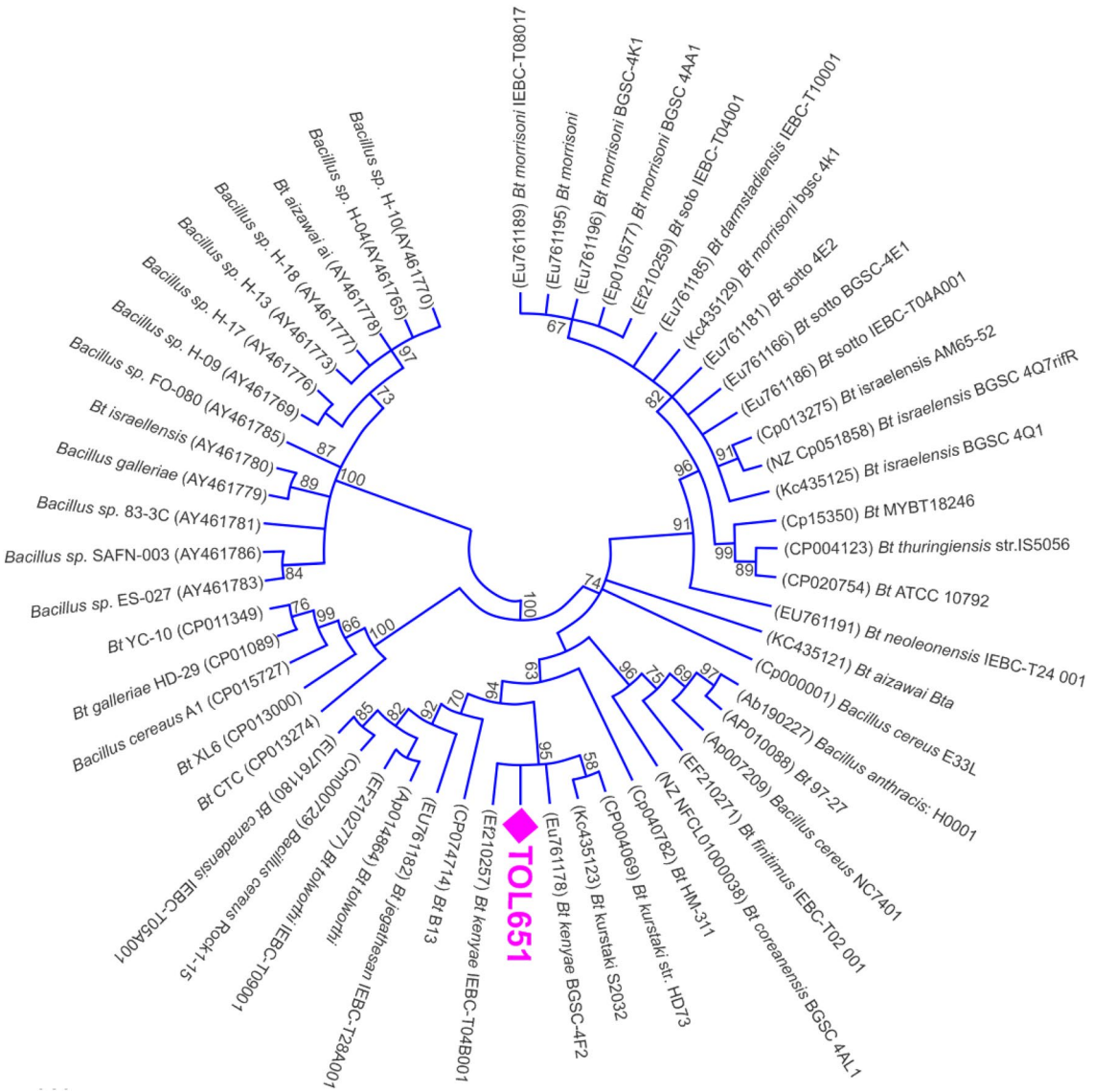

Fig. 4 Phylogenetic analysis of the TOL651 and other *Bacillus* ssp. strains. Bootstrap values (%) presented at the branches were calculated from 1000 replications. Bootstrap values < 50% were disregarded for branches in the graphical representation of the tree

Table 3 Identification of genes coding pesticidal proteins-like in the *Bt* TOL651

Sequence_id	CDS_id	Length (aa)	Hit_id	Coverage (%)	Identity (%)	*E*-value	Accession
Scaffold_5	peg.3270	513	Spp1Aa1[a,b]	99.21[a]	80.48[a]	0.0	BAF62176
				97.47[b]	80.50[b]	0.0	
Scaffold_18	peg.5608	634	Cry2Aa9[a,b]	100[a]	100[a]	0.0	ABR68091.1
				99.84[b]	96.7[b]	0.0	
Scaffold_18	peg.5616	720	Cry1Ia44[a,b]	100[a]	100[a]	0.0	QBO24620
				99.86[b]	100[b]	0.0	
Scaffold_18	peg.5617	521	Cry1Aa18[b]	98.66[a]	100[a]	0.0	AEH31438
Scaffold_21	peg.6113	1178	Cry1Ac5[a,b]	100[a]	99.92[a]	0.0	AAA22339
				99.92[b]	98.60[b]	0.0	

[a]Btoxin_Digger

[b]Customized *Bt* database

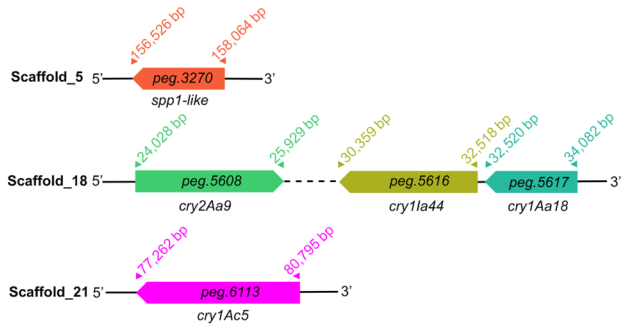

Fig. 5 Representation of the genome position of pesticidal protein-like identified in the scaffolds of the *Bt* TOL651 strain

and Cry1Aa18 (Table 4) (Table S2). Except for Cry1Ia44 (peg.5616), which had no unique peptide, all Cry proteins identified in the genome were expressed in the sporulation phase (72 h of incubation). Cry1Ac5 (peg.6113) showed the highest number of peptides detected, with 85 in total and 82 unique sequences (Table 4) (Table S2). Additionally, regarding the pesticidal proteins, the inhibitor A metalloprotease (InhA1), a potential pathogenic factor, was also found in the proteome of *Bt* TOL651 (Table 4) (Table S2). Other spore-associated proteins (spore coat and forespore-specific proteins), peptides associated with protein metabolism (elongation factor Tu, shock protein Hsp20 family, and chaperone protein DnaK), and other functions (aminopeptidase, glycerophosphoryl phosphodiesterase, choline-binding protein, DUF3915 domain containing, acid endopeptidase, neutral protease B, and DNA-binding protein) were also detected (Table 4).

Discussion

Bt TOL651 strain analyses presented a Cry protein profile in SDS-PAGE gel with two major protein bands of approximately 130 and 65 kDa in size, which is also associated with Cry1 and Cry2 proteins, respectively (Ganesh et al. 2018; Monnerat et al. 2007; Schnepf et al. 1998; Singh et al. 2021). In concordance with the protein profile, the crystal morphology of *Bt* TOL651 revealed bipyramidal forms associated with Cry1 proteins and cuboids formed by Cry2 proteins similar to the HD-1 strain (Monnerat et al. 2007; Schnepf et al. 1998).

The TOL651 genome similarity analysis confirmed that this isolate is a *Bt* species, since ANI values ≥ 95% (98–99%) were assigned in comparison with other genomes of this species (Richter and Rosselló-Móra 2009). However, a high ANI value between *Bt* TOL651 and the *B. cereus* (*Bc* B4158) was also found, which corroborates the complex separation between *Bt* and *B. cereus* at the genomic level (Helgason et al. 2000; Lechuga et al. 2020; Zhou et al. 2022). However, in the subspecies classification, the phylogenetic study showed that the *Bt* TOL651 strain might have a close relationship with *Bt kenyae* strains.

Bt strains identified as *kenyae* subspecies have been reported to harbor c*ry1Ab*, *cry1Ac*, *cry1E*, and *cry2Aa* genes and showed toxic activity against lepidopteran insects

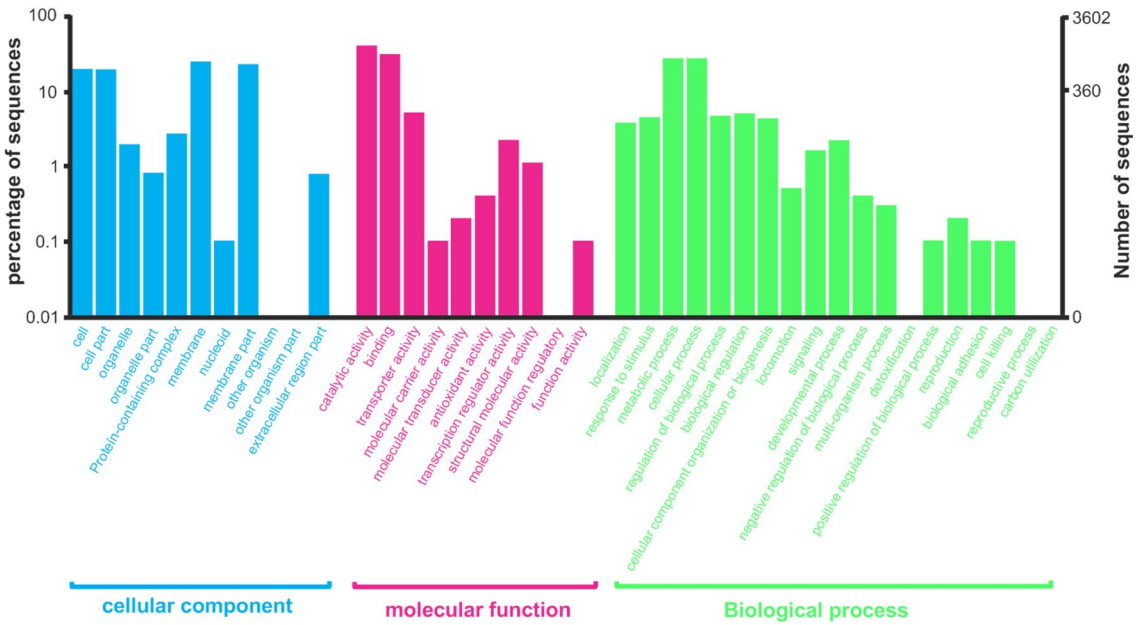

Fig. 6 Functional annotation and classification for LC–MS/MS identified proteins of TOL651 in the spore–crystal mixture

Table 4 Identification of pesticidal and other proteins in the spore/crystal mixture of the *Bt* TOL651 strain detected by LC–MS/MS analysis

CDS id	Description[a]	Length (bp)	Peptide sequence (no.)	Unique peptides (no.)[d]	Coverage[e]	Protein score[f]	NSAF[g]
peg.6113	**Cry1Ac5**[b]	1177	85	82	0.5582	311.262	0.3234803
peg.5608	**Cry2Aa9**[b]	633	46	46	0.5987	149.316	0.1884978
peg.5617	**Cry1Aa18**[b]	520	27	24	0.5635	101.849	0.2065138
peg.5616	Cry1Ia44	750	2	0	0.0167	6.19	0.0489361
peg.4938	**Inhibitor A metalloprotease (InhA1)**	796	8	8	0.0804	21.097	0.0054508
peg.4935	Spore coat-associated protein 1	197	6	6	0.1777	16.659	0.0330371
peg.6532	Spore coat protein CotG	179	5	5	0.1229	19.754	0.0212096
peg.1595	Glycerophosphoryl phosphodiesterase	314	5	5	0.1783	14.459	0.0086363
peg.3277	Choline-binding protein (PcpA)[c]	310	5	5	0.1548	16.069	0.0122468
peg.4409	Spore coat protein (CotB)	169	5	5	0.2781	15.245	0.0192554
peg.2864	Shock protein, Hsp20 family	154	4	4	0.2597	10.137	0.0140873
peg.920	Hypothetical protein	247	4	4	0.1093	12.996	0.0109789
peg.969	DUF3915 domain-containing[c]	122	4	4	0.4016	12.488	0.0222278
peg.570	Forespore-specific protein	213	4	4	0.1549	10.307	0.0101852
peg.3555	Aminopeptidase	466	4	4	0.1438	13.59	0.0046554
peg.4408	Spore coat protein CotB	149	4	4	0.4966	13.189	0.0182
peg.4933	Spore coat-associated protein 2	195	4	4	0.2	10.628	0.016688
peg.688	Chaperone protein DnaK	611	3	3	0.0426	10.54	0.0035506
peg.1529	Uncharacterized protein (YxeE)	109	3	3	0.2018	6.706	0.0199031
peg.3676	DNA-binding protein	170	2	2	0.1882	4.262	0.0063807
peg.2225	Acid endopeptidase	333	2	2	0.0961	6.225	0.0032574
peg.3226	Neutral protease B (NprB)	591	2	2	0.0592	7.248	0.0036708
peg.65	Hypothetical protein	155	2	2	0.2065	6.864	0.0139964
peg.5901	Elongation factor Tu	395	2	2	0.1038	8.342	0.0041192

The main pesticidal proteins and the virulent factor are shown in bold

[a]Annotation based on RASTtk

[b]Classification based on Btoxin_Digger and/or Customized *Bt* database

[c]Descrition based on BLASTx

[d]The number of peptide sequences that are unique to protein

[e]The percentage of the protein sequence covered by the identified peptides

[f]The sum of the ion scores of all peptides that were identified

[g]Normalized spectral abundance factor, calculated using the number of spectra divided by the protein length and then normalized over the total of spectral counts/length for all the proteins in the sample

(Chang et al. 1999; Hire et al. 2008, 2009). *Bt* TOL651 harbors *cry2Aa9*, *cry1Ia44,* and *cryAc5* genes. HD-1 strain, a *kurstaki* subspecies, harbors *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ia, cry2Aa*, *cry2Ab*, and *vip3Aa* genes of pesticidal proteins (Zhu et al. 2015).

In addition, *Bt* TOL651 harbored enterotoxins such as hemolysins genes (hemolysins I, III, and IV) presented in other species of *Bacillus cereus* sensu lato group and detected in new *Bt* strains (Kim et al. 2015; Ma et al. 2020; Lechuga et al. 2020). Although *Bt* is considered a safe bioinsecticide for non-target organisms (Pohare et al. 2021), the presence of these genes in *Bt* TOL651 requires their validation for safe use in crops, as well as this toxicity for humans, since we propose its use as a spore–crystal mixture. Considering the potential antibiotic resistance of *Bt* TOL651, the putative antibiotic resistance genes were screened in the genome sequence, indicating the presence of genes resistant to beta-lactam antibiotics, corroborating other studies (Luna et al. 2007; Kaze et al. 2021). Banik et al. (2019) have demonstrated the sensitivity of *Bt* strains against clinically important antibiotics, indicating it is a safe biocontrol agent for crop application without any harm to consumers. However, additional experimental antibiogram tests need to be conducted to validate the resistance or sensitivity of the TOL651 to antibiotics.

The combined genomic and proteomic analysis described in this work revealed that the spore–crystal mixture of the *Bt* TOL651 has a high proportion of the pesticidal proteins of Cry1Ac5, Cry2Aa9, and Cry1Aa18. These Cry proteins were shown in other studies to be toxic to lepidopteran insect pests (Dammak et al. 2015; dos Santos et al. 2009; Sun et al. 2022). Similarly, genomic and proteomic analysis of the *Bt* 4.0718 strain indicated the expression of Cry2Aa, Cry1Aa, and Cry1Ac (Rang et al. 2015; Huang et al. 2012). The *Bt* HD-1 strain in Caballero et al.'s (2020) study using the same omics approach indicated the expression of Cry1Aa, Cry1Ac, Cry1Ab, and Cry2Aa.

Although present in the genome of *Bt* TOL651, the Cry1Ia44 protein was not detected in the spore–crystal mixture by proteomic analysis. Despite harboring the *cry1Ia* gene sequence, in other *Bt* strains the expression of this protein was also not detected, based on the LC–MS/MS approach (Huang et al. 2012; Quan et al. 2016). This result, corroborating with other studies, has suggested the cryptic nature of the *cry1I* genes based on their absence in parasporal crystals (Gleave et al. 1993; Tailor et al. 1992). However, the genomic and proteomic study of the *Bt* AB1 strain revealed three unique peptides of Cry1Ia, their expression being considered at a trace level (Baragamaarachchi et al. 2019).

Cry1Ac5 was the most abundant spore–crystal of *Bt* TOL651. The gene coding Cry1Ac5 has been described in *Bt* isolates from warehouses in China (Hongyu et al. 2000) and in *Bt* strain isolated from India, toxic to *Spodoptera frugiperda* (Sathyan et al. 2022). In contrast to TOL651, another wild-type *Bt* isolates and commercial HD-1 strain expressed Cry2Aa in greater proportion (Huang et al. 2012; Caballero et al. 2020), indicating that the abundance of Cry-like proteins may change according to the strain.

Bioassays with the spore–crystal mixture showed, in comparison with the commercial strain *Bt* HD-1, that *Bt* TO651 was more toxic for both insects, with *A. gemmatalis* being the most susceptible. Studies showed that the toxicity level of each Cry protein varies with the insect species. For example, Bel et al. (2017) found that Cry1Ac was more toxic than Cry1Aa against *A. gemmatalis*, but the opposite occurred when these proteins were tested on *Chrysodeixis includens* (Walker 1858) (Noctuidae: Lepidoptera). *D. saccharalis* was susceptible to Cry1Aa and Cry1Ac, of which Cry1Ac was more effective (Davolos et al. 2015; Tan et al. 2011). However, this insect was more susceptible to a mixture of Cry1 and Cry2 proteins than when tested separately (Macedo et al. 2012). This can happen because Cry1Ac and Cry2Aa proteins do not share the same midgut receptor binding sites; therefore, a synergic effect can be expected when both proteins are present (Macedo et al. 2012). Cry1Aa, Cry1Ab, and Cry1Ac proteins share binding sites (Davolos et al. 2015). In contrast to HD-1 (Caballero et al. 2020), TOL651 expressed only Cry1Aa18 and Cry1Ac5. This finding has suggested that Cry1Ac has a high affinity in comparison to Cry1Aa for the shared binding site, due to divergences in domain II of proteins (Hernández-Rodríguez et al. 2013). So, the presence of Cry1Aa18 and Cry1Ac5 in the spore–crystal mixture of TOL651 may not affect the action of Cry1Ac5. Thus, our results suggest that a major proportion of Cry1Ac5 protein in the spore–crystal mixture along Cry1Aa18 and Cry2Aa9 proportions may explain the toxicity of *Bt* TOL651 against *A. gemmatalis* and *D. saccharalis*.

In addition to Cry proteins, the inhibitor A metalloprotease (InhA1) was also detected in the spore–crystal mixture of *Bt* TOL651. InhA1 produced early in the sporulation phase allows the neutralization of the host immune system by specifically degrading the insect attacin and cecropin proteins (Miyoshi and Shinoda 2000; Pohare et al. 2021). Besides, Dammak et al. (2015) mentioned that InhA1 within a spore–crystal mixture can enhance the pathogenic effect of Cry1–Cry2 proteins, since it can cause disorganization of the intestinal epithelium and delay a possible resistance caused by the intensive use of Cry proteins. Although InhA1 expression has been reported in a spore mixture of *Bt* strains (Banik et al. 2019; Khorramnejad et al. 2020), researches have indicated the absence of InhA1 in the late sporulation phase (Li et al. 2012), and different levels in transcription and expression of InhA1 between *Bt* strains, suggesting a possible strategy to adapt to various hosts (Zhu et al. 2015). The deficiency in the expression of the virulence factor camelysin also produced for *Bt* could be involved in the lack of expression of InhA (Yin et al. 2011).

Neutral protease B (NprB) (also named NprA and Npr99) was also present in the spore–crystal mixture of *Bt* TOL651 and associated with the virulence of *Bacillus cereus*, degrading host tissues and resulting in increasing tissue permeability to the pathogen (Chung et al. 2006). Heat shock protein Hsp20 and the elongation factor Tu were also detected in the spore–crystal mixture of *Bt* TOL651 and are necessary for the formation of crystals in *Bt* strains (Ding et al. 2009). The Hsp20 protein supports other proteins in refolding and preventing protein degradation (Xie et al. 2019).

Biopesticidesare commonly used in multi-strain consortia and represent better cost-effectiveness than constructing recombinant or purified toxins for the development of products (Sreshty et al. 2011). The synergism between different crystalline proteins produced by two *Bt* strains that do not compete for the same binding site has shown enhanced activity against lepidopteran pests (Konecka et al. 2012). The genes and proteins identified in the genome–proteomic step study of *Bt* TOL651 will experimentally facilitate the determination of the potential of synergism between TOL651 and other strains.

In conclusion, our findings showed the potential use of the Brazilian *Bt* TOL651 strain in the control of *D. saccharalis* and *A. germmatalis*, of which *A. germmatalis* was most susceptible. *Bt* TOL651 was closely related to *kenyae*

subspecies and expressed mainly Cry1Aa18, Cry1Ac5, and Cry2Aa9 pesticidal proteins in the spore–crystal mixture, with Cry1Ac5 being the most abundant protein. The virulence factor InhA1 may contribute to the pathogenicity of *Bt* TOL651. The genomic–proteomic approach used in this study allowed a better understanding of *Bt* TOL651 pathogenicity, representing an important step for the development and monitoring of potential new bioinsecticides.

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Author contributions RWSA, GBA, and EEO designed the experiments. GBA, RWSA, and BMR performed the genomic and proteomic data analysis. GBA, LOVJ, GRS, BMR, and RWSA drafted the manuscript. RWSA, EEO, LOVJ, BMR, MAO, and MMS revised the manuscript. All authors read and approved the final manuscript.

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Data availability All data and code generated appear in the submitted article.

Declarations

Conflict of interest The authors declare no financial interest.

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