Whole Genome Sequencing Analysis of *Bacillus thuringiensis* GR007 Reveals Multiple Pesticidal Protein Genes

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*Bacillus thuringiensis* (Bt) are soil ubiquitous bacteria. They produce a great variability of insecticidal proteins, where certain of these toxins are used worldwide for pest control. Through their adaptation to diverse ecosystems, certain *Bt* strains have acquired genetic mobile elements by horizontal transfer, harboring genes that encode for different virulent factors and pesticidal proteins (PP). Genomic characterization of *Bt* strains provides a valuable source of PP with potential biotechnological applications for pest control. In this work, we have sequenced the complete genome of the bacterium *Bt* GR007 strain that is toxic to *Spodoptera frugiperda* and *Manduca sexta* larvae. Four replicons (one circular chromosome and three megaplasmids) were identified. The two largest megaplasmids (pGR340 and pGR157) contain multiple genes that codify for pesticidal proteins: 10 *cry* genes (*cry1Ab*, *cry1Bb*, *cry1Da*, *cry1Fb*, *cry1Hb*, *cry1Id*, *cry1Ja*, *cry1Ka*, *cry1Nb*, and *cry2Ad*), two *vip* genes (*vip3Af* and *vip3Ag*), two binary toxin genes (*vpa2Ac* and *vpb1Ca*), five genes that codify for insecticidal toxin components (Tc’s), and a truncated *cry1Bd*-like gene. In addition, genes that codify for several virulent factors were also found in this strain. Proteomic analysis of the parasporal crystals of GR007 revealed that they are composed of eight Cry proteins. Further cloning of these genes for their individual expression in *Bt* acrystalliferous strain, by means of their own intrinsic promoter showed expression of seven Cry proteins. These proteins display differential toxicity against *M. sexta* and *S. frugiperda* larvae, where *Cry1Bb* showed to be the most active protein against *S. frugiperda* larvae and *Cry1Ka* the most active protein against *M. sexta* larvae.

Keywords: genome sequencing, *Bacillus thuringiensis*, Cry toxin, MS/MS, biocontrol

INTRODUCTION

The *Bacillus cereus* sensu lato group encompasses several ubiquitous, endospore-forming Gram-positive bacteria including *B. cereus*, *B. anthracis*, and *Bacillus thuringiensis* (*Bt*). Despite their close phylogenetic relationship, their pathogenic properties are contrasting, since *B. anthracis* causes anthrax disease, *B. cereus* produces food poisoning in mammals, and *Bt* is a well-known pathogen...
of invertebrates (Helgason et al., 2000). Phylogenetic analysis showed that among the species of B. cereus sensu lato group, B. cereus and Bt are the closest bacteria. The main differences between them are found in their mobile genetic elements (MGE), such as insertion sequences, prophages, and plasmids. It was proposed that the acquisition of such MGE in B. cereus and Bt was facilitated by the absence of a functional CRISPR-Cas systems in many of the B. cereus sensu lato strains, allowing incorporation of novel genetic elements that may help for their better adaptation to diverse ecological niches (Zheng et al., 2020).

Due to their insecticidal properties, Bt have been exploited worldwide as biological pesticides for the control of multiple insect pests (Sanahuja et al., 2011). These bacteria are characterized by the production of pesticidal proteins (PPs) during their sporulation phase of growth. Most of these PPs accumulate as parasporal inclusions within the mother cell compartment and have been recently renamed in the Bacterial Pesticidal Protein Resource Center (BPPRC) as follows: PPs belonging to the three domain crystal proteins retained the mnemonic name of Cry, while proteins related to Cyt family retained the mnemonic name of Cyt. PPs with homology to Etx-Mtx2 family are now named Mpp. The PPs with homology to aegerolysin are Gpp. Those with homology to the ‘Toxin10/Bin family received the name of Tpp and the PPs with a predominantly alpha helical structure are named App (Crickmore et al., 2020). In addition, some Bt bacteria also produce and secrete additional PPs during their vegetative growth, such as proteins related to the vegetative insecticidal protein Vip3 that retained the mnemonic name of Vip, while the proteins related to the catalytic component of Vip2 are now named Vpa, and the PPs related to the Vip1 binding partner from the binary toxin are now recognized as Vpb (Crickmore et al., 2020). All these proteins are encoded in large megaplasmids as individual genes or grouped in pathogenic islands (PAI), accompanied by repeat sequences, insertion elements, and transposases, which may allow a higher recombination rate among diverse Bt strains (Mahillon and Chandler, 1998; Fiedoruk et al., 2017).

In searching novel PPs produced by Bt, that could be useful for pest control in agriculture or farming activities, a large number of Bt strains have been isolated from insect corpses, soil, and phyloplane. Characterization of such strains is not an easy task. Probably, the faster and low-cost approach to screen insecticidal toxins from a Bt strain collection is the traditional PCR strategy, either using a set of universal primers or designed specific primers (Cerón et al., 1994; Cerón et al., 1995; Porcar and Juárez-Pérez, 2003). In addition, also DNA-hybridization and DNA-microarray techniques were shown to be useful for the identification of specific cry genes (Beard et al., 2001; Letowski et al., 2005). However, these DNA-based techniques are limited to detect genes that were previously identified and lack information regarding to their expression. Proteomic analysis of parasporal crystal is more accurate to determine the presence and abundance of PPs expressed in specific Bt crystal inclusions, and such studies have been performed to identify the PPs produced by Bt subsp

1https://www.bpprc.org/

Materials and Methods

Genomic DNA Purification

Bt GR007 strain was obtained from a recent screening of soil samples from the state of Morelos, Mexico, and selected for its toxicity against S. frugiperda larvae. Bt GR007 was cultured in LB liquid medium for 12 h at 30°C and bacteria were harvested by centrifugation. Genomic DNA was purified using AxyPrep Bacterial genomic DNA miniprep kit from Axygen (Corning Life Sciences, Glendale AZ) following the manufacturer’s instructions. DNA was analyzed by

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Pacheco et al.
electrophoresis in agarose gel and quantified in NanoDrop 2000 spectrophotometer (Thermo Scientific). The 20 kb templates used for sequencing were prepared by using Blue Pippin Size-Selection System (Sage Science, Beverly, MA) that allows resolving and collecting high molecular weight DNA, according to the manufacturer's instructions.

DNA Sequencing and Assembly
The genomic sequence of *Bt* GR007 was obtained by Beijing Sinobiocore Biological Technology Co., using PacBio RSII platform. A total of 138,274 reads were obtained which represent 928,053,411 bp and total 151X fold coverage of the genome. All reads were assembled by using the Single Molecule, Real-Time (SMRT) sequencing data SMRTPipe v 2.3.0 from PacBio.\(^2\) Annotation of coding sequences (CDS), ribosomal RNA (rRNA), transfer RNA (tRNA), and miscellaneous RNA (miscRNA) were performed using Prokka v1.11 software (Seemann, 2014). The identity of CDS was searched using SwissProt and BLAST (nr/nt) databases. Classification of protein in Clusters of Orthologous Groups (COG) was performed using eggNOG-mapper (Huerta-Cepas et al., 2017). Insertion sequences were identified using ISFinder database (Sigier et al., 2006). Identification of ProPhages regions was performed using Phage_Finder (Fouts, 2006). Identification of CRISPR arrays were predicted using PILERCR v1.06 software (Edgar, 2007). Circular genome maps and plasmid comparison were generated using GView server (Petkau et al., 2010).

Construction of Single Nucleotide Polymorphism Phylogenomic Tree
In this work we selected to work with the reported assembled genomes from different *Bt* strains that were found in the NCBI database. In this database we found genomic information of 670 Bt strains. However, only 63 genomes and 21 chromosomes are completely assembled. After taking out sequences that were duplicated, we downloaded 73 completely assembled genomes from this database. We used the Parsnp tool from the Harvest Suite software for fast multiple alignment of genomic sequences based on single nucleotide polymorphism (SNP) (Treangen et al., 2014). We ran Parsnp version 1.2 with the -c parameter. The phylogenomic tree was visualized and stylized in iTOL server (Letunic and Bork, 2019).

Cloning of Cry Toxins
With the aim to clone the regulatory and terminator regions of each cry gene, PCR oligonucleotides were designed to amplify a fragment containing the cry gene flanked by ~450 bp at 5’ and 3’ ends (Table 1). All PCR were performed with Phusion HD DNA Polymerase (Thermo Scientific Fisher) using as template the genomic DNA from *Bt* GR007 strain. The cry genes were cloned into pHT315 shuttle vector (Arantes and Lereclus, 1991) using In-Phusion HD Cloning Kit ( Takara, Shiga, Japan) following the manufacturer's instruction. Briefly, pHT315 linearized with *SmaI* endonuclease was fused with the PCR fragments using In-Phusion Kit enzyme premix during 15 min at 50°C, and the cloning reaction was electrotransformed into *E. coli* DH5α cells. Transformant cells were analyzed by colony PCR and plasmids of positive clones were purified and further sequenced.

| Primer | Sequence (5’→3’) |
|---|---|
| 1AbF | CTCTAGAAGATTCCTGCTAGATAATTTTTGTCATCGC |
| 1AbR | TCGAGCTCGGTACCCTTAAATGTAATTACAAGTAAATGG |
| 1BbF | CTCTAGAAGATTCCTGCTAGATAATTTTTGTCATCGC |
| 1BbR | TCGAGCTCGGTACCCTTAAATGTAATTACAAGTAAATGG |
| 1DaF | CTCTAGAAGATTCCTGCTAGATAATTTTTGTCATCGC |
| 1DaR/FbR | TCGAGCTCGGTACCCTTAAATGTAATTACAAGTAAATGG |
| 1DaR | TCGAGCTCGGTACCCTTAAATGTAATTACAAGTAAATGG |
| 1DaR/FbR | TCGAGCTCGGTACCCTTAAATGTAATTACAAGTAAATGG |
| 1DaR | TCGAGCTCGGTACCCTTAAATGTAATTACAAGTAAATGG |
| 1DaR/FbR | TCGAGCTCGGTACCCTTAAATGTAATTACAAGTAAATGG |
| 1DaR | TCGAGCTCGGTACCCTTAAATGTAATTACAAGTAAATGG |
| 1DaR/FbR | TCGAGCTCGGTACCCTTAAATGTAATTACAAGTAAATGG |
| 1DaR | TCGAGCTCGGTACCCTTAAATGTAATTACAAGTAAATGG |
| 1DaR/FbR | TCGAGCTCGGTACCCTTAAATGTAATTACAAGTAAATGG |

Parasporal Crystal Production
Plasmids harboring cry genes were electrotransformed into the acrylstitialiferous *Bt* strain 407, as previously described (Macaluso and Mettus, 1991). *Bt* strains were grown during 72 h at 30°C in HCT medium (Lecadet et al., 1980) supplemented with 10 µg/ml erythromycin. After sporulation, spore/crystal mixture was recovered and washed three times with wash solution (300 mM NaCl, 10 mM EDTA) and three times with 1 mM PMSF. After these washing steps the spore/crystal mixture was then suspended in ddH₂O. These samples were directly suspended in SDS-PAGE Laemmli buffer, heated 3 min at 100°C, and analyzed by 10% SDS-PAGE. Protein concentration of the final spore/crystal mixtures suspended in ddH₂O was estimated by Bradford method using a BSA standard curve as reference.

Liquid Chromatography-Mass Spectrometry Analysis
Spore/crystal inclusion suspensions of *Bt* GR007 were mixed in Laemmli loading buffer, boiled for three min, and proteins were separated on a 10% SDS-PAGE. Protein bands were excised, digested “in gel” with trypsin, and desalted. Cleaved peptides were subsequently analyzed by Liquid Chromatography-Mass Spectrometry (LC-MS) system composed of a nanoflow pump EASY-nLC II and mass spectrophotometer LTQ-Orbitrap Velos (Thermo Fisher) at the proteomic facility of the Institute of Biotechnology from National Autonomous University of Mexico. For protein identification, data were screened against the Cry and Vip protein sequences obtained from genome sequencing of this work using the Proteome Discoverer software. A minimum False-positive rate of 0.01 was used for accepting protein identifications.

\(^2\)https://www.pacb.com/support/software-downloads/
and the 50% lethal concentration (LD50) for the negative control. Mortality was analyzed after 7 days, thus in each bioassay 432 larvae were analyzed for the 6 concentration of spore/crystal dose in triplicate (a total of 72 24 well polystyrene plates and one plate was used per each control was water added to the surface of the diet. We used 2^Cry1Hb proteins were tested up to 5000 ng/cm^2 larvae we used the following concentrations: 70, 35, 17.5, 8.7, 4.3, and 2.1 ng/cm^2. For bioassays against M. sexta larvae we used the following concentrations: 2000, 1000, 500, 250, 125, and 62 ng/cm^2. The negative control was water added to the surface of the diet. We used 24 well polystyrene plates and one plate was used per each concentration of spore/crystal dose in triplicate (a total of 72 larvae were used per toxin concentration in each repetition, thus in each bioassay 432 larvae were analyzed for the 6 different concentrations of spore/crystal mixtures and 72 larvae for the negative control). Mortality was analyzed after 7 days and the 50% lethal concentration (LC50) was calculated with Probit LeOra software. Mortality of the control was lower than 5%. Complete bioassays with three technical replicates were performed in duplicate (864 larvae in total per bioassay performed with each toxin).

RESULTS

Genome and Phylogenetic Analysis of Bt GR007

Bt GR007 strain was selected due to its toxicity against S. frugiperda during preliminary screening assays compared to other native strains. In order to gain access to the gene sequences of potential PP in Bt GR007 strain, the whole genome of this strain was obtained and assembled (BioProject: PRJNA736034). The genome of this strain consists of four replicons, the chromosome and three plasmids (Figure 1A). The circular chromosome contains 5,659,016 bp with 36.2% GC content (Gene Bank Accession number CP076539). A total number of 5,714 CDS were predicted, from which 4,746 genes were identified using the database of SwissProt and BLAST analysis. The predicted proteins were further classified with COG functional assignation using eggNOG database. The chromosome also contains 30 rRNAs (10 23S, 10 16S, and 10 5S), 104 tRNAs, 145 miscrRNAs, and one tmRNA. DNA regions related with genetic mobility were found along the chromosome, among these we found seven large prophage regions and 89 insertion sequences for transposases (Tnp). In agreement with most of the Bt strains that have been sequenced previously, Bt GR007 lacks a functional CRISPR system (Zheng et al., 2020; Table 2).

The three extrachromosomal plasmids pGR340, pGR157, and pGR55 consist of 340,862, 157,685, and 55,760 bp, respectively (Figure 1A) (Gene Bank accession numbers CP076541, CP076540, and CP076542). The two largest plasmids, pGR340 and pGR157, harbor the genes that encode for potential PPs, which are grouped in PAIs, and their annotation was done according to the new database of PPs (see text footnote 1). The plasmid pGR340 contains three PAIs designed as PAI-1.1, PAI-1.2, and PAI-1.3 (Figure 1B). The length of PAI-1.1 is 88.2 kbp and contains seven cry genes encoding for lepidopteran specific Cry proteins (Cry1Da, Cry1Id, Cry1Ja, Cry1Nb, Cry1Ab, Cry1Bb, and Cry1Hb) and a cluster of five genes coding for insecticidal toxin components (Tcs) (TccC1, TccC6, TcC, TdC2, and TcA). The PAI-1.2 of 17.3 kbp contains two genes encoding for PPs, named Vip3Aa and Cry2Ad. The PAI-1.3 with a length of 16.5 kbp has an homologous gene that codifies for a protein related to the Etx/Mtx2 toxin family (recently designed as Mpp). This PAI-1.3 island also contains genes that codify for the binary toxins Vpa2Ac and Vpb1Ca (old names were Vip2Ac and Vip1Ca, respectively) arranged in an operon. The plasmid pGR157 contains a single PAI named PAI-2.0 of 26.5 kbp that contains two genes encoding for Cry proteins (Cry1Fb and Cry1Ka) and one gene encoding for a Vip protein (Vip3Ag). In addition, pGR157 contains a gene for a non-functional truncated Cry1Bd-like protein since it lacks the N-terminal region that is present in the Cry1Bd protein (NCBI Acc. No AAD10292). All PAIs were associated with Tnp’s (displayed as blue arrows in Figure 1B). Supplementary File 1 contains the detailed information of Bt GR007 genome annotation.

Bt encodes a large number of virulence-associated genes. We searched for these genes and found that most of them were found in the chromosome of GR007 strain (Table 3), while some virulence factors were found in PAI 1.1 such as cytolysin operon (hblL2, hblL1, and hblB), the phospholipase C (plc-6), subtilisin (sub-8), the cell wall binding/hydrolisis protein (cwl-9), and internalin (inlA-5) (Table 3). The PAI2.0 island also codifies for a cell wall hydrolase (cwl-3) (Table 3).

To investigate the phylogenetic relationship of Bt GR007 strain with other previously sequenced Bt strains, we constructed a phylogenomic tree based on SNP of their circular chromosomes (Figure 2). Figure 2 shows that Bt GR007 strain was grouped in a clade of Bacillus strains that kill insects and nematodes, such as Bt subsp. israelensis (Dipteran-specific), Bt subsp. morrisoni BGSC 4AA1 (Coleptaran-specific), and Bt YBT-1518 (Nematoda-specific). However, the overall structure of this phylogenomic tree showed a low phylogenetic correlation among the lineage of Bt GR007 with other well characterized Lepidopteran-specific Bt strains, such as the HD1, HD73, and YBT1250 strains that were grouped in a different branch. Based on the data of the phylogenomic tree, the closest Bt strain to GR007 was Bt HD12 strain. The Cry proteins content of Bt GR007 is similar to Bt HD12 (BioProject: PRJNA302106), with the exception that the latter strain lacks cly1Fb and vip3Ag genes. In addition, the number of plasmids is different between these two strains, since Bt HD12 contains six plasmids (named as pHD120017, pHD120038, pHD120039, pHD120112, pHD120161, and pHD120345) (Gene Bank Accession Numbers CP014848.1 to CP014853.1). A detailed analysis of the plasmids present in these two strains showed that pGR340 from GR007 strain was essentially identical to pHD120345 from HD12 strain.
(AN CP014853.1) and contains the same number of the PAIs, while pGR157 and pGR55 from GR007 strain matched with pHD120161 from HD12 strain (AN CP014852.1), suggesting a recombination of these two plasmids in Bt GR007 to form a single plasmid lacking cry1Fb and vip3Ag genes (pHD120161) in Bt HD12 strain (Supplementary Figure 1).
TABLE 2 | Features of the genome of Bacillus thuringiensis GR007 strain.

| Chromosome | pGR340 | pGR157 | pGR55 |
|------------|--------|--------|-------|
| Size (bp)  | 5,659,016 | 340,862 | 157,685 | 55,760 |
| GC content (%) | 36.2 | 34.5 | 33.5 | 35.5 |
| CDS  | 5,714 | 290 | 154 | 77 |
| tRNA  | 30 | – | – | – |
| tRNAs | 104 | – | – | – |
| miscRNA | 145 | 8 | 1 | – |
| tmRNA | 1 | – | – | – |
| COG assignment | 4,746 | 150 | 75 | 25 |
| Pre-phages | 7 | – | – | – |
| Insertion sequences | 89 | 19 | 9 | – |
| CRISPR arrays | – | – | – | – |

Proteomic Analysis of the Proteins Expressed in the Crystal Inclusion of GR007

A proteomic analysis was performed to analyze the PP's composition of the parasporal crystal produced by Bt GR007 strain. The strain was grown until the sporulation phase and parasporal crystals were separated by SDS-PAGE. Proteins with apparent molecular weight of 130, 95, 72, and 32 kDa, which correspond to the molecular weight of PPs identified in the megaplasmids, were excised directly from the gel after Coomassie blue staining (Figure 3, lane Bt GR007) and analyzed by LC-MS analysis. Peptides identified by LC-MS matched to eight Cry proteins (Cry1Ab, Cry1Bb, Cry1Da, Cry1Fb, Cry1Hb, Cry1Ja, Cry1Ka, and Cry1Nb) showing high sequence coverage in the 130 and 72 kDa bands, that correspond to protoxin and activated toxin, respectively (Table 4).

Characterization of Pesticidal Cry Proteins

To further analyze the Cry proteins expressed in Bt GR007, we cloned all cry genes identified in this strain to evaluate their toxicity when expressed in the acrystalliferous Bt strain 407. The cry genes codifying for Cry1Ab, Cry1Bb, Cry1Da, Cry1Fb, Cry1Hb, Cry1Ja, Cry1Ka, and Cry1Nb proteins were identified in the pGR340 and pGR157 megaplasmids. Oligonucleotides were designed to amplify via PCR the complete gene including a DNA fragment approximately 450 bp upstream of the start codon of the gene, and 450 bp after the stop codon. We expected that these DNA fragments should contain the intrinsic promotor and terminator regions from each cry gene for their individual expression. These DNA fragments were cloned in pHt315 shuttle vector and electrotransformed into acrystalliferous Bt 407 strain. All transformant Bt strains were grown until sporulation and the spore/crystal mixtures were analyzed by SDS-PAGE. Cry1Ab, Cry1Bb, Cry1Da, Cry1Fb, Cry1Hb, Cry1Ja, and Cry1Ka proteins were successfully produced (Figure 3). The Cry1Id and Cry2Ad that were not identified in the parasporal crystal were also cloned, but these proteins were not found as inclusion bodies when transformed into Bt 407 (Supplementary Figure 2).
We analyzed the promoter region of these genes, the upstream nucleotide sequence was aligned with the cry1Ab promoter region that is depend in SigK or SigK factors for its expression. The promoter regions of cry1Da, cry1Ja, cry1Bb, cry1Hb, cry1Fb, and cry1Ka are similar to cry1Ab, while promoter regions from cry1Nb, cry1Id, and cry2Ad showed different sequences,
suggesting that their regulation is not dependent on SigE and SigK factors (Supplementary Figure 3).

The toxicity of GR007 strain was tested against two lepidopteran insects, *S. frugiperda* and *M. sexta*. We also assayed insecticidal activity against the dipteran insect, *Aedes aegypti*. Toxicity of this strain was confirmed only for the two lepidopteran larvae while no toxicity was observed against dipteran insects (data not shown). Finally the toxicity of the individual Cry1Ab, Cry1Bb, Cry1Da, Cry1Fb, Cry1Hb, Cry1Ja, and Cry1Ka proteins was analyzed in bioassays against *S. frugiperda* and *M. sexta* larvae. Table 5 shows that all Cry toxins were highly toxic to *M. sexta* larvae but showed differential toxicity against *S. frugiperda*. The Cry1Bb protein was the most active toxin against *S. frugiperda* with an LC50 value of 128.5 ng/cm² while Cry1Hb and Cry1Ka showed no toxicity even at the highest concentration of protein that was used in the bioassay (5000 ng/cm²) (Table 5).

**DISCUSSION**

Whole genome sequence screening of *Bt* strains has been one of the most successful approaches for PP gene discovery in recent years. In addition, it has provided data for the analysis of the
showed that in different plasmids. Phylogenetic analysis of whole genomes system, which correlated with its high PP gene content, encoded and other PP genes found in PAI-1.3 island of this strain, such as against lepidopteran insects (Crickmore et al., 2020). However, Cry1 proteins expressed in the crystal inclusions that are specific insects. This insect specificity correlated with the identification of Bt GR007 strain showed insecticidal activity against lepidopteran toxic activity against different insect targets (Wang et al., 2020). The genomic analysis allowed the identification of multiple PAI islands. The PAI-1.1 is the longest PAI region in this strain, containing seven different cry genes. All of them codify for lepidopteran specific Cry proteins (Cry1Da, Cry1Id, Cry1Ja, Cry1Nb, Cry1Ab, Cry1Bb, and Cry1Hb) (Crickmore et al., 2020). This PAI-1.1 also codifies for a cluster of five insecticidal toxin components (Tcs). The Tcs proteins were originally identified in enterobacteria P. luminescens and Xenorhabdus nematophila, which are symbiont of nematodes (Waterfield et al., 2001). These Tcs proteins are pore-forming toxins that kill lepidopteran and dipteran insects. It was previously shown that tcs genes may be found in other bacteria such as Serratia entomophila that is also an insect pathogen or Yersinia pestis, that is mammalian pathogen transmitted by an insect vector (Waterfield et al., 2001). It was also reported that some Bt strains may harbor these genes and that tcaA and tcaB genes were expressed during the infection of gypsy moth larvae by these Bt strains (Blackburn et al., 2011). In the case of GR007 strain, it still remains to be demonstrated if these Tcs proteins participate in toxicity.

The PAI-1.2 contains only two genes encoding for PPs (Vip3Af and Cry2Ad), which are also recognized for their toxicity against lepidopteran insects (Estruz et al., 1996; Liao et al., 2015). In contrast the PAI-1.3 codifies for proteins that have been associated with toxicity against different insect orders, like the PP that shows 34.6% identity with Mpp4Aa from Lysinibacillus sphaericus that belongs to the Mtx2-protein family active against mosquito larvae (Rey et al., 2016). In Bt the Mtx2 like-toxins (now named as Mpp or Gpp) are toxic evolution of the Bt genome and analysis of horizontal gene transfer events that have contributed to the evolution of Bt strains (Zheng et al., 2017). Bt bacteria are characterized by the presence of insecticidal proteins that accumulate in crystal inclusions during their sporulation phase of growth and such proteins are codified in different plasmids. It was shown that the B. cereus group selectively inactivated the CRISPR Cas system, which correlates with acquisition of mobile elements, such as different plasmids containing genetic information that could help in the adaptation of these bacteria to diverse environments (Zheng et al., 2020). Bt GR007 strain did not contain a functional CRISPR Cas system, which correlated with its high PP gene content, encoded in different plasmids. Phylogenetic analysis of whole genomes showed that Bt GR007 strain (BioProject: PRJNA736034) is closely related with Bt HD12 strain (BioProject: PRJNA302106). Interestingly, the genome sequence comparison between both strains suggested that HD12 might had a genome rearrangement where information contained in plasmids pGR157 and pGR55, that are present in Bt GR007 strain, recombined to form a single large plasmid named pH12016 in HD12 strain, loosing cry1Fb and vip3Ag genes during this recombination process.

It is now recognized that different Bt strains harbor several PP that in theory may expand their host specificity by improving its toxic activity against different insect targets (Wang et al., 2020). Bt GR007 strain showed insecticidal activity against lepidopteran insects. This insect specificity correlated with the identification of Cry1 proteins expressed in the crystal inclusions that are specific against lepidopteran insects (Crickmore et al., 2020). However, other PP genes found in PAI-1.3 island of this strain, such as vpa2Ac and vpb1Ca genes and the putative novel mpp gene, suggest that it may also display also coleopteran toxicity, but this hypothesis remains to be studied in the future.

| Protein | Manduca sexta | Spodoptera frugiperda |
|---------|---------------|----------------------|
| Cry1Ab  | 133.3         | 52                   |
| Cry1Bb  | 145.0         | 46                   |
| Cry1Da  | 131.9         | 46                   |
| Cry1Fb  | 132.3         | 46                   |
| Cry1Hb  | 132.5         | 24                   |
| Cry1Ja  | 132.6         | 50                   |
| Cry1Ka  | 137.1         | 63                   |
| Cry1Nb  | 76.9          | 6                    |

Table 5: Toxicity of parasporal crystals against insect larvae.
to other insect orders, such as the Mpp64Ba and Mpp64Ca toxins (previously known as Cry64Ba and Cry64Ca) that are highly active against hemipteran pests (Liu et al., 2018), or Gpp34Aa/Tpp35Aa proteins (previously known as Cry34/Cry35) that showed toxicity against coleopteran pests (Kelker et al., 2014). However, the new PP found in GR007 strain was not classified in the BPPRC web site (see text footnote 1), since we lack information of its pesticidal activity and its identity with other Bt PPs is lower than 35%. This PAI-1.3 island also contains genes that codify for the binary Vpa2Ac and Vpb1Ca toxins, that were previously shown to be toxic to coleopteran larvae (Bi et al., 2015). However, all these PP proteins were not detected in the parasporal crystal inclusion of Bt GR007 so their expression and insect specificity remains to be analyzed. Finally The PAI2.0 island contains three genes encoding for PP that have been shown to be toxic to lepidopteran larvae (Cry1Fb, Cry1Ka, and Vip3Ag) (Koo et al., 1995; Estruch et al., 1996; DaSilva et al., 2016). The Vip3A proteins are expressed in the vegetative phase of growth and are secreted into the medium. The expression of Vip3Ag and Vip3Af in GR007 strain remains to be characterized.

All PAIs from GR007 strain showed multiple Tnp’s sequences (blue arrows in Figure 1B). It has been proposed that Tnp sequences have contributed to the transfer of PP genes and their recombination among different Bt strains to generate the great variability of these proteins (Mahillon and Chandler, 1998).

Analysis of non-toxin virulence factors produced by Bt GR007 strain showed that most of them are codified in the chromosome of this bacterium (Table 3). It has been proposed that Chitinases, collagensases, cell wall hydrolases, phospholipases, and proteases such as metalloproteases, camelysin (a surface metalloproteaseinase), bacilloylins (a neutral proteinase), and subtilisin (a serine protease) may be important for efficient larval body utilization during the infection process (Malovichko et al., 2019). Enhancin and collagensase proteins may also participate in the utilization of insect tissues during the last stages of saprophytic colonization (Malovichko et al., 2019). The cytolsins such as hblL2, hblL1, and hblB, a three-component hemolitic complex, was shown to be produced by B. cereus sensu lato strains and has been implicated as a cause of diarrhea associated with food poisoning (Worthy et al., 2021). These cytolsin genes and other cytolsin genes such as hly II and hly III, xhlA, ilyA were found in the chromosome of GR007 strain. Finally, the internalxin proteins were initially described in Listeria monocytogenes, playing an important role in cell invasion (Dramsi et al., 1997). In the case of B. cereus it was proposed that internalins may play a role during pathogenesis and that they are induced in insects after oral ingestion (Fedhila et al., 2006). All these virulence factors may potentially add competitive advantages to the Bt strains, improving their toxicity to the target insect as previously suggested (Malovichko et al., 2019).

Here, we cloned the cry genes found in GR007 strain and analyzed their expression and toxicity against two lepidopteran insects, M. sexta and S. frugiperda. The toxicity of the Bt GR007 strain to these insect pests was relatively low compared to the toxicity of some Cry proteins expressed individually (Table 5). Seven proteins were successfully expressed in Br cells. The toxicity of Cry1Ab against M. sexta (LC50 8.6 ng/cm2) was relatively low when compared to previous reports that have showed LC50 values two or threefold lower (Gómez et al., 2018; Pacheco et al., 2018). In contrast, the toxicity of this protein against S. frugiperda showed similar values to other reports (Gómez et al., 2018). However, it is important to mention that it was shown that different S. frugiperda populations showed great variability in their susceptibility to Cry1Ab, ranging from not susceptible at all, up to highly susceptible (Gorser et al., 2017; Gómez et al., 2018; Figueiredo et al., 2019). The Cry1Bb protein showed to be highly active against M. sexta with LC50 value of 5.4 ng/cm2 and this protein was the most active toxin against S. frugiperda among all tested proteins in this work, with a LC50 value of 128.5 ng/cm2 (Table 5), showing two times higher toxicity to S. frugiperda than previously reported (Luo et al., 1999). The toxicity of Cry1Da against M. sexta and S. frugiperda was similar to previous reports (Höfte and Whiteley, 1989; Wang et al., 2019). In the case of Cry1Fb, Cry1Hb, and Cry1Ka there are no reports previous in the literature about the toxicity of these proteins against these target insects. It was only shown that Cry1Fb was active against Agrotis ipsilon and was not toxic against Heliothis virescens (de Maagd et al., 2003; Karlova et al., 2005); and that Cry1Ka was active against Afogeia rupae, but not active against Plutella xylostella, Spodoptera exigua, or Bombbyx mori (Koo et al., 1995). Finally the toxicity of Cry1Ja was previously analyzed against S. exigua showing high toxicity (Herrero et al., 2001; Ibarguti et al., 2006) and the reported toxicity of Cry1Ja against M. sexta was much higher than the LC50 value reported in this work (Herrero et al., 2001).

Spodoptera frugiperda has been shown to be highly susceptible to Cry1Fa and transgenic maize expressing Cry1Fa has been shown to be effective in controlling this insect pest. However, S. frugiperda has evolved resistance to Cry1Fa-maize in Puerto Rico, United States, Brazil, and Argentina (Storer et al., 2012; Farias et al., 2014; Chandrasena et al., 2018). Thus, additional Cry proteins that show no cross-resistance to Cry1Fa are likely to provide tools to counter resistance of this pest to transgenic maize. In the case of Cry1Bb, it was shown that this toxin and Cry1Fa share a binding site in the brush border membranes of S. frugiperda suggesting that Cry1Bb and Cry1Fa should show cross-resistance in this insect species (Luo et al., 1999). However, the Vip3Aa has been shown to be effective against the Cry1Fa resistant populations (Welch et al., 2015). The toxicity of the other PP proteins codified in Bt GR007 strain such as Cry1Id, Cry1Nb, Cry2Ad, the new Mpp, Vpa2Ac, Vpb1Ca Vip3Af, and Vip3Ag remains to be analyzed against different target insects including insect populations that have evolved resistance to other Cry proteins in the field.

Finally, we analyzed the promotor region of GR007 PP genes. We found that cry1Da, cry1Ja, cry1Bb, cry1Hb, cry1Fb, and cry1Ka genes have promotor regions highly similar to cry1Ab gene suggesting that all of them may be regulated by SigF factors. Previously the regulation of some cry genes from HD12 strain was analyzed showing that the expression of cry1Ac, cry1Bb, cry1Fb, and cry1Ja genes is regulated by both sigma factors, while cry1Da was only regulated by SigE. However, the problem was that the upstream sequence of cry1Da gene was not fully analyzed (Song et al., 2017). They did not included the −35 region of SigF promoter, resulting in the loss of SigK function when fused to LacZ gene (Song et al., 2017). Here we
show that the cry1Da gene that is present in GR007 strain has the complete promoter region including −35 and −10 DNA sequence for binding of both sigma factors, suggesting that its expression is determined by both sigma factors. Our analysis also showed that promoter regions from cry1Nb, cry1Id, and cry2Ad showed different sequences, suggesting that they do not share the same regulation as the other cry genes present in this strain (Supplementary Figure 3). Blast analysis showed that the promoter region from cry2Ad is similar to the upstream sequence of cry1Fa gene. The promoter region of cry1Id is similar to the upstream sequence of cry1La, while the promotor region of cry1Nb has no similarities to any other sequence including all known upstream sequences from other cry genes.

Overall, our data indicate that in nature certain Bt strains contain and express multiple PP genes codifying for proteins that display activities against different target pest. These strains could be excellent candidates to generate novel formulations. The information provided here regarding the genome of Bt GR007 strain will help with the understanding Bt toxin gene diversity and regulation of its PPs.

DATA AVAILABILITY STATEMENT

The data for this study can be found in BioProject: PRJNA736034 and Gene Bank Accession numbers CP076539, CP076541, CP076540, and CP076542.

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AUTHOR CONTRIBUTIONS

JS collected the sample. SP, IG, and JS conducted the experiments. SP and MC analyzed the data. AB and MS conceived of the idea and designed the study. SP, MS, and AB participated in drafting the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.758314/full#supplementary-material
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