Genome Analysis of Entomopathogenic *Bacillus* sp. ABP14 Isolated from a Lignocellulosic Compost

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Abstract

We report the complete genome sequence of *Bacillus* sp. strain ABP14 isolated from lignocellulosic compost and selected by its ability in hydrolyzing carboxymethyl cellulose. This strain does not produce a Cry-like protein but showed an insecticidal activity against larvae of *Anticarsia gemmatalis* (Lepidoptera). Genome-based taxonomic analysis revealed that the ABP14 chromosome is genetically close to *Bacillus thuringiensis* serovar *finitimus* YBT020. ABP14 also carries one plasmid which showed no similarity with those from YBT020. Genome analysis of ABP14 identified unique genes related to cell surface structures, cell wall, metabolic competence, and virulence factors that may contribute for its survival and environmental adaptation, as well as its entomopathogenic activity.

Key words: *Bacillus cereus sensu lato*, insecticidal activity, entomopathogenic activity.

Introduction

Bacillus spp. comprises Gram-positive, rod-shaped, endospore forming bacteria. Ubiquitous in nature, *Bacillus* species show great catabolic and biosynthetic versatility allowing their survival in several and diverse environments (Slepecky and Hemphill 2006). Several members of the genus *Bacillus* are able to produce and secrete a wide variety of enzymes and biologically active compounds with potential application in medicine, industry, and agriculture (Slepecky and Hemphill 2006).

In agriculture, the bacterium *Bacillus thuringiensis* (*Bt*) has been successfully used by its bioinsecticide properties and it is the best-known *Bacillus* species used as a biopesticide (Bravo et al. 2011). *Bt* is active against the larval stages of lepidopteran, dipteran and coleopteran insects by producing insecticidal pore forming proteins known as Cry and Cyt toxins during sporulation (Bravo et al. 2011). In addition to Cry, some *Bt* strains also produce other insecticidal factors, such as heat-stable beta-exotoxin and vegetative insecticidal proteins which are produced during the vegetative growth phase (Liu et al. 2014; Chakroun et al. 2016). The effective insecticidal properties of Cry toxins allowed their expression in genetic engineered plants leading to commercial crops either resistant to insect attack or with a lower requirement for chemical insect control (Bravo et al. 2011). These crops are commercially available, although resistance to those toxins have been described (McGaughey and Whalon 1992), and several insect pests are not susceptible to Cry toxins, therefore reinforcing the necessity in finding new strains and toxins.

In this work, we report a new *Bacillus* strain, named ABP14, isolated from a lignocellulosic compost and selected by its ability to hydrolyze carboxymethylcellulose (CMC).

This strain showed insecticidal activity against Lepidoptera, nonetheless, analysis indicated that ABP14 does not produce Cry-like toxins. Therefore, in order to investigate the metabolic capacity and the insecticidal activity of ABP14, the whole-genome sequencing and analysis were performed.

Materials and Methods

Bacteria Isolation and Growth

A compost sample was collected from a pile of agricultural waste, on a rural estate at municipality of Tupãssi, Paraná, Brazil (location: –24.728411, –53.511011). Compost sample (10 g) was added to 190 ml 0.5% CMC Medium (K_2HPO_4 1.6 g l^{-1}; KH_2PO_4 0.2 g l^{-1}; $(NH_4)_2SO_4$ 1 g l^{-1}; $MgSO_4{\cdot}7H_2O$ 0.2 g l^{-1}; $FeSO_4{\cdot}7H_2O$ 0.01 g l^{-1}; NaCl 0.1 g l^{-1}; $CaCl_2{\cdot}2H_2O$ 0.02 g l^{-1}; yeast extract 1 g l^{-1}; CMC 5 g l^{-1}; pH 7.2) in 500 ml erlenmeyer flasks and incubated for 4 days in a shaker incubator at 37 °C at 120 rpm. Enrichment for cellulose-degrading bacteria was performed 5 times by inoculating 10 ml of the culture into fresh 190 ml 0.5% CMC medium, every 5 days. Then, a dilution of the fifth enrichment step was plated onto 0.5% CMC solid medium (with 12 g l^{-1} bacteriological agar). Plates were incubated at 37 °C for 24 h and morphologically different colonies were picked, restreaked on 0.5% CMC solid medium, and incubated at 37 °C for 3 days. Bacterial colonies able to degrade CMC were visualized upon staining with 0.1% Congo Red solution (Teather and Wood 1982). Isolates were kept at 4 °C on solid medium and stored in skim milk at –80 °C (Cody et al. 2008). One strain showed higher CMC degrading activity was named ABP14 and further analyzed.

Bacterial Phenotypic Characterization

Morphology and cell wall properties were verified by Gram staining. For temperature growth assessment, one fresh colony was inoculated onto nutrient medium (NA) plates, and incubated individually at 4 up to 50 °C. Plates were monitored daily for 14 days. Motility, rhizoid growth, hemolytic activity, and production of crystal toxin were performed based on FDA Bacteriological Analytical Manual (Tallent et al. 2012).

For scanning electron microscopy *Bacillus* sp. ABP14 cultures were harvested and cells fixed with Karnovsky's fixative (Karnovsky 1965). After fixation and dehydration, gold metallization was carried out in a Balzers SCD – 030, and the material was observed with JEOL-JSM 6360 LV scanning electron microscope in the Electron Microscopy Center at Federal University of Paraná.

For all tests two independent assays were performed in duplicate.

Insect Bioassay

The free ingestion method was used to determine the toxicity of selected strain culture to laboratory-reared velvetbean caterpillar *Anticarsia gemmatalis* Hübner, 1818 (Lepidoptera, Erebidae). Larvae used in the experiment were obtained from cultures of the Department of Zoology, Federal University of Paraná.

Cells were cultivated in GYS medium (Yousten and Rogoff 1969) in a rotary shaker (120 rpm) at 37 °C during 24 or 72 h. Cell culture (500 µL) was loaded onto an antibiotic-free insect artificial diet (20 g) (Greene et al. 1976) to feed a third-instar *A. gemmatalis* larva. Each larva was placed in a container with artificial diet and held at 25 °C and 12 h light:dark photoperiod for ~20 days until pupation. The bioassay comprised three experiments (each containing a single third-instar *A. gemmatalis* larva per container, with six larvae total). The control group was fed with artificial diet added of GYS medium. Viability of larval and pupal stages was assessed. The assay was repeated twice to confirm the result.

Genome Sequencing, Assembly, and Annotation

The whole-genome sequence was carried out using *Nextera DNA Library Preparation Kit* and paired-end sequencing in the Illumina MiSeq platform. An additional DNA sequencing was performed using a genomic DNA library constructed with *Ion Express Plus Library Kit* and the Ion Torrent Technology platform.

Raw reads quality was checked by FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/; last accessed June 6, 2019). DNA sequence was de novo assembled using CLC Genomics Workbench 6.5.1 (CLC Bio) and GS de novo Assembler 2.8 (454 Life Sciences). Large contigs obtained from Illumina data were oriented based on the *B. thuringiensis* serovar *finitimus* strain YBT020 (Zhu et al. 2011) (used as reference genome and hereafter indicated as YBT020) to generate scaffolds. Gaps were filled with contigs from Ion Torrent assembly using *GFinisher* (Guizelini et al. 2016). Genome sequences of ABP14 and YBT020 were aligned using NUCmer in MUMmer 3.0 package (Kurtz et al. 2004).

Functional annotation and metabolic reconstruction was carried out by RAST (Aziz et al. 2008), COG (Tatusov et al. 2000), and KAAS (KEGG Automatic Annotation Server) (Moriya et al. 2007). Assigned functions were checked with BLASTp (Altschul 1997) and InterProScan (Zdobnov and Apweiler 2001). rRNA and tRNA were predict by RNAmmer 1.2 (Lagesen et al. 2007) and tRNAscan-SE (Lowe and Eddy 1997).

Comparative Genomics

To investigate species affiliation of ABP14 within *Bacillus cereus sensu lato* group, the complete genome sequences of 36 organisms were subjected to digital DNA–DNA hybridization (dDDH) analysis. Values of genomic distances were calculated using Genome-to-Genome Distance Calculator (GGDC) 2.1 server with the recommended settings (Meier-Kolthoff et al. 2013). Results of formula II were used to construct a genomic

distance matrix and a heatmap was used for ordering the strains in a symmetric matrix and clustered them at species-level with a dDDH threshold of 70%.

ABP14 and other *Bacillus* strains proteomes were compared by sequence similarity using an all-against-all BLAST search. Homology between proteins was established with 80% amino acid identity over at least 90% of the length of the longest protein. Proteins present in the selected bacteria without homology with other strains analyzed were separated for study and manually checked.

Nucleotide Sequence Accession Numbers

This *Bacillus* sp. ABP14 genome project has been deposited at GenBank under the accession number CP017016 (chromosome) and CP017017 (plasmid).

Results and Discussion

The ABP14 strain was isolated from a lignocellulosic compost of agricultural waste by its cellulose-degrading activity. ABP14 showed white cream colony morphology with filamentous margins when grown on NA medium. Cells were motile Gram positive rods with subterminal endospore formation, showed no pigment and were positive for hemolytic, oxidase and catalase activities. Cells were able to grow from 10 to 45°C. During sporulation phase, no crystal proteins were observed using scanning electron microscopy of endospore (supplementary fig. 1, Supplementary Material online).

ABP14 showed insecticidal activity against *Lepidoptera* (supplementary fig. 2, Supplementary Material online). Cultures of 24 or 72 h of growth were tested and similar toxic effects were observed after 15 days, with larvae showing initially paralysis, followed by hemocytic melanization and death. A longer period was necessary to observe larval death as compared *B. thuringiensis* (Salamitou et al. 2000) probably due the absence of Cry-like toxins in ABP14.

The genome sequence of *Bacillus* sp. ABP14 was obtained using a combination of Illumina and Ion Proton technologies. The whole-genome sequenced by Illumina MiSeq system generated a total of 2,968,082 paired-end reads with 228.92 bp average read length and 131.86-fold genome coverage. DNA sequencing by Ion Proton system, generated a total of 9,882,626 reads, with 123.66 bp average read length, and 237.18-fold genome coverage. After assembly, ABP14 showed one circular chromosome and one circular plasmid. The overall ABP14 genome feature is shown in table 1.

In order to evaluate species affiliation of ABP14 within the *B. cereus sensu lato* group, a dDDH was carried out and a heat map was used for ordering 35 strains in the symmetric matrix and clustered them at species-level with a dDDH threshold of 70% (fig. 1). Phylogenetic tree grouped organisms into two clusters, C1 and C2, with the thermotolerant *Bacillus cytotoxicus* (Guinebretiere et al. 2013) into a separated clade. All analyzed *Bacillus anthracis* and most of the *B. cereus* were nested in C1, whereas C2 mainly grouped the insecticidal strains of *B. thuringiensis*. C1 was divided into five subclusters (*SC1-5*) with seven strains of *B. anthracis* nested into *SC*1 and the attenuated strain (CDC684) (Okinaka et al. 2011) in *SC*2 together with *B. cereus* pathogenic strains, and the human pathogenic isolate *B. thuringiensis* serovar Konkukian strain 97-27 (Han et al. 2006) but apart from pathogenic *cereulide* toxin producers *B. cereus* NC7401 and AH187 (*SC3*). ABP14 was grouped with YBT020 into a separated subcluster (*SC*5), but apart from the others *B. thuringiensis* strains nested into C2. These results confirmed the genetic homogeneity of *B. anthracis* species and demonstrated its more remote relationship with *B. thuringiensis*.

Table 1
Sequence Features of Replicons from *Bacillus* sp. ABP14

	Replicons	
Features	***Chromosome***	***Plasmid (pABP14)***
Size (bp)	5,141,367	11,199
GC content (%)	35.4	30.4
Total number of genes	5,369	10
Coding sequences	5,238	10
Average ORFs size (bp)	823.8	769.2
Protein coding regions (%)	83.9	68.7
Genes with functional assignment	3,708	5
Function unknown	1,530	5
rRNA operon	13	0
tRNA	92	0

ABP14 and YBT020 showed a 81.3% dDDH value, suggesting them as subspecies according to Meier-Kolthoff et al. (2014). A closer comparison between ABP14 and YBT020 identified 278 unique genes in ABP14 (96 with functional assignment and 192 as hypothetical or conserved hypothetical). These genes are involved with cell wall composition, exosporium proteins, restriction–modification system, pH homeostasis and bacterial competence. ABP14 genome also encodes a cellulase, allowing its use as carbon source. No homology was observed between plasmids pABP14 and pBMB26 or pBMB28 (YBT020).

YBT020 is an insecticidal crystal toxin producer strain, carrying plasmid borne genes *cry26Aa* and *cry28Aa*. On the other hand, ABP14 showed insecticidal ability but genes coding for Cry/Cyt proteins or for other insecticidal factors were not found.

ABP14 and YBT020 genome comparison showed that both strains share a set of virulence factors common in *B. cereus sensu stricto*, such as the nonhemolytic enterotoxin genes (*nheABC*), a perfringolysin-O (*hlyI*), two channel-forming type III haemolysin (*hlyIII*), and metalloproteases. However, gene coding for phosphatidylcholine-specific

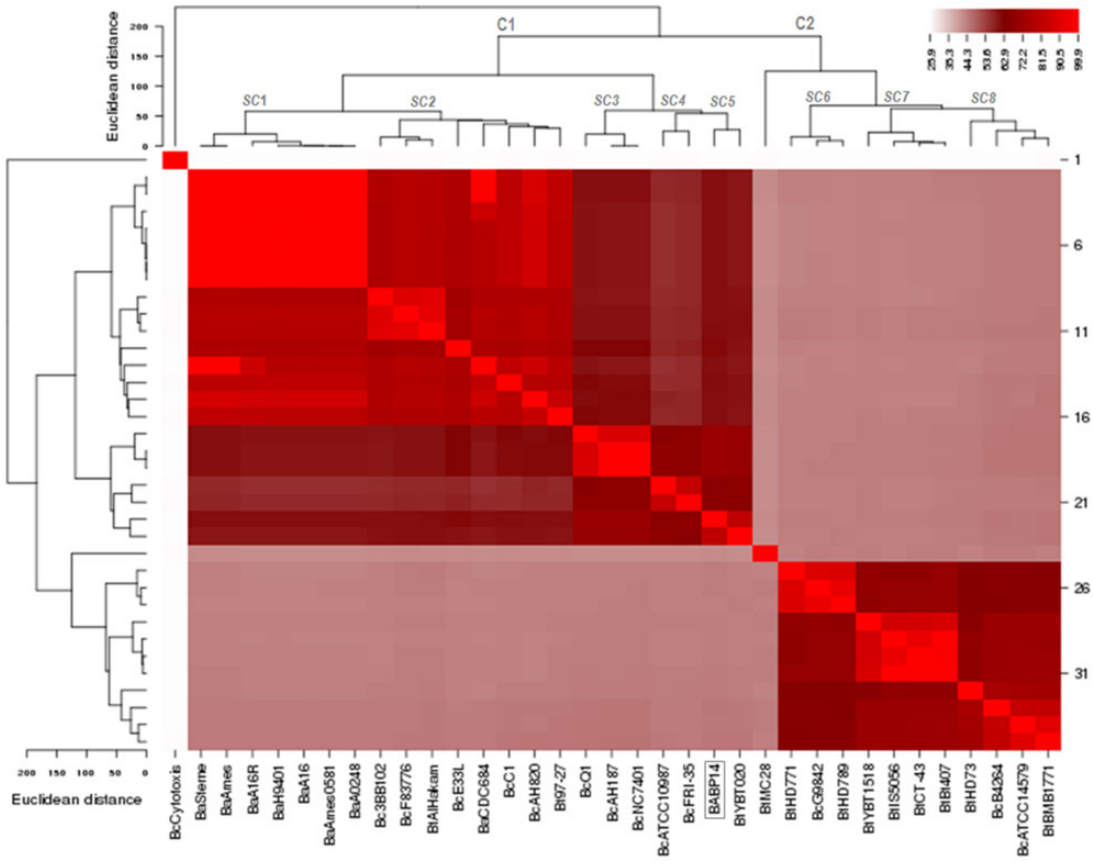

Fig. 1.—Heatmap of dDDH analysis using GGDC method from genome sequence comparison of *Bacillus* sp. ABP14 and *Bacillus cereus sensu lato* strains. The dendrogram of genome similarity based on Euclidean distance to dDDH values clustered according to the average linkage method. Clustered image map was generated with CIMminer tool. The colored bar indicates genome similarity. C1 and C2 indicate Clusters 1 and 2; SC1-8: Subclusters 1–8. Strains and accession numbers are listed. BcCytotox (*Bacillus cytotoxicus* NVH 391-98; NC_009674.1); BaSterne (*Bacillus anthracis* str. Sterne; NC_005945.1); BaAmes (*B. anthracis* str. Ames; NC_003997.3); BaA16R (*B. anthracis* str. A16R; NZ_CP001974.1); BaH9401 (*B. anthracis* str. H9401; NC_017729.1); BaA16 (*B. anthracis* str. A16; NZ_CP001970.1); BaAmes0581 (*B. anthracis* str. Ames Ancestor; NC_007530.2); BaAO248 (*B. anthracis* str. AO248; NC_012659.1); Bc3BB102 (*B. cereus* 03BB102; NC_012472.1); BcF83776 (*B. cereus* F837/76; NC_016779.1); BtAlHakam (*Bacillus thuringiensis* str. Al Hakam; NC_008600.1); BcE33L (*B. cereus* E33L; NC_006274.1); BaCDC684 (*B. anthracis* str. CDC684; NC_012581.1); BcCI (*B. cereus* biovar anthracis CI; NC_014335.1); BcAH820 (*B. cereus* AH820; NC_011773.1); Bt97-27 (*B. thuringiensis* serovar konkukian 97-27; NC_005957.1); BcQ1 (*B. cereus* Q1; NC_011969.1); BcAH187 (*B. cereus* AH187; NC_011658.1); BcNC7401 (*B. cereus* NC7401; NC_016771.1); BcATCC10987 (*B. cereus* ATCC10987; NC_003909.8); BcFRI-35 (*B. cereus* FRI-35; NC_018491.1); BtYBT020 (*B. thuringiensis* serovar finitimus YBT020; NC_017200.1); BtMC28 (*B. thuringiensis* MC28; NC_018693.1); BtHD771 (*B. thuringiensis* HD-771; NC_018500.1); BcG9842 (*B. cereus* G9842; NC_011772.1); BtHD789 (*B. thuringiensis* HD-789; NC_018508.1); BtYBT1518 (*B. thuringiensis* YBT-1518; NC_022873.1); BtIS5056 (*B. thuringiensis* serovar thuringiensis IS5056; NC_020376.1); BtCT-43 (*B. thuringiensis* serovar chinensis CT-43; NC_017208.1); BtBt407 (*B. thuringiensis* Bt407; NC_018877.1); BtHD73 (*B. thuringiensis* serovar kurstaki HD73; NC_020238.1); BcB4264 (*B. cereus* B4264; NC_011725.1); BcATCC14579 (*B. cereus* ATCC14579; NC_004722.1); BtBMB171 (*B. thuringiensis* BMB171; NC_014171.1).

phospholipase C (PC-PLC) was not observed in both strains. In the ABP14 genome it was not observed genes coding for Hemolysin BL (*hblCD* and *hblA*) and hemolysin type II (*hlyII*); whereas, ABP14 produces cytotoxin K (CytK) which is absent in YBT020.

ABP14 showed tools for protection against host defense system such as the immune inhibitor A protein (InhA), which selectively cleaves insect antibacterial peptides (Ivanova et al. 2003). It also showed genes encoding proteins that may have a role in the host invasion process such as chitinases and several proteases which could damage the peritrophic membrane of larval midgut, thus allowing the invasion to the haemocoel. Moreover, CytK which is a necrotic, hemolytic and pore-forming toxin, may also play a role to ABP14 cytotoxicity.

Bacillus sp. ABP14 can be classified as an insect pathogen lacking Cry toxins, but it is able to produce proteins that may play a role in the host death showing potential study targets for application on new systems for protection or defense against insects.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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