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Genomic Analysis of *Bacillus* sp. Strain B25, a Biocontrol Agent of Maize Pathogen *Fusarium verticillioides*

Nadia R. Douriet-Gámez[1] · Ignacio E. Maldonado-Mendoza[1] · Enrique Ibarra-Laclette[2] · Jochen Blom[3] · Carlos L. Calderón-Vázquez[1]

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Abstract *Bacillus* sp. B25 is an effective biocontrol agent against the maize pathogenic fungus *Fusarium verticillioides* (*Fv*). Previous in vitro assays have shown that B25 has protease, glucanase, and chitinase activities and siderophores production; however, specific mechanisms by which B25 controls *Fv* are still unknown. To determine the genetic traits involved in biocontrol, B25 genome was sequenced and analyzed. B25 genome is composed of 5,113,413 bp and 5251 coding genes. A multilocus phylogenetic analysis (MLPA) suggests that B25 is closely related to the *Bacillus cereus* group and a high percentage (70–75%) of the genetic information is conserved between B25 and related strains, which include most of the genes associated to fungal antagonism. Some of these genes are shared with some biocontrol agents of the *Bacillus* genus and less with *Pseudomonas* and *Serratia* strains. We performed a genomic comparison between B25 and five *Bacillus* spp., *Pseudomonas* and *Serratia* strains. B25 contains genes involved in a wide variety of antagonistic mechanisms including chitinases, glycoside hydrolases, siderophores, antibiotics, and biofilm production that could be implicated in root colonization. Also, 24 genomic islands and 3 CRISPR sequences were identified in the B25 genome. This is the first comparative genome analysis between strains belonging to the *B. cereus* group and biocontrol agents of phytopathogenic fungi. These results are the starting point for further studies on B25 gene expression during its interaction with *Fv*.

Keywords *Bacillus* · Biocontrol · Sequencing · Comparative genomics

Introduction

Several members of the *Bacillus cereus* group, including *B. thuringiensis* and *B. cereus*, are effective in controlling important plant diseases [56, 64]. Given the importance of disease control in agriculture, efforts have been made to clarify the genetic contents of biocontrol agents by genome sequencing of *Bacillus* spp. [16, 35]. *Pseudomonas* spp. [41] and *Serratia* spp. [22, 49] strains identify the genes involved in antifungal activity, including antibiotics, siderophores, lytic enzymes, proteases, non-ribosomal peptides, polyketides, etc. Also, genomic information on biocontrol agents has allowed comparing between related bacteria and bacteria that perform similar functions regarding biological control [38]. For example, a comparative genomics study between *Pseudomonas* spp. strains reported common genes related to antagonism being *P. protegens* Pf-5, the strain with the highest content of biocontrol genes. A significant fraction of these genes were strain-specific. In fact, it has been reported that this group of bacteria only shares 45–52% of all the genetic information [41].

Nadia R. Douriet-Gámez and Ignacio E. Maldonado-Mendoza have contributed equally to this work.

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✉ Carlos L. Calderón-Vázquez
carlosligne@yahoo.com; ccalderon@ipn.mx

[1] Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional Unidad Sinaloa, Instituto Politécnico Nacional, Blvd. Juan de Dios Bátiz Paredes No. 250, Col. San Joachin, 81101 Guasave, Sinaloa, Mexico

[2] Instituto de Ecología A. C. (INECOL), Red de Estudios Moleculares Avanzados, Carretera Antigua a Coatepec No. 351, Col. El Haya, 91070 Xalapa, Veracruz, Mexico

[3] Bioinformatics and System Biology, Justus-Liebig University, 35392 Giessen, Hesse, Germany

The disease control activity exerted by members of the *Bacillus* spp. is complex, with several mechanisms, including competition for nutrients (including iron) and space, antibiosis, mycoparasitism, and biofilm formation [51]. To date, the genetic basis of biocontrol mechanisms in members of the *B. cereus* group is not fully understood.

Bacillus sp. B25 is an effective control agent against the phytopathogenic fungus *Fusarium verticillioides* (*Fv*) [18]. A decrease in *Fusarium* severity of stalk and ear rot in field trials, as well as fungal fumonisin levels in grain, were observed when this strain was inoculated [40]. Additional in vitro assays demonstrated that B25 is able to produce protease, glucanase, and chitinase activities, as well as siderophores [18], so it was presumed that this strain owns a wide range of genes reported as related to biocontrol.

B. cereus, B. thuringiensis, and *B. anthracis* are the main closely related species belonging to the *B. cereus* group [52]. Although research on them has been focused on other topics, including human pathology and industrial applications [55, 63], it is known that the first two species are effective in fungal biocontrol, with the production of chitinases and siderophores as antagonistic mechanisms [29, 50], or the production of parasporal crystal proteins that can act as insecticide [28]. Thus, strains of this group could possess coding genes for both insecticidal and antifungal activity.

To date, some mechanisms by which B25 controls *Fv* have been evidenced at the biochemical level; however, its effectiveness against the phytopathogenic fungus both in the field and in vitro experiments, suggests that B25 owns a wider range of mechanisms that makes it an interesting biocontrol agent. Genomic information of B25 could be shared with phylogenetically related strains and other biocontrol agents. Hence, a comprehensive knowledge of gene content of B25 would elucidate the genetic basis involved in controlling fungal activities. A genomic comparison of B25 against related species with similar functional characteristics would elucidate the shared and unique genetic components in B25. Here, we analyze the genome of the B25 strain and describe a genomic comparison of B25 against five *Bacillus* spp. strains, *Pseudomonas protegens* pf-5, and *Serratia plymuthica* AS9, some of them with biocontrol activities. This is the first comprehensive comparative genome analysis addressed at their genetic potential to control fungal pathogens among strains belonging to this taxonomic group and other biocontrol agents.

Materials and Methods

Genome Sequencing and Assembly

Genomic DNA isolation was achieved with the DNeasy Blood & Tissue® kit (Qiagen, CA, USA). Libraries of 550 and 1000 base pairs were prepared and sequenced in a 2×300 format (MiSeq platform, Illumina, San Diego, CA, USA). Adapter trimming and paired-end filtering (with a quality of ≥25) were performed using SeqPrep [61]. Reads were assembled de novo using Newbler 3.0 [45], and SSPACE [7] was used to reduce the number of scaffolds, and gaps were closed with GapFiller [8]. To define if resulting scaffolds were part of a bacterial chromosome, they were compared to *B. thuringiensis* strain BMB171 (CP001903.1). The SynMap tool of the CoGe database [43] was used for aligning and orienting the scaffolds using the genome of BMB171 [27] as reference (Fig. S1). The B25 sequence was deposited in the GenBank (CP016285) as *Bacillus* sp. B25 (2016b).

Genome Annotation and Identification of Genes of Interest in B25

Protein-coding sequences and RNA genes were predicted by Glimmer V.3.0 [17]. Automatic assignment of functionality was carried out by the Rapid Annotations using Subsystems Technology (RAST) server [2] using BMB171 genome as reference. Selection of antagonistic and housekeeping genes for phylogenetic analysis was carried out browsing the genome with the SEED-Viewer tool of the RAST server. Non-ribosomal peptide synthesis clusters were identified with antiSMASH [48], and their function was deduced according to their homology to the known antibiotics using NCBI's conserved domain database [44]. Genomic islands (GIs) were identified using IslandPath-DIMOB in IslandViewer 4 platform [5]. Clustered regularly interspaced short palindromic repeats (CRISPR) domains were screened using CRISPR finder [24].

Phylogenetic Analysis

A multilocus phylogenetic analysis (MLPA) was carried out using a 15 kilobase pair (kbp) concatenated sequence including *16S rRNA*, *gyrB*, *groEL*, *polC*, *rpoB*, and *purH* genes. Gene sequences of B25 were compared with the corresponding concatenated sequences of ten strains of the *B. cereus* group: *B. cereus* strains ATCC 14579 (CP001903.1) [28], FORC 005 (CP009686.1) [31], B4264 (CP001176.1), and Q1 (CP000227.1) [63]; *B. thuringiensis* strains BMB171 [25], HD-29 (CP010089.1) [66], HD73 (CP004069.1) [39], Bt407 (CP003889.1), and CT-43 (CP001907.1), and *B. anthracis* BA1015 (CP009544.1) [60]. A genome sequence from *B. bombysepticus* Wang (CP007512.1) [11] closely related to this group was also included. Sequences of *B. subtilis* strains 168 (AL009126.3) [34], BAB-1 (CP004405.1) [25], *B. amyloliquefaciens* strains M27 (AMPK01000000.1) [34], and FZB42 (CP000560.1) [9] were used as outgroups. Concatenated sequences were aligned with MUSCLE [17]

and MEGA 5.2 [62] was used to generate the phylogenetic analysis using the neighbor-joining (NJ) method [58] and Kimura 2-parameter model for the phylogenetic tree construction (bootstrap of 1500 replicates). MLPA was supported by means of an average nucleotide identity (ANI) calculation using B25 sequences against the 11 strains related to *Bacillus cereus* group, using the 15 kbp concatenated sequence and also the complete genome sequence of compared strains. Both analyses were carried out using the ANI calculator [57] with the cut-off value to delimit bacterial species set to 95% [4].

Comparative Genomic Analysis

To determine conserved sequence segments between B25 in *Bacillus cereus* group, a whole genome alignment was carried out using MAUVE [14]. To compare the genetic content of B25 against genetically related strains (*B. bombysepticus* Wang, *B. thuringiensis* BMB17, and *B. cereus* Q1), at the structural and functional levels, a functional gene annotation with the subsystem feature category-based comparison of the RAST server was performed. Shared and unique genes between B25 and *B. cereus* group were identified using EDGAR [6]; the pan-genome and core-genome were obtained. A comparative analysis addressed at identifying genes involved in bacterial antagonism was performed using the proteomes of *Bacillus* sp. B25, *B. bombysepticus* Wang, *B. thuringiensis* BMB171, *B. cereus* Q1, *B. subtilis* BAB-1, *B. amyloliquefaciens* M27, *Serratia plymuthica* AS9 (CP002773.1), and *Pseudomonas protegens* Pf-5 (CP000076.1). The complete dataset contained 33,353 protein-coding genes. An all-against-all comparison was performed using BLAST-P (E-value 1×10^{-10}) followed by clustering with OrthoMCL v1.4 [37] using a default MCL inflation parameter of 1.5.

Results

Genome Features of *Bacillus* sp. B25

The *Bacillus* sp. B25 genome contains a circular chromosome of 5,113,413 bp with a GC-content of 35.6%. It comprises 5360 genes, 5251 are coding sequences (CDS), and 109 are non-coding RNAs (Table 1 and Fig. S2). 2334 CDS (45%) were classified into 27 functional categories and 470 subsystems (Fig. S3) and a putative function was assigned to 2214 CDS (Fig. S3, Dataset S1). Besides, 18 scaffolds were not aligned with the reference genome and 17 of those showed identity to *Bacillus* spp. plasmids and contained 1063 CDS (Table S1). These contained 22 genes associated with sporulation, 26 genes related to transcriptional regulators, multidrug resistance, and genes related to fungal antagonism such as siderophores and 1 chitinase. Three CRISPR domains were identified in the non-assembled scaffold ppB25_2 along a 8 kbp region that contains the *Cas3, Cas5d, Csd1*, and *Cas7* genes (Fig. S7). According to CRISPR finder, other "questionable" CRISPR sequences were identified in the B25, Wang, BMB171, and Q1 chromosomes. Furthermore, no Cas proteins were identified close to these repeats.

Table 1 *Bacillus* sp. B25 genome assembly and annotation statistics

Feature	Value
Genome assembly	
Size	5,113,413 bp
GC-content	35.6%
Scaffolds	30
Larger scaffold	863 kbp
Average scaffold size	170 kbp
Number of gaps	42
Genome annotation	
Total number of genes	5360
Number of coding sequence (CDS)	5251
Number of RNA genes	109
Extra chromosomal sequences (not assembled)	18

Phylogenetic Analysis

The B25 strain was previously identified as *Bacillus cereus sensu lato* [18] based only on the 16S rRNA gene sequence. Here an MLPA was performed and clustered B25 close to *B. thuringiensis* (BMB171, HD-29, and HD73), *B. cereus* (ATCC 14579, FORC 005, B4264) all related to the *Bacillus cereus* group, and *B. bombysepticus* Wang (Fig. S4). This was also confirmed with ANI values obtained using both whole genome and concatenated sequences analysis (Table S2).

Genome Properties of the *Bacillus cereus* Group

The genome size and number of predicted genes of B25 is slightly lower but similar to BMB171, Q1, and Wang and all strains have similar GC-content percentage (35–36% GC) (Table S3). All compared strains have a large proportion of CDS (9.8–10.5%) related to responses against phytopathogens including the subsystems carbohydrates, virulence, disease and defense, stress response, regulation and cell signaling, iron acquisition and metabolism, as well as dormancy and sporulation (Fig. S5, Dataset S2).

Pan- and core-genome analysis (Fig. 1; Dataset S3) revealed that the four strains shared 3964 CDS, corresponding to approximately 70–75% of all CDS in these genomes. Genes that code for virulence factors and those involved

with antifungal activity are allocated here. A total of 387 CDS integrate the list of single genes in B25, including transporter genes, mobile elements, phages, transcriptional regulators, membrane proteins, motility, and lytic enzymes. Interestingly, two chitinases and one chitin-binding protein were specific for B25 (Dataset S3).

All compared chromosomes presented high collinearity among their sequences (Fig. S6). Q1 was the strain with more translocations in its genome sequence. 15 GIs were identified in the B25 chromosome (Fig. S2) and only 7 GIs were well conserved along the Wang, BMB171, and Q1 chromosomes (Table S4). These GI regions include genes related to mobile element proteins, phages, transcription/translation factors, sporulation, multidrug resistance, and lytic enzymes (chitinase and chitosanase). Also 11 GIs are distributed in 8 of the 17 sequences similar to plasmids (Table S5).

Genes Involved in Antagonism

To compare the genetic information related to antagonism between B25 and the *Bacillus cereus* group strains (BMB171 and Q1), *B. bombysepticus* Wang and the biocontrol agents *B. amyloliquefaciens* M27, *B. subtilis* BAB-1, *Pseudomonas protegens* Pf-5, and *Serratia plymuthica* AS9, their proteomes were compared. The comparison was then focused on 36 genes related to biofilm production, siderophores (bacillibactin and petrobactin), extracellular enzymes (glycoside hydrolase, chitinase, and chitosanase) and antibiotics (surfactin, fengycin, and iturin) (Table 2). All strains share genes involved in bacillibactin production except Pf-5, which lacks two of the five genes comprising this operon. B25, Wang, and BMB171 also contain all genes related to petrobactin production. M27 and AS9 contain only one and two genes involved in petrobactin production, respectively (Table 2). Regarding lytic activity, the *B. cereus* group strains, *B. bombysepticus* Wang and *S. plymuthica* AS9 share chitinases. The *B. cereus* group, including the Wang strain share a chitosanase, excluding Q1. B25, BMB171, and *B. subtilis* BAB-1 that contain a possible endoglucanase, with a domain of glycoside hydrolase family 1 (GH1) (Table 2).

B25, Wang, BMB171 and *B. amyloliquefaciens* M27 own the complete operon for surfactin production (*SrfAABCD*). Pf-5 and BMB-1 presented only one and two out of the four

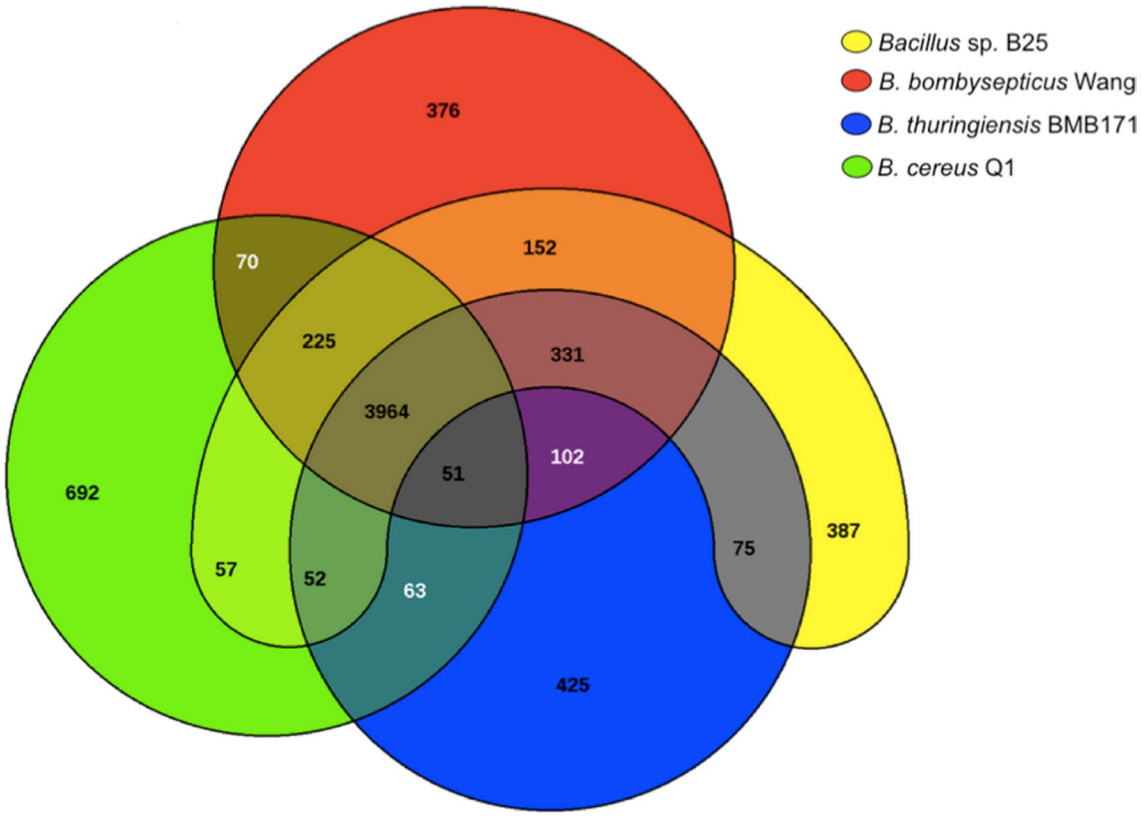

Fig. 1 Number of shared and genome-specific genes of strains related to *Bacillus cereus* group. The Venn diagram shows all CDS of *Bacillus* sp. B25, *B. bombysepticus* Wang, *B. thuringiensis* BMB171, and *B. cereus* Q1. Numbers of overlapped regions represent the CDS shared between the genomes compared. The number that is not in an overlapped region indicates the number of CDS in each genome without homologs in other genome included in the analysis

Table 2 Comparative analysis of key genes involved in potential fungal antagonism activities in *Bacillus, Pseudomonas*, and *Serratia* strains

Functional protein		Genes	B25	Wang	BMB171	Q1	M27	BAB-1	Pf-5	AS9
Siderophores	Bacilibactin[a]	*DhbC*	+	+	+	+	+	+	−	+
		DhbB	+	+	+	+	+	+	+	+
		DhbA	+	+	+	+	+	+	−	+
		DhbE	+	+	+	+	+	+	+	+
		DhbF	+	+	+	+	+	+	+	+
	Petrobactin[a]	*AsbA*	+	+	+	−	−	−	−	+
		AsbB	+	+	+	−	−	−	−	+
		AsbC	+	+	+	−	−	−	−	−
		AsbD	+	+	+	−	−	−	−	−
		AsbE	+	+	+	−	−	−	−	−
		AsbF	+	+	+	−	+	−	−	−
Lytic enzymes	Endoglucanase[b]	Glycoside hydrolase	++	−	+	−	−	+	−	−
	Chitosanase[b]	*Csn*	++	+	+	−	−	−	−	−
		Chitinase A	+	+	+	+	−	−	−	+
	Chitinases[b]	*Chitinase B*	+	+	+	+	−	−	−	++
Antibiotics	Surfactin[a]	*SrfAA*	+	+	+	−	+	−	−	−
		SrfAB	+	+	+	−	+	−	−	−
		SrfAC	+	+	+	−	+	+	−	−
		SrfAD	+	+	+	−	+	+	+	−
	Fengycin[a]	*FenA*	−	−	−	−	+	−	−	−
		FenB	−	−	−	−	+	−	−	−
		FenC	−	−	−	−	+	−	−	−
		FenD	−	−	−	−	+	−	−	−
	Iturin[a]	*ItuA*	−	−	−	−	+	−	−	−
		ItuB	−	−	−	−	+	−	−	−
		ItuC	−	−	−	−	+	−	−	−
	SFP[b, c]	*sfp*	+	+	+	−	+	+	+	−
Biofilm formation	TasA[a]	*SipW*	+	+	+	+	+	+	−	−
		TasA-like 2	+	+	+	+	+	+	−	−
		Hypothetical protein	+	−	+	+	−	−	−	−
		TasA-like 1	+	+	+	+	−	−	−	−
		SinR	+	+	+	+	+	+	−	−
		SinI	+	+	+	+	−	−	−	−

B25: *Bacillus* sp. B25, Wang: *B. bombysepticus* Wang, BMB171: *B. thuringiensis* BMB171, Q1: *B. cereus* Q1, M27: *B. amyloliquefaciens* M27, BAB-**1**: *Bacillus subtilis* BAB-1, Pf05: *Pseudomonas protegens* Pf05, AS9: *Serratia plymuthica* AS9

\+ Gene present in the genome sequence. ++ means a duplicate gene

− Gene absence in the genome sequence

[a]Operon genes components

[b]Individual genes

[c]SFP = 4′-phosphopantetheinyl transferase

genes for surfactin production. Despite that fengycin and iturin are common in *Bacillus* sp., genes for these antibiotics were not identified in any other strain. M27 was the only strain with all genes for production of the antibiotics surfactin, iturin, and fengycin (Table 2). B25, BMB171 and Q1 have all the genes of the operon for biofilm formation *sipW-TasA*, Wang genome lacks an hypothetical protein, and BAB-1 and M27 have only *SipW, TasA*-like 2, and *SinR*, whereas strains Pf-5 and AS9 lack all biofilm formation genes (Table 2).

Discussion

B25 Genome Properties and Phylogeny

The complete genome of *Bacillus* sp. B25 provided new insights into the potential metabolites that it can produce related to biocontrol activity. The genome size, gene and GC-content are consistent with those of previously published *Bacillus* spp. genomes. Based on the performed phylogenetic analysis, B25 is part of the *B. cereus* group, located close to *B. thuringiensis* (BMB171, HD-29, and HD73) and *B. cereus* (ATCC 14579, FORC 005, and B4264), and also to the *B. bombysepticus* Wang strain. A significant synteny between B25, BMB171, and Wang and non-significant synteny with Q1 was found. Although synteny has been demonstrated among the *B. cereus* group, some studies have evidenced differences in gene order, chromosomal rearrangements, and nucleotide variations [54]. *B. bombysepticus* has not been included in the *B. cereus* group but our MLPA and genome comparison results suggest that Wang is close to the *B. cereus* group, but this requires further confirmation.

Despite their phenotype and particular properties, strains of the *B. cereus* group share a considerable amount of genetic information [23, 52]. In this work, B25 shares 70–75% of its genome with *B. thuringiensis, B. cereus*, and *B. bombysepticus* Wang, a high percentage compared to other bacteria [41], so their differences could be based only on a few genes.

Unique genes from B25, including transcriptional regulators, membrane proteins, and motility, as well as those genes involved in lytic enzymes activity, should be analyzed further to determine their differential role in biocontrol activity. These are allocated in the GIs (Table S4) along with integrative and conjugative elements, transposons, multidrug resistance and some phage-related genes [30]. Those are commonly acquired by horizontal gene transfer encoding adaptative traits that may be beneficial under certain growth or environmental conditions [59].

Type I CRISPR-Cas system is considered as a unique defense mechanism against foreign DNA and in bacterial stress responses and virulence [42, 46]. The presence of three CRISPR elements in B25 confirms the importance of resistance to exogenous DNA in this strain. It remains to learn if the CRISPR spacers play a role in controlling horizontal gene transfer of plasmids and prophages found in B25 genome. The fact that the other strains do not contain a CRISPR-Cas system suggest that B25 could represent a separated phylogenetic sub-lineage within *B. cereus* group.

Genes Associated with Fungal Antagonism

We identified common and specific genes related to biocontrol activities in B25. At the genetic level, the most conserved mechanisms among all *Bacillus, Pseudomonas*, and *Serratia* strains were bacillibactin, some lytic enzymes, surfactin, and biofilm formation. Siderophores such as bacillibactin play a key role for antagonism and have been reported in several species [12] including B. *amyloliquefaciens* SQR9 [36]. The siderophore petrobactin has also been considered as vital for iron acquisition and virulence in *B. anthracis* [32]. The fact that B25 has 11 genes involved in siderophore synthesis suggests that this antagonistic mechanism may be of importance against fungal pathogens in B25.

Most bacteria produce lytic enzymes that can hydrolyze a wide variety of polymeric compounds, including chitin, proteins, cellulose, lipids, and glucans, the major components of the fungal cell wall [1], and the presence of these enzymes is expected to suppress phytopathogen growth [33]. B25 possesses two complete genes coding for chitinases, which may be playing a key role in fungal growth inhibition [15]. B25 chitinase genes transcript levels are induced in response to the addition of chitin and *Fv* fungal lysates [19] confirming a potential role for these genes to control this plant fungal pathogen. In parallel, B25 could also degrade the fungal cell wall with the help of a chitosanase gene. Orthologous chitosanase sequences have been reported as involved in biocontrol against *Fusarium oxysporum* and *Rhizoctonia solani* [21, 47]. Considering that B25 also has a putative endoglucanase, and the reported effectiveness of this lytic enzyme against a wide range of fungi [53], it is possible that B25 possesses a strong mechanism based on cell wall degradation to control the fungal development through chitinase, chitosanase, and glucanase activity. Further analyses are necessary to investigate the role of these genes in lytic enzyme activity.

Antibiotic production seems to be the less conserved biocontrol mechanism among compared strains. *Bacillus amyloliquefaciens* M27 is the only strain that has the necessary genes for the surfactin, iturin, and fengycin biosynthesis. B25, Wang, and BMB171 have the necessary genes for surfactin biosynthesis. Surfactin synthesis requires the expression of four structural genes present in the operon *SrfAABCD* and is a key biocontrol mechanism of *B. amyloliquefaciens* SQR9 [36]. The activity of the biocontrol agent *B. subtilis* SG6 against *F. graminearum* has been associated with the co-production of chitinase, fengycin, and surfactin [65]. Even though fengycin is not present in B25, the activity of chitinase and surfactin could be an important complement for B25 exerting fungal antagonism. Bacillibactin, surfactin, and other non-ribosomal peptides also require the presence and expression of the *sfp* gene (a phosphopantetheinyl transferase) [36]. *Sfp* was identified in

B25 (Table 2), and its role in bacillibactin production in this strain remains to be known.

Biofilm formation is the first step of endophytic bacteria to colonize roots [20] and it can help to mediate plant protection against environmental factors [26] and plant diseases [3, 10]. In B25, the identified *sipW-tasA* operon and regulatory genes *sinR* and *sinI* could be determinant for *Fv* biocontrol mechanisms involving cell–cell communication via *quorum sensing* and production of antifungal metabolites [13]. Once *B. subtilis* colonizes roots, it forms a stable and extensive biofilm and produces surfactin, which could protect the plant from pathogenic organisms [3]. Hence, the discovery of genes related to biofilm formation in B25 is crucial to understand its role as an effective biocontrol agent.

As far as we know, this work is the first comparative genome analysis between strains belonging to the *Bacillus cereus* group and other biocontrol agents. The data here reported demonstrate that B25 contains the genes necessary for controlling the growth of phytopathogenic fungus, including competence for nutrients, mycoparasitism, antibiosis, and biofilm formation. Despite its genera and species, these mechanisms are ubiquitous among biocontrol strains. The B25 genomic analysis provides the basis for further studies on gene expression of B25 during its interaction with *Fv*, as well as to analyze the activity of the genes identified here to elucidate the mechanisms that B25 uses to control *Fv*.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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