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ORIGINAL ARTICLE

Whole-genome analysis of *bacillus velezensis* ZF2, a biocontrol agent that protects *cucumis sativus* against *corynespora* leaf spot diseases

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Abstract

Bacillus spp. have been widely described for their potentials to protect plants against pathogens. Here, we reported the whole genome sequence of *Bacillus velezensis* ZF2, which was isolated from the stem of a healthy cucumber plant. Strain ZF2 showed a broad spectrum of antagonistic activities against many plant bacterial and fungal pathogens, including the cucumber leaf spot fungus *Corynespora cassiicola.* The complete genome of *B. velezensis* ZF2 contained a 3,931,418-bp circular chromosome, with an average G + C content of 46.50%. Genome comparison revealed closest similarity between ZF2 and other *B. velezensis* strains. Genes homologous to 14 gene clusters for biosynthesis of secondary metabolites were identified in the ZF2 genome. Also identified were a number of genes involved in bacterial colonization, including the genes for motility, biofilm formation, flagella biosynthesis, and capsular biosynthesis. Numerous genes associated with plant–bacteria interactions, including cellulase or protease biosynthesis, and plant growth promotion were also identified in the ZF2 genome. Overall, our data will aid future studies of the biocontrol mechanisms of *B. velezensis* ZF2 and promote its application in vegetable disease control.

Keywords Antagonistic activity · *Bacillus velezensis* · Biological control · Comparative genomic analysis · Secondary metabolites

Introduction

Bacillus velezensis is a gram-positive, rod-shaped, motile, spore-forming, and aerobic bacterium, and forms creamy white, rough colonies with slightly irregular edges on agar-solidified culture medium (Cristina et al. 2005). *B. velezensis* was first isolated and described in southern Spain in 2005 (Cristina et al. 2005), and later reported as a heterotypic synonym of *B. amyloliquefaciens* based on DNA–DNA relatedness values (Wang et al. 2008). *B. velezensis* was distinguished from *B. amyloliquefaciens* based on core genome sequences (Dunlap et al. 2015), and these two species were separated from the "original *Bacillus* member" *Bacillus subtilis* in a recent study (Fan et al. 2017a, b). To date, many studies have investigated *B. velezensis* for the potential for controlling plant diseases. For example, *B. velezensis* CC09, which was isolated from *Cinnamomum camphora*, has been reported as a biocontrol agent for controlling wheat powdery mildew disease (Cai et al. 2017). *B. velezensis* 2A-2B, isolated from the rhizosphere of *Sporobolus airoides*, has been shown to exhibit a strong inhibition toward root rot (Martínez-Raudales et al. 2017). Furthermore, *B. velezensis*

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LDO2 (a peanut endophyte), *B. velezensis* BAC03 (isolated from potato common scab suppressive soil), and *B. velezensis* RC 218 (isolated from wheat anthers) have been explored for biological control of diverse plant diseases (Chen et al. 2019; Meng et al.2016; Chulze 2016).

Cucumber *Corynespora* leaf spot disease caused by *C. cassiicola* is one of the most important foliar diseases of cucumber. *C. cassiicola* is a major plant fungal pathogen that causes significant economic losses for many plant species, including cucumber. (Déon et al. 2014). Currently, chemical control is the primary method to control *Corynespora* leaf spot disease; however, *C. cassiicola* has developed resistance to many commonly used fungicides, including benzimidazoles, dicarboximides and *N*-phenylcarbamates (Miyamoto et al. 2010). Recently, biological control has been explored as an alternative solution for the prevention and suppression of plant diseases. For example, the strain *B. megaterium* was reported to control *Aspergillus flavus* on peanut kernels (Kong et al. 2010); *B. amyloliquefaciens* L-1 could be used to control pear ring rot (Sun et al. 2017); *B. subtilis* BSCBE4, *Pseudomonas chlororaphis* PA23 and *P. fluorescens* ENPF1 were reported to inhibit the growth of *C. cassiicola* mycelia in vitro (Mathiyazhagan et al. 2004). Nevertheless, few studies have explored the biocontrol potential of *B. velezensis* for *Corynespora* leaf spot disease, and the biocontrol mechanisms of *B. velezensis* against the disease still need to be elucidated.

Many strains of *Bacillus* spp. have been used in biocontrol strategies as antagonists of plant pathogens and/or plant growth promoters. These claimed activities mostly rely on the production of various secondary metabolites, including lipopeptides, polypeptides, macrolactones, fatty acids, polyketides, and isocoumarins, most of which exhibit a wide range of antimicrobial activities (Omura et al. 2001). These structurally diverse compounds can also affect the microflora in the rhizosphere or trigger host defense responses (Velusamy and Gnanamanickam 2008). *B. velezensis* was reported to harbor a large number of gene clusters involved in the biosynthesis of secondary metabolites mentioned above. For instance, the gene clusters *srf*, *bmy*, and *fen* were shown to direct the synthesis of the cyclic lipopeptides surfactin, bacillomycin, and fengycin; while, the gene clusters *mln*, *bae*, and *dfn* have been linked with the synthesis of the polyketides macrolactin, bacillaene, and difficidin, respectively (Chen et al. 2008). Furthermore, most of these secondary metabolites have been reported to facilitate the colonization in rhizosphere soil of *B. velezensis* strains (Abdellaziz et al. 2018). For instance, *B. velezensis* FZB42^T showed strong colonizing ability and antimicrobial activities, one of the major factors was owing to the production of many kinds of lipopeptides including surfactin, iturin, bacillomycin, fengycin, mycosubtilin, bacillaene, bacilysin (Bochow et al. 2001; Hua et al. 2007; Alvarez et al. 2015). *B. velezensis* QST713, an antagonist against green mold disease, contained 15 gene clusters for the synthesis of secondary metabolites, all of which showed antimicrobial or antibacterial activities (Pandin et al. 2018). *B. velezensis* M75, which was isolated from cotton waste, had a number of genes associated with the synthesis of various secondary metabolites with antimicrobial activities or suppressing plant pathogens (Hua et al. 2007; Sang et al. 2017). Two antifungal compounds isolated from *B. velezensis* G341, which had been identified as bacillomycin and fengycin, have also been observed to inhibit the mycelial growth of various phytopathogenic fungi (Lim et al. 2017). Importantly, colonization and biofilm formation are closely associated with biological control by *Bacillus* spp. (Fan et al. 2017a, b). Based on previous studies, a number of genes associated with biofilm formation and colonization have been shown to play an important role in biological control, including the flagellar motility-associated genes, *motA*, *motB*, and *flgM* (Domka et al. 2007) and the *Bacillus*-specific biofilm formation pathway genes *kinB*, *spo0A*, *spo0F*, *degU*, and *degS* (Grossman et al. 1992). Besides these activities, plant growth promotion by *Bacillus* spp. was reported using growth-promoting substances, including auxins, cytokinins and gibberellins (Santner et al. 2009). For example, *B. velezensis* FZB42^T can produce indole-3-acetic acid (IAA) and cytokinin, both of which are associated with plant growth promotion (Idris et al. 2007).

In this study, *B. velezensis* strain ZF2 with antagonistic activities against a broad range of plant bacterial and fungal pathogens was isolated from the stem of healthy cucumber. The entire genome of ZF2 was sequenced, annotated and compared with representative genomes of other *Bacillus* species. Phylogenetic analysis was performed to determine the taxonomic position of ZF2 and its relationship with other representative *Bacillus* spp. Genome comparison revealed that various genes involved in the biosynthesis of secondary metabolites and functional genes associated with beneficial plant–bacteria interactions occurred in the ZF2 genome. Taken together, these results provided novel insights into the biocontrol mechanisms of *B. velezensis* and could benefit the practical application of strain ZF2 in biocontrol of plant diseases.

Materials and methods

Bacterial isolation, antagonism assays and biocontrol assays

Bacillus velezensis ZF2 was isolated from the stem of a healthy cucumber plant in a greenhouse at Beijing, China, in November 2017, as described by Caulier et al (2018). Briefly, the stem tissues were surface sterilized in 70% ethanol for 1 min, and washed with sterile distilled water for

three times. 1 mL of the fragmentized sample was subjected to heat treatment (80 °C, 15 min), 1000-fold diluted, plated on LB plate, and incubated at 28 °C for 24 h. After selective isolation, *Bacillus*-like strains were retained for further investigations. The antagonistic activity of *B. velezensis* ZF2 against plant pathogenic bacteria and fungi was assessed through plate bioassays, and the control efficacy of *B. velezensis* ZF2 against *Corynespora* leaf spot disease was tested through bioassays experiment with potted cucumbers. Plate assays were performed to assess the inhibition of *C. cassiicola* colony growth by *B. velezensis* ZF2, which was cultured for different fermentation time (48 h, 96 h, or 120 h) and in seven different fermentation media (NB: 10 g peptone, 3 g beef powder, 5 g NaCl per liter, pH 7.0; LB: 10 g tryptone, 5 g yeast extract, 10 g NaCl per liter, pH 7.0; BPY: 5 g beef extract, 10 g peptone, 5 g yeast extract, 5 g NaCl, 10 g glucose per liter; CP: 500 g corn powder per liter; SP3: 30 g soybean powder, 10 g starch, 10 g glucose, 1 g KH_2PO_4, 1 g K_2HPO_4, 0.02 g $FeSO_4{\cdot}7H_2O$, 1 g $CaCO_3$ per liter; SP8: 30 g soybean powder, 2 g peptone, 0.2 g KH_2PO_4, 1 g $CaCO_3$, 5 g glucose, 3 g yeast extract per liter; and SP + CP: 30 g corn powder, 30 g soybean powder, 0.3 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4$, 1 g $CaCO_3$ per liter) (Yu et al. 2007). In addition, plate assays to assess cellulase (Cel) and protease (Prt) activity were conducted according to previously reported methods with modifications (Murata et al. 1991; Chatterjee et al. 1995). Bacterial cells were grown in LB medium overnight at 28 °C and adjusted to an OD_{600} of 0.8. Samples were applied to the cells, and the plates were incubated for 24 h at 28 °C to evaluate Cel and Prt activity. The Cel plates were stained with a 0.1% (w/v) Congo red solution for 30 min and then washed with a 1 M NaCl solution for 15 min. Haloes in the Prt plates became visible without any further treatment. Each treatment was repeated three times, and all of the above experiments were repeated three times.

Culture conditions, and genomic DNA extraction

The strain ZF2 was cultivated in LB (Luria broth) or NB (Nutrient broth) media at 28 °C with shaking for 24 h. The strain morphologies were observed by scanning electron microscope (SEM) and transmission electron microscopy (TEM 1230 microscope, JEOL). Genomic DNA was extracted from cultured ZF2 cells ($OD_{600} = 0.8$) using a TIANamp Bacteria DNA kit (Tiangen Biotech (Beijing) Co., Ltd.).

Genome sequencing, assembly and annotation

The genome of *B. velezensis* ZF2 was sequenced by Allwegene Technologies Corporation, Beijing, China. Whole-genome sequencing was performed using a Pacific Biosciences (PacBio) RS II platform, and a 10-kb SMRT Bell was used for template library construction. The sequences were assembled de novo using SMRT Link v.5.1.0 programs (https://soap.genomics.org.cn/soapdenovo.html) (Table S1). The graphical views of genome alignments were generated using CGView (Tatusova et al. 2016). Identification and annotation of the functional genes was performed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP, https://www.ncbi.nlm.nih.gov/genome/annotation_prok/) (Tatusova et al. 2016). Transfer RNA (tRNA) and ribosome RNA (rRNA) genes were identified using tRNAscan-SE version 2.0 and RNAmmer version 1.2, respectively (Lagesen et al. 2007; Lowe and Chan 2016). Small nuclear RNAs (snRNAs) were predicted by BLAST searching against the R fam database (https://rfam.xfam.org) (Stanke et al. 2008). The functions of the predicted proteins were assigned through comparisons against multiple databases, including NR (nonredundant) protein databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Li et al. 2002), the RAST (Rapid Annotation using Subsystem Technology) analysis platform (Aziz et al. 2008), Pfam (https://pfam.xfam.org/), SwissProt and the enhanced COG (clusters of orthologous groups of proteins) database (https://www.ncbi.nlm.nih.gov/COG/) (Tatusov et al. 2000). In addition, SignalP 4.0 (Bendtsen et al. 2004) and TMHMM 2.0 (Krogh et al. 2001) were used to predict putative signal peptides and transmembrane helices, respectively. PHAST was used for prophage prediction (Arndt et al. 2016), and clustered regularly interspaced short palindromic repeats (CRISPR) were identified using CRISPR finder (Ibtissem et al. 2008).

Phylogenetic analysis and genome comparisons

The taxonomic position of *B. velezensis* ZF2 was determined by multilocus gene sequence analysis (MLSA) based on four housekeeping genes (16S rRNA, *rpoD*, *pgk*, and *gyrB*). The gene sequences were aligned using MUSCLE and trimmed to remove ambiguously aligned regions. Subsequently, the phylogenetic tree was constructed using the maximum likelihood method in MEGA 6.0 (Tamura et al. 2013). Other available gene sequences of closely related species for phylogenetic tree construction were downloaded from the NCBI database (Table S2). According to the phylogenetic analysis, four closely related *Bacillus* species with released complete genomes, including *B. velezensis* LS69 (GenBank: CP015911.1), *B. velezensis* FZB42^T (GenBank: CP000560.1), *B. amyloliquefaciens* DSM 7 ^{T} (GenBank: FN597644.1) (Rückert et al. 2011) and *B. subtilis* 168 ^{T} (GenBank: AL009126.3) (Nakamura et al. 1999) were selected for genome comparison. Average nucleotide identities (ANI) (Michael and Ramon 2009) and in silico DNA–DNA hybridization (DDH) (Meier-Kolthoff et al. 2013) were calculated using the OrthoANIu algorithm (https

://www.ezbiocloud.net/tools/orthoaniu) and the Genome-to-Genome Distance Calculator (GGDC) (https://ggdc.dsmz.de/ggdc.php), respectively. Furthermore, complete genome comparisons were conducted with the progressive alignment option of the Mauve 2.3.1 comparison software using the ZF2 genome as the reference genome (Michael and Ramon 2009). Venn diagrams were generated using R package (Michael and Ramon 2009).

Analysis of gene clusters for biosynthesis of secondary metabolites

Gene clusters for biosynthesis of secondary metabolites were predicted using the antiSMASH 2.0 program. The analysis was performed via the authors' Web servers using the default parameters (https://antismash.secondarymetabolites.org). Comparative analysis of gene clusters identified in *B. velezensis* ZF2, *B. velezensis* LS69, *B. velezensis* FZB42^T, *B. amyloliquefaciens* DSM 7 ^{T}, and *B. subtilis* 168 ^{T} was performed based on the Kyoto Encyclopedia of Genes and Genomes database (KEGG, https://www.genome.jp/kegg/) and the GenBank database.

Genome mining for genes encoding plant beneficial traits

Functional genes involved in plant–bacterial interactions such as biofilm formation, flagellar biosynthesis, motility, capsular biosynthesis were searched by NCBI database. The analysis of sequence homology of different functional genes in *B. velezensis* ZF2, *B. velezensis* FZB42^T, *B. velezensis* LS69, *B. amyloliquefaciens* DSM 7 ^{T}, and *B. subtilis* 168 ^{T} were conducted using the KEGG database at amino acid level.

Results and discussion

Biocontrol effect of *B. velezensis* ZF2 against the *Corynespora cassiicola*

B. velezensis ZF2 showed strong antagonistic activities against the plant pathogens *Pectobacterium carotovorum* subsp. *brasiliense*, *Pseudomonas syringae* pv. *lachrymans*, *P. syringae* pv. *tomato*, *Ralstonia solanacearum*, *Xanthomonas campestris* pv. *campestris*, *Clavibacter michiganensis* subsp. *michiganensis*, *Corynespora cassiicola*, *Colletotrichum* sp., *Phytophthora capsici*, *Botrytis cinerea*, *Fusarium oxysporum*, and *Alternaria solani* (Fig. S1). Importantly, strain ZF2 exhibited a strong inhibitory activity against *C. cassiicola*. Notable inhibition zones were observed surrounding the strain ZF2 in plate assays, with an inhibition ratio of up to 60.10% (Fig. S1 G). Interestingly, microscopic observations showed that the mycelia of *C. cassiicola* on the antagonistic plate gathered into clusters and were significantly enlarged; whereas, the mycelia in the control plate appeared a normal state of growth (Fig. S2 A and B). Extracellular enzyme assays showed that strain ZF2 produced protease (Prt) and cellulase (Cel) (Fig. S2 C and D), which might result in mycelial expansion of *C. cassiicola* and suppression of cucumber leaf spot disease. Moreover, strain ZF2 showed a significant effect in controlling *Corynespora* leaf spot disease on potted cucumbers (Fig. S3). The control efficiency was 90.81 ± 1.62, close to the efficacy of chlorothalonil (96.09 ± 1.72), a fungicide with strong sterilizing activity (Sigler and Turco 2002) (Table S3).

Furthermore, the inhibitory effect of *B. velezensis* ZF2 toward *C. cassiicola* was evaluated following fermentation for different times or in different mediums. The results showed that strain ZF2 displayed a maximal inhibitory effect after 48 h of fermentation with a 10% of seed culture addition (1, 5, 10%) (Fig. S4A). The inhibitory effect became enhanced with the increase of the ratio of seed culture addition. Moreover, the inhibitory effect appeared most obvious (63.98%) when strain ZF2 was incubated in corn meal medium, among seven different media, with a 10% seed culture addition (Fig. S4 B).

Organism information

B. velezensis ZF2 was determined to be a motile, gram-positive, endospore-forming, and aerobic bacterium belonging to the *Bacillaceae* family. The strain grew readily on LB plate at 28 °C and produced creamy white colonies with irregular margins after 24 h of incubation (Fig. S5 A), showing colony morphology and cultural characteristics similar to other *Bacillus* spp. (Das et al. 2018; Lim et al. 2017; Martínez-Raudales et al. 2017). Strain ZF2 displayed rod-shaped cells with a length of 3–5 µm and a diameter of 0.8–1.2 µm (Fig. S5 B, C). The strain could grow in 2.0–10.0% NaCl (w/v) and over a wide temperature range (15–37 °C), with an optimal pH of 7.0. Biolog assays showed that strain ZF2 could utilize diverse carbon sources, including α-D-glucose, D-mannose, D-mannitol, D-sorbitol, L-alanine, L-glutamic acid, myo-inositol and sodium butyrate (Table S4). Pathogenicity tests showed that strain ZF2 was nonpathogenic toward cucumber (Fig. S6). Minimum information about the genome sequence (MIGS) of *B.velezensis* ZF2 is summarized in Table S1 (Field et al. 2008).

General genomic features of *Bacillus velezensis* ZF2

The complete genome of *B. velezensis* ZF2 comprised a circular 3,931,418-bp chromosome with an average G + C content of 46.50%, without plasmid. A graphical circular genomic map showing the genome structure and functions

of strain ZF2 is presented in Fig. 1. A total of 4,058 open reading frames (ORFs) were predicted in the genome of ZF2. The ZF2 genome contained 3,808 protein-coding genes (CDS), 86 tRNA genes, 27 rRNA genes, 5 ncRNA genes and 123 pseudogenes (Table S5). Using the Pfam, SignalP, and TMHMM databases, 2,617 (64.49%), 150 (3.70%) and 1,054 (25.97%) of the ORFs could be classified into different groups, respectively. In addition, the ZF2 genome encoded 88 secreted proteins, 9 genomic islands, 2 prophage regions and 2 CRISPR loci (Table S5).

The functional categories of the 3,808 CDS in the ZF2 genome were further analyzed using the Cluster of Orthologous Groups of proteins (COG) database. The results showed that 3,335 CDS were assigned to different COG categories (Table S6), among which 7.10% were associated with transcription, 5.45% with ribosomal structure and

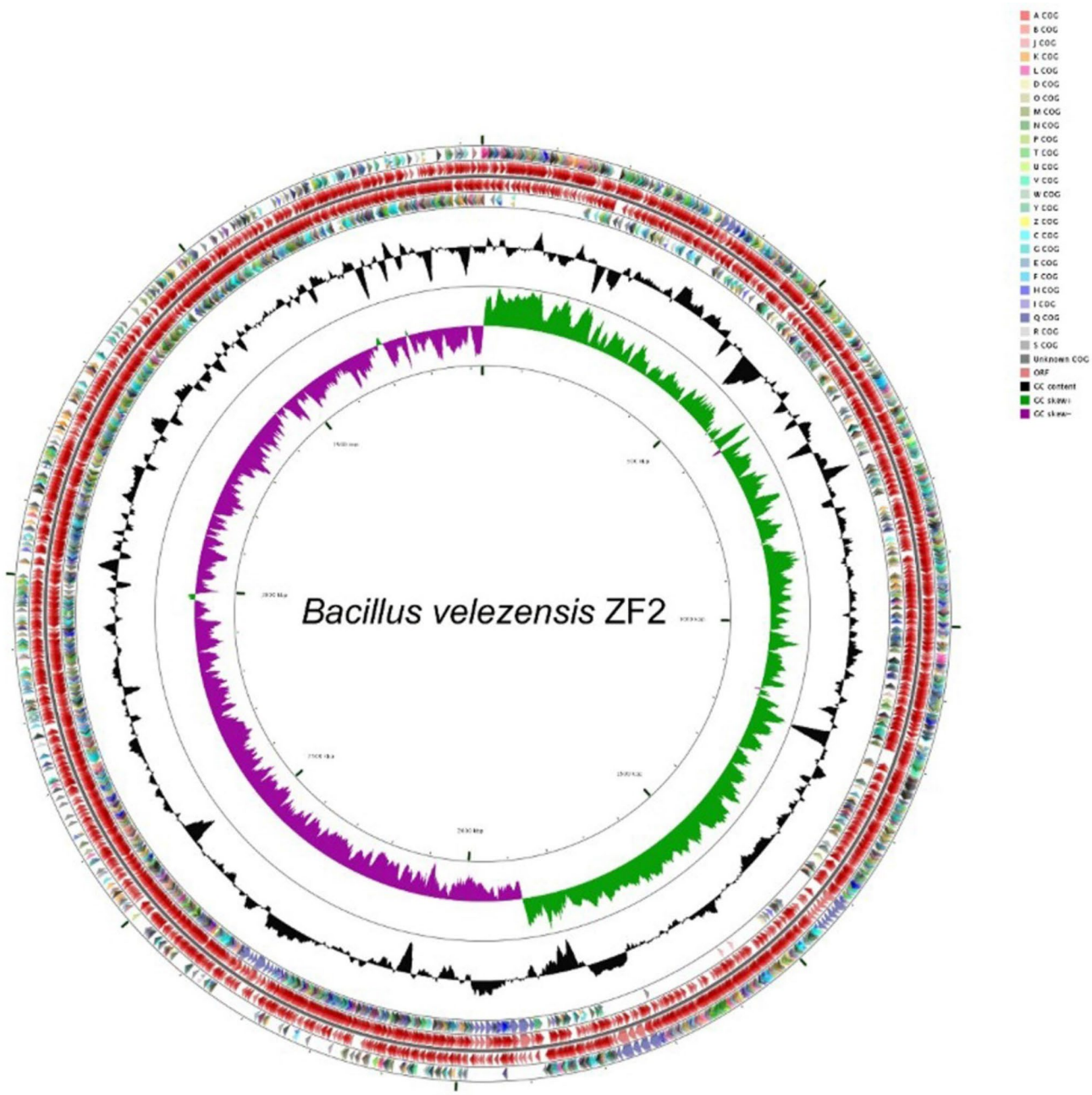

Fig. 1 Graphical circular map of the *B. velezensis* ZF2 genome generated using the CGview server. From outside to center, rings 1 and 4 show protein-coding genes oriented in the forward (colored by COG categories) and reverse (colored by COG categories) directions, respectively. Rings 2 and 3 show genes on the forward and reverse strands. Ring 5 shows the G+C% content plot (black), and the inner most ring shows the GC skews, where green indicates positive values and purple indicates negative values

biogenesis, 6.55% with carbohydrate transport and metabolism, and 7.47% with amino acid transport and metabolism. In addition, 723 genes were not assigned to COG categories, and their features and functions need to be determined.

Comparative genomics analysis of *Bacillus velezensis* ZF2 with other *Bacillus strains*

To understand the genetic relationships between *B. velezensis* ZF2 and other *Bacillus* strains, a phylogenetic tree was constructed based on four housekeeping genes (16S rRNA, *rpoD*, *pgk*, and *gyrB*) (Table S2). As expected, three primary monophyletic clades including *B. velezensis*, *B. amyloliquefaciens*, and *B. subtilis,* respectively, were corroborated by bootstrap values (Fig. 2) *B. velezensis* was closest to *B. amyloliquefaciens*, and both species were separated from *B. subtilis*. These results were consistent with the former notion that *B. velezensis* and *B. amyloliquefaciens* were classified as *B. subtilis* (Palazzini et al. 2016). In the study, strain ZF2 was classified as *B. velezensis*, and closest to JYYP2, followed by LS69 and SQR9, among 17 *B. velezensis* strains (Fig. 2).

Genome comparison was conducted between *B. velezensis* ZF2 and other four available complete genomes of *Bacillus* strains (*B. velezensis* LS69, the strain closely related to ZF2; *B. velezensis* FZB42^T, the type strain of *B. velezensis*; *B. amyloliquefaciens* DSM 7 ^{T}, the type strain of *B. amyloliquefaciens*; and *B. subtilis* 168 ^{T}, the type strain of *B. subtilis*) (Table 1). The results indicated that the genome size of strain ZF2 (3,929,773 bp) was larger than FZB42^T and LS69 (3,918,589 and 3,917,761 bp, respectively) (Chen et al. 2009; Liu et al. 2017), but smaller than *B. amyloliquefaciens* DSM 7 ^{T} (3,980,199 bp) (Christian et al. 2011) and *B. subtilis* 168 ^{T} (4,215,606 bp) (Harwood and Wipat 1996) (Table 1). The average G + C content of ZF2 was similar to that of *B. velezensis* FZB42^T (46.50%), *B. velezensis* LS69 (46.50%) and *B. amyloliquefaciens* DSM 7 ^{T} (46.10%), and was higher than that of *B. subtilis* 168 ^{T} (43.50%) (Table 1). Furthermore, all the five *Bacillus* genomes contained a single chromosome, without plasmid.

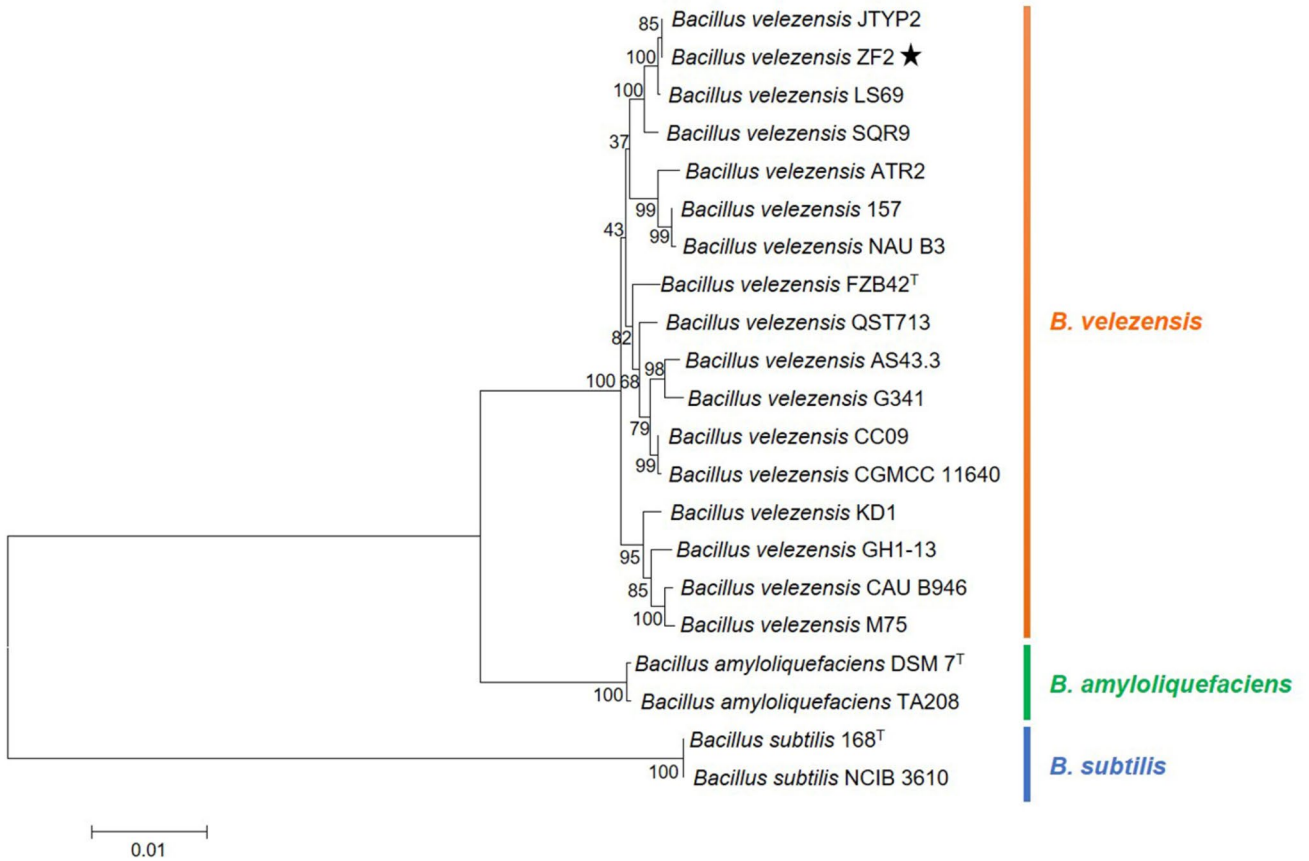

Fig. 2 Phylogenetic tree highlighting the relative position of *B. velezensis* ZF2 among other *Bacillus* species. The phylogenetic tree was constructed based on four housekeeping genes (16S rRNA, *rpoD*, *pgk*, and *gyrB*) according to the aligned gene sequences using the maximum likelihoods method in MEGA 6.0. Bootstrap values (1,000 replicates) are shown at the branch points. The scale bar indicates 0.01 nucleotide substitution per nucleotide position. GenBank accession numbers associated with the housekeeping loci for all strains are presented in Table S2

Table 1 Genomic features of *Bacillus velezensis* ZF2 and other *Bacillus* spp

Features	*Bacillus velezensis* ZF2	*Bacillus velezensis* FZB42^T	*Bacillus velezensis* LS69	*Bacillus amyloliquefaciens* DSM 7 ^{T}	*Bacillus subtilis* 168 ^{T}
Size (bp)	3,929,773	3,918,589	3,917,761	3,980,199	4,215,606
G + C content (%)	46.50	46.50	46.50	46.10	43.50
Replicons	One chromosome	One chromosome	One chromosome	One chromosome	One chromosome
Total genes	3927	3.892	3870	4120	4536
Predicted no. of CDS	3685	3687	3678	3870	4237
Ribosomal RNA	27	29	21	30	30
Transfer RNA	86	88	72	94	86
Other RNA	5	4	5	5	95
Pseudogene	124	84	94	121	88
GenBank sequence	CP032154.1	CP000560.1	CP015911.1	FN597644.1	AL009126.3

ANI and DDH analyses are widely used to evaluate the similarities between bacterial strains based on whole-genome sequence, and the compared strains with ANI values ≥ 96% and DDH values ≥ 70% are typically regarded as being the same species (Fujikawa and Sawada 2016; Zhang et al. 2016). In this study, the ANI and DDH values between strains ZF2 and LS69 were 99.95 and 100.00%, respectively. Similarly, the ANI and DDH values between strains ZF2 and FZB42^T were 98.30 and 95.70%, respectively. The ANI value between ZF2 and *B. amyloliquefaciens* DSM 7 ^{T} was 93.98%, although the DDH values was 82.50%. These results indicated that *B. velezensis* and *B. amyloliquefaciens* had a close relationship, but they were different species. Moreover, lower ANI and DDH values were obtained when the *B. subtilis* 168 ^{T} genome was used as reference. Altogether, these findings revealed that strains ZF2, LS69 and FZB42 were closely related to each other, and could be assigned to the same taxonomic position (Table 2).

The evolutionary distance among these five *Bacillus* strains was further evaluated using their whole-genome sequences with the Mauve program. Compared to FZB42^T and LS69, no gene insertions or deletions were detected in *B. velezensis* ZF2. The synteny plot of the pairwise alignments from the Mauve analysis strengthened the classification of the three strains as the same species. At the species level, the genome sequence of strain ZF2 was aligned to *B. amyloliquefaciens* DSM 7 ^{T} and *B. subtilis* 168 ^{T}. The results showed that large local collinear block (LCB) inversion occurred among the three species, especially between DSM 7 ^{T} and 168 ^{T}, and the ZF2 genome was much more similar to DSM 7 ^{T} than to 168 ^{T}, supporting the relationship described above (Fig. 3a).

Table 2 Percentage of average nucleotide identities (ANI) and in silico DNA–DNA hybridization (DDH)among the selected *Bacillus* genomes

	Bacillus velezensis ZF2	*Bacillus velezensis* LS69	*Bacillus velezensis* FZB42^T	*Bacillus amyloliquefaciens* DSM 7 ^{T}	*Bacillus subtilis* 168 ^{T}
Bacillus velezensis ZF2		**99.95**	**98.30**	**93.98**	**77.23**
		100.00	95.70	82.50	33.70
Bacillus velezensis LS69	**99.95**		**98.37**	**94.07**	**77.14**
	100.00		95.60	82.40	33.70
Bacillus velezensis FZB42	**98.30**	**98.37**		**94.20**	**77.12**
	95.70	95.60		80.70	33.40
Bacillus amyloliquefaciens DSM 7	**93.98**	**94.07**	**94.20**		**77.10**
	82.50	82.40	80.70		31.30
Bacillus subtilis 168	**77.23**	**77.14**	**77.12**	**77.10**	
	33.70	33.70	33.40	31.30	

ANI values were computed for pairwise genome comparison with using the OrthoANIu algorithm. The percentage of ANI was shown on the top and bolded

In silico DNA–DNA hybridization was calculated using Genome-to-Genome Distance Calculator (GGDC). The percentage of DDH was shown on the bottom

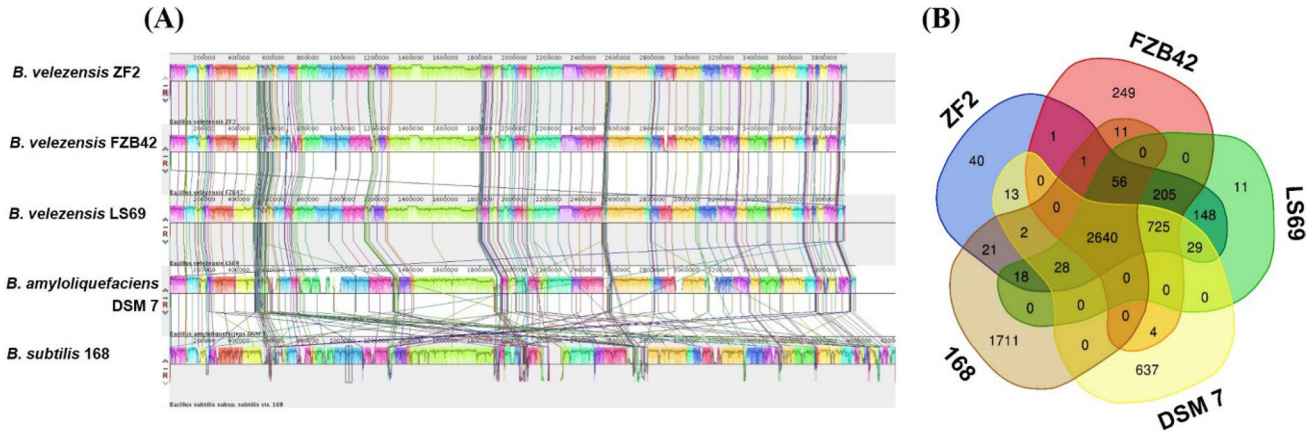

Fig. 3 Comparison of *B. velezensis* ZF2 genome sequences against other four *Bacillus* genome sequences. **a** Synteny analysis of the *B. velezensis* ZF2 and *B. velezensis* FZB42^{T}, *B. velezensis* LS69, *B. amyloliquefaciens* DSM 7 ^{T} and *B. subtilis* 168 ^{T} genomes. Pairwise alignments of genomes were generated using Mauve. ZF2 genome as the reference genome. Boxes with same color indicate syntenic regions. Boxes below the horizontal strain line indicate inverted regions. Rearrangements are shown with colored lines. The scale is in nucleotides. **b** Venn diagram showing the number of shared and unique clusters of orthologous genes

To identify specific genes in *B. velezensis* ZF2, its genome sequence was compared to the complete genome sequences of the four *Bacillus* strains. As shown in Fig. 2b, 6,550 protein clusters and 2,640 core genes present in ZF2 were shared with strains FZB42^{T}, LS69, DSM 7 ^{T} and 168 ^{T}. In addition, 3,849 orthologous genes were shared between ZF2 and LS69, while 3,628 orthologous genes were shared between ZF2 and FZB42^{T}. Additionally, 3,437 orthologous genes were shared between ZF2 and DSM 7 ^{T}, and 2,766 orthologous genes were shared between ZF2 and 168 ^{T}. These findings indicated that strain ZF2 showed a higher level of similarity with strain LS69. Furthermore, 40 unique genes were identified in the genome of *B. velezensis* ZF2, and the functions of most of these genes need further confirmation.

Gene clusters involved in the synthesis of secondary metabolites

Secondary metabolites produced by *Bacillus* spp. exhibited broad-spectrum biological activities, including antimicrobial activity against various phytopathogens (Karlovsky et al. 2008; Mondol et al. 2013). For example, surfactin, a cyclic lipopeptide antibiotic and biosurfactant produced by *Bacillus* strains, showed antimicrobial and antiviral activity by altering membrane integrity (Vollenbroich et al. 1997) and could protect plants against infection by the pathogen *Pseudomonas syringae* (Peypoux et al. 1999). The surfactin-encoding locus comprised of four open reading frames (*srf*A-D). SrfA and B were three amino acid activating modules, while SrfC was a one-module enzyme and SrfD mediated the transfer of the β-hydroxy fatty acid substrate (Jaruchoktaweechai et al. 2000). Fengycin, a lipopeptide complex, was first discovered in *B. subtilis* strain F-29–3 and could effectively inhibit the growth of filamentous fungi (Vanittanakom et al. 1986). A five-gene cluster (*fenA–E*) has been shown to be responsible for the biosynthesis of fengycin. Bacilysin was reported as a dipeptide antibiotic containing a L-Ala residue at the N-terminus and a nonproteinogenic amino acid, L-anticapsin, at the C-terminus (Jaruchoktaweechai et al. 2000), and exhibited activity against a wide range of bacteria and the yeast *Candida albicans* due to an anticapsin moiety (Zimmerman et al. 1987). The *bacABCDE* operon was responsible for the biosynthesis of bacilysin (Chen et al. 2009).

In this study, 14 gene clusters involved in the production of secondary metabolites were detected in the genome of strain ZF2, including two encoding NRPSs (nonribosomal peptide synthetases), two transATPKSs (trans-Acyl transferase polyketide synthetases), three transATPKS-NRPSs, and two bacteriocin-NRPSs, a saccharide, a lantipeptide, a putative NRPS, a fatty acid and an unknown type. In addition, gene clusters were identified that were specifically involved in the synthesis of surfactin, butirosin, macrolactin, bacillaene, fengycin, difficidin, bacillibactin, teichuronic acid, bacilysin, citrulline, molybdenum cofactor, iturin, amylolysin and amylocyclicin (Table 3). These compounds have been reported to have antimicrobial activities (Adnan et al. 2014; Dion et al. 1972b). Moreover, each of the 14 gene clusters was searched for homologues in the other four *Bacillus* strains (*B. velezensis* LS69, *B. velezensis* FZB42^{T}, *B. amyloliquefaciens* DSM 7 ^{T}, and *B. subtilis* 168 ^{T}) through an antiSMASH genome analysis and the KEGG database. The results showed that the 14 gene clusters associated with the biosynthesis of secondary metabolites in *B. velezensis* ZF2 were also present in FZB42^{T} and LS69 strains, and

Table 3 Comparative analysis of secondary metabolites clusters of *Bacillus velezensis* ZF2 identified in genome with plant-associated strains and reference genomes

Bacillus velezensis ZF2						Presence (+) or absence (−) of secondary metabolites clusters in *Bacillus* strains			
Metabolites	Synthetase	Core gene cluster	Size	Position	Bioactive spectrum	FZB42^T	LS69	DSM7^T	168 ^{T}
Surfactin	NRPS	*srfAABCD*, *bsdAB*	66,231 bp	321,977–388,208	Virus, mycoplasma and fungi	+	+	+	+
Butirosin	OtherKS	*yhdFR*, *pksAF*, *citR*, *rpoE*, *cueR*	42,049 bp	923,781–965.830	Bacteria, Cyanobacteria	+	+	+	−
Macrolactin	TransATPKS	*mlnABCDEFGH*, *kinC*, *speA*	87,409 bp	1,385,009–1,472,418	Bacteria	+	+	−	−
Bacillaene	TransATPKS-NRPS	*pksCDEJLMNRS*, *miaB*, *aprX*	102,336 bp	1,698,684–1,801,020	Bacteria	+	+	+	+
Fengycin	TransATPKS-NRPS	*kdgR*, *yvrGH*, *ituABCD*, *yxiF*, *bioBFI*, *fenABCDE*,*yngI*	138,886 bp	1,865,753–2,004,639	Filamentous fungi	+	+	+	+
Difficidin	TransATPKS	*dfnABCDEF-GHIJK*, *yqkD*, *lysR*, *yqiL*	100,585 bp	2,270,261–2,370,846	Bacteria	+	+	−	−
Bacillibactin	Bacteriocin-NRPS	*dhbABEF*, *besA*, *ublA*, *yuxL*, *lipA*	68,245 bp	3,000,664–3,068,909	Microbial competitors	+	+	+	+
Teichuronic acid	Saccharide	*degSU*, *tagO*, *tuaABCEGH*, *mnaA*, *ggaA*, *opuBCD*	56,166 bp	3,389,585–3,445,751	Bacteria	+	+	+	+
Bacilysin	NRPS	*bacABCDE*, *cysL*, *ycbL*, *rfbBCD*, *spsABCG*, *ywdF*, *vpr*, *nfrA*	73,132 bp	3,589,054–3,662,186	Bacteria and Candida albicans	+	+	+	+
Citruline	Fatty acid	*fabHF*	24,684 bp	1,080,144–1,104,828	Bacteria	+	+	+	+
Molybdenum cofactor	Putative NRPS	*metBC*, *galE*	17,703 bp	1,144,555–1,162,258	Bacteria	+	+	−	−
Iturin	TransATPKS-NRPS	*xynD*, *ituABCD*, *yxjCDEF*	44,809 bp	1,883,810–1,928,619	Fungi	+	+	+	+
Amylolysin	Lantipeptide	*amlFEKRAMT*	9,630 bp	1,194,266–1,203,896	Gram-positive bacteria	+	+	+	−
Amylocyclicin	Bacteriocin-NRPS	*acnBACDEF*	4,171 bp	3,044,117–3,048,288	Gram-positive bacteria	+	+	+	−

these strains possessed similar core biosynthetic genes with very high identity (approximately 100%) at the amino acid level (Table 3 and Fig. 4). These three strains had the primary gene clusters *srfAA*, *srfAB*, *srfAC*, *srfAD* and *bsdB* involved in the biosynthesis of surfactin. Similarly, the key gene clusters *mlnABCDEFGH* and *speA*, which play roles in the synthesis of macrolactin, were also detected in the FZB42^T and LS69 genomes. The 68.2-kb gene clusters associated with the synthesis of bacillibactin were similar in the ZF2, FZB42^T and LS69 genomes. The *dfnABCDEF-GHIJK* gene cluster involved in the synthesis of difficidin within the ZF2 genome was collinear with the *dfn* gene cluster of strains FZB42^T and LS69 (Chen et al. 2009; Liu et al. 2017). However, some secondary metabolites were not present in strain DSM 7 ^{T} or 168 ^{T}, including macrolactin, difficidin, molybdenum cofactor, butirosin, amylolysin and amylocyclicin. Indeed, the genes encoding molybdenum cofactor and butirosin were reported rarely and exhibited very lower similarity (11 and 7%, respectively) in different strains. Butirosin (with the core biosynthetic genes *ydhFR* and *pksF*) was reported as a new aminoglycoside antibiotic in *Bacillus circulans* with activity against gram-positive and gram-negative bacteria (Dion et al. 1972). Molybdenum cofactor (with the core biosynthetic genes *metBC* and *galE*)

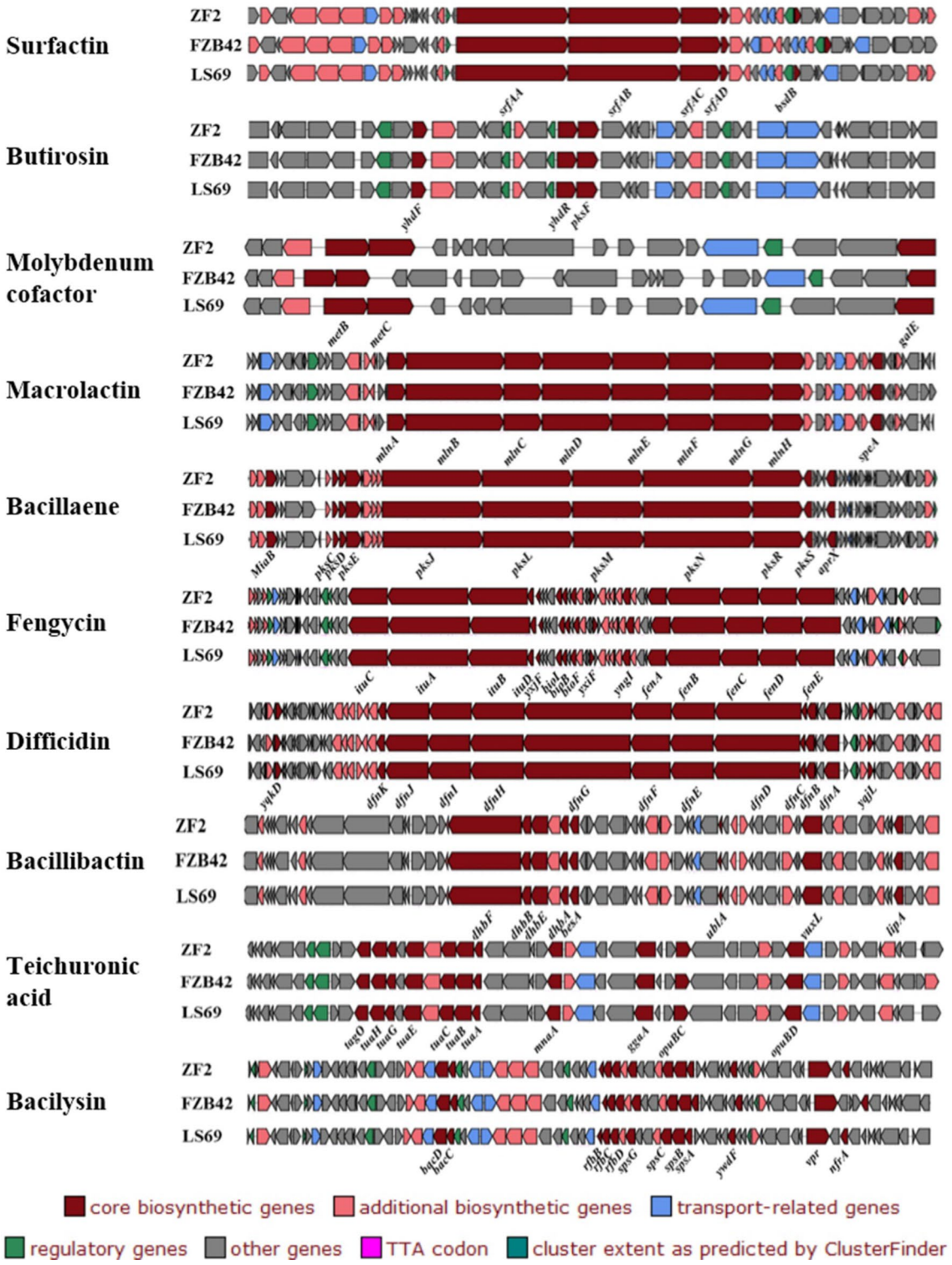

Fig. 4 Comparisons of NRPS, transATPKS and saccharide clusters in ZF2 (above), FZB42^T (middle), LS69 (below). Dark red indicates the core biosynthetic genes in different gene clusters among three *Bacillus* strains. The core biosynthetic genes are labeled in each gene cluster

was observed to be associated with nitrogenase (Shah and Brill 1977) and has not been reported in *B. velezensis*. In summary, the genomic features of ZF2 suggested that it had the potential to be used as a biocontrol agent against plant pathogenic microbes.

Mining for functional genes potentially associated with plant–bacteria interactions

Bacillus strains have recently become widely used as biocontrol agents because of their strong ability to tolerate adverse environmental conditions, such as high temperature, pressure and salinity, and to promote beneficial plant–bacteria interactions. These properties were largely attributed to various functional genes which were linked with biocontrol, including those responsible for sporulation, biofilm formation, bacterial colonization, flagella biosynthesis, plant growth promotion, and plant defense induction (Rampelotto 2010). As expected, *B. velezensis* ZF2 genome contained large numbers of genes coding for these biological functions (Table S7). Specifically, diverse genes for sporulation occurred in the ZF2 genome. The homologous Spo0A (AXY70842.1) served as the master transcription regulator of sporulation in *B. subtilis* (Virginie et al. 2010). SinR (AXY70880.1) and its antagonist SinI (AXY70879.1) were identified as pleiotropic DNA binding proteins and are essential for sporulation and subtilisin synthesis (Mandic-Mulec et al. 1995). The YqxM-SipW-TasA (AXY70883.1) operon encodes the TasA protein that binds cells together in biofilms (Diego et al. 2010) and is associated with sporulation (Stöver and Driks 1999). In addition, many regulatory genes associated with biofilm formation were detected in the ZF2 genome, such as *sigEFGH* (encoding RNA polymerase sporulation sigma factor), *pgsABC* (involved in poly-γ-glutamate synthesis), and *escABC* (encoding a transporter permease).

To be effective biocontrol agents, *B. velezensis* strains are required for motility and colonization in plant tissues (Ben et al. 2011; Wu et al. 2015). Interestingly, our results showed that ZF2 possessed *swrABC* genes, encoding swarming motility proteins, an exopolysaccharide operon (*epsA-O*) associated with capsular biosynthesis (Hua et al. 2007), and a number of *fli* and *flg* genes involved in flagella biosynthesis. All these functions have been suggested to enhance swarming motility and colonization (Emilia et al. 2012; Mordini et al. 2013). Moreover, these genes were highly similar in ZF2 and FZB42^T, with an amino acid identity of 97–100% (Table S7). However, many functional genes of ZF2 were not found in *B. subtilis* 168 ^{T}, or with a lower similarity, and the reason may be that the two strains belonged to different species.

Furthermore, a variety of genes encoding proteins associated with plant growth promotion were detected in the ZF2 genome, including putative indole-3-acet-aldehyde (IAA) dehydrogenase (*dhaS*), IAA acetyl-transferase (*ysnE*), auxin efflux carrier (*ywkB*), phytase and nitrilase (*yhcX*). These genes are responsible for the production of IAA, which has been suggested to contribute to the plant growth-promoting abilities of *Bacillus* strains (Shao et al. 2015). Strain ZF2 also had various genes encoding proteins involved in the synthesis of 3-hydroxy-2-butanone, including acetolactate decarboxylase (*alsD*), acetolactate synthase (*alsS*), a transcriptional regulator (*alsR*) and 2,3-butanediol dehydrogenase (*bdhA*) (He et al. 2013), and showed high similarity in ZF2 and FZB42^T (95–100% amino acid identity). This compound was reported to improve plant growth and trigger systemic resistance (Renna et al. 1993; Nicholson 2008). In addition, ZF2 also harbored a number of genes associated with biosynthesis of cellulase and protease, including those encoding serine protease (*spr*, *ispA*, *aprX*, *yyjD*, and *yyxA*), glucanase (*bglC* and *bglS*), galactokinase (*galK*, *galE*, and *galT*), phosphotransferase (*lacE* and *lacF*), and numerous intramembrane proteases (*ydcA*, *ydiL*, *prsW*, *ypbD*, and *yyaK*). Moreover, these genes displayed a high level of similarity in ZF2 and FZB42^T (96 to 100% amino acid identity) (Table S7). These enzymes were suggested to be associated with carbon source and cellulose utilization by *Bacillus* spp. in plants (Dardanelli et al. 2000). Altogether, these findings demonstrated that *B. velezensis* ZF2 was adapted to diverse environments and of the potential to promote plant growth.

Conclusions

B. velezensis ZF2, which was isolated from the stem of a healthy cucumber plant, had a broad range of antagonistic activities against 14 plant bacterial and fungal pathogens, and could be used for biological control of cucumber *Corynespora* leaf spot disease. Whole genome sequencing and comparative genomic analysis confirmed the taxonomic classification for strain ZF2 as a member of *B. velezensis*. *B. velezensis* ZF2 harbored 14 gene clusters involved in the production of secondary metabolites that have been shown to possess antimicrobial activities. Furthermore, a number of genes involved in bacterial colonization and plant growth promotion were also present in the ZF2 genome, and all these genes were highly homologous to *B. velezensis* FZB42^T. All these features indicated that strain ZF2 could be a promising biocontrol agent for plant disease control and advance a better understanding of biocontrol mechanisms of *B. velezensis*.

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Author contributions SX, XWX, LL, and BJL conceived and designed the experiments. SX, XWX, YRZ, and LL performed the experiments and analyzed the data. SX, LL, and BJL wrote the manuscript. YXS

and ALC revised manuscript. All authors have read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest in the publication.

Nucleotide sequence accession number The complete genome sequence of *Bacillus velezensis* ZF2 has been deposited in NCBI under the GenBank accession number CP032154.1. The strain has also been deposited in the China General Microbiological Culture Collection Center (CGMCC) for Type Culture Collection under the accession number 16013.The complete genome sequence of *Bacillus velezensis* ZF2 has been deposited in NCBI under the GenBank accession number CP032154.1. The strain has also been deposited in the China General Microbiological Culture Collection Center (CGMCC) for Type Culture Collection under the accession number 16013.

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