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The final publication is available at Springer via http://dx.doi.org/10.1007/s00248-018-1247-4.

Xu, Wei-Fang, Hui-Shuang Ren, Ting Ou, Ting Lei, Jun-Hong Wei, Chuan-Shu Huang, Tian Li, Gary Strobel, Ze-Yang Zhou, and Jie Xie. "Genomic and Functional Characterization of the Endophytic Bacillus subtilis 7PJ-16 Strain, a Potential Biocontrol Agent of Mulberry Fruit Sclerotiniose." Microbial Ecology (September 2018): 1-13. DOI:10.1007/s00248-018-1247-4.

Genomic and Functional Characterization of the Endophytic *Bacillus subtilis* 7PJ-16 Strain, a Potential Biocontrol Agent of Mulberry Fruit Sclerotiniose

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Abstract

Bacillus sp. 7PJ-16, an endophytic bacterium isolated from healthy mulberry stems and previously identified as *Bacillus tequilensis* 7PJ-16, exhibits strong antifungal activity and the capacity to promote plant growth. This strain was studied for its effectiveness as a biocontrol agent to reduce mulberry fruit sclerotiniose in the field and as a growth-promoting agent for mulberry in the greenhouse. In field studies, the cell suspension and supernatant of strain 7PJ-16 exhibited biocontrol efficacy and the lowest disease incidence was reduced down to only 0.80%. In greenhouse experiments, the cell suspension (1.0×10^6 and 1.0×10^5 CFU/mL) and the cell-free supernatant (100-fold and 1000-fold dilution) stimulated mulberry seed germination and promoted mulberry seedling growth. In addition, to accurately identify the 7PJ-16 strain and further explore the mechanisms of its antifungal and growth-promoting properties, the complete genome of this strain was sequenced and annotated. The 7PJ-16 genome is comprised of two circular plasmids and a 4,209,045 bp circular chromosome, containing 4,492 protein-coding genes and 116 RNA genes. This strain was ultimately designated as *Bacillus subtilis* based on core genome sequence analyses using a phylogenomic approach. In this genome, we identified a series of gene clusters that function in the synthesis of non-ribosomal peptides (surfactin, fengycin, bacillibactin and bacilysin) as well as the ribosome-dependent synthesis of tasA and bacteriocins (subtilin, subtilosin A), which are responsible for the biosynthesis of numerous antimicrobial metabolites. Additionally, several genes with function that promote plant growth, such as indole-3-acetic acid biosynthesis, the production of volatile substances, and siderophores synthesis, were also identified.

The information described in this study established a good foundation for understanding the beneficial interactions between endophytes and host plants, and facilitates the further application of *B. subtilis* 7PJ-16 as an agricultural biofertilizer and biocontrol agent.

Keywords

Bacillus subtilis · Control efficiency · Plant growth promotion · Genome sequence · Antimicrobial metabolites

Introduction

Mulberry sclerotiniose, also known as white fruit disease, is a widespread destructive disease of mulberry fruit that is responsible for huge economic losses each year [1, 2]. The disease is divided into three types distinguished by the symptoms that appear on infected fruits in association with different causal agents: 1) mulberry sorosus hypertrophic sclerote disease, 2) mulberry sorosus parvulling sclerote disease, and 3) mulberry sorosus diminuting sclerote disease [3]. The first type is the most common both in China and in other parts of the world [4, 5]. There are four species in the family Sclerotiniaceae that have been implicated in these three types of symptoms: *Ciboria shiraiana* [6, 7], *Ciboria carunculoides* [8, 9], *Sclerotinia sclerotiorum* [4] and *Scleromitrula shiraiana* [6], each of which can infect mulberry fruit. In recent decades, chemical and non-chemical treatments have been successfully used to control the fruit disease [10, 11], and most reports described chemical interventions. Unfortunately, the long-term overuse of chemical pesticides has many side-effects on environmental safety and the balance of the agroecosystem. Furthermore, human health is potentially threatened by the presence of fungicide residues in fruits. Therefore, biological control of plant pathogens through the application of antagonistic probiotics is a promising plant protection strategy because it is safe for human beings and is environmentally friendly [12, 13].

Bacillus is a large, heterogeneous group of gram-positive, rod-shaped, endospore-forming aerobic or facultatively anaerobic bacteria that are able to grow in various environments, including plant tissues [14]. Many different communities of

Bacillus spp. have been isolated from a variety of plant hosts such as maize [15], mulberry [16], and rice [17]. The endophytic *Bacillus* species, especially *Bacillus subtilis*, suppress phytopathogenic fungal growth by producing a variety of bioactive compounds, with 4 ~ 5% of the bacterial genome being dedicated to the synthesis of these antimicrobial compounds [18]. Among these bioactive substances, cyclic lipopeptides (CLPS), non-ribosomally synthesized peptide derivatives, play an important role in antagonizing phytopathogen growth. Furthermore, some strains of *B. subtilis* enhance plant nutrition and stimulate plant growth while colonizing the tissues of the host plant [19].

The genus *Bacillus* is genetically diverse, so it has been difficult to classify *Bacillus* species accurately thus far, such as the taxonomy of the *B. subtilis*-like organisms *B. subtilis* and *Bacillus tequilensis* [20]. The analysis of 16S rRNA gene sequences is currently the most commonly used method for bacterial identification, but its usefulness is limited due to the high similarity among related taxa [21]. Recently, genome comparisons and phylogenomic analyses have been common approaches for the reconfirmation of some *Bacillus* species [22].

Previously, we isolated and identified an endophytic bacterium *Bacillus* sp. 7PJ-16, originally classified as *B. tequilensis* based on comprehensive analysis of its morphological, physiological, biochemical and 16S rRNA gene sequence characteristics (accession numbers, KR708875) [23]. However, 16S rRNA analysis of the 7PJ-16 strain also revealed a similarity of ≥ 99% to several *B. subtilis* isolates (e.g., accession numbers NR113265, NR112629 and NR027552.) deposited in NCBI.

Therefore, further experimental verification was needed for the taxonomic clarification of our 7PJ-16 strain. The 7PJ-16 strain exhibited strong antagonistic activity against various plant pathogens, including *S. shiraiana*, *S. sclerotiorum*, *Botryotinia fuckeliana*, *Cercospora beticola*, and *Fusarium oxysporum*, and cell-free supernatant of 7PJ-16 promoted mulberry tissue culture seedlings growth [23]. Our previous work revealed that the 7PJ-16 isolate is a promising biocontrol agent for mulberry fruit sclerotiniose, but its mechanisms of antimicrobial and growth-promoting properties remained to be explored. The aims of this study were (i) to evaluate the biocontrol efficiency of *Bacillus* sp. 7PJ-16 for mulberry fruit sclerotiniose in the field; (ii) to assess the growth-promoting ability of this strain in greenhouse conditions; (iii) to confirm the taxonomy of the 7PJ-16 strain using phylogenomic analysis and subsequently detect genes or gene clusters encoding secondary metabolites by sequencing and genome annotation of *Bacillus* sp. 7PJ-16; and (iv) to determine the potential of *Bacillus* sp. 7PJ-16 for secondary metabolite production. Ultimately, the goal is to have enough solid scientific information to the application of 7PJ-16 strain in the biological control mulberry fruit sclerotiniose.

Materials and Methods

Bacterial Strain and the Culture Medium

The 7PJ-16 strain was originally isolated from the surface-sterilized stem of a healthy Chuan Sang 7637 (mulberry cultivar which tolerant to mulberry fruit sclerotiniose) sampled from the mulberry experimental field of the Sericulture Science and Technology Institute, Chongqing, China (29°50′39″ N, 106°25′55″ E)

[23]. It was stored at -80 ˚C in 30% glycerol. The bacterial cells were streaked onto LB agar plates, and a single colony was inoculated into LB broth (100 mL in a 250 mL Erlenmeyer flask) with constant shaking at 180 revolutions per minute (rpm) at 28 ˚C for 18 h before using.

Determination of the 7PJ-16 Strain Biocontrol Properties in the Field

Fresh *Bacillus* sp. 7PJ-16 cultures were obtained as described above, and then they were inoculated into modified potato dextrose broth (PDB) for bioactive compounds production (1% v/v, inoculation amount) (MPDB medium, 200 g potato, 20 g maltose, 10 g peptone, 5 g $(NH_4)_2SO_4$, 1.5 g Na_2HPO_4 per liter) and incubated at 30 ˚C for 4 days in a fermentation tank (300 rpm). The bacterial cultures were centrifuged at 8,000 rpm for 20 min to generate the supernatant. On the other hand, *Bacillus* sp. 7PJ-16 was incubated in PDB for 18 ~ 24 h, and then the cultures were centrifuged at 5,000 rpm for 8 min, finally the bacterial pellets were resuspended in sterilized distilled water (SDW). Fresh prepared cell suspension were adjusted to 1.0×10^9, 1.0×10^8 and 1.0×10^7 colony forming units per microliter (CFU/mL) with SDW and the supernatant were diluted in SDW as original, 10-fold and 100-fold dilution before being applied in the field experiments.

The field experiments were performed at the Sericulture Science and Technology Institute, Chongqing, China (29°50′39″ N, 106°25′55″ E) during the spring of two consecutive years (2016 and 2017) using mulberry cultivar *Morus atropurpurea* Roxb cv. ‘ Zhongsang 5801’. It has been known for the past 5 years that the orchard was naturally infested, and sclerotium and apothecia were also found in

the orchard. There are 1 m separation belts between different experimental plots and the size of each plot was about 4 × 10 m. Approximately 36 ~ 42 plants were grown in each plot.

In 2016, the experiment consisted of four treatments: (1) water control; (2) fungicide thiophanate-methyl (1000-fold dilution); (3) *Bacillus* sp. 7PJ-16 supernatant (original) and (4) *Bacillus* sp. 7PJ-16 supernatant (100-fold dilution). All treatments were applied three times at the initial, full and end of flowering stage, respectively. In 2017, the biocontrol effects of *Bacillus* sp. 7PJ-16 bacterial suspension and supernatant were evaluated, and the effects of different application time and various treatment concentration on disease incidence of mulberry fruits were also assessed. The following sixteen treatments were included in this experiment (1) water control; (2) application of the fungicide thiophanate-methyl (1000-fold dilution) three times at the initial, full and end of flowering stage respectively; (3)-(5) application of the 7PJ-16 cell suspension at the initial flowering stage with three concentrations which were 1.0×10^9, 1.0×10^8 and 1.0×10^7 CFU/mL respectively; (6)-(8) 7PJ-16 cell suspension was applied twice (at the initial and full flowering stage) with three concentrations which were 1.0×10^9, 1.0×10^8 and 1.0×10^7 CFU/mL respectively; (9) 7PJ-16 cell suspension of 1.0×10^9 CFU/mL was applied three times at the initial, full and end of flowering stage respectively; (10)-(12) 7PJ-16 supernatant of three different dilutions (original, 10-fold and 100-fold dilution) were applied at the initial flowering stage; (13)-(15) 7PJ-16 supernatant of three different dilutions (original, 10-fold and 100-fold dilution) were applied twice at the initial and full flowering

stage respectively; (16) applications of original 7PJ-16 supernatant three times at the initial, full and end of flowering stage respectively. The 7PJ-16 supernatant was collected after being cultured in MPDB medium for 48 h. All treatments were applied at 9:00-10:00 AM. As for all treatments applied three times, included all treatments in 2016 and treatments (1), (2), (9) and (16) in 2017, a randomized block design with 3 replicates (12 trees per replicate) of each treatment was performed, rather than 3 replicates (3 trees per replicate) of each treatment for other treatments.

The disease incidence of mulberry fruits was investigated approximately one week before harvest. For each treatment, the total and diseased fruits from three independent trees (two or three branches per tree) were visually recorded. The disease incidence of each treatment was calculated as the percentage of diseased fruits. The disease incidence and biocontrol efficacy of each treatment were calculated as follows:

Disease incidence (%) = the number of diseased fruits / the total number of fruits examined × 100;

Control efficacy (%) = (the mean disease incidence of the control - the mean disease incidence of the treatment) / the mean disease incidence of the control × 100.

Effects of the 7PJ-16 Strain on Mulberry Seed Germination and Seedling Growth

Fresh cell suspension and cell-free supernatant of 7PJ-16 strain were prepared as described above in the King's B medium [24] rather than MPDB or normal PDB medium, and the cell-free supernatant in this section was obtained by being passed

through 0.22 μm micropore filter. Different concentrations of 7PJ-16 cell-free supernatant (original, 10-fold, 100-fold and 1000-fold dilution) and different concentrations of 7PJ-16 suspension (1.0×10^8, 1.0×10^7, 1.0×10^6 and 1.0×10^5 CFU/mL) were diluted in SWD.

Mulberry seeds (Gui Sangyou No.12) were disinfected and germinated according to the methods described by Xie et al. [25]. Briefly, the surface-disinfected seeds were respectively soaked in 7PJ-16 cell-free supernatant of different dilutions and 7PJ-16 cell suspension of different cell concentrations for 24 h at 25 ˚C. The treated seeds were eventually germinated on sterile moist filter paper in Petri dishes at 26 ˚C for 15 days with 12 h of light. On the fourth day of cultivation, the germination potential was calculated as the percentage of germinated mulberry seeds. The seed germination rate was recorded at the eighth day. Furthermore, the length of plumule and radicle were measured after the seedlings were cultivated for 15 days. Surface-disinfected seeds treated with King's B medium and SWD were served as controls. Each treatment was replicated three times with 20 seeds apiece.

The seedlings used for pot experiments were obtained using the water-treatment seed germination described above. The seedlings were aseptically transplanted into 8 × 8 cm diameter pots containing autoclaved soil, and planted 3 ~ 4 seedlings in each pot. And then all seedlings were cultivated in a greenhouse at 28 ˚C, relative humidity 70%, and a 12 h photoperiod. When the seedling grew to 4 leaves, 10 mL of each treatment samples were applied to one pot. Each treatment was replicated three times. Forty five days after inoculation, five seedlings were randomly selected from each

treatment to measure parameters of plant growth, including root and shoot length, fresh weight of root and shoot, and dry weight of root and shoot.

Statistical Analysis

All treatments for each experiment were compared using analysis of one-way analysis of variance (ANOVA), and the means were compared using a least significant difference (LSD) test at $P \leq 0.05$. The program Excel and SPSS, version 17.0 (SPSS Inc., Chicago, IL, USA) were used for statistical analysis.

Genomic DNA Preparation, Sequencing and Analysis

The 7PJ-16 strain was cultured in LB medium as described above, and bacterial cells were subsequently collected via centrifugation. Genomic DNA was extracted with Prepman Ultra Sample Preparation Reagent (Applied Biosystems, USA) according to the manufacturer's instructions. The entire genome of 7PJ-16 was examined on a Pacific Biosciences RSII sequencer (PacBio, Menlo Park, CA) according to standard protocols at Guangzhou Genedenovo Biotechnology Co., Ltd. (Guangdong, China).

De novo sequence assembly of the genome was performed using the software hierarchical genome-assembly process (HGAP) [26]. The open reading frame was predicted using GeneMarkS [27], which is a well-studied gene-finding program used for prokaryotic genome annotation. The protein sequences were annotated based on observed genes and searched using various function-related databases (Clusters of Orthologous Groups, COG; Kyoto Encyclopedia of Genes and Genomes, KEGG; Non-Redundant Protein Database, Nr; and Gene Ontology, GO). A circular genome

map with COG functional annotation was plotted with Circos [28]. The phylogenetic tree based on the core genomes of 18 strains with known genome sequences related to members of the *Bacillus* genus was constructed using MEGA 6.0 software using the neighbor-joining method and default parameters [29]. Bioactive secondary metabolites were predicted with antiSMASH 4.0 (http://antismash.secondarymetabolites.org/#!/start).

Production of Secondary Metabolites

The production of siderophores, cellulase and volatile compounds was detected on standard chrome Azurol-S (CAS) agar medium [30], carboxymethylcellulose agar medium [31] and a two-compartment Petri dish [32], respectively. The qualitative detection of indole acetic acid was inferred by the methods of Jasim, Joseph & John [30].

Results

Biocontrol Efficacies of 7PJ-16 on Mulberry Fruit Sclerotiniose in the Fields

In the 2016 experiment, the disease incidence for the 7PJ-16 supernatant treatment, regardless of original (19.57%) or 100-fold dilution (52.83%), were much lower than that of the water control (70.22%) (Table 1). The biocontrol efficacy of the original 7PJ-16 supernatant was up to 72.13%, although it was lower than that of the fungicide treatment (98.16%) (Table 1). Whereas, when original 7PJ-16 supernatant was applied twice on the mulberry flowers, the leaves were decrescent and slightly curled, and the flowers were marginally withered. Interestingly, these leaf and flower disease symptoms were not observed in the mature fruit stage. The phenomenon

might suggest that the original 7PJ-16 supernatant, collected on 4 days after inoculation, contained high concentration of antibiotics, and they can effectively suppress phytopathogen growth but with obviously negative effects on mulberry. Thus, the fermentation time of harvesting 7PJ-16 supernatant was adjusted to 2 days in the field experiment of 2017, which might decrease the concentration of antibiotics.

The field experimental results in 2017 indicated 7PJ-16 cell suspension or supernatant could control the disease incidence of mulberry fruit sclerotiniose, but the biocontrol efficacy decreased gradually when the 7PJ-16 cell suspension or supernatant were diluted and the application times were shortened (Table 2). The biocontrol efficacy of the 7PJ-16 cell suspension of 1.0×10^9 CFU/mL (original) was equivalent to the original 7PJ-16 supernatant regardless of application times: one application (84.33% vs 85.88%), two applications (87.33% vs 87.99%) and three applications (90.84% vs 87.78%), respectively (Table 2). Additionally, applying 7PJ-16 cell suspension of 1.0×10^9 CFU/mL three times resulted in the lowest disease incidence (0.80%), performing better than that of the fungicide (0.83%). Interestingly, as for the treatments applied three times, the fruits which were treated with the 7PJ-16 cell suspension of 1.0×10^9 CFU/mL matured earlier no matter turned red or finally matured compared with the other treatments (Fig. 1A). Meanwhile, mulberry trees treated with the 7PJ-16 supernatant had the same growth in comparison with other treatments, without any phytotoxicity symptoms (Fig. 1C). Additionally, the 7PJ-16 cell suspension of 1.0×10^9 CFU/mL could accelerate the ripening of mulberry fruits. This observation prompted us to evaluate its capacity of plant growth promotion on

mulberry seed and seedling in greenhouse conditions.

Growth-Promotin Assay of 7PJ-16 on Mulberry Seed Germination and Mulberry Seedling Growth in Greenhouse Conditions

Compared with the two control treatments, the 7PJ-16 cell suspension of 1.0×10^5 CFU/mL and the 7PJ-16 cell-free supernatant of 1000-fold dilution significantly ($P \leqslant 0.05$) increased the germination potential and germination rate of mulberry seeds (Table 3). The seeds treated with the cell-free supernatant (original and 10-fold dilution) or cell suspension of 1.0×10^7 CFU/mL did not seem to stimulate mulberry seeds germination. In addition, the seedlings in the all treatments of the 7PJ-16 cell-free supernatant exhibited longer plumule length and radicle length than those for treatments of the 7PJ-16 cell suspension, and the most striking effect was observed in the treatment with 7PJ-16 cell-free supernatant at a 10-fold dilution. It resulted in an remarkably increase radicle length from 30.18 mm to 43.71 mm and promoted the plumule length from 11.10 mm to 15.26 as compared with the water control (Table 3).

The 7PJ-16 suspension greatly promoted mulberry seedlings growth, especially the treatment with the 7PJ-16 cell suspension of 1.0×10^6 CFU/mL. All growth parameters, including the root length, the shoot length and the fresh/dry weight of root and shoot of seedlings, were significantly ($P \leqslant 0.05$) higher in this treatment compared to those of water control (CK) plants (Fig. 2 and Fig. S1). Notably, compared with the CK, this treatment increased root length from 2.22 mm to 4.14 mm (Fig. 2A), and increased shoot fresh weight from 55.18 mg to 128.82 mg (Fig. 2B). Furthermore, the 1.0×10^5 CFU/mL suspension also facilitated root growth in comparison with the CK,

as the dry weight of these seedlings increased by 120.39% (from 1.52 mg to 3.35 mg) (Fig. 2C).

Similarly, the 7PJ-16 cell-free supernatant also partly stimulated the growth of mulberry seedlings, particularly the treatment with a 100-fold dilution of 7PJ-16 supernatant, which clearly enhanced root length from 2.08 mm to 4.01 mm compared to the water control (Fig. 2D). However, the original cell-free supernatant produced a slightly negative effect on the growth of mulberry seedlings. The shoot length, root fresh weight and shoot fresh weight of plants grown in this treatment decreased by 4.17%, 5.26%, and 11.42%, respectively (Fig. 2D, E and Fig. S2).

Genome Sequencing and Phylogenetic Analysis of *Bacillus* sp. 7PJ-16

The complete genome of *Bacillus* sp. 7PJ-16 consists of a 4,209,045 bp circular chromosome and two circular plasmids unnamed 1 (21,679 bp) and unnamed 2 (8,581 bp) (Fig. 3). The G+C contents of the chromosome and the two plasmids are 43.28%, 40.30% and 38.54%, respectively. The chromosome contains 4,492 predicted coding sequences (CDSs), 30 rRNA genes and 86 tRNA genes; the two plasmids contain 29 and 12 predicted CDSs, respectively (Fig. 3).

The general features of *Bacillus* sp. 7PJ-16 genome and other representative *Bacillus* strains are summarized in Table 4. Phylogenetic analysis revealed that 7PJ-16 forms a distinct branch with *B. subtilis* Bsn5 (CP002468), *B. subtilis* SG6 (CP009796), *B. subtilis* QB928 (CP003783) and *B. subtilis* subsp.*subtilis* str.168 (NC_000964) (Fig. 4), which suggested that it may be more appropriately identified as a *B. subtilis* strain. To confirm this hypothesis, we performed a BLAST search on the sequence of the

gryA gene of the subject strain (data not given), which this gene can be used to differentiate *B. subtilis* and related *Bacillus* species [33]. The BLAST search indicated that *Bacillus* sp. 7PJ-16 has a greater than 99% sequence similarity to several *B. subtilis* isolates (accession numbers AY663694 and EU138617) rather than *B. tequilensis* for the *gryA* gene sequence. Therefore, we propose that *Bacillus* sp. 7PJ-16 be recognized as a strain of *B. subtilis*, as an update to its final classification status [23].

Secondary Metabolite Gene Clusters in *B. subtilis* 7PJ-16

Genome analysis revealed that there are two different pathways for the biosynthesis of secondary metabolites in the *B. subtilis* 7PJ-16 genome. This strain harbors ten gene clusters related to antimicrobial activities (Table 5). Among these gene clusters, four are related to non-ribosomal peptide synthetases including surfactin, fengycin, bacillibactin and bacilysin, covering over 213.7 kb in total. Additionally, two of the gene clusters direct the synthesis of the bacteriocins, subtilin and subtilosin A, which mainly inhibit the growth of similar or closely related bacterial strains. In contrast to other closely related strains, the *tasA* gene was also found.

Surfactin has been reported to be a cyclic lipopeptide antibiotic with a strong inhibitory effect on tumors, viruses and mycoplasma [34]. The biosynthesis of surfactin in *B. subtilis* 7PJ-16 was attributed to a large 65.4 kb DNA sequence, consisting of four core biosynthetic genes (*srfABCD*) and four additional transport-related genes (*ycxABCD*). Fengycin and the closely related plipastatin are

also CLPs, which are specifically active against filamentous fungi [35]. The *pps* operon was assigned to fengycin biosynthesis, but *B. subtilis* 7PJ-16 only has partial genes (*ppsE, ppsD*) in this gene cluster. A similarly incomplete fengycin cluster was previously discovered in *Bacillus amyloliquefaciens* DSM-7 [36]. Bacilysin, one of the simplest known peptide antibiotics, is active against a variety of bacteria and the yeast *Candida albicans* [37]. In addition, the functional *bacABCDE* gene cluster responsible for bacilysin synthesis was detected in the 7PJ-16 genome. Additionally, *B. subtilis* 7PJ-16 also might be an excellent bacteriocin producer, potentially producing two common lantipeptides, subtilin and subtilosin A, which are capable of inhibiting the growth of gram-positive bacteria [38]. Notably, we also found the *tasA* gene from the coding region, and it can express an antimicrobial protein which has a significant antagonistic effect on numerous phytopathogens [39].

Identification of Genetic Elements in *B. subtilis* 7PJ-16 Associated With the Promotion of Plant Growth

In addition to the diverse genes related to antimicrobial metabolites, strain 7PJ-16 also harbors genes involved in the synthesis of various substances that promote plant growth, including auxin (e.g., IAA), bacillibactin and volatile compounds (e.g., 2,3-butanediol and acetoin) (Table 6). Bacillibactin, an iron-siderophore encoded by the *dhb* gene cluster, can help *Bacillus* cells to take up iron ions from the natural environment under iron-limited conditions. Indole acetic acid (encoded by the *yhcX* and *dhaS genes*), acetoin (encoded by the *alsD*, *alsS* and *alsR* genes) and 2,3-butanediol (encoded by the *bdhA* gene) have been indicated to

enhance plant growth and trigger systemic resistance [40]. Notably, the *bglC* and *bglS* genes (encoding cellulase) and *xynA* and *xynD* (encoding xylanase), which play a key role in the degradation of plant cell walls [40], were also found in the *B. subtilis* 7PJ-16 genome. Accordingly, the results regarding metabolite production showed that 7PJ-16 possesses the ability to secrete iron-siderophores, indole acetic acid and volatile compounds (Fig. 5).

Discussion

Compared with chemical control, biological control has been considered to be a potential and sustainable alternative method due to the rare environment pollution and low health risk. Several bacterial strains showing high antagonistic activity towards phytopathogens, such as *Bacillus* [17, 19, 22, 32, 41], *Pseudomonads* [42], and *Pantoea* [25] can be successfully employed as potential biocontrol agents in plant disease management. Our results of the two-year field experiments indicated that the application of 7PJ-16 was able to suppress the occurrence of mulberry fruit sclerotiniose under field conditions, even in 2016 with the high disease incidence in case of the water treatment (70.22%) (Table 1). In 2017, the biocontrol efficacies in the high-concentration treatments were better than that of the relatively low-concentration treatments, and the efficacy was affected by application times to a certain extent (Table 2). Similar results were obtained by the application of endophytic *B. subtilis* EDR4 controlling *S. sclerotiorum* on rapeseed [41]. In fact, it has been reported that the cell suspension of *B. thuringiensis* C25 and the wettable powder of *Trichoderma* sp. can be used to control the mulberry fruit disease in fields,

but the disease prevention efficacy remained only at 51.98% ~ 84.02% [13, 43], which was less than the control efficacy of present study (90.84%). This paper revealed that the 7PJ-16 strain have the huge potential to control the sclerotial disease of mulberry fruit, and therefore makes itself a good candidate of biological control in modern agricultural systems.

Bacillus species produce a structurally diverse group of antimicrobial compounds, among which the three families of lipopeptides (surfactin, fengycin and iturin) exhibit highly potent antifungal activity and are very heat stable and insensitive to pH [44]. Numerous studies have demonstrated that the antifungal effect of *Bacillus* strains against phytopathogenic fungi was closely associated with lipopeptides synthesis [45–47]. Although the molecules responsible for the antagonistic activity of *B. subtilis* 7PJ-16 have not yet been identified, genes involved in lipopeptides synthesis, such as *srfABCD*, *ycxABCD* and *ppsDE*, were detected in the 7PJ-16 strain genome. Furthermore, the stability assay indicated that when the cell-free supernatant containing crude bioactive substances from the 7PJ-16 strain was subjected to different pH and thermal conditions, the antifungal activity was not affected [23]. Therefore, one underlying antimicrobial mechanism of the 7PJ-16 strain may be the presence of genes encoding surfactin and other lipopeptides in its genome.

Apart from controlling diseases, the genus *Bacillus* also directly or indirectly promote plant growth by several mechanisms, including production of phytohormones like IAA, siderophores production and secretion of the active enzymes, such as cellulose and protease [19, 48, 49]. Our pot experiment results demonstrated that *B.*

subtilis 7PJ-16 did have positive effects on the mulberry growth, and both 7PJ-16 cell suspension and cell-free of this strain supernatant could stimulate the mulberry seed germination and mulberry seedling growth, especially for the relatively low-concentration treatments, such as the cell suspension of 1.0×10^6 CFU/mL and cell-free supernatant of 100-fold dilution (Fig. 2, Fig. 1S and Fig. 2S). Mulberry growth promotion might be related to the siderophore, cellulose, IAA and volatile compounds produced by the 7PJ-16 strain (Table 6, Fig. 5). These results indicated that the antagonistic bacterium 7PJ-16 can also serve as a plant growth stimulator or biofertilizer.

Since *Bacillus* species have the ability to control plant diseases and improve the plant growth by several mechanisms, these beneficial properties have resulted in the isolation, characterization and application of many strains of this organism for use in plant protection [17, 19, 41, 48]. However, many *Bacillus* strain have very similar phenotypic, physiological properties and 16S rRNAs gene sequences, making the definitive taxonomy of these strains very difficult [50]. Owing to the development of sequencing technique and bioinformatics, the whole genome of numerous *Bacillus* genus have been sequenced and some strains were re-categorized into other species by the genome phylogenomic analysis [22, 51]. The final classification status as *B. subtilis* of our 7PJ-16 strain was also reconfirmed by this approach (Fig. 4). Our study verified that the genome-based analysis could be used as a useful alternative technique in taxonomy of genetic-closely related species, such as the *B. subtilis*-like organisms [20].

Conclusions

In this study, we report the complete genome of *Bacillus* sp. strain 7PJ-16 and its final classification status as *B. subtilis* was further confirmed by the phylogenomic analysis. *B. subtilis* 7PJ-16 not only could control mulberry fruit sclerotiniose under fields, but stimulate the mulberry seed germination and mulberry seedling growth in greenhouse conditions. In addition, six gene clusters related with biosynthesis of antimicrobial metabolites were identified in the *B. subtilis* 7PJ-16 genome and it provided us a molecular basis to understand the strain's extraordinary biocontrol capacity against mulberry fruit sclerotiniose. And the 7PJ-16 strain possesses numerous genes related to the synthesis of plant growth-promoting substances, such as indole-3-acetic acid, volatile substances and siderophores. Genome sequencing and analysis of the *B. subtilis* 7PJ-16 strain does provide valuable information revealing its biological control mechanisms at the genetic level. As such this information has the potential to the application of *Bacillus* strains as agricultural biocontrol and biofertilizer agents. However, additional studies are needed to assess the safety status and potential health benefits of this strain before it can be applied at the field level.

Nucleotide Sequence Accession Numbers

The complete genome sequence of *B. subtilis* 7PJ-16 has been deposited in GenBank under accession number CP023409. In addition, this strain has been deposited at the China Center for Type Culture Collection under accession number CCTCC M 2015319.

Acknowledgments

The authors acknowledge the financial support of the National Natural Science Foundation of China (31601678), the Fundamental Research Funds for the Central Universities (XDJK2018D020) to Ting Ou, and the Natural Science Foundation of Chongqing (cstc2015jcyjys80001) to Jie Xie.

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Table 1 The biocontrol efficacies of 7PJ-16 supernatant in the field in 2016

Treatments	Disease incidence ± SE (%)	Biocontrol efficacy ± SE (%)
Water	70.22 ± 2.72 a	—
7PJ-16 supernatant (original)	19.57 ± 1.60 c	72.13 ± 2.28 b
7PJ-16 supernatant (100-fold dilution)	52.83 ± 5.45 b	24.77 ± 7.76 c
Thiophanate-methyl (1000-fold dilution)	1.29 ± 0.13 d	98.16 ± 0.18 a

SE, standard error of means

Different letters on each number define groups of treatments that showed significant differences at the $P \leqslant 0.05$ by one-way analysis of variance (ANOVA) and least significant difference (LSD) tests

Table 2 The biocontrol efficacies of 7PJ-16 cell suspension and supernatant in the field in 2017

Treatments	Disease incidence ± SE (%)	Biocontrol efficacy ± SE (%)
Water	8.73 ± 0.30 a	—
Application once at initial flowering stage		
7PJ-16 suspension (1.0×10^9 CFU/mL)	1.37 ± 0.31 de	84.33 ± 3.55 ab
7PJ-16 suspension (1.0×10^8 CFU/mL)	6.05 ± 0.49 bc	30.66 ± 5.65 cd
7PJ-16 suspension (1.0×10^7 CFU/mL)	6.67 ± 0.42 b	23.58 ± 4.88 d
7PJ-16 supernatant (original)	1.23 ± 0.14 e	85.88 ± 1.62 a
7PJ-16 supernatant (10-fold dilution)	2.28 ± 0.39 d	73.90 ± 4.44 b
7PJ-16 supernatant (100-fold dilution)	5.86 ± 0.63 bc	32.90 ± 7.24 c
Application twice at initial and full flowering stage		
7PJ-16 suspension (1.0×10^9 CFU/mL)	1.11 ± 0.23 e	87.33 ± 2.61 a
7PJ-16 suspension (1.0×10^8 CFU/mL)	5.29 ± 0.31 c	39.37 ± 3.59 c
7PJ-16 suspension (1.0×10^7 CFU/mL)	7.28 ± 0.32 b	16.58 ± 3.67 d
7PJ-16 supernatant (original)	1.05 ± 0.11 e	87.99 ± 1.31 a
7PJ-16 supernatant (10-fold dilution)	1.72 ± 0.11 de	80.34 ± 1.24 ab
7PJ-16 supernatant (100-fold dilution)	2.04 ± 0.42 de	76.68 ± 4.79 ab
Application three times at total flowering stage		
7PJ-16 suspension (1.0×10^9 CFU/mL)	0.80 ± 0.12 e	90.84 ± 1.41 a
7PJ-16 supernatant (original)	1.07 ± 0.03 e	87.78 ± 0.32 a
Thiophanate-methyl (1000-fold dilution)	0.83 ± 0.04 e	90.52 ± 0.40 a

SE, standard error of means

Different letters on each number define groups of treatments that showed significant differences at the $P \leqslant 0.05$ by one-way analysis of variance (ANOVA) and least significant difference (LSD) tests

Table 3 Effects of the endophyte 7PJ-16 on mulberry seed germination and growth

Treatments	Dilution time	Germination potential (%)	Germination rate (%)	Radicle length (mm)	Plumule length (mm)
King·s B medium	—	55.00 ± 4.18 de	71.00 ± 3.67 cd	40.88 ± 2.70 a	13.42 ± 0.94 ab
Water	—	58.00 ± 2.55 cd	73.00 ± 5.70 cd	30.18 ± 0.70 bc	11.10 ± 0.75 cd
7PJ-16 suspension	0	71.00 ± 3.32 ab	79.00 ± 6.40 abc	32.34 ± 2.10 bc	11.09 ± 0.60 cd
(10^8 CFU/mL)	10	45.00 ± 2.24 f	74.00 ± 11.55 bcd	28.76 ± 1.38 c	10.99 ± 0.65 d
	100	65.00 ± 2.24 bc	88.00 ± 3.00 ab	33.43 ± 1.57 b	11.54 ± 0.65 bcd
	1000	79.00 ± 1.87 a	90.00 ± 3.16 a	32.55 ± 0.99 bc	10.26 ± 0.40 d
7PJ-16 cell-free	0	54.00 ± 3.67 def	71.00 ± 3.67 cd	40.32 ± 2.62 a	13.26 ± 1.40 abc
supernatant	10	46.00 ± 2.45 ef	61.00 ± 4.58 d	43.71 ± 1.89 a	15.26 ± 0.82 a
	100	67.00 ± 1.22 bc	83.00 ± 3.39 abc	40.08 ± 2.20 a	11.81 ± 1.08 bcd
	1000	73.00 ± 6.04 ab	84.00 ± 2.45 abc	39.33 ± 2.35 a	14.94 ± 0.69 a

Note: Values are the results of the mean plus standard error, and different letters in the same column indicate a significant difference at $P \leqslant 0.05$ by one-way analysis of variance (ANOVA) and least significant difference (LSD) tests

Table 4 Genomic features of *Bacillus* sp.7PJ-16 and related members of the *Bacillus* genus

Features	*Bacillus* sp.7PJ-16	*B. tequilensis* FJAT-14262a	*B. subtilis* 168	*B. subtilis* Bsn5	*B. amyloliquefaciens* L-S60	*B. velezensis* 9912D
Genome size (bp)	4,209,045	4,038,551	4,215,606	4,093,599	3,903,017	4,241,576
G+C content (%)	43.28%	43.71%	43.50%	43.85%	46.67%	45.99%
Plasmid	2	-	-	-	-	1
rRNA operons	30	6	30	31	26	27
tRNA operons	86	66	86	83	91	86
Total predicted CDS[a]	4,492	3,919	4,174	4,177	3,909	4,436

CDS[a]: Coding DNA Sequences

Table 5 Identification of gene clusters potentially involved in antimicrobial metabolite synthesis by *B. subtilis* 7PJ-16

Clusters[a]	Types[b]	Position[c]	Metabolite[d]	Function[e]
1	Nrps	391449-456840	Surfactin	Antivirus, antimycoplasma, antitumor
2	Terpene	1197446-1218258	Unknown	Unknown
3	Lantipeptide	1765179-1805797	Unknown	Unknown
4	Nrps	1918705-1975814	Fengycin	Antifungal
5	Terpene	2053475-2075397	Unknown	Unknown
6	T3pks	2254102-2295199	Unknown	Unknown
7	Nrps	3228426-3278167	Bacillibactin	Accumulate and take up iron ions
8	Lantipeptide	3426439-3452493	Subtilin	Antibacterial(G^+)
9	Lantipeptide	3813741-3835352	Subtilosin A	Antibacterial(G^+)
10	Nrps	3838655-3880073	Bacilysin	Antibacterial and *Candida albicans*

[a] Clusters identified using the default settings of antiSMASH 4.0

[b] Classes of gene cluster according to antiSMASH 4.0

[c] Location of gene clusters in the *B. subtilis* 7PJ-16 genome

[d] Secondary metabolites potentially produced based on the gene clusters

[e] Bioactive function of metabolites synthesized by the gene clusters

Table 6 Representative genes of *B. subtilis* 7PJ-16 likely involved in plant growth promotion

Gene	Position	Protein	Description
dhbF	3,248,426-3,255,562	2,3-dihydroxybenzoate-glycine-threonine trimeric ester	Bacillibactin biosynthesis
dhbB	3,255,582-3,2565,20	Isochorismatase	Bacillibactin biosynthesis
dhbE	3,256,548-3,258,167	2,3-dihydroxybenzoate-AMP ligase	Bacillibactin biosynthesis
dhbC	3,258,196-3,259,392	Isochorismate synthase	Bacillibactin biosynthesis
dhbA	3,259,418-3,260,203	2,3-dihydroxybenzoate dehydrogenase	Bacillibactin biosynthesis
alsD	3,696,405-3,697,184	Acetolactate decarboxylase	Acetoin biosynthesis
alsS	3,697,234-3,698,949	Acetolactate synthase	Acetoin biosynthesis
alsR	3,699,104-3,700,012	Transcriptional regulator	Regulator of the *alsDS* operon
bdhA	704,601-705,641	Acetoin reductase	2,3-butanediol biosynthesis
treP	900,870-902,282	Phosphotransferase	Trehalose biosynthesis
treA/C	902,353-904,038	Trehalose-6-phosphate hydrolase	Trehalose biosynthesis
treR	904,059-904,775	Transcriptional regulator (GntR family)	Trehalose biosynthesis
phy	2,241,654-2,242,802	Phytase	Phosphate availability
bglS	1,924,777-1,926,225	Endo-1,4-beta-glucanase	Cellulose degradation
bglC	3,998,347-3,999,057	Endo-beta-1,3-1,4 glucanase	Cellulose degradation
xynA	2,011,681-2,012,322	Endo-1,4-beta-xylanase	Xylanase degradation
xynD	1,928,213-1,929,616	Endo-1,4-beta-xylanase	Xylanase degradation
yhcX	1,044,946-1,046,487	Amidohydrolase	Trp-dependent IAA synthesis
dhaS	2,061,925-2,063,397	Aldehyde dehydrogenase	Trp-dependent IAA synthesis

Fig. 1 Biocontrol efficacies of the endophyte 7PJ-16 on mulberry fruit sclerotiniose after the third application in the fields in 2017. (A) 7PJ-16 cell suspension; (B) thiophanate-methyl; (C) 7PJ-16 supernatant; (D) water control. The number 1 ~ 4 respectively represent the control efficacies at 7, 15, 22 and 30 days after treatment

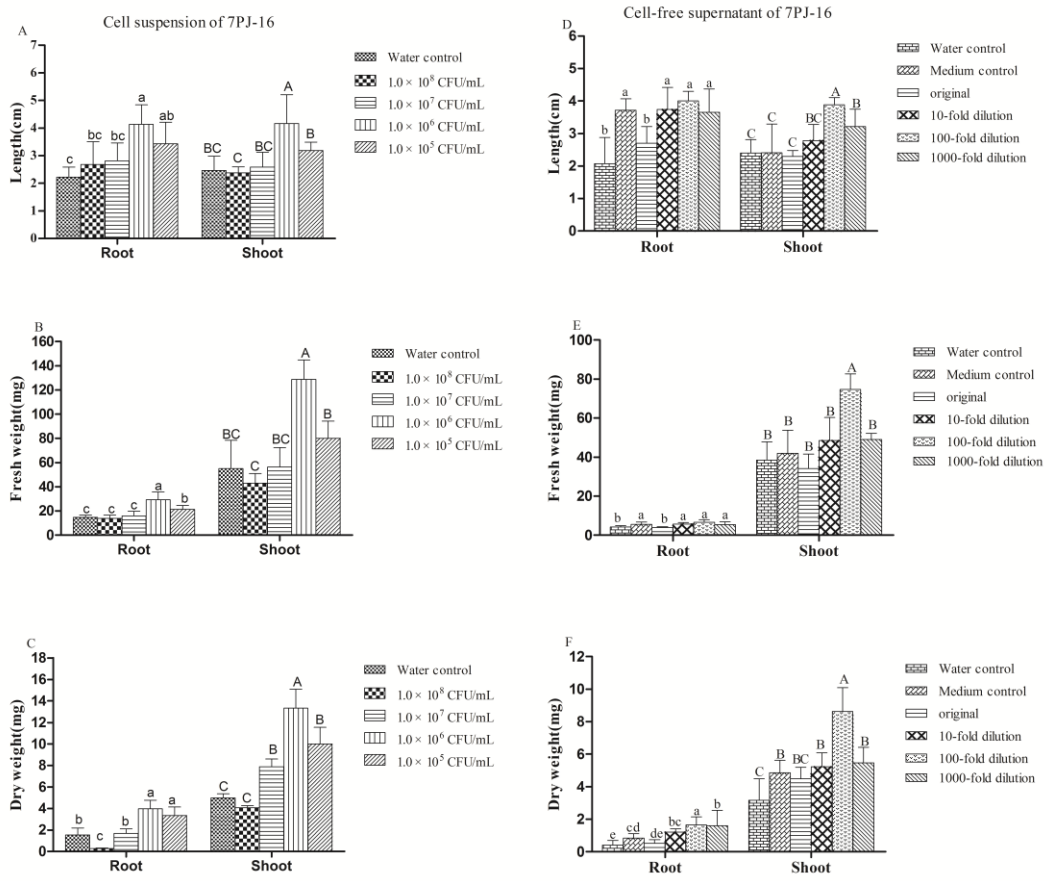

Fig. 2 The effects of 7PJ-16 cell suspension (A~C) and 7PJ-16 cell-free supernatant (D~F) on the growth of mulberry seedlings 45 days after treatment. (A) and (D) root and shoot lengths; (B) and (E) root and shoot fresh weights; (C) and (F) root and shoot dry weights. Bars with the different letters indicate a significant difference between means by one-way analysis of variance (ANOVA) and least significant difference (LSD) tests ($P \leqslant 0.05$). Error bars indicate ± SD of five replicates

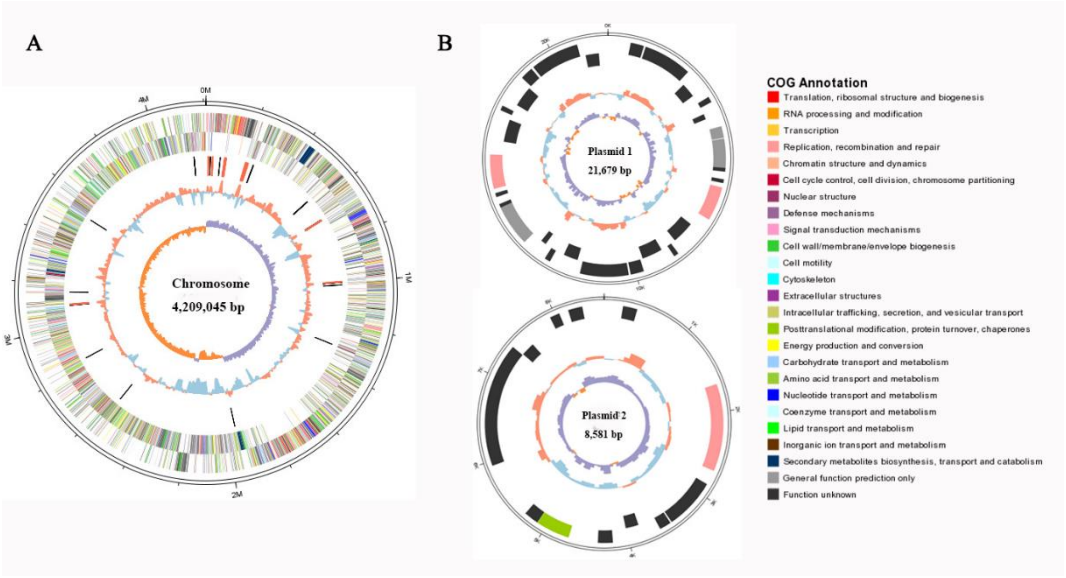

Fig. 3 Circular genome maps of the *Bacillus* sp. 7PJ-16 genome. (A) circular chromosome; (B) plasmids. From outside to inside: (1) size of the complete genome; (2-3) predicted protein-coding genes on the + and − strands, with different colors representing different COG functional classifications; (4) rRNA (red) and tRNA (black); (5) G + C content, with > 43.28% G + C indicated in red and ≤ 43.28% G + C in blue; (6) G + C skew, with G% > C% in purple and G% < C% in orange

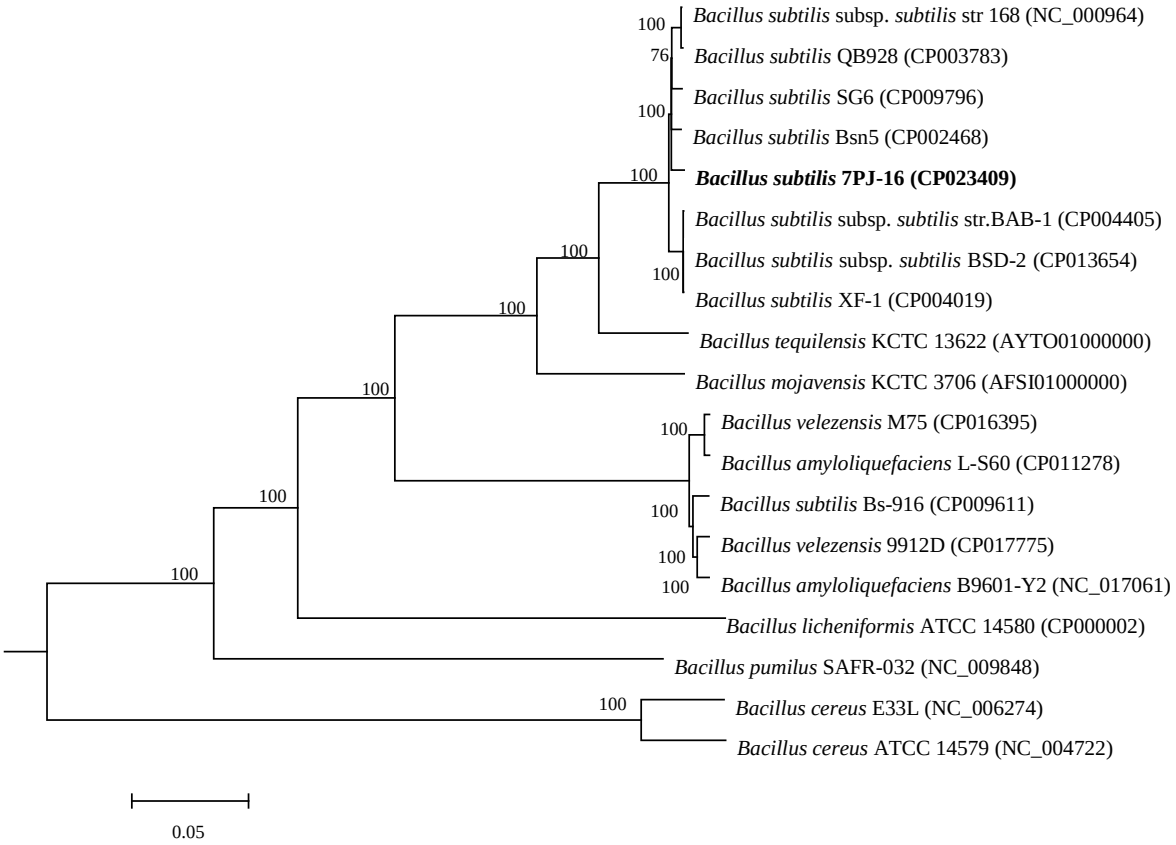

Fig. 4 Phylogenetic tree of *Bacillus* sp. 7PJ-16 based on the core genomes of 18 representative *Bacillus* strains. The percentages at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1,000 resampled data sets

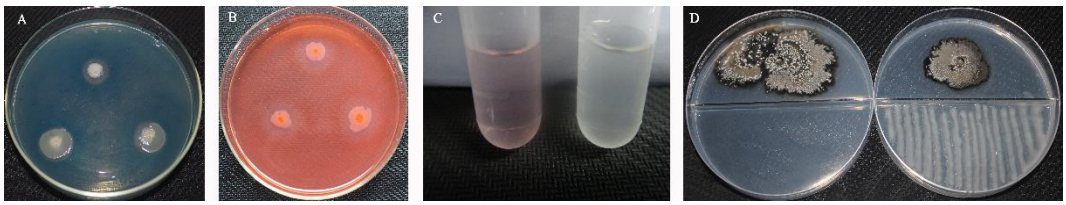

Fig. 5 Secretions of *B. subtilis* 7PJ-16. (A) iron-siderophore: the zone on chrome Azurol-S medium; (B) cellulase: the zone on carboxymethylcellulose medium; (C) indole acetic acid: left, IAA produced following 7PJ-16 inoculation of PDB medium supplemented with L-tryptophan, and right, non-inoculated PDB medium; (D) volatile compounds: left, PDA medium inoculated with *Scleromitrula shiraiana*, and right, PDA medium inoculated with *Scleromitrula shiraiana* and the antagonistic bacterium 7PJ-16

Fig. S1 The effects of 7PJ-16 cell suspension on the growth of mulberry seedlings 45 days after treatment in pots. (A) water control (CK); (B1-B4) 7PJ-16 cell suspension (1.0×10^8, 1.0×10^7, 1.0×10^6, 1.0×10^5 CFU/mL)

Fig. S2 The effects of 7PJ-16 cell-free supernatant on the growth of mulberry seedlings 45 days after treatment in pots. (A) water control (CK1); (B) medium control (CK2); (C1-C4) 7PJ-16 supernatant (original, 10-fold, 100-fold and 1000-fold dilution)