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Full Length Research Paper

Identification and characterization of a *Bacillus subtilis* strain TS06 as bio-control agent of strawberry replant disease (*Fusarium* and *Verticilium* wilts)

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Replant disease is a major limitation for strawberry production in greenhouse. Bio-control may be a good way to cope with the replant diseases. Here, we report identification and characterization of a bacterial strain TS06 that may be used as a bio-control agent against the replant diseases in strawberry. TS06 was identified as a new strain of *Bacillus subtilis* based on the homology of its 16S rRNA and gyrA sequences to the reference strains in Genbank. It has a 7 bp difference from the most homologous strain *B. subtilis* NRRL B-23978. TS06 formed a monophyletic group with species of the *B. subtilis* complex group in the 16S rDNA sequence analysis, and shared similarity values of 99.9% with the *B. subtilis* type strains in the sequence analysis of the gyrA genes. TS06 was demonstrated to be a potent inhibitor to *Fusarium oxysporum* and *Verticillium dahliae*. There were 89.6 and 84.5% growth inhibition of TS06 on *F. oxysporum* and *V.dahliae* respectively in the antagonism test of TS06 strain *in vitro*. The mycelia growth inhibition rate of TS06 for *F. oxysporum* and *V.dahliae* reached 65.96 and 68.45% respectively, in the 40% TS06 culture filtrate treatments. Strain TS06 reduced the average percentage of strawberry diseases (*Fusarium* and *Verticillium* Wilts) by 88.94 and 79.94% respectively in greenhouse. In summary, *B. subtilis* strain TS06 has a broad antifungal spectrum and synchronously bio-control the two severe replant diseases (*Fusarium* and *Verticilium* Wilts), and TS06 can be developed as a bio-control agent to be applied in strawberry production.

Key words: *Bacillus subtilis,* strain TS06, biological control, strawberry *Fusarium* wilt, strawberry *Verticillium* wilt.

INTRODUCTION

Strawberry (*Fragaria ananassa* Duch.) is a very popular fruit. An increase in strawberry production has been recorded worldwide in recent years (FAO, Statistical Databases) (Kurze et al., 2001). China is one of the main strawberry producing countries; in that 2,000,000 tons of strawberry fruits were produced in over 133,300 ha in 2009. Recently, both the output and area of strawberry in China have ranked 1st in the world (http://www.agri.gov.cn/). The term replant disease of strawberry plants (RDS) describes the unexplained poor growth and serious disease of strawberry plants, which occurs after replanting on a site that was previously planted with strawberry plants. RDS persists in soil, after plants have been removed. Above ground symptoms include reduction in plant vigor, yield and quality of plant products. Typically, root systems are small, with discoloured feeding roots and few functional root hairs. Substantial economic losses, resulting from RDS, have been reported in most strawberry plant growing regions across the world.

Strawberry *Fusarium* and *Verticilium* wilts caused by *Fusarium oxysporum Shl.f.sp.fragariae* Wiinks et willams and *Verticillium dahliae* Kelb respectively, are the most serious RDS in China. Strawberry *Fusarium* and *Verticilium* wilts are economically important soil-borne diseases. RDS

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is very difficult to control due to: 1) the long viability of the resting structures (microsclerotia); 2) the broad host range of the pathogen and 3) the inability of fungicides to affect the pathogen. Once it enters into the xylem (Fradin et al., 2006), *F. oxysporum* and *V. dahliae* can infect plant roots from the soil. In addition, transplants are infected through runners from infected mother plants (Matuo et al., 1980). Strawberry cultivars are highly infected by *Fusarium* and *Verticilium* wilts. The control of strawberry *Fusarium* and *Verticilium* wilt disease in commercial orchards in the north of China is currently accomplished primarily through the use of soil fumigation. Materials such as methyl bromide, metam sodium, chloropicrin and fungicide are applied before planting. However, the increasing environmental pollution, caused by pesticide and fumigant use and frequent appearance of fungicide resistant strains of *Fusarium* and *V. dahliae* (Safiyazov et al., 1995; Zhengjun et al., 1996; Berg et al., 2001; Tjamos et al., 2004; Çubukçu et al., 2007) are of increasing concern. As a result, alternative control measures such as bio-control agents have been tried (Weller, 1988; O'Sullivan and O'Gara, 1992; Whipps, 1997; Emmert and Handelsman, 1999).

One of the bacteria bio-control agents, genus *Bacillus,* has received more attention than any other bacterial group (Powell et al., 1993; Merritt et al., 1989; Shoda, 2000; Israel et al., 2005; Dawar et al., 2010). *Bacillus* species produce broad-spectrum antibiotics and maintain viability for a long time, as a result of endospores production (Emmert et al., 1999). The use of bacteria to control *Fusarium* or *Verticilium* wilt in strawberries has not been reported as much as in other crops. It is noted that generally, only one bacterial species suppresses only one of the *Fusarium* or *Verticilium* wilt. The result, that one species of bacteria can control both *Fusarium* and *Verticilium* wilts in strawberries, at the same time, has not been previously reported. In this work, our research focused on the identification and evaluation of strain TS06 for the disease control of *Fusarium* and *Verticilium* wilts. The aim was to develop a new and effective bio-control agent, specifically for the control of strawberry replant disease, "*Fusarium* wilt" and "*Verticilium* wilt," in protective planting conditions.

MATERIALS AND METHODS

Culturing of fungal and pathogenicity tests on strawberry plants

F. oxysporum Shl.f.sp.fragariae Wiinks et willams and *V. dahliae* Kelb were kindly provided by professor Keqiang Cao of Hebei Agricultural University. *F. oxysporum* and *V.dahliae* were cultivated on plates of potato dextrose agar (PDA; Difco, Detroit, MI, U.S.A) for 6 to 10 days at 25°C until sporulation. A monospore isolate was maintained on PDA at 4°C and was sub-cultured onto fresh PDA plates at two months intervals (Sadfi-Zouaoui et al., 2008). Pathogenicity tests were conducted on strawberry plants at six leaf stages in 12 × 10 cm plastic pots containing sterilized soil (humus soil: sand = 2:1, pH = 6.8) mixture. For inoculum preparation, *V. dahliae* and *F. oxysporum* isolates were grown separately on PDA at 25°C in dark conditions, for 6 to 10 days until sporulation. Conidia were washed once with sterile water and diluted to a conidia concentration of 2 x 10^7 CFU/ml. Plants were wound inoculated, using a 5 µl drop of each of the conidial suspensions, by puncturing the stem 2 cm above the soil with an 18 gauge needle. The experiments were conducted under greenhouse conditions (16 h of light, 25±1°C), and the plants were watered with liquid fertilizer (MS nutrient solution) once a week. Control treatments were inoculated with sterile water. Each treatment consisted of three plants. 14 days after inoculation, disease severity was assessed for each plant on a 0 to 4 rating scale according to the percentage of foliage, roots and crowns affected by; acropetal chlorosis, necrosis, wilt, defoliation, and brown or dark brown colours (0 = healthy plant, 1 = 1 to 33%, 2 = 34 to 66%, 3 = 67 to 97%, 4 = dead plant) (Bejarano-Alcazar et al., 1995).

Isolating and selecting of bacteria and antagonism test *in vitro*

56 bacterial isolates were isolated from replanted soil in strawberry fields. The bacterial strains were maintained on an NB (NB; Difco, Detroit, MI,U.S.A) plate at 30°C (Nam et al., 2009). To evaluate their antagonistic activity against *F. oxysporum Shl.f.sp.fragariae* and *V. dahlia,* each plate of the bacterial isolate suspension at 10^7 CFU/ml was streaked across the centre of the plate. Two mycelia plugs of 5 mm in diameter of *F. oxysporum* and *V. dahlia* were placed respectively, on either side of the bacterial strip. The distance between the two micro-organisms was 2.5 cm. Plates were then incubated at 25°C for 7 days. The percentage growth inhibition of the fungus was calculated by the formula (Whipps et al., 1987):

(R1-R2)/R1×100

Where, R1 is the farthest radial distance (measured in millimeter) grown by the fungus after seven days of incubation in the direction of the antagonist (a control value). R2 is the distance of fungal growth from the point of inoculation to the colony margin in the direction of the antagonist. Growth inhibition was categorized on a scale (Korsten et al., 1995) from 0 to 4, where 0 = no growth inhibition, 1 = 1 to 25% growth inhibition, 2 = 26 to 50% growth inhibition, 3 = 51 to 75% growth inhibition, and 4 = 76 to 100% growth inhibition. All *in vitro* antagonism assays were made in triplicate (Sadfi-Zouaoui et al., 2008). The bacterial strains that showed obvious inhibition to both *F. oxysporum* and *V. dahlia* were selected for further evaluation and stored in 20% glycerol at -80°C.

The inhibition of TS06 culture filtrates on fungi spore germination of *F. oxysporum* Shl.f.sp.fragariae and *V. dahlia*

Growth rate method (Zhong, 1998): The pathogenic fungi *F. oxysporum* and *V. dahlia* were inoculated to PDA plates and cultured for three days at 28°C. The different concentrations (5, 10, 20 and 40%) of 36 h culture filtrate of TS06, were prepared for the inhibition tests. Sterilized PDA medium was cooled to 50～55°C, the TS06 culture filtrate was added, rapidly shaken well and poured into a Petri dish. The incubated 5 mm diameter mycelia disk of *F. oxysporum* or *V. dahlia* was placed on the centre of the PDA plate and then incubated at 25°C in darkness. Each treatment was repeated three times. The added 40% sterile NB medium treatment was set as a control. When the colony growth was assessed to be close to the edge of the Petri dish in the control group, the colony diameter was measured by Cross method (Chun, 1991). The growth and inhibition rates were calculated as follows:

Growth = Colony diameter - mycelia disk diameter.

Table 1. Strain types of *Bacillus* as reference for 16S rDNA sequence determination.

Species	Genbank accession number
Bacillu licheniformis	NC_006322
Bacillus subtilis	AJ276351
Bacillu pumilus	AY876289
Bacillus megaterium	X60629
Bacillus coagulans	DQ297928
Bacillus cereus	AE016877
Bacillus firmus	AB271750
Bacillus lentus	AB271746
Bacillus thuringiensis	AF290545
Bacillus thermoaerophilus	X94196
Bacillus amyloliquefaciens	X60605
Bacillus circulans	AY724690
Bacillus sphaericus	DQ286299
Brevibacillus laterosporus	AB112720
Brevibacillus brevis	AB101593
Paenibacillus polymyxa	AJ320493
Virgibacillus pantothenticus	AB305195

$$\text{Inhibition rate (\%)} = \frac{(\text{The growth of control group} - \text{The growth of treatment group})}{\text{The growth of control group}} \times 100$$

Phenotypic identification of the antagonistic bacteria strain TS06 Isolate TS06 showed potential as a biological control agent for *Fusarium* and *Verticilium* wilts, based on its phenotypic characteristics, as previously described by the Bacteriology Committee of the American Phytopathological society (Nam et al., 2009; Ruiz-Garcia et al., 2005).

16S rRNA gene and gyrA sequence determination of the antagonistic bacteria strain TS06

Genomic DNA of strain TS06 was extracted as described by Costa et al. (2006). The 16S rRNA gene of strain TS06 was amplified using universal primers 27f/1492r (Lane, 1991) by adding of 0.5 µl DNA extract to a thermocycler microtube containing 5 µl 10X Taq buffer (Promega), 5 µl 25 mM $MgCl_2$, 0.5 µl 25 nm primers, 37.7 µl sterile distilled water and 0.3 µl 5U Taq polymerase/µl (Promega). PCR was performed by an initial denaturation at 94°C for 5 min followed by 30 cycles of annealing at 56.2°C for 30 s, extension at 72°C for 2 min and denaturation at 94°C for 30 s and finally, an extension cycle of 72°C for 7 min. The gyrA genes were amplified using the primer pairs [(p-gyrA-f (50-CAG TCA

GGA AAT GCG TAC GTC CTT -30) and p-gyrA-r (50-CAA GGT AAT GCT CCA GGC ATT GCT -30)] to identify the *Bacillus* spp, the amplification system is similar to16S rRNA (Chun et al., 2000). PCR products were purified with a QIAquick gel extraction Kit (Tiangen, China). Direct sequencing of the PCR product was performed by Genome Express (sangon, China). The resulting sequences were aligned manually, with representative species sequences of Bacillus, or strains sequences of *Bacillus subtillis* or related taxa obtained from the GenBank database (Tables 1 and 2). All of the analyses were made using the MEGA program, Version 4.1.

Table 2. Strain types of *Bacillus subtilis* as reference for gyrA sequences determination.

Species	Genbank accession number
B. subtilis subsp.*subtilis* KCTC 3014	AF272024
B. subtilis subsp.*subtilis* KCTC 3239	AF272023
B. subtilis NRRL B-41008	AY663693
B. subtilis NRRL B-23972	AY663694
B. subtilis NRRL B-41005	AY663692
B. subtilis NRRL B-23978	AY663697
B. subtilis NRRL B-23968	AY663696
B. subtilis NRRL B-23967	AY663695
B.subtilis subsp.*spizizenit* NRRL B-23049	DQ995271
B. inaquosorum NRRL-B14697	EU138597
B. inaquosorum NRRL B-23052	EU138597
B. vallismortis NRRL B-14890	EU138601
B.amyloliquefaciens DJ-5	AY822026

Bio-control of *Fusarium* or *Verticilium* wilt of strawberry plants in the greenhouse

The preparation of antagonistic bacteria suspension

Antagonistic bacteria TS06 was cultivated at 37°C for 21 to 24 h on a rotary shaker in 250 ml conical flasks containing 50 ml nutrient broth: (30 g corn, 60 g soybean oil meal and 3 g $K_2HPO_4 \cdot 3H_2O$ in 1 L distilled water, 37°C, pH 6.0). This culture solution served as seed culture. The pH of the production medium was adjusted to 6.0 before sterilization. The fermentation was carried out in 500 ml Erlenmeyer flask with 50 ml production medium inoculated with 5% seed culture and incubated at 35°C, and 180 r/min for 60 to 66 h. The biomass density of 10^{10} CFU/ml was obtained and sterile water was added, leaving the bacteria suspension of TS06 at 10^7 CFU/ml.

The preparation of spore suspension of F. oxysporum and V. dahliae

F. oxysporum and *V.dahliae.* were cultivated on plates of potato dextrose agar (PDA; Difco,Detroit,MI,U.S.A) for 6 to10 days at 25°C until sporulation, the spores were washed with sterile water, leaving the spore suspension of *F. oxysporum* and *V.dahliae* at 10^6 CFU/ml.

Effects of strain TS06 on the disease *Fusarium* and *Verticilium* wilts of strawberry plants

Artificially infected *F. oxysporum Shl.f.sp.fragariae* Wiinks et willams and *V. dahliae* Kelb were used to evaluate the biological control potential of strain TS06. The soil mix (humus soil: vermiculite = 2:1) for greenhouse trials was sterilized. The experiment was conducted on healthy strawberry plants at five leaf stages in 12 × 12 cm diameter plastic pots. The bio-control trials, containing five treatments was as follows:

SF1: Prior to planting, the plant roots were dipped in a suspension of 10^6 CFU/ml *F. oxysporum* and *V. dahlia* for 20 min. Soil was drenched with a 20 ml suspension of TS06 (10^7 CFU/ml), and then the plants were planted. Soil drenching with 20 ml suspension of TS06 (10^7CFU/ml) was applied twice, at 10 days intervals, after planting.

SF2: Prior to planting, the plant roots were dipped in a

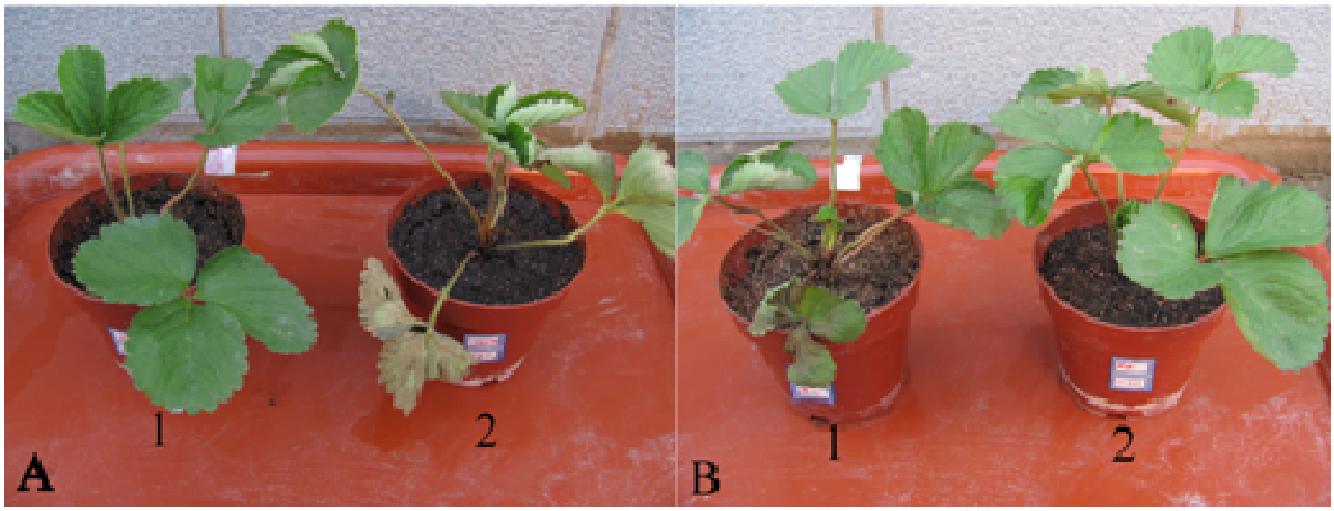

Figure 1. Pathogenicity tests of *F. oxysporum* and *V.dahliae* on strawberry leaves. A:1 Control; 2, *V. dahliae* inoculated; B: 1, *F. oxysporum* inoculated; 2, Control.

suspension of 10^6 CFU/ml *F. oxysporum* and *V. dahliae* for 20 min. The soil was drenched with 20 ml of sterile water and then the plants were planted. Soil drenching with 20 ml sterile water was applied twice at 10 days intervals, after planting.

SF3: Negative control: prior to planting, the plant roots were dipped in sterile water for 20 min. Soil was drenched with 20 ml sterile water and then the plants were planted. Soil drenching with 20 ml sterile water was applied twice, at 10 days intervals, after planting.

SF4: Prior to planting, the plant roots were dipped in sterile water for 20 min. Soil was drenched with a 20 ml suspension of TS06 (10^7 CFU/ml), and then the plants were planted. Soil drenching with 20 ml suspension of TS06 (10^7 CFU/ml) was applied twice at 10 days intervals, after planting.

SF5: Prior to planting, the plant roots were dipped in a suspension of 10^6 CFU/ml *F. oxysporum* and *V. dahliae* for 20 min. Soil was drenched with 20 ml of the fungicide Hymexazol (5 g of 98% hymexazol in 15 kg of water), and then the plants were planted. Soil drenching with 20 ml sterile water was applied twice, at 10 days intervals, after planting.

30 replicates of each treatment were performed in a completely randomized block design. All treatment combinations were repeated three times. The experiments were conducted under greenhouse conditions (18 h of light, 25 ± 1°C) during a seven-week period. The disease development was rated using the following disease rating scale: 0 = no symptoms; 1= 1 to 2 leaves rolled and yellowed leaves; 2 = all leaves rolled and deformed; 3 = chlorosis and early plant wilting; 4 = necrosis and entire plant wilting and 5 = plant dead (Nam et al., 2005). The disease index and control effect was calculated as follows:

Disease index = {[Σ(Number of plants of each disease degree × corresponding disease degree)]/(the total number of plants investigated × highest disease degree)}× 100

Control effect = {(Disease index of control –Disease index of treatment)/Disease index of control} × 100

Data analysis

All the data were processed and analyzed by software SAS8.0, and the significance of different treatments was analyzed by ANOVA and Duncan's procedures.

RESULTS

Pathogenicity tests of *F. oxysporum* and *V. dahliae* on strawberry plants

Pathogenicity of the two strains of *F. oxysporum* and *V. dahliae* was tested on strawberry plants. Both *F. oxysporum* and *V. dahliae* led the upper leaves to be wilted (Figure 1), and led the roots and crowns to be brown or dark brown (Figure 2), and finally the whole plant died. The results show that the two tested strains of *F. oxysporum* and *V. dahliae* can infect strawberry and have obvious pathogenicity to strawberry plants resulting in replant diseases of *Fusarium* and *Verticilium* wilts of strawberry plants in the greenhouse.

Antagonism test of TS06 strain to *F. oxysporum* and *V. dahliae in vitro*

56 bacterial isolates were isolated from the replanted strawberry fields, the most effective strain was TS06, with inhibition zone of 15 and 14 mm for *F. oxysporum* and *V. dahliae*, and 89.6 and 84.5% growth inhibition of TS06 on *F. oxysporum* and *V.dahliae* respectively. Based on the results, bacterial isolate TS06 has been selected, because it has the potential as a microbial antagonist against *Fusarium* and *Verticillium* wilts in strawberries. The inhibition of strain TS06 culture filtrates, on fungi spore germination of *F. oxysporum* and *V. dahliae* was significant (Figure 3, Tables 3 and 4). The inhibitory effects of each treatment group were increased with the increasing concentration of TS06 culture filtrates. The effect of culture filtrate of strain TS06 on *V. dahliae* was little more obvious than on *F. oxysporum* in all the treatments (Figure 4). The mycelia growth inhibition rate of TS06 on

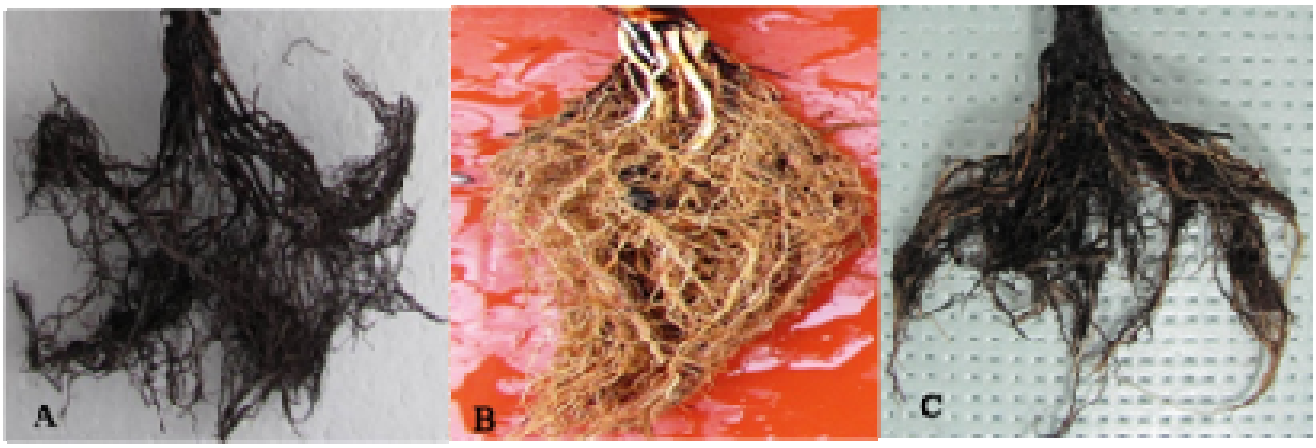

Figure 2. Pathogenicity tests of *F. oxysporum* and *V.dahliae* on strawberry roots and crowns. A, *F. oxysporum* inoculated; B, control; C, *V. dahliae* inoculated.

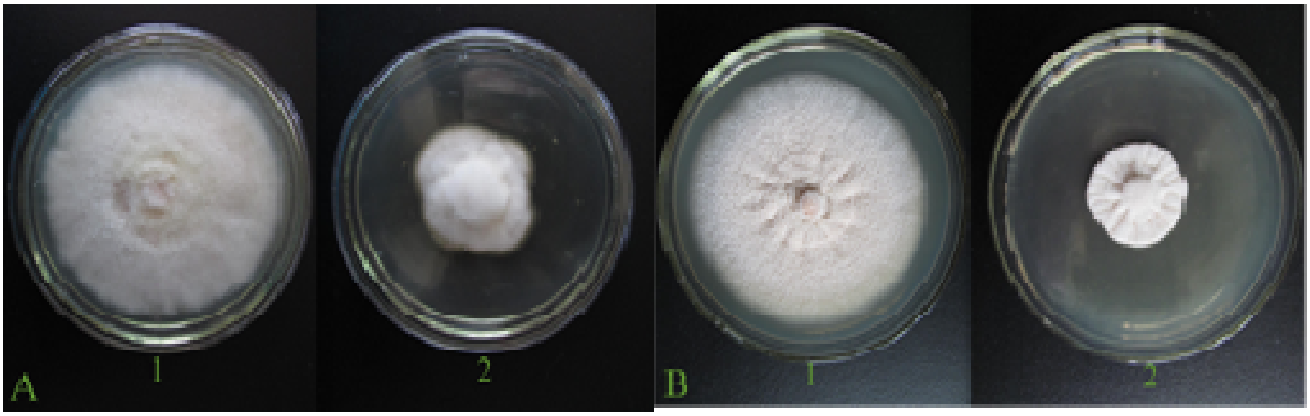

Figure 3. The inhibition of TS06 culture filtrates on fungi spore germination of *F. oxysporum* and *V. dahliae*. **A,** TS06 and *F. oxysporum;* 1, control; 2, 40% TS06 culture filtrate treatment; **B,** TS06 and *V. dahlia;* 1, control; 2, 40% TS06 culture filtrate treatment.

Table 3. Effects of culture filtrate of strainTS06 on the hyphal growth of *F. oxysporum*.

Addition ratio (%)	Growth (mm)(SE)	Inhibition rate (%)(SE)
5	62.12±0.09	13.43±0.67[a]
10	59.54±0.084	14.72±0.08[a]
20	44.93±0.045	35.57±0.72[b]
40	22.76±0.013	65.96±0.31[c]
Control	80.12±0..27	-

The data in the table are average of three repetitions, the same letters in the same column mean no significant differences among treatments (P<0.05).

F. oxysporum and *V. dahliae* reached 65.96 and 68.45% respectively in the 40% TS06 culture filtrate treatments. The anti-fungal effect of TS06 was very significant, helping to explain the potential of TS06 as a microbial antagonist against *Fusarium* and *Verticillium* wilts in strawberries.

Identification of the antagonistic bacteria strain TS06

The isolate TS06 exhibited a phenotypic similarity with *Bacillus* spp., based on its biochemical, morphological, and cultural characteristics (Bergey's Manual of Systemaic Bacteriology). In the 16S rDNA sequence analysis,

Table 4. Effects of culture filtrate of strainTS06 on the hyphal growth of *V. dahliae.*

Addition ratio (%)	Growth (mm) (SE)	Inhibition rate (%) (SE)
5	58.15±0.055	14.27±0.35[a]
10	57.72±0.093	15.42±0.63[a]
20	43.92±0.067	38.31±0.14[b]
40	20.81±0.081	68.45±0.27[c]
Control	70.99±0.052	-

The data in the table are average of three repetitions, the same letters in the same column mean no significant differences among treatments (P<0.05). Values are mean ±SE.

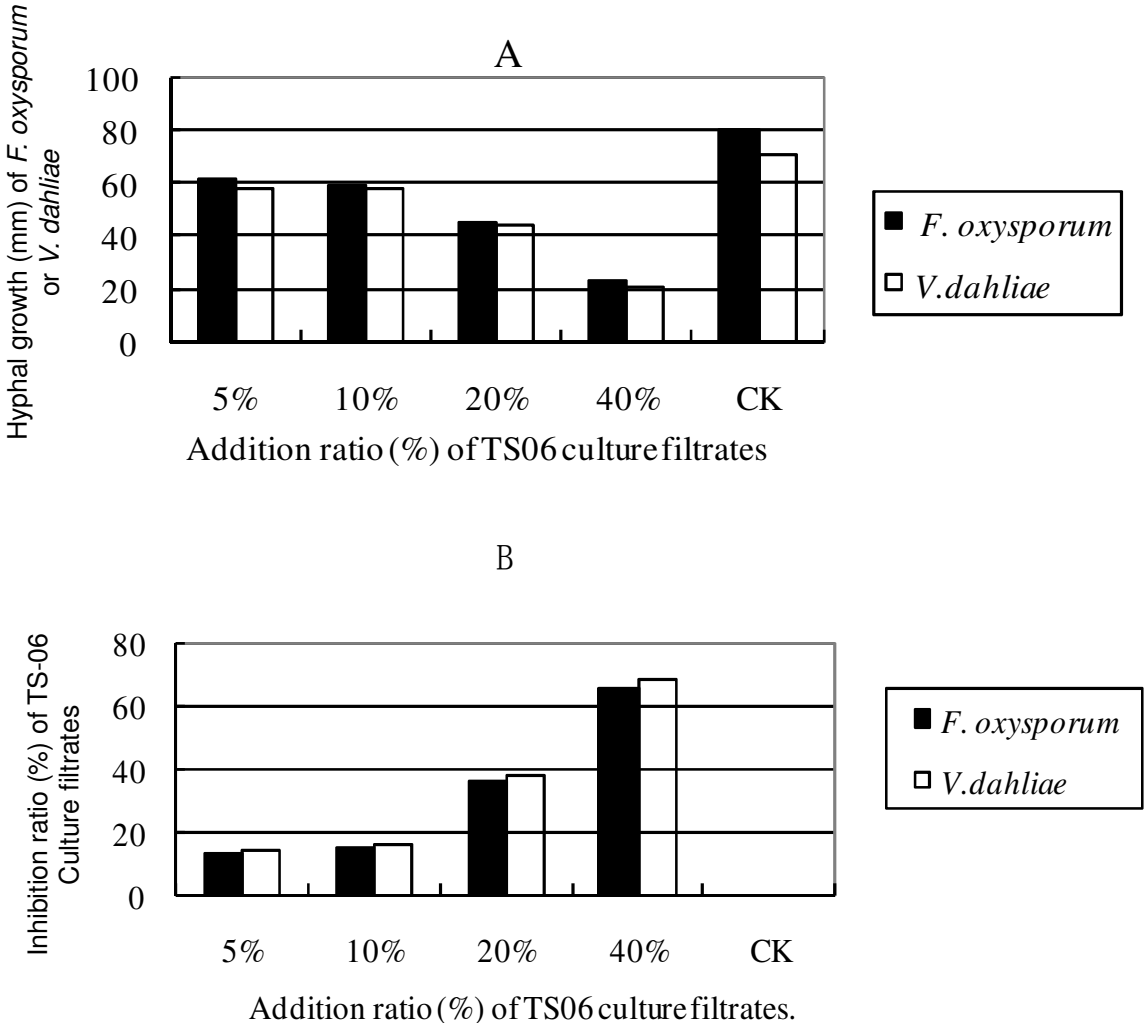

Figure 4. Comparison of the effects of culture filtrate of strainTS06 on the *V. dahliae* and *F. oxysporum.* A) Hyphal growth of *V. dahliae* and *F. oxysporum* with different addition ratio (%) of TS06 culture filtrates; B) inhibition ratio (%) of TS06 culture filtrates with different addition ratio (%) of TS-06 culture filtrates.

strain TS06 formed a monophyletic group with species of the *B. subtilis* complex group (Figure 5). The sequence analysis of the *gyr*A genes revealed that the TS06 strain share similarity values of 99.9% with the *B. subtilis* type strain *B. subtilis* NRRL B-23978 (Figure 6). TS06 is a new strain; it has a 10 bp difference in the 16S rDNA sequence and a 7 bp difference in the *gyr*A genes compared to the strain *B. subtilis* NRRL B-23978. Phylogenetic trees (Figures 5 and 6) were established, based on the BLAST results with 16S rDNA and *gyr*A genes sequence, which

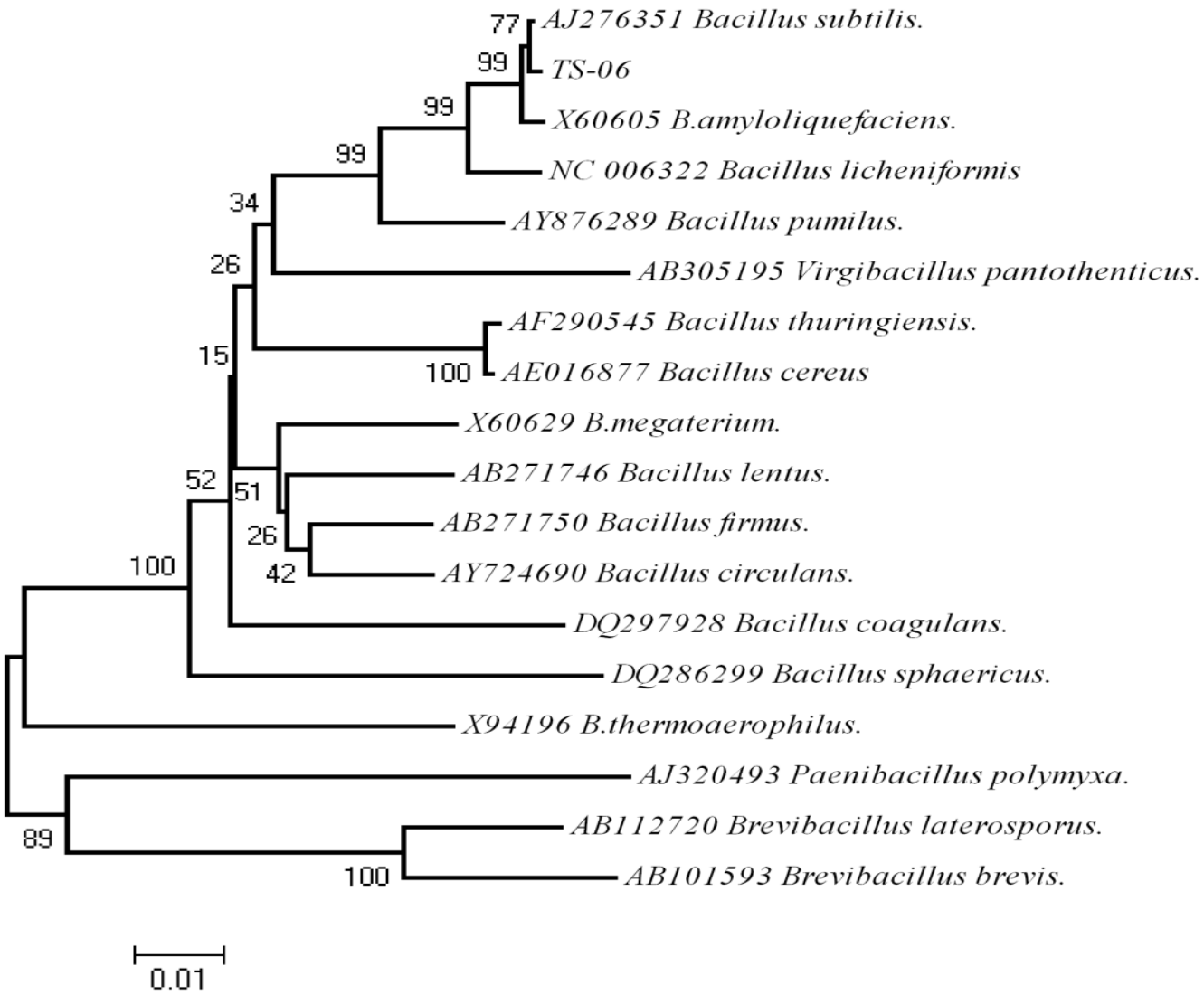

Figure 5. Neighbor-joining tree based on 16S rDNA gene sequences showing relationships between strain TS06 and species of the *Bacillus* spp. complex group. The percentage numbers above each branch indicate the levels of bootstrap support (>50%) for the branch point based on 1,000 resamplings. The bar represents 0.01 substitutions per site.

could identify strain TS06 as a strain of *B. subtilis*.

Effects of strain TS06 on *Fusarium* and *Verticilium* wilts of strawberry plants

In the greenhouse trials, TS06 reduced the disease incidence of *Fusarium* wilt of strawberry plants when compared with the non-treated TS06 control treatment (Table 5). Both disease incidence and disease index of SF2 treatment were the highest; 86.54 and 75.90% respectively, in the treatments SF1 to 5. *Fusarium* wilt of strawberry plants in the SF2 treatment was more serious compared with the other treatments. This situation occurred mainly because the plants or soil were not treated with TS-06 or any other fungicide treatment, before strawberry plants were inoculated with *Fusarium*. Even though we used the fungicide Hymexazol, the disease incidence (32.43%) and disease index (21.25) of SF5 treatment were also higher than that (10.25 and 8.00%) of SF1 treatment. There were significant differences between TS06 treatment and fungicide Hymexazol, and the control effect of TS06 was much better than the fungicide Hymexazol. Both the disease incidence (5.59%) and disease index (3.00) of SF4 treatment were the lowest in all the treatments.

In addition, the plant height and the number of leaves of strawberries in the SF4 treatment were better than the other treatment plants. It suggests that TS06 includes plant-growth-promoting substances that affect plant

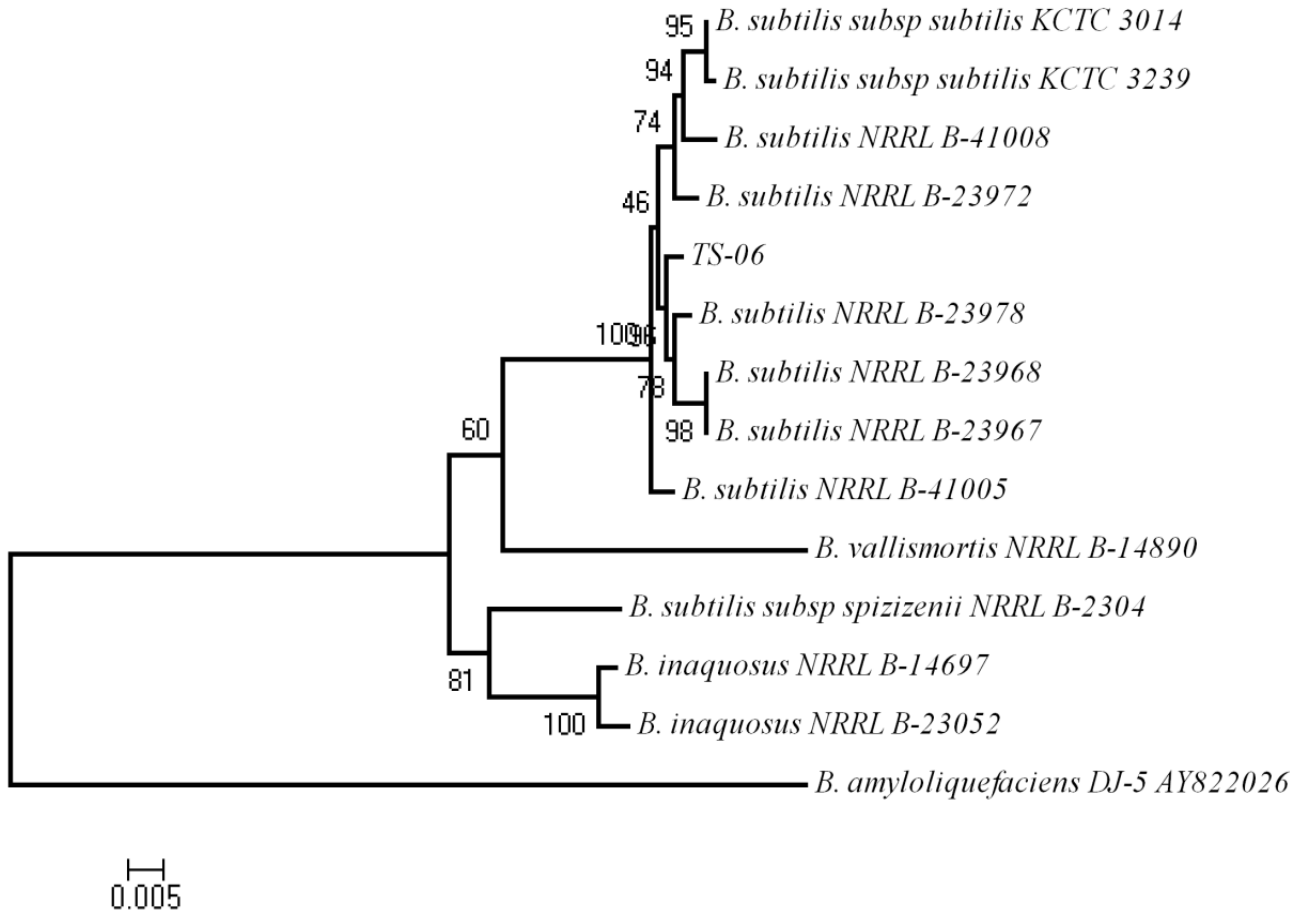

Figure 6. Neighbor-joining tree based on gyrA gene sequences showing relationships between strain TS06 and strains of the *Bacillus subtilis* complex group. The percentage numbers above each branch indicate the levels of bootstrap support (>50%) for the branch point based on 1,000 resamplings. The bar represents 0.005 substitutions per site.

Table 5. Control effects of *Bacillus subtilis* TS06 on strawberry *Fusarium* wilt disease.

Treatment	Number of strawberry plants	Disease incidence (%)	Disease indix	Control effect (%)
SF-1	30	10.25±0.45	8.00±0.12	88.94±0.62[a]
SF-2	30	86.54±0.90	75.90±0.49	----
SF-3	30	20.13±0.27	11.44±0.33	-----
SF-4	30	5.59±1.13	3.00±0.89	-----
SF-5	30	32.43±0.78	21.25 ±0.58	67.42±0.74[b]

The data in the table are average of three repetitions; the same letters in the same column mean no significant differences among treatments (P<0.05). Values are mean ±SE.

growth. Such substances have been reported in promoting plant growth through the; fixation of atmospheric nitrogen, production of siderophores, solubilization of minerals and synthesis of phytohormones (Glick, 1995; Parke et al., 2001; Nelson, 2004). This may be a valuable area for further study. The same situation is equally suitable for the bio-control of *Verticilium* wilt using TS06 (Table 6). The control effect of *B. subtilis* TS06 on strawberry *Fusarium* wilt disease was better than on strawberry *Verticilium* wilt disease in greenhouse, which is consistent to the results of antagonism test of TS06 strain to *F. oxysporum* and *V.dahliae in vitro* (Figure 7). The greenhouse trails showed that the formulations of *B. subtilis* TS06 reduced the diseases of *Fusarium* and *Verticilium* wilts of strawberry plants in greenhouse conditions. This suggests that the strain TS06, has the potential for commercial use, as a bio-control agent, for reducing *Fusarium* and *Verticilium* wilts of strawberry

Table 6. Control effects of *Bacillus subtilis* TS06 on strawberry *Verticilium* wilt disease.

Treatments	Number of strawberry plants	Disease incidence (%)	Disease indix	Control effect (%)
SF-1	30	15.34±0.59	9.21±0.75	79.94±0.68[a]
SF-2	30	76.43±0.84	63.50±0.96	----
SF-3	30	16.46±1.08	10.56±0.47	-----
SF-4	30	4.39±0.34	2.30±0.19	-----
SF-5	30	29.55±0.57	19.24±0.55	69.05±0.38[b]

The data in the table are average of three repetitions, the same letters in the same column mean no significant difference among treatments (P<0.05). Values are mean ±SE.

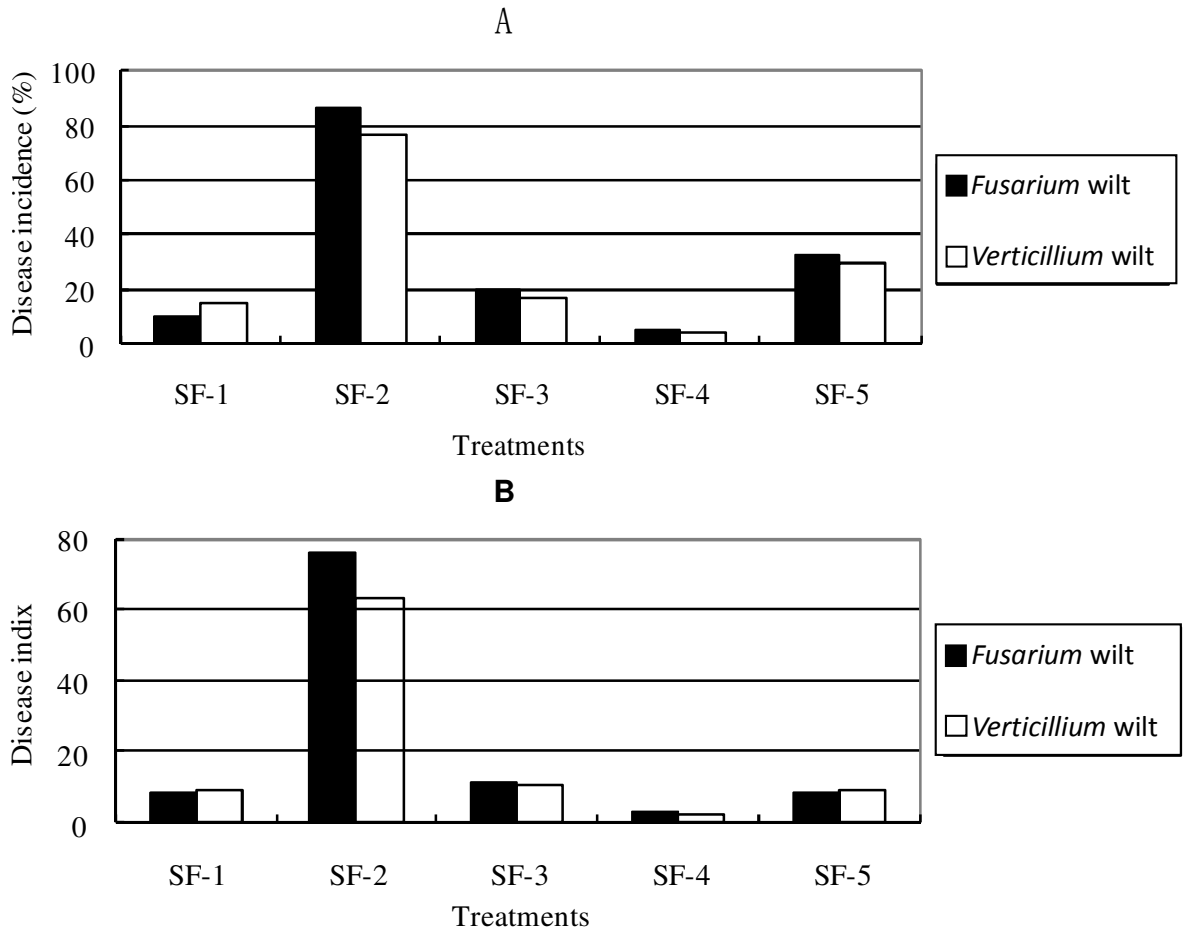

Figure 7. Comparison of the control effects of *Bacillus subtilis* TS06 on strawberry *Fusarium* and *Verticilium* wilts disease. A) The disease incidence (%) of strawberry *Fusarium* and *Verticilium* wilts disease varied with the treatments; B) the disease index of strawberry *Fusarium* and *Verticilium* wilts disease varied with the treatments.

plants planted in greenhouse conditions.

DISCUSSION

The identification technology of bacterial antagonism needs to be developed. Identification of *B. subtilis*-like organisms has become difficult and laborious, as they cannot be distinguished from each other by conventional phenotypic tests. The application of molecular methods has greatly changed the conventional taxonomic classification of bacilli. 16S rRNA sequencing is often used to define species (Wu et al., 2006) but the presence of highly conserved sequences in the 16S rRNA gene does not permit the discrimination among some species and subspecies of this group (Shaver et al., 2002). This observation was also true for the nine reference strains (including TS06) of the *B. subtilis* group in our study that

clustered together on 16S rRNA gene sequence analysis. Based on previous works (Wang et al., 2009) in our laboratory, the amplified ribosomal DNA restriction analysis (ARDRA) assay was also employed to identify TS06. The 16S rDNA PCR amplicon of TS06 was digested by six restriction enzymes (*Alu* I, *Taq I*, *Mse* I, *Bst* U I, *Hha* I and *Tsp*509 I), and ARDRA results were able to differentiate TS06 strain from other species of the *Bacillus*, but unable to differentiate TS06 from other type strains of the *B. subtilis* phylogenetic cluster. These data reaffirm the need for the development of methods to provide a convenient system for the identification of TS06-like *B. subtilis* strains.

Recently, partial gyrA sequences, coding for DNA gyrase subunit A, was found to provide a firm framework for the rapid and accurate classification and identification of *B. subtilis* and related taxa (Chun et al., 2000). The partial *gyrA* sequences of representatives of *B. subtilis* and allied taxa were able to identify TS06 in this study. The results showed that the protein-coding genes exhibit much higher genetic variation; it was able to differentiate TS06 strain from the other eight strains within the specie of the *B. subtilis*. The *gyrA* gene sequences have been shown to be a more efficient phylogenetic tool, than the 16S RNA gene sequences or ARDRA, for discriminating between species and subspecies of the *B. subtilis* group. But we could consider the 16S RNA gene sequence a suitable method to identify some unknown Bacillus isolates, or at least in the primary differentiation of species from those of other *Bacillus* groups. The combination of 16S rDNA and *gyr*A sequences in this study could precisely identify the TS06 strain. More importantly, it was able to differentiate the TS06 strain from other type strains within the *B. subtilis* group. TS06 formed a monophyletic group with species of the *B. subtilis* complex group in the 16S rDNA sequence analysis. It shares a similarity value of 99.9%, with the *B. subtilis* type strains in the sequence analysis of the *gyr*A genes. However, *B. subtilis* TS06 is a new bio-control strain; it has a 7 bp difference from the most homologous strain *B. subtilis* NRRL B-23978.

For the credible application of bacterial antagonism in strawberry fields, a good combination result of *in vitro* and *in vivo* inhibition test of antagonists is needed. *In vitro* inhibition tests, for screening antagonists, are not always adequate for exploiting bio-control agents. In primary screening of antagonists, some of the 56 bacterial isolates showed an *in vitro* antagonistic effect against *F. oxysporum Shl.f.sp.fragariae* Wiinks et willams or *V. dahliae* Kelb, but they did not reduce the pathogen produced diseases in the greenhouse experiment. Antibiosis activity observed in agar media might be affected by the pathogen, plant and surrounding medium. One of the possible reasons for the reduced effectiveness of a microorganism *in vivo,* is its response to different stimuli in the medium into which it is introduced (Weller, 1988). The antifungal properties exhibited by antagonists *in vitro* might be altered, when they are introduced into the substrate, where the plant is grown (Cook, 1992). The inhibition of TS06 on pathogens was observed significantly, not only *in vitro* but also *in vivo* performance in greenhouse trials. The control effect of *B. subtilis* TS06 on strawberry *Fusarium* and *Verticilium* wilt disease *in vivo* is consistent with the results of antagonism test of TS06 strain on *F. oxysporum* and *V. dahliae in vitro* .The strain TS06, had a broad antifungal spectrum against the two different pathogens; it reduced the average percentage of *Fusarium* wilt disease by 88.94% and *Verticillium* wilt disease by 79.94% respectively, compared to the strawberry plants not treated with TS06. The precise inhibition effect of TS06, *in vitro* and *in vivo*, gives TS06 the potential to be exploited as a bio-control agent for strawberry replant disease, in strawberry production.

Previous studies have shown that the reasons for antagonism between bacteria and fungi may be substrate competition, a possible trade off between fungal growth and tolerance towards bacteria (Mille-Lindblom et al., 2006). In the 40% TS06 culture filtrate treatments, the growth inhibition rate of TS06 on *F. oxysporum* and *V. dahliae* reached 65.96 and 68.45% respectively. The actual mechanism responsible was not investigated in this study but the results suggest that there are some components in the TS06 culture filtrates that produced the antagonistic inhibition effect, on fungi spore germination of *F. oxysporum Shl.f.sp.fragariae* and *Verticillium*. Schreiber (1988) study showed that *B. subtilis* can inhibit the growth of *V. dahliae*, mainly as a result of *B. subtilis* production of cyclic peptide antibiotics. Berg et al. (1994, 2005) confirmed that the production of antibiotics, is the main mechanism of antagonistic bacterial inhibition, in the growth of *V. dahliae*. Our experimental results are consistent with these viewpoints. The inhibition effect of strain TS06 culture filtrates on fungal spore germination of *F. oxysporum* and *V. dahliae* was significant. The precise mechanism of this inhibition may be antibiotic, or may involve components which need further study.

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