Characterization of Antagonistic *Bacillus methylotrophicus* Isolated From Rhizosphere and Its Biocontrol Effects on Maize Stalk Rot

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ABSTRACT

Stalk rot is one of the most serious and widespread diseases in maize, and effective control measures are currently lacking. Therefore, this study aimed to develop a new biological agent to manage this disease. An antagonistic bacterial strain, TA-1, was isolated from rhizosphere soil and identified as *Bacillus methylotrophicus* based on morphological and biochemical characterization and 16S ribosomal RNA and *gyrB* gene sequence analyses. TA-1 exhibited a strong antifungal effect on the growth of *Fusarium graminearum* mycelium, with 86.3% inhibition at a concentration of 10⁸ CFU per ml. Transmission electron microscopy showed that TA-1 could disrupt the cellular structure of the fungus, induce necrosis, and degrade the cell wall. Greenhouse and field trials

were performed to evaluate the biocontrol efficacy of TA-1 on maize stalk rot, and the results of greenhouse experiment revealed that the bacterium significantly reduced disease incidence and disease index. Seeds treated with a 10⁸ CFU ml⁻¹ cell suspension had the highest disease suppression at 86.8%. Results of field trials show that seed bacterization with TA-1 could not only reduce maize stalk rot incidence but also increase maize height, stem diameter, and grain yield. The lipopeptide antibiotics were isolated from the culture supernatants of TA-1 and identified as surfactins and iturins. Consequently, *B. methylotrophicus* TA-1 is a potential biocontrol agent against maize stalk rot.

Maize (*Zea mays* L.) is one of the most important cereals in the world, and it plays a critical role in global agriculture, human diets, animal feed, and cultural customs (Nuss and Tanumihardjo 2010). However, maize production is influenced severely by stalk rot, one of the most widespread and destructive soilborne diseases. Infected roots become brown and rotten; plants may dry up while remaining green. Furthermore, the stalks become hollow and soft, showing discoloration and tissue deterioration (Santiago et al. 2007). Stalk rot is mainly caused by several species of *Fusarium* fungi, such as *F. graminearum*, *F. verticillioides*, and *F. proliferatum* (Yu et al. 2017; Zhang et al. 2018). Among these, *F. graminearum* is the predominant pathogen causing stalk rot in maize, because it produces a variety of mycotoxins that further degrade grain quality (Hao et al. 2017). Moreover, *F. graminearum* can survive in the soil for several years, making it difficult to control. In China, losses caused by maize stalk rot range from 5 to 80%, and therefore, control of toxigenic *F. graminearum* is challenging (Yu et al. 2017).

Traditionally, the management of maize stalk rot has been dependent on the use of resistant cultivars and application of chemical fungicides (Shin et al. 2014). However, the current agronomic practices are not sufficiently effective; maize varieties

with high resistance to stalk rot are still insufficient, and breeding resistant maize varieties is time consuming. Furthermore, pesticide usage is very complicated in China; many pesticides are not available to growers because of high cost, and pesticides may pose dangers to humans and the environment (van der Werf 1996). Moreover, to reduce pesticide use, China initiated the 2020 Zero-Growth Action Plan for Pesticides, which emphasizes the role of green pest prevention and control. Therefore, it is important to find a safer and more efficient biocontrol agent to manage maize stalk rot.

Biological control is an accepted environmentally safe approach and has shown potential for managing maize stalk rot as well as having the capacity to reduce the reliance by growers on chemicals (Mahmood et al. 2017). *Bacillus* strains can form endospores and resist hostile conditions, such as desiccation, heat, and ultraviolet irradiation. Thus, the genus *Bacillus*-based products predominate over other biocontrol microorganisms and represent the best candidates for the development of efficient biopesticides (Torres et al. 2017). There have been >100 commercial products of *Bacillus* formulations registered in China so far. Among *Bacillus* species, *B. methylotrophicus* strains, formerly *B. amyloliquefaciens* subsp. *plantarum* (Dunlap et al. 2015), are known for their methanol-utilizing and plant growth-promoting properties, and they are considered promising biocontrol agents against a wide range of plant-associated diseases (Madhaiyan et al. 2010; Zhi et al. 2017).

One of the underlying antagonistic mechanisms of the genus *Bacillus* is the production of antimicrobial lipopeptides, such as surfactin, iturin, and fengycin, which have been used widely in biotechnological and biopharmaceutical applications because of their surfactant properties (Zhao et al. 2017). Studies have proven that these antimicrobial lipopeptides not only can inhibit phytopathogen growth but also, can induce host resistance potential (Ongena and Jacques 2008). Furthermore, the production of antimicrobial peptides (AMPs) is a major defense mechanism against pathogen infestation (Reddy et al. 2004). Therefore, the objectives of this study were to (i) isolate and identify *Bacillus* strains antagonistic to maize stalk rot; (ii) evaluate the antagonistic efficacy of the strain for controlling maize stalk rot in vitro, in the

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greenhouse, and in the field; and (iii) analyze the antifungal peptides produced by the strain to explore its potential biocontrol mechanism by polymerase chain reaction (PCR) amplification of AMP genes and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) techniques.

MATERIALS AND METHODS

Isolation and screening of antifungal bacteria. Soil samples were collected from the rhizosphere of field-grown tomato in Tai'an (Shandong, China). The soil was silt loam, with a mean particle size distribution of 36% sand, 60% silt, and 4% clay; organic matter content was 34.2 g kg⁻¹ of soil, and pH was 6.9. Within each sampling area, soil samples were collected from five randomly selected locations from 0- to 30-cm columns by a cylinder and mixed to form a composite sample. Soil samples were sieved (2 mm), transported to the laboratory in an ice chest (4°C), and stored at 4°C until strains of bacteria were isolated by the serial dilution method (Kumar et al. 2012). Soil samples (10 g) were heat treated (80°C) to eliminate nonsporulated microbes and then thoroughly mixed with 90 ml of sterile distilled water on a rotating shaker for 5 min. Next, 0.1 ml of the suspension was spread uniformly and cooled on nutrient agar (NA) plates in triplicate, and the inoculated plates were incubated at 30 ± 1°C for 48 h. Rough and abundant colonies with waxy growth (1 to 4 mm in diameter) and irregularly spreading edges were obtained. The selected isolates were maintained on NA medium slants at 4°C. The purified bacteria were all tested for growth inhibition of five plant pathogens on plates by the dual-culture method (Chan et al. 2003). Plant pathogens tested were *Rhizoctonia solani*, *Botrytis cinerea*, *Phytophthora parasitica* var. *nicotianae*, and *F. oxysporum* (all from the Key Laboratory of Pesticide Toxicology and Application Technique, College of Plant Protection, Shandong Agricultural University). The fungi were stored at 4°C on potato dextrose agar (PDA) and grown in culture medium at 25°C for 3 to 7 days before antagonistic experiments. Fungal cakes were placed in the center of PDA plates; four pure bacterial colonies were cultured at equal distance (3 cm) from pathogenic cakes (72 h at 30°C), and inhibition diameter was calculated. The inhibition rates of mycelium growth of three antagonistic isolates against five pathogens were calculated with the following formula: Inhibition rate (%) = (C - E)/C × 100%, where C is the diameter of the control colony and E is the diameter of the treatment colonies. The strain showing the strongest antifungal effect was coded as TA-1 and selected for additional study.

Preparation of bacterial inoculum and the fungal pathogen strain spore suspensions. A bacterial suspension of antagonistic strain TA-1 was prepared as follows. Based on previous references, bacterial isolates were added to Luria-Bertani liquid medium and incubated for 24 h at 30°C with shaking until the logarithmic growth phase was reached (Lin et al. 2014). Then, all cultures were harvested by centrifugation at 5,000 rpm for 5 min with a centrifuge (CS150GXL; Hitachi, Tokyo, Japan), the supernatant was discarded, and the pellets were washed and resuspended in sterile distilled water to obtain a final bacterial population density of 10⁹ CFU per ml. Finally, isotonic saline water (0.85% NaCl) was used to dilute the suspension from 10⁹ to 10⁴ CFU ml⁻¹ (Zhou et al. 2011). The bacterial suspensions were used for greenhouse and field experiments evaluating disease suppression of *F. graminearum*.

The fungal pathogen *F. graminearum* was isolated from infected maize roots and identified using the morphological criteria described by Nelson et al. (1983). The isolate was cultured on PDA medium at 25°C in the dark and stored at 4°C until use. The pathogenicity of this fungus has been tested in multiple assays and in different maize hybrids.

To prepare pathogen inoculum for field trials, *F. graminearum* was cultured in a 250-ml conical flask with 100 ml of maize sucrose

medium. Maize sucrose medium was produced by soaking 200 g of maize seeds for 12 h, boiling them to the cracking point, and discarding the seeds; then, the leachate was collected, sterile distilled water was added to reach 1,000 ml, and 10 g of sucrose was dissolved in the solution. Flasks were incubated on a rotating shaker (Guohua Electric Appliance Co., Ltd., Changzhou, Jiangsu, China) at 160 rpm and 28°C for 7 days to produce macroconidia, and a macroconidial suspension was prepared by filtering the culture through sterile filter paper to eliminate mycelial fragments. The concentration of the suspension was adjusted to 10⁵ spores per ml using a hemocytometer (Zhao et al. 2014). Twenty *F. graminearum* mycelial plugs (5 mm) were put into sterile plastic bags containing sterile cracked maize, incubated at 25°C for 10 days, and shaken daily. Then, the maize-fungus mixture was obtained and used for field trials only (Lizárraga-Sánchez et al. 2015).

Phenotypic and molecular characterization of the TA-1 strain. Physiological and biochemical tests were performed using universal methods (Xue et al. 2006), including gram staining, morphology, growth properties, catalase and oxidase activities, methyl red testing, and the Voges-Proskauer (V-P) reaction as well as tests for H₂S production, biopolymer hydrolysis, indole production, nitrate reduction, and anaerobic growth.

DNA was extracted from strain TA-1 using a Bacterial DNA Kit (Omega Bio-Tek Inc., Guangzhou, China) according to the manufacturer's instructions. Universal primer sets (27F and 1492R; UP1 and UP2r) were used to amplify the 16S ribosomal RNA (rRNA) gene and the *gyrB* gene, respectively (Frank et al. 2008; Yamamoto and Harayama 1995). PCR was performed in a 25-μl reaction mixture with an initial denaturation at 95°C (3 min) followed by 35 and 30 cycles of denaturation at 95°C (30 s), annealing at 52°C (30 s), extension at 72°C (2 min), and a final extension at 72°C (15 min) in an Eppendorf gradient thermocycler (Eppendorf). The PCR fragments were sequenced at Shanghai Sangon Biological Technology and Services Co., Ltd. (Shanghai, China), and the similarity values of the 16S rRNA and *gyrB* gene sequences were assessed using the Basic Local Alignment Search Tool program against the GenBank database. Phylogenetic trees were constructed using the neighbor-joining method with the Molecular Evolutionary Genetics Analysis software (Tamura et al. 2013). The stability of the nodes was tested by using the Kimura two-parameter model with bootstrap value of 1,000 replications. Strain TA-1 (CCTCC M 2018362) was registered by the China Center for Type Culture Collection.

In vitro antagonistic activity of *B. methylotrophicus* TA-1 against *F. graminearum*. The antagonistic effects of the TA-1 isolate on the growth of *F. graminearum* were assessed using a dual-culture assay (Chan et al. 2003). A 7-mm agar plug cut from a lawn of the test fungus was inoculated in the middle of each PDA plate, and the single colony of TA-1 was picked up by a toothpick and placed on the PDA plate at a distance of 2.5 cm from the fungal disc. Control plates were inoculated with the same *F. graminearum* colony, but no bacteria were introduced. The plates were cultured at 28°C for 5 days, and the antagonistic effect was evaluated by measuring the distance between the edges of the growing mycelium and the antagonistic bacterium.

The mycelial linear growth rate method was used. Briefly, sterilized PDA was prepared and cooled to 55°C. One milliliter of strain TA-1 was added to the PDA at different cell concentrations to reach final concentrations of 10⁸, 10⁷, 10⁶, 10⁵, and 10⁴ CFU ml⁻¹. Then, the PDA media were poured into 9-cm sterile petri dishes. After that, an agar plug (diameter: 0.5 cm) taken from the growing edge of a 4-day-old *F. graminearum* culture was inoculated to the center of every plate. Petri dishes prepared with 1 ml of sterile distilled water added to PDA were used as controls. All procedures were conducted in a laminar flow cabinet. Plates were cultured at 28°C for 5 days in darkness. Pathogen survival was determined by measuring the diameter of each fungal colony at 24-h intervals for 3 days. The tests were replicated three times, with 15 replicates at

each concentration. The inhibition rate of *F. graminearum* mycelial growth was calculated using the following formula: Inhibition rate (%) = (A – B)/A × 100, where A is the fungal growth in the control and B is the fungal growth with treatment (Zhou et al. 2011).

Greenhouse experiment. A pot trial experiment was conducted under greenhouse conditions with temperatures ranging from 25 to 30°C and relative humidity ranging from 60 to 90% in a greenhouse at Shandong Agricultural University. Maize hybrid Zhengdan 958, which was widely grown in China, without seed-coating treatment was chosen for the pot trial experiment, and the estimated duration of the hybrid to maturity was about 114 days. Before sowing, maize seeds of consistent size and shape were surface sterilized with 1% NaClO and then washed with sterile distilled water. Next, the seeds were soaked in bacterial suspensions of different concentrations (10⁸ to 10⁴ CFU ml⁻¹) for 2 h and air dried overnight. The concentration of the bacterial cultures after drying was assessed by the serial dilution method (Supplementary Table S1). Then, 10 seeds per pot were planted in pots (15 × 12 cm) containing 300 g sterile soil and nursery substrate (peat:vermiculite:perlite = 4:3:2 [vol/vol/vol]). Seeds soaked in sterile distilled water and a 25% fludioxonil seed-coating suspension served as controls. The tests were conducted twice, with each treatment repeated five times.

The treated pots were inoculated with *F. graminearum* 2 days postseeding with 30-ml suspensions of conidia (10⁵ spores per 1 ml). Pathogen-inoculated pots were arranged randomly with five replications and hand watered as needed. Disease incidence was determined at reproductive stage R4. During sampling, 20 plants were destructively sampled: they were split longitudinally from the ear down to the soil line from each plot and washed with running tap water. The severity of disease was evaluated by assessing the degree of decay in the seedling radicles and coleoptiles. Stalk rot severity was rated on a standard scale from zero to four, with slight modifications (Gai et al. 2017): zero indicated healthy plants with a well-developed root and coleoptile, one indicated plants with 25% of the radicle and coleoptile exhibiting decay symptoms, two indicated >25 to 50% of the radicle and coleoptile exhibiting decay symptoms, three indicated >50 to 75% of the radicle and coleoptile exhibiting decay symptoms, and four indicated radicles and coleoptiles with ≥75% decay. The disease incidence, disease index, and disease control effects were calculated using the following formulas:

$$\text{Disease incidence(\%)} = (\text{Total number of diseased seedlings} / \text{Total number of emerged seedlings}) \times 100$$

$$\text{Disease index} = \sum[(\text{Rating} \times \text{Number of plants rated}) / (\text{Total number of plants} \times \text{Highest rating})] \times 100$$

$$\text{Disease control effect (\%)} = [(\text{Disease incidence of the control} - \text{Disease incidence of the treatment}) / \text{Disease incidence of the control}] \times 100$$

Field trials. Field trials were conducted at the Maize Technological Innovation Center in the Huanghuaihai plain (36°10'19" N, 117°9'03" E, 128 m above sea level) in Tai'an, China during two consecutive maize-growing seasons (2015 and 2016). The experimental soil type was brown loam with organic matter content 10.6 g kg⁻¹ in the tillage layer (0 to 20 cm), and its pH was 6.5. The total nitrogen, available phosphorous, and available potassium amounts were 1.1 g kg⁻¹, 25.8 mg kg⁻¹, and 90.2 mg kg⁻¹, respectively. The mean total amounts of temperature and precipitation of summer maize growth periods during 2015 and 2016 were 26.7°C and 478.5 mm as well as 27.2°C and 524.6 mm, respectively. Maize hybrid Zhengdan 958 without seed-coating

treatment was chosen for the field trials. Nitrogen (180 kg of N ha⁻¹ as urea), phosphorus (80 kg of P₂O₅ ha⁻¹ as superphosphate), and potassium (150 kg of KCl ha⁻¹ as potassium chloride) were applied as basic fertilizers.

Treatments in both years were planted in a randomized complete block design with four replications per treatment. Each experimental plot was 10 × 3 m with four furrows. Two central rows of maize plants were used to avoid “border effects” for each experimental plot. Two kilograms of the maize-fungus inoculum mixture (prepared as described above) was inoculated as previously described (Venancio et al. 2003) to the furrows of each plot before sowing. Maize seeds were treated with different concentrations of bacterial suspensions (10⁸, 10⁷, 10⁶, 10⁵, and 10⁴ CFU ml⁻¹) for 2 h, air dried overnight, and sown by a manual maize planter at a depth of 5 to 8 cm at a density of 65,000 plants ha⁻¹ on June 15 in both 2015 and 2016. Noninoculated TA-1 suspensions maize seeds were used as controls, and a 25% fludioxonil seed-coating suspension served as the fungicide control. Additional nitrogen, in the form of urea, was applied at rates of 60 kg of N ha⁻¹ at the jointing and tasseling stage. Plots were hand weeded and irrigated as needed.

Incidence of maize stalk rot was evaluated in 60 plants per treatment selected randomly by handpicking from the center of each usable area at reproductive stage R4 (Lizárraga-Sánchez et al. 2015). The disease incidence, severity, and control effects were calculated as described in the greenhouse experiment. At the same time, plant height was measured from the base of the stalk to the apex of the plant using a measuring tape, and stem diameter was measured using a vernier caliper. When plants reached the physiological maturity stage (R6), all cobs present in each individual plot were harvested to determine kernel yield (moisture content was ~14%). Antifungal activity of TA-1 and its control effect against maize stalk rot were evaluated above, and then, the action mechanisms of TA-1 were explored.

Transmission electron microscopy observation. Transmission electron microscopy (TEM) was used to observe *F. graminearum* hyphae growing toward colonies of *B. methylotrophicus* TA-1 on the fourth day of interaction on PDA medium. Briefly, 100 µl of 10⁵ spores per ml of *F. graminearum* spore suspensions were spread uniformly on PDA plates containing TA-1 cells at the concentration of 10⁸ CFU ml⁻¹, and 0.85% NaCl was used as a control. Fungal hyphae of *F. graminearum* were harvested and fixed in 2% glutaraldehyde for 24 h at 4°C, rinsed three times with phosphate buffer (0.02 M), and subsequently fixed with 2% osmium tetroxide for 2 h at 20°C. The hyphae were then dehydrated in a graded series of ethanol concentrations (45, 55, 70, 85, 95, and 100%) for 20 min each. After dehydration, the samples were embedded in Epon 810 (Polysciences, Warrington, PA), and ultrafine sections (80 nm) of tissue were prepared at room temperature using an RMC-MT7000 ultramicrotome and a diamond knife. The tissue was mounted on a copper grid, and poststaining was performed (uranyl acetate for 30 min and lead citrate for 20 min). Samples were kept in a desiccator until examination by TEM (HITACHI, H-600) operated at 120 kV (Zhao et al. 2014).

PCR amplification of AMP genes. The TA-1 antagonistic strain was further screened for the presence of common lipopeptide genes, including genes for surfactin, iturin, fengycin, and bacillomycin D, and these genes were amplified by PCR under the following cycling conditions: 95°C for 5 min and 40 cycles of 94°C for 1 min, the annealing temperature for 1 min, and 70°C for 1 min. A final extension step at 70°C for 10 min was followed by maintenance at 4°C (Gond et al. 2015). Primers used for the respective lipopeptide genes are listed in Supplementary Table S2.

Isolation, purification of lipopeptides, and MALDI-TOF MS analysis. Lipopeptide products were isolated by adding 6 N HCl to the cell-free supernatants of TA-1 until pH 2.0 was reached. Precipitates were collected, dried, and extracted with methanol. White solids were generated and collected after the solvents were

removed under reduced pressure, and then, they were dissolved in methanol followed by charcoal treatment and passed through a 0.22- μ m filter. The MeOH extractions were added to a threefold volume of H₂O and neutralized to pH 7.0 with 5 M NaOH (Luo et al. 2014b). Separation of the filtrates was achieved using an Agilent 1200 high-performance liquid chromatography (HPLC) system (Agilent Technologies) attached to a reverse-phase C18 column (4.6 $\times$ 250-mm internal diameter, 120 Å; YMC, Allentown, PA) with a linear gradient elution of deionized water and acetonitrile (containing 0.1% trifluoroacetic acid). The flow rate was 0.8 ml min⁻¹ with a detection wavelength of 210 nm, and fractions were collected manually (Perez et al. 2017). During the purification, the antagonism assay against *F. graminearum* guided the collection of active fractions by the plate confrontation method (Chan et al. 2003). Briefly speaking, a 7-mm agar plug cut from a lawn of the test fungus was inoculated in the middle of each PDA plate, and 2 μ l compounds were placed at two sites in the PDA plate at a distance of 2.5 cm from the fungal disc. The HPLC-purified compounds with the highest antagonistic activity were analyzed on a 4800 Plus MALDI-TOF MS system (Bruker Daltonics Omniflex instrument). For MALDI-TOF MS analysis, HPLC fractions extracted from TA-1 were mixed with α -cyano-4-hydroxycinnamic acid saturated in 70% aqueous acetonitrile containing 0.1% (vol/vol) trifluoroacetic acid in equal amounts, spotted on the matrix-assisted laser desorption/ionization sample target, and left to air dry. Ions were produced with a nitrogen laser (337 nm) and accelerated at 20 kV. Each mass spectrum was produced by averaging >100 individual laser shots (Dunlap et al. 2011).

Statistical analyses. Before analysis, data expressed as percentages were arcsine transformed to homogenize variances. Sources of variation were treatments and blocks. The effects of different treatments were examined using analysis of variance, and when the *F* test was significant at *P* $\leq$ 0.05, treatment means were compared using the Student-Newman-Keuls test (SPSS V22; IBM SPSS Statistics, Chicago, IL).

RESULTS

Screening of TA-1. In total, 31 bacteria were isolated from rhizosphere soil. All of them were tested for growth inhibition of five plant pathogens on plates by the dual-culture method, and the three most effective antagonistic isolates were obtained. The strain showing the strongest antifungal effect was coded as TA-1 and selected for additional study. The inhibition rates of TA-1 to *F. graminearum*, *R. solani*, *B. cinerea*, *P. parasitica* var. *nicotianae*, and *F. oxysporum* were 72.8, 62.3, 59.7, 64.5, and 70.6%, respectively (Table 1).

Characterization and identification of the TA-1 isolate. The morphological, biochemical, and physiological characteristics of strain TA-1 are shown in Supplementary Table S3. The cells were gram-positive, endospore-forming, aerobic rods. The colonies were creamy white and convex with regular margins. TA-1 colonies initially exhibited a smooth surface on NA, but after a few days, the surfaces of the colonies became dry and wrinkled. Oxidase, catalase, and urease reactions, V-P test, and nitrite reduction were positive. TA-1 could utilize glucose, mannose, citrate, and hydrolyzed starch and casein. The bacterium did not produce acids or gas from glucose, and the methyl red test was negative. All of these properties agreed with those described for the *B. methylotrophicus*-type strain CBMB205^T, except urease activity (Madhaiyan et al. 2010).

The 16S rRNA phylogenetic tree revealed that strain TA-1 formed a distinct group with the *B. methylotrophicus*-type strain CBMB205^T (GenBank accession number EU194897) with 97% bootstrap support (Fig. 1A). Based on *gyrB* gene sequence analysis, strain TA-1 displayed the highest sequence similarity (99%) to the *B. methylotrophicus*-type strain NCCB 100236^T (=CBMB205^T; GenBank accession number KC608571) (Fig. 1B). To summarize, strain TA-1 was identified as *B. methylotrophicus* based on the above morphological, biochemical, and molecular characterization. The partial 16S rRNA gene and *gyrB* gene sequences of strain TA-1 were submitted to the National Center for Biotechnology

TABLE 1. Inhibition rates of mycelium growth of three antagonistic isolates against five plant pathogens^a

Strain	<i>Fusarium graminearum</i>	<i>Rhizoctonia solani</i>	<i>Botrytis cinerea</i>	<i>Phytophthora parasitica</i> var. <i>nicotianae</i>	<i>F. oxysporum</i>
TA-1	72.8 $\pm$ 1.2 a	62.3 $\pm$ 2.4 a	59.7 $\pm$ 2.9 a	64.5 $\pm$ 1.6 a	70.6 $\pm$ 1.5 a
TA-2	48.8 $\pm$ 3.1 b	56.3 $\pm$ 1.6 a	18.1 $\pm$ 1.7 b	52.2 $\pm$ 2.3 b	45.1 $\pm$ 2.9 c
TA-3	43.0 $\pm$ 1.4 b	54.6 $\pm$ 2.3 a	55.3 $\pm$ 1.7 a	45.2 $\pm$ 2.7 b	21.1 $\pm$ 4.6 d

^a The inhibition rates of mycelium growth of three antagonistic isolates against five pathogens were calculated with the following formula: Inhibition rate (%) = (C – E)/C $\times$ 100%, where C is the diameter of the control colony and E is the diameter of the treatment colonies. Values are the means $\pm$ standard error of five replicates. Values in each column followed by different letters are statistically different by the Student-Newman-Keuls test (*P* $\leq$ 0.05).

Fig. 1. Neighbor-joining phylogenetic tree based on A, 16S ribosomal RNA and B, *gyrB* (gene sequences showing the phylogenetic relationship of strain TA-1 to other members of the genus *Bacillus*.

Information database with the accession numbers MF425821 and MF425820, respectively.

In vitro antagonistic activity of *B. methylotrophicus* TA-1 against *F. graminearum*. In a dual-culture assay on PDA plates,

the width of the inhibition zone ranged from 10 to 15 mm after incubation, which showed a high level of antifungal activity of TA-1 against *F. graminearum*; the *F. graminearum* hyphae in the control plates grew actively and spread over the entire PDA plate

TABLE 2. Effect of *Bacillus methylotrophicus* TA-1 against maize stalk rot under greenhouse condition^y

Treatment ^z	Disease incidence (%)	Disease index	Disease control effect (%)
10 ⁴ CFU ml ⁻¹	55.0 ± 2.9 b	36.7 ± 3.3 b	37.7 ± 5.7 d
10 ⁵ CFU ml ⁻¹	38.3 ± 1.7 c	29.2 ± 3.4 b	56.6 ± 5.0 c
10 ⁶ CFU ml ⁻¹	33.3 ± 1.6 cd	24.2 ± 2.7 cd	62.3 ± 4.9 bc
10 ⁷ CFU ml ⁻¹	25.0 ± 0.9 de	17.5 ± 4.7 de	71.7 ± 6.5 abc
10 ⁸ CFU ml ⁻¹	11.7 ± 2.3 f	10.4 ± 3.3 e	86.8 ± 4.1 a
Fludioxonil FS	16.7 ± 1.7 ef	14.6 ± 2.2 de	81.1 ± 1.9 ab
CK	88.3 ± 5.6 a	75.8 ± 1.1 a	—

^y Values are the means ± standard error of five replicates. Values in each column followed by different letters are statistically different by Student-Newman-Keuls test ($P \leq 0.05$).

^z 10⁴ CFU ml⁻¹ = TA-1 concentration of 10⁴ CFU ml⁻¹, 10⁵ CFU ml⁻¹ = TA-1 concentration of 10⁵ CFU ml⁻¹, 10⁶ CFU ml⁻¹ = TA-1 concentration of 10⁶ CFU ml⁻¹, 10⁷ CFU ml⁻¹ = TA-1 concentration of 10⁷ CFU ml⁻¹, and 10⁸ CFU ml⁻¹ = TA-1 concentration of 10⁸ CFU ml⁻¹, Fludioxonil FS = 25% fludioxonil seed-coating suspension, and CK = control.

Fig. 2. Effect of *Bacillus methylotrophicus* TA-1 against maize stalk rot in the field in **A**, 2015 and **B**, 2016; 10⁴ CFU ml⁻¹ = TA-1 concentration of 10⁴ CFU ml⁻¹, 10⁵ CFU ml⁻¹ = TA-1 concentration of 10⁵ CFU ml⁻¹, 10⁶ CFU ml⁻¹ = TA-1 concentration of 10⁶ CFU ml⁻¹, 10⁷ CFU ml⁻¹ = TA-1 concentration of 10⁷ CFU ml⁻¹, 10⁸ CFU ml⁻¹ = TA-1 concentration of 10⁸ CFU ml⁻¹, fludioxonil FS = 25% fludioxonil seed-coating suspension, and CK = control. Data are means ± SE of replicates of the experiment, and the means are separated with the Student-Newman-Keuls test ($P = 0.05$).

(Supplementary Fig. S1). In contrast, the hyphal growth of *F. graminearum* was obstructed by the presence of the TA-1 strain and could not break through its limitation.

In the mycelial growth assay using PDA plates with different concentrations of *B. methylotrophicus* TA-1, the diameter of the *F. graminearum* mycelium decreased significantly with increasing concentrations of *B. methylotrophicus* TA-1 (Supplementary Fig.

S2 and Supplementary Table S4). Inhibition of *F. graminearum* was highest (86.3%) at the 10^8 CFU ml⁻¹ concentration of *B. methylotrophicus* TA-1.

Effect of TA-1 on maize stalk rot in the greenhouse. Maize seeds treated with different concentrations (10^4 to 10^8 CFU ml⁻¹) of TA-1 suspension had significantly lower disease incidence and disease index values than the untreated control, and the efficacy

Fig. 3. Effect of *Bacillus methylotrophicus* TA-1 on maize **A**, plant height, **B**, stem diameter, and **C**, yield; 10^4 CFU ml⁻¹ = TA-1 concentration of 10^4 CFU ml⁻¹, 10^5 CFU ml⁻¹ = TA-1 concentration of 10^5 CFU ml⁻¹, 10^6 CFU ml⁻¹ = TA-1 concentration of 10^6 CFU ml⁻¹, 10^7 CFU ml⁻¹ = TA-1 concentration of 10^7 CFU ml⁻¹, 10^8 CFU ml⁻¹ = TA-1 concentration of 10^8 CFU ml⁻¹, fludioxonil FS = 25% fludioxonil seed-coating suspension, and CK = control. Data are means $\pm$ SE of replicates of the experiment, and the means are separated with the Student-Newman-Keuls test ($P = 0.05$).

of the TA-1 suspension was similar to that of chemical fungicide fludioxonil (Table 2). Maize stalk rot incidence was reduced from 88.3% in the control to incidences ranging from 55.0 to 11% in the treated plants depending on the concentration of the TA-1 strain suspensions. Thus, the biocontrol efficacy ranged from 37.7 to 86.8%. Treatment with the 10^8 CFU ml^{-1} TA-1 suspensions showed the greatest effect, with no significant difference from that of fludioxonil.

Effect of TA-1 on maize stalk rot in the field. Field trials conducted in 2015 and 2016 showed that all of the bacterial applications on maize seeds significantly reduced the disease incidence of maize stalk rot and exhibited remarkable control efficiency. In the 2015 trial, maize stalk rot incidence was reduced from 35.6% in the control to incidences ranging from 22.8 to 5.6% in the treated plants depending on the concentration of the TA-1 strain suspensions (Fig. 2A).

Fig. 4. Transmission electron microscopy of *Fusarium graminearum* hyphae growing toward colonies of *Bacillus methylotrophicus* TA-1 on the fourth day of interaction on potato dextrose agar medium. **A**, A cross-section and a longitudinal section of a control hypha. **B**, A cross-section of an affected hypha. **C**, A longitudinal section of a control hypha. **D**, A longitudinal section of an affected hypha. **E**, An intact cell wall of a control hypha. **F**, A degrading cell wall of an affected hypha.

Almost all treatments improved maize height, stem diameter, and grain yield compared with the untreated controls (Fig. 3). In the 2015 experiment, the greatest plant heights and stem diameters were observed in plots treated with the TA-1 concentration of 10^8 CFU ml⁻¹ (221.3 cm and 24.8 mm, respectively). Other treatments had intermediate height and stem diameter, but they were still significantly better than the untreated control (except for TA-1 at concentration of 10^4 CFU ml⁻¹). As for grain yields, seed treatments with TA-1 could remarkably improve maize yields, and the maximum came up to 10.9 t ha⁻¹, far exceeding the untreated control yields of 8.8 t ha⁻¹. There were no significant differences between the 10^8 CFU ml⁻¹ TA-1 suspensions and the 25% fludioxonil seed-coating suspension in disease incidence, control effect, and yield.

A similar trend was observed in 2016 trial results (Figs. 2B and 3). Maize seeds treated with different concentrations of TA-1 suspension had significantly lower disease incidence and excellent biocontrol efficacy compared with the untreated control. Maize stalk rot incidence decreased from 22.2 to 5.0%, and control effect increased from 34.4 to 85.3% accordingly. Maize height, stem diameter, and yield presented the upward trend associated with rising concentrations of TA-1 suspension compared with the untreated control.

Ultrastructure of the direct bacteria-pathogen interaction in vitro. TEM analysis elucidated the morphological changes in the hyphal ultrastructure of *F. graminearum* induced by *B. methylotrophicus* TA-1. Longitudinal and cross-section TEM showed that the structure of *F. graminearum* cells remained intact when grown alone (Fig. 4A, C, and E), but growing with the bacteria, *F. graminearum* hyphae showed some degree of necrosis and degradation in the cell walls, cytoplasm, or cellular organelles (Fig. 4F). In both the longitudinal and cross-sections of treated hyphae, the organelles and cytoplasm in the hyphal cells were irregular and degenerate, even appearing as empty holes (Fig. 4B and D). These results indicated that some cyclic lipopeptides produced by *B. methylotrophicus* TA-1 initially degraded the cell walls of *F. graminearum* and then invaded the cells, leading to the release of the cell contents, which is consistent with previous studies (Gong et al. 2015).

AMP genes in *B. methylotrophicus* strain TA-1. AMP biosynthetic genes have been related to the biocontrol of plant pathogens by several *Bacillus* strains. The presence of five AMP gene markers was checked by PCR, and amplification of these markers showed that each gene had one specific band of the

expected size (Supplementary Fig. S3). The results showed that the genes *Sfp*, *ItuD*, *SrfC*, *FenD*, and *BamC* were present in the bacteria genome and might responsible for the antifungal activity of TA-1.

Identification of the active compounds by MALDI-TOF MS analysis. Two pure antifungal compounds (labeled C1 and C2) were obtained by using reverse-phase HPLC. Both C1 and C2 showed desirable antifungal activity against the pathogenic fungus *F. graminearum* (Fig. 5A). C1 and C2 were subjected to MALDI-TOF MS analysis, and molecular masses were searched in the m/z ranges from 1,035 to 1,080 and from 900 to 1,200, respectively. Results showed that C1 possessed three peaks. The masses of molecular ions at m/z were 1,043.499, 1,057.500, and 1,071.524, differing by 14 Da; this suggests that they were homologous molecules of a methylene group ($-CH_2-$) differing in fatty acid chain, which belonged to iturins (Fig. 5B). MALDI-TOF MS analysis of C2 showed that it had three peaks (m/z were 1,010.612, 1,032.620, and 1,054.599), which corresponded with the surfactin (m/z 994 to 1,065). C2 yielded a mass at 1,010.612, and therefore, C2 was identified as surfactin (Fig. 5C).

DISCUSSION

Fungal diseases are widespread among all plant species and cause extensive economic damage to agricultural crops (Oliver and Solomon 2008). Because of regulatory constraints and public resistance to chemical pesticides, development of viable biocontrol methods is needed. Use of biological fungicides is attractive, because they are cost effective and environmentally friendly; therefore, numerous antagonist bacteria with antifungal activities have been promoted as safe biocontrol agents to control fungal diseases (Liu et al. 2017). Among these, *Bacillus* species are attractive because of their biology and proven potential for use; in recent years, various strains, such as *B. subtilis*, *B. polymyxa*, *B. amyloliquefaciens*, *B. cereus*, and *B. pumilus*, have been used as potential biocontrol agents against different *F. graminearum* infections (Zalila-Kolsi et al. 2016).

Antagonistic *Bacillus* strains frequently have been found and isolated from soil, and in this study, the antagonistic strain TA-1 was isolated from rhizosphere soil and identified as *B. methylotrophicus*. TA-1 exhibited high levels of antagonism against *F. graminearum* and significantly reduced radial mycelial growth in vitro. Wang et al. (2013) reported that *B. amyloliquefaciens* W19 inhibited the growth of *F. oxysporum* significantly, and Zhao et al. (2014) found that *B. subtilis* strain SG6 reduced *F. graminearum* mycelial

Fig. 5. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of the purified antifungal compounds. **A**, Antagonistic activities of peptide antibiotic purification fraction against *Fusarium graminearum* after incubation at 28°C for 5 days. **B**, MALDI-TOF MS analysis of compound 1 produced by strain TA-1. **C**, MALDI-TOF MS analysis of compound 2 produced by strain TA-1.

growth by 87.9%. Inhibition of hyphal growth may be one of the main control mechanisms of *Bacillus* strains against *Fusarium* pathogens.

Coating seeds with antagonist bacteria has proven to be an effective method for suppressing plant pathogens and improving plant resistance (Estevez de Jensen et al. 2002). Pereira et al. (2009) showed that treatment of maize seeds with *B. amyloliquefaciens* resulted in excellent antagonism against *F. verticillioides* and positively affected the length of the stem and the weight of the root system in the resultant seedlings. Khabbaz et al. (2015) reported that cucumber seeds treated with *B. subtilis* Bs 8B-1 had decreased genus *Rhizoctonia* damping-off severity and increased plant weight compared with those of infested controls. Numerous attempts have been made to control maize stalk rot using biological control measures. Lizárraga-Sánchez et al. (2015) proved that *B. cereus* sensu lato strain B25 was capable of controlling maize stalk rot. Saravanakumar et al. (2017) found that *Trichoderma harzianum* strain CCTCC-RW0024 displayed an 86.66% disease reduction and could develop a biocontrol agent against maize stalk rot. However, there are currently no registered biocontrol agents to prevent the disease in China. In this study, we evaluated the efficacy of TA-1 for controlling maize stalk rot under greenhouse and field conditions, and the results indicated that treating maize seeds with a TA-1 suspension at different concentrations could decrease disease index and disease incidence effectively while improving seed germination rates, seedling length, fresh weight, and dry weight of the above- and underground parts and root length (data not shown). These observed effects suggest that TA-1 acted as a plant growth-promoting rhizobacterium and produced antifungal substances leading to the suppression of maize stalk rot.

However, because of the similar environmental condition of field trials in 2015 and 2016, data from the 2 years' trials exhibited similar trends. Also, linear increase of biocontrol efficacy with increasing bacterial inoculation was observed. Moreover, there was no significant difference between the 10^8 CFU ml⁻¹ TA-1 suspensions and the 25% fludioxonil seed-coating suspension in disease incidence, control effect, and yield. The results in this study are in agreement with previous results. A recent study showed that *B. methylotrophicus* BC79 culture filtrate (4,000 g/667 m²) exhibited the highest efficiency for *Magnaporthe oryzae*, with 84.8% biocontrol effect, followed by of BC79 culture filtrates (2,000 g/667 m²; 80.0%) and 20% tricyclazole (76.1%) (Shan et al. 2013). Another study revealed that *B. subtilis* SG6 significantly reduced disease incidence, *Fusarium* head blight index, and deoxynivalenol in the field trials and that it was more effective than the widely used chemical fungicide carbendazim (Zhao et al. 2014).

Chitin, β -1,3-glucan, and cellulose are the most common constituents of fungal cell walls, which are essential structures that determine cell shape and protect the cells against internal turgor pressure or adverse environmental effects (Arroyo et al. 2016). In this study, TEM analysis revealed that TA-1 could induce cell wall degradation in *F. graminearum*. Degrading the fungal cell wall requires the participation of various extracellular lysis enzymes (Marcello et al. 2010), and the TEM results of this study indicated that TA-1 might degrade the cell wall of *F. graminearum* by producing hydrolytic enzymes. Therefore, TA-1 is a potential candidate for the biocontrol of soilborne diseases, but additional research is needed to identify these compounds.

AMPs, especially lipopeptides, are the major antimicrobial compounds secreted by *Bacillus* species, and they play important roles in the management of plant pathogens (Omardien et al. 2016). Many studies have reported that *B. methylotrophicus* strains can produce one or more antifungal lipopeptides, which have been proven to effectively suppress pathogenic plant fungi (Zhang et al. 2015). The presence of AMP biosynthetic genes in strain TA-1 was determined by PCR, and the results showed that all five AMP genes (*Sfp*, *ItuD*, *SrfC*, *FenD*, and *BamC*) were present. However, only

surfactin and iturin were detected in our MALDI-TOF MS analysis. The complexity of natural fermentation products and the losses that occurred in process of crude extraction, separation, and drying may be the reason why other antimicrobial lipopeptides were not found in our research.

Surfactin and iturin are two well-studied antimicrobial cyclic lipopeptides produced by *Bacillus* species, and they have significant prospective applications in many fields (Yang et al. 2015). Surfactin consists of a heptapeptide moiety with variable amino acid at positions 2, 4, and 7 linked to a C₁₃-C₁₆ β -hydroxy fatty acid (Bonmatin et al. 1995). It has advantages of inducing hemolysis, having low toxicity, being highly biodegradable, and maintaining antagonistic activity against multiple pathogens (Nitschke and Costa 2007). Acting as an elicitor, surfactin can trigger an induced systemic resistance of plants against various diseases (Henry et al. 2011). Le Mire et al. (2018) confirmed that surfactin stimulated wheat defense mechanisms by inducing the expression of various defense genes coding for antimicrobial compounds, regulators of oxidative stress, and enzymes involved in defense signaling. Iturin possesses a heptapeptide backbone connected to a C₁₄-C₁₇ β -amino fatty acid chain and contains many variants, such as iturins A, C, D, and E; bacillomycins D, F, and L; bacillopeptins; and mycosubtilin (Yang et al. 2015). Iturin not only exhibits strong in vitro fungi toxicity but also, induces defense response in plants, thereby reducing plant disease severity (Gu et al. 2017). Kawagoe et al. (2015) reported the defense signaling pathways triggered by iturin A and showed that both salicylic acid and jasmonic acid signaling pathways were involved in the defense response. In this study, in view of other nonlipopeptide metabolites produced by the fermentation broth of TA-1, the lipopeptides were relatively complex and difficult to purify. Consequently, liquid chromatography-tandem mass spectrometry and nuclear magnetic resonance will be applied in future work to analyze purified compounds C1 and C2 to further confirm results on the inhibitory mechanism of TA-1 on *F. graminearum*. In addition, by studying *B. subtilis* 916 mutants without the productions of lipopeptides, Luo et al. (2014a) showed that bacillomycin Ls and fengycins contributed mainly to the antifungal activity. Therefore, mutants of TA-1 that is deficient in antibiotic production should be tested to assess the extent to which the lipopeptides contribute to the biocontrol activity against stalk rot.

In conclusion, this study showed that the biocontrol strain *B. methylotrophicus* TA-1 could suppress maize stalk rot effectively in vitro, in the greenhouse, and in the field. Therefore, the use of strain TA-1 may represent an efficient alternative to chemical fungicides for sustainable maize cultivation, because this strain simultaneously produces antifungal lipopeptides and performs other plant growth-promoting activities. Additional studies are needed to address the mode of action, safety, and cost effectiveness to develop TA-1 as a promising biocontrol agent.

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