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Exploration of a multifunctional biocontrol agent *Streptomyces* sp. JCK-8055 for the management of apple fire blight

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Abstract

Apple fire blight, caused by the bacterium *Erwinia amylovora*, is a devastating disease of apple and pear trees. Biological control methods have attracted much attention from researchers to manage plant diseases as they are eco-friendly and viable alternatives to synthetic pesticides. Herein, we isolated *Streptomyces* sp. JCK-8055 from the root of pepper and investigated its mechanisms of action against *E. amylovora*. *Streptomyces* sp. JCK-8055 produced aureothricin and thiolutin, which antagonistically affect *E. amylovora*. JCK-8055 and its two active metabolites have a broad-spectrum in vitro activity against various phytopathogenic bacteria and fungi. They also effectively suppressed tomato bacterial wilt and apple fire blight in in vivo experiments. Interestingly, JCK-8055 colonizes roots as a tomato seed coating and induces apple leaf shedding at the abscission zone, ultimately halting the growth of pathogenic bacteria. Additionally, JCK-8055 can produce the plant growth regulation hormone indole-3-acetic acid (IAA) and hydrolytic enzymes, including protease, gelatinase, and cellulase. JCK-8055 treatment also triggered the expression of salicylate (SA) and jasmonate (JA) signaling pathway marker genes, such as *PR1*, *PR2*, and *PR3*. Overall, our findings demonstrate that *Streptomyces* sp. JCK-8055 can control a wide range of plant diseases, particularly apple fire blight, through a combination of mechanisms such as antibiosis and induced resistance, highlighting its excellent potential as a biocontrol agent.

Key points

- *JCK-8055 produces the systemic antimicrobial metabolites, aureothricin, and thiolutin.*
- *JCK-8055 treatment upregulates PR gene expression in apple plants against E. amylovora.*
- *JCK-8055 controls plant diseases with antibiotics and induced resistance.*

Keywords Apple fire blight · *Streptomyces* sp. · Aureothricin · Thiolutin · Plant growth-promoting bacterium · Plant defense resistance

Introduction

Phytopathogenic microorganisms have detrimental impacts on crop production as well as the economic and political stability of countries (Ristaino et al. 2021). Fire blight caused by the bacterial pathogen *Erwinia amylovora* was first noted in Hudson Valley, New York (USA) in 1780 (Bonn and Van der Zwet 2000). Since its discovery, fire blight has been recognized as a devastating disease of pome fruit trees, leading to substantial economic losses worldwide (Zhao et al. 2019). Traditionally, fire blight has been managed through cultural practices and preventative sprays containing copper and antibiotics (Norelli et al. 2003). Streptomycin, an aminoglycoside antibiotic, is the most effective chemical in fire blight management, reducing disease incidence by more than 90% in clinical trials conducted in the northeastern states

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of the USA (Aldwinckle et al. 2001; Norelli et al. 2003). Nevertheless, considering pesticide spray drift with antibiotics may induce the spread of antibiotic-resistant bacteria, the use of antibiotics for plant protection is under scrutiny (McManus 2014). Therefore, various strategies have been implemented to find alternative antibiotics by inducing plant resistance through breeding, genetic engineering, and biological control (Adhikari et al. 2020). Among these strategies, biological control is an eco-economic and evolutionary approach that enhances plant immunity and/or modifies the environment by utilizing beneficial microbes, substances, or healthy cropping practices (He et al. 2021). Numerous bacterial isolates taken from diverse environmental samples have been examined to discover appropriate candidates that exhibit antagonistic properties toward *E. amylovora* (Dagher et al. 2020; Esteban-Herrero et al. 2023; Mechan Llontop et al. 2020). It is essential that candidate bacteria survive well on or in plants, are non-phytotoxic, possess a broad range of effectiveness, synthesize various hydrolases associated with biocontrol, and induce plant defense (Dagher et al. 2020; Esteban-Herrero et al. 2023; Mechan Llontop et al. 2020; Zeng et al. 2023). However, the exploration of bacteria possessing all these traits in the control of *E. amylovora* is quite limited.

Plant growth-promoting rhizobacteria (PGPR) are a group of free-living soil bacteria that directly and/or indirectly enhance the growth of plants (Backer et al. 2018; Hayat et al. 2010). The direct promotion of plant growth by PGPR involves the production of plant growth-promoting substances or facilitating the absorption of specific nutrients by the plants from the environment (Denancé et al. 2013). As PGPR mitigate phytopathogenic organisms, they can indirectly promote plant growth (Beneduzi et al. 2012). The production of hydrolytic enzymes by PGPR is also an important mechanism directed against phytopathogens for sustainable plant disease management (Jadhav et al. 2017). *Streptomyces* species, a major group of PGPR, found in soil or associated with plants coexist in a competitive environment where resources and space are limited (Dow et al. 2023). The group accounts for more than two-thirds of the 23,000 bioactive secondary metabolites produced by microbes, highlighting it as a prolific producer of secondary metabolites and volatile organic compounds (Sousa and Olivares 2016). However, even though *Streptomyces* species are promising candidates for controlling phytopathogenic microorganisms (Olanrewaju and Babalola 2019), very few studies have investigated their use to control apple fire blight. Many *Streptomyces* species, such as *S. celluloflavus*, *S. kasugaensis*, *S. luteoreticuli*, *S. pimprina*, and *S. thioluteus*, have been reported to produce thiolutin (Lamari et al. 2002). In addition, aureothricin is a by-product of thiolutin fermentation in various *Streptomyces* species (Celmer and Solomons 1955). Aureothricin and thiolutin are dithiolopyrrolone (DTP) compounds with broad-spectrum antibacterial activity against various microorganisms (Qin et al. 2013). However, their application in controlling phytopathogens remains unknown.

For millions of years, plants and pathogenic microbes have engaged in a hostile relationship, leading to the evolution of different mechanisms by plants to combat infections. These mechanisms include several layers of constitutive and inducible defenses (Syed Ab Rahman et al. 2018). Salicylic acid (SA) and jasmonate (JA) are hormones critical for plant immune responses, with SA and JA biosynthesis and signaling linked historically to defense against biotrophic or necrotrophic pathogens, respectively (Shigenaga et al. 2017). Inducible and functionally diverse pathogenesis-related (PR) proteins are known to accumulate in response to pathogen attacks. They have been implicated in active defense, potentially limiting pathogen development and spread (Edreva and Kostoff 2005). However, few studies have demonstrated the expression of a *PR* gene in apple trees in the complex interaction between *E. amylovora* and *Streptomyces*.

During screening for microorganisms that have antibacterial activity against *E. amylovora*, we isolated strain JCK-8055, which showed a wide range of antimicrobial activity, leading us to conduct this study. The study objectives were (1) to isolate and characterize the traits of JCK-8055, (2) to extract and identify the active secondary metabolites of JCK-8055, (3) to assay the antimicrobial activity of secondary metabolites against various phytopathogenic bacteria and fungi, (4) to determine the effectiveness of its secondary metabolites in preventing and curating plant bacterial disease, and (5) to evaluate the induced resistance of JCK-8055 in plants against bacterial diseases and the expression of PR genes induced by *Streptomyces* sp. JCK-8055 in apple trees.

Materials and methods

Streptomyces isolation and culture conditions

The pepper root samples collected in Daejeon, Republic of Korea (100 mg), were surface sterilized by soaking in 70% ethanol for 5 min, followed by 2% NaOCl containing 250 µg/mL Tween 20 for 2 min. After five rinses with sterile distilled water, the sterilized plant sample was homogenized in 10 mL of sterile distilled water and agitated for 10 min at 150 rpm. The sample was diluted by the 10-fold serial dilution method and was spread onto tryptic soy agar (TSA) (Difco, Detroit, USA) plates. Distinct colonies were picked, maintained in TSA medium, and preserved in 30% glycerol at −80 °C. A single colony from each isolate was transferred to a 20 mL test tube containing 4 mL of tryptic soy broth (TSB) (Difco, Detroit, USA) and incubated at 28 °C and 180 rpm for 7 days. The resulting culture filtrate from each isolate was obtained by filtering through a 0.2 µm membrane.

Fermentation conditions of JCK-8055

A single colony of JCK-8055 was transferred to TSB. After 48 h of incubation, 1% of the seed culture was inoculated into a 1 L Erlenmeyer flask containing 200 mL of TSB and fermented for 7 days at 28 °C and 180 rpm. The resulting culture broth was centrifuged to separate the cell-free (CF) supernatant, and this supernatant was then filtered through a 0.2 µm membrane to prepare the culture filtrate for further analysis.

Plant materials

Transgenic *Arabidopsis thaliana* Col-0 seeds (containing the *pathogenesis-related 1* (*PR1*) *pro::β-glucuronidase (GUS)* gene construct) were surface sterilized by soaking in 70% ethanol for 5 min, followed by 2% NaOCl containing 250 µg/mL Tween 20 for 2 min. After five rinses, the seeds were soaked in sterilized water at 4 °C for 2 days. The seeds were then germinated on ½ Murashige-Skoog (MS) agar medium containing 50 µg/mL kanamycin under a 16-h/8-h daylight/night cycle in a growth chamber at 25 °C for 2 weeks.

Seokwang tomato seeds (FarmHannong Co., Ltd., Seoul, Republic of Korea) were planted in vinyl trays filled with nursery soil and were cultivated in an incubation room with a 12-h/12-h daylight/night cycle per day. The seedlings were then transplanted into 7.5 cm diameter plastic pots 24 h before sample treatment or pathogen inoculation.

The M9 apple seedlings (15 ± 3 cm in height) were procured from the Korea Technology Promotion Agency (Iksan, Republic of Korea). The seedling was maintained in a greenhouse with a temperature range of 18–25 °C.

In vitro antibacterial assay against *E. amylovora* TS3128

The microdilution method was used to test the antibacterial activity of the culture filtrates (Le et al. 2021). In brief, suspensions of *E. amylovora* in TSB media (100 µL) with inocula of 10^5 colony forming units (CFU)/mL were added to the wells of sterile 96-well plates. A concentration of 10% of culture filtrates were added in the wells and followed by two-fold dilution in the growth media. Streptomycin sulfate was used as a positive control, while the untreated bacterial solution and TSB were used as the negative controls. The test plates were then incubated at 30 °C for 1 day, and the growth of the pathogen was measured as an optical density (OD) at 600 nm using a microplate reader (iMark; Bio-Rad, Hercules, CA, USA). The minimum inhibitory concentration (MIC) value was determined as the lowest concentration capable of inhibiting the growth of microorganisms, and the inhibition rate was calculated as follows:

$$\text{Inhibition rate (\%)} = \frac{\text{OD of untreated control} - \text{OD of treated sample}}{\text{OD of untreated control} - \text{OD of negative control}} \times 100\%$$

Preparation of DNA, PCR amplification, and phylogenetic analysis

The genomic DNA of the selected JCK-8055 strain was extracted using the i-genomic BYF DNA Extraction mini kit (iNtRON Biotechnology, Inc., Seongnam, Republic of Korea). The amplification of the 16S rRNA and *gyrB* gene was carried out using the primer pairs listed in Supplementary Table S1. The PCR thermal profile consisted of an initial denaturation step of 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, annealing at 55 or 65 °C for 30 s, an extension at 72 °C for 90 s, and a final extension at 72 °C for 10 min. The sequences of the 16S rRNA and *gyrB* of the strain were compared for similarity with the reference *Streptomyces* species in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (Supplementary information). Phylogenetic trees were generated using three algorithms, including neighbor-joining (NJ) (Saitou and Nei 1987), maximum-likelihood (ML) (Felsenstein 1981), and minimum-evolution (ME) (Nei et al. 1998), in the MEGA X software (Kumar et al. 2018).

Characterization of phenotypes, biochemical tests, and physiological characteristics

The growth of JCK-8055 was investigated at 28 °C for 7 days on different media, including tryptone-yeast extract (ISP1), yeast-malt agar (ISP2), oatmeal agar (ISP3), inorganic salt-starch agar (ISP4), glycerol asparagine agar (ISP5), peptone yeast extract iron agar (ISP6), marine agar (MA), Bennet, potato dextrose agar (PDA), and TSA. The composition of each medium is described in the Supplementary information. The morphological features of JCK-8055 were observed under a scanning electron microscope (SEM) (Quanta™ 250 FEG; FEI Company, Hillsboro, Oregon, USA). The carbon utilization and enzyme activity of JCK-8055 were evaluated by API 50 CH and API ZYM (bioMérieux, Craponne, France). In addition, the production ability of amylase, cellulase, gelatinase, and protease by JCK-8055 was evaluated based on the methodology described by Hossain et al. (2020). The production of indole-3-acetic acid (IAA) by JCK-8055 also was tested according to the protocol outlined by Gang et al. (2019). The optimal growth conditions were determined at various NaCl concentrations (1%, 3%, 5%, 7%, and 9%), temperatures (4 °C, 20 °C, 28 °C, 37 °C, and 45 °C), and pH levels (4, 6, 8, and 10). Growth media were adjusted to the desired pH with NaOH and HCl by Thermo Scientific™ Orion Star™ A211 Benchtop pH meter (Seoul, Republic of Korea).

Extraction and isolation of the antibacterial metabolites of JCK-8055

To isolate the active antibacterial compounds, the culture broth of JCK-8055 was filtered through four layers of sterile gauze. The CF supernatant was partitioned twice with ethyl acetate (EtOAc) and then butanol (BuOH). Next, the EtOAc, BuOH, and aqueous layers were separately concentrated to dryness and re-dissolved in acetone, methanol, and distilled water (DW), respectively. The antibacterial activity of the three layers against *E. amylovora* TS3128 was then tested, and the layer having the highest activity was selected for further investigation.

The selected layer was subjected to chromatography on a silica gel column (Kieselgel 60, 230–400 mesh: E. Merck, Darmstadt, Germany), a Sephadex LH-20 column (70–100 µm; Sigma-Aldrich, Vienna, Austria), and preparative thin layer chromatography (TLC) on Kieselgel 60 GF_{254} (0.5 mm film thickness, E. Merck, Darmstadt, Germany) while using $CHCl_3$:MeOH (99:1, v/v), 100% MeOH, and $CHCl_3$:MeOH (9:1, v/v) as the eluents, respectively. The fractions were visualized by TLC (Kieselgel 60 GF_{254}, 0.2 mm film thickness, E. Merck, Darmstadt, Germany) with $CHCl_3$:MeOH (9:1) as the mobile phase. Based on the TLC profiles, the fractions with similar retardation factor (Rf) values were combined. Finally, each group was dissolved in 1 mL of acetone and tested for antibacterial activity against *E. amylovora* TS3128, with 1% acetone as the untreated control.

The purity of the isolated compounds was assessed by high-performance liquid chromatography (HPLC) using an Atlantis T3 C_{18} column (4.6 × 250 mm; Waters, Milford, MA, USA) with a Waters 515 HPLC pump and a Waters 996 photodiode array (PDA) detector. The mobile phase consisted of a gradient solvent system of 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile, with a 1 mL/min flow rate. The gradient profile was as follows: 0 min (20% acetonitrile), 25 min (100% acetonitrile), and 30 min (100% acetonitrile). Detection was carried out at a wavelength of 360 nm, and UV spectra were recorded using a PDA detector.

Structure determination of antibacterial metabolites

The chemical structures of the antibacterial metabolites were determined using a combination of liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy. For LC-MS analysis, a Shimadzu 6AD HPLC system (Shimadzu, Tokyo, Japan) coupled with an API2000 mass spectrometer (Applied Biosystems, Waltham, MA, USA) was used in positive mode (100–1800 m/z) with a ZORBAX C_{18} column (4.6 × 250 mm, Agilent, Santa Clara, CA, USA). The mobile phases were distilled water and acetonitrile (ACN) containing 0.05% TFA, and a linear gradient of 0–8% over 16 min was used with a flow rate of 1 mL/min. For NMR spectroscopy, ^1H spectra were acquired in $CDCl_3$ using a Bruker Avance III HD 500 MHz instrument (Bruker Biospin GmbH, Rheinstetten, Germany) at 500 MHz, with tetramethylsilane as an internal standard for chemical shift calculations.

Selection of optimal medium for antibacterial metabolite production

To determine the liquid medium for the optimal production of active compounds, JCK-8055 was cultured in six different liquid media (ISP2, ISP4, Bennet, GSS, M3, and TSB) (Supplementary information). Liquid cultures were incubated at 28 °C or 37 °C and grown for 4–9 days. After incubation, the culture filtrates were obtained using a 0.2 µm membrane filter. They then performed a quantitative analysis of the antibacterial metabolites produced under each condition using HPLC. Standard compounds, aureothricin and thiolutin, were purchased from Biosynth Carbosynth (Staad, Switzerland) and Sigma-Aldrich (St. Louis, MO, USA).

In vitro antimicrobial assay

The antimicrobial activity of the JCK-8055 culture filtrates and the secondary metabolites against phytopathogenic microorganisms was evaluated using a broth dilution method following a previous report by Le et al. (2021). This study used 21 phytopathogenic bacteria and fungi (Supplementary information).

Histological GUS (β-glucuronidase) assay

The culture broth of JCK-8055 was centrifuged at 4000 rpm for 15 min to obtain the CF supernatant and cells. The cells were washed three times with sterilized distilled water (SDW) and then resuspended with SDW at a 1:1 (v:v) ratio. The culture broth, CF supernatant, and cell suspension were diluted to 100-fold, 500-fold, and 1000-fold concentrations using SDW. The expression of GUS activity was examined when transgenic *A. thaliana* seedlings were treated with each sample for 48 h at room temperature. The seedlings were stained using a chemical solution according to the procedure described by Kondo et al. (2014). SA and TSB medium were used as a positive control and a negative control, respectively.

RNA isolation and qRT-PCR

Apple plants were sprayed with the 500-fold-diluted CF supernatant of JCK-8055 10 days and 3 days before inoculation (DBI) with *E. amylovora*. An untreated control group was sprayed with water. At 0, 1, and 2 DBI, leaves from three plants in each group were separately collected and ground in liquid nitrogen. Total RNA was extracted using the iNtRON Iqeasy™ Plant RNA Extraction mini kit (iNtRON Biotechnology, Gyeonggi-do, Republic of Korea). cDNA was synthesized from 5000 ng of each RNA sample using the Invitrogen SuperScript™ IV First-Strand Synthesis System kit (Thermo Fisher Scientific, Vilnius, Lithuania). qRT-PCR was conducted using a Bio-Rad CFX96™ Real-Time System on a 96-well plate iCycler iQ thermal cycler (Bio-Rad, Hercules, CA, USA). Each reaction contained 1 µL of cDNA template, 4 µL of a 400 nM primer mix, and 5 µL of 2× iQ™ SYBR Green supermix (Bio-Rad, Hercules, CA, USA). The PCR cycling parameters included an initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s, and 55 °C for 5 s, and a final extension at 95 °C for 30 s. Primers for qRT-PCR analysis are listed in Supplementary Table S1. The relative expression of the target genes was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and was normalized with the expression of the *EF1* gene (NCBI-ID: DQ341381).

In planta bioassays

Colonization of JCK-8055 in tomato

Because JCK-8055 was originally isolated from pepper roots, the colonization ability of this strain was evaluated by re-isolating *Streptomyces* from coated tomato roots, utilizing a modified method based on the procedures described by Mattei et al. (2022) and Colombo et al. (2019). Strain was incubated in a 250 mL flask containing 50 mL of TSB for 2 days at 28 °C and 180 rpm. The cells were harvested by centrifugation (4000 rpm, 15 min), washed three times, and resuspended with 5 mL of SDW (7×10^7 CFU/mL). Tomato seeds were disinfected with 1% sodium hypochlorite and 70% ethanol and then rinsed with SDW 3 times. The seeds were then soaked in these cell suspensions for 24 h. Coated seeds were dried overnight in a horizontal laminar flow.

To evaluate the colonization ability of JCK-8055 on a plant, both coated and uncoated tomato seeds were planted in sterilized nursery soil and maintained under 25 °C, 75% humidity, and 12 h of daylight per day. After 2 weeks of sowing, the roots of each tomato plant seedlings were collected and surface sterilized with 1% sodium hypochlorite (1 min) and 70% ethanol (1 min), followed by rinsing with SDW three times. *Streptomyces* strains showing a similar morphology to JCK-8055 were isolated and identified following the same procedures described in the *Streptomyces* isolation and JCK-8055 identification section. The 16S RNA sequence of the re-isolated *Streptomyces* strain was then compared with that of JCK-8055.

Tomato bacterial wilt

To assess the biocontrol efficacy of the strain when it acts as colonizers of the roots of tomato seeds, the coated and uncoated tomato seeds were planted in nursery soil and maintained under 25 °C, 75% humidity, and 12 h of daylight per day. The positive control was the uncoated plant treated with a 1000-fold dilution of seongbocycline (oxytetracycline 17% WP; Sungbo Chemicals Co., Ltd., Gyeonggi, Republic of Korea) 1 day before bacterial inoculation. The untreated control was an uncoated plant treated with SDW. An uninoculated control was prepared by sowing uncoated seeds in uninoculated pots.

JCK-8055 was cultured in a TSB medium to test the antibacterial activity of its secondary metabolites. Its CF culture filtrate was diluted with water at 5, 10, and 20-fold concentrations. A mixture of two antibacterial compounds, referred to as Sephadex yellow fraction (SYF), was prepared at concentrations of 20 µg/mL for SYF 20 and 100 µg/mL for SYF 100. For a preventive activity test, the tomato plants were treated with 20 mL of the samples 1 DBI through soil drenching. For a curative activity test, the plants were treated with 20 mL of the samples 1 day after inoculation (DAI) through soil drenching. All samples were mixed with Tween 20 at 250 µg/mL concentrations. As a positive control, buramycin (streptomycin sulfate 20% wettable powder (WP); Farm-Hannong Co., Seoul, Korea) was used and diluted 1000-fold according to the manufacturer's instructions. The untreated control was treated with TSB mixed with Tween 20 solution. The uninoculated control was prepared with plants in uninoculated pots.

The potential of induced resistance in tomatoes by the fermentation supernatant of JCK-8055 against *Ralstonia solanacearum* SL341 was also evaluated. The CF supernatant of JCK-8055 was applied to the plants separately through soil drenching or foliar spraying. Briefly, the CF culture filtrate of this strain was diluted 250-fold, 500-fold, and 1000-fold with SDW. All samples were mixed with Tween 20 at 250 µg/mL concentrations. A 20 mL volume of CF supernatant was added to the pots through soil drenching. During the foliar spraying, the soil surface of each pot was covered with plastic, and all leaves were sprayed with the solution. These samples were treated 3 DBI. A 1000-fold dilution of buramycin (streptomycin sulfate 20% WP; Farm Hannong Co., Seoul, Korea) was used as the positive control and applied on plant 1 DBI through soil drenching. The

untreated control was treated by TSB mixed with Tween 20 solution. The uninoculated control consisted of plants in uninoculated pots.

All samples against tomato bacterial wilt were evaluated using tomato plants at the fourth-leaf stage. On the day of bacterial infection, the plants were inoculated with a cell suspension of *R. solanacearum* SL341, 10^8 colony forming units (CFU)/mL, through soil drenching and kept in the dark for 24 h at 30 ± 2 °C. The plants were maintained with 75% humidity and 12 h of daylight per day, and the disease symptoms were observed at 7 DAI. The disease severity on the tomato plants was rated on a scale of 0–4: 0, no leaf symptoms; 1, one leaf wilted; 2, two or three leaves wilted; 3, more than four leaves wilted; 4, a dead plant (Vu et al. 2017).

Apple fire blight

The efficacy against fire blight was evaluated using the JCK-8055 CF supernatant and its two wettable powder (WP)-type formulations. The freeze-dried culture broth (CB FD) or the EtOAc extract of JCK-8055 was mixed with synthesized hydrated silicon dioxide (white carbon; Rhodia Asia Pacific Pte Ltd., Kallang, Singapore), sodium dodecyl sulfate, (CR-SDS; Yoosung Chemical R&T Co., Ltd., Chungnam, Republic of Korea), sodium poly (naphthalene formaldehyde) sulfonate (CR-100; Yoosung Chemical R&T Co., Ltd., Chungnam, Republic of Korea), and kaoline to create WP-type formulations. Briefly, 2 g of the freeze-dried culture broth was mixed with 1.5 g of silicon dioxide, 0.5 g of sodium dodecyl sulfate, 0.5 g of sodium poly (naphthalene formaldehyde) sulfonate, and 5.5 g of kaoline to create the JCK-8055 CB-FD WP20. Similarly, 1 g of the EtOAc extract culture filtrate was mixed with 1.5 g of silicon dioxide, 0.5 g of sodium dodecyl sulfate, 0.5 g of sodium poly (naphthalene formaldehyde) sulfonate, and 6.5 g of kaoline to create the JCK-8055-EtOAc WP10. The formulations were finely mixed in a blender.

To evaluate the antibacterial activity of JCK-8055, the plants were treated with a 500-fold dilution of JCK-8055 EtOAc WP10 by foliar spray. The positive control was Agrepto (streptomycin 20%; Kyung Nong, Seoul, Korea) used according to the manufacturer's guidelines. The untreated control was treated with SDW, and the uninoculated control consisted of plants in uninoculated pots. All the samples for the antibacterial activity assay were treated 1 DBI.

To assess the induced resistance activity, the plants were treated with a 500-fold dilution of JCK-8055 CF supernatant with Tween 20 at 250 µg/mL concentrations by either soil drench or foliar spray. In addition, the plants were treated with a 1000-fold dilution of JCK-8055 CB-FD WP20 by foliar spray. The positive control was Serifel (containing *Bacillus* sp.; BASF, Seoul, Korea) used according to the manufacturer's guidelines. The untreated control was treated with 250 µg/mL of Tween 20, and the uninoculated control consisted of plants in uninoculated pots. All samples for the induced resistance assay were treated twice: 10 DBI and 3 DBI.

The *E. amylovora* TS3128 solution (100 µL) was spread on TSA and incubated at 30 °C. After 24 h, the bacterium was isolated from TSA by swabbing with SDW. The bacterial concentration in the suspension was then adjusted to 3.3 × 10^7 CFU/mL using a spectrophotometer at a wavelength of 600 nm. On the day of bacterial infection, the leaves of the apple seedlings were inoculated with a cell suspension of *E. amylovora* TS3128 (10 mL) using a sprayer. The apples were then covered with wetted plastic bags for 2 days at 25 °C. After 7, 10, and 14 days, the presence of fire blight symptoms was evaluated using a disease index based on the modified scale of Hevesi et al. (2000). The scale ranges from 0 to 5: 0, no leaf symptom; 1, blackening spot on 1 leaf; 2, blackening spot on 2 or 3 leaves; 3, blackening spot on 4 or 5 leaves or browning from the shoot; 4, blackening spot on more than 5 leaves or halfway from the leaf vein; and 5, blackening spot on all leaves or bacterial exudate appearing on the plant.

The data were evaluated by disease rating (DR) calculations described by Hevesi et al. (2000).

$$\text{Disease rating} = \frac{\Sigma\left[(N1 \times 1) + (N2 \times 2) + (N3 \times 3) + (N4 \times 4) + (N5 \times 5)\right.}{\Sigma N}$$

$N1-5$ is the number of plants in each disease category.

The disease severity (DS) was expressed as a percentage of the untreated control, along with the standard deviation. The calculation of the control value was performed using the following equation:

$$\text{Control value } (\%) = \frac{(\text{DS of untreated control} - \text{DS of treatment})}{\text{DS of untreated control}} \times 100\%.$$

Statistical analysis

The results of the pot experiments were analyzed by one-way or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The statistical analysis and graph design were performed in GraphPad Prism 8.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Characterization of JCK-8055

JCK-8055 grew on all tested media and produced a yellow pigment when cultured on ISP2, ISP3, ISP4, and TSA (Supplementary Table S2). In particular, the strain

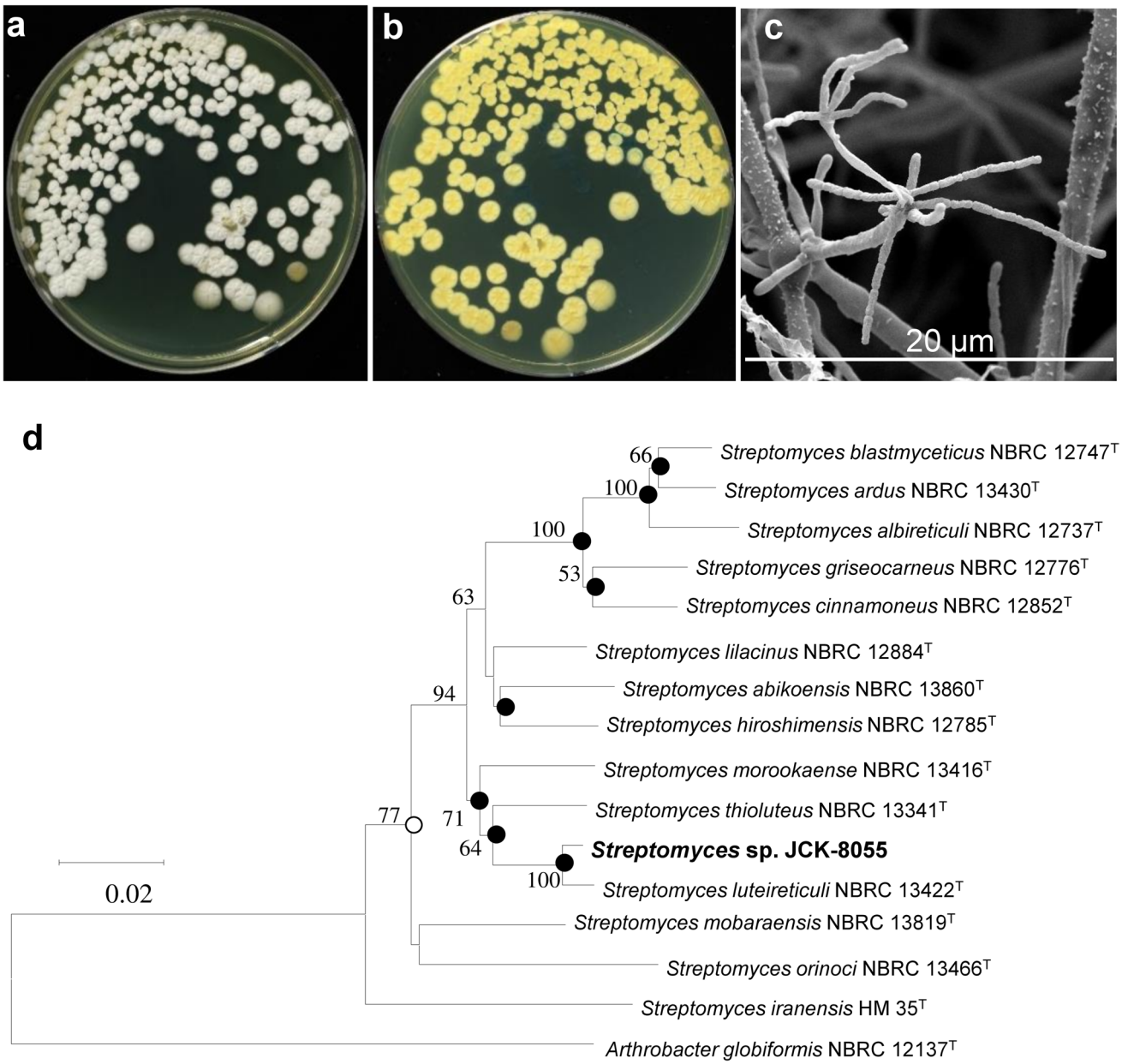

Fig. 1 Growth and morphology of JCK-8055 on TSA plates. **a** The top side view and **b** the bottom side view of TSA plates showing the growth of JCK-8055 colonies after 7 days of growth at 28 °C. **c** Spore chain morphology of JCK-8055 by SEM. **d** A neighbor-joining phylogenetic tree based on the 16S rRNA and *gyrB* gene sequences of strain JCK-8055 from bacteria related to the *Streptomyces* species. *Arthrobacter globiformis* NBRC 12137^T was used as the outgroup. The sequence accession numbers of these strains were provided in Supplementary Tables 6 and 7. Filled circles at nodes indicate that the corresponding nodes were recovered in the trees reconstructed with three algorithms (NJ, ME, and ML methods) whereas the nodes with empty circles were recovered by two algorithms. Bootstrap values (≥50%) based on 1000 replications are indicated at branch nodes. Scale bar, 0.02 nucleotide substitutions per nucleotide position. TSA, tryptic soy agar; SEM, scanning electron microscope

produced a light-yellow diffusible pigment on TSA (Fig. 1a, b). It also grew well in a temperature range of 20–37 °C, a pH range of 6–10, and a NaCl concentration range of 0–3% (Supplementary Table S3). JCK-8055 utilized several carbon sources, including glycerol, D-ribose, and esculin ferric citrate, and produced enzymes, such as cellulase, gelatinase, and protease, and the plant growth regulator IAA (Supplementary Tables S4 and S5 and Fig. S1). In addition, JCK-8055 formed sporulating mycelia, which were white or gray, on most agar media. The spore chain morphology of JCK-8055 was umbellate monoverticilate (Fig. 1c).

Phylogenetic analysis of JCK-8055

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene and *gyrB* sequence of strain JCK-8055 is OP872729.1 and OP886867.1, respectively. The 16S rRNA and *gyrB* sequences of JCK-8055 shared the highest similarity with the sequences of *S. luteireticuli* NBRC 13422^{T} (99.71% and 98.66%, respectively) (Supplementary Tables S6 and S7). All phylogenetic trees indicated that the strain forms a monophyletic clade with *S. luteireticuli* NBRC 13422 ^{T} (Fig. 1d and Supplementary Figs. S2 and S3). Despite their close genetic relationship, there were some differences between the two strains in the mycelial growth phenotypes and carbon source utilization (Supplementary Table S8). Overall, the combination of genotypic and phenotypic differences supported JCK-8055 as a *Streptomyces* sp. The strain has been deposited in the Korean Collection for Type Cultures (Jeongeup, Republic of Korea) as KCTC 15168BP.

Isolation and identification of antimicrobial metabolites

When 5% of the *Streptomyces* sp. JCK-8055 culture filtrate was treated, antibacterial activity against *E. amylovora* TS3128 was shown in the treatment group. Bioautography analysis using 10 mg/mL of each solvent extract showed that the ethyl acetate extract exhibited a clear zone (Supplementary Fig. S4), indicating that the antibacterial compounds were present in the EtOAc layer. To isolate the active antibacterial compounds, repeated column chromatography and preparative-TLC were performed under the guidance of a bioassay. As a result, it obtained two yellow compounds separated through Sephadex LH-20 column chromatography, named Sephadex yellow fractions 1 and 2 (SYF1, SYF2), with yields of 3.2 mg for SYF1 and 2.8 mg for SYF2, respectively. The HPLC analysis of the two compounds showed only one peak on each chromatogram. The UV spectra of the two compounds showed three maxima peaks at approximately 243.0, 313.8, and about 390 nm (Supplementary Fig. S5). The LC/MS analysis of the positive-ion mode of compounds SYF1 and SYF2 showed protonated molecular ions ($[M+H]^{+}$) at m/z 243.02 and 229.01, respectively (Supplementary Figs. S6 and S7). The ^{1}H-NMR spectra of the two compounds revealed singlets at 7.34 and 3.25 ppm, typical for one isolated olefinic proton and one N-CH_3 group included in an amide functional group, respectively (Supplementary Figs. S8 and S9). Compound SYF2 had a singlet at 2.02 ppm, accounting for one isolated methyl (NH-CO-CH_3) group, while compound SYF1 had a triplet and quartet at 1.17 and 2.40 ppm, respectively, demonstrating one isolated ethyl (NH-CO-CH_2-CH_3) group. Based on the mass analysis and ^{1}H-NMR, compounds SYF1 and SYF2 were identified as aureothricin and thiolutin, respectively (Fig. 2).

Optimization of culture medium for enhancing antibacterial metabolite production

JCK-8055 exhibited vigorous growth in various media with a broad range of nutrient sources at 28 °C and 37 °C (Fig. 3). To achieve the maximum yield of compounds SYF1 and SYF2, *Streptomyces* sp. JCK-8055 was cultivated in several liquid media. SYF1 was produced at the

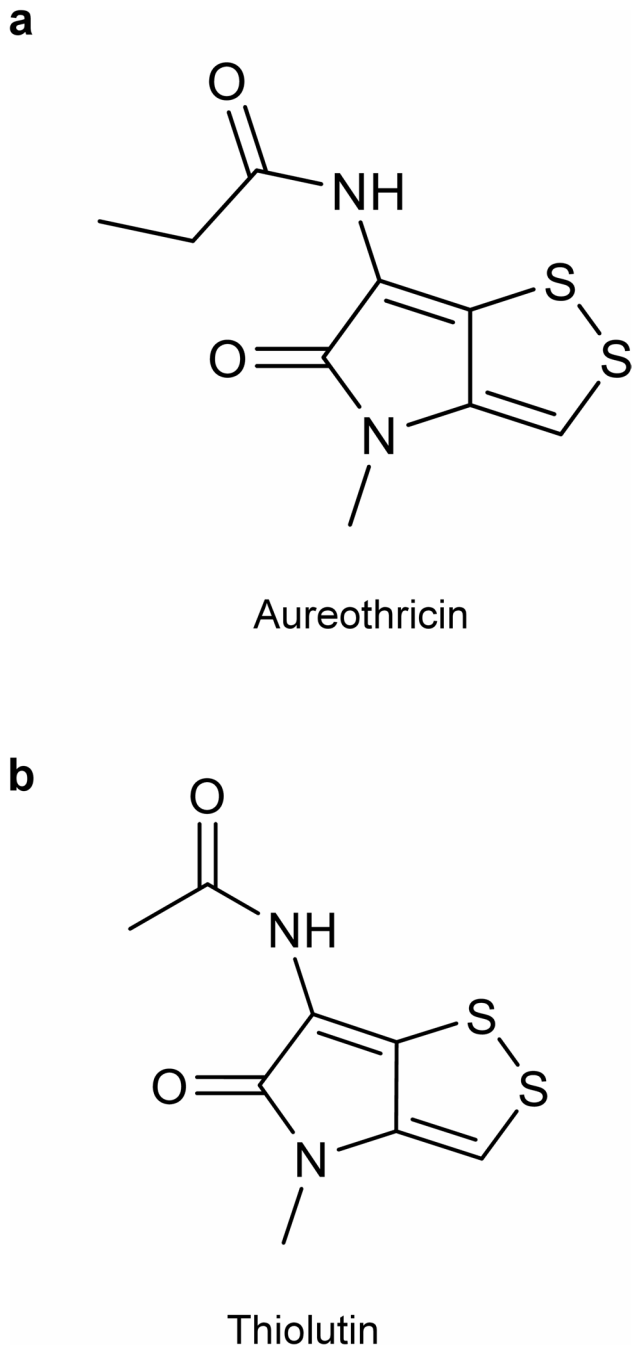

Fig. 2 Chemical structures of two antibacterial metabolites produced from JCK-8055

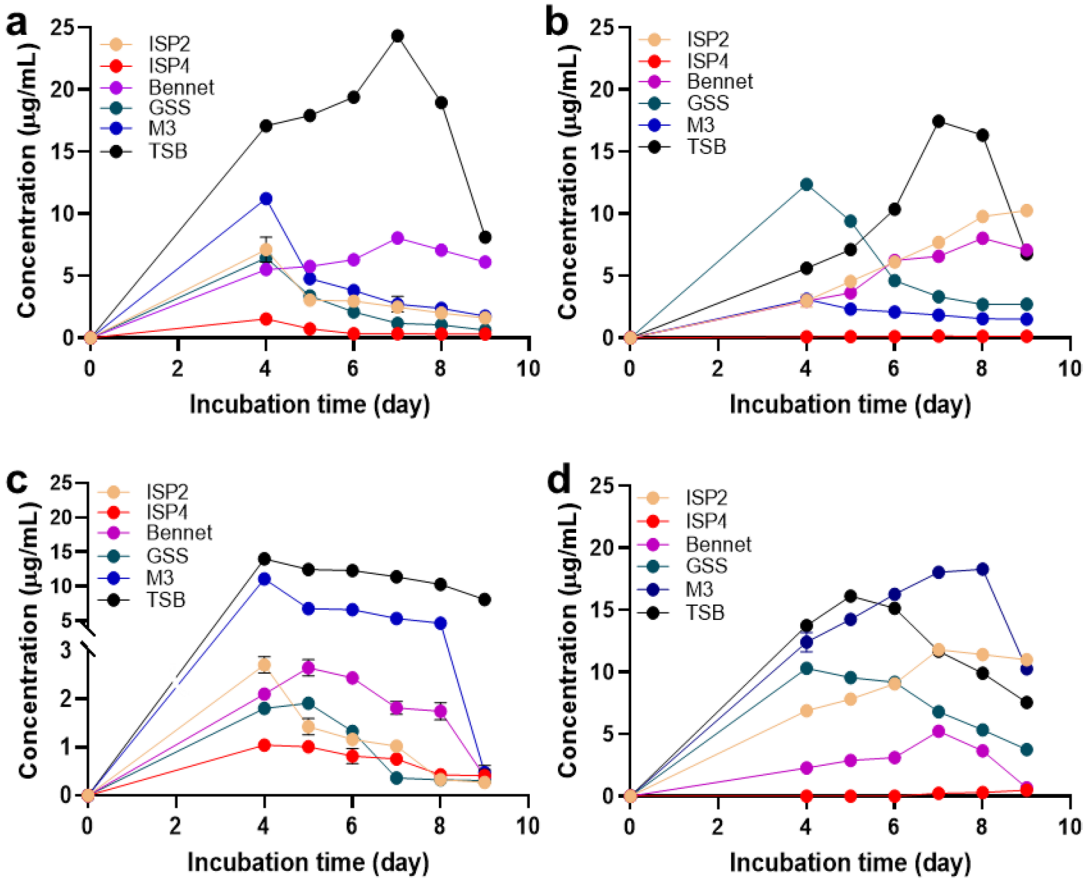

Fig. 3 Production of antibacterial metabolites by JCK-8055 in various conditions. **a** Production of aureothricin at 28 °C, **b** thiolutin at 28 °C, **c** aureothricin at 37°C, and **d** thiolutin at 37 °C. The values are represented as the average values ± standard deviation obtained from three separate runs, each having three replicates

highest concentration of 24.33 ppm in TSB medium at 28 °C 7 DAI, followed by 14.10 ppm in TSB medium at 37 °C 4 DAI. Similarly, SYF2 was produced at the highest concentration of 18.39 ppm in M3 medium at 37 °C 8 DAI, followed by 17.20 ppm in TSB medium at 28 °C 7 DAI. Therefore, JCK-8055 produced high levels of both compounds when grown in TSB medium (Supplementary Fig. S10 and Fig. 3).

In vitro antimicrobial assay against phytopathogens

The culture filtrate of JCK-8055 exhibited broad-spectrum antibacterial activity against various phytopathogenic bacterial strains in TSA (Tables 1 and 2). However, it did not show antifungal activity against any of the tested fungi, but reacted antibiotic against the oomycete *Phytophthora infestans*. At MICs indicated within the tables, the active compounds, aureothricin and thiolutin, exhibited antibiotic activity against selected bacteria, fungi, and oomycetes, except for *Fusarium oxysporum* f. sp. *cucumerinum*.

GUS staining assay

To investigate the induction of defense gene expression in transgenic *A. thaliana* Col-0 by *Streptomyces* sp. JCK-8055, a transgenic line carrying a fused construct of the *PR1* promoter to the *GUS* reporter gene was utilized (Park et al. 2020). The positive control, SA, led to the detection of vascular-associated GUS activity in leaves, stems, and roots. GUS activity was observed in both *A. thaliana* seedlings treated with culture broth and the filtrate of *Streptomyces* sp. JCK-8055 (Fig. 4). Conversely, the seedlings treated with the cells of JCK-8055 did not show similar signs to those treated with TSB medium.

Efficacy of *Streptomyces* sp. JCK-8055 against tomato bacterial wilt

Both *Streptomyces* sp. JCK-8055 and its two metabolites possess preventive and curative activity against *R. solanacearum* SL341 (Fig. 5a, b). However, the preventive activity exhibited better antibacterial efficacy than the curative activity. The CF supernatant of the strain

Table 1 Minimum inhibitory concentrations (MICs) of the fermentation filtrate obtained from *Streptomyces* sp. JCK-8055 and its antibacterial metabolites against plant pathogenic bacteria

Plant pathogenic bacteria	MIC			
	Fermentation filtrate (%)	Aureothricin (μg/mL)	Thiolutin (μg/mL)	Streptomycin sulfate (μg/mL)
Acidovorax avenae subsp. *cattleyae*	6.67 ± 2.36	6.25 ± 0.00	12.50 ± 0.00	>200.00 ± 0.00
Acidovorax konjaci	10.00 ± 0.00	6.25 ± 0.00	6.25 ± 0.00	10.42 ± 2.95
Agrobacterium tumefaciens	>10.00 ± 0.00	100.00 ± 0.00	50.00 ± 0.00	200.00 ± 0.00
Burkholderia glumae	5.00 ± 0.00	6.25 ± 0.00	4.16 ± 1.48	20.83 ± 5.89
Clavibacter michiganensis subsp. *michiganensis*	10.00 ± 0.00	50.00 ± 0.00	25.00 ± 0.00	20.83 ± 5.89
Erwinia amylovora FB302	5.00 ± 0.00	1.56 ± 0.00	0.78 ± 0.00	3.12 ± 0.00
Erwinia amylovora TS3128	4.17 ± 1.18	4.16 ± 1.48	3.12 ± 0.00	3.12 ± 0.00
Erwinia pyrifoliae KI115	4.17 ± 1.18	2.08 ± 0.74	0.78 ± 0.00	10.42 ± 2.95
Pectobacterium carotovorum subsp. *carotovorum*	>10.00 ± 0.00	100.00 ± 0.00	50.00 ± 0.00	16.67 ± 5.89
Pectobacterium chrysanthemi	5.00 ± 0.00	12.50±0.00	3.12 ± 0.00	>200.00 ± 0.00
Pseudomonas syringae pv. *lachrymans*	>10.00 ± 0.00	100.00 ± 0.00	50.00 ± 0.00	>200.00 ± 0.00
Ralstonia solanacearum SL341	5.00 ± 0.00	25.00 ± 0.00	25.00 ± 0.00	2.08 ± 0.74
Xanthomonas arboricola pv. *pruni*	10.00 ± 0.00	0.62 ± 0.00	0.41 ± 0.15	20.83 ± 5.89
Xanthomonas euvesicatoria	>10.00 ± 0.00	25.00 ± 0.00	12.50 ± 0.00	20.83 ± 5.89

[a]The names of bacteria are presented in alphabetical order

Table 2 Minimum inhibitory concentrations (MICs) of the fermentation filtrate obtained from *Streptomyces* sp. JCK-8055 and its antibacterial metabolites against plant pathogenic fungi and oomycetes

Plant pathogenic fungi and oomycetes	MIC		
	Fermentation filtrate (%)	Aureothricin (μg/mL)	Thiolutin (μg/mL)
Fusarium graminearum	>10.00 ± 0.00	50.00 ± 0.00	12.50 ± 0.00
Fusarium asiaticum	>10.00 ± 0.00	50.00 ± 0.00	12.50 ± 0.00
Fusarium oxysporum f. sp. *cucumerinum*	>10.00 ± 0.00	>200.00 ± 0.00	>200.00 ± 0.00
Phytophthora infestans	5.00 ± 0.00	4.16 ± 1.47	2.60 ± 0.74
Pythium ultimum	>10.00 ± 0.00	50.00 ± 0.00	12.50 ± 0.00
Rhizoctonia solani AG 2-2	>10.00 ± 0.00	50.00 ± 0.00	12.50 ± 0.00
Rhizoctonia solani AG-4	>10.00 ± 0.00	50.00 ± 0.00	25.00 ± 0.00
Sclerotinia homoeocarpa	>10.00 ± 0.00	3.12 ± 0.00	1.04 ± 0.37

[a]The names of fungi and oomycetes are presented in alphabetical order

demonstrated preventive activity by controlling *R. solanacearum* SL341 by 42.42%, 57.58%, and 72.73% at 20-, 10-, and 5-fold dilutions, respectively. The fraction containing the two metabolites exhibited control rates of approximately 59.09% and 100% at 20 and 100 μg/mL concentrations, respectively. The curative activity of the culture filtrate inhibited *R. solanacearum* SL341 by 27.27%, 41.41%, and 42.42% at 20-, 10-, and 5-fold dilutions, respectively. The two compounds provided protective rates of 54.54% and 72.73% at 20 and 100 μg/mL concentrations, respectively. The positive control, buramycin, only showed preventive activity with a control value of about 59.09% against *R. solanacearum* SL341 at a 1000-fold dilution (Supplementary Fig. S11).

Initially, the colonization of inner root tissues by specific *Streptomyces* strains was confirmed. The tomato seedling roots were successfully colonized by the selected *Streptomyces* strain on TSA plates. The morphology and 16S RNA sequence of this strain were similar to those of *Streptomyces* sp. JCK-8055 (Supplementary Fig. S12). These strains could internally colonize the plant, acting as endophytic bacteria. In the seed coating experiment, the control values of JCK-8055 (62.80%) and the positive control (71.33%) were similar, indicating that JCK-8055-coated seeds had excellent control efficacy against tomato bacterial wilt (Fig. 6a).

The pretreatment experiment aimed to evaluate the efficacy of the CF supernatant of *Streptomyces* sp. JCK-8055 against tomato bacterial wilt through foliar spraying and soil

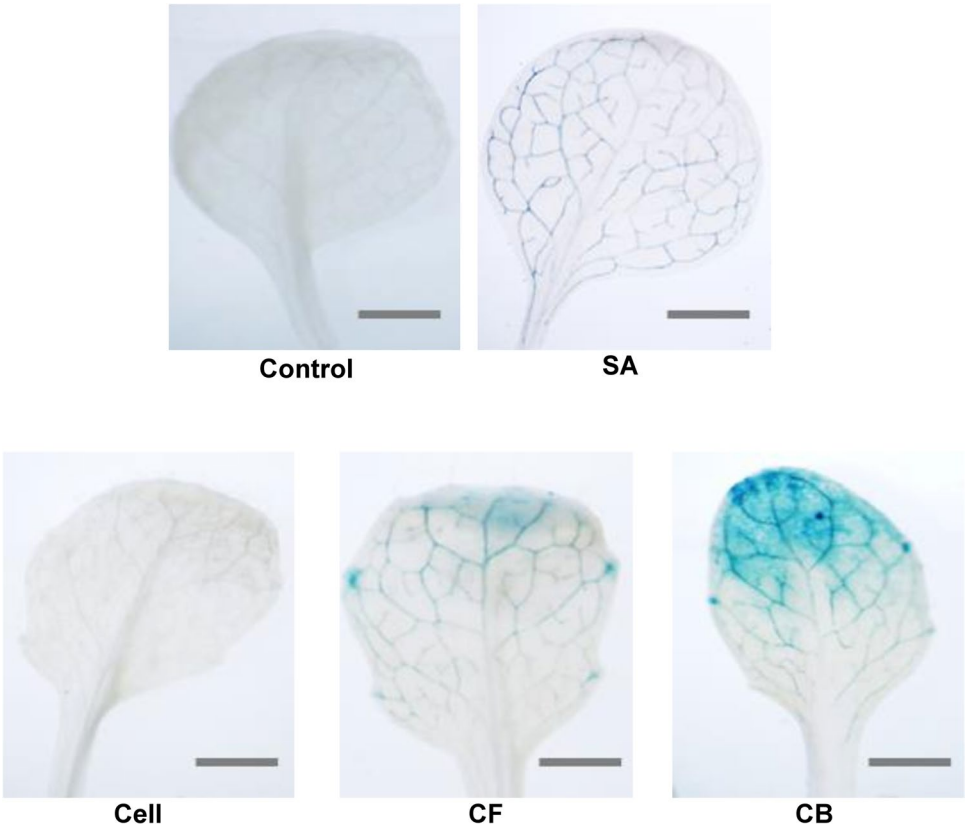

Fig. 4 GUS activity in transgenic *A. thaliana* rosette leaves responded to treatment with control, SA, cell, CF, and CB. Control, 0.05% TSB medium; SA, salicylic acid (0.1 mM); cell, cells of JCK-8055; CF, culture filtrate of JCK-8055; CB, culture broth of JCK-8055; GUS, *β-glucuronidase*; TSB, tryptic soy broth. Scale bar is 1.0 mm

drenching. There was no significant difference in disease control between the treatment with the 250-fold diluted CF supernatant through foliar spraying (70.00%) and soil drenching (68.89%). However, in the 500-fold and 1000-fold dilution treatments, JCK-8055 was more effective against *R. solanacearum* SL341 when applied through soil drenching than through foliar spraying, with values of 100.00% and 82.22% as compared to 24.44% and 22.22%, respectively (Fig. 6b). No phytotoxic symptom was observed on any samples (Supplementary Fig. S13 and Fig. 6c).

Efficacy of *Streptomyces* sp. JCK-8055 against apple fire blight disease

Disease symptoms in the untreated control and JCK-8055 CF filtrate-treated plants were observed 7, 10, and 14 DAI with the pathogen. The disease ratings in the untreated control group gradually increased, with indexes of 2.2, 2.9, and 3.7. In contrast, the disease ratings of plants treated with the JCK-8055 CF filtrate slightly increased or remained unchanged with values of 0.47, 0.73, and 1.13 for foliar spraying and 0.53 for soil drenching, respectively (Fig. 7a). The control values of JCK-8055 against *E. amylovora* TS3128 showed no significant difference between foliar spraying and soil drenching at 14 DAI, with control values of 69.37% and 85.59%, respectively (Fig. 7b).

The TSB culture filtrate of JCK-8055 exhibited antibacterial activity and induced resistance against apple fire blight caused by *E. amylovora* TS3128. Formulations containing JCK-8055, namely, JCK-8055-EtOAc WP10 and JCK-8055 CB-FD WP20, effectively suppressed the development of apple fire blight at 14 DAI with control values of 78.38% and 97.30%, respectively (Fig. 7c, d and Supplementary Fig. S14). JCK-8055 CB-FD WP20 showed a significantly higher disease efficacy than the positive control, Serifel. *E. amylovora* TS3128 could penetrate the leaves treated with JCK-8055 CB-FD WP20, but the diseased leaves dropped at the internode, and bacterial penetration into nearby leaves was inhibited (Supplementary Fig. S15). No phytotoxic symptom was observed on any samples.

Time-course expression of differential genes

At 0 DAI, before pathogen inoculation, the expression of the *PR1*, *PR2*, and *PR3* genes in apple seedlings treated with the fermentation filtrate of JCK-8055 was increased by 3.14, 1.33, and 1.31 folds compared to untreated plants, respectively. At 1 DAI, the expression of the *PR1*, *PR2*, and *PR3* genes was increased by 16.14, 7.55, and 3.37 in apple plants treated with the fermentation filtrate of JCK-8055 compared to the untreated control, whereas at 2 DAI, their expression levels were decreased (Fig. 8).

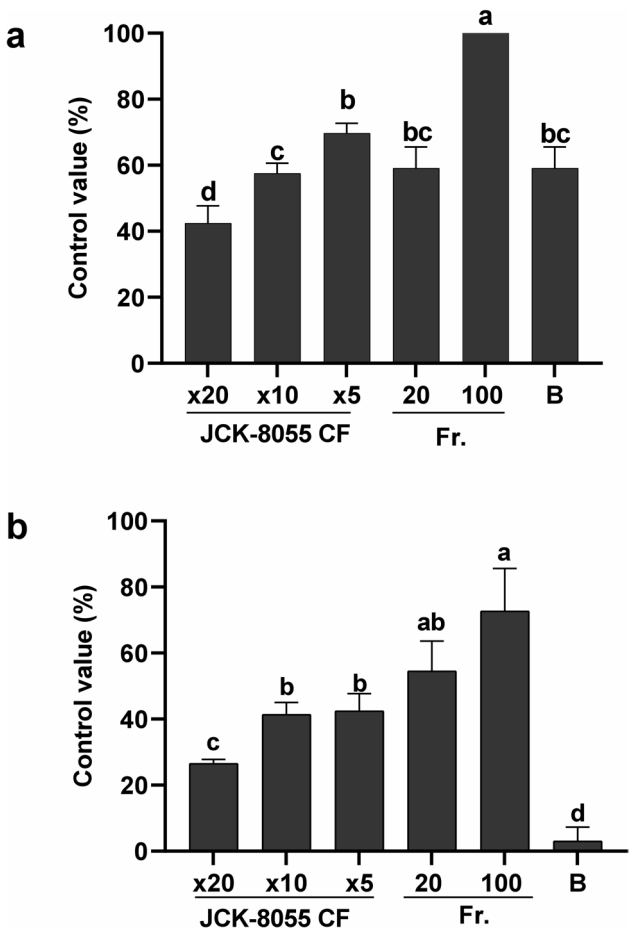

Fig. 5 Disease control efficacy of JCK-8055 against tomato bacterial wilt caused by *R. solanacearum* SL 341. **a** Preventive and **b** curative activity of the culture filtrate of JCK-8055 and its antibacterial metabolites, aureothricin, and thiolutin, against tomato bacterial wilt. CF, culture filtrate of JCK-8055; ×5, 5-fold dilution; ×10, 10-fold dilution; ×20, 20-fold dilution; Fr. 20, 20 µg/mL of the fraction contained antibacterial metabolites mix; Fr. 100, 100 µg/mL of the fraction contained antibacterial metabolites mix; B, positive control buramycin. The values are presented as the mean ± standard error of three runs, with three replicates each. Different letters indicate statistically differences ($P < 0.05$)

Discussion

Fire blight, a highly destructive bacterial disease, can cause significant damage to economically important crops such as apples (*Malus domestica*) and pears (*Pyrus pyrifolia*) (Lee et al. 2022). Since its first reported occurrence in Korea in 2015 (Park et al. 2017), the disease has rapidly spread to other orchards. Consequently, there were 744 orchards infected with fire blight in 2020 (Lee et al. 2022). Researchers have increasingly focused on biological control methods for managing plant diseases due to their eco-friendliness and potential as alternatives to synthetic pesticides. Diverse strains of *Streptomyces* have shown promise as biocontrol agents for controlling phytopathogenic microorganisms, but their effectiveness in managing apple fire blight has not been extensively studied. In this study, we demonstrate that *Streptomyces* sp. JCK-8055 exhibits high potential for controlling tomato bacterial wilt and apple fire blight. *E. amylovora* often enters the tree through natural openings, especially flowers and wounds (Vanneste 2000). Rain, wind, pruning tools, and insects can spread fire blight to healthy plants (Bonn and Van der Zwet 2000). Once established in a tree, fire blight quickly invades new and older parts during the growing season (Aćimović et al. 2015); therefore, effective fire blight management requires precise timing in parallel with conventional control measures (Evans et al. 1999; Rademacher et al. 2005). The findings of this study indicate that JCK-8055 has suppressive effects on the development of tomato bacterial wilt and apple fire blight as a seed coating reagent, thus acting as a preventive and curative bactericide. Therefore, JCK-8055 may effectively control phytopathogenic bacteria during the differentiation stage of plant growth and disease infection.

PGPR strains can control plant diseases directly by producing antagonistic metabolites toward the pathogens or indirectly by enhancing the defense mechanisms of a host (Pandit et al. 2022). *Streptomyces* is recognized as the largest genus of antibiotic-producing bacteria, as many antibiotics are derived from it (Procopio et al. 2012). The culture filtrate of strain JCK-8055 exhibited antibacterial activity against *E. amylovora*. Therefore, we assumed that strain JCK-8055 directly affects *E. amylovora* by synthesizing metabolites. To determine which metabolites synthesized by JCK-8055 exhibited antibacterial properties against *E. amylovora*, we employed a combination of LC-MS and NMR techniques to elucidate their chemical structures. Based on the mass analysis and ^{1}H-NMR, the metabolites of JCK-8055 were identified as aureothricin and thiolutin. Thiolutin and aureothricin were first isolated from *Streptomyces albus* and *Streptomyces* sp. 26A, respectively, in the late 1940s (Celmer et al. 1952; Umezawa et al. 1948), and their structures were then discovered in 1955 (Celmer and Solomons 1955). Both compounds belong to the DTP group of antibiotics (Li et al. 2014) and have shown high activity against fungi, ameboid parasites, Gram-positive, Gram-negative, acid-fast bacteria, and cancer cell lines (Li et al. 2014; Seneca et al. 1952). However, limited research has been conducted on their activity against phytopathogens. To the best of our knowledge, this is the first study to demonstrate that thiolutin and aureothricin could inhibit the growth of plant pathogens, including *E. amylovora* and *R. solanacearum*. JCK-8055 cultivation and its two active metabolites suppressed the development of tomato bacterial wilt through preventive and curative methods. However, we found that the preventive method was more effective than the curative method. This finding agrees with a previous study

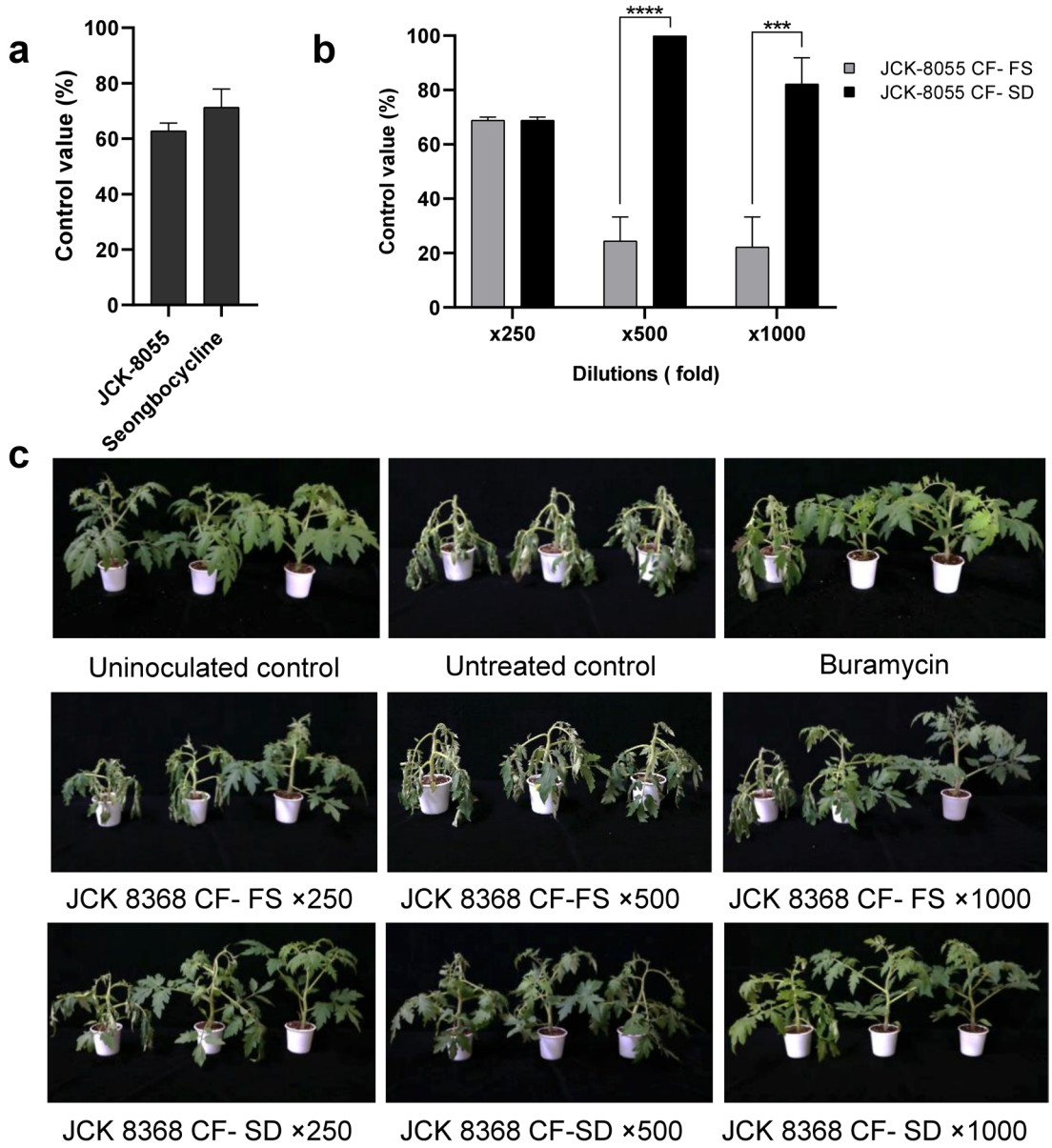

Fig. 6 Disease control efficacy by **a** seed coating and **b, c** pretreatment of JCK-8055 against tomato bacterial wilt caused by *R. solanacearum* SL 341. CF, culture filtrate of JCK-8055; ×250, 250-fold dilution; ×500, 500-fold dilution; ×1000, 1000-fold dilution; FS, foliar spraying; SD, soil drenching. The values are presented as the mean ± standard error of three runs, with three replicates each. *** (*P<0.001*) and **** (*P<0.0001*) represent significant differences

by Tsiantos and Psallidas (2002). These results are attributed to the fact that the compounds that have preventive activity penetrated the plant before phytopathogenic bacterial infection. The compounds then come into contact with bacteria and inhibit them from penetrating the plant. In addition, the preventive compounds could persist within the plant tissue, effectively halting the early growth of the pathogen. On the other hand, the term "curative properties" refers to the ability of compounds to combat pathogens that have already infected a plant (Mueller and Robertson 2008). However, if the pathogen successfully colonizes the plant prior to curative compound treatment, its population may rapidly increase and spread throughout all plant tissues, potentially limiting the effectiveness of the compounds (Mueller and Robertson 2008). The formulation based on the ethyl acetate extract of JCK-8055, which contained aureothricin and thiolutin, successfully prevented the severity of apple fire blight. Thus, these two compounds can inhibit the disease development even after the introduction of bacteria, as they can be absorbed into the plant and distributed throughout its tissues, including the roots, stem, and leaves. Controlling targets by translocating into treated plants is a characteristic

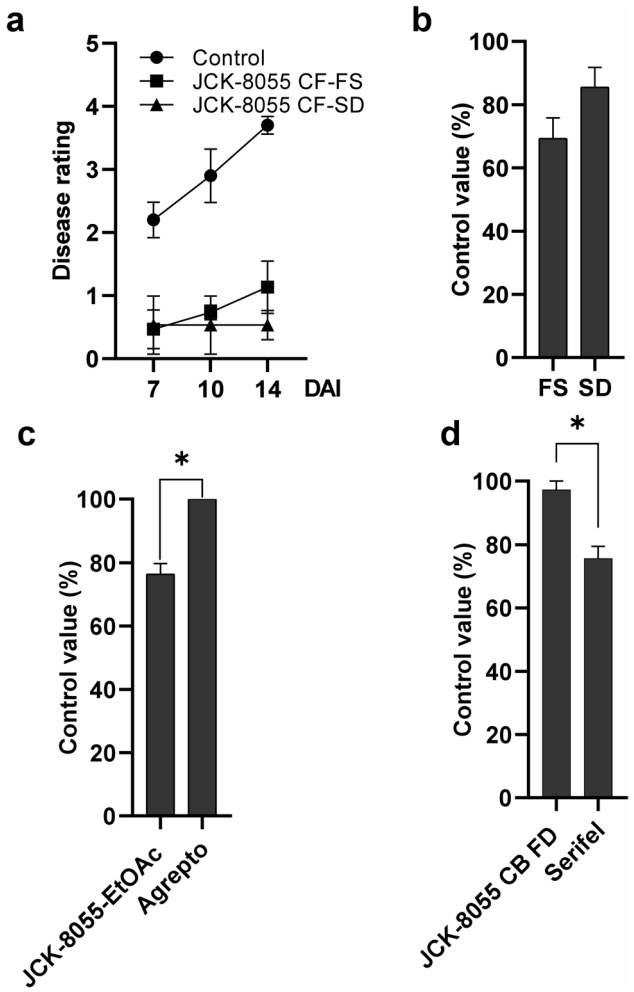

Fig. 7 Disease control efficacy of JCK-8055 against apple fire blight caused by *E. amylovora* TS3128. **a** The disease development of treatments is shown by the disease rating on M9 apple plants inoculated with *E. amylovora* TS3128. **b** Pretreatment of JCK-8055 via foliar spraying and soil drenching against apple fire blight. Disease control efficacy of JCK-8055 formulation against apple fire blight caused by *E. amylovora* TS3128 by **c** EtOAc WP10-wettable powder formulation with 10% ethyl acetate partition and **d** CB FD WP20-wettable powder formulation with 20% freeze-dried culture broth. FS, foliar spraying; SD, soil drenching. The values are presented as the mean ± standard error of five runs, with two replicates each. * ($P < 0.05$) represent significant differences

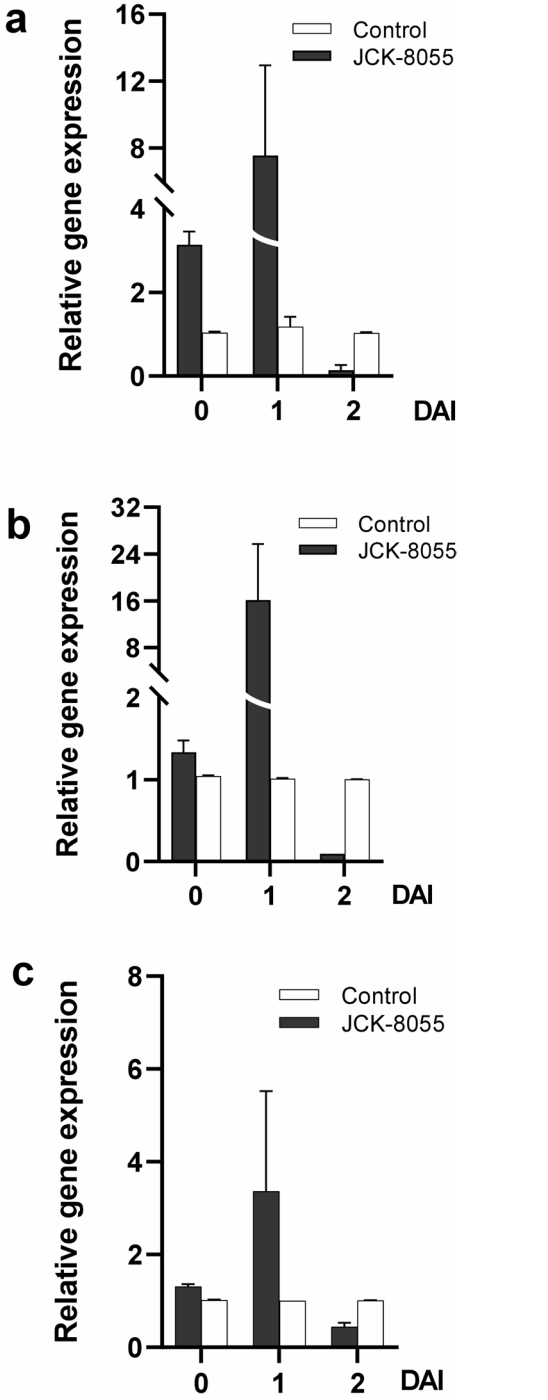

Fig. 8 Effect of the *Streptomyces* sp. JCK-8055 culture filtrate on the gene expression levels of apple plants before and after inoculation with *E. amylovora* TS3128. The expressions of the **a** *PR1*, **b** *PR2*, and **c** *PR3* genes were analyzed from plants sprayed with 0.5% TSB (untreated) or treated with the 500-fold-diluted culture filtrate at different times, including 0, 1, and 2 DAI. The values are presented as the mean ± standard deviation ($n = 3$) of three runs, with three replicates each. TSB, tryptic soy broth; CF_500 Fold, 500-fold-diluted; DAI, days after inoculation

of a systemic bactericide (Kumar et al. 2019). To the best of our knowledge, there are a few systemic bactericides, with synthesis compounds as the main active ingredients, especially copper-based products (Pscheidt and Ocamb 2022). Therefore, JCK-8055 could be a potential candidate to develop as a bio-systemic bactericide. The secondary metabolite production of *Streptomyces* can be influenced by various factors, such as nutrient availability, incubation

duration, and temperature. However, no prior research has been conducted to optimize the medium for aureothricin and thiolutin production by *Streptomyces*. Thus, we conducted an experiment to identify the optimal medium for enhancing the production of these compounds. When JCK-8055 was cultivated in TSB medium, both compounds were produced at concentrations of 24.33 µg/mL for aureothricin and 17.20 µg/mL thiolutin after 7 days of incubation at 28 °C. Even though the two compounds show antifungal activity, 10% culture filtrate of JCK-8055 did not inhibit the mycelial growth of various phytopathogenic fungi, but the oomycetes *P. infestans*. This may be due the low concentrations of the two compounds present in 10% TSB culture filtrate of JCK-8055; their concentrations were 2.43 µg/mL for aureothricin and 1.72 µg/mL for thiolutin, which are much lower than MIC values of the two compounds required for blocking selected fungi (Table 2). In case of *P. infestans*, the MIC values were 4.16 µg/mL for aureothricin and 2.60 µg/mL for thiolutin. The mycelial growth of *P. infestans* was completely inhibited by treatment with 5% of TSB culture filtrate of JCK-8055. This may be due to the synergistic activity of the two compounds present in 5% culture filtrate or by presence of other antibiotic metabolites in the culture filtrate of JCK-8055. In contrast, even though the MIC values of the two compounds against the fungus *Sclerotinia homeocarpa* were very low, namely, 3.12 µg/mL for aureothricin and 1.04 for thiolutin, the mycelial growth of *S. homeocarpa* was not completely inhibited by treatment of 10% of culture filtrate of JCK-8055. This may be due to the presence of antagonistic metabolites in the culture filtrate of JCK-8055.

Hydrolytic and carbohydrate-binding enzymes, such as cellulases, xylanases, pectinases, chitinases, amylases, proteases, and lectins, play a vital role in biocontrol against pathogens. They are essential in colonizing plant surfaces and inducing plant resistance (Carro and Menéndez 2020; Chernin and Chet 2002). The *Streptomyces* spp. are able to produce hydrolytic enzymes, which enable them to effectively compete in the rhizosphere environment and interact with the cell walls of pathogens (Jadhav et al. 2017). These enzymes facilitate the hydrolysis of the cell wall, proteins, and DNA of the pathogen. The production of hydrolytic enzymes, including cellulase, gelatinase, and protease, by JCK-8055 may be associated with the ability of JCK-8055 to colonize plants and inhibit the growth of the bacteria *R. solanacearum* and *E. amylovora*.

The foliar application of the *Streptomyces* spp. culture filtrate can effectively control various plant diseases, such as potato-virus Y, tomato bacterial wilt, cucumber *Fusarium* wilt, and tomato *Fusarium* wilt (Abbasi et al. 2019; Le et al. 2021; Nasr-Eldin et al. 2019). In line with these studies, we found that the treatment with the 500-fold dilutions of the JCK-8055 fermentation supernatant suppressed the growth of tomato bacterial wilt by soil drenching up to 100%. Furthermore, regardless of the application method (foliar spraying or soil drenching), the JCK-8055 fermentation supernatant exhibited excellent disease control efficacy against apple fire blight, even at a very low concentration (500-fold dilution). From these results, we hypothesized that *Streptomyces* sp. JCK-8055 could not only suppress pathogens but also enhance defense resistance in plants. This hypothesis was supported by GUS expression in transgenic *Arabidopsis* (*PR1pro::GUS* gene) plants treated with JCK-8055 and the increased transcriptional levels of defense-related genes (*PR1*, *PR2*, and *PR3*).

Plants exhibit specific defense responses depending on the type of attacker encountered (Spoel et al. 2007). These responses encompass systemic acquired resistance (SAR) and induced systemic resistance (ISR), which are mechanisms by which plants activate defense responses not only at the site of induction but also in other locations throughout the plant (Conrath et al. 2006). SAR is dependent on the signaling molecule SA and is induced by pathogens interacting with leaves, whereas ISR is reliant on JA and triggered by beneficial microbes interacting with the roots (Vlot et al. 2021). Both are linked to the protein buildup involved in pathogenesis, which is hypothesized to be a factor in the resistance. *PR1* and *PR2* transcripts are markers for SA signaling and are related to SAR (Fan et al. 2009). Similar to the SA positive control, the fermentation supernatant of JCK-8055 induced the expression of GUS in transgenic *Arabidopsis* plants. This study is the first report to investigate the expression of the *PR* gene in response to *E. amylovora* using *Streptomyces* as a biocontrol agent. One day after inoculation of *E. amylovora* TS3128, the JCK-8055-treated plants exhibited the transcriptional upregulation of *PR1*, *PR2*, and *PR3*. Since *PR1* and *PR2* are the critical components of the SA pathway, *PR3* plays an essential role in the JA pathway (Thomma et al. 1998). Therefore, JCK-8055 may enhance systemic resistance induction by activating the SA and JA signaling pathways in apple trees. During the infection of *E. amylovora*, the expression of *PR1*, *PR2*, and *PR3* was upregulated when the apple seedling was treated with JCK-8055. This result suggested that *Streptomyces* sp. JCK-8055 may produce induced resistance (IR) elicitors by a combination of SA- and JA-related signaling pathways. PR proteins are found in all plant parts, including the leaves, stems, roots, and flowers (Van Loon and Van Strien 1999). Several PR protein families, such as β-1,3-endoglucanases (PR-2) and endochitinases (PR-3), are involved in plant defense against fungal and/or oomycete pathogens (Ghag et al. 2012; Van Loon et al. 2006). Overexpression of the *PR* genes significantly boosted the defense response in plants against a broad range of pathogens (Ali et al. 2018). In addition, these genes play a crucial role in developing the hypersensitive response, a programmed cell death, that occurs when the plant recognizes harmful non-self agents (Salguero-Linares

et al. 2022). Further investigation is needed to determine the full potential of JCK-8055 in enhancing plant defense mechanisms. It should be noted that once *E. amylovora* penetrates the host and spreads into the host xylem or cortical parenchyma, external control methods become ineffective (Koczan et al. 2009). However, *Streptomyces* sp. JCK-8055 not only directly antagonizes the pathogen but also interferes with plant defense systems. Thus, *Streptomyces* sp. JCK-8055 has a high potential to be used as a biocontrol agent to manage fire blight.

Auxins, such as indole-3-acetic acid (IAA), 4-chloroindole-3-acetic acid (4-CI-IAA), phenylacetic acid (PAA), and indole-3-butyric acid (IBA), play a crucial role in the growth and development of plants by controlling many developmental processes, including gametogenesis, embryogenesis, seedling growth, root formation, vascular patterning, and flower development (Zhao 2010). In addition, auxins directly and indirectly impact the regulation of plant pathogen resistance responses (Kieffer et al. 2010). For example, the elevated IAA level causes plant cell wall expansion and stomatal opening (Fu and Wang 2011). Auxins facilitate the survival of auxin-secreting bacteria themselves and their effective colonization of leaves and roots, which enables them to hinder other phytopathogenic infections and enhances plant health (Tzipilevich et al. 2021; Zhang et al. 2019). In addition, bacteria that produce auxins participate in a reciprocal interaction with the plant immune system by SAR- and ISR-influenced phytohormone crosstalk, thus facilitating their integration with the plant and enhancing their competitiveness against other bacteria (Tzipilevich et al. 2021; Vlot et al. 2021). For example, numerous research studies have consistently demonstrated the critical role of actinobacteria in enhancing plant growth by increasing nutrient availability in the soil and plant growth hormones, particularly IAA (Lin and Xu 2013). In addition, *Streptomyces* sp. JCK-8055 can produce IAA, suggesting it is a promising candidate for plant growth promotion. Moreover, once the host recognizes the pathogens trying to suppress its immune system, a hypersensitive response is launched (Heath 2000) where the host intentionally destroys the infected tissues to limit further pathogen colonization (Balint-Kurti 2019). For example, infected leaves can be shed at the abscission zone to eliminate 100% of the bacteria from their body (Patharkar et al. 2017). Our study observed leaf shed in the abscission zone of apple leaves in the in vivo experiment of JCK-8055 CB-FD WP20, suggesting that JCK-8055 could cause a hypersensitive response. Jin et al. (2015) reported that auxin is a plausible long-range signal regulating abscission, and the balance of auxin levels between leaves and branches is critical to determining whether the leaf is shed. Taken together, IAA produced by JCK-8055 may contribute to shedding apple tree leaves in the abscission zone by the hypersensitive response against tomato bacterial wilt. Further research is necessary to clarify the mode of action of the two antibacterial metabolites, identify the active metabolites responsible for plant resistance, decipher the potential mode of action and molecules involved in the bacterial-plant interaction, and assess the efficacy of these metabolites in controlling various plant diseases in the field.

In conclusion, *Streptomyces* sp. JCK-8055 has demonstrated its effectiveness as a plant protectant against bacterial diseases, particularly apple fire blight, through two main mechanisms: (1) it produces two systemic compounds, aureothricin, and thiolutin and (2) JCK-8055 triggers plant immunity by producing hydrolytic enzymes, IAA, and upregulating plant *PR* genes. *Streptomyces* sp. JCK-8055 could be developed as a high potential bio-bactericide. However, more research is required prior to commercialization.

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Author contribution The authors LTTN and JCK designed the study. LTTN, VVL, and ARP designed and performed experiments and analyzed the data. LTTN, IH, and VVL performed the RNA isolation and identified the *Streptomyces* species. ^{1}H-NMR and LC-MS/MS analyses were done by LTTN and JCK. LTTN, ARP, and VVL wrote, and JCK revised the manuscript. All authors read and approved the final version of this manuscript.

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Data availability The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

Declarations

Ethical approval This article does not contain studies conducted on human participants or animals by any of the authors.

Conflict of interest The authors declare no competing interests.

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