Diffusible and Volatile Antifungal Compounds Produced by *Pseudomonas chlororaphis* subsp. *aurantiaca* ST-TJ4 against Various Phytopathogenic Fungi

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Original article

Abstract

Plant growth-promoting rhizobacteria can potentially be used as an alternative strategy to control plant disease. In this study, strain ST-TJ4 isolated from the rhizosphere soil of a healthy poplar was found to have strong antifungal activity against 11 phytopathogenic fungi in agriculture and forestry. Strain ST-TJ4 was identified as *Pseudomonas chlororaphis* subsp. *aurantiaca* based on 16S rDNA sequences. The bacterium can produce siderophores, cellulase, and protease, and has genes involved in the synthesis of phenazine, 1-phenazinecarboxylic acid, pyrrolnitrin, and hydrogen cyanide. Moreover, the volatile compounds released by strain ST-TJ4 can inhibit the mycelial growth of plant pathogenic fungi more than diffusible substances can. Based on volatile compound profiles of strain ST-TJ4 obtained from headspace collection and GC-MS/MS analysis, 1-undecene was identified. In summary, the results suggested that *P. chlororaphis* subsp. *aurantiaca* ST-TJ4 can be used as a biocontrol agent for various plant diseases caused by phytopathogenic fungi.

1. Introduction

Severe crop loss remains inevitable due to plant diseases, particularly those caused by pathogenic fungi, which are responsible for an estimated 70–80% of plant diseases (Berendsen et al., 2012). Phytopathogenic fungi reduce both crop yield and quality. They are major restraints to sustainable agriculture production, especially in intensive cropping systems (Yang et al., 2019). Synthetic fungicides have been used extensively to control diseases caused by these pathogenic agents (Fletcher et al., 2006). However, these chemicals may lead to toxic residues in treated products (Davies et al., 1997; Wang et al., 1999). Synthetic pesticides can also pollute the environment due to their slow biodegradation, and can induce resistance or reduce the susceptibility of pathogenic fungi. Furthermore, the species and numbers of plant fungal pathogens in the rhizosphere change with environmental conditions and evolution, which increases the difficulty of controlling plant diseases (Pieterse et al., 2016). Facing the severe threat to global crop security caused by plant diseases, it is important to develop environmentally friendly and highly effective fungicides against plant pathogens.

Compared with application of synthetic chemical fungicides, the use of microorganisms and their metabolites is a promising and environmentally friendly alternative for the prevention and control of plant diseases (Sahni et al., 2008). Biological control factors such as Bacillus, Pseudomonas, Burkholderia, and Paenibacillus spp. play important roles in inhibiting pathogens (Li et al., 2017; Fan et al., 2018; Zhang et al., 2019; Wang et al., 2019). Bacillus species can produce a wide variety of antimicrobial compounds with different chemical structures, including lipopeptides, polyketides and bacteriocins (Olfa et al., 2015). The siderophore-mediated competition for iron gives beneficial microbes a competitive advantage in suppressing the proliferation and root colonization of plant pathogens (Maindad et al., 2014; Kong et al., 2019).

In addition, microbial volatile organic compounds (VOCs) have attracted more attention because they can spread over long distances, mediating indirect contact interactions between organisms. Low

concentrations of VOCs can be sensed, so they can directly inhibit the growth of pathogenic fungi and induce systemic resistance in plants (Cordero et al., 2014; Velivelli et al., 2015). The VOCs produced by Bacillus can inhibit mycelial growth of Alternaria solani and Botrytis cinerea, which cause early blight and grey mold, respectively (Gao et al., 2017). They can also control Ceratocystis fimbriata in postharvest sweet potatoes (Zhang et al., 2010). It is suggested that the application of microbial volatiles has great potential in plant diseases.

Current research regarding biocontrol bacteria is mainly focused on crop applications and rarely considers forest diseases. In preliminary analyses prior to this study, we screened a bacterial strain with high siderophore production from the poplar rhizosphere; however, it remains unclear whether this strain has antagonistic effects on fungi causing forest diseases or produces additional antagonistic substances. Therefore, in this study, we identified diffusible substances and VOCs produced by this strain to determine whether they may be applied as antagonistic substances. We also determined the taxonomic status of this strain via morphological identification and molecular biology. The results of this study may provide a new and effective antagonistic bacterial strain for the biological control of forest fungal diseases, and lay a foundation for molecular analyses of the bacteriostatic mechanism of this strain.

2. Materials And Methods

2.1. Bacterial and fungal strains

In this study, bacterial strain ST-TJ4 was isolated from poplar rhizosphere soil in 2018 at Tianjin, China. Strain ST-TJ4 was stored at − 80 °C in Luria−Bertani (LB) medium with 50% (v/v) glycerol for long-term use. The fungal plant pathogens examined in this study were Botryosphaeria berengeriana (causes apple ring rot); Colletotrichum tropicale (causes Ficus binnendijkii var. variegate anthrax); Cytospora chrysosperma, Fusicoccus aesculi, and Phomopsis ricinella (cause Poplar canker); Fusarium oxysporum (causes cotton wilt); Fusarium graminearum (causes wheat head blight); Phytophthora cinnamomi (causes cedar root rot); Pestalotiopsis versicolor (causes tea round spot); Rhizoctonia solani (causes pine seedling damping-off) and Sphaeropsis sapinea (causes pine shoot blight). These fungal plant pathogens were preserved in the Forest Pathology Laboratory of Nanjing Forestry University. The isolates were maintained on Potato dextrose agar (PDA) plates at 25 °C before use.

2.2. In vitro antifungal activity

Using in vitro dual-culture analysis, strain ST-TJ4 was subjected to in vitro antifungal activity assays against the above 11 phytopathogenic fungi. PDA medium was used as basal medium. A 0.6-cm-diameter plug containing mycelia was taken from 7-day-old target fungi and placed at the center of PDA dual plates. Single ST-TJ4 colonies were patched about 2.5 cm from the fungus. After 3−7 days, the width of the inhibition zone between the bacterial colony and fungal pathogen was measured. Each treatment was replicated four times. The experiment was also repeated twice.

2.3. Analysis of the antagonistic substances in vitro

Siderophore production was determined using the Chrome Azurol S (CAS) assay (Schwyn and Neilands 1987). The method proposed by Han et al. (2015) was modified as follows to examine the chitinase and protease activities of the metabolites from ST-TJ4. Colloidal chitin and skim milk powder (SMP) were added to PDA plates to final concentrations of 1 mg/mL and 3% (w/v), respectively. To examine the glucanase activity of ST-TJ4, the method described by Suryadi et al. (2014) was used, adding yeast β-glucan at a final concentration of 0.2% (w/v) to PDA plates. Individual ST-TJ4 colonies were cultured in liquid LB medium overnight at 28 °C with shaking. When the OD_{600} was 0.6, 2 μL of the culture was plated on PDA plates containing colloidal chitin, SMK, or β-glucan and placed in an incubator at 28 °C (Huang et al., 2017). Three days later, the PDA plates containing colloidal chitin and SMP were observed and evaluated for the presence of a clear zone surrounding the bacterial colonies caused by chitinase and protease degradation. The plates containing β-glucan were stained, and glucanase activity was observed using the method of Suryadi et al. (2014).

2.4. Detection of genes encoding antibiotics and HCN in ST-TJ4

Total DNA was isolated from ST-TJ4 cells by the cetyltrimethylammonium bromide (CTAB) method (Ausubel et al., 2001). Then, polymerase chain reaction (PCR) assays were used to detect the phzCD, phz, and prnC genes according to protocols described in Raaijmakers et al. (1997) and Hu et al. (2014). The hcnAB gene was detected as previously described (Svercel et al., 2007). The primers used in the experiment are shown as Table 1:

Table 1
Oligonucleotide primers used in this study

Primer	Sequence	Target	Antibiotic or related pathways	Size (bp)
pca2a	TTGCCAAGCCTCGCTCCAAC	phzCD	1-Phenazinecarboxylic acid	1150
pca3b	CCGCGTTGTTCCTCGTTCAT			
PHZ1	GGCGACATGGTCAACGG	phz	Phenazine biosynthesis	1408
PHZ2	CGGCTGGCGGCGTATTC			
PRNCF	CCACAAGCCCGGCCAGGAGC	prnC	Pyrrolnitrin	786
PRNCR	GAGAAGAGCGGGTCGATGAAGCC			
PM1	TGCGGCATGGGCGTGTGCCATTGCTGCCTGG	hcnAB	Hydrogen cyanide	570
PM2	CCGCTCTTGATCTGCAATTGCAGGCC			

2.5. Antifungal activity of VOCs of strain ST-TJ4

The method using two sealed base plates was employed to test the antifungal activity of VOCs from strain ST-TJ4. One base plate contained 20 mL LB, and the other contained 20 mL PDA. The LB medium was coated with 100 μL of ST-TJ4 suspension, and a 6-mm-diameter spot of plant pathogenic fungus was placed on the PDA plate. Next, the two base plates were sealed with Parafilm and cultured in a mold cultivation cabinet for 5–7 d at 25°C. Every experiment was repeated three times. The plant pathogenic fungus inhibition rate was calculated as Inhibition rate (%) = $(C_d - T_d) \times 100\%/C_d$, where C_d is the fungal colony diameter on the control PDA base plate, and T_d is the fungal colony diameter on the treatment PDA base plate.

2.6. Analysis of volatile organic compounds (VOCs)

Volatile organic compounds from LB liquid medium inoculated with strain ST-TJ4 were analyzed using solid phase microextraction-gas chromatography–mass spectrometry (SPME-GC-MS/MS) with a SPME fiber assembly (CAR/PDMS; Supelco, Bellefonte, USA). Strain ST-TJ4 was grown in LB liquid medium for 48 h in the dark at 28 °C on a rotary shaker at 180 rpm. To prevent VOCs from escaping, the tapered bottle was sealed with tin foil. Non-inoculated LB liquid medium served as a control. The GC (7890A gas chromatograph, Agilent Technologies, USA) was programmed at an initial temperature of 60 °C for 5 min and then gradually increased by 5 °C/min to a temperature of 325 °C, which was held for 50 s. The GC transfer line was maintained at 280 °C, and the detector temperature at 250 °C. The inlet pressure was 67 kPa; He flow, 20 mL/min; filament voltage, 70 eV; and transfer line, 280 °C. The VOCs were identified by comparing the mass spectra obtained from the ST-TJ4 sample with reference spectra in the Wiley 9th edition spectral libraries and National Institute of Standards and Technology 2014 V2.20 (NIST, USA, http://www.nist.gov).

2.7. Identification of strain ST-TJ4

The biochemical tests were completed following the standard methods in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). For molecular identification of ST-TJ4, bacterial genomic DNA was extracted and purified using the CTAB method (Ausubel et al., 2001). The 16S rRNA gene was PCR amplified using the universal primers 27f (5'AGAGTTTGATCATGGCTCAG3') and 1492R (5'TACGGYTACCTTGTTACGACTT3'). Sequence homology searches were performed using the online blast search engine in EzTaxon-eDatabase–EzBioCloud (http://www.ezbiocloud.net/eztaxon). The sequences were aligned and a phylogenetic tree constructed using Mega 7.0 Beta (Tamura, et al., 2013). A tree was obtained using the neighbor-joining method based on the Tamura 3-parameter model with the 'gaps complete deletion' option, and the tree was drawn with branch lengths measured in number of substitutions per site. The interior-branch test using 1000 bootstrap replications was used to assess the relative stability of the branches.

2.8. Statistical analysis

Statistical significance was determined using SPSS 17.0, with one-way analysis of variance (ANOVA) followed by Duncan's multiple range test.

3. Results

3.1. Antagonistic effects of diffusible substances produced by ST-TJ4

Strain ST-TJ4 inhibited the mycelial growth of all fungal pathogens tested to varying degrees. ST-TJ4 significantly inhibited the mycelial growth of B. berengeriana, F. graminearum, F. aesculi, F. oxysporum, P. versicolor, P. cinnamomi, P. ricinella and S. sapinea, but only weakly affected that of C. chrysosperma, C. tropicale and R. solani (Fig. 1).

3.2. Siderophore and cell wall degradation enzyme activity of ST-TJ4

To examine the antimicrobial substances produced by ST-TJ4, including cellulase, chitinase, protease, and glucanase, ST-TJ4 was inoculated on PDA plates containing cellulose, colloidal chitin, SMP, or β-glucan. After 3 days, an obvious hydrolysis zone was observed on the agar plates containing cellulose and SMP, but not on those containing chitin or β-glucan (Fig. 2). These results indicated that ST-TJ4 has strong siderophore, cellulase, and proteolytic activities, but no chitinolytic or glucanolytic activity.

3.3. Detection of antibiotic and HCN encoding genes in ST-TJ4

We tested strain ST-TJ4 for the presence of operons for the biosynthesis of the antibiotics phenazine-1-carboxylic acid (PCA), phenazine (PHZ), pyrrolnitrin (PRN), and hydrogen cyanide (HCN) by PCR using specific primers. A fragment of the predicted size for each of these compounds was observed from the DNA of the reference Pseudomonas strains, which produces these compounds. Fragments of the predicted sizes for PCA (~ 1150 bp), PHZ (~ 1408 bp), PRN (~ 786 bp), and HCN (~ 570 bp) were amplified from strain ST-TJ4 DNA (Fig. 3).

3.4. The antifungal spectrum of ST-TJ4 VOCs

The two-sealed-base-plates method was used to determine the antifungal spectrum of ST-TJ4 VOCs. Strain ST-TJ4 VOCs showed significant antifungal activity against 11 plant pathogenic fungi. Although the effects against P. versicolor, F. graminearum, and F oxysporum were weak, the mycelial growth and pigment secretion of the other 8 pathogens were significantly inhibited by 40.4–91.35% (Fig. 4; Table 2). Moreover, comparing the antagonistic effects of VOCs and diffusible substances, the inhibitory effect of VOCs on all pathogens exceeded those of diffusible substances, suggesting that volatile compounds predominate in biological control by ST-TJ4.

Table 2
Mycelia growth of phytopathogenic fungi inhibited by strain ST-TJ4

Target pathogens	Percent inhibition (%)	Mean ± SE
	Diffusible	Volatile
Botryosphaeria berengeriana	53.49 ± 4.8	73.76 ± 1.7
Colletotrichum tropicale	21.46 ± 2.0	86.25 ± 5.1
Cytospora chrysosperma	23.18 ± 6.9	82.79 ± 0.5
Fusarium graminearum	28.04 ± 8.4	55.62 ± 7.1
Fusarium oxysporum	41.11 ± 5.3	40.4 ± 5.5
Fusicoccus aesculi	35.98 ± 9.9	72.95 ± 13.38
Pestalotiopsis versicolor	49 ± 4.9	62.31 ± 15.6
Phomopsis ricinella	59.91 ± 3.9	73.03 ± 7.4
Phytophthora cinnamomi	50.58 ± 5.2	91.35 ± 1.1
Rhizoctonia solani	19.87 ± 4.4	90.63 ± 0.4
Sphaeropsis sapinea	56.73 ± 2.6	71.97 ± 3.6

3.5. GC-MS/MS analysis of VOCs produced by ST-TJ4

Volatiles from strain ST-TJ4 were collected in a SPME syringe and analyzed with a GC-MS/MS system. The same volatiles as in the LB medium and substances with relative contents less than 0.5% were filtered out. This revealed very clear separation between the control and strain ST-TJ4, as indicated in Fig. 5. Five different VOCs emitted by strain ST-TJ4 that were absent in the control were identified. The most abundant volatile produced by strain ST-TJ4 was 1-undecene, which had a large peak (75.97%) at a retention time (RT) of 8.65 minutes (Table 3). The other compounds were l-Ala-l-Ala-l-Ala (1.42%), octamethylcyclotetrasiloxane (1.14%), 4-hydroxybenzoic acid (1.06%), and phosphonoacetic acid (0.54%).

Table 3
GC-MS/MS volatile profile of strain ST-TJ4

Retention time (min)	Relative peak area (%)	CAS#	Compound
2.04	1.42	5874-90-8	l-Ala-l-Ala-l-Ala
6.85	1.14	541-05-9	octamethylcyclotetrasiloxane
8.65	75.97	821-95-4	1-undecene
8.95	1.06	2078-13-9	4-hydroxybenzoic acid
12.04	0.54	53044-27-2	phosphonoacetic acid

3.6. Identification of strain ST-TJ4

Colonies of ST-TJ4 on LB plates appeared thin, flat, orange, opaque, round with smooth edges, and approximately 1.2–3 mm in diameter (Fig. 6a). The oxygen demand test showed that ST-TJ4 is an aerobic bacterium. As shown in the picture, it grows well outside the cover glass, but hardly grows under the cover glass (Fig. 6b). Gram staining showed that ST-TJ4 is a Gram-negative bacterium (Fig. 6c). The phylogenetic analysis showed that the 16S rRNA nucleotide sequence of strain ST-TJ4 shared high similarity (89%) with the reference species Pseudomonas chlororaphis subsp. aurantiaca (Fig. 7). Based on the morphological characteristics and phylogenetic analysis, strain ST-TJ4 was identified as Pseudomonas chlororaphis subsp. aurantiaca.

4. Discussion

One of the biggest ecological challenges facing microbiologists and plant pathologists in the near future is the development of environmentally friendly alternatives to chemical pesticides for combating plant diseases (Roquigny et al., 2018). The use of beneficial microorganisms is one of the most promising methods for safe, rational crop-management practices (Ongena and Jacques, 2008). Thus, we attempted to isolate antagonistic bacteria with strong antifungal activity against 11 phytopathogenic fungi. We found that strain ST-TJ4 inhibited the mycelial growth of various plant pathogenic fungi. This suggested that strain ST-TJ4 has a wide fungicide spectrum and could produce antifungals. Based on the morphological characteristics and phylogenetic analysis, strain ST-TJ4 was identified as P. chlororaphis subsp. aurantiaca.

Bacteria can produce cell wall-degrading enzymes and secondary metabolites to hinder the growth of other microorganisms. Fungal cell walls play important roles during cell division and growth and in maintaining hyphal morphology and adaptation to the environment (Adams, 2004; Cabib et al., 2001). In pathogenic fungi, the ability to maintain cell wall integrity is critical for establishing diseases in the host (Jeon et al., 2008). The fungal cell wall is a matrix of three main components: chitin, glucans, and proteins (Webster and Weber, 2007; Lenardon et al., 2010; Pareek et al., 2014). The destruction of these components usually leads to changes in morphology and loss of the biological function of fungal

hyphae. In this study, ST-TJ4 had strong cellulase and protease activities on culture plates containing β-glucan and SMP, but no hydrolytic activity on the plates containing colloidal chitin. This indicates that ST-TJ4 has the potential to degrade the cellulose and proteins of fungal hyphae.

Antibiotics are toxins produced by microbes that can kill other microorganisms (Rovera et al., 2014; Chen et al., 2018). Antibiosis is one of the most studied mechanisms of biological control and a selection trait to consider when screening potential biological control agents (BCAs) (Raio et al., 2011; Filion et al., 2018). The production of pigments in a medium indicates that bacteria are producing metabolites. We performed experiments to detect biosynthetic genes implicated in the production of antifungal compounds in P. chlororaphis subsp. aurantiaca ST-TJ4. The PCR results suggested that ST-TJ4 contains genes for the biosynthesis of PCA and PRN. Phenazine type antibiotics, such as PCA, are particularly active against lower fungi (Fang et al., 2013; Sharma et al., 2018). Park et al. (2011) demonstrated that PRN produced by P. chlororaphis O6 plays a key role in the inhibition of phytopathogenic fungi such as Phytophthora infestans, which causes tomato late blight disease. Genes for the biosynthesis and production of HCN were also detected in strain ST-TJ4. HCN is involved in the biocontrol of wheat sharp eyespot disease by several Pseudomonas spp. (Jiao et al., 2013).

Volatile organic compounds produced by beneficial bacteria that contribute to inhibiting plant pathogen growth and spore germination have received considerable attention (Lim et al., 2017; Gong et al., 2019). Compared with diffusion antibiotics, VOCs can spread over long distances, and the fungistatic microenvironment around the antagonist communities expands. Therefore, microbial antagonist strains capable of producing volatile compounds with potent inhibitory activity against plant pathogens are more likely to prevent pathogenic fungi from infecting plants, kill surviving spores in the soil, and limit both the production and establishment of the disease (Gao et al., 2017). Our study analyzed the VOCs produced by strain ST-TJ4 using SPME−GC-MS/MS and detected five compounds. Hunziker et al. (2015) and Guevara-Avendaño et al. (2019) reported that 1-undecene, which has strong antifungal activity, was the main active compound released by Pseudomonas fluorescens. Similarly, in our analysis, 1-undecene was the most abundant volatile in strain ST-TJ4. The antifungal activity of 1-undecene against R. solani AG-1(IA), which causes banded leaf and sheath blight (BLSB) of maize, has been reported (Tagele et al., 2019). The volatile methyl siloxane octamethylcyclotetrasiloxane (D4) has been reported to have antibacterial and antifungal activity (Lin et al., 2014) and was found in the present study. To our knowledge, this is the first report on the emission of 1-undecene and octamethylcyclotetrasiloxane by P. chlororaphis subsp. aurantiaca, which has a broad bacteriostatic spectrum.

Interestingly, the diffusible substances produced by ST-TJ4 showed weak antagonistic effects against C. chrysosperma, R. solani, and C. tropicale, whereas its volatiles almost completely inhibited the growth of these three pathogenic fungi. Because we used LB medium to culture bacteria in the two-sealed-base-plates method, while strain ST-TJ4 was streaked onto PDA plates in the dual-culture experiment. It is possible that the inoculation strategy led to different antagonistic effects; that is, the inhibition rate of spreading small cells may not have been as great as that of the spread cells exposed to larger surfaces of the medium, which would allow the single cells to obtain more nutrients (Rajer et al., 2017). The results

of Valentina et al. (2017) support our speculation. Undoubtedly, it is beneficial for the bacteria to proliferate quickly. Only when a variety of biocontrol mechanisms are combined and complement each other can microorganisms play the greatest role in biological control.

To conclude, our results suggest that P. chlororaphis subsp. aurantiaca ST-TJ4 is a good biocontrol agent candidate, although it is unclear how effective this antagonist would be under field conditions. Diffusible and volatile compounds produced by ST-TJ4 has the potential to be used in agriculture and forestry as direct contact biofungicides and biofumigants.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All the data and materials have been provided in main manuscript.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' contributions

Weiliang Kong completed the data analysis and the first drafat of the paper; Weiliang Kong and Pusheng Li were the finishers of the experimental research; Tianyu Wu participated in the experimental result analysis; Xiaorui Sun directed the experimental design and result analysis; Xiaoqin Wu directed experimental design, data analysis, paper writing and revision. All the authors read and agree on the final text.

Acknowledgements

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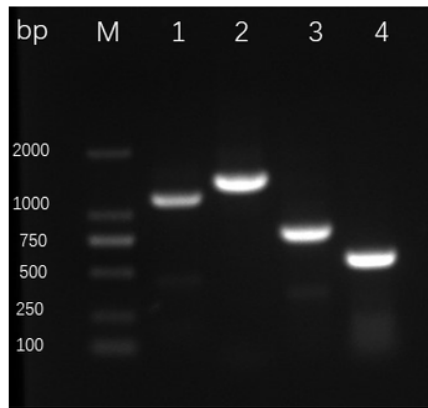
Figures

Figure 1

In vitro antifungal activity of ST-TJ4 against various phytopathogenic fungi in dual-culture assays.
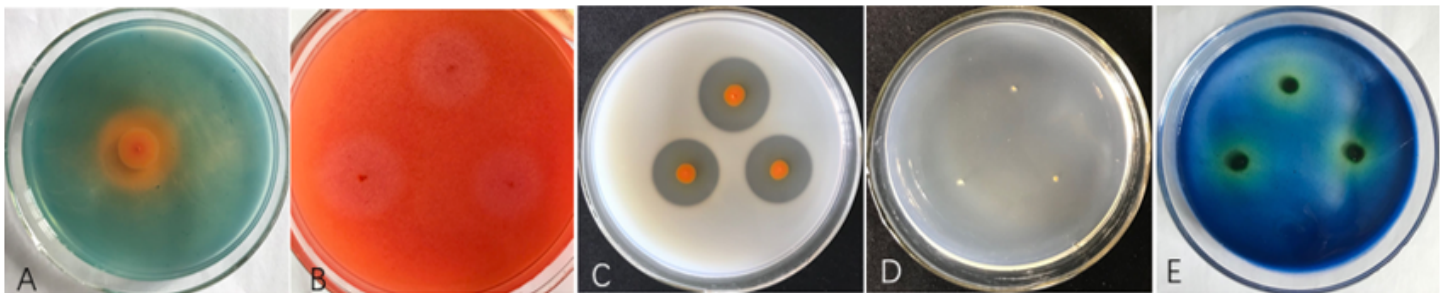
Figure 2

Fungal cell-wall-degrading enzymes produced by ST-TJ4 cells. (A) Siderophores determined using CAS agar plates; (B) cellulase activity determined using carboxyl methyl cellulose (CMC) agar plates; (C) protease activity determined using SMP agar plates; (D) chitinase activity determined using colloidal chitin agar plates; and (E) glucanase activity determined using Pachyman solid medium supplemented with 6% aniline blue.
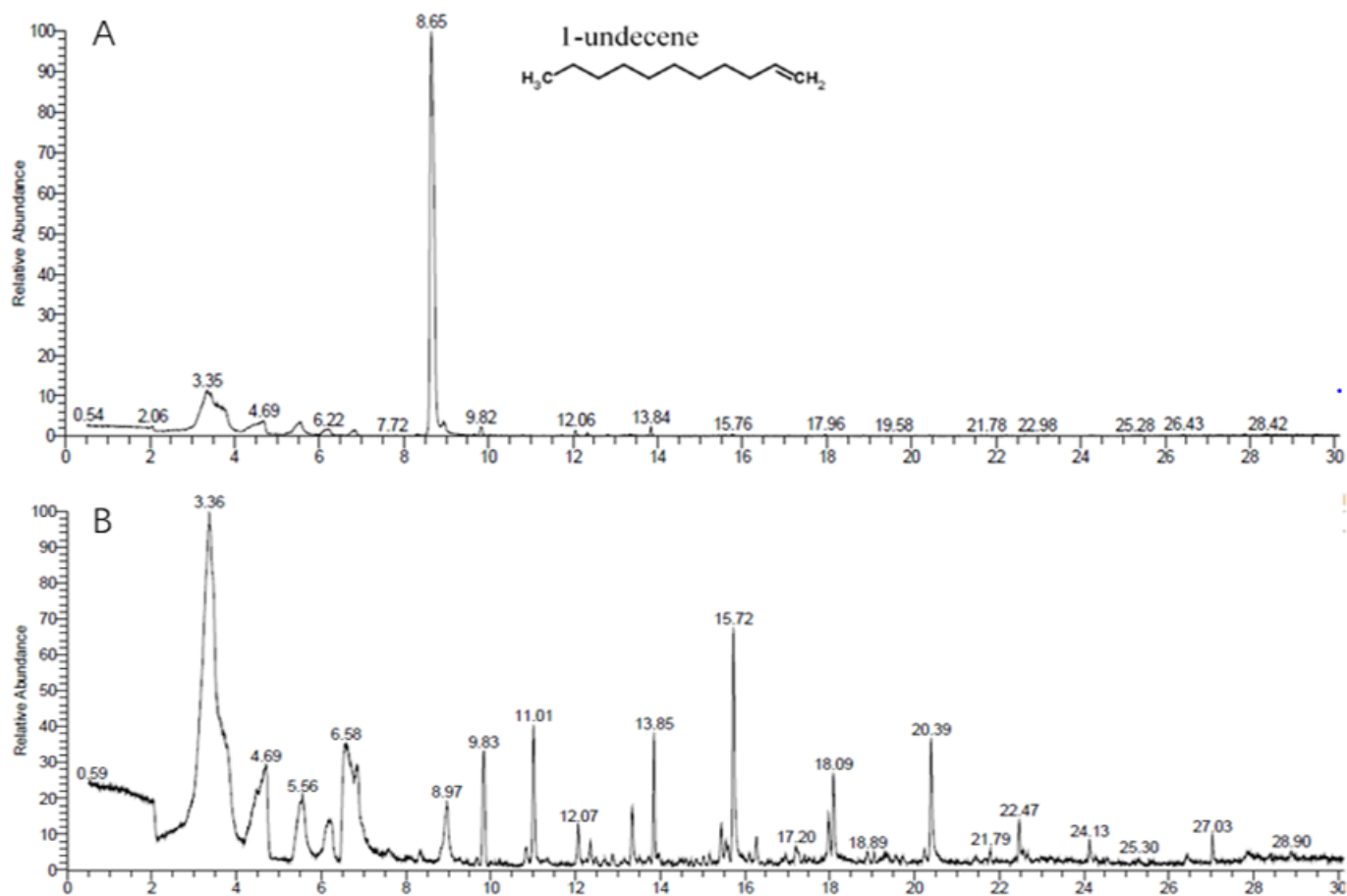
Figure 3

Detection of antibiotic genes in ST-TJ4 by PCR amplification (lane M, DNA marker; 1, PCA; 2, PHZ; 3, PRN; 4, PM)
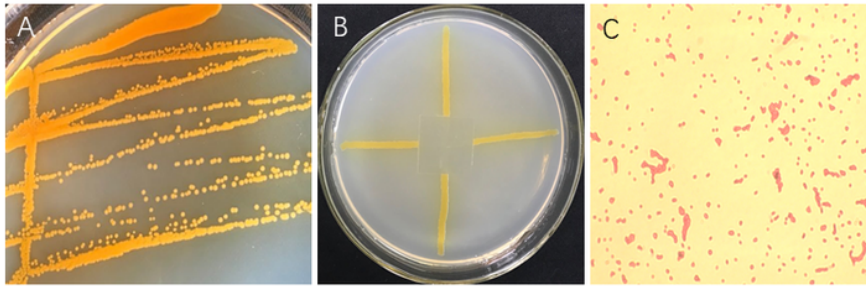

Figure 4

The antifungal spectrum of strain ST-TJ4 VOCs. The right side is treated with VOCs, and the left side is untreated.
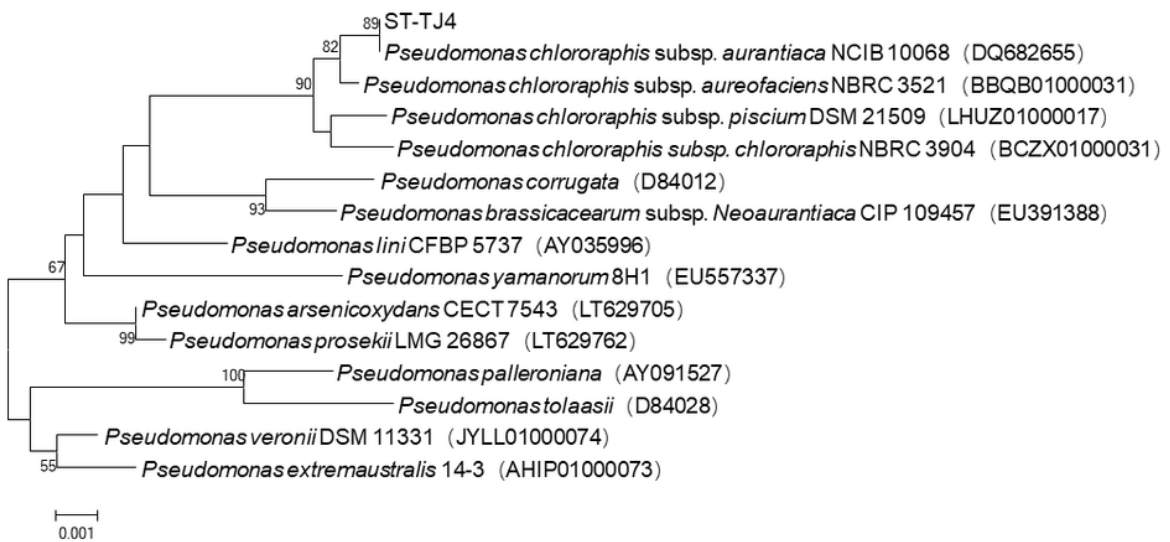
Figure 5

Chromatographic profiles of the VOCs of (A) strain ST-TJ4 incubated for 48 h in LB medium and (B) uninoculated LB medium.

Figure 6

Morphological identification: colony morphology (a), oxygen demand test results (b), and Gram staining (c) of Pseudomonas chlororaphis subsp. aurantiaca strain ST-TJ4.

Figure 7

Phylogenetic tree derived from the 16S rDNA sequence of Pseudomonas chlororaphis subsp. aurantiaca strain ST-TJ4. The tree was constructed using the neighbor-joining method with 1000 bootstrap resamplings using MEGA 7.0.