



Antifungal spectrum characterization and identification of strong volatile organic compounds produced by *Bacillus pumilus* TM-R



Toshiyuki Morita^{a,b}, Issei Tanaka^a, Noriko Ryuda^a, Masao Ikari^c, Daisuke Ueno^{a,b}, Takashi Someya^{a,b,*}

^a Department of Environmental Biosciences, Faculty of Agriculture, Saga University, Japan

^b The United Graduate School of Agricultural Sciences, Kagoshima University, Japan

^c T.M Enterprise Ltd., Japan

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ABSTRACT

To obtain biocontrol agents for suppression of food-deteriorating fungi during storage of agricultural products, bacteria producing volatile organic compounds (VOCs) with strong antifungal activity were screened and isolated from various environmental samples. Among 136 bacterial isolates, strain TM-R showed the strongest and broadest antifungal activity. Based on physiological and genetical characterization, the bacterium was identified as *Bacillus pumilus*. The effects of VOCs produced by the bacterium, which was grown on four types of agar media (nutrient, Trypto-Soya, Luria–Bertani, and TM Enterprise), were examined against six species of fungi (*Alternaria alternata*, *Aspergillus niger*, *Cladosporium cladosporioides*, *Curvularia lunata*, *Fusarium oxysporum*, and *Penicillium italicum*) in both small- and large-scale tests (plate and 12-L tests, respectively). In the plate test, the bacterium markedly suppressed the mycelial growth of five fungi (*Alternaria alternata*, *Cladosporium cladosporioides*, *Curvularia lunata*, *F. oxysporum*, and *P. italicum*) but promoted the growth of *Aspergillus niger*. In the 12-L test, the degree of growth inhibition decreased; however, the bacterium grown on TMEA still exhibited the strongest inhibition, especially against *P. italicum* (growth inhibition rate of 93%). Surprisingly, the growth of *Aspergillus niger* was promoted even more strongly (−36%) by the bacterium on TMEA than in the plate test (−9%). Twenty-two of 32 VOCs detected by GC-MS were identified using three databases (NIST 2011, AromaOffice, and AroChemBase). The species and concentration of detected VOCs differed greatly among growth media. To identify causative antifungal VOCs, we estimated the correlation between growth inhibition of *P. italicum* by the bacterium grown on each of the four media vs. the relative abundance of individual VOCs. As a result, four VOCs (methyl isobutyl ketone, ethanol, 5-methyl-2-heptanone, and S-(−)-2-methylbutylamine) were determined to be the predominant antifungal VOCs. To the best of our knowledge, this study is the first to specify causative antifungal VOCs using such an approach.

1. Introduction

Plant pathogenic fungi are one of the main causes of agricultural losses during cultivation and storage. Although chemical fungicides have been used in pre- and/or post-harvest treatments to control plant pathogens for more than seven decades, their application has gradually declined because of concerns regarding deleterious effects on human health and the environment (Usall et al., 2016) as well as the emergence of resistant fungal strains (Droby et al., 2016).

As an alternative measure, biological control using antagonistic microorganisms against plant pathogens has been a focus of interest

(Fridlender et al., 1993; Hassan et al., 2017; Larkin and Fravel, 1998). More recently, microorganisms that produce antifungal volatile organic compounds (VOCs) have received much attention. VOCs are low-molecular-weight organic compounds that easily vaporize and diffuse at ordinary temperatures and pressures. Many microorganisms have been reported to generate VOCs with suppressive effects on fungi. These microorganisms include *Bacillus* spp. (Arrebola et al., 2010; Chaurasia et al., 2005; Liu et al., 2008), *Streptomyces* spp. (Li et al., 2010; Wang et al., 2013), various other bacteria (Garbeva et al., 2014), and the fungus *Muscodor albus* (Strobel et al., 2001). VOC-producing microorganisms have also been exploited for use as biofumigants of stored

* Corresponding author.

E-mail address: someyat@cc.saga-u.ac.jp (T. Someya).

agricultural products (Mercier and Smilanick, 2005).

Some VOCs, such as dimethyl disulfide, dimethyl trisulfide, and acetoin, have been determined to be the responsible antifungal compounds (Arrebola et al., 2010; Li et al., 2010; Tyc et al., 2015), but reports of successful identification of such causative VOCs are limited. This scarcity is probably because most bacterial VOCs are present in low concentrations (Elkahoui et al., 2014) and collectively have complex compositions; in addition, their concentrations and relative abundances are influenced by many factors, such as the type of substrate, cultivation conditions, and physiological states of microorganisms (Tyc et al., 2015).

Taking into account our ultimate goal—the application of VOC-producing bacteria for biofumigation—the objectives of this study were fourfold: (i) to isolate and identify a novel bacterium producing strong antifungal VOCs from environmental samples; (ii) to reveal the spectrum of antifungal activity of this bacterium grown on various culture media against various species of fungi; (iii) to identify VOCs produced by the bacterium when grown on various media; and (iv) to identify the causative VOCs by closely examining the correlation between antifungal activity and individual concentrations of VOCs.

2. Materials and methods

2.1. Screening for bacteria producing VOCs with strong antifungal activity

To screen for bacteria producing VOCs with strong antifungal activity, bacteria were isolated from various environmental samples (soil, manure, and grease traps) and subjected to dual plate tests as described below. Strain TM-R, first isolated as a lipolytic bacterium from a restaurant grease trap, was selected for further study. The bacterium was incubated on nutrient agar (NA; 5.0 g L⁻¹ meat extract, 10.0 g L⁻¹ peptone, 5.0 g L⁻¹ sodium chloride, and 15.0 g L⁻¹ agar, pH 7.0) at 30 °C for 24 h before use.

2.2. Identification of *Bacillus* TM-R

Bacillus TM-R was subjected to Gram staining, and its cell morphology and spore formation were observed under a light microscope. Oxidase and catalase production were examined with oxidase test paper (Nissui Pharmaceutical Co., Japan) and a 3% hydrogen peroxide solution, respectively. Hemolysin production was detected on both horse blood agar and sheep blood agar (Nissui) by observing clear zones around colonies after incubation at 37 °C for 24 h. DNase production was assessed by observation of clear zones around colonies after flooding DNA agar plates (Eiken Chemical Co., Japan) with a 3% HCl solution.

Bacillus TM-R was identified on the basis of its pattern of utilization of API50CHB substrate (bioMérieux Japan, Japan) as well as by 16S rRNA gene sequencing. A universal primer set (10F and 1541R) was used to amplify the 16S rRNA gene of the bacterium, and the sequence was obtained on a Genetic Analyzer 3130 DNA sequencer (Applied Biosystems, USA). Homology studies were performed using DDBJ BLAST (<http://blast.ddbj.nig.ac.jp/blastn>).

2.3. Test fungi

For antifungal activity tests, six fungal strains were used: *Aspergillus niger* NBRC33023, *Alternaria alternata* NBRC31188, *Cladosporium cladosporioides* NBRC4459, *Curvularia lunata* NBRC100182, *Fusarium oxysporum* NBRC30701, and *Penicillium italicum* NBRC32032. These fungi were grown on potato dextrose agar (PDA; 4.0 g L⁻¹ potato infusion, 20 g L⁻¹ dextrose, and 15.0 g L⁻¹ agar, pH 5.6) at 30 °C for 1–3 d before assays. For screening VOC-producing bacteria, *Cladosporium cladosporioides* was used as a test fungus.

2.4. Test of antifungal activity of VOCs produced by *Bacillus* TM-R

Fresh culture (200 µL) of *Bacillus* TM-R was inoculated into 1.8 mL of

each of four types of media, namely, nutrient broth (NB; 3.0 g L⁻¹ meat extract, 10.0 g L⁻¹ peptone, and 5.0 g L⁻¹ sodium chloride, pH 7.0), Trypto-Soya broth (TSB; 17.0 g L⁻¹ casein peptone, 3.0 g L⁻¹ soy peptone, 5.0 g L⁻¹ sodium chloride, 2.5 g L⁻¹ K₂HPO₄, and 2.5 g L⁻¹ glucose, pH 7.3), Luria-Bertani broth (LB; 10.0 g L⁻¹ tryptone, 5.0 g L⁻¹ yeast extract, and 5.0 g L⁻¹ sodium chloride, pH 7.0), and TM Enterprise broth (TMEB; proprietary composition), and incubated upright at 30 °C for 18 h. A 100-µL aliquot of bacterial culture grown in each of the four media was inoculated by spreading onto fresh agar medium made from the corresponding medium supplemented with 15 g L⁻¹ agar (i.e., NA, Trypto-Soya agar [TSA], Luria-Bertani agar [LBA], and TM Enterprise agar [TMEA]).

In a dual plate test, fungal cultures on PDA were excised with a sterile cork borer (7 mm diameter), and the fungal plug was placed in the center of a fresh PDA plate. A Petri dish containing one of the four types of media inoculated with *Bacillus* TM-R as described above was uncovered and positioned facing an uncovered Petri dish containing PDA with a fungal plug. These two Petri dishes were then connected with surgical tape. The dual plates were incubated at 30 °C for 5 d with the fungal-culture side on the bottom to avoid the propagation of fungal growth to the bacterial culture. Media without *Bacillus* TM-R were used as controls.

After incubation, fungal colony diameters (mm) were measured. The mycelial growth inhibition rate (%) was calculated as $100 \times [(C - 7) - [T - 7]] / (C - 7)$, where C and T are fungal growth diameters (mm) on control and treated plates, respectively.

In a 12-L test, sealable plastic containers with rubber airtight packings (outer size: 216 × 309 × 300 mm, corresponding to approximately a 12-L internal volume) were used. The inside of each container was sterilized with 99% ethanol and dried on a clean bench under UV radiation. Three PDA plates inoculated with each test fungus were attached to the inner wall of the container with adhesive tape, and a Petri dish containing one of the four types of media inoculated with *Bacillus* TM-R as above was uncapped and inserted therein. The container was tightly sealed and incubated at 30 °C for 5 d. As a control, a plate of medium without *Bacillus* TM-R was used. After incubation, the mycelial growth inhibition rate (%) was calculated as described above.

2.5. Collection of VOCs with a MonoTrap[®]

VOCs produced by *Bacillus* TM-R were collected using a MonoTrap[®] (RGC18 TD, GL Sciences, Japan), which is a graphite carbon-containing solid-phase extraction device (diameter, 2.9 mm; length, 10 mm) designed for a thermal desorption (TD) system. *Bacillus* TM-R was pre-incubated on agar plates containing one each of the four types of above-described media at 30 °C for 18 h. Each Petri dish was subsequently uncovered and placed open-side up against an uncovered Petri dish containing two MonoTrap[®] units; the two Petri dishes were then joined with surgical tape. The dual plates were incubated at 30 °C for 24 h with the MonoTrap[®] side on the bottom. To avoid excess adhesion of vapor from the medium that could interfere with gas chromatography–mass spectrometry (GC-MS) analysis, the exposure period was limited to 24 h in accordance with the MonoTrap[®] manufacturer's recommendations. In regard to the timing of sampling, an incubation period of 18 to 42 h was selected to avoid lag and early exponential growth phases, when the production of VOCs would be lower. The two MonoTrap[®] units were then removed and immediately placed in an adsorption tube (diameter, 7 mm; length, 90 mm) of a TD system (GL Sciences, Japan). As an internal standard, 10 ng of toluene-d (99.6%; Wako Pure Chemical, Japan) was spiked onto the MonoTrap[®] prior to GC-MS analysis.

2.6. VOC identification procedures

Bacterial VOCs were analyzed on a GC-MS instrument (a 6890N gas chromatograph and a 5973N mass spectrometer; Agilent, USA) equipped

Table 1
Characteristics of *Bacillus* TM-R.

Character	Reaction or state
Gram staining	+
Cell morphology	Short rod
Spore formation	+
Oxidase	-
Catalase	+
Hemolysin, horse blood	-
Hemolysin, sheep blood	-
DNase	-

with a TurboMatrix650 TD system (Perkin Elmer, USA) for desorption of VOCs from the MonoTrap[®]. VOCs were separated on a DB-5MS capillary column (60 m × 0.32 mm ID × 0.5 μm; Agilent J&W, USA). After 2 min at 50 °C, the oven temperature was increased by a rate of 5 °C min⁻¹ to 100 °C and then by a rate of 10 °C min⁻¹ to 250 °C, where it was subsequently held for 30 min. Helium was used as the carrier gas. Mass spectra were collected in full-scan mode (m/z range 30–350) with an electron impact (EI) energy at 70 eV. Chromatograms were processed using Chemstation (Agilent, USA), with the peak processing threshold set to S/N > 3. The comprehensive aroma chemical databases AromaOffice (Nishikawa Keisoku, Japan) and AroChemBase (Alpha M.O.S., France), which include names, formulas, molar masses, and retention index (RI) values, were used for the first step of chemical identification. The retention times of *n*-alkanes (C8–C33), which were injected into the TD-GC-MS system prior to the sample analysis, were used to calculate RIs for the database search. Compounds identified using the aroma chemical databases were confirmed by a mass spectral library search (NIST 2011). Identified and annotated compounds were defined as follows. Compounds with a RI match in aroma chemical databases and high hit probabilities (≥70%) in the NIST mass spectral library were considered to be identified. Compounds with matches in one database (an aroma chemical database or the NIST mass spectral library) were considered to be annotated, while those with no matches were defined as unidentified.

2.7. Screening of VOCs for antifungal activity

To identify putative antifungal volatiles, mycelial growth inhibition rates against *P. italicum* were measured by the 12-L test, as this fungal species showed the highest sensitivity to VOCs. The correlation between mycelial growth inhibition and the abundance of individual VOCs relative to the internal standard was analyzed. The relative abundance of VOCs was obtained by dividing the abundance (peak area) of each compound by that of toluene-d.

2.8. Statistical methods

To compare differences among means, Tukey's honestly significant difference (HSD) test ($P < 0.05$) was carried out using the statistical analysis program JMP8 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Screening for bacteria producing VOCs with strong antifungal activity

A dual plate test was used to screen bacterial isolates from various environmental samples (soil, manure, and grease traps) for the ability to produce VOCs showing antifungal activity. Strain TM-R was selected for further studies from 136 isolates because it exhibited the strongest antifungal activity (data not shown). Strain TM-R was a Gram-positive, rod-shaped, spore-forming, catalase-positive, and oxidase-negative bacterium. It displayed no hemolysis on either horse or sheep blood agar and no DNase production (Table 1).

Table 2
Carbohydrate utilization pattern of *Bacillus* TM-R.

Carbohydrate	Reaction
Glycerol	-
Erythritol	-
D-Arabinose	-
L-Arabinose	+
D-Ribose	+
D-Xylose	-
L-Xylose	-
D-Adonitol	-
Methyl-β D-xylopyranoside	-
D-Galactose	-
D-Glucose	+
D-Fructose	+
D-Mannose	+
L-Sorbose	-
L-Rhamnose	-
Dulcitol	-
Inositol	-
D-Mannitol	+
D-Sorbitol	-
Methyl-α D-xylopyranoside	-
Methyl-α D-glucopyranoside	-
N-Acetylglucosamine	+
Amygdalin	+
Arbutin	+
Esculin ferric citrate	+
Salicin	+
D-Cellobiose	+
D-Maltose	-
D-Lactose	-
D-Melibiose	-
D-Sucrose	+
D-Trehalose	+
Inulin	-
D-Melezitose	-
D-Raffinose	-
Starch	-
Glycogen	-
Xylitol	-
Gentiobiose	+
D-Turanose	-
D-Lyxose	-
D-Tagatose	+
D-Fucose	-
L-Fucose	-
D-Arabitol	-
L-Arabitol	-
Gluconate	-
2-Ketogluconate	-
5-Ketogluconate	-

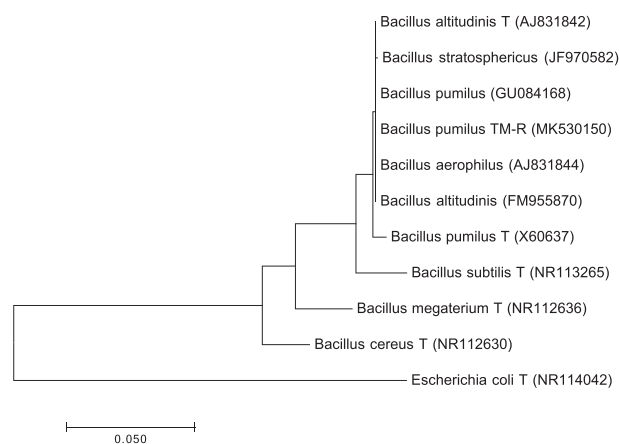


Fig. 1. Phylogenetic tree of *Bacillus* TM-R and other bacteria based on maximum-likelihood analysis of 16S rRNA gene sequences.

Table 3
Substrate utilization of *Bacillus* TM-R, *B. pumilus*^T, and *B. altitudinis*^T.

Substrate	<i>Bacillus</i> TM-R	<i>B. pumilus</i> ^T NBRC12092*	<i>B. altitudinis</i> ^T JCM13350*
Glycerol	-	-	+
D-Arabinose	-	-	+
L-Sorbose	-	-	+
Inositol	-	-	+
D-Sorbitol	-	-	+
N-Acetylglucosamine	+	-	+
D-Cellobiose	+	-	+
Inulin	-	-	+
D-Raffinose	-	+	-

* Data from Shivaji et al. (2006).

3.2. Taxonomic identification of strain TM-R

The carbohydrate utilization pattern of strain TM-R was determined using API50CHB (Table 2). A query via API Web™ revealed a 99% similarity to *Bacillus pumilus*. A comparison of 16S rRNA full-length gene sequences indicated a 100% (1,513/1,513) similarity with *B. pumilus* and *B. altitudinis*, 99.9% (1,482/1,484) with *B. altitudinis*^T JCM13350, and 99.6% (1,468/1,474) with *B. pumilus*^T NBRC12092. In a phylogenetic tree based on 16S rRNA gene sequences (Fig. 1), *Bacillus* TM-R was shown to have a very close relationship with *B. pumilus*, *B. altitudinis*, and several other species.

An examination of the carbohydrate utilization patterns of *Bacillus pumilus*^T NBRC12092 and *B. altitudinis*^T JCM13350 reported by Shivaji et al. (2006) revealed that the carbohydrate utilization pattern of *Bacillus* TM-R was much closer to that of *Bacillus pumilus*^T NBRC12092 than *B. altitudinis*^T JCM13350 (Table 3). Consequently, *Bacillus* TM-R was identified as *B. pumilus* (accession number: MK530150).

3.3. Antifungal activity of VOCs produced by *Bacillus pumilus* TM-R

The effects of VOCs produced by *B. pumilus* TM-R on six species of fungi were examined using both plate and 12-L tests and four kinds of bacterial growth media. For example, *B. pumilus* TM-R markedly suppressed the mycelial growth of *Alternaria alternata* but unexpectedly and greatly promoted that of *Aspergillus niger* (Fig. 2).

In the plate test (Fig. 3), *B. pumilus* TM-R exhibited the strongest growth inhibition against *P. italicum* (95%–100%, depending on the growth medium), strong growth inhibition against *Cladosporium clado-sporioides* and *Alternaria alternata* (82%–100% and 81%–97%, respectively), and moderate growth inhibition against *Curvularia lunata* and *F. oxysporum* (46%–86% and 36%–71%, respectively). In contrast, the growth of *Aspergillus niger* was promoted to varying degrees (–72% to –9%) depending on the medium. In regard to bacterial growth media, TMEA supported the strongest growth inhibition (71%–100%) of *B. pumilus* TM-R against all six fungi except for *Aspergillus niger*. The growth promotion of *Aspergillus niger* was strongest when the bacterium

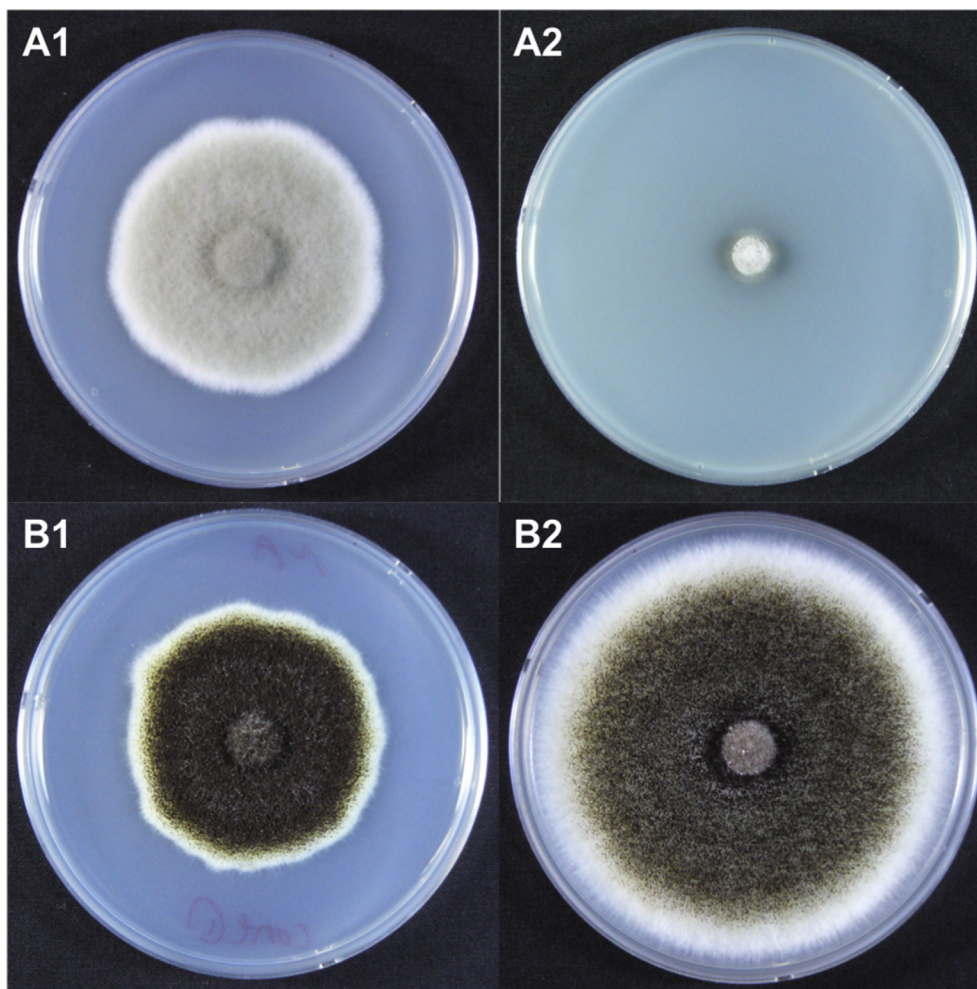


Fig. 2. The effect of volatile organic compounds emitted by *Bacillus pumilus* TM-R on the growth of fungal mycelia in a dual plate test. The fungi *Alternaria alternata* and *Aspergillus niger* were cultivated in the absence (A1 and B1, respectively) and presence (A2 and B2, respectively) of *B. pumilus* TM-R. Nutrient agar was used for the cultivation of *B. pumilus* TM-R.

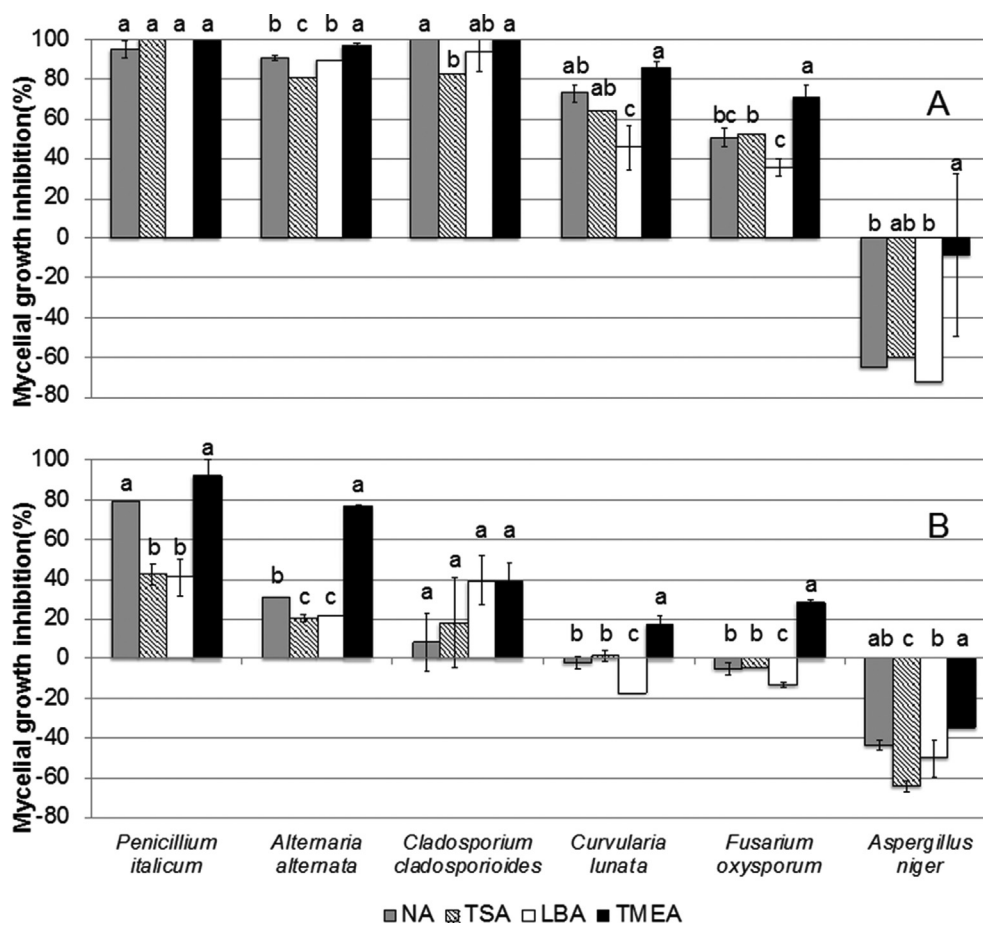


Fig. 3. The effect of volatiles produced by *Bacillus pumilus* TM-R on fungal mycelial growth. (A) Dual plate test. (B) 12-L test. Data are means of triplicate plates. Different letters indicate significant differences among treatments within each species ($P < 0.05$, Tukey's HSD test).

was grown on NA, TSA, or LBA (−60% to −72%), with only weak promotion (−9%) observed on TMEA.

Although the degree of growth inhibition in the 12-L test (Fig. 3) was less than that in the plate test, the bacterium still exhibited the strongest inhibition against all fungi (except for *Aspergillus niger*) when grown on TMEA. Growth inhibition of the bacterium on TMEA was strongest against *P. italicum* (93%), followed by *Alternaria alternata* (77%), with moderate inhibition observed against *Cladosporium cladosporioides*, *Curvularia lunata*, and *F. oxysporum* (17%–39%). As for *Aspergillus niger*, the bacterium on TMEA displayed moderate growth promotion (−36%); this increase was even higher than that (−9%) observed in the plate test, even though the concentration of VOCs would be lower because of the larger test volume.

The degree of suppression among the four media differed between the plate and 12-L tests. Fungal growth inhibition by the bacterium cultured on NA and TMEA decreased to a lesser extent, from ca. 100% in the plate test to 80% or more in the 12-L test, while that on TSA and LBA decreased markedly, from 100% to ca. 40%.

3.4. Identification of VOCs produced by *B. pumilus* TM-R

VOCs produced by *B. pumilus* TM-R that was grown on four different media were detected by GC-MS. VOCs not present in the control (Table 4) were identified using three databases (NIST library, AromaOffice, and AroChemBase). Out of 32 detected VOCs, 22 were identified. With respect to growth media, the number of identified compounds was as follows: 17 out of 24 detected on NA, 15 out of 17 on TSA, 12 out of 12 on LBA, and 17 out of 18 on TMEA. The types of detected VOCs differed greatly among growth media, with only nine VOCs detected on all four

media: methyl isobutyl ketone, ethyl 2-methylpropanoate, 3-methylbutanoic acid, methyl pentanoate, ethyl 2-methylbutanoate, 5-methyl-2-hexanone, 2-heptanone, 6-methyl-2-heptanone, and 5-methyl-2-heptanone. Three VOCs (3-methylbutanol, unidentified peak 4, and octadecane) were detected only on TMEA, the medium giving the strongest inhibition spectrum.

3.5. Identification of VOCs responsible for antifungal activity

To identify causative antifungal volatiles, a correlation analysis was performed between the rate of mycelial growth inhibition of *P. italicum* in the 12-L test and the abundance of each VOC relative to an internal standard. *Penicillium italicum* was selected because this fungus had the highest sensitivity to VOCs. As shown in Table 5, the relative abundance of eight VOCs (methyl isobutyl ketone, ethanol, 5-methyl-2-heptanone, S(−)-2-methylbutylamine, ethyl 2-methylpropanoate, 6-methyl-2-heptanone, ethyl phenylacetate, and 3-methyl-1-butanamine) had relatively high correlations with the mycelial growth inhibition rate ($R^2 > 0.7$). The four compounds with the highest correlations ($R^2 = 0.97$ – 0.99) were methyl isobutyl ketone, ethanol, 5-methyl-2-heptanone, and S(−)-2-methylbutylamine (Fig. 4).

4. Discussion

In this study, *Bacillus* TM-R from an environmental sample was selected as a strong VOC-producing bacterium and tentatively assigned as *Bacillus pumilus* or a related species. Using only 16S rRNA gene sequencing, however, *B. pumilus* cannot be differentiated from its close congeners, especially *B. altitudinis* (Liu et al., 2013). A detailed

Table 4

Identification of volatile organic compounds produced by *B. pumilus* TM-R grown on four different media.

Peak	Retention Index	Compound	Identification*	Detection in Medium			
				NA	TSA	LBA	TMEA
1	458	Ethanol	annotated	+			+
2	671	2-Pentanone	identified		+	+	+
3	681	3-Methyl-1-butanamine	annotated	+	+		+
4	701	S-(−)-2-methylbutylamine	annotated	+			+
5	709	unidentified peak 1	unidentified		+		
6	709	unidentified peak 2	unidentified	+			
7	718	unidentified peak 3	unidentified	+			
8	724	3-Methylbutanol	annotated				+
9	729	unidentified peak 4	unidentified				+
10	734	Methyl isobutyl ketone	identified	+	+	+	+
11	750	Ethyl 2-methylpropanoate	identified	+	+	+	+
12	755	Dimethyl disulfide	identified			+	
13	803	unidentified peak 5	unidentified	+			
14	814	3-Methyl-butanoic acid	annotated	+	+	+	+
15	825	Methyl pentanoate	identified	+	+	+	+
16	835	unidentified peak 6	annotated	+			
17	849	Ethyl 2-methylbutanoate	identified	+	+	+	+
18	851	Ethyl 3-methylbutanoate	annotated		+	+	+
19	852	unidentified peak 7	unidentified	+			
20	860	5-Methyl-2-hexanone	annotated	+	+	+	+
21	892	2-Heptanone	identified	+	+	+	+
22	925	2,5-Dimethylpyrazine	identified	+			
	925	2,6-Dimethylpyrazine	identified	+			
23	958	6-Methyl-2-heptanone	annotated	+	+	+	+
24	969	5-Methyl-2-heptanone	annotated	+	+	+	+
25	981	1-Octen-3-ol	identified	+	+		
26	1160	2-Decanone	identified	+	+		
27	1167	unidentified peak 8	unidentified	+	+		
28	1262	Ethyl phenylacetate	identified	+	+		+
29	1345	Indole	annotated	+			
	1345	5H-1-Pyridine	annotated	+			
30	1367	unidentified peak 9	unidentified	+			
31	1373	unidentified peak 10	unidentified	+			
32	1799	Octadecane	identified				+

* Compounds with a retention index match in aroma chemical databases (AromaOffice and/or Arochem Search) and high hit probabilities ($\geq 70\%$) in the NIST library were considered to be identified. Compounds with matches in one of the three databases were considered to be annotated, while those with no matches were designated as unidentified.

comparison of carbohydrate utilization patterns based on API50CHB suggested a closer similarity to *B. pumilus* than to *B. altitudinis*. TM-R was thus identified as *B. pumilus* on the basis of both 16S rRNA gene sequencing results and carbohydrate utilization patterns. *Bacillus pumilus* has been isolated from various environments, including soil, water, and food. Some isolates of this species have been reported to possess antifungal activity due to water-soluble metabolites or VOCs (Liu et al., 2008; Munimbazi and Bullerman, 1998), while others are plant pathogens (Peng et al., 2013) or food-poisoning bacteria (From et al., 2007). In this study, *B. pumilus* TM-R was confirmed to produce neither hemolysin nor DNase, which suggests a high probability of non-pathogenicity to humans.

Many studies have been published on the growth suppression of plant pathogenic fungi by VOCs produced by bacteria such as *Bacillus amyloliquefaciens* (Arrebola et al., 2010; Gotor-Vila et al., 2017; Yuan et al., 2012), *Bacillus subtilis* (Arrebola et al., 2010; Chaurasia et al., 2005; Fiddaman and Rossall, 1994; Liu et al., 2008), *Collimonas* spp. (Garbeva et al., 2014), *Chryseobacterium* sp. (Tyc et al., 2015), *Dyella* sp. (Tyc et al., 2015), *Janthinobacterium* sp. (Tyc et al., 2015), *Paenibacillus polymyxa* (Liu et al., 2008), *Pseudomonas* spp. (Elkhoui et al., 2014; Fernando et al., 2005; Rojas-Solís et al., 2018), *Streptomyces* spp. (Li et al., 2010; Wang et al., 2013), and *Tsukamurella* sp. (Tyc et al., 2015). Fungi sensitive to these bacteria include *Alternaria brassicae* (Liu et al., 2008), *Botrytis cinerea* (Fiddaman and Rossall, 1994; Gotor-Vila et al., 2017; Liu et al., 2008; Rojas-Solís et al., 2018), *Fusarium oxysporum* (Garbeva et al., 2014; Liu et al., 2008; Yuan et al., 2012), *Penicillium italicum* (Li et al., 2010), *Pythium ultimum* (Garbeva et al., 2014; Tyc et al., 2015), *Rhizoctonia solani* (Elkhoui et al., 2014; Fiddaman and Rossall, 1994; Garbeva

et al., 2014; Liu et al., 2008; Tyc et al., 2015), *Sclerotinia sclerotiorum* (Fernando et al., 2005; Fiddaman and Rossall, 1994; Liu et al., 2008), *Trichoderma harzianum* (Garbeva et al., 2014), and *Verticillium dahliae* (Liu et al., 2008).

Bacillus pumilus, the same species as strain TM-R, is reported to produce VOCs and suppress many kinds of fungi, including *Ascochyta citrullina*, *Alternaria brassicae*, *Alternaria solani*, *Botrytis cinerea*, *Cercospora kikuchii*, *Fusarium graminearum*, *Fusarium oxysporum*, *Phoma arachidicola*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Verticillium dahliae* (Liu et al., 2008). To the best of our knowledge, however, fungal growth promotional effects due to *B. pumilus*, such as those confirmed for our strain, have not been reported.

The antifungal activity strength of VOCs produced by bacteria has been found to change depending on the growth medium (Arrebola et al., 2010; Fiddaman and Rossall, 1994; Gotor-Vila et al., 2017), which in turn has also been found to influence VOC composition (Garbeva et al., 2014). The relationship between VOC composition and antifungal activity strength, however, has not been addressed. In this study, we demonstrated that the composition of VOCs produced by *B. pumilus* TM-R, and thus its antifungal activity, changed according to the growth medium. Four compounds (methyl isobutyl ketone, ethanol, 5-methyl-2-heptanone, and S-(−)-2-methylbutylamine) were determined to be the predominant VOCs responsible for the observed antifungal activity. This type of trial to specify causative compounds is, to our knowledge, the first such approach in the study of microbial VOCs.

In addition to the popular dual plate test, we also performed a 12-L test, the first reported application of a large-scale antifungal activity assay of bacterial VOCs. We expected that *B. pumilus* TM-R would exert

Table 5

Correlation between the rate of mycelial growth inhibition of *Penicillium italicum* and the relative abundance of each volatile organic compound.

Peak No.	Compound	R ²	Slope	y-intercept
10	Methyl isobutyl ketone	0.99	190	15
1	Ethanol	0.98	11	43
24	5-Methyl-2-heptanone	0.97	160	11
4	S(-)-2-methylbutylamine	0.97	230	42
11	Ethyl 2-methylpropanoate	0.88	79	19
23	6-Methyl-2-heptanone	0.86	64	6.3
28	Ethyl phenylacetate	0.85	110	34
3	3-Methyl-1-butanamine	0.72	100	36
17	Ethyl 2-methylbutanoate	0.59	200	30
7	unidentified peak 3	0.54	180	54
9	unidentified peak 4	0.54	240	54
32	Octadecane	0.54	22	54
21	2-Heptanone	0.49	34	49
20	5-Methyl-2-hexanone	0.46	200	27
12	Dimethyl disulfide	0.34	-190	71
5	unidentified peak 1	0.30	-280	71
25	1-Octen-3-ol	0.30	-120	71
2	2-Pentanone	0.25	53	52
14	3-Methyl-butanolic acid	0.21	-83	85
6	unidentified peak 2	0.16	120	59
8	3-Methylbutanol	0.16	120	59
13	unidentified peak 5	0.16	140	59
16	unidentified peak 6	0.16	100	59
19	unidentified peak 7	0.16	190	59
22	2,5-Dimethylpyrazine 2,6-Dimethylpyrazine	0.16	6.9	59
29	Indole 5H-1-Pyridine	0.16	91	59
30	unidentified peak 9	0.16	110	59
31	unidentified peak 10	0.16	140	59
18	Ethyl 3-methylbutanoate	0.08	79	55
15	Methyl pentanoate	0.00	-28	68
27	unidentified peak 8	0.00	14	62
26	2-Decanone	0.00	13	63

strong antifungal activity by generating amounts of VOCs in the 12-L test sufficient to affect fungi. Larger differences in the degree of antifungal activity among media as well as test fungi were observed in the 12-L test

than in the plate test, thus contributing to differences in the causative VOCs identified above. With respect to large-scale examination of antifungal activity, the potential of the fungus *Muscodor albus* as a biofumigant against *Penicillium digitatum* has been previously demonstrated in practical experiments on a storage-room scale (1.8 × 3.0 × 2.1 m; Mercier and Smilanick, 2005).

In regard to fungal growth promotion by bacterial VOCs, *Burkholderia cepacia* and *Staphylococcus epidermidis* have been reported to promote the growth of *Rhizoctonia solani* (Vespermann et al., 2007).

When TMEA was used as the growth medium for *B. pumilus* TM-R, a very weak growth-promoting effect on *Aspergillus niger* was observed in the plate test (-9% growth suppression), whereas a dramatic enhancement was obtained in the 12-L test (-40%); in other words, the growth-promoting effect increased with test volume. The volume of the container used in the 12-L test was approximately 130 times larger than that of the dual plate test (90 mL). Thus, the VOCs would be diluted accordingly. The VOCs produced by the bacterium likely included both suppressive and promotional compounds. As the volume was increased, the concentration of suppressive compounds probably decreased below a certain threshold, whereas promotional compounds may have still remained above the concentration threshold required to be effective.

Noteworthy, the VOC mixture emitted by *B. pumilus* TM-R suppressed most tested fungi but promoted *Aspergillus niger*. This result is most likely because VOCs contain both inhibitory and promoting substances and *Aspergillus niger* is tolerant to inhibitory VOCs. This conclusion is partially supported by the findings of a previous study in which *Aspergillus niger* showed no growth inhibition caused by VOCs produced by many species of bacteria (Vespermann et al., 2007).

We also note that the above-mentioned enhancement of the growth-promoting effect by an increase in the test volume was limited to TMEA medium. This result may be due to a possible difference in the proportion of concentrations of promoting and suppressive VOCs among growth media.

Of the 32 detected VOCs produced by *B. pumilus* TM-R, only two (3-methylbutanol and dimethyl disulfide) have been previously determined to have antifungal activity (Li et al., 2010; Toffano et al., 2017). These VOCs did not appear to be major fungal suppressors in our study,

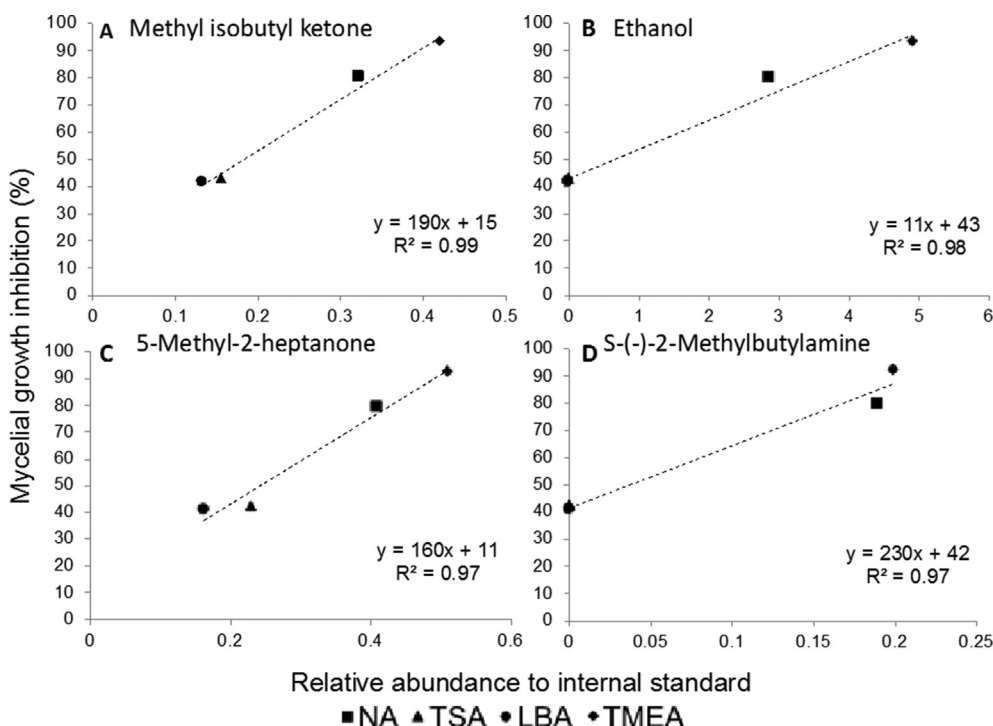


Fig. 4. Correlation between the rate of mycelial growth inhibition against *Penicillium italicum* and the relative abundance of the four major VOCs.

however, as correlations between their relative abundance and mycelial growth inhibition were very low. Instead, four VOCs (methyl isobutyl ketone, ethanol, 5-methyl-2-heptanone, and S-(-)-2-methylbutylamine) were determined to be the major volatiles responsible for antifungal activity. Other than ethanol, these VOCs have not been reported as antifungal agents. To confirm the antifungal activity of these VOCs, further research involving fumigation experiments using purchased pure chemicals are required.

Out of the 24 VOCs identified in this study, three (methyl pentanoate, ethyl phenylacetate, and 5H-1-pyridine) have not been previously reported as bacterial products. Some of the identified VOCs are likely rarely detected, as databases specific to VOCs (AromaOffice and ArochemBase) were used in addition to the popular NIST library.

In the next stage of our research, we plan to test the effectiveness of *B. pumilus* TM-R as a biofumigant placed in storage boxes or rooms housing crops such as citrus fruits.

Declarations

Author contribution statement

Toshiyuki Morita: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Issei Tanaka, Masao Ikari: Performed the experiments.

Noriko Ryuda: Performed the experiments; Analyzed and interpreted the data.

Daisuke Ueno: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Takashi Someya: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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