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Root-associated bacteria influencing mycelial growth of *Tricholoma matsutake* (pine mushroom)

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Tricholoma matsutake **is an ectomycorrhizal fungus usually associated with *Pinus densiflora* in South Korea. Fruiting bodies (mushrooms) of *T. matsutake* are economically important due to their attractive aroma; yet, *T. matsutake* is uncultivatable and its habitat is rapidly being eradicated due to global climate change. Root-associated bacteria can influence the growth of ectomycorrhizal fungi that co-exist in the host rhizosphere and distinctive bacterial communities are associated with *T. matsutake*. In this study, we investigated how these bacterial communities affect *T. matsutake* growth by isolating bacteria from the roots of *P. densiflora* colonized by ectomycorrhizae of *T. matsutake* and co-culturing root-associated bacteria with *T. matsutake* isolates. Thirteen species of bacteria (27 isolates) were found in pine roots, all belonging to the orders Bacillales or Burkholderiales. Two species in the genus *Paenibacillus* promoted the growth of *T. matsutake* in glucose poor conditions, likely using soluble metabolites. In contrast, other bacteria suppressed the growth of *T. matsutake* using both soluble and volatile metabolites. Antifungal activity was more frequent in glucose poor conditions. In general, pine rhizospheres harbored many bacteria that had a negative impact on *T. matsutake* growth and the few *Paenibacillus* species that promoted *T. matsutake* growth. *Paenibacillus* species, therefore, may represent a promising resource toward successful cultivation of *T. matsutake*.**

Keywords: *Paenibacillus*, pine mushroom, growth promoting bacteria, metabolite, volatile organic compound, fairy ring

Introduction

Tricholoma matsutake, pine mushroom (PM), is an ectomycorrhizal fungus associated with trees belonging to the families Fagaceae (e.g. *Quercus serrata*) and Pinaceae (e.g. *Pinus densiflora*) (Yamanaka *et al.*, 2014). Pine mushrooms are edible, and are highly prized in Asia due to their attractive appearance, sublime taste and pine-like aroma (Wang *et al.*, 1997). PM, however, has yet to be successfully cultivated (Yamada *et al.*, 2006) and its productivity and habitats are declining due to global climate changes (Guo *et al.*, 2017). Thus, it is urgent issue to investigate PM ecology associated with fruiting body formation. Previous studies of PM have focused on abiotic factors such as climate (Kang *et al.*, 1989; Yang *et al.*, 2012) and nutritional conditions (Guerin-Laguette *et al.*, 2003; Kim *et al.*, 2005; Jeon and Ka, 2015), yet limited studies have investigated the role bacterial communities and their associated metabolites that play in PM growth and development. One of major drawbacks is slow growth rate of PM isolate in artificial media. Therefore, enhancing PM growth rate in media will be fundamental step for PM cultivation.

The rhizosphere is a suitable habitat for bacteria because nutrients are supplied by the plant, through the root system, as plant exudates and deposits, which are easily accessible to the bacteria (Raaijmakers *et al.*, 2009). In the rhizosphere, root-associated bacteria co-exist with ectomycorrhizal fungi with complex interactions resulting between these communities (Garbaye, 1991; Hampp and Maier, 2008; Nazir *et al.*, 2010). Several bacterial taxa isolated from ectomycorrhizae are known as mycorrhiza helper bacteria (MHB), and they can promote mycelial growth and ectomycorrhizal formation (Frey-Klett *et al.*, 2007). In contrast, other bacterial species suppress growth and retard colonization of ectomycorrhizal fungi (Varese *et al.*, 1996; Bending *et al.*, 2002). Thus, ectomycorrhizal fungi may select beneficial bacteria for their development.

Bacteria secrete a diverse suite of secondary metabolites that play important roles in bacterial-fungal interactions (Frey-Klett *et al.*, 2011; Schmidt *et al.*, 2016). Bacterial metabolites can have a positive or negative impact on fungal growth. Some bacterial metabolites, such as nutrients or stimuli, promote fungal development, while others act as antifungal compounds that suppress fungal growth or spore germination. Generally, secondary metabolites can be divided into two types: soluble compounds and volatile organic compounds (VOC) (Tyc *et al.*, 2017). Soluble compounds (e.g. carbohydrates, peptides, and organic acids) can dissolve in liquid and exert a strong influence within a short distance. VOCs, on the other hand (e.g. terpenes and nitrogen compounds) are relatively small molecules that readily evaporate and can diffuse long distances through air (Schmidt *et al.*, 2016; Tyc *et al.*, 2017).

PM form dense mycelia in soil around the root system of host trees called a fairy ring (Wang *et al.*, 1997). In PM fairy rings, bacterial communities were altered, which suggests intimate interaction between PM and bacterial communities (Vaario *et al.*, 2011; Kim *et al.*, 2014; Oh *et al.*, 2016, 2018).

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In order to identify growth promoting bacteria, we isolated members of the bacterial community from the root system of *Pinus densiflora* within PM fairy rings, and investigated how different bacterial taxa affected PM growth.

Materials and Methods

Sampling and isolation of bacteria

Roots of *Pinus densiflora* were collected under PM fairy rings at two locations in South Korea: A forest in Hongcheon County (N37° 41′ 35″, E127° 58′51) that is maintained by the National Institute of Forest Science (Seoul, South Korea) and a forest in Uljin County (N37° 02′ 09″, E129° 17′ 62″), that historically exhibits high production of PM fruiting bodies. Sample collection was carried out with permission from the Institute. At both sites, *Pinus densiflora* is dominant and *Rododendron* spp. are abundant as understory vegetation. Six lateral roots (c.a. 10 cm) colonized with PM ectomycorrhizae were collected from three fairy rings in each site and transferred to the laboratory in an ice box. Root samples were carefully rinsed with sterile distilled water to remove soil and debris attached to the roots. For bacterial isolation, 5 mm of root slices were placed on Petri dishes containing Tryptic soy agar (TSA; Difco) and Reasoner's 2A agar medium (R2A; Difco) and incubated at 30°C for 2–7 days. A total of 324 root slices (9 slices × 3 replicate × 2 media × 3 fairy rings × 2 sites) was used for isolation. In order to obtain pure culture, each isolate was sub-cultured onto TSA, and then kept in 20% glycerol at -80°C for subsequent co-culture experiments.

Molecular experiment and identification

Bacterial isolates were identified using the 16S ribosomal RNA region. The PCR was performed using Maxime PCR PreMix kit (iNtRON Biotechnology) containing 1 μl of diluted bacteria isolate, 1 μl of 27F and 1492R primers (Weisburg *et al.*, 1991), and 16 μl of distilled water. PCR conditions were as follows: 95°C for 10 min, and 35 cycles of 95°C for 40 sec, 55°C for 40 sec, 72°C for 60 sec, and final extension at 72°C for 5 min. The PCR products were checked using 1% agarose gel electrophoresis and purified using Expin^{TM} PCR Purification Kits (GeneAll Biotechnology). Sequencing of the full 16S region was conducted at Macrogen using an ABI Prism 3730 genetic analyzer (Life Technologies).

Sequence chromatograms were proofread and assembled using MEGA v. 5.0 (Tamura *et al.*, 2011). Phylogenetic analysis was conducted with the reference sequences based on results from BALST on EzBioCloud (Yoon *et al.*, 2017). After aligning sequences using MAFFT (Katoh and Standley, 2013), a phylogenetic tree was constructed using the neighbor-joining method with the Kimura-2-parameter model and 1,000 bootstrap replications. Sequences generated from the analysis were deposited in GenBank under the accession numbers MG457677-MG457703.

Screening bacterial effect on PM growth

Because forest soil is generally a nutrition limited environment (Ekblad and Nordgren, 2002; Schmidt *et al.*, 2016), the mode of interaction can change between bacteria and PM depending on nutritional condition (nutrient rich vs. nutrient poor). Since PM growth depends on glucose as a major energy source, two kinds of growth media were used to determine the effect bacteria on PM growth: high and low glucose concentration media. 'Tricholoma matsutake' media (TMM) (glucose 20 g/L, yeast extract 1.5 g/L, soytone 1.5 g/L, and agar 20 g/L) (Kim *et al.*, 2005) was used for the high glucose concentration medium (HTMM), and the low glucose concentration media (LTMM) had the same components except the glucose concentration was reduced by a factor of 10 (2 g/L).

Bacterial effects on PM growth were screened using single plate co-culture experiments when testing bacterial metabolite effects and dual plate co-culture experiment when testing VOC effects (Fig. 1). For single plate co-culture experiments, we cultured PM isolates and bacteria together on a single plate (Fig. 1A). PM isolate was pre-incubated in potato dextrose broth (Difco) at 25°C for six months. Before inoculation on medium, PM isolate was homogenized using a HG-15A homogenizer (DAIHAN Scientific) with 30 ml of sterile distilled water. Homogenized PM isolate was inoculated on the center of a 60 mm Petri dish containing growth media. Representative isolates of bacterial species were streaked along a 30 mm line located 15 mm away from PM isolate on the center of plate using a sterile 1 μl inoculating loop (SPL Life Science). The effect of bacterial VOCs was tested with dual plate co-culture experiments using a bi-partition Petri dish (90 mm; SPL Life Science) (Fig. 1B). Growing media were poured in half part of bi-partition Petri dish for PM isolates, and in a 35 mm Petri dish (SPL Life Science) for bacteria. After streaking bacterial isolates, uncovered bacteria culture plates were placed in an empty part of bi-partitioned dish.

All experiments were conducted in quadruplicate and incubated at 25°C for four weeks (28 days). Radial growth change of PM isolates was estimated by comparing the diameter of PM isolate of bacteria co-cultured plates with that of plates where PM was grown alone (control) using a pairwise *t*-testing adjusted by the false discovery rate of Benjamini and Hochberg (Benjamini and Hochberg, 1995).

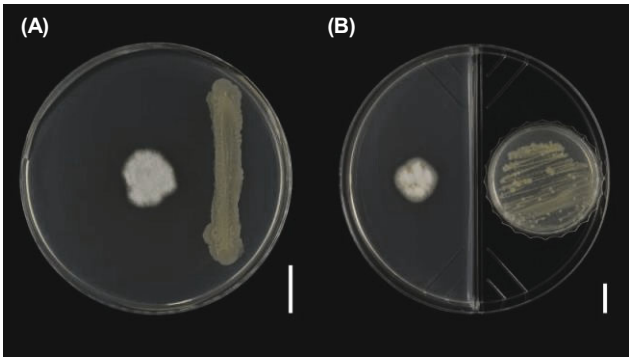

Fig. 1. PM growth experiment design for (A) single plate co-culture and (B) dual plate co-culture experiment. Scale bar indicates 10 mm.

Results

Isolation and identification of pine root-associated bacteria

A total of 27 bacterial isolates was obtained from 27 root slices in R2A medium, while fungal contamination or no microbes were detected from 297 root slices. All strains were identified based on the 16S rRNA region, and initial identification using BLAST showed high similarity between isolate sequences and type sequences (97.6–100%) (Table 1). Final identification was conducted based on phylogenetic analysis, which showed that eight species belong to Bacillales (Firmicutes; Bacilli) and five species to Burkholderiales (Proteobacteria; Betaproteobacteria) (Fig. 2). *Bacillus* had the highest number of species (6 species), followed by *Paenibacillus* (2 species) and *Paraburkholderia* (2 species). The number of isolates was highest for *Bacillus* sp. 1 (6 strains), followed by *Paraburkholderia sediminicola* (4 strains), *Bacillus* sp. 3 (3 strains), and *Herbaspirillum chlorophenolicum* (3 strains).

Difference of radial growth of PM isolate in co-culture

Single plate co-culture experiments showed different bacterial effects on PM growth depending on glucose concentration (Fig. 3). In HTMM, seven bacterial species showed significant negative effects on PM growth: plates cultured with *Achromobacter* sp., *Bacillus* sp. 1, *Bacillus* sp. 2, and *Paraburkholderia terrae* exhibited no growth of PM isolate, while *Bacillus* sp. 3, *Burkholderia* sp., and *Paraburkholderia sediminicola* reduced PM growth to 2.9%, 12.0%, and 4.0%, respectively, compared to the control. For PM cultured in LTMM, all bacteria tested showed significant effects on PM growth. PM isolates cultured (separately) with 11 bacterial species showed no growth in LTMM. In contrast, two bacterial species, *Paenibacillus latus* and *Paenibacillus* sp., increased PM growth by 478.5% and 570.9%, respectively, compared to the control plate.

The dual plate co-culture experiment that tested bacterial VOC effects also showed variable results (Fig. 4). In the HTMM, there was no effect on PM growth by any of the bacteria tested except for one species, *Achromobacter* species. PM isolate co-cultured with *Achromobacter* sp. showed zero growth. In LTMM, VOCs of 11 bacterial species significantly suppressed PM growth; however, VOCs of *Paenibacillus latus* and *Paenibacillus* sp. increased PM growth by 230% and 130%, respectively, although this difference was not statically significant.

Discussion

Ectomycorrhizae have a significant effect on their surrounding environment, the ectomycorrhizosphere (Linderman, 1988; Calvaruso *et al.*, 2007), including the ability to shape

Table 1. BLAST results of bacterial strains isolated from roots of *Pinus densiflora*
BLAST conducted against ezBioCloud database. The letter ^{T} indicates ex-type strain. The representative strains used for co-culture experiments represent in bold

No	ID	Best match result	Similarity (%)	Final identification	Accession number
1	PRB205	*Bacillus drentensis* LMG 21831^{T}	99.71	*Bacillus drentensis*	MG457690
2	**PRB256**	*Bacillus drentensis* LMG 21831^{T}	99.85	*Bacillus drentensis*	MG457696
3	**PRB360**	*Bacillus megaterium* NBRC 15308^{T}	100.00	*Bacillus megaterium*	MG457703
4	PRB063	*Bacillus wiedmannii* FSL W8-0169^{T}	99.93	*Bacillus* sp.1	MG457678
5	**PRB119**	*Bacillus wiedmannii* FSL W8-0169^{T}	99.93	*Bacillus* sp.1	MG457684
6	PRB120	*Bacillus wiedmannii* FSL W8-0169^{T}	99.93	*Bacillus* sp.1	MG457685
7	PRB121	*Bacillus wiedmannii* FSL W8-0169^{T}	99.93	*Bacillus* sp.1	MG457686
8	PRB122	*Bacillus wiedmannii* FSL W8-0169^{T}	99.93	*Bacillus* sp.1	MG457687
9	PRB335	*Bacillus wiedmannii* FSL W8-0169^{T}	99.93	*Bacillus* sp.1	MG457701
10	**PRB062**	*Bacillus toyonensis* BCT-7112^{T}	100.00	*Bacillus* sp.2	MG457677
11	**PRB107**	*Bacillus weihenstephanensis* NBRC 101238^{T}	100.00	*Bacillus* sp.3	MG457680
12	PRB108	*Bacillus weihenstephanensis* NBRC 101238^{T}	100.00	*Bacillus* sp.3	MG457681
13	PRB270	*Bacillus weihenstephanensis* NBRC 101238^{T}	100.00	*Bacillus* sp.3	MG457697
14	**PRB221**	*Bacillus butanolivorans* DSM 18926^{T}	98.57	*Bacillus* sp.4	MG457691
15	**PRB332**	*Paenibacillus lautus* NRRL NRS-666^{T}	99.49	*Paenibacillus lautus*	MG457700
16	**PRB331**	*Paenibacillus uliginis* N3/975^{T}	97.59	*Paenibacillus* sp.	MG457699
17	PRB336	*Paenibacillus uliginis* N3/975^{T}	97.59	*Paenibacillus* sp.	MG457702
18	**PRB106**	*Achromobacter spanius* LMG 5911^{T}	100.00	*Achromobacter* sp.	MG457679
19	**PRB248**	*Burkholderia contaminans* LMG 23361^{T}	99.21	*Burkholderia* sp.	MG457695
20	PRB241	*Paraburkholderia sediminicola* HU2-65W^{T}	99.64	*Paraburkholderia sediminicola*	MG457693
21	**PRB109**	*Paraburkholderia sediminicola* HU2-65W^{T}	99.71	*Paraburkholderia sediminicola*	MG457682
22	PRB200	*Paraburkholderia sediminicola* HU2-65W^{T}	99.78	*Paraburkholderia sediminicola*	MG457689
23	PRB189	*Paraburkholderia sediminicola* HU2-65W^{T}	99.86	*Paraburkholderia sediminicola*	MG457688
24	**PRB227**	*Paraburkholderia terrae* NBRC 100964^{T}	99.57	*Paraburkholderia terrae*	MG457692
25	**PRB110**	*Herbaspirillum chlorophenolicum* CPW301^{T}	98.85	*Herbaspirillum chlorophenolicum*	MG457683
26	PRB247	*Herbaspirillum chlorophenolicum* CPW301^{T}	98.85	*Herbaspirillum chlorophenolicum*	MG457694
27	PRB294	*Herbaspirillum chlorophenolicum* CPW301^{T}	98.85	*Herbaspirillum chlorophenolicum*	MG457698

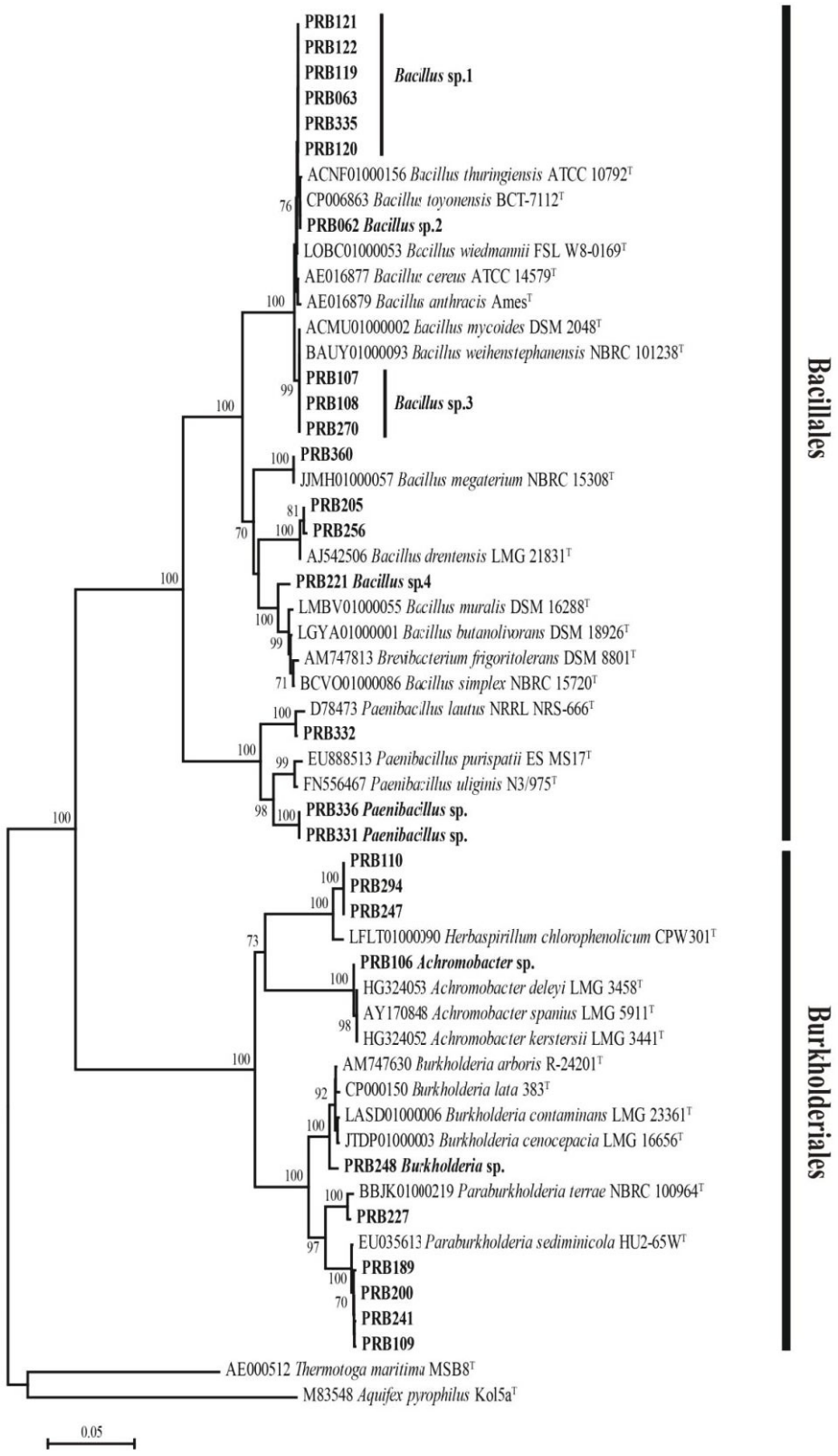

Fig. 2. Neighbor-joining phylogenetic tree for final identification based on bacterial 16S rRNA region. Phylogeny was constructed by Kimura-2-parameter model with 1,000 bootstrap replicates. High bootstrap values (≥ 70%) are only presented on branches. The letter ^{T} indicates ex-type strain.

associated bacterial communities (Kataoka *et al.*, 2008; Izumi and Finlay, 2011; Marupakula *et al.*, 2016). Several distinctive bacterial species have been detected in PM-associated environments such as the fairy ring and fruiting bodies using culture-dependent or culture-independent methods (Kim and Whang, 2007; Vaario *et al.*, 2011; Kataoka *et al.*, 2012;

Kim *et al.*, 2014; Jiang *et al.*, 2015; Li *et al.*, 2016; Oh *et al.*, 2016). We isolated 13 bacteria species (27 strains) from pine roots in PM fairy rings from two locations that have historically had high PM productivity (Table 1). One explanation for this relatively small number of bacterial isolates may be due to the use of artificial media. In addition, since the media lacked antifungal components, many of the samples were contaminated with other fungi. The bacterial diversity reflected in the isolates we did recover, however, is consistent with previous studies of PM-associated bacteria that showed that *Bacillus, Burkholderia*, and *Paenibacillus* was abundant in PM fairy ring soil (Kataoka *et al.*, 2012; Kim *et al.*, 2014; Oh *et al.*, 2016) and PM fruiting bodies (Li *et al.*, 2016). These results suggest that some bacteria associated with pine root systems have important relationships with PM. Many of these bacteria, however, were also commonly detected in other ectomycorrhizosphere of *Pinus* roots (Bending *et al.*, 2002; Izumi *et al.*, 2006, 2007, 2008; Timonen and Hurek, 2006; Marupakula *et al.*, 2016). Consequently, it is unclear that the bacteria isolated from pine roots are either MHB for PM (Li *et al.*, 2016; Oh *et al.*, 2016) or just favor *Pinus* ectomycorrhizosphere because frequency of bacterial detection or isolation cannot provide any proof of relationship with PM.

Co-culture experiments showed that only two *Paenibacillus* species promoted PM mycelial growth and that the other 11 species inhibited PM mycelia growth (Fig. 3). These growth-inhibiting bacteria might share their habitat with PM as rhizospheric bacteria that compete with PM for nutrient resources provided by *Pinus densiflora.* Previous studies showed that *Paenibacillus* species promote mycorrhizal colonization of *Lactarius rufus* (Poole *et al.*, 2001), while they reduce growth of *Laccaria bicolor* (Deveau *et al.*, 2007). Although *Bacillus* and *Burkholderia* are also known as MHB in *Laccaria* species (Duponnois and Garbaye, 1991; Dunstan *et al.*, 1998; Poole *et al.*, 2001), the *Bacillus* and *Paraburkholderia* species isolated in this study showed strong negative effects toward growth of PM isolates (Fig. 3). Therefore, the effect of root-associated bacteria may vary depending on the mycorrhizal species. Among the root-associated bacteria we isolated, two *Paenibacillus* species have MHB effects on PM growth.

Differences in bacterial effect as a function of nutrient condition were noticeable in this study (Figs. 3 and 4). Growth promoting effects of *Paenibacillus* was only apparent in the glucose poor treatment, which is consistent with a previous study of the MHB effect of *Pseudomonas fluorescens* (Brulé *et al.*, 2001). *Pseudomonas fluorescens* promoted the growth of *Laccaria bicolor* in nutrition poor medium, but suppressed the growth in nutrient rich medium. One possible reason is that growth promoting effects may be limited to initiation of growth. Generally, growth initiation is self-induced in by fungi in preferable environments, such as a nutrient rich media. Therefore, PM growth promoting effects may not have been observed in HTMM because growth was already independently initiated by PM. In case of the interaction

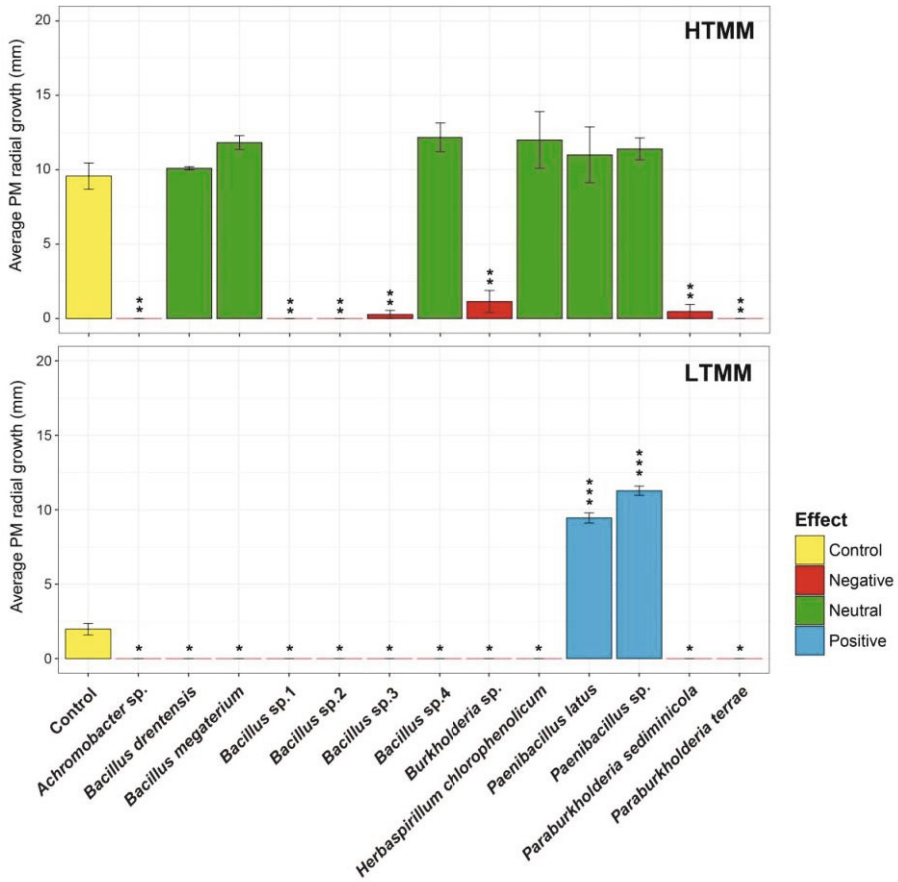

Fig. 3. Average radial growth (mm) of PM from single plate co-culture experiments in a glucose rich medium (HTMM) and a glucose poor medium (LTMM). Difference of PM growth cultured with bacteria was tested against control plate (PM grow alone) by pairwise-*t*-test adjusted by the false discovery rate of Benjamini and Hochberg. Asterisks indicate a significant difference (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Significant bacterial effect is indicated by color (yellow, control; red, negative; green, neutral; blue, positive).

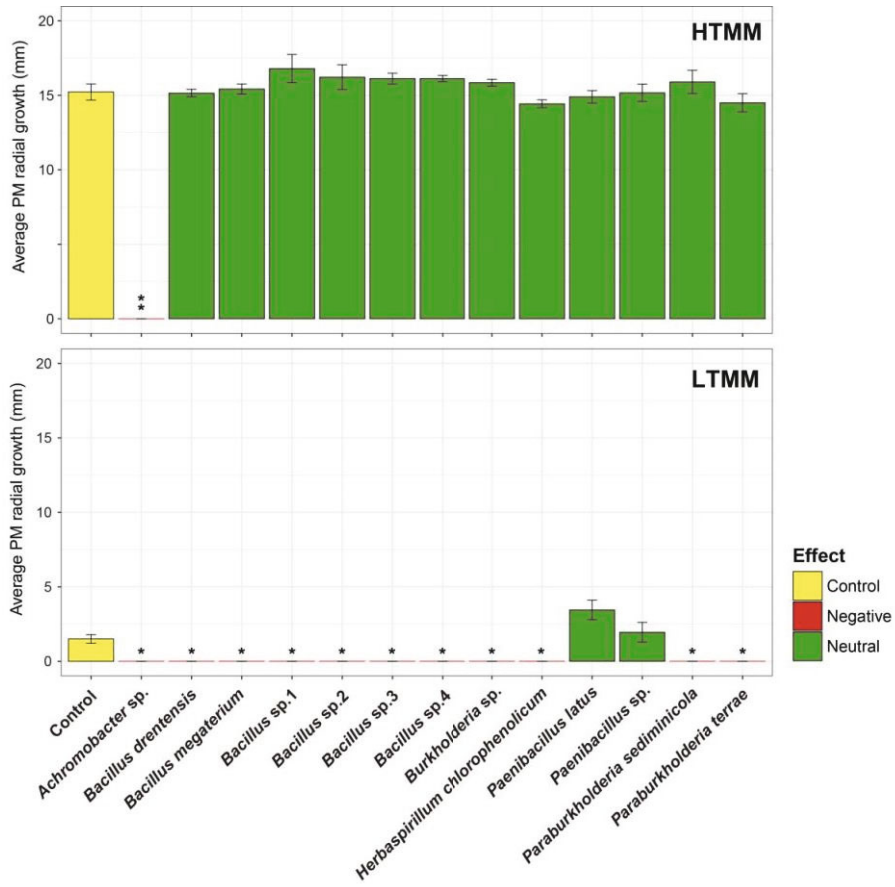

Fig. 4. Average radial growth (mm) of PM from dual plate co-culture experiments in a glucose rich medium (HTMM) and a glucose poor medium (LTMM). Difference of PM growth cultured with bacteria was tested against control plate (PM grow alone) by pairwise-*t*-test adjusted by the false discovery rate of Benjamini and Hochberg. Asterisks indicate a significant difference (*$P < 0.05$, **$P < 0.01$). Significant bacterial effect is indicated by color (yellow, control; red, negative; green, neutral).

between MHB *Streptomyces* sp. and *Amanita muscaria*, the growth promoting effect of *Streptomyces* is similar to glucose effect that stimulates expression of the acetoacyl-CoA synthetase gene (*Aacs*) which is associated with ergosterol synthesis, an important component of cell structure (Schrey *et al.*, 2005). The other explanation is that bacteria secrete nutrients that increase PM growth. Frey-Klett *et al.* (2007) suggested that nitrogen-fixing bacteria provide nitrogen for mycelial growth in ectomycorrhizal fungi, and many *Paenibacillus* species have nitrogen-fixing ability (Berge *et al.*, 2002; Bal and Chanway, 2012); however, nitrogen-fixing ability was not reported in the *Paenibacillus* species we isolated (Behrendt *et al.*, 2010). With respect to the bacteria that conferred a negative effect on PM growth, antifungal activity was more frequent in LTMM than it in HTMM (Figs. 3 and 4). Many bacterial species produced different metabolites depending on environmental conditions, especially in different nutrient conditions. This variation in metabolite production was likely due to nutrient competition with other co-existing microbes (Garbeva and de Boer, 2009). The PM growth experiments revealed that bacteria that had a neutral effect on PM growth exhibited strong antifungal activity in LTMM (Figs. 3 and 4). Thus, the mode of interaction between bacteria and PM may be variable depending on nutrient conditions. Considering nutrient-limited conditions in soil, most bacteria had an antagonistic relationship with PM, while two *Paenibacillus* species appeared to promote PM growth in forest soil.

Many metabolites associated with the interaction of bacteria and fungi act as communication agents (Frey-Klett *et al.*, 2011; Schmidt *et al.*, 2016). Among soluble compounds secreted from MHB, auxofuran promotes mycelia growth of the ectomycorrhizal fungus, *Amanita muscaria* (Riedlinger *et al.*, 2006). Bacterial peptides may also influence fungal growth. The Type III secretion system (T3SS) mediates entry of bacterial peptides into the eukaryotic cytoplasm and also increases gene expression (Preston, 2007). Previous studies have shown that the T3SS gene, which is essential for effective MHB action of *Pseudomonas fluorescens* on the growth of *Laccaria bicolor* (Cusano *et al.*, 2011) and that bacteria encoding the T3SS gene were increased in fungal-associated environment (Warmink and van Elsas, 2008; Viollet *et al.*, 2011). In PM fairy ring soil, predicted functional profiling revealed that the number of the T3SS gene is increased compared to it in bulk soil (Oh *et al.*, 2016).

Bacterial VOCs can also stimulate fungal growth (Wheatley, 2002; Adams *et al.*, 2009). VOCs of *Pseudomonas* promote growth and sporulation of bark beetle symbiotic fungus, *Leptographium procerum* (Adams *et al.*, 2009). In the dual plate co-culture experiments, the growth promoting effects of *Paenibacillus* disappeared (Fig. 4), thus *Paenibacillus* metabolites that promote PM growth may be soluble compounds. Indeed, diverse soluble and VOC antifungal metabolites are produced by bacteria (Kai *et al.*, 2009; Raaijmakers *et al.*, 2009). Root-associated bacteria secrete various soluble antifungal compounds as they compete with other root-

associated microorganism for plant-derived nutrients or space in the rhizosphere (de Boer *et al.*, 2005; Raaijmakers *et al.*, 2009). For example, *Pseudomonas* secretes antifungal compounds such as 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide, and phenazines that target various cellular structures of fungi (Haas and Défago, 2005; Raaijmakers *et al.*, 2006). Recently, antifungal VOCs have garnered increased attention with the aim to control pathogenic fungi, and various bacteria (e.g. *Bacillus*, *Burkholderia*, and *Pseudomonas*) are known to emit VOCs that suppress fungal growth (Kai *et al.*, 2009). *Bacillus cereus* and *Bacillus weihenstephanensis* secrete antifungal VOCs (acetamide, benzothiazole, and benzaldehyde) that inhibited spore germination and growth of nematophagous fungus *Paecilomyce lilacinus* (Zou *et al.*, 2007). In the dual plate co-culture experiments, no antifungal effects were observed in any bacteria in the HTMM treatment, except for those of *Achromobacter* sp. (Fig. 4). In LTMM, however, antifungal activity was present in all bacteria except for *Paenibacillus*, which suggests that antifungal VOC production is induced in LTMM, likely due to competition for limited nutrients. Interestingly, *Achromobacter* sp. did not follow this pattern that VOCs from *Achromobacter* sp. suppressed PM growth in all glucose conditions (Fig. 4). Further study is needed to accurately identify bacterial metabolites in order to improve our understanding of the interactions between root-associated bacteria and PM.

Some limitations in this study are required to attention for interpreting the results. As mentioned above, we only obtained the small number of bacteria (27 isolates), which can reflect small portion of bacterial diversity in pine roots within PM fairy rings. Thus, bacterial isolation from various culture conditions (e.g. incubation temperature, media, or micro-aerobic condition) will improve the range of bacterial isolation. In addition, we did not test the strain specificity of bacterial effect on the PM growth. Previous studies showed that effect of bacteria on the fungal growth can be different depend on the genotype pair between bacteria and fungi (Duponnois and Garbaye, 1990; Labbé *et al.*, 2014). Therefore, co-culture experiments with multiple strains are needed to generalize the effect of PM-associated bacteria on the PM growth.

In conclusion, we isolated root-associated bacteria under the PM fairy ring that influenced PM growth either in a negative or positive fashion. Most bacteria suppressed PM growth by producing both soluble compounds and VOCs, depending on nutrient conditions. Two *Paenibacillus* species, *Paenibacillus latus* and *Paenibacillus* sp., increased PM growth in glucose deficient conditions. Matsutake-infected or -inoculated pine seedlings may be a promising approach toward introducing PM to novel areas where PM fruiting bodies are not currently produced (Ka *et al.*, 2017). According to our results, however, rhizospheres of pine roots have large numbers of bacteria that suppress PM growth and probably ectomycorrhizal colonization as well, leading to lower rates of successful PM colonization and maintenance. Therefore, we suggest that utilization of *Paenibacillus* or its metabolites can help to improve pine seedling colonization methods and thus increase overall PM production.

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