Soil Biology & Biochemistry 41 (2009) 2147–2152

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Quantification of *Wautersia* [*Ralstonia*] *basilensis* in the mycorrhizosphere of *Pinus thunbergii* Parl. and its effect on mycorrhizal formation

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ARTICLE INFO

Article history:
Received 10 December 2008
Received in revised form
19 July 2009
Accepted 23 July 2009
Available online 4 August 2009

Keywords:
Mycorrhiza helper bacteria
Pinus thunbergii
Wautersia basilensis
Real-time PCR

ABSTRACT

The bacterium *Wautersia* [*Ralstonia*] *basilensis* has been shown to enhance the mycorrhizal symbiosis between *Suillus granulatus* and *Pinus thunbergii* (Japanese black pine). However, no information is available about this bacterium under field conditions. The objectives of this study were to detect *W. basilensis* in bulk and mycorhizosphere soils in a Japanese pine plantation in the Tottori Sand Dunes, determine the density of *W. basilensis* in soil, and determine the optimal cell density of *W. basilensis* for mycorrhizal formation in pine seedlings. We designed and validated 16S rRNA gene-targeted specific primers for detection and quantification of *W. basilensis*. SYBR Green I real-time PCR assay was used. A standard curve relating cultured *W. basilensis* cell density (10^3–10^8 cells ml^{-1}) to amplification of DNA showed a strong linear relationship ($R = 0.9968$). The specificity of the reaction was confirmed by analyzing DNA melting curves and sequencing of the amplicon. The average cell density of *W. basilensis* was $>4.8 \times 10^7$ cells g^{-1} of soil in the mycorrhizosphere and 7.0×10^6 cells g^{-1} in the bulk soil. We evaluated the *W. basilensis* cell density required for mycorrhizal formation using an *in vitro* microcosm with various inoculum densities ranging from 10^2 to 10^7 cells g^{-1} soil (10^4–10^9 cells ml^{-1}). Cell densities of *W. basilensis* of $>10^6$ cells g^{-1} of soil were required to stimulate mycorrhizal formation. *In vivo* and *in vitro* experiments showed that *W. basilensis* was sufficiently abundant to enhance mycorrhizal formation in the mycorrhizosphere of Japanese black pine sampled from the Tottori Sand Dunes.

1. Introduction

Pinus thunbergii Parl. (Japanese black pine) has been planted in afforestation efforts in many coastal regions of Japan. It has important roles as windbreaks and prevention of tide-water inundation, and in stabilizing sand. Our previous study on pines growing in the Tottori Sand Dunes showed that more than 95% of pine roots had ectomycorrhizal (ECM) fungi (Kataoka et al., 2008). Ectomycorrhizal fungi are often associated with plants growing on poor soil, such as Japanese black pine growing on sand dunes, because they facilitate nutrient and water uptake (Smith and Read, 2008) and enhance disease resistance of host plants (Whipps, 2004). Both ECM fungi and rhizosphere bacteria generally modify root functions (Garbaye, 1991). Soil associated with mycorrhizas is known as the "mycorrhizosphere". Recently, bacteria inhabiting the mycorrhizosphere have been shown to enhance the symbiosis between plant roots and ECM fungi (Garbaye, 1994). Garbaye (1994) designated bacteria with positive effects on ECM fungi as "mycorrhiza helper bacteria (MHB)". Kataoka and Futai (2008) also isolated two rhizobacteria, *Wautersia* [*Ralstonia*] *basilensis* and *Bacillus subtilis*, which were able to enhance the mycorrhizal symbiosis between *Suillus granulatus* and Japanese black pines from the Tottori Sand Dunes. *Ralstonia basilensis* has been named by Steinle et al. (1998) as a degrader of 2,6-dichlorophenol. After that, a taxonomic revision has occurred in recent years, and *R. basilensis* is now a basonym for *W. basilensis* (Vaneechoutte et al., 2004).

To understand the relationship between MHB abundance and mycorrhizal formation, it is important to develop an accurate method to analyze the microbial populations. Generally, the dilution plating method is used to detect bacteria. Green fluorescent protein (GFP)-tagged *Pseudomonas fluorescens* Aur6 colonizing a mycorrhizal root was monitored using the dilution plating method and epifluorescence microscopy (Rincon et al., 2005). Frey-Klett et al. (1999) monitored changes in the population of the rifampin-resistant mutant MHB using the dilution plating method. However, this method has some limitations; first, it takes several days to detect bacteria, and second, it is difficult to specifically detect an individual bacterial species. Therefore, culture-independent methods based on extraction and analyses of DNA from environmental samples have

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doi:10.1016/j.soilbio.2009.07.027

been used (Mavrodi et al., 2007). Real-time PCR is used to quantify amplified PCR products, and has a high specificity when specific primers are used. To date, there have been no reports on the use of real-time PCR to measure indigenous bacteria associated with ECM fungi under field conditions.

The objectives of this study were to (1) detect *W. basilensis* in bulk and mycorrhizosphere soils in a Japanese black pine plantation using SYBR Green I real-time PCR; (2) determine the density of *W. basilensis* in soil; and (3) determine the optimal cell density of *W. basilensis* for mycorrhizal formation in pine seedlings.

2. Materials and methods

2.1. Collection and preparation of soil samples

Soil samples were collected on 24 July 2007 from 1-year-old Japanese black pine seedlings in a pine stand at Tottori Sand Dunes. The annual mean temperature and precipitation are 14.6 °C and 1897.7 mm, respectively. These data were obtained from a meteorological station at the Arid Land Research Center, Tottori University, Tottori, Japan. The soil texture in this region is sand.

Each plant was kept for 20 min in distilled water, and then carefully shaken in distilled water to remove loose (non-adhering) soil (Suzuki and Ishizawa, 1965). The soil adhering to the root was defined as mycorrhizosphere soil and the non-adhering soil was defined as bulk soil. The mycorrhizosphere soil was then divided into four 2-cm sections; 0–2, 2–4, 4–6, and 6–8 cm from the apex of the taproot. The bulk soil was collected from four points in a cross shape at distances 3- and 6-cm and depth 5 cm from each pine seedling. After collection of soil at each distance, the four soils collected were mixed into one sample. Thus, 10 soil samples obtained from 10 plants were used in this study. All soil samples were taken to our laboratory at Kyoto University and kept at −80 °C until use.

DNA was extracted from soil using a modified CTAB method (Takeuchi et al., 2005). The soil samples (each 0.1 g) were ground in liquid nitrogen using a mill (2000 rpm for 30 s; Multi-Beads Shocker, Yasui Kikai Co., Osaka, Japan), and treated with 800 μl 2 × CTAB buffer (2.0% (w/v) CTAB; 1.4 M NaCl; 100 mM Tris–HCl (pH 8.0); 20 mM EDTA (pH 8.0)). After vortexing, the sample solution was incubated for 30 min at 60 °C. Then, 1 ml chloroform/isoamyl alcohol (24:1) was added and the solution was emulsified by careful mixing. The mixture was centrifuged at 15 300 × *g* for 15 min at 4 °C, and the supernatant was transferred to a new tube. Then 600 μl isoamyl alcohol was added and the solution was incubated at room temperature for 20 min. After centrifugation at 15 300 × *g* for 15 min at 4 °C, the resulting DNA pellet was rinsed with 70% ethanol and dried. Each dried DNA pellet was dissolved in TE (1 mM Tris–HCl; pH 8.0; 0.1 mM EDTA; pH 8.0).

2.2. Microbial cultures

W. [*Ralstonia*] *basilensis*, which was isolated from mycorrhizosphere soil of Japanese black pine, positively affects mycorrhizal formation in *S. granulatus* (Kataoka and Futai, 2008). *W. basilensis* was cultured in potato dextrose broth (PDB; Difco) (pH 7.0). The mycorrhizal fungus *S. granulatus* was cultured on Modified Melin–Norkrans (MMN) medium (Marx, 1969) at 25 °C for 2 weeks.

2.3. Design and evaluation of primers specific for W. basilensis

The sequences of primers designed in this study are RalF11 (ATG GCG CIT GTG ACT GCA AGG CTA), and RalR117 (GCA TGA GCG TCA GTG ACG TCC CAG). Using the program ClustalW, we used 16S rRNA sequences obtained from the DDBJ/GenBank/EMBL databases to construct multiple alignments of the target groups and reference organisms. We identified potential target sites for specific detection by comparing sequences unique to groups with large numbers of reference strains (Table 1). The specificity of the primers shown in Table 1 was then checked against the database by submitting the sequences to the Check Probe program of the Ribosomal Database Project (www.cme.msu.edu/RDP) (Maidak et al., 1999). Moreover, the specificity of primers was assessed with DNA extracted from eight other bacterial taxa; *Bacillus* sp. 1, *Bacillus* sp. 2, *Rhizobium* spp., *Burkholderia* sp. 1, *Burkholderia* sp. 2, *Burkholderia* sp. 3, *Serratia* spp., and *Mycobacterium* spp. These bacteria were originally isolated from a pine stand at the Tottori Sand Dunes, southwest Japan (35°32′ N, 134°13′ E). A negative control without a DNA template and a positive control with DNA of *W. basilensis* were included in each PCR run. The 16S rRNA of each test bacterial strain was amplified on a Gene Amp PCR System 9700 (Applied Biosystems, CA, USA), with one cycle at 95 °C for 5 min, 40 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The 25 μl reaction volume contained 1.0 μl DNA extract, 1.0 μl each primer set (10 μM), 0.2 μl Gene Taq NT (Nippon Gene, Toyama, Japan), and 1.6 μl 2.5 mM dNTP mixture. After real-time PCR, the melting temperature and sequence of the amplicon were determined to check the specificity of primers.

2.4. Extraction of bacterial DNA from liquid cultures

Bacterial DNA was extracted by freeze-thaw lysis of cells with 100 μl 2 × CTAB buffer [2.0% (w/v) CTAB; 1.4 M NaCl; 100 mM Tris–HCl (pH 8.0); 20 mM EDTA (pH 8.0)]. Bacterial cells were lysed by three cycles of rapid freezing in liquid N_2 followed by transfer to a boiling water bath. The sample solution was vortexed and then incubated for 30 min at 60 °C. An equal volume of chloroform/isoamyl alcohol (24:1) was added and the solution was mixed to emulsify the mixture. After centrifugation at 15 300 × *g* for 20 min at room temperature, the supernatant was transferred to a new tube. Then 75 μl of isoamyl alcohol was added and the mixture was incubated at room temperature for 20 min. It was then centrifuged at 15 300 × *g* for 20 min at 4 °C. The resulting DNA pellet was rinsed with 70% ethanol and dried. Each dried DNA pellet was dissolved in 100 μl TE (1 mM Tris–HCl; pH 8.0; 0.1 mM EDTA; pH 8.0).

2.5. Real-time PCR

Quantitative PCR based on SYBR Green I labeling was performed on a LightCycler 1.5 (ST300) instrument (Roche, Mannheim, Germany). The 20-μl reaction mixture contained 5 μl DNA sample, each primer at 10 μM, and 4 μl LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche). The amplification program started with denaturation at 95 °C for 10 min followed by 45 cycles with denaturation at 95 °C for 0 s, annealing at 64 °C for 8 s, extension at 72 °C for 10 s, and fluorescence acquisition at the end of extension. The temperature transition rate was 20 °C/s in all steps. After real-time PCR, a melting curve was plotted by continuously measuring fluorescence during heating from 65 to 95 °C at a transition rate of 0.1 °C/s. Threshold cycle (C_T) values were determined with LightCycler software (version 3.5), using the second derivative method. The standard curve was generated by plotting the C_T values as a function of the log of initial DNA concentration, using DNA templates obtained from *W. basilensis* cells at known concentrations (6.6×10^4–6.6×10^9 cells ml^{-1}).

2.6. Effects of W. basilensis inocula on mycorrhizal formation in vitro

Ectomycorrhizas were synthesized in 100 ml polypropylene containers (40-mm diameter). Seeds of Japanese black pine were

Table 1
Multiple sequence alignment of bacterial 16S rRNA. Probe sequence is specific for *Wautersia basilensis*.

Bacteria (accession number)	Forward primer	Reverse primer
	Probe sequence (5′–3′)	Probe sequence (5′–3′)
Bacillus subtilis (AB301922)	GG-T-AT-G-AA−−GGGAA—T	−−TCT–A————G-G-A
Rhizobium sp. (EF100525)	-CT–CT—AT−−GGTAT—T	−−TC-A-T————G-G-T
Serratia sp. 1 (AB301924)	-CT–AT—AA−−GC-A−−	−−ACGAAG————————G-T
***Wautersia basilensis* Ptrs (AB301921)**	ATGGCGCTTGTGACTGCAAGGCTA	CTGGGACGTCACTGACGCTCATGC
Ralstonia sp. BP2 (EF198469)	–T–AT-G————C−−−	−−−−TAA—————————
Ralstonia eutropha (EU827495)	–T–AT-G————C−−−	−−−−−A-G—————————
Ralstonia solanacearum (AY642432)	———————————	−−−−TAA—————————
Burkholderia sp. (EF622219)	-CT–AT————————TC—T	−−−T-AAT—————————
Pseudomonas sp. (EF641267)	GCA–AG-G-G–A-ATTG-A-A-	AGATT-ATA-T—CAAT-TTG–

soaked in distilled water for 20 h and surface-sterilized in 10% $Ca(Cl_2O)_2$ for 45 min. The seeds were washed in sterile distilled water for 5 min before being germinated on 1% distilled water agar in 9-cm diameter Petri dishes for 7 days at 25 °C. Sterile seedlings prepared in this way were used for ectomycorrhizal synthesis when the taproot was 0.5–1.0 cm long.

Sandy soil from the Tottori Sand Dunes was sieved through a 2-mm mesh sieve, and 70 g soil was added to each container. Then 8 ml of mineral nutrient solution (N; 0.03 mg l^{-1}, P; 0.05 mg l^{-1}, K; 0.025 mg l^{-1}, glucose; 10 g l^{-1}) was added, and the container was autoclaved at 121 °C for 120 min. Discs (6-mm diameter) were cut from the growing margin of *S. granulatus*, which was grown on Modified Melin–Norkrans (MMN) agar. The discs were then transferred into liquid MMN medium for 3 days to activate the fungus. One disc was inoculated into each container of soil, which was then incubated for 2 weeks at 25 °C, and then a 1-week-old seedling of Japanese black pine was transplanted into the soil.

The test bacteria were first cultured in potato dextrose broth (PDB) (pH 7.0) for 24 h at 28 °C. A cell suspension of *W. basilensis* (6.6×10^{10} cells ml^{-1}) was prepared. The bacterial suspension was diluted from 10^2 cells g^{-1} to 10^7 cells g^{-1} with sterilized water. An aliquot (1 ml) of bacterial suspension was pipetted onto the soil between the fungal disk and the root. The propagators were placed in a growth chamber at 25 °C with a 12 h light–12 h dark cycle. Each cell density of *W. basilensis* was replicated seven times. Parallel control plates were also prepared, in which PDB (pH 7.0) replaced the bacterial inoculum. After 2 months, all plants were harvested to determine the numbers of uninfected and mycorrhizal lateral roots. Roots were observed under a dissecting microscope, and were defined as ECM roots when they were devoid of root hairs and had a visible mantle. Nonmycorrhizal roots had root hairs and no mantle. The percentage of ECM roots was calculated as follows: (number of ECM root tips/total number of root tips) × 100 (Taniguchi et al., 2007).

2.7. Statistical analyses

Differences in bacterium densities 3 and 6 cm away from pine seedling roots, and between mycorrhizosphere and the bulk soil was determine by Tukey's test ($P < 0.05$) using a computer program, StatView 5.0 (SAS Institute Inc., Cary, NC, USA). Also a *t*-test ($P < 0.05$) was conducted to determine if there were differences between individual bacterial cell density and control treatments.

3. Results

3.1. Primer specificity

To analyze *W. basilensis* in the soil of Japanese black pine, we designed specific primers and then analyzed their specificity (Table 1). The specificity of the primers was tested against DNA extracts from eight bacterial taxa. The RalF11/RalR117 primers specifically amplified target DNA only from *W. basilensis* (Fig. 1). *Burkholderia* spp., which are closely related to *Wautersia* spp., were detected as fluorescence, but the banding positions of their DNA amplicons differed from that of the target. Melting temperature analysis was used to evaluate the PCR results.

3.2. Real-time PCR detection of W. basilensis

A standard curve was constructed by preparing suspensions of *W. basilensis*. The relationship between the number of starting cells (x) and the cycle number (y) at which the fluorescence surpassed a defined threshold was linear over a 5-log range ($y = 44.344–1.2701x$) (Fig. 2a). The correlation coefficient (R) of this standard curve was >0.99. This indicates that the linear range for the procedures used in this study is 10^4–10^8 cells ml^{-1}. Almost identical results were obtained for *W. basilensis* with the specific primers (data not shown). We also determined the specific melting temperature of *W. basilensis* (T_m; 86.28 ± 0.08 °C) (Fig. 2b).

3.3. Quantification of W. basilensis in soil samples

We used the real-time PCR assay to determine the density of *W. basilensis* in each soil type. The average density of *W. basilensis* in bulk soil was 6.9×10^6 cells g^{-1}. There was no significant difference between the densities of bacteria 3- and 6-cm away from *P. thunbergii* seedling roots (Fig. 3a). The density of *W. basilensis* in mycorrhizosphere soil averaged for the four distances from root tip apex (0–2, 2–4, 4–6, 6–8 cm) was 4.8×10^7 cells g^{-1}. There was no

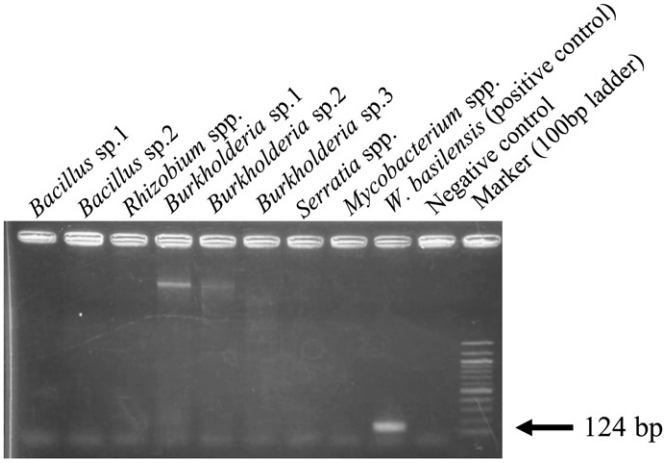

Fig. 1. Specificity of primers used in this study. *Bacillus* sp. 1, *Bacillus* sp. 2, *Rhizobium* spp., *Burkholderia* sp. 1, *Burkholderia* sp. 2, *Burkholderia* sp. 3, *Serratia* spp., and *Mycobacterium* spp. were used to confirm specificity of primers for *Wautersia basilensis* on the gel. A *Wautersia* spp. and D.W. were used as positive and negative controls, respectively.

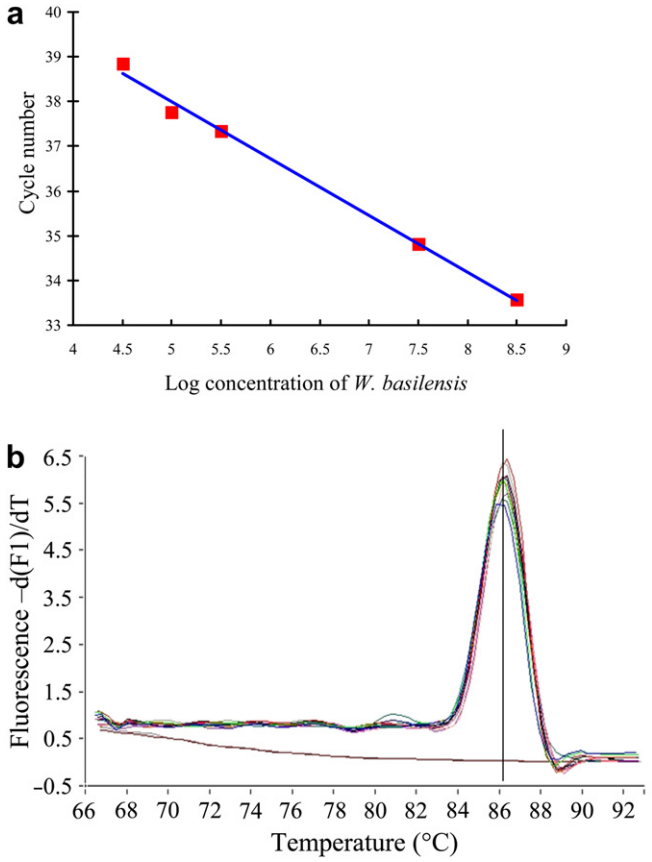

Fig. 2. Real-time PCR standard curve. Curve was constructed from six values obtained from known concentrations of the Mycorrhiza helper bacteria (MHB), (a) *Wautersia basilensis*, and (b) melting curves.

significant difference in densities among the four sections (Fig. 3b). The highest density of *W. basilensis* was detected in the area surrounding the root tip, but there were no significant differences in densities among the four parts of the taproot (Fig. 3b). Furthermore, the density of *W. basilensis* in the mycorrhizosphere was significantly higher than that in bulk soils (Fig. 3c). We analyzed melting curves in all 120 samples, and found that they all had the same average melting peak, T_m; 86.75 ± 0.87 °C. DNA melting curves can be used to differentiate PCR products, as the shape of the curves is a function of GC content, length, and sequence (Kirk et al., 1997). Therefore, our results confirmed that only the target DNA from *W. basilensis* was amplified (Fig. 4a and b).

3.4. Effects of W. basilensis inocula on mycorrhizal formation in vitro

No significant differences were observed in mycorrhizal formation between the control (without *W. basilensis*) and *W. basilensis* treatments at densities from 10^2 to 10^5 cells g^{-1}. However, inoculation with *W. basilensis* at densities greater than 10^6 cells g^{-1} significantly enhanced mycorrhizal formation (Fig. 5).

4. Discussion

4.1. Primer specificity

Real-time PCR has been used to quantify bacterial cell densities in various environments (Lloyd-Jones et al., 2005; Monnet et al., 2006; Pujol et al., 2006). The specificity of SYBR Green I assays

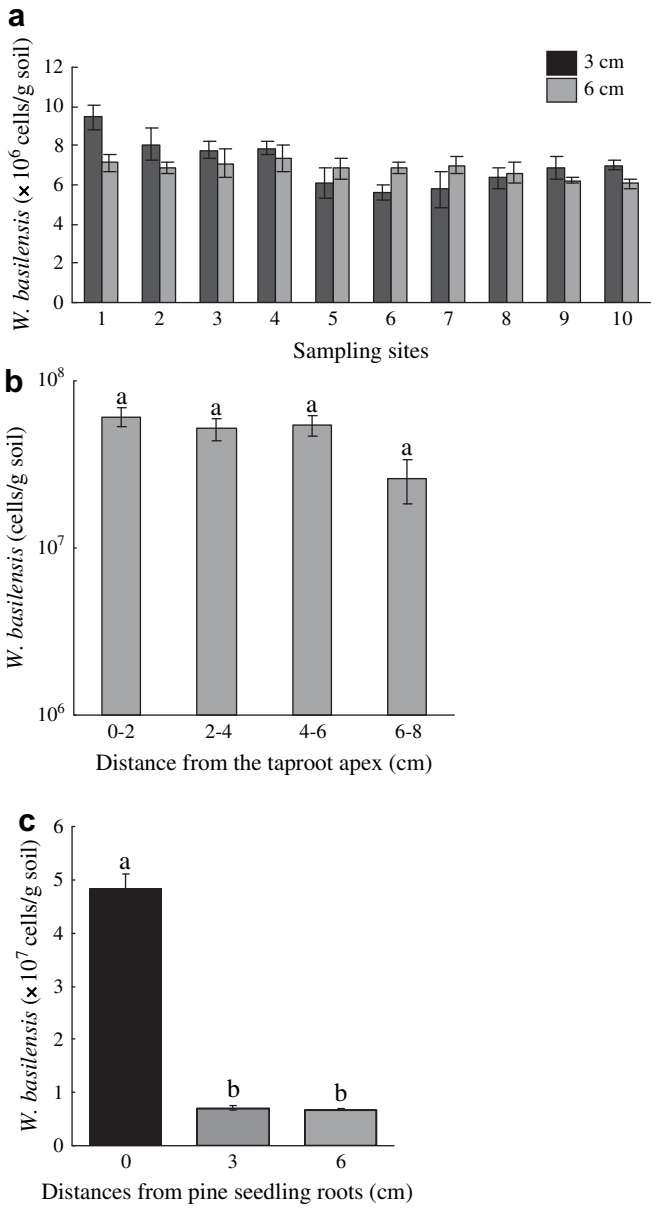

Fig. 3. (a) Density of *Wautersia basilensis* in bulk soil surrounding 10 pine seedlings grown in Tottori Sand Dunes. Bulk soil was collected from positions 3 and 6 cm from a pine seedling. Error bars indicate S.D. (b) Density of *Wautersia basilensis* detected in mycorrhizosphere soil sampled from 10 pine seedlings in the Tottori Sand Dunes. Soils were collected from four sections along the taproot; 0–2 cm, 2–4 cm, 4–6 cm and 6–8 cm from the apex. (c) Comparison of *Wautersia basilensis* density in mycorrhizosphere (0 cm) and bulk soils (3 and 6 cm from pine seedling). Data were analyzed using Tukey's test and different letters denote significant differences among treatments ($p < 0.05$). Error bars indicate S.D.

depends entirely on the PCR primers. Therefore, we constructed a specific RalF11/RalR117 primer set from *W. basilensis* (Table 1). The Probe Match tool compared the primers against 739397 ribosomal sequences and found only 308 bacterial sequences that matched the primer's sequences. Among the matches, some were matched to bacteria in the genera *Cupriavidus* (111), *Ralstonia* (40), *Wautersia* (7), and *Alcaligenes* (2), while some were matched to other groups within the Burkholderiaceae (15). Only one sequence matched to other bacteria; e.g., *Rhizobium* (1). In the phylogenetic tree drawn by Kataoka and Futai (2008) there was a bootstrap value of 999 with *R. basilensis* RK1 (AF312022) (Steinle et al., 1998). A taxonomic revision has occurred in recent years, and bacteria

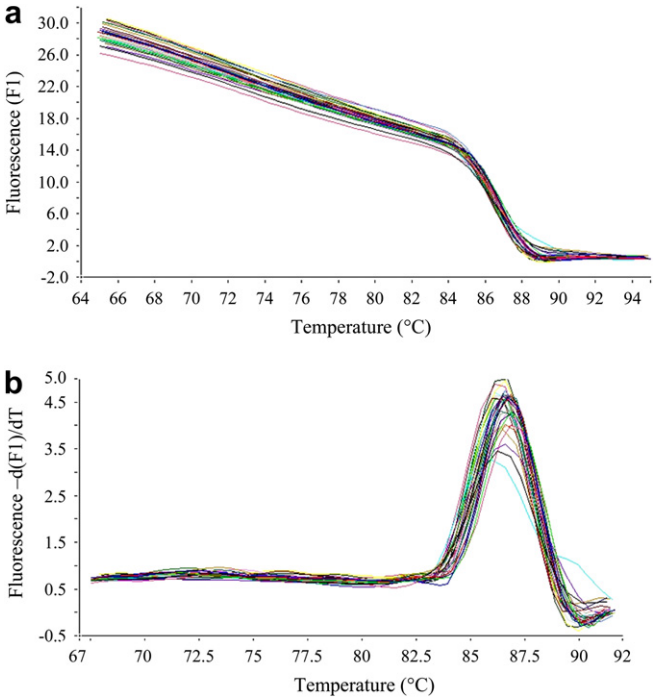

Fig. 4. Real-time PCR melting curves (a) and melting peak curves (b) from reactions using DNA templates extracted from soil samples.

formerly in the genera *Ralstonia*, *Cupriavidus* and *Alcaligenes* are now a basonym for the genus *Wautersia* (Vaneechoutte et al., 2004). The specificity of the primers was then confirmed by comparison with eight bacterial taxa (Fig. 1). These were isolated from the same mycorrhizosphere of Japanese black pine as *W. basilensis*.

Real-time PCR products were also checked by analyzing DNA melting curves (Figs. 2b, 4a and b). The melting temperature (T_m) of DNA in the standard was 86.28 ± 0.08 °C, which was similar to that of DNA obtained from soil samples (86.75 ± 0.24 °C) (Figs. 2a and b, 4a and b). Our results showed that this primer set achieved high amplification efficiency for the target, and did not result in nonspecific amplification of 16S rRNA genes from other microorganisms in the mycorrhizosphere soil.

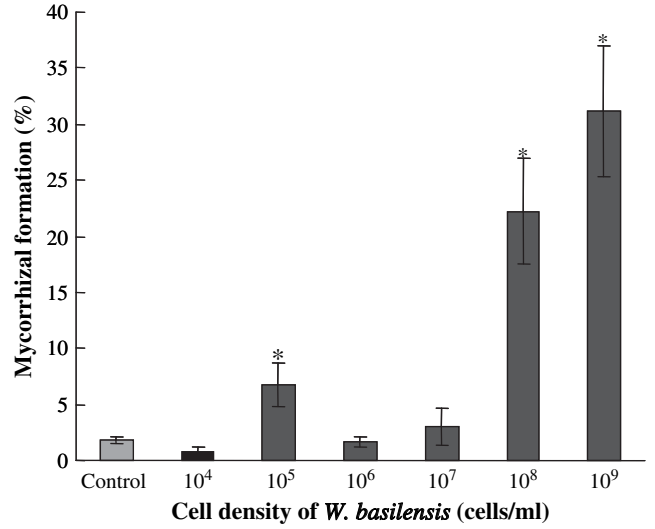

Fig. 5. Effect of *Wautersia basilensis* cell density on mycorrhizal formation in *Pinus thunbergii–Suillus granulatus* symbiosis (90 days). * indicates values that differ significantly between mycorrhizal formation with bacterial inoculation and the control (no bacterial inoculation) (*t*-test, $P < 0.05$). Error bars indicate S.E. Analyses are based on seven replicates per treatment.

4.2. Quantification of the target bacteria in soil samples

The density of *W. basilensis* 3 cm from *P. thunbergii* seedling roots did not significantly differ from that 6 cm from seedling roots, and the average cell density was 8×10^6 cells g^{-1} of soil. Bacteria can adhere non-specifically to solid surfaces as a mechanism to avoid dispersion through the soil by water (Perotto and Bonfante, 1997). Therefore, *W. basilensis* was distributed uniformly in bulk soil. In contrast, the average density of *W. basilensis* in mycorrhizosphere soil was 4.8×10^7 cells g^{-1}. Thus, *W. basilensis* can aggregate and/or propagate very effectively in the limited volume very close to the mycorrhizal root surface (Fig. 3c). Rincon et al. (2005) showed that hyphae within the mantle of *S. granulatus* could mechanically retain bacteria. Furthermore, the fact that bacteria can form biofilms around the hyphae of mycorrhizal fungi has been demonstrated for both ecto- and endomycorrhizal fungi (Bianciotto et al., 1996; Frey-Klett et al., 2007). Thus, the chemical and physical attributes of the mycorrhizosphere enable a high rate of bacterial retention, allowing bacteria to associate closely with both mycorrhizal fungi and the host plant.

4.3. Effects of W. basilensis inocula on mycorrhizal formation in vitro

We evaluated the cell density required for mycorrhizal formation using an *in vitro* microcosm with various inoculum densities of *W. basilensis*, ranging from 10^2 to 10^7 cells g^{-1} (10^4–10^9 cells ml^{-1}). Mycorrhizal formation was stimulated by *W. basilensis* densities exceeding 10^6 cells g^{-1} (Fig. 5). Aspray et al. (2006) reported that *Burkholderia* sp. EJP67 stimulated mycorrhizal formation within a narrow range of inocula densities (10^7 and 10^9 cfu ml^{-1}), a result which is consistent with our data. Bacteria use quorum sensing to coordinate certain behaviors based on the local density of the bacterial population (Whitehead et al., 2001; Gonzalez and Keshavan, 2006; Khmel, 2006). It is unknown whether such sensing underpins the enhancement of mycorrhizal formation facilitated *W. basilensis* (this study) or *Burkholderia* sp. EJP67.

The density of *W. basilensis* in the mycorrhizosphere of *P. thunbergii* was more than 10^7 cells g^{-1}. Therefore, it is clear that the amount of *W. basilensis* associated with *P. thunbergii* trees in the Tottori sand dunes is adequate to enhance mycorrhizal formation.

In conclusion, this study has shown that *W. basilensis* was specifically and rapidly detected from the mycorrhizosphere using real-time PCR. However, the ability of *W. basilensis* to stimulate mycorrhizal formation depended on cell densities. *In vivo* and *in vitro* experiments showed that the abundance of *W. basilensis* in the mycorrhizosphere soil from the Tottori Sand Dunes was adequate to stimulate mycorrhizal formation. These results and the new method described here will increase our understanding of *W. basilensis* in field conditions.

Acknowledgments

We thank Yuko Takeuchi, Laboratory of Environmental Myco-science, Kyoto University, for her critical suggestions.

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