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Community structures of total bacterial DNA, cultivable bacteria and prototrophs in bulk soil and rhizospheres

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ABSTRACT

Aims: It has been hypothesized that root exudates can be a nutritional factor influencing the bacterial community structure as well as the occurrence of prototrophs and auxotrophs in rhizospheres. The present study was performed to examine the community structures of total bacterial DNA, cultivable bacteria and prototrophs in 3 soil samples with different levels of abundance of root exudates.
Methodology and results: Denaturing gradient gel electrophoresis (DGGE) was performed to examine the community structures of total bacterial DNA, cultivable bacteria and prototrophs in 3 soil samples including bulk soil, rhizosphere of a single plant species and rhizosphere of multiple plant species. For clustering analysis, a dendrogram generated from the DGGE patterns revealed the different bacterial community structures in these soil samples. Both rhizospheres claded together, separating from bulk soil. The DGGE patterns of cultivable bacteria showed particular fingerprints corresponding to kinds of media and soil samples. Nutrient agar (NA) medium, isolation medium for prototroph (IMP) and IMP supplemented with soil extracts were used for bacterial cultivations. Prototrophs were isolated and examined by random amplified polymorphic DNA (RAPD) and 16S rRNA gene sequence analysis. The genetic diversity of prototrophs in 3 soil samples was similar (approximately 5% to 10% similarities) and most of them (13 of 28 strains) were members of *Pseudomonas* with 97% to 100% identities.
Conclusion, significance, and impact of study: The present study provides a strong evidence of the influence of root exudates and plant species on bacterial community structures.

Keywords: bacterial diversity, denaturing gradient gel electrophoresis (DGGE), prototroph, random amplified polymorphic DNA (RAPD), 16S rRNA gene.

INTRODUCTION

Many microorganisms isolated from natural niches have different nutritional requirements. This implies that the microbial community and the microbial diversity in a specific environment highly depend on the abilities of microorganisms to utilize nutrients and adjust themselves to nutritional supplementation. The important nutritional factors determining the bacterial community and the bacterial diversity include amino acids and vitamins that are essential to the cell metabolisms. A specific group of microorganisms, termed as “prototrophs”, have shown an astonishing growth requirement with the abilities to synthesize their metabolites including numerous amino acids and vitamins. Prototrophs can grow in minimal media containing simple carbon and nitrogen sources, phosphate buffer and inorganic salts. In contrast, auxotrophs require exogenous metabolites for their growth. The distribution of prototrophs and auxotrophs in some preferable habitats have been reported. *Lactobacillus lactis* subsp. *lactis* strains isolated from dairy products were auxotrophs for branched-chain amino acids (leucine, isoleucine and valine), while most strains isolated from non-dairy media were prototrophs. It might be expected that a change of the ecological niche of a microorganism from a less to a richer environment may lead to the loss of functions which have become superfluous (Godon *et al.*, 1993). Tryptophan prototrophs were recovered from a wild type tryptophan-requiring strain of *Lactobacillus plantarum* after 8 days of incubation in a tryptophan-depleted medium (Thompson *et al.*, 1997). *L. plantarum* strains associated with milk products were more likely to lose the ability to synthesize arginine than *L. plantarum* strains isolated from plant products or humans were. This result suggests that an association with dairy products favored the selection of natural *L. plantarum*

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arginine auxotrophs. The difficulty in correlating the occurrence of auxotrophy with specific habitat adaptations may result from the complexity inherent in numerous metabolic pathway interconnections and nutritional exchange between organisms present simultaneously in fermented foods or ecological niches (Bringel and Hubert, 2003).

In soil habitats, soil bacteria are primarily limited in distribution, density and persistence by the nature and amount of carbon and energy available to them (Viteri and Schmidt, 1987). Root exudate amount and composition are the key drivers for the difference in bacterial community structure (Marschner *et al.*, 2004). Plants produce root exudates that are able to stimulate the microbial community in the rhizosphere by providing nutrients and easily degradable energy sources from root exudates and dead root cells to soil microorganisms, then root exudates can also create a selective pressure on the microbial community (Kaksonen *et al.*, 2006). Nimnoi *et al.* (2011) demonstrated that different plant species and soil characteristics synergically affected the rhizosphere bacterial community and the plants appeared to have a much stronger influence on the bacterial community rather than the inoculants. As plant root exudates consist of a complex mixture of sugars, organic acids, phytosiderophores, purines, nucleosides, inorganic ions, gaseous molecules, amino acids and vitamins (Dakora and Phillips, 2002), it has been hypothesized that different root exudates produced by distinct plant species can be a nutritional factor influencing the occurrence of prototrophs and auxotrophs in rhizospheres.

In this study, denaturing gradient gel electrophoresis (DGGE) was performed to examine the community structures of total bacterial DNA, cultivable bacteria and prototrophs in 3 soil samples with different levels of abundance of root exudates. Bulk soil, rhizosphere of a single plant species and rhizosphere of multiple plant species were analyzed for pH, moisture content, urease activity, phosphatase activity and soil particle-size distribution. The cultivable bacteria and prototrophs were enumerated. Prototrophs were isolated and examined by random amplified polymorphic DNA (RAPD) and 16S rRNA gene sequence analysis. Their plant growth-promoting characteristics were also examined.

MATERIALS AND METHODS

Soil sample collection

Bulk soil amended with organic residues had been left in pots without planting for 3 years. Rhizosphere of a single plant species was collected from pots, in which *Euphorbia milii* Des Moul had been planted for 3 years. Rhizosphere of multiple plant species was collected from pots, in which several plants including *E. milii* Des Moul, *Hippeastrum johnsonii* and *Codiaeum variegatum*, had been planted for 3 years.

Determination of soil characteristics and preparation of soil extracts

pH, moisture content, urease activity, phosphatase activity and soil particle-size distribution of soil samples were analyzed as described by Renshaw *et al.* (2003), Mazzoleni and Dickmann (1988), Pongsilp *et al.* (2012), Leelahawonge and Pongsilp (2009) and Kettler *et al.* (2001), respectively. Soil characteristics were measured in 3 replicates. Soil extracts were prepared by the method described by Narisawa *et al*. (2008).

Isolation and enumeration of cultivable bacteria and prototrophs

Bacteria were extracted from soils by the method described in Pongsilp *et al.* (2012). Cultivable bacteria were enumerated by the standard plate count method using nutrient agar (NA) medium, isolation medium for prototroph (IMP) and IMP supplemented with 20% soil extracts. IMP was modified from arabinose-gluconate (AG) medium (Sadowsky *et al.*, 1987) and consisted of 1.0% HEPES-MES buffer (pH 6.8), 1.0 g/L arabinose, 1.0 g/L glucose, 1.0 g/L NH_4Cl and 1.0% each of salt solutions (0.7 g/L $FeCl_3 \cdot 6H_2O$, 18.0 g/L $MgSO_4 \cdot 7H_2O$, 1.3 g/L $CaCl_2 \cdot 2H_2O$, 25.0 g/L $NaSO_4$, 32.0 g/L NaCl and 12.5 g/L Na_2HPO_4). Plates were incubated at 30 °C for 2 weeks. Bacterial counts were estimated in 3 replicates. Single colonies on IMP were selected. Pure cultures of prototrophs were maintained on IMP slants at 4 °C. The prototrophic isolates were designated by abbreviations: PRO-BS, PRO-SP and PRO-MP are used to refer to the isolates derived from bulk soil, rhizosphere of a single plant species *E. milii* Des Moul and rhizosphere of multiple plant species *(E. milii* Des Moul, *H. johnsonii* and *C. variegatum*), respectively.

Random amplified polymorphic DNA (RAPD) analysis

Prototrophic isolates recovered from 3 soil samples were selected to investigate genetic diversity based on RAPD fingerprinting. Genomic DNA was extracted from an exponentially grown culture by using a GF-1 Bacterial DNA extraction kit (Vivantis, USA) according to the manufacture's instruction. The polymerase chain reaction (PCR) was carried out using an arbitrary primer RAPD1 (5' GGT GCG GGA A 3') (Nuntagij *et al.*, 1997). RAPD-PCR amplification was carried out as described previously (Pongsilp and Nuntagij, 2009). Negative controls (no DNA added) were included in all sets of reactions. Approximately 2,000 ng of PCR products were loaded into the wells of 1% agarose gels and unweighted pair groups using mathematical averages (UPGMA) dendrograms were constructed using the Image Master 1D Elite Software version 5.20 (GE Healthcare, Munich, Germany). The strains generated individual RAPD patterns were further examined for 16S rRNA gene sequences and plant growth-promoting characteristics.

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Sequence analysis of partial 16S rRNA gene

Genera of prototrophs were identified by sequence analysis of 16S rRNA gene. Partial 16S rRNA gene (approximately 500 bp) of each strain was amplified using a pair of universal primers UN16S 926f (5' AAA CTY AAA KGA ATT GAC GG 3') and UN16S 1392r (5' ACG GGC GGT GTG TRC 3') (Y, C or T; K, G or T; R, A or G) (Lane, 1991). PCR reaction was carried out as described by Pongsilp *et al.* (2002). PCR products were visualized by 1% agarose gel electrophoresis and purified using a Qiaquick gel extraction kit (Qiagen Inc., Valencia, CA). The purified PCR products were sequenced by Bio Basic, Inc. (Markham, Ontario, Canada). The obtained sequences were checked for chimeric sequences by using the Chimera Check program (version 2.7) of the Ribosomal Database Project (RDP). A sequence similarity search was performed by using BLASTN program (http://www.ncbi.nlm.nih.gov/).

Determination of plant growth-promoting characteristics

Prototrophic strains were determined for plant growth-promoting characteristics including the productions of IAA, ammonia and siderophores. IAA, ammonia, catecholate siderophores and hydroxamate siderophores produced by each strain were assayed by the methods of Gordon and Weber (1951), Barnes and Sugden (1990), Arnow (1937) and Csaky (1948), respectively. The concentrations of such substances were determined by comparisons with their own standard curves. The experiments were carried out in 3 replicates.

Statistical analysis

Experimental data was compared by using the SPSS program version 16.0 (SPSS Inc., Chicago, IL).

Soil DNA, culture DNA extraction and 16S rRNA gene amplification

The total soil DNA was extracted from each soil sample by using a PowerSoil™ DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA) according to the manufacture's instruction. Pooled colonies grown on each medium were inoculated into broth of the same medium. The culture DNA was extracted from an exponentially grown culture of pooled colonies by using a GF-1 Bacterial DNA extraction kit (Vivantis, USA) according to the manufacture's instruction. The total soil DNA and culture DNA were amplified by PCR with a MyCycler™ Thermal Cycler (Bio-Rad, Hercules, CA). The variable region 3 (V3) within the 16S rRNA gene [corresponding to positions 341–534 of the 16S rRNA gene in *Escherichia coli*; Keyser *et al.* (2006)] was amplified by using a pair of primers, F341 (5' CCT ACG GGA GGC AGC AG 3') with the GC-clamp (5' CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G 3') and R534 (5' ATT ACC GCG GCT GCT GG 3')]. PCR reactions and conditions were performed as described in Muyzer *et al.* (1993). The amplified products were separated on 1% agarose gels containing ethidium bromide (EtBr) and visualized under UV light.

Denaturing gradient gel electrophoresis (DGGE)

The PCR fragments were separated by using DGGE performed with the BioRad DCode TM Universal Mutation Detection System (Bio-Rad, Hercules, CA). Twenty-five microliters (approximately 2,500 ng) of mixed PCR products from each soil sample and pooled colonies obtained from each medium were applied to 8% polyacrylamide gel with a liner gradient of 25–50% denaturant [100% denaturant corresponds to 40% (vol/vol) of formamide plus 7 M of urea]. Electrophoresis was performed at 200 V for 4 h at a constant temperature of 60 °C. Gels were then stained with GelStar® Nucleic Acid Gel Stain (Cambrex Bio Science, Rockland, ME) for 30 min and visualized. The PCR-DGGE of each sample was performed in 3 replicates. The SPSS program (version 16.0) (SPSS Inc., Chicago, IL) was used to analyze the DGGE patterns. The bands in each sample were scored in a binary matrix based on the presence (1) or the absence (0) of each band. The Pearson correlation index (r) for each pair of lanes within a gel was calculated as a measure of similarity between the community fingerprints. Cluster analysis was performed by applying the Between-groups linkage method to the matrix of similarities.

RESULTS AND DISCUSSION

Determination of soil characteristics

Soil characteristics were determined for 3 soil samples including bulk soil, rhizosphere of a single plant species and rhizosphere of multiple plant species. The results are presented in Table 1. Both pH and moisture content were highest in rhizosphere of a single plant species, followed by rhizosphere of multiple plant species and bulk soil, respectively. Youssef and Chino (1989) suggested that the extent to which rhizosphere pH can differ from that of bulk soil depends mainly on plant species and initial pH of bulk soil. The moisture contents of both rhizospheres were more than that of bulk soil and this may due to dynamics of mucilage exuded by roots (Carminati, 2013). Urease activity in 3 soil samples was not significantly different from each other. Urease is a nickel-containing, multisubunit enzyme that catalyzes the hydrolysis of urea to ammonia and carbonic acid (Morou-Bermudez and Burne, 1999). Urease expression in bacteria is often regulated in response to environmental parameters such as nitrogen availability and pH (Mobley *et al*., 1995). Acid phosphatase activity was highest in rhizosphere of multiple plant species, followed by bulk soil and rhizosphere of a single plant species, respectively. Neutral phosphatase activity in rhizosphere of multiple plant species and rhizosphere of a single plant species was not

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significantly different from each other. Neutral phosphatase activity in rhizosphere of a single plant species and bulk soil was not significantly different from each other. While neutral phosphatase activity in rhizosphere of multiple plant species was significantly higher than that in bulk soil. Alkaline phosphatase activity in rhizosphere of a single plant species and rhizosphere of multiple plant species was not significantly different from each other. Alkaline phosphatase activity in rhizosphere of multiple plant species and bulk soil was not significantly different from each other. While alkaline phosphatase activity in rhizosphere of a single plant species was significantly higher than that in bulk soil. Phosphatase is one of the enzymes able to release phosphorus from organic compounds by dephosphorylation of phospho-ester or phosphoanhydride bonds in organic matter (Rodriguez *et al.*, 2006). Acid-, neutral- and alkaline phosphatases catalyze such a reaction at acid, neutral and alkaline conditions, respectively. It is shown in the previous studies that both urease and phosphatase activities in soils depend on soil type (Renella *et al*., 2007) and plant species (Trasar-Cepeda *et al*., 2008).

Table 1: Characteristics of soil samples.

Soil characteristics	Soil samples		
	bulk soil	rhizosphere of a single plant species	rhizosphere of multiple plant species
pH	6.75 ± 0.06^{a}	7.69 ± 0.02^{c}	7.55 ± 0.02^{b}
Moisture content (%)	8.67 ± 0.72^{a}	51.95 ± 2.45^{c}	16.40 ± 1.08^{b}
Urease activity (unit/g soil)	14.12 ± 2.89^{a}	16.09 ± 3.13^{a}	11.01 ± 2.68^{a}
Acid phosphatase activity (milliunit/g soil)	19.26 ± 3.07^{b}	10.74 ± 2.18^{a}	26.60 ± 2.77^{c}
Neutral phosphatase activity (milliunit/g soil)	14.14 ± 3.46^{a}	25.18 ± 7.64^{ab}	31.86 ± 6.34^{b}
Alkaline phosphatase activity (milliunit/g soil)	19.39 ± 8.02^{a}	50.00 ± 14.91^{b}	36.93 ± 15.58^{ab}
Soil particle-size distribution			
%sand	30.00	29.73	39.40
%silt	17.10	9.20	15.53
%clay	52.90	61.07	45.07

The values shown are the means of 3 replicates ± standard deviations.
Means followed by the same letters in a row are not significantly different.

Isolation and enumeration of cultivable bacteria and prototrophs

Bacterial counts in 3 soil samples obtained from 3 kinds of media are shown in Table 2. NA is a rich medium containing carbon, nitrogen sources and unidentified growth factors. It is used for general-purpose isolation, allowing the growth of many bacteria. The highest cell numbers of total cultivable bacteria were obtained from rhizosphere of multiple plant species and bulk soil, which were not significantly different from each other, but higher than that of rhizosphere of a single plant species. The lower bacterial count in rhizosphere of a single plant species may due to the effect of root exudates on selection of certain bacterial species as observed by Haichar *et al.* (2008). IMP is a minimal medium consisting of carbon, nitrogen sources and mineral salts only. This medium was formulated without amino acids, vitamins and other growth factors. Bacteria able to grow on IMP were thus prototrophs. Cell numbers of prototrophs in 3 soil samples recovered from IMP were significantly different from each other. The highest cell number of prototrophs was obtained from rhizosphere of a single plant species, followed by rhizosphere of multiple plant species and bulk soil, respectively. Prototrophs were presented as a large portion (93.35%) of total cultivable bacteria in rhizosphere of a single plant species. For all soil samples, cell numbers of bacteria recovered from IMP and IMP supplemented with soil extracts were not significantly different from each other. The result suggests that compounds present in soil extracts did not promote overall bacterial growth. The prototrophic isolates were selected from each soil sample for RAPD analysis.

Random amplified polymorphic DNA (RAPD) analysis

Total 28 prototrophic isolates generated particular RAPD profiles with amplified fragments ranging between 200 bp to 5,000 bp in size. These isolates that generated specific individual RAPD patterns could be identified as the individual strains and were selected for further studies. The RAPD patterns are strain dependent and therefore, they are useful in analyzing the intra-specific diversity of several genera in bacterial community and for typing many different organisms (Nimnoi and Pongsilp, 2009). The dendrograms constructed from RAPD profiles of strains derived from bulk soil, rhizosphere of a single plant species and rhizosphere of multiple plant species are shown in Figures 1, 2 and 3, respectively. The genetic diversity of prototrophs in 3 soil samples was similar

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(approximately 5% to 10% similarities). A dendrogram generated from RAPD profiles of 7 strains recovered from bulk soil composed of 2 main clusters. The strains PRO-BS2 and PRO-BS5 generated closest profiles, separating at approximately 58% similarity (Figure 1). Among 10 strains recovered from rhizosphere of a single plant species, the strain PRO-SP1 generated a profile that formed a different cluster in a dendrogram. The closest profiles were observed with the strains PRO-SP6 and PRO-SP9 that linked together at approximately 80% similarity (Figure 2). A dendrogram generated from RAPD profiles of 11 strains recovered from rhizosphere of multiple plant species was separated into 2 main clusters. The most similarity (approximately 63%) was observed with the strains PRO-MP7 and PRO-MP8 (Figure 3).

Table 2: Bacterial counts in 3 soil samples obtained from 3 kinds of media.

Media	Bacterial counts in soil samples (log CFU/g soil)		
	bulk soil	rhizosphere of a single plant species	rhizosphere of multiple plant species
Nutrient agar (NA)	8.00 ± 0.00^{e}	6.01 ± 0.07^{d}	8.08 ± 0.15^{e}
Isolation medium for prototroph (IMP)	5.26 ± 0.24^{a}	5.98 ± 0.14^{cd}	5.73 ± 0.05^{b}
IMP supplemented with soil extracts	5.39 ± 0.12^{a}	5.78 ± 0.12^{bc}	5.86 ± 0.12^{bcd}

The values shown are the means of 3 replicates ± standard deviations.
Means followed by the same letters are not significantly different.

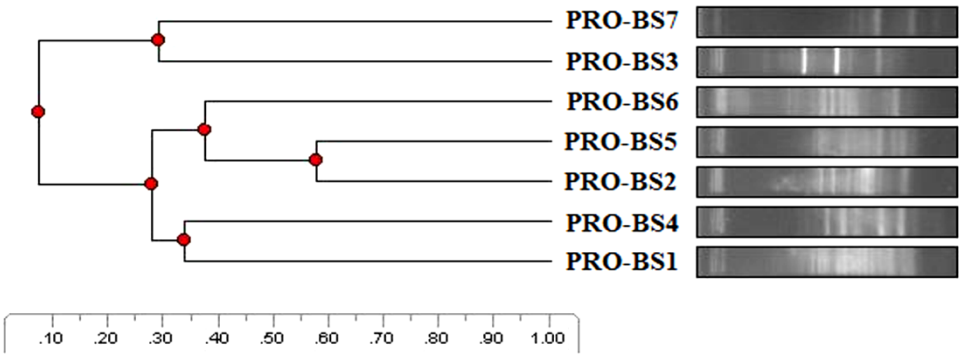

Figure 1: Dendrogram generated from RAPD profiles of the selected bacteria from bulk soil.

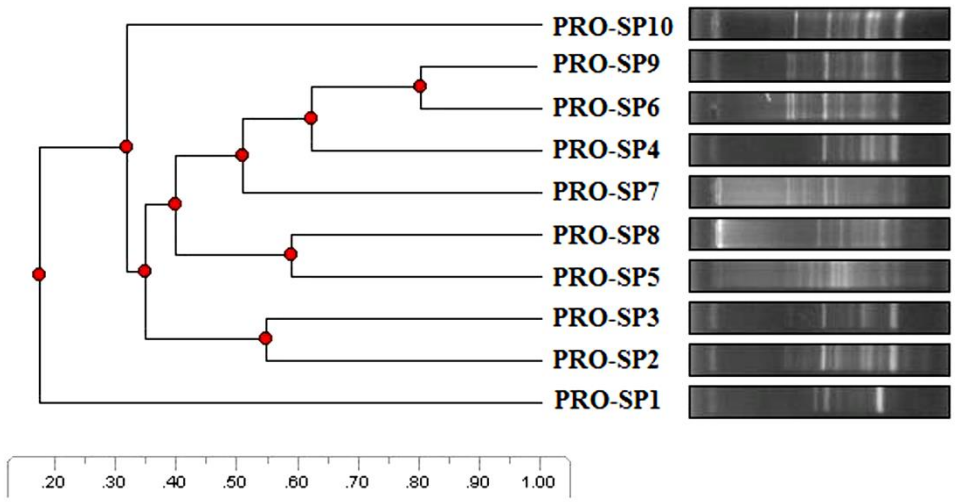

Figure 2: Dendrogram generated from RAPD profiles of the selected bacteria from rhizosphere of a single plant species.

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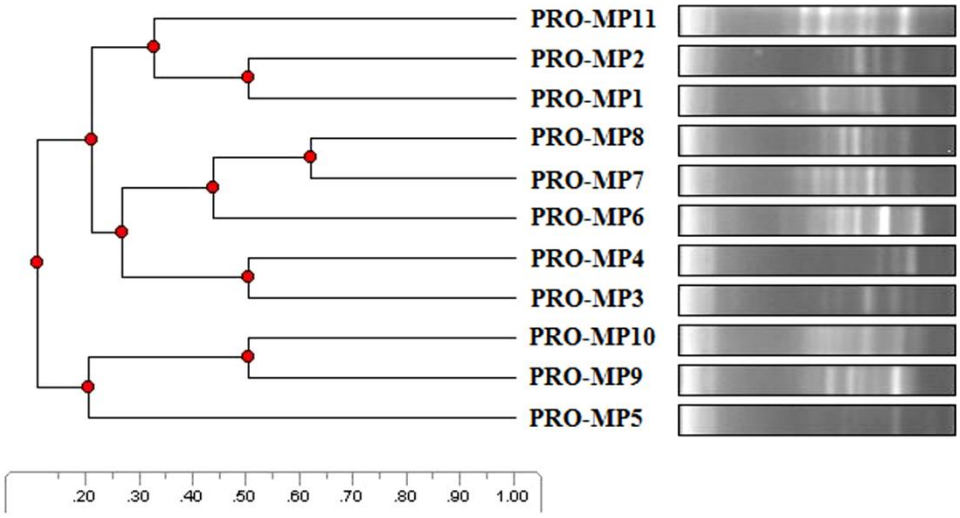

Figure 3: Dendrogram generated from RAPD profiles of the selected bacteria from rhizosphere of multiple plant species.

Sequence analysis of partial 16S rRNA gene

Partial sequences of 16S rRNA gene were obtained from 28 prototrophic strains. These sequences were confirmed to be non-chimeric. The most popular microbial core gene by far has been the single 16S rRNA gene (Pace, 2009), which fits the criteria of ubiquity, regions of strong conservation and regions of hypervariability (Segata and Huttenhower, 2011). Even though the 16S rRNA gene is very useful for estimating the evolutionary relationships and identifying bacterial genera (van Berkum and Eardly, 1998), in some cases, 16S rRNA gene sequencing cannot differentiate among a limited number of genera. Based on the sequences of 16S rRNA gene, the closest genus/genera of each prototrophic strain and percentage of identity are presented in Table 3. Most of them (13 strains including PRO-SP2, PRO-SP3, PRO-SP4, PRO-SP6, PRO-SP7, PRO-SP8, PRO-SP9, PRO-SP10, PRO-MP1, PRO-MP3, PRO-MP7, PRO-MP10 and PRO-MP11) were members of *Pseudomonas* with 97% to 100% identities. Four strains including PRO-BS3, PRO-BS5, PRO-MP5 and PRO-MP8 were very closely related to *Rhizobium* with 97% to 99% identities. *Pseudomonas* and *Rhizobium* have commonly been isolated from soils (Nimnoi and Pongsilp, 2009; Lehmann *et al*., 2011; Doornbos *et al.*, 2012) and proved to exert plant growth-promoting characteristics (Sharma *et al.*, 2003; Nimnoi and Pongsilp, 2009). Three strains including PRO-SP1, PRO-SP5 and PRO-MP4 showed high identities (97% to 99%) with those of *Acidovorax* (the former *Pseudomonas*), *Alicycliphilus* and *Geobacter*. The strains PRO-BS1 and PRO-BS4 were identified as *Chitinophaga* with 99% identity.

Chitinophaga, a chitinolytic bacterium, has been reported to be isolated from several samples such as a soil sample collected from a cliff in Republic of Korea (Lee *et al*., 2009), rhizospheres of rice (*Oryza sativa* L.) (Chung *et al*., 2012), ginseng (Lee and Whang, 2014) and *Arabidopsis thaliana* (Lin *et al*., 2014) as well as roots of *Cymbidium goeringii* (Li *et al*., 2013) and tree trunk of *Pinus pinaster* (Proenca *et al*., 2014). The strain PRO-BS6 was closely related to *Caulobacter*, *Hephaestia* and *Sphingomonas* with 96% identity. The strain PRO-BS7 was closely related to *Arthrobacter*, *Corynebacterium* and *Sinomonas* with 99% identity. The strain PRO-MP2 was closely related to *Bacillus* and *Geobacillus* with 99% identity. The remaining strains PRO-BS2, PRO-MP6 and PRO-MP9 were closely related to *Promicromonospora*, *Chitinimonas* and *Flavobacterium*, respectively, with 97% to 99% identities. The partial 16S rRNA gene sequences of these strains can be retrieved from the GenBank database under accession numbers JX135581-JX135608.

Determination of plant growth-promoting characteristics

The 28 prototrophic strains were characterized for plant growth-promoting characteristics including the productions of IAA, ammonia and siderophores. The results are presented in Table 4. IAA is the main plant growth hormone with auxin activity (Davies, 1995). IAA plays a key role in the control of many physiological processes in plants such as root proliferation (Lambrecht *et al.*, 2000; Patten and Glick, 2002), cell division and shoot growth (Davies, 1995). The capacity to produce IAA is widespread among bacteria that inhabit diverse environments such as soils, fresh and marine waters, and plant and animal hosts (Patten *et al*., 2013). Some bacteria can provide ammonia to environments via ammonification, which can occur from the degradation of nitrogen compounds such as nitrate (Hoffmann *et al.*, 1998), casamino acid, trypticase peptone (McSweeney *et al.*, 1999) and urea (Xing *et al*., 1995). Siderophores are iron chelators, chemically capable of binding and solubilizing iron (Xiao and Kisaalita, 1997). Iron is an essential nutrient for the growth and the proliferation of bacteria (Hotta *et al.*, 2010). Although relatively abundant, in its normal ferric ion (Fe^{3+}) form, it is very insoluble at

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normal pH (Stevens *et al.*, 1999). When grown in iron-deficient conditions, many bacteria can synthesize siderophores and then provide available iron. The productions of IAA, ammonia, catecholate siderophore and hydroxamate siderophore were highest in the strains closely related to *Rhizobium*, *Promicromonospora*, *Pseudomonas* and *Chitinophaga*, respectively. The productions of IAA and siderophore are common characteristics of rhizosphere bacteria such as rhizobia and *Pseudomonas*, which stimulate and facilitate plant growth. A *Promicromonospora* strain isolated from soil was found to exert several characteristics of plant growth-promoting rhizobacteria (PGPR) (Kang *et al*., 2012). The results reveal that the prototrophic strains varied in their abilities to produce these plant growth-promoting substances and some strains possibly exerted positive effects on plant growth and health by providing nutrients and plant hormones.

Table 3: The closest genera and % identities of 16S rRNA gene of the prototrophic strains.

Sources and strains	Accession numbers	Closest genera	% identities of 16S rRNA gene
Bulk soil			
PRO-BS1	JX135581	*Chitinophaga*	99
PRO-BS2	JX135582	*Promicromonospora*	97
PRO-BS3	JX135583	*Rhizobium*	98
PRO-BS4	JX135584	*Chitinophaga*	99
PRO-BS5	JX135585	*Rhizobium*	99
PRO-BS6	JX135586	*Caulobacter*, *Hephaestia* and *Sphingomonas*	96
PRO-BS7	JX135587	*Arthrobacter*, *Corynebacterium* and *Sinomonas*	99
Rhizosphere of a single plant species			
PRO-SP1	JX135588	*Acidovorax*, *Alicycliphilus* and *Geobacter*	99
PRO-SP2	JX135589	*Pseudomonas*	99
PRO-SP3	JX135590	*Pseudomonas*	100
PRO-SP4	JX135591	*Pseudomonas*	99
PRO-SP5	JX135592	*Acidovorax*, *Alicycliphilus* and *Geobacter*	99
PRO-SP6	JX135593	*Pseudomonas*	100
PRO-SP7	JX135594	*Pseudomonas*	99
PRO-SP8	JX135595	*Pseudomonas*	99
PRO-SP9	JX135596	*Pseudomonas*	99
PRO-SP10	JX135597	*Pseudomonas*	99
Rhizosphere of multiple plant species			
PRO-MP1	JX135598	*Pseudomonas*	99
PRO-MP2	JX135599	*Bacillus* and *Geobacillus*	99
PRO-MP3	JX135600	*Pseudomonas*	97
PRO-MP4	JX135601	*Acidovorax, Alicycliphilus* and *Geobacter*	97
PRO-MP5	JX135602	*Rhizobium*	97
PRO-MP6	JX135603	*Chitinimonas*	99
PRO-MP7	JX135604	*Pseudomonas*	99
PRO-MP8	JX135605	*Rhizobium*	99
PRO-MP9	JX135606	*Flavobacterium*	99
PRO-MP10	JX135607	*Pseudomonas*	99
PRO-MP11	KX135608	*Pseudomonas*	99

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Table 4: Plant growth-promoting characteristics of the prototrophic strains.

Sources and strains	Amounts of plant-growth promotors produced by strains			
	IAA (µg/mL)	ammonia (µg/mL)	catecholate siderophore (µmol/L)	hydroxamate siderophore (µmol/L)
Bulk soil				
PRO-BS1	1.07 ± 0.06	9.47 ± 0.23	5.67 ± 0.92	6.83 ± 2.18
PRO-BS2	5.37 ± 0.18	36.88 ± 2.97	4.31 ± 0.45	13.33 ± 5.45
PRO-BS3	2.82 ± 0.54	8.98 ± 1.34	3.04 ± 0.76	4.31 ± 1.57
PRO-BS4	2.35 ± 0.43	11.80 ± 6.87	2.78 ± 0.80	27.58 ± 8.94
PRO-BS5	16.15 ± 0.18	21.95 ± 2.61	2.35 ± 1.12	10.00 ± 5.49
PRO-BS6	0.83 ± 0.25	11.23 ± 1.83	4.57 ± 0.81	7.88 ± 4.09
PRO-BS7	0.38 ± 0.02	28.48 ± 4.45	4.87 ± 0.65	10.46 ± 3.64
Rhizosphere of a single plant species				
PRO-SP1	4.24 ± 0.13	18.09 ± 1.07	2.90 ± 0.63	5.02 ± 3.82
PRO-SP2	0.59 ± 0.10	16.82 ± 0.81	9.57 ± 1.42	5.46 ± 5.36
PRO-SP3	1.09 ± 0.26	16.68 ± 0.53	2.52 ± 0.35	3.43 ± 0.93
PRO-SP4	0.65 ± 0.18	16.50 ± 0.04	3.55 ± 0.84	1.95 ± 0.34
PRO-SP5	0.79 ± 0.26	14.75 ± 0.23	3.09 ± 0.05	0.08 ± 0.05
PRO-SP6	1.14 ± 0.09	16.17 ± 0.70	3.95 ± 1.06	6.82 ± 1.94
PRO-SP7	0.65 ± 0.28	18.66 ± 0.87	6.78 ± 0.54	7.58 ± 0.86
PRO-SP8	2.70 ± 0.66	15.35 ± 0.35	5.39 ± 0.56	7.88 ± 9.86
PRO-SP9	0.80 ± 0.40	17.73 ± 1.92	3.08 ± 0.61	11.52 ± 2.14
PRO-SP10	1.01 ± 0.05	17.65 ± 0.24	2.89 ± 0.17	8.54 ± 3.92
Rhizosphere of multiple plant species				
PRO-MP1	0.94 ± 0.55	18.68 ± 1.27	3.40 ± 0.98	4.85 ± 2.14
PRO-MP2	3.93 ± 0.85	29.54 ± 10.33	4.02 ± 0.75	12.43 ± 1.28
PRO-MP3	0.44 ± 0.16	16.97 ± 1.37	3.55 ± 0.71	3.42 ± 3.03
PRO-MP4	0.54 ± 0.35	20.75 ± 2.04	4.05 ± 0.18	3.22 ± 2.58
PRO-MP5	1.56 ± 0.73	19.95 ± 2.72	2.34 ± 0.45	14.55 ± 5.31
PRO-MP6	2.98 ± 0.05	16.54 ± 0.68	2.56 ± 0.04	7.52 ± 3.76
PRO-MP7	1.36 ± 0.64	16.62 ± 0.52	6.02 ± 0.23	6.34 ± 5.50
PRO-MP8	12.15 ± 0.34	27.33 ± 1.26	3.09 ± 1.35	4.02 ± 3.84
PRO-MP9	0.16 ± 0.13	14.34 ± 0.55	2.74 ± 0.05	2.57 ±1.07
PRO-MP10	1.72 ± 0.10	18.63 ± 0.98	3.01 ± 0.04	2.12 ± 1.98
PRO-MP11	1.10 ± 0.23	17.76 ± 0.66	8.41 ± 4.46	7.27 ± 4.83

The values shown are the means of 3 replicates ± standard deviations.

DGGE community fingerprinting of soil samples

Total bacterial DNA was directly extracted from 3 soil samples and the V3 region of 16S rRNA gene was amplified. The V3 region was found to be hypervariable and most suitable for distinguishing all bacteria to the genus level except for closely related enterobacteriaceae (Chakravorty *et al*., 2007). The identical patterns were obtained from 3 replicates of the PCR-DGGE of each sample (Figure not shown). The different DGGE patterns were displayed in Figure 4. These patterns exhibited the dominant species and the intensity of each band indicated its relative abundance. As soil characteristics and plant species have been reported as major factors in determining bacterial community structure (Kowalchuk *et al.*, 2002; Marschner *et al.*, 2004; Nimnoi *et al.*, 2010; Pongsilp *et al.*, 2012), the difference in bacterial community obtained from these soil samples was possibly affected by both soil characteristics and plants. The DGGE pattern of total bacterial DNA in bulk soil (lane 1 Figure 4) exhibited 8 bands with almost equal intensity. Similarly, the DGGE pattern of total bacterial DNA in rhizosphere of a single plant species consisted of 2 stronger bands and 6 less intensive bands (lane 2 Figure 4). The DGGE pattern of total bacterial DNA in rhizosphere of multiple plant species consisted of 1 stronger band and 5 less intensive bands (lane 3 Figure 4). The result indicates that a few dominant ribotypes were present in both rhizospheres but not in bulk soil, suggesting the specific promotion of root exudates on the growth of certain bacterial species in rhizospheres. The specific promotion may result from kinds of nutrients and energy sources in root exudates that can be competitively utilized by some bacterial species.

For clustering analysis, a dendrogram generated from the DGGE patterns (Figure 5) revealed the different bacterial community structures in these soil samples. Both rhizospheres cladded together at a Euclidean distance of 4,

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separating from bulk soil. Both rhizospheres and bulk soil linked together at a Euclidean distance of 21. It is obvious that the community structure in bulk soil was distantly divergent from those of both rhizospheres. This is a strong evidence showing the influences of root exudates and plant species on bacterial community structure. Several factors have been reported for their strong influences on soil bacterial community. These factors include plant species, amount and composition of root exudates as well as soil characteristics, soil fertility and locations (Smalla *et al.*, 2001; Marschner *et al.*, 2004; Costa *et al.*, 2006; Ge *et al*., 2010; Nimnoi *et al.*, 2010; Pongsilp *et al.*, 2012).

DGGE community fingerprinting of cultivable bacteria

Three kinds of media, including NA, IMP and IMP supplemented with soil extracts, were used for enumeration and cultivation of bacteria in different groups. The total DNA was extracted from pooled colonies grown on each medium. The V3 region of 16S rRNA gene was amplified and PCR-DGGE was performed. As displayed in Figure 4, the DGGE patterns of cultivable bacteria (lanes 4–12) showed particular fingerprints corresponding to kinds of media and soil samples. The differences in genetic diversity and community of cultivable bacteria were demonstrated. The DGGE patterns of cultivable bacteria consisted of multiple bands, in which several bands were much more intensive than those of soil samples. The result suggests that these media were useful for isolation and enrichment of some bacterial groups. The DGGE patterns of total cultivable bacteria in bulk soil (lane 4 Figure 4), rhizosphere of a single plant species (lane 5 Figure 4) and rhizosphere of multiple plant species (lane 6 Figure 4) consisted of 7, 4 and 3 stronger bands, respectively. Several fainter bands were present in the DGGE patterns of total cultivable bacteria in all soil samples (lanes 4-6 Figure 4). Certain bacterial ribotypes in each soil sample were found to be uncultivable as several bands of the DGGE patterns of total bacterial DNA disappeared in the DGGE patterns of total cultivable bacteria (Figure 4, compare lane 1 to lane 4; lane 2 to lane 5; lane 3 to lane 6). IMP showed the selectivity for specific groups of bacteria. In bulk soil, the DGGE pattern of cultivable bacteria recovered from IMP was totally different from that of total cultivable bacteria (Figure 4, compare lane 4 to lane 7). In both rhizospheres, a few ribotypes of total cultivable bacteria remained and several fainter bands appeared in the DGGE patterns of cultivable bacteria recovered from IMP (Figure 4, compare lane 5 to lane 8; lane 6 to lane 9). The effect of soil extracts on cultivable bacteria was most obviously observed in bulk soil as all bands of the DGGE pattern of cultivable bacteria recovered from IMP were different from the DGGE pattern of cultivable bacteria recovered from IMP supplemented with soil extract (Figure 4, compare lane 7 to lane 10). While 6 and 4 bacterial ribotypes in the DGGE patterns of cultivable bacteria recovered from IMP in rhizosphere of a single plant species and rhizophere of multiple plant species, respectively, remained in the DGGE patterns of cultivable bacteria recovered from IMP supplemented with soil extracts (Figure 4, compare lane 8 to lane 11; lane 9 to lane 12). Even though all major bands present in the DGGE patterns of cultivable bacteria in both rhizospheres recovered from IMP remained in the DGGE patterns of cultivable bacteria recovered from IMP supplemented with soil extract, some faint bands present in the DGGE pattern of cultivable bacteria recovered from IMP disappeared and a few additional bands occurred upon the addition of soil extract to IMP (Figure 4, compare lane 8 to lane 11; lane 9 to lane 12). These results indicate the influence of soil extract on bacterial community.

A dendrogram constructed from clustering analysis (Figure 5) presented the levels of similarities among samples. Total cultivable bacteria of 3 soil samples recovered from NA claded together at a Euclidean distance of 20. As NA allowed the growth of bacteria in diverse groups, total cultivable bacteria of 3 soil samples recovered from NA should represent diverse bacterial ribotypes that were more similar to each other than to any other samples. However, high Euclidean distances indicated that these total cultivable bacteria were not relatively close to each other. The positions in a dendrogram indicated that total cultivable bacteria recovered from NA were not closely related to their total bacterial communities, suggesting that uncultivable bacteria were considerable populations in all soil samples. The DGGE patterns of total bacterial DNA in bulk soil and cultivable bacteria in bulk soil recovered from IMP supplemented with soil extract claded together, separating from the DGGE pattern of cultivable bacteria in bulk soil recovered from IMP. The result suggests that IMP shaped the community of cultivable bacteria, while soil extract partially retrieved the community of cultivable bacteria to be close to that of total bacterial DNA. The closest neighbors in a dendrogram were cultivable bacteria in rhizosphere of a single plant species recovered from IMP and cultivable bacteria in such a soil sample recovered from IMP supplemented with soil extract. Both DGGE patterns claded together at the lowest Euclidean distance of 1, separating from total bacterial DNA and total cultivable bacteria in the same soil sample. In this case, soil extract slightly affected the bacterial community. For rhizosphere of multiple plant species, the DGGE patterns of cultivable bacteria recovered from IMP and cultivable bacteria recovered from IMP supplemented with soil extract linked together at a Euclidean distance of 14, indicating the influence of soil extract on bacterial community.

The chemical analysis revealed that natural soil extract is composed of organic carbons and inorganic ions including ammonium, nitrate, nitrite, phosphate, potassium, sodium, calcium, magnesium and iron (III) (Lorenz and Wackernagel, 1991). It has been reported that soil extract can replace some ingredients in media used for enumeration and isolation of specific bacteria (Aagot *et al.*, 2001). Even though the widely accepted medium for viable counts of bacteria from soil is soil extract agar, a major drawback is its variability because of the use of different soils by different investigators (Larkin,

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1972). The deficiency of nutrients and growth factors in soil extracts has also been reported in previous studies (Bhagwat and Keister, 1992; Lorenz and Wackernagel, 1992). Sørheim *et al.* (1989) demonstrated that the carbon source of a general soil extract medium markedly influenced the bacterial diversity. In this study, the different levels of influence of soil extracts on bacterial community were observed. This difference may due to amount and composition of nutrients and growth factors present in soil extract as well as nutrient requirement of cultivable bacteria present in soil. When compare the community structures of cultivable bacteria grown on a minimal medium with those of cultivable bacteria grown on such medium supplemented with soil extracts, the most influence of soil extracts on bacterial community was observed with bulk soil, followed by rhizosphere of a multiple plant species. While community structures exhibited almost the same in the DGGE patterns associated with rhizosphere of a single plant species.

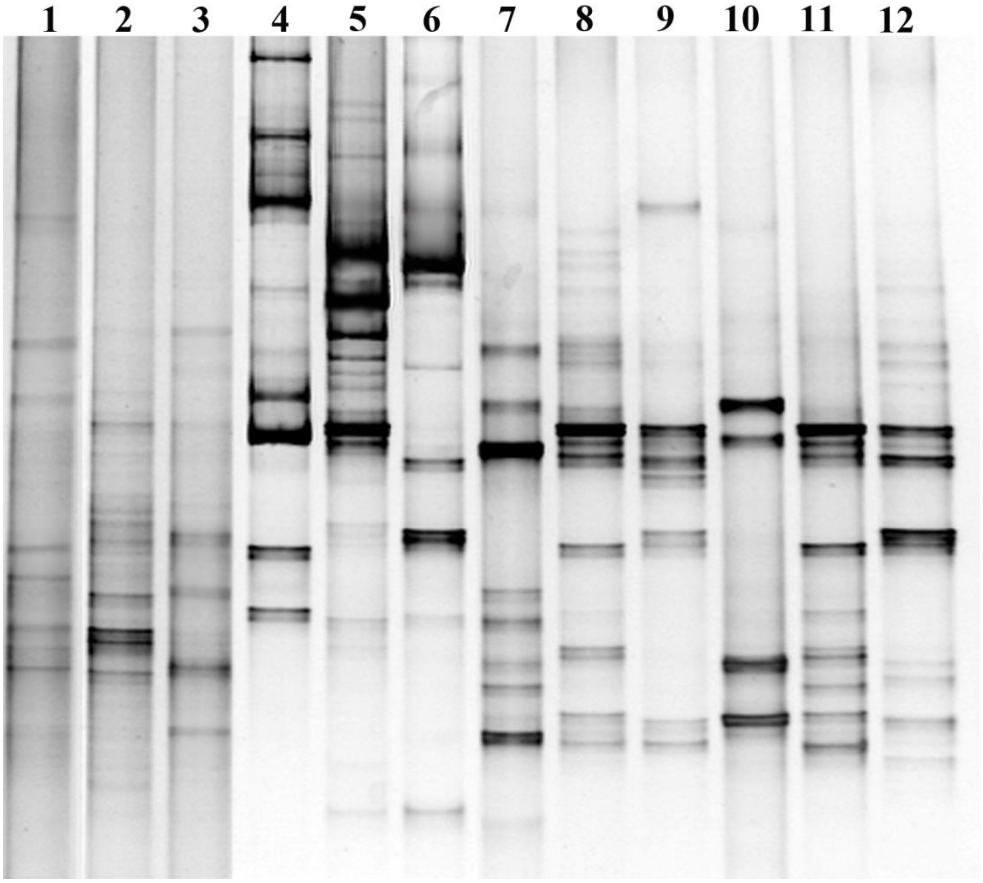

Figure 4: DGGE patterns of soil samples and pooled colonies recovered from 3 kinds of media
Lane 1, bulk soil; 2, rhizosphere of a single plant species; 3, rhizosphere of multiple plant species; 4, cultivable bacteria in bulk soil recovered from NA; 5, cultivable bacteria in rhizosphere of a single plant species recovered from NA; 6, cultivable bacteria in rhizosphere of multiple plant species recovered from NA; 7, cultivable bacteria in bulk soil recovered from IMP; 8; cultivable bacteria in rhizosphere of a single plant species recovered from IMP; 9, cultivable bacteria in rhizosphere of multiple plant species recovered from IMP; 10, cultivable bacteria in bulk soil recovered from IMP supplemented with soil extract; 11, cultivable bacteria in rhizosphere of a single plant species recovered from IMP supplemented with soil extract; 12, cultivable bacteria in rhizosphere of multiple plant species recovered from IMP supplemented with soil extract.

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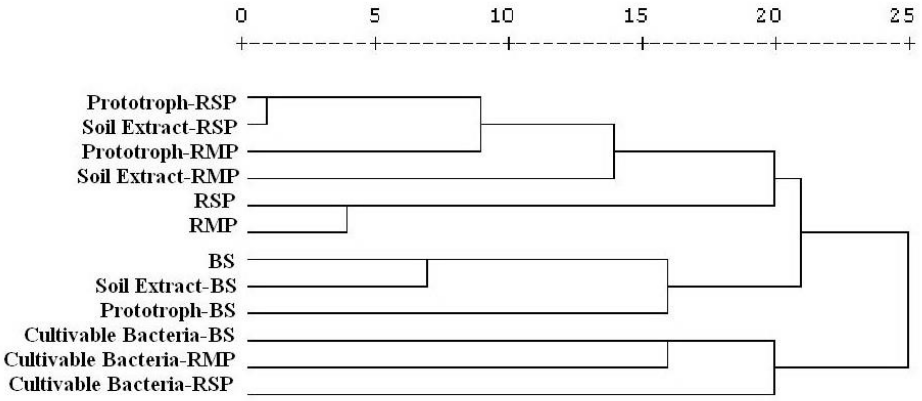

Figure 5: Cluster analysis of DGGE patterns of 3 soil samples and cultivable bacteria recovered from 3 kinds of media
Dendrogram generated by using Ward's cluster analysis. Scale indicates Euclidean distance. BS, bulk soil; RSP, rhizosphere of a single plant species; RMP, rhizosphere of multiple plant species; Cultivable Bacteria-BS, cultivable bacteria in bulk soil recovered from NA; Cultivable Bacteria-RSP, cultivable bacteria in rhizosphere of a single plant species recovered from NA; Cultivable Bacteria-RMP, cultivable bacteria in rhizosphere of multiple plant species recovered from NA; Prototroph-BS, prototrophs in bulk soil recovered from IMP; Prototroph-RSP, prototrophs in rhizosphere of a single plant species recovered from IMP; Prototroph-RMP, prototrophs in rhizosphere of multiple plant species recovered from IMP; Soil Extract-BS, cultivable bacteria in bulk soil recovered from IMP supplemented with soil extract; Soil Extract-RSP, cultivable bacteria in rhizosphere of a single plant species recovered from IMP supplemented with soil extract; Soil Extract-RMP, cultivable bacteria in rhizosphere of multiple plant species recovered from IMP supplemented with soil extract.

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