Rhizosphere effect has no effect on marker genes related to autotrophic CO₂ fixation in paddy soils?

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Abstract

Purpose The rhizosphere is a hotspot for microbial activities as well as microbial ecology studies. This study aimed to explore the “rhizosphere effect” on marker genes of CO₂ fixation autotrophic microorganisms.

Materials and methods Microcosm experiments were conducted using two different types of paddy soil, planted with rice and added with urea. At tillering and ripening stages, bulk and rhizosphere soils were sampled separately for DNA extraction and quantitative PCR analyses.

Results and discussion Six marker genes (*cbbLG*, *cbbLR*, *cbbM*, *acIB*, *oorA*, *accA*) of three autotrophic pathways (the Calvin cycle, the reductive tricarboxylic acid cycle, and the 4-hydroxybutyrate cycle) were detected, suggesting their pervasiveness in paddy soil. Redundancy analysis and variation partitioning based on partial redundancy analysis revealed higher contributions of growth stage (explaining 37 %) and soil type (explaining 19 %) on the variation of these genes, lower but significant impact of rhizosphere effect (explaining 12 %), and no significant effect of urea addition ($P>0.05$).

Conclusions These results suggested that the composition of CO₂ fixation autotrophic microorganisms in the paddy soil was subject to combined actions of soil type and growth stage as well as rhizosphere effect.

Keywords CO₂ fixation · Marker genes · Paddy soil · Rhizosphere effect

1 Introduction

Over the past 60 years, five CO₂ fixation pathways have been found successively (Berg 2011). The Calvin cycle (Benson et al. 1950), occurring in plants as well as in photo- and chemoautotrophic bacteria, is known to be the major pathway for CO₂ fixation. The key enzyme in this cycle is the ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), and its large-subunit coding gene, *cbbL*, has been used as a marker gene ubiquitously in microbial ecology studies (Alfreider et al. 2003; Selesi et al. 2005; Xiao et al. 2014; Yuan et al. 2012; Watson and Tabita 1997). The reductive tricarboxylic acid (rTCA) cycle (Evans et al. 1966), found in microaerophiles and anaerobes, reverses the reactions of the oxidative citric acid cycle (Krebs cycle). The key enzymes involved are fumarate reductase, ferredoxin-dependent 2-oxoglutarate synthase, and ATP-citrate lyase, and two marker genes, *acIB* for the β -ATP citrate lyase (Campbell et al. 2003) and *oorA* for the 2-oxoglutarate: acceptor oxidoreductase (Campbell and Cary 2004), have been developed. The reductive acetyl-CoA pathway (Wood et al. 1986), found in strictly anaerobic bacteria and archaea, is thought to be the oldest pathway among the five (Berg 2011). The key enzyme is CO dehydrogenase/acetyl-CoA synthase, due to shared pathways between autotrophic and heterotrophic acetogens, it is hard to find marker genes to distinguish them. The 3-hydroxypropionate/methyl-CoA cycle (Holo 1989) occurs in

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some green non-sulfur bacteria of the family *Chloroflexaceae*. Although some primers targeting key enzyme biotin-dependent acetyl-CoA have been developed (Auguet et al. 2008), they were found to be of low specificity. The 3-hydroxypropionate/4-hydroxybutyrate and dicarboxylate/4-hydroxybutyrate cycle (named together as the 4-hydroxybutyrate cycle) (Berg et al. 2007; Huber et al. 2008) were detected in aerobic *Crenarchaeota* like *Acidianus*, *Metallosphaera*, and *Sulfolobales* and anaerobic *Crenarchaeota* like *Ignicoccus*, *Thermoproteus*, and *Pyrolobus*. The marker gene *accA*, targeting acetyl-CoA carboxylase, has been used and well described in marine microbial ecology researches (Hu et al. 2013; Yakimov et al. 2009; Hu et al. 2011).

The rhizosphere is defined as the volume of soil adjacent to and influenced by plant root, primarily through the release of root exudates, such as oxygen, carbonic acids, amino acids, or sugars (Rovira 1969; Sørensen 1997; Schröder and Hartmann 2003). Regardless of soil or plant type, rhizosphere conditions sustain microbial communities distinctive from those found in the bulk soil, which is termed as the “rhizosphere effect” (Rovira 1956; Buchan et al. 2010). The rhizosphere is different from bulk soil in terms of physiochemical properties, microbial composition, as well as activities (Jia et al. 2013). The paddy soil is an ideal model system for the description and prediction of microbiological and biogeochemical processes, due to its well-defined micro-scale chemical gradient, presence of extensive root system, and the resulting differential spatial distribution of functional groups of microorganisms, and also their temporal development during the growth season (Liesack et al. 2000; Chen et al. 2008). Very little, however, is known regarding the abundance and composition of populations of CO₂ fixation autotrophic microorganisms in bulk and rhizosphere soils in the paddy soil.

Therefore, this study aimed to explore the “rhizosphere effect” on marker genes of CO₂ fixation autotrophic microorganisms. Six common marker genes for three pathways (the Calvin cycle, the reductive tricarboxylic acid cycle, and the 4-hydroxybutyrate cycle) were used for quantitative PCR (qPCR), and statistical analyses were performed to explore the rhizosphere effect on CO₂ fixation autotrophic microorganisms.

2 Materials and methods

2.1 Microcosm experiments

Original soil samples (0–20 cm) were collected from two distinct sites in South China, located in Leizhou (LZ, clay) and Yingtan (YT, clay loam), as described in Table 1 and previous study (Xiao et al. 2014). Prior to the start of the experiment, the soils were air-dried then homogenized and

Table 1 Two types of paddy soil in this study

	Location	Parent material	Soil type (USDA soil classifications)
LZ	N20°33'57.9" E110°04'25.2"	Latosol	Clay
YT	N28°12'22.2" E116°56'02.2"	Quaternary red clay	Clay loam

Abbreviation: LZ, Leizhou; YT, Yingtan

sieved (<2 mm). After that, the soils were incubated at 105 % water holding capacity (WHC) at 25 °C for 1 month. Then root bags (30-μm nylon mesh, 7.5-cm diameter, 12-cm height), filled with 475 g sieved soil, were placed in the center of PVC pots (15-cm diameter, 23-cm height), which were then filled with 3 kg soil. Rice seeds (cv. Xiangzaoshan 45) were sterilized in 30 % H₂O₂ for 10 min and thoroughly washed with de-ionized water. After 3 days of germination, uniform seedlings were transplanted into the root bags and incubated in a greenhouse (illumination 1,500 lx, ambient temperature 28 °C, 16 °C night; humidity, ambient ~90 %). The base fertilizer with N (100 mg N kg⁻¹ soil, CO(NH₂)₂), P, and K (200 mg K₂HPO₄·3H₂O kg⁻¹ soil) was applied for all soils. Urea (CO(NH₂)₂), a common fertilizer in agricultural lands of China, was additionally applied during the experiment (400 mg N kg⁻¹ soil), and controls without urea addition were setup. Each treatment was conducted in triplicates. At two of the rice growth stages, tillering stage (50 days after transplanting) and ripening stage (113 days after transplanting), rhizosphere and bulk soils were separately sampled and immediately stored at -80 °C for following analyses.

2.2 Microbial DNA extraction

Genomic DNA was extracted from soil samples using the FastDNA SPIN Kit for Soil (Qbiogene, USA) following the manufacturer's manual. The integrity and yield of extracted nucleic acids were checked by agarose gel electrophoresis and UV quantification (NanoDrop ND-1000, Germany).

2.3 Quantification of genes

For the Calvin cycle, quantitative PCR (qPCR) was performed using primer pairs *cbbLG1F/cbbLG1R* for the green-like ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) genes (*cbbLG*), *cbbLR1F/cbbLR1R* for the red-like RubisCO form I gene (*cbbLR*) (Selesi et al. 2005), and *cbbM-F/cbbM-R* for the RubisCO form II gene (*cbbM*) (Alfreider et al. 2003). For the rTCA pathway, qPCR was performed using primer pairs *acIB-892F/acIB-1204R* for the β-ATP citrate lyase *acIB* gene (Campbell et al. 2003) and *oorA-67F/oorA-345R* for the

2-oxoglutarate: acceptor oxidoreductase *oorA* genes (Campbell and Cary 2004). For the 4-hydroxybutyrate cycle, Crena_529F/Crena_981R was used for the *Crenarchaeal* carboxylase *acca* genes (Yakimov et al. 2009; Hu et al. 2013).

PCR cloning products were ligated into the pGEM-TEasy Vector (Promega, USA) and transformed into *Escherichia coli* JM109 competent cells following the manufacturer's manual. Positive clones were selected for sequencing, and those with the target gene insert were used to extract plasmid DNA with a MiniBEST Plasmid Purification Kit (TaKaRa, Japan). The plasmid DNA concentration was determined by a UV-vis spectrophotometer (NanoDrop Technologies, Germany), and the copy numbers of gene were calculated accordingly. Ten-fold serial dilutions of a known copy number of the plasmid DNA were used to generate an external standard curve. The reaction was performed in a 25 μ l volume containing 12.5 μ l SYBR Premix ExTaq (TaKaRa Biotechnology, Japan), 1 μ M of each primer and 1 μ l of five-fold dilution DNA template (1–10 ng μ l⁻¹). Amplification conditions were similar to that in original references except that number of cycles was increased to 45, and a plate that reads at 83 °C was added (Bio-Rad iQ5, USA). The assay efficiency was 95–105 % with R^2 values more than 0.997. Melting curve analysis was performed in the end to check the specificity of amplification products.

2.4 Statistical analysis

The effects of experimental variables (rhizosphere effect, soil type, growth stage, urea addition) on the microbial composition were explored by redundancy analysis (RDA) using Canoco 4.5 for Windows (Microcomputer Power, USA). RDA was chosen according to the result of detrended correspondence analysis. RDA and partial redundancy analysis with manual forward selection were performed, and significance of Monte Carlo permutation test was defined at $P < 0.05$ (Guo et al. 2013).

3 Results

3.1 Abundance of six marker genes

For the Calvin cycle, the abundances of *cbbL* genes (*cbbLG*, *cbbLR*, and *cbbM*) of the Calvin cycle were shown in Fig. 1, and the copy numbers of three genes followed the order: *cbbLR* > *cbbM* > *cbbLG*, at 10^4 , 10^6 , 10^7 copies g⁻¹ soil, respectively. Gene copies were generally higher in ripening stage than that in tillering stage except for *cbbLG*.

For the reductive tricarboxylic acid cycle, copies of *oorA* (above 10^6 copies g⁻¹ soil) were approximately one magnitude higher than that of *aclB* (above 10^5 copies g⁻¹ soil) (Fig. 2a, b). Copy numbers of both genes were higher at

Fig. 1 Abundance of marker genes of the Calvin cycle. **a** for *cbbLG*, **b** for *cbbLR*, **c** for *cbbM*. Error bars represent standard errors of triplicate samples. Abbreviation: C control, N urea addition, B bulk soil, R rhizosphere soil

ripening stage than at tillering stage, and the abundance of *aclB* was correlated positively with that of *oorA* ($r=0.984$, $P < 0.01$).

For the 4-hydroxybutyrate cycle, copy numbers of *acca* were generally above 10^5 copies g⁻¹ soil. The highest *acca* gene copies appeared in bulk soil of Leizhou without urea addition (Fig. 3), reaching 1.11×10^6 copies g⁻¹ soil and $1.81 \times$

Fig. 2 Abundance of marker genes of the reductive tricarboxylic acid (rTCA) cycle. **a** for *acIB*, **b** for *oorA*. Error bars represent standard errors of triplicate samples. Abbreviation: C control, N urea addition, B bulk soil, R rhizosphere soil

10^6 copies g^{-1} soil at tillering stage and ripening stage, respectively. Similarly, higher copies of *accA* were detected at ripening stage.

Fig. 3 Abundance of marker gene (*accA*) of the 4-hydroxybutyrate cycle. Error bars represent standard errors of triplicate samples. Abbreviation: C control, N urea addition, B bulk soil, R rhizosphere soil

3.2 Relationships between gene abundances and environmental variables

Redundancy analysis (RDA) showed that 68 % of the variation in gene abundance could be explained by environmental variables (Fig. 4a). Variation partitioning based on partial redundancy analysis (pRDA) showed that growth stage, soil type, and rhizosphere effect all contributed significantly ($P < 0.05$) to variations of gene abundance, explaining 37 %, 19 %, and 12 % of total variations, respectively (Fig. 4b), whereas urea addition had no significant effect ($P > 0.05$).

4 Discussion

Various autotrophic pathways reflect the diversity and adaptation of organisms in different environments in the process of the Earth evolution (Berg 2011). To date, most studies have

Fig. 4 Redundancy analysis of gene abundances (**a**) and Venn Diagram of variation partitioning based on partial redundancy analysis (**b**). The open and closed symbols represent tillering stage and ripening stage, respectively. Abbreviation: L Leizhou, Y Yingtan, C control, N urea addition, B bulk soil, R rhizosphere soil

emphasized on the Calvin cycle, while few studies covered other alternative pathways at the same time (Kellermann et al. 2012). The present study detected three prevailing autotrophic pathways in paddy soils using six common marker genes. Similar to our previous study, gene copy numbers of *cbbLR* were generally higher than those of *cbbLG* and *cbbM*, it could be speculated that generally *cbbLR*-bearing bacteria were dominant in paddy soils compared with microorganisms harboring other two *cbbL* genes (Xiao et al. 2014). While quantification of microbial populations is important in many aspects of microbial ecology (He et al. 2007), it is hard to calculate their exact numbers based on gene copies as some cell might carry more than one copy of the gene, and some bacteria, like *Ammonifex degensii* and *Ferroglobus placidus*, possess two or more autotrophic pathways concurrently (Markert et al. 2007; Berg 2011). Nevertheless, copy numbers of these marker genes were comparable to that of marker genes of many known important processes in paddy soils, such as *amoA*, *nirK*, and *pmoA*, indicating their potential important role in soil nutrient cycling (Xiao et al. 2014).

The composition of microorganisms in soils is subject to many environmental factors, such as soil properties, plant type, rhizosphere effect, and growth time (Grayston et al. 1998; Markert et al. 2007; Jia et al. 2013; Smalla et al. 2001; Chen et al. 2010). In our experiment, growth stage, soil type, and rhizosphere effect were found to affect the variations of abundances of six marker genes (Fig. 4a, b). In comparison with growth stage and soil type, rhizosphere effect contributed relatively lower (Fig. 4b), but its role could not be ignored, especially in paddy soils. In flooded paddy soils, although oxygen depletes rapidly in a few millimeters beneath the soil surface, it can leak from aerenchymatous tissue in rice roots, creating an oxic rhizosphere differing from the surrounding bulk soils (Revsbech et al. 1999; Chen et al. 2008). Oxygen affected the activity of enzymes involved in microbial CO₂ fixation, like ferredoxin-dependent 2-oxoglutarate synthase and ATP-citrate lyase, which were quite sensitive to oxygen, while ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) was known for its resistance to oxygen (Berg 2011). Moreover, during the growth period, nutrient uptake, root exudates, and oxygen release can change over space and time (Revsbech et al. 1999; Ramesh and Chandrasekaran 2004; Zhang et al. 2006), affecting microbial composition directly or indirectly. Soil type, deriving from combined effects of parent material, biology, time, climate, and topography, always influences microbial population size and community structure and sometimes even acts as dominant factor irrespective of rice-planted or unplanted in paddy soil (Chen et al. 2010). In contrast with previous studies on gene abundance like 16S rDNA and *amoA* (Zhang et al. 2012; Chen et al. 2008), urea addition showed no significant effect on variations of gene abundance in our study (Fig. 4a). Application of inorganic fertilizers generally enhanced growth

of rice crops and accumulation of soil organic carbon through increasing root turnover and rhizodeposition, and thus influenced abundance and composition of microorganisms in paddy soils (Zhong and Cai 2007). However, such an effect was usually indirect and depended on the simultaneous usage of other element such as phosphate (P) or potassium (K) (Zhong and Cai 2007). In fact, most microbial parameters were found to be mainly correlated with soil organic carbon content rather than P and N (Zhong and Cai 2007; Xiao et al. 2014; Yuan et al. 2012). Another possible explanation is that nitrogen was not limiting for these CO₂ fixation autotrophic microorganisms in these soils, as the base fertilization used in this experiment (100 mg N kg⁻¹ soil) might provide enough available nitrogen to them (Marschner et al. 2001).

5 Conclusions

In summary, this study showed variations of marker genes for three autotrophic pathways in paddy soils at different growth stages. Statistical analysis revealed higher contributions of growth stage and soil type on the variation of gene abundances and also lower but significant impact of rhizosphere. It can be concluded that the CO₂ fixation autotrophic microorganisms in the paddy soil were affected by complex interactions between soil type, growth stage, and rhizosphere effect.

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