Diversity of aerobic anoxygenic phototrophic bacteria in paddy soil and their response to elevated atmospheric CO$_2$

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Summary

Aerobic anoxygenic phototrophic bacteria (AAnPB) are recognized as an important group driving the global carbon cycling. However, the diversity of AAnPB in terrestrial environment remains largely unknown as well as their responses to the elevated atmospheric CO$_2$. By using culture-independent techniques, the diversity of AAnPB in paddy soil and the changes in response to the rising atmospheric CO$_2$ were investigated within China FACE (Free-air CO$_2$ enrichment) platform. There was a phylogenetically diverse AAnPB community with large population size residing in paddy soil. The community structure of AAnPB in bulk and rhizospheric soils stayed almost identical, while the population size was higher in rhizospheric (2.0–2.5 $\times$ 10$^8$ copy number of $pufM$ genes g$^{-1}$ dry weight soil (d.w.s.)) than that in bulk (0.7–0.8 $\times$ 10$^8$ g$^{-1}$ d.w.s.) soils. Elevated atmospheric CO$_2$ appeared to significantly stimulate AAnPB abundance (up to 1.4–1.5 $\times$ 10$^8$ g$^{-1}$ d.w.s.) and result in a higher AAnPB percentage in total bacterial community (from 0.5% up to 1.5%) in bulk soil, whereas no significant effect was observed in rhizospheric soil. Our results would extend the functional ecotypes of AAnPB and indicate that environmental changes associated with the rising atmospheric CO$_2$ might affect AAnPB community in paddy soil.

Introduction

It was widely recognized that purple phototrophic bacteria (PPB) thrive only in anoxic environment, and oxygen inhibits the expression of light harvesting system, which hampers their growth (Pfennig, 1967; Bauer and Bird, 1996). However, this concept has been changed by the discovery of aerobic bacteriochlorophyll $\alpha$-containing bacteria similar to known PPB. These obligately aerobic species of bacteria are termed as aerobic anoxygenic phototrophic bacteria (AAnPB) (Shimada, 1995; Yurkov and Beatty, 1998; Kolber et al., 2001). Sequencing analysis of 16S rRNA and light harvesting genes suggested that AAnPB have evolved from PPB (Xiong et al., 2000) and they share the conservative regions of $pufM$ gene encoding the reaction centre of light harvesting system (Nishimura et al., 1996; Yurkov and Beatty, 1998). However, the growth of AAnPB does not strictly depend on light. The oxidative phosphorylation is the major pathway for AAnPB propagation. Light energy is utilized by AAnPB only for reducing energy requirements of growth (Beatty, 2002). Due to the highly heterogeneous constituents of AAnPB and no selective medium for culture-reliant approaches, the $pufM$ gene is widely used as a biomarker for investigating the biogeographical distribution of AAnPB in nature (Waidner and Kirchman, 2008).

AAnPB are found in a wide variety of environments (Yurkov, 2006) and thought to be of ecological significance (Kolber et al., 2001; Jiao et al., 2007). Most researches have focused on the ecophysiological distribution of AAnPB in marine ecosystem, and little is known in terrestrial environment. Paddy field may represent the most important agricultural ecosystems in Asia since rice is the main source for food supply. Flooding management makes paddy field a unique aquatic system because of the co-occurrence of both anoxic and oxic niches. For instance, during rice growing period, soil is mainly characterized as an anaerobic system, while there are oxic zones such as rhizospheric and oxidized surface soils (Roger, 1996). In addition, soil is basically oxic during wheat growing and winter fallow seasons. Therefore, paddy soil could be an ideal environment for the growth of AAnPB. In the meantime, AAnPB require more organic carbon for growth due to the oxidative phosphorylation pathway when compared with the phototrophic lifestyle of other anoxygenic phototrophs. In fact, AAnPB outcompete other phototrophs, become the dominant anoxygenic
phototrophs in oceans and are responsible for the ocean’s carbon cycling (Kolber et al., 2000; 2001). This new physiological group of bacteria might thus play an important ecological role in carbon cycling in paddy soil (Beaty, 2002).

Atmospheric CO$_2$ concentration has increased dramatically since pre-industrial times, and a further twofold increase (540–970 µmol mol$^{-1}$) is expected by the end of the 21st century (Cheng et al., 2010). Elevated atmospheric CO$_2$ concentration can result in higher biomass of plants and root exudates (Ainsworth, 2008), which in turn influences soil microorganisms (Sadowsky and Schortemeier, 1997). The China free-air CO$_2$ enrichment (FACE) project began on 2002 to explore the responses of crops and soil microorganisms in a paddy field to this climate change by artificially elevating atmospheric CO$_2$ in paddy soil. We hypothesized that the community of heterotrophically growing AAnPB might be indirectly affected by the rising atmospheric CO$_2$ in paddy soil. Therefore, by using culture-independent methods on the basis of the pufM gene (Waidner and Kirchman, 2008), we investigated the community composition and the population size of AAnPB and their responses to elevated atmospheric CO$_2$ and N fertilization under in situ conditions in the China FACE platform.

Results

DGGE fingerprinting of pufM genes in paddy soil

DGGE revealed that two dominant bands (2 and 5) consistently appeared in soil samples from all treatments, while the digitalized band intensity of band 5 increased by 3.1–37.5% under elevated atmospheric CO$_2$ (Fig. 1). Indeed, the intensities of 10 less dominant bands (1, 3, 4, 6, 7, 12, 13, 15, 16 and 17) also appeared to be enhanced by elevated atmospheric CO$_2$, while the intensities of bands 14 and 18 decreased by 9.5–100% and 7.1–100% respectively. Shannon's diversity indices were 2.56–2.93 for bulk soils and 2.63–2.98 for rhizospheric soils, which indicated a subtle difference of AAnPB composition between bulk and rhizospheric soils. The effect of N fertilization on pufM genes was also hardly differentiated in paddy soil.

Phylogenetic identification of pufM genes in paddy soil

Phylogenetic analysis indicated the presence of a phylogenetically diverse AAnPB community in paddy soil (Fig. 2). The pufM gene sequences could be phylogenetically placed within three groups. The Alphaproteobacteria phyla contained the sequences of bands 1, 10, 13, 16, 17 and 18, while DGGE bands 8 and 14 were affiliated within the phyla of Gammaproteobacteria. These pufM genes were closely related to the members of isolated AAnPB. The third group was formed by the dominant DGGE bands 2 and 5, which clustered with Methylobacterium- and Bradyrhizobium-like AAnPB respectively. However, the growths of the isolated Methylobacterium or Bradyrhizobium do not depend on aerobic anoxygenic photosynthetic metabolism, despite the fact that both of them are capable of producing bacteriochlorophyll $\alpha$ and carotenoids in cells (Urakami and Komaga, 1984; Evans et al., 1990).

Real-time PCR quantification of pufM and 16S rRNA genes in paddy soil

Because primer sets pufM 557F/WAW and pufM 557F/750R were both used to investigate AAnPB diversity in the previous reports (Hu et al., 2006; Waidner and Kirchman, 2008), they were chosen and validated each other in this study. The higher copy number of soil pufM gene was detected by pufM 557F/WAW than pufM 557F/750R assays (Fig. 3A), although both assays revealed the similar changing pattern of pufM genes in paddy soil. This indicated the former assay might be better suited for pufM gene quantification in paddy soils than the latter. The pufM gene abundance by pufM 557F/WAW assay varied from $7.0 \times 10^4$ to $1.5 \times 10^5$ d.w.s. in bulk soil and $2.0 \times 10^5$ to $2.5 \times 10^6$ d.w.s. in rhizospheric soil (Fig. 3A). It indicated that AAnPB abundance in rhizospheric soil was significantly larger than that in bulk soil ($P<0.05$). Furthermore, the copy numbers of pufM genes in bulk soil were higher under FACE treatment than Ambient treatment ranging from $7.0-8.0 \times 10^4$ to $1.4-1.5 \times 10^5$ d.w.s., whereas no apparent difference was observed in rhizospheric soil. In addition, N fertilization treatment had little effect.

Fig. 1. DGGE fingerprinting pattern of pufM genes in paddy soils. R1 and R2 indicate the duplicate of DGGE fingerprints. The DGGE bands denoted by arrow from number 1 to 18 were excised for sequencing.
There were $0.9-1.6 \times 10^{10}$ copy number of 16S rRNA gene g$^{-1}$ d.w.s. in bulk soil and $2.7-3.7 \times 10^{10}$ g$^{-1}$ d.w.s. in rhizospheric soil respectively (Fig. 3B), which is consistent with the data from other soil (Pittl et al., 2010). No significant difference of bacterial 16S rRNA gene copy numbers was observed between FACE and Ambient conditions for all treatments (Fig. 3B). However, elevated CO$_2$ appeared to result in a higher AAnPB percentage in total bacterial community (from 0.5% up to 1.5% for pufM 557F/WAW assay) in bulk soil, but not in rhizospheric soil.

**Discussion**

There was a diverse AAnPB community with large population size residing in paddy surface soil. Although the depth of photic paddy soil is usually between 0.2 and 2 cm (Roger, 1996), the unilluminated zone of surface paddy soil that is ploughed as a result of agricultural practices will become the illuminated zone following plowing the next year. Thus surface soil at a depth of 0–5 cm is presumably suitable for the investigation of phototrophs. In fact, the investigations of the diversities of algae (Fujita and Nakahara, 2006) and cyanobacteria (Song et al., 2005) are also conducted at such depth in paddy surface soil. AAnPB are found to be widespread in almost all extant ecosystems (Yurkov, 2006). Culture-independent analysis in this study indicated some pufM genes of AAnPB could occur in geographically distinct ecosystems, while certain unique AAnPB could be particularly favoured in paddy soil. The pufM genes in paddy soil within the phyla of Alpha- and Gammaproteobacteria were similar to those of AAnPB isolated from ocean such as *Rubrivivax gelatinosus* and *Rhodobacter blasticus* and from lake water such as *Acidiphilium cryptum* (Hu et al., 2006; Waidner and Kirchman, 2008). However, the deeply branching cluster of pufM genes related to *Methyllobacterium* sp. and *Bradyrhizobium* sp. was detected only in paddy soil, which might extend the functional ecotypes of AAnPB. Although several non-AAnPB species appeared in the phylogenetic tree (Fig. 2), the genotypes related to these non-AAnPB were possibly from some unclassified AAnPB due to the low bootstrap values. The mix of alpha and gamma subdivisions of *Proteobacteria* in the phylogenetic tree built on the pufM gene might result from the
horizontal transfer of photosynthetic gene (Nagashima et al., 1997). The process of horizontal gene transfer is accelerated in particular in eutrophic environment (van Elsas and Bailey, 2002). Besides distinct genotypes, AAnPB community had large population size in paddy soil (Fig. 3A), which was generally 2 to 3 orders of magnitude higher than those detected in aquatic environments (Du et al., 2006; Waidner and Kirchman, 2007). Compared with the oligotrophic condition of lake or marine ecosystem, paddy soil is much eutrophic and is presumably in support of the growth of strictly heterotrophic AAnPB (Yurkov, 2006), which is supported by the result from aquatic environments that higher AAnPB abundance is found in the region richer in nutrient (Jiao et al., 2007; Waidner and Kirchman, 2008).

The abundance of AAnPB was higher in rhizospheric soil than that in bulk soil despite the subtle difference in their compositions. Flooded paddy soil can be considered a unique ecosystem characterized by oxidation-reduction status through the soil profiles, where microbial community structures might differ greatly between bulk and rhizospheric soils (Liesack et al., 2000). It was thus surprising that DGGE fingerprinting analysis showed no dramatic change in the composition of pufM genes between bulk and rhizospheric soils (Fig. 1). The intensified agricultural practice such as plowing and tillage management could enhance the homogeneity of not only soil but also AAnPB distribution between bulk and rhizospheric soils. For instance, AAnPB from surface soil might be dispersed with depth during plowing, while AAnPB in deep soil might persist for a long time so that they could inoculate the bulk surface soil following plowing the next year. However, the copy numbers of pufM genes were significantly higher in rhizospheric soil than those in bulk soil, implying the stimulated growth of certain AAnPB (Fig. 3) in rhizospheric soil. The promoted growth of AAnPB in rhizospheric soil might be explained by the elevated concentrations of oxygen and labile organic carbon released from rice root. Given the highly versatile metabolic ability of AAnPB to adapt to a large variety of conditions, further studies at mRNA level probably will allow the better differentiation of active AAnPB composition in so complex environments.

The stimulated growth of AAnPB and their higher percentage in total bacterial community resulting from elevated CO₂ implied AAnPB could be ecophysiology important to carbon cycling in paddy soil. Elevated atmospheric CO₂ stimulates the photosynthesis of plants (Ainsworth, 2008). This in turn results in more carbon input into soil by stimulated root exudates and the decomposition of increased plant biomass (Rogers et al., 1994; Daep et al., 2000; Jastrow et al., 2000). The studies...
reveal that elevated atmospheric CO$_2$ leads to the increases in SOC by 14% (Feng et al., 2009) and dissolved organic C (DOC) by 42% (Cheng et al., 2010) in paddy soil. This process might enhance the bioavailability of organic matters and final stimulate the heterotrophic growth of AAnPB in paddy soil. The increase in AAnPB abundance and the pattern of AAnPB percentages under elevated atmospheric CO$_2$ in bulk soil indeed are in agreement with the conclusion from aquatic ecosystems that higher nutrient results in higher AAnPB abundance and percentage (Jiao et al., 2007; Waidner and Kirchman, 2008). In addition, the higher percentage indicated that AAnPB could be more sensitive to the changes in soil organic matters in paddy soil than other bacteria and implied that they might play an important ecological role in carbon cycling in paddy ecosystem.

Interestingly, it was noteworthy that no similar results were obtained in rhizospheric soil. The microbial processes are more complicated in rhizospheric soil than those in bulk soil due to the interference of plant root activities (Paterson et al., 1997). Many factors might result in the variation in the responses of AAnPB to elevated atmospheric CO$_2$ between bulk and rhizospheric soils. Soil sulfide concentration was suggested to be one of influencing factors. In general, sulfate-reducing bacteria (SRB) are attached to plant roots (Nielsen et al., 2001). The SRB population size 4–8 times larger and sulfide concentration 4–6 times higher are detected in paddy rhizospheric soil than those in paddy bulk soil, and elevated atmospheric CO$_2$ enhances the population size of SRB by 250% in rhizospheric soil (Feng et al., 2009).

Thus, despite the fact that higher amount of root exudates released into rhizospheric soil under elevated CO$_2$ (Paterson et al., 1997) could stimulate AAnPB growth when compared with ambient condition, we hypothesize that the stimulative effect might be counteracted to a greater extent by the higher sulfide concentration as well as other unknown environmental factors. In fact it seemed that AAnPB abundance in paddy soil was affected by the presence of rice root to a greater extent than elevated atmospheric CO$_2$.

**Experimental procedures**

**Site description**

A FACE experiment was carried out with a rice–wheat rotation system at the Nianyu Experimental Station, Jiangsu Province, China (31°35′N, 120°30′E). The soil is classified as stagnic anthrosols. There has been no substantial change of agronomic practice in the field for more than 50 years and this region is the representative of the majority of agricultural lands in Jiangsu province, a key area for rice production in China. The soil had a 9.2% concentration of sand (1–0.05 mm), a 65.7% concentration of silt (0.05–0.001 mm), a 25.1% concentration of clay (< 0.001 mm), a 1.2 g cm$^{-3}$ bulk density, a 15.0 g kg$^{-1}$ soil organic C (SOC), a 1.59 g kg$^{-1}$ total N, a 1.23 g kg$^{-1}$ total P, a 10.4 mg kg$^{-1}$ available P and a pH of 6.8. The station sits in the subtropical climatic zone with a mean annual precipitation of 900–1000 mm, mean annual temperature 16°C, the average daily integral radiation of 12.3 MJ m$^{-2}$, a total sunshine time of more than 2000 h and a frost-free period of more than 230 days per year.

**FACE system**

The China FACE project began on 2002 rice cultivation and has been carried out for 6 years. A detailed description in the FACE system has been given before (Okada et al., 2001; Liu et al., 2002). Briefly, it consists of three octagonal rings with a target CO$_2$ concentration of 200 ± 40 µmol mol$^{-1}$ above the current concentration of 355 ± 15 µmol mol$^{-1}$ (hereinafter referred to as the ‘FACE ring’), and three comparison rings without CO$_2$ enrichment (hereinafter referred to as the ‘Ambient ring’). FACE rings are at least 90 m apart from Ambient rings. Each ring has a diameter of 12.5 m. Facing the centre of FACE rings, pure CO$_2$ at high pressure was released about 50 cm above the crop canopy throughout the daylight hours from tubes surrounding crops. CO$_2$ release was strictly controlled by a computer program with an algorithm based on the wind-speed and direction to keep the target pCO$_2$ level within the FACE ring. Both FACE and Ambient rings were divided into two identical plots, to which low nitrogen (LN) (150 kg N ha$^{-1}$) and high nitrogen (HN) (250 kg N ha$^{-1}$) were applied. To prevent N transportation between HN and LN plots within each ring, a polyvinyl chloride (PVC) board was inserted 25 cm below the plow layer and was extended 30 cm above the water. A 50-cm-long strip next to either side of the PVC was set as a buffer zone from which no samples were taken.

**Outline of cropping systems**

The rice–wheat rotation system in this study is a popular cropping system in the regions under a comparable subtropical climate in China. Wheat is sown in early November and harvested in late May or early June of the following year. Rice seeds are sown on a nursery bed in mid May and the seedlings transplanted in mid June and harvested in late October. Urea and compound fertilizer are applied as N fertilizer. For rice cultivation, N fertilizer is applied three times: 36% of nitrogen is basally applied in mid June, and the remainder nitrogen is top-dressed in mid June (24%) and mid July (40%). For wheat cultivation, N fertilizer is applied also three times: 50% of nitrogen is basally applied in early November, and the remainder nitrogen is top-dressed in mid February (10%) and mid April (40%). Detailed information on rice and wheat cultivation has been given before (Xu et al., 2006; Zhu et al., 2009).

**Samples collection and determination**

On 23 September 2008, one of the three ambient rings and one of the three FACE rings were randomly chosen for soil sampling at rice anthesis. Soil samples from each plot treated with HN or LN were collected in triplicate. The rhizospheric
soil was defined as soils adhering to rice root up to 2-mm-thick layer and recovered by gently washing away most soil particles around rice root. The bulk soil was collected from 0–5 cm depth surface layer, which was undisturbed by rice growth with 20–30 cm away from rice plant.

**DNA extraction, PCR-DGGE, sequencing and phylogenetic analysis**

For each of the triplicate soil samples 0.5 g of soil was used for DNA extraction using a FastDNA SPIN Kit for soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer’s instructions. The extracted soil DNA was dissolved in 50 μl TE buffer, quantified by spectrophotometer and stored at −20°C before use. The *pufM* gene fragment of 277 bp in length was amplified with the forward primer *pufM* 557F: CCGCACCTGGACTGGAC, and reverse primer *pufM* WAW: AYNGCRAACCACCANGC in the DDBJ database under accession numbers AB510450 to AB510467.

Three random clones containing the correct gene size for *pufM* and maximum parsimony. The phylogenetic relationship of *pufM* gene sequences was used to build a basic phylogenetic tree by the neighbour-joining method using the software package MEGA 4.0 version (Molecular Evolutionary Genetics Analysis) controlling photosynthesis gene expression. (Feng et al., 1994). Dominant DGGE bands were excised and eluted overnight in sterilized distilled water at 4°C. The eluted DNA was used as a template to re-amplify the *pufM* gene as described above for the mobility check, then cloned into a pMD18-T vector (TaKaRa) and transformed into *Escherichia coli* DH5α. Three random clones containing the correct gene size for each DGGE band were sequenced by the Invitrogen Sequencing Department in Shanghai. DNASTAR software package was used to manually check and compare the clone sequences. One representative clone sequence with high quality after sequence comparison from each band was used for phylogenetic analysis.

Together with the top BLAST hit, the second and third highest BLAST hits of homologous gene sequences, the *pufM* gene sequences were used to build a basic phylogenetic tree by the neighbour-joining method using the software package MEGA 4.0 version (Molecular Evolutionary Genetics Analysis) (Tamura et al., 2007). The tree topology was further evaluated by different methods including minimum evolution and maximum parsimony. The phylogenetic relationship of *pufM* gene sequences to the closest homologue in the GenBank was then inferred. The sequences generated in this study have been deposited in the DDBJ database under accession numbers AB510450 to AB510467.

**Real-time quantitative PCR of *pufM* and 16S rRNA genes in paddy soil**

The copy numbers of soil *pufM* gene from AAnPB and bacterial 16S rRNA gene in paddy soil were quantified by real-time quantitative PCR analysis with an Opticon 2 continuous fluorescence detection system (MJ Research) by the assays of the *pufM* 557F/NAW and bacterial 519F/907R (Biddle et al., 2008). The reliability of soil *pufM* genes quantification was further assessed by the *pufM* 557F/750R as described previously (Waidner and Kirchman, 2008). The detailed protocol for qPCR was shown below. To generate real-time PCR standard curve, a single clone containing the correct insert was grown in Luria–Bertani medium and then plasmid DNA was extracted, purified and quantified. A 10-fold dilution series of the plasmid DNA was made to generate a standard curve covering seven orders of magnitude from 10² to 10⁸ copies of template per assay. Assays were set up using the SYBR *Premix Ex Taq* Kit (TaKaRa). The 25 μl reaction mixture contained 12.5 μl of SYBR *Premix Ex Taq*, 0.5 μM of each primer, 200 ng BSA μl⁻¹, 1.0 μl template containing approximately 2–9 ng DNA. A negative control was always run with water as template instead of soil DNA extract. Specific amplification were confirmed by agarose gel electrophoresis of real-time PCR amplicons showing an expected band of PCR amplicons and melting curve analysis always resulting in a single peak. Real-time PCR was performed in triplicate and amplification efficiencies of 97.4–104% were obtained with R² values of 0.966–0.977. Based on the standard curve plotted by using the known gene copy number against the cycle threshold (Cₗ), the copy numbers of *pufM* and bacterial 16S rRNA genes were calculated by extrapolating its Cₗ value at which fluorescence emission crosses a threshold within the logarithmic increase of target genes. The threshold was defined as 10 times the standard deviation around the average intensity of background fluorescence. The differences in copy number of *pufM* and bacterial 16S rRNA genes between treatments were statistically evaluated using one-way analysis of variance under significant differences P < 0.05 using SPSS 13.0 statistical package.

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