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Effects of lead upon the actions of sulfate-reducing bacteria in the rice rhizosphere

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ABSTRACT

Microbe–mineral interactions play an important role in affecting geochemical transformations of heavy metals in the soil environment. The formation of metal sulfide, which is mediated by sulfate-reducing bacteria (SRB) through contributing to sulfate reduction is an important pathway for heavy metal stabilization in anoxic soil. In oxic rice rhizospheres, there are abundant sulfur oxidizing bacteria (SOB) which can enhance sulfur oxidation and hence the availability of heavy metals, resulting in the uptake of such metals by the plant and a potential risk to human health. In this study, the potential existence of SRB in oxic rice rhizospheres, their contribution to sulfate reduction, and potential to reduce the availability of heavy metal was investigated. PCR-DGGE fingerprinting and real-time PCR results showed increasing numbers of SRB with Pb addition, which corresponded with increases in soil pH and reduction in Eh, suggesting the enhancement of sulfur reduction and SRB activity. Sulfur K-edge XANES, which characterized sulfur speciation in situ, revealed reduced states of sulfur. The SRB mediated the sulfate reduction and contributed to the formation of reduced sulfur which interacted with Pb, leading to the formation of stable metal sulfide and reduction of Pb availability. In return, acclimated SRB populations developed in Pb-polluted conditions. Hence stabilization of reduced sulfur by Pb enhanced the activity of SRB and sulfate reduction in rice rhizosphere.

1. Introduction

Contamination of heavy metals in soil and its subsequent accumulation along the food chain is a potential risk to human health. Great concern has been paid to the behavior of metals in the soil–plant system, particularly on the availability of such metals to plants and their consumers. Rice is an important food crop in China and paddy soils play a significant role in sulfur cycling. It is confirmed that cycling of sulfur takes play at the interface between the oxygenated rhizosphere and the anoxic bulk soil. Sulfur oxidizing bacteria (SOB) which enhance sulfur oxidation and the availability of heavy metals are abundant and active in rice rhizosphere soil. They could boost the uptake of heavy metal in rice, resulting in serious threats to human health through the food chain (Kayser et al., 2000; Wang et al., 2008; Wind and Conrad, 1995). By contrast, sulfate-reducing bacteria (SRB) are likely to exist in bulk soil, which in paddies is typically anoxic, as they are considered to be strict anaerobes. Bioprecipitation of heavy metals as metal sulfides mediated by SRB is a promising strategy for heavy metal pollution control as it is a ubiquitous process in anaerobic environment (Hoa et al., 2007; Liamleam and Annachhatre, 2007). The oxygenated rhizosphere effect of rice can potentially prevent activity of SRB and hence the precipitation of heavy metals.

However, some subgroups of SRB could defense oxygen. This feature may favor bioprecipitation of heavy metals in situ in oxic conditions (Dolla et al., 2006). On the other hand, heavy metals also can have great impact on SRB activity in polluted conditions. The objective of this study was to investigate the existence of SRB and the possibility of reduction of sulfate in the oxic rice rhizosphere, as well as their interaction with Pb. The investigation of SRB was conducted by molecular tools (nested PCR-DGGE and phylogenetic analysis of 16S rRNA gene) that specifically monitor the diversity of SRB. Sulfur K-edge XANES was conducted to detect sulfur species in situ effectively, attached to classical analytical methods (Eh, pH measurements).

2. Materials and methods

2.1. Experimental design and treatments

Waterloggogenic paddy soil was collected from the top layer (0–15 cm) of a rice field in Shaoxing, Zhejiang Province, China.

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The soil had an organic matter content = 2.26%; pH 5.63 (water); total $P = 0.54$ g kg^{-1}; total $K = 13.0$ g kg^{-1}; total $S = 0.25$ g kg^{-1}. Aliquots (2.5 kg) of air dried soil were put into 30 plastic pots and each treated with 0.4 g kg^{-1} urea and 0.4 g kg^{-1} K_2HPO_4. The experimental design was employed with $Na_2S_2O_3$ and $Pb(NO_3)_2$. The chemicals were dissolved in distilled water, sprayed on the soil samples, and mixed evenly. Pb treatments were 0, 500 and 1200 mg kg^{-1} dry soil separately. Sulfur treatments were 1 g kg^{-1} dry soils on each Pb concentration, to provide substrate for SOB and SRB. Deionized water was added daily to maintain flooded conditions. After 3 months of soil aging, 3 rice seedlings (*Oryza sativa* L. Hybrid Zhenong 7) were transplanted to each nylon mesh bag per pot to keep roots in a small space as described by Lu et al. (2000). All treatments were carried out in triplicate in a greenhouse with a randomised block design.

2.2. Eh measurement, sampling and detection of physical–chemical characterization

Eh measurements were carried out in situ before sampling by inserting the electrodes into the rhizosphere and bulk soil at the same depth using Orion Epoxy Sure-Flow Combination Rdeox/ORP (9678BN) following the manufacturer's instructions. The rhizosphere soil was collected after 45 days growth. Part was stored at −20 °C for PCR-DGGE and the rest was freeze-dried for detection of physical-chemical characterization and sulfur XANES analysis.

Soil pH value was measured (H_2O: soil = 5). The speciation of Pb in the soil was determined by a sequential extraction procedure, which was based on the method reported by Chen et al. (2004). This sequential extraction procedure divided heavy metals in sediments into five binding forms, namely, exchangeable, carbonate-bound, Fe/Mn oxide-bound, organic matter-bound and residual. The Pb contents were determined by flame atomic absorption spectrophotometry (PerkinElmer AA100) after extraction with appropriate chemical agents associated with the forms above.

2.3. Direct PCR and nested PCR-DGGE

Total soil DNA of the rhizosphere soils was extracted and purified using a bead beating method (FastDNA™ SPIN Kit for Soil, Bio101 Inc., USA) following the manufacturer's instructions.

A direct amplification with primers specific for the six different groups of SRB was attempted on the extracted DNA. The six pairs of the SRB group-specific primers were DFM140 and DFM842 for *Desulfotomaculum* (Group 1), DBB121 and DBB1237 for *Desulfobulbus* (Group2), DBM169 and DBM1006 for *Desulfobacterium* (Group 3), DSB127 and DSB1273 for *Desulfobacter* (Group 4), DCC305 and DCC1165 for *Desulfonema–Desulfocarcina–Desulfococcus* (Group 5), DSV230 and DSV838 for *Desulfovibrio–Deuslfomicrobium* (Group 6; Daly et al., 2000).

A strategy of three-step nested PCR-DGGE was then used to analyze the SRB diversity in rice rhizosphere (Dar et al., 2005). Firstly, the nearly complete sequence of 16S rRNA was amplified using the primer GM3F/GM4R (Muyzer et al., 1995). Then a following amplification with each of the six pairs of the SRB group-specific primers was carried out using the product obtained as a template. Finally, the product obtained in Step 2 was used as a template for the amplification with primers F341GC and R518 (Muyzer et al., 1993) in order to generate products suitable for DGGE. Details of the different oligonucleotides and the reaction condition used in this study were as the description of Dar et al. (2005).

The obtained PCR products were then subjected to DGGE analysis using a Dcode™ Universal Detection System instrument according to the manufacturer's instructions (Bio-Rad, USA). DGGE was performed using a denaturing gradient of 25%–60% denaturants (100% denaturant contained 7 M urea and 40% (v/v) formamide) in 8% polyacrylamide gel in 1×TAE buffer (pH 8.0) at 60 °C for 6.5 h at a constant voltage of 160 V (Dcode™ Universal Detection System, Bio-Rad, USA). After electrophoresis, the gels were stained with SYBR™ GREEN I (Sigma, USA) for 30 min following the manufacturer's instructions.

2.4. Cloning, sequencing and phylogenetic analysis

Individual bands were excised, reamplified. The product was then purified using Qiaquick PCR Clean-up columns (Qiagen, Valencia, CA). The purified products were ligated into the pMD19-T easy cloning vector (Takala, Japan) and transformed to *E. coli* DH5α competent cells. Clones grew for 12–16 h in Luria–Bertani agar adding 100 μg ml^{-1} ampicillin, and were identified based on blue-white screening. Plasmid DNA was purified with UNIQ-10 column Plasmid Mini-prep Kit (Sangon, Canada) and sequenced by the Invitrogen Corporation (USA).

Sequences recovered from the excised bands were analyzed using Premier Version 5.0. Sequences were compared using the National Center for Biotechnology Information (NCBI) BLAST program and the Ribosomal Database Project II (RDPII) Chimera Check program. Sequences were aligned with the CLUSTAL X 1.83 program and the resulting alignments were optimized by using Paup v.4.0b.8.a (Sinauer Associates, Inc., Sunderland, Mass.) to construct phylogenetic trees. The neighbor-joining algorithms were used to generate optimal tree topologies, confirmed by 1000-fold bootstrapping.

2.5. Real-time PCR

For the quantification SRB in the soil, two primers (B-F: 5′-CCTACGGGAGGCAGCAGTG-3′; B-R: 5′-TACCGCGGCTGCTGGCAC-3′) were designed according to the results of cloning and sequencing with Primer Express 2.0 and Beacon designer. The sensitivities of PCR assays were determined with dilution series (10^5, 10^4, 10^3, 10^2 and 10^1) of template to make standard curve. The amplification was done in iQTM5 Multi-instrument real-time fluorescence quantitative PCR (Bio-Rad, the USA). The reaction was carried out as follows:10 s initial denaturation at 95 °C, followed by 45 cycles of denaturation at 95 °C for 5 s, annealing at 62 °C for 20 s and DNA extension at 72 °C for 1 min. The 16S rDNA target numbers were then calculated according to the standard curve.

2.6. Sulfur speciation using sulfur K-edge XANES

Sulfur species in rhizosphere soil were analyzed by X-Ray absorption Spectroscopy at Beijing Synchrotron Radiation Facility, Institute of High Energy Physics of China. The freeze-dried soil samples were pressed into thin films before analysis. The storage ring was operated at the energy of 2.5 GeV with Si (111) double crystals. Spectra were recorded at 4B7A beamline (medium X-ray beamline, 2100–6000 eV) and scanned at step widths of 0.3 eV in the region between 2420 and 2520 eV, with fluorescence mode using a fluorescent ion chamber Si (Li) detector (PGT LS30135). Additional filters were placed between the sample and the detector to reduce the fluorescence signal derived from Si in the soil samples. Reference compounds for exploring different sulfur oxidation states and chemical structures are shown in Table 1. The X-ray energy was calibrated with reference to the spectrum of the highest resonance energy peak of Na_2SO_4 at 2.4804 KeV. The speciation of sulfur was identified by the energy position of Gaussian peak and the relative abundance of each sulfur species