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Nitrate Stimulates Anaerobic Microbial Arsenite Oxidation in Paddy Soils

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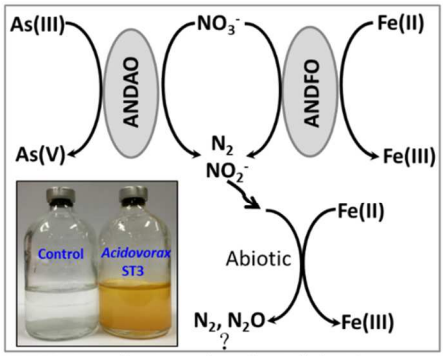
ABSTRACT

Arsenic (As) bioavailability to rice plants is elevated in flooded paddy soils due to reductive mobilization of arsenite [As(III)]. However, some microorganisms are able to mediate anaerobic As(III) oxidation by coupling to nitrate reduction, thus attenuating As mobility. In this study, we investigated the impact of nitrate additions on As species dynamics in the porewater of four As-contaminated paddy soils. The effects of nitrate on microbial community structure and the abundance and diversity of the As(III) oxidase (*aioA*) genes were quantified using 16S rRNA sequencing, quantitative PCR and *aioA* gene clone library. Nitrate additions greatly stimulated anaerobic oxidation of As(III) to As(V) and decreased total soluble As in the porewater in flooded paddy soils. Nitrate additions significantly enhanced the abundance of *aioA* genes and changed the microbial community structure by increasing the relative abundance of the operational taxonomic units (OTUs) from the genera *Acidovorax* and *Azoarcus*. The *aioA* gene sequences from the *Acidovorax*-related OTU were also stimulated by nitrate. A bacterial strain (ST3) belonging to *Acidovorax* was isolated from nitrate-amended paddy soil. The strain was able to oxidize As(III) and Fe(II) under anoxic conditions using nitrate as the electron acceptor. Abiotic experiments showed that Fe(II), but not As(III), could be oxidized by nitrite. These results show that nitrate additions can stimulate As(III) oxidation in flooded paddy soils by enhancing the population of anaerobic As(III) oxidizers, offering a potential strategy to decrease As mobility in As-contaminated paddy soils.

ToC Graphic



ANDAO: Anaerobic nitrate-dependent As(III)-oxidizers
ANDFO: Anaerobic nitrate-dependent Fe(II)-oxidizers

INTRODUCTION

Contamination of paddy soils with arsenic (As) is widespread in South and Southeast Asia due to mining activities and irrigation with As-laden groundwater,[1, 2] resulting in elevated levels of As in rice grain that may pose a significant risk to public health for populations consuming rice as the staple food.[2-6] In more severe cases, As contamination can lead to phytotoxicity and substantial yield losses in rice crops.[7] Arsenic bioavailability to rice is strongly dependent on the biogeochemical processes in the paddy soil.[8, 9] Microbes are key drivers for mediating the transformations of As species in the environment,[10-12] but these processes remain poorly understood in paddy systems.

In flooded paddy soil, As is mobilized mainly as arsenite [As(III)] as a result of the reductive dissolution of iron (oxyhydr)oxides and the reduction of As(V) to As(III); the latter is less strongly adsorbed and therefore more mobile.[13] As(V) reduction is carried out by microbes employing either the detoxification or the dissimilatory pathway.[10] In the detoxification pathway, As(V) is reduced by enzymes such as ArsC and the product As(III) is extruded out of the cell via efflux transporters.[14] In the dissimilatory pathway, As(V) is used as a terminal electron acceptor during anaerobic respiration with a carbon (C) source as the electron donor.[15, 16] Functional genes encoding ArsC and the dissimilatory As(V) reductases ArrA are abundant in paddy soils,[17, 18] but their relative contributions to As(V) reduction in paddy soils remain unknown.

Despite the reduced redox status in flooded paddy soil, As(V) still accounts for considerable proportions (typically 10 – 30%) of the total soluble As in the porewater,[19, 20] even though thermodynamic calculations would predict negligible As(V) in the system. One possibility is that As(III) is oxidized by anaerobic As(III) oxidizers, which use different electron acceptors (e.g. NO_3^-, Fe(III)) for respiration in anoxic environments.[21-23] Anaerobic As(III) oxidation coupled to

denitrification has been observed in anoxic sediments, lake water columns and bioreactors.[21, 24-26] It has been shown that the availability of nitrate is a key factor regulating As biogeochemical cycle in lake water column.[27] A number of anaerobic As(III) oxidizers have been isolated and characterized.[21, 22, 24] These microbes couple As(III) oxidation with nitrate reduction under anoxic conditions. Some of the anaerobic As(III) oxidizers are autotrophic bacteria (e.g. *Alkalilimnicola ehrlichii* MLHE-1, *Azoarcus* sp. DAO1, *Sinorhizobium* sp. DAO10, and *Paracoccus* sp. SY), which use As(III) as the electron donor for respiration, whereas others are heterotrophic bacteria relying on organic C sources for growth.[28] By transforming As(III) to As(V), anaerobic As(III)-oxidizers play an important role in attenuating As mobility and bioavailability.[22, 29] In addition to As(III) oxidation, some anaerobic microorganisms can also oxidize Fe(II) by coupling to the reduction of nitrate.[30-34] The formation of ferric precipitates can facilitate the adsorption of As species, thus decreasing their mobility.[11, 13]

Despite the importance of As biogeochemical cycle in paddy systems, there are few studies on anaerobic As(III) oxidation in paddy soils. In the present study, we hypothesized that nitrate can promote anaerobic As(III) oxidation in flooded paddy soils by enhancing the population of nitrate-dependent anaerobic As(III) oxidizers. Microcosm experiments were conducted to investigate the effect of nitrate additions on As species dynamics in porewater and the diversity and abundance of As(III)-oxidizing microbial communities in a number of As-contaminated paddy soils. Furthermore, a nitrate-dependent anaerobic As(III) and Fe(II)-oxidizer was isolated and characterized. The results suggest the possibility of using nitrate to manipulate the biogeochemical cycle of As in paddy soils.

MATERIALS AND METHODS

Soil sampling and characterization. Four As-contaminated paddy soils (0 – 20 cm) were collected from south or south-central China in the present study. The soils have been contaminated with As due to nearby mining activities (Chenzhou, CZ; Hechi, HC; Shantou, ST) or geogenic reasons (Qiyang, QY) (Supporting Information, Table S1). Soils were air-dried, disaggregated and passed through a 2 mm sieve before being stored in plastic containers in the dark. Soil properties, including pH, texture, organic matter content, cation exchange capacity (CEC), total N, available P, and total As concentrations were determined as described previously.[35] Amorphous and crystalline Fe oxides and the associated As were extracted by acid ammonium oxalate and dithionite-citrate-bicarbonate (DCB), respectively.[36]

Microcosm experiments. In the first microcosm experiment, ST soil was used to investigate the effects of nitrate additions on the transformation of As species in soil porewater and the abundance of the microbial functional genes involved in As transformation. This soil has a relatively high capacity for anaerobic As(III) oxidation according to a preliminary experiment. Nine microcosms were constructed each with 300 g of soil being placed in a 500 mL glass vial (6 cm diameter, 25 cm height) and flooded with 300 mL deionized water. A 10-cm-long soil solution sampler (Rhizon-MOM, Rhizosphere Research Products, The Netherlands) was inserted into the center of the soil layer for collecting soil porewater. The headspace in the vials were purged with O_2-free N_2 gas for 30 min and sealed with gastight glass covers. The vials were incubated at room temperature (25±1 ^{o}C) in the dark for 4 weeks. On day 14, 1 or 3 mmol NO_3^- kg^{-1} soil (dry weight) was injected into the center of the microcosms (3 replicates per nitrate treatment) with a PVC porous pipe. The other 3 replicates served as the control (no addition of NO_3^-). Soil porewater was collected at weekly intervals during the 4-week incubation. Porewater samples were acidified

105 to pH < 2 with HCl (10 mL solution with 0.1 mL concentrated HCl) immediately after
106 collection,[37] and filtered through a 0.2 μm membrane filter (mixed cellulose ester) before the
107 determination of soluble Fe and Mn and As speciation within 24 h of collection. The redox
108 potential (Eh) was measured in the middle of the soil (10 cm below the surface of the soil layer)
109 using a Pt electrode and an Ag-AgCl reference electrode. Soil samples were destructively
110 sampled for microbial analysis at the end of the experiment. Soils were frozen in liquid N_2
111 immediately and stored at -80 °C prior to microbial analysis.

112 CZ, QY, and HC soils were used in the second microcosm experiment. The experimental
113 setup was the same as Experiment 1, except that only two treatments of nitrate additions (0 and 1
114 mmol NO_3^- kg^{-1} soil) were included with the injection of nitrate being made on day 14 and 28,
115 and the incubation lasted 42 days. Soil porewater was collected at weekly intervals. Soil samples
116 were collected at the end of the experiment for microbial analysis.

117

118 **Quantification of 16S rRNA, *aioA*, *arxA*, *arrA*, and *arsC* gene copy numbers.** DNA was
119 extracted from soil samples using the MOBIO PowerSoil DNA Isolation Kit according to the
120 manufacturer's protocol. The abundances of bacterial 16S rRNA genes and the genes coding for
121 the As(III) oxidases (*aioA* and *arxA*), As(V) respiratory reductase (*arrA*), and the As(V) reductase
122 (*arsC*) were quantified using real-time quantitative PCR (iQ5 Thermocycler; BioRad, U.S.A.) as
123 described previously.[38-42] The primers and PCR conditions are given in Supporting Information
124 Table S2.

125

126 **Cloning of As(III) oxidase gene *aioA* and DNA sequencing.** To assess the effect of nitrate
127 additions on the diversity of As(III) oxidizers, three independent clone libraries of *aioA* genes

were generated from each of the control and NO_3^--amended ST soil after 28 days of anaerobic incubation. The *aioA* genes were amplified from the genomic DNA as described previously.[41] The amplified products (approximately 550 bp) from three replicates of each treatment were pooled and purified with a QIAquick PCR Purification kit (Qiagen). The purified products were ligated into the pEASY-T3 plasmid vector and transformed into *Escherichia coli* TOP10 competent cells according to the manufacturer's instructions. Fifty clones per nitrate treatment were randomly selected for sequencing, resulting in a total of 150 *aioA* sequences. High-quality sequences were then assigned to taxonomic groups by Blast analysis against the NCBI-nr database and comparisons with the Functional Gene Pipeline/Repository[43] within a 90% confidence threshold. Representative sequences were selected from each operational taxonomic units (OTUs) determined with a cutoff value of 97% using Mothur.[44] The representative sequences were transferred into amino acid sequences and aligned with the AioA sequences retrieved from NCBI website using MEGA 6.0.[45]

High-throughput Illumina sequencing of 16S rRNA gene amplicons. The V4-V5 hypervariable region of bacterial 16S rRNA genes was amplified using the 515F/907R primer set.[46] A sample-specific 12-bp barcode was added to the reverse primer. 16S rRNA gene tag-encoded ultrahigh-throughput sequencing was carried out using the Illumina Hiseq platform with PE250 mode at Biozeron (Shanghai, China). Sequences were analyzed with QIIME software package (Quantitative Insights Into Microbial Ecology) and UPARSE pipeline.[47] The sequence reads were first filtered by QIIME quality filters. OTUs were identified with uclust at the 97% sequence similarity level,[48] and a representative sequence from each OTU was aligned using PyNAST.[47] Taxonomic classification of each OTU was determined using the Ribosomal Database

Project (RDP) classifier.[49] Relative abundance (%) of individual taxa within each community was estimated by comparing the number of sequences assigned to a specific taxon versus the number of total sequences obtained for that sample. The α microbial biodiversity of the samples was estimated by the abundance-based indices of Chao1, Shannon indices and Simpson. The β diversity (unweighted and weighted UniFrac distances) was calculated on the basis of a subset of randomly selected sequences per community. The genomic datasets have been deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP075803).

Isolation and identification of As(III)-oxidizing bacteria. To isolate bacterial strains capable of As(III)-oxidation under anoxic nitrate-reducing conditions, 1 g of homogenized NO_3^--amended ST soil was diluted with 9 mL of a anoxic sterile phosphate-buffered saline (PBS) solution (50 mM phosphate, pH 7.5) and agitated for 30 min under anoxic conditions in a Coy anaerobic chamber (N_2 : H_2 atmosphere; 95:5). The supernatant was serially diluted with anoxic sterile PBS buffer and 100 µL aliquots were spread onto R2A agar plates[50] containing 5 mM nitrate. The plates were incubated in a 3.5-L anaerobic jars (Oxoid) at 28°C for 10 days for heterotrophic colony development. Single colonies were streaked onto the same solid medium. The AroAdeg2F/2R primers were used as a selective marker to amplify the *aioA* genes from the isolates as described by Inskeep et al.[41] The isolates with *aioA* genes were selected and transferred into anoxic low-phosphate (LP) medium[51] containing 5 mM $NaNO_3$, 0.5 mM As(III), and 5 mM sodium acetate under anoxic condition. After anaerobic incubation for 1 week at 28 °C, arsenic species in the solution were determined using HPLC-ICP-MS. To test the autotrophic growth abilities of the isolates under anaerobic conditions, the isolates were transferred into anoxic LP medium containing 0.5 mM As(III) and 5 mM nitrate with CO_2 as the sole carbon

source.

Genomic DNAs of the isolates were extracted according to the standard procedures.[52] The 16S rRNA genes of the isolates were amplified by PCR using a universal bacterial 16S rRNA primers.[53] The partial 16S rRNA gene and *aioA* gene sequences were identified using the BLAST-N tool (www.ncbi.nlm.nih.gov/blast) on the NCBI website. Phylogenetic trees were constructed as described above.

Pure culture studies. One of the isolates (strain ST3), which was found to be abundant in the nitrate-amended ST soil, was tested for the abilities to oxidize As(III) and Fe(II) under anoxic conditions. Cultures grown anaerobically in anoxic low-phosphate (LP) medium were harvested by centrifugation (6,000 × *g*, 5 min), washed twice with anaerobic PIPES [piperazine-*N*,*N*-bis(2-ethanesulfonic acid)] buffer (10 mM, pH 7.0), and the resuspension served as an inoculum for assays of the As(III) and Fe(II) oxidation abilities.

For the anaerobic As(III) oxidation assay, an aliquot of the ST3 seed culture (5%, vol/vol) was inoculated into 50 mL anoxic LP medium containing 0.5 mM As(III), 10 mM sodium acetate, and 5 mM $NaNO_3$ under anoxic conditions. Cells were incubated for 6 days with periodic sampling (0.2 mL) for As speciation measurements. The experiment was performed in three replicates, and three vials were also prepared without ST3 inoculum to serve as a control. The ability of strain ST3 to oxidize As(III) under aerobic conditions was also tested in the CDM medium containing 0.5 mM As(III) as described previously.[54] For the anaerobic Fe(II)-oxidation assay, 4 mM $FeCl_2$ was added to the anoxic LP medium. The precipitates of whitish, poorly crystalline Fe(II) carbonates and Fe(II) phosphates were removed by filtration inside a Coy anaerobic chamber as described by Kappler et al.[55] The final Fe(II) concentration of the

precipitate-free medium was approximately 3 mM. 50 mL the filtered medium was filled anaerobically into each 100 mL serum bottle, which were sealed with butyl stoppers and crimped. Bottles were amended with anoxic $NaNO_3$ (10 mM) and sodium acetate (10 mM) as described by Klueglein et al.,[56] and inoculated with 5% of ST3 culture ($OD_{600nm} \approx 0.2$). The experiment was performed in three replicates with no ST3 inoculum as a control. All cultures were incubated at 28 °C in the dark.

To test if nitrite, an intermediate of denitrification, could oxidize As(III) or Fe(II) abiotically, serum bottles containing sterile and anoxic LP medium and 0.5 mM As(III) or 3 mM Fe(II) were amended with different nitrite concentrations (0 – 4 mM), and incubated for up to 6 days at 28 ^{o}C under anaerobic conditions. Changes in As(III) and Fe(II) concentrations were determined.

Analytical Methods. The concentrations of As species in the soil porewater samples were determined using HPLC-ICP-MS (Perkin Elmer NexION 300X, USA). Arsenic species were separated using an anion exchange column (Hamilton PRP X-100, 250 mm diameter). Details of the As speciation method were described previously.[57] The concentrations of total soluble Fe and Mn in soil porewater samples were determined using flame atomic absorption spectrometry (PinAAcle 900T, PerkinElmer, USA). Dissolved Fe(II) and Fe(III) were quantified by the ferrozine colorimetric assay.[58] Total Fe was determined by reducing an aliquot of the sample with hydroxylamine hydrochloride (10% w/v in 1 M HCl) before the addition of the ferrozine reagent. The purple ferrozine - Fe(II) complex was quantified at 562 nm using a SpectraMax M5 Plate reader (Molecular Devices, Sunnyvale, CA, USA). For the determination of Fe(II) and Fe(III) in the experiment with strain ST3, 100 µL of solution was withdrawn anoxically with a syringe and dissolved in 900 µL of 40 mM sulfamic acid for 1 h at the room temperature as described by

Klueglein and Kappler.[56] Nitrate and nitrite concentrations were measured colorimetrically.[59]

Statistical analysis. The significance of the treatment effects was assessed by analysis of variance (ANOVA), followed by comparisons between treatment means using the least significant difference (LSD) at $P < 0.05$. All statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, Illinois, USA).

Nucleotide Sequence Accession Numbers. The 16S rRNA gene and *aioA* gene sequences identified in this study have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers KX243306−KX243322.

RESULTS

Soil characteristics. The four soils used in the present study were contaminated by As due to mining or geogenic sources (Supporting Information, Table S1). Total As concentration ranged from 86 to 554 mg kg^{-1}, with 27 – 64% of the total As being extractable by ammonium oxalate indicative of an association with poorly crystalline iron (oxyhydr)oxides. ST and HC soils were more contaminated with As than the other two soils, with the majority of As (61 – 64%) being ammonium oxalate extractable. Soil organic matter content varied from 18.5 to 51.1 g kg^{-1}, whilst soil pHs were circumneutral in the four soils.

Effect of nitrate addition on As(III) oxidation in flooded paddy soils. The effect of nitrate addition (0, 1 and 3 mmol kg^{-1}) on As speciation in the porewater was first tested in ST soil. As expected, flooded incubation resulted in a rapid increase in the porewater As concentration, with

As(III) being the predominant As species (93 –95% of the total soluble As) (Fig. 1a-c). Additions of nitrate on day 14 had no significant effect on porewater Eh or pH (Supporting Information, Fig. S1). Compared with the control, nitrate additions decreased the concentration of As(III) in porewater markedly over the next 14 days (Fig. 1a). This effect was also dependent on the nitrate dose. At the higher level of nitrate addition (3 mmol kg^{-1}), most of the porewater As(III) disappeared from the porewater 1 week after nitrate addition. Concurrently, As(V) concentration increased sharply (Fig. 1b), although the increase in As(V) concentration was smaller than the decrease in As(III) concentration, resulting in an overall decrease in the total As in porewater (Fig. 1c). Additions of nitrate also significantly decreased the concentration of Fe(II), but increased the concentration of Fe(III), in porewater (Fig. 1d, e). Fe(III) accounted for 1.3 – 2.3% of the total soluble Fe in the control porewater on days 21 and 28, and the proportion increased to 3.5-3.9% and 9.0-10.9% in the 1 and 3 mmol NO_3^- kg^{-1} soil treatments, respectively. Because the increase in Fe(III) was much smaller than the decrease in Fe(II), total soluble Fe decreased significantly in the nitrate amended treatments (Fig. 1f). In the experiment, there was an excellent agreement between the total soluble Fe concentrations measured by the ferrozine colorimetric method and the atomic absorption spectrometry method (Supporting Information, Fig. S2). Total soluble Mn (Supporting Information, Fig. S3) also decreased in response to nitrate additions, although the extent of decrease was smaller than that for As or Fe.

The experiment was then repeated on three other paddy soils (CZ, HC, QY) with or without addition of 1 mmol NO_3^- kg^{-1} on day 14 and 28. Similar to the experiment with ST soil, nitrate addition decreased As(III) concentration significantly, but increased As(V) concentration concurrently in the porewater sampled on days 21 – 42 in all three soils (Supporting Information, Fig. S4). However, the increase in As(V) concentration did not compensate for the decrease in

As(III) concentration after nitrate addition, resulting in an overall decrease in the total As concentration in the porewater. Nitrate addition also decreased total soluble Fe concentration in porewater, with the effect being significant in most sampling points (Supporting Information, Fig. S4).

Small concentrations of dimethylarsinate (DMA) were detected in the porewater of CZ and QY soils, but not in ST or HC soils (data not shown). There was no significant effect of nitrate additions on DMA concentrations in CZ or QY soils (data not shown).

Abundances of 16S rRNA, *aioA, arsC* and *arrA* genes. The abundances of 16S rRNA, reflecting the total bacterial population, and four functional genes involved in As transformation were quantified at the end of the incubation experiments. In ST soil, additions of 1 and 3 mmol kg^{-1} nitrate significantly increased the copy number of the As(III) oxidase gene *aioA* by approximately 2 and 10 fold, respectively (Fig. 2b). No PCR products were obtained with the primer set tested for *arxA* in any of the four soils. In contrast, there was no significant effect on the copy numbers of bacterial 16S rRNA, *arrA* (encoding As(V) respiratory reductase) or *arsC* (encoding arsenate reductase) (Fig. 2a, c, d). Similarly, additions of 1 mmol kg^{-1} nitrate significantly increased the copy number of *aioA* in CZ, QY, HC soils, but had no significant effect on either bacterial 16S rRNA or *arrA* genes (Supporting Information, Fig. S5). In the case of *arsC,* the effect was inconsistent, with one soil (QY) showing a significant positive effect, one soil (HC) showing a significant negative effect and the other soil (CZ) showing no significant effect.

Diversity of *aioA* gene. Because AioA is involved in microbial As(III) oxidation under anoxic

conditions,[22, 60] the diversity of *aioA* gene was investigated in the control and nitrate-amended treatments of ST soil after 28 days of flooded incubation. PCR amplification yielded an *aioA* gene product of the expected size (550 bp). A total of 143 *aioA* gene clone sequences were confirmed as As(III) oxidase genes by blasting against the NCBI database. These sequences could be grouped into 6 phylotypes and 14 OTUs (Supporting Information, Table S3). There were 48, 47 and 48 *aioA* sequences in the clone libraries from the control, 1 and 3 mmol kg^{-1} nitrate-amended ST soil, respectively, belonging to 14, 14 and 12 OTUs, respectively. These sequences were clustered with the *aioA* gene sequences from both cultured and uncultured organisms available in the GenBank database and were under the classes *α-proteobacteria* and *β-proteobacteria*, with 87.4% of clones having the closest cultured relatives with the latter class. (Supporting Information, Table S3). Nitrate amendments increased the proportions of *aioA* in phylotype I (unidentified *β*-proteobacteria-1 OTUs) and phylotype IV (*Acidovorax*-like OTU) (Fig. 3a; Supporting Information, Fig. S6 and Table S3). Amendments of 1 and 3 mmol kg^{-1} nitrate increased the percentage of phylotype I clones in the total clones to 40% and 67%, respectively, compared with 33% in the control. *Acidovorax*-related OTU (phylotype IV) was detected only in the 3 mmol kg^{-1} nitrate amended microcosm, accounting for 13% of the total *aioA* clones (Supporting Information, Fig. S6 and Table S3). In contrast, nitrate amendments decreased the number of *aioA* clones in phylotype III belonging to the bacterial genera *Hydrogenophaga* (Supporting Information, Fig. S6 Table S3). The closest relative in this phylotype was the *aioA* gene from *Hydrogenophaga*, which was isolated from gold mine environments in Australia.[61]

Characterization of total microbial community. Total bacterial community was evaluated by

Illumina sequencing of 16S rRNA genes (Supporting Information, Table S4). A total of 283,749 sequences were obtained from 9 ST soil samples from the three nitrate treatments, resulting in an average of 31,528 sequences per library. Analyses of the sequence data using principal coordinate analysis (PCoA) showed that nitrate additions resulted in a shift in microbial community structure along PC2 (Supporting Information, Fig. S7). The Illumina 16S rRNA gene sequences could be grouped into 1777 OTUs. The abundance of OTUs in the soil bacterial community was not significantly affected by nitrate amendments. Neither was the bacterial diversity, measured by the Shannon or Chao1 index, affected by nitrate amendments. However, significant differences were found in the phylum- or class-level distribution of soil bacterial 16S rRNA (Supporting Information, Fig. S8 and Table S5). The abundance of the phylum *Bacteroidetes* was significantly increased by nitrate amendments (from 15.26% to 20.53%; Supporting Information, Table S5). At the class level, nitrate amendments significantly increased the abundance of *β-proteobacteria* from 2.35% to 3.67%. Within the *β-proteobacteria* class, the relative abundance of *Acidovorax*-related OTU in the *Comamonadaceae* family was significantly enhanced by nitrate amendments (Fig. 3b, Supporting Information, Table S5). In particular, the relative abundance of the genus *Acidovorax* was increased by more than 20 fold by the amendment of 3 mmol kg^{-1} nitrate. The genus *Azoarcus* of the family *Rhodocyclaceae* also displayed a significant increase in relative frequency in nitrate-amended soils (Supporting Information, Table S5), increasing from 0.01% in the control to 0.52% in the soil amended with 3 mmol kg^{-1} nitrate. With the rise in *β-proteobacteria*, declines in the relative abundance of the *γ-proteobacteria* (from 2.85 % to 1.78 %) and *Clostridia* (from 20.66 % to 17.1 %) were also apparent.

Isolation and characterization of an anaerobic As(III) oxidizer. To further understand

nitrate-stimulated As(III) oxidation, we isolated a heterotrophic As(III)-oxidizing bacterial strain (ST3) from the nitrate-amended ST soil under the anaerobic and nitrate-reducing conditions. Strain ST3 shares the highest 16S rRNA gene sequence similarity with a facultative anaerobic As(III) oxidizer *Acidovorax* sp. NO1 (99%) (Supporting Information, Fig. S9).[62] The deduced amino acid sequence of *aioA* from strain ST3 showed 99% identity to AioA from *Acidovorax* strain NO1 (Supporting Information, Fig. S6).

The ability of strain ST3 to oxidize As(III) under the anaerobic nitrate-reducing conditions was tested. As(III) (0.5 mM) was completely oxidized by strain ST3 within 6 days (Fig. 4). Concurrently, strain ST3 reduced approximately 3.5 mM NO_3^- (initial concentration 5 mM) to NO_2^-. No As(III) oxidation in the medium occurred in the sterile control without strain ST3 inoculum. In the presence of ST3 but without nitrate, there was also no oxidation of As(III) (data not shown). Strain ST3 was also capable of oxidizing 0.5 mM As(III) in the CDM medium (with lactate as the sole carbon source) under aerobic conditions (Supporting Information, Fig. S10), consistent with the strain being a facultative anaerobe. As(III) oxidation by strain ST3 was faster under aerobic conditions than under anaerobic conditions.

Strain ST3 was also able to oxidize 0.6 mM Fe(II) (initial concentration 3 mM) within 10 days under anoxic nitrate-reducing conditions, while approximately 3.6 mM NO_3^- (initial concentration 10 mM) was reduced (Fig. 5). 0.4 mM nitrite accumulated in the medium on day 2 and the concentration remained stable to the end of the experiment.

To test if As(III) and Fe(II) could be oxidized abiotically by nitrite which was reduced from nitrate by strain ST3, As(III) or Fe(II) was reacted with nitrite under anoxic conditions without strain ST3. Oxidation of Fe(II) was observed, and the oxidation increased with the concentration of nitrite added (Supporting Information, Fig. S11). With 2 mM nitrite, approximately 0.6 mM

Fe(II) was oxidized in 6 days, which was similar to the extent of Fe(II) oxidation by strain ST3. In contrast, no abiotic oxidation of As(III) by nitrite was observed at any of the nitrite concentrations (0.5 – 4 mM) tested (Supporting Information, Fig. S12).

DISCUSSION

Previous studies have shown that some microbes can mediate anaerobic As(III) oxidation coupled to denitrification in anoxic lake water,[21] sediment[24] and paddy soil.[22] There is evidence that the availability of nitrate has a strong influence on As speciation and mobility in lake water column[23, 27] and sediment.[63] In the present study, we show that the additions of nitrate markedly enhanced anaerobic As(III) oxidation in flooded paddy soils, resulting in decreased As mobility likely due to a stronger adsorption of As(V). This effect was observed in all four As-contaminated paddy soils tested, although the magnitude of the effect varied among the soils (Fig. 1 and Fig. S4). In one of the soils tested (ST), increasing the nitrate dose from 1 to 3 mmol kg^{-1} increased the extent of anaerobic As(III) oxidation, suggesting a dose-dependent effect. The doses of nitrate used in our experiments were modest compared to the typical amounts of nitrogen fertilizers applied to paddy rice in China (around 200 kg N ha^{-1}, equivalent to 5.5 mmol kg^{-1} soil in the 0 – 20 cm plow layer assuming a bulk density of 1.3 g cm^{-3}). The typical form of N fertilizer applied to paddy rice is urea, which is hydrolyzed to ammonium in the soil. It has been reported that ammonium can be nitrified to nitrate in the rhizosphere of rice because of the release of oxygen from the aerenchyma of rice roots.[64, 65] As(III) may be oxidized by oxygen without the involvement of microorganisms, but this process is known to be very slow.[66] In the presence of nitrate and As(III) oxidizing bacteria, the rate of As(III) oxidation would be enhanced greatly. Therefore, nitrate-enhanced microbial As(III) oxidation may be expected to occur in the

rhizosphere of rice under normal paddy management conditions.

Microbial As(III) oxidation is catalyzed by As(III) oxidases consisting of the AioA and AioB subunits.[67] This has been shown to be the case for both aerobic and anaerobic As(III) oxidation.[68, 69] In the four paddy soils tested, we found that the abundance of *aioA* gene was significantly enhanced by nitrate additions (Fig. 2 and Fig. S5), indicating an increase in the microbial population capable of anaerobic As(III) oxidation. In contrast, there was no significant effect of nitrate additions on the total abundance of bacteria measured as the copy number of 16S rRNA. Furthermore, nitrate additions had insignificant or inconsistent effect on the abundance of the microbial functional genes responsible for As reduction, such as *arrA* and *arsC* genes (Fig. 2 and Fig. S5). Distinct from AioA, ArxA is a new clade of As(III) oxidases found in strain MLHE-1 from Mono Lake.[42] However, no PCR products were obtained using the primer set that has been successfully used to amplify *arxA*-like genes from saline lake, sediment, and hot spring environments,[42, 70, 71] suggesting a lack of these genes in the paddy soils tested.

To understand why nitrate additions enhanced the abundance of *aioA* in flooded paddy soils, we characterized the microbial community structure in ST soil amended with or without nitrate by 16S rRNA gene sequencing. Nitrate additions significantly increased the abundance of the sequences related to the members of *β-proteobacteria.* Within *β-proteobacteria*, the abundances of the genera *Acidovorax* and *Azoarcus* displayed significant increases in their relative frequency compared with the control treatment without nitrate addition (Table S5). Many members in the genera *Acidovorax* and *Azoarcus* are denitrifiers.[24, 32, 56] It is therefore not surprising that their abundances were increased by nitrate additions. Previous studies have shown that some members of *Acidovorax* and *Azoarcus* can mediate anaerobic As(III) oxidation by respiring nitrate as the terminal electron acceptor.[24, 28, 62] Examples include the *Acidovorax* strain NO1 isolated from

As-contaminated gold mine soils[62] and the *Azoarcus* strain DAO1 isolated from an As-contaminated soil.[24] A recent study showed that *Acidovorax*-like *aioA* genes are distributed widely in paddy soils.[18] Nitrate additions were found to influence the diversity of *aioA* gene sequences. In particular, *aioA* sequences belonging to the unidentified *β-proteobacteria*-1 OTUs (phylotype I) and *Acidovorax*-like OTU (phylotype IV) became more numerous in the nitrate-amended ST soil (Fig. 3, Fig. S6 and Table S3). These results are consistent with the 16S rRNA sequencing data, supporting the notion that the *aioA* phylotypes I and IV may play an important role in anaerobic As(III) oxidation in the nitrate-amended ST soil. *γ-proteobacteria* are well known for their capability to reduce As(V) in diverse environments.[72] Nitrate additions were found to decrease the relative abundance of *γ-proteobacteria* in ST soil. This may imply a decreased As(V) reduction capacity in nitrate amended soils. However, this possibility can be largely ruled out because there was no significant or consistent effect of nitrate on the abundances of the As(V) reductase genes *arrA* and *arsC* (Fig. 2, and Fig. S5).

To further investigate the mechanism of nitrate-enhanced As(III) oxidation in flooded paddy soils, we isolated a bacterial strain, ST3, from the ST soil amended with 3 mmol nitrate kg^{-1}. Strain ST3 belongs to *Acidovorax* and possesses a strong ability to oxidize As(III) under anoxic conditions coupled to the reduction of nitrate to nitrite (Fig. 4). Unlike the chemoautotrophic As(III)-oxidizing bacterium *Paracoccus* strain SY isolated previously from an As-contaminated paddy soil,[22] strain ST3 is a heterotrophic As(III) oxidizer. Phylogenetic analysis of the putative AioA indicated that AioA sequences closely related to strain ST3 were present at relatively high frequency in the ST soil amended with 3 mmol kg^{-1} nitrate (Fig. S6). The presence of the distinctive *aioA* gene in both strain ST3 and the soil microcosm strongly suggests that the *Acidovorax*-like AioA catalyzes anaerobic As(III) oxidation *in situ*. Interestingly, strain ST3 was

also able to carry out nitrate-dependent Fe(II) oxidation under anoxic conditions (Fig. 5). The ability of some denitrifying microorganisms to oxidize Fe(II) with nitrate as the electron acceptor is well documented.[73] Nitrite, which is the product of nitrate reduction by anaerobic nitrate-reducing bacteria, can also oxidize Fe(II) abiotically at circumneutral pH.[56] This abiotic oxidation was also observed in our experiment (Supporting Information, Fig. S11). It is possible that the oxidation of Fe(II) by strain ST3 might result from the abiotic reaction with nitrite. In contrast, As(III) could not be oxidized by nitrite (Supporting Information, Fig. S12), which is not surprising given that AioA has been shown to be required for As(III) oxidation.[22, 24] In ST soil, nitrate additions were found to decrease the concentration of Fe(II) in the soil porewater and increase the concentration of Fe(III) (Fig. 1), suggesting that nitrate stimulated Fe(II) oxidation. This result is consistent with previous studies on lake water[27] and paddy soils.[31] Formation of Fe(III) precipitates from anaerobic oxidation of Fe(II) would provide additional sorption phase for both As(V) and As(III) in flooded paddy soils, which may also contribute to decreased As mobility in nitrate-amended soils.

Environmental significance. The results from the present study demonstrate that anaerobic As(III)-oxidation in paddy soil can be greatly enhanced by nitrate additions. This effect can be attributed to both the enhanced population of anaerobic As(III) oxidizers in the soils and the enhanced activity of As(III) oxidation coupled to nitrate reduction. The availability of nitrate is therefore an important factor in influencing the biogeochemical cycle of As in paddy soils. Additions of nitrate may be useful way to decrease As mobility and bioavailability in As-contaminated paddy soils.

ASSOCIATED CONTENTS

Supporting Information Available

Figures showing the effects of nitrate additions on soil pH and redox potential in Shantou soil; the relationship between the total soluble Fe concentrations in porewater determined by the atomic absorption spectroscopy and ferrozine colorimetric methods; effects of nitrate additions on the dynamics of porewater Mn concentrations in Shantou soil, the dynamics of porewater As(III), As(V), total soluble As, Fe and Mn concentrations, and the abundance of *aioA*, *arsC*, *arrA* and 16S rRNA genes in CZ, QY and HC paddy soils; neighbor-joining phylogenetic trees of bacterial AioA protein sequences retrieved from the nitrate-amended and control Shantou soil after anoxic incubation for 28 days; principal coordinate analysis (PCoA) of 16S rRNA gene sequences from Shantou soil amended with different concentrations of nitrate; taxonomic distributions of bacterial phyla and classes in nitrate-amended and control Shantou soil; phylogenetic tree based on 16S rRNA gene sequences from the strain ST3 isolated in the present study and other As(III)-oxidizers; oxidation of As(III) in CDM medium by *Acidovorax* strain ST3 under aerobic condition; effect of nitrite on abiotic oxidation of Fe(II) and As(III) at neural pH under anoxic conditions. Tables showing the locations and selected properties of the soils used in the study; details of primer pairs and qPCR thermal cycling parameters for 16S rRNA, *aioA, arxA*, *arsC* and *arrA* genes; contributions of various phylotypes to *aioA* gene-based clone libraries derived from the nitrate-amended and control Shantou soil; richness and diversity of bacterial communities in the nitrate-amended and control Shantou soil analyzed by pyrosequencing; effect of nitrate additions on the percentages of different bacterial phylogenetic groups in the total numbers of OTUs based on 16S rRNA gene sequencing in Shantou soil. The Supporting Information is available free of charge on the ACS publications Web site.

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Fig. 1. Effect of nitrate additions on the concentrations of As(III) (a), As(V) (b), total soluble As (c), Fe(II) (d), Fe(III) (e), and total soluble Fe (f) in the porewater of Shantou (ST) soil incubated under anoxic conditions. Data are mean ± SE (n = 3). Arrow indicates the time of nitrate addition.

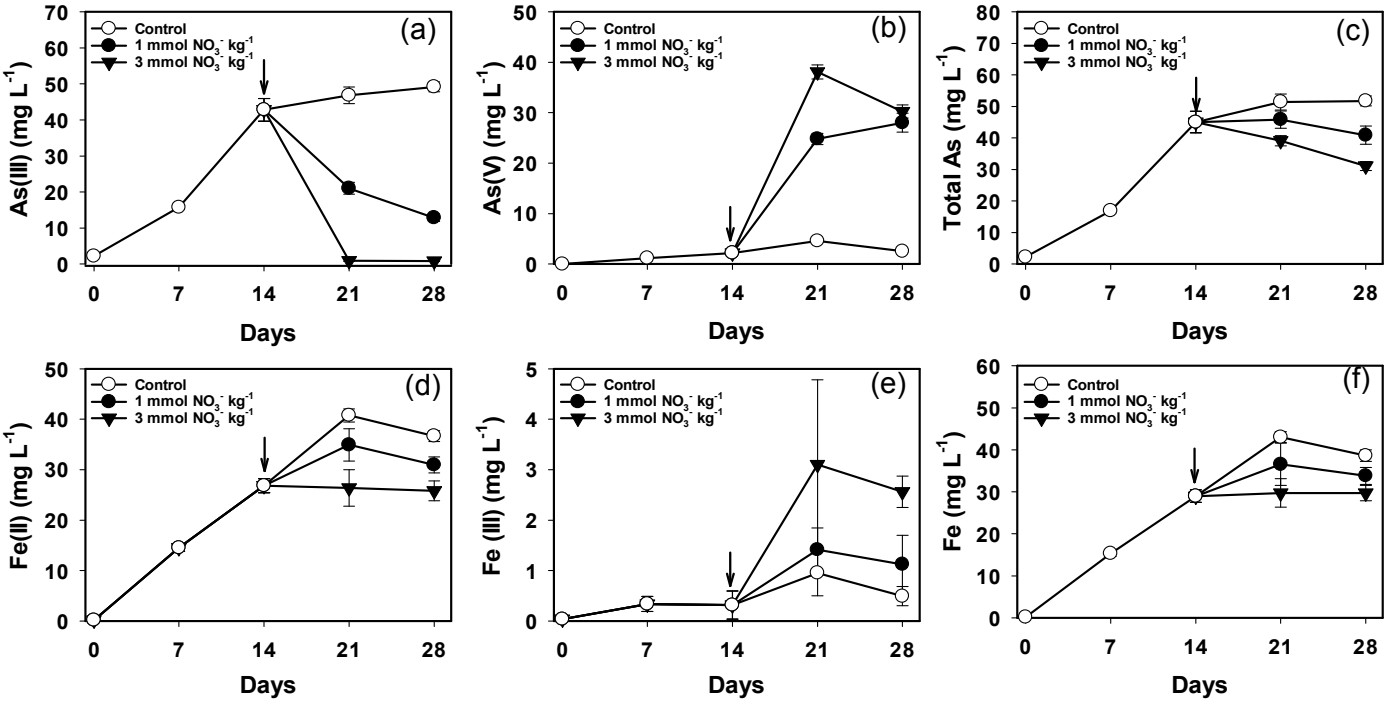

Fig. 2. Effect of nitrate additions on the abundance of 16S rRNA (a), *aioA* (b), *arsC* (c) and *arrA* (d) genes in Shantou (ST) soil after anoxic incubation for 28 days. Data are mean ± SE (n = 3). Different letters indicate significant differences at $P < 0.05$.

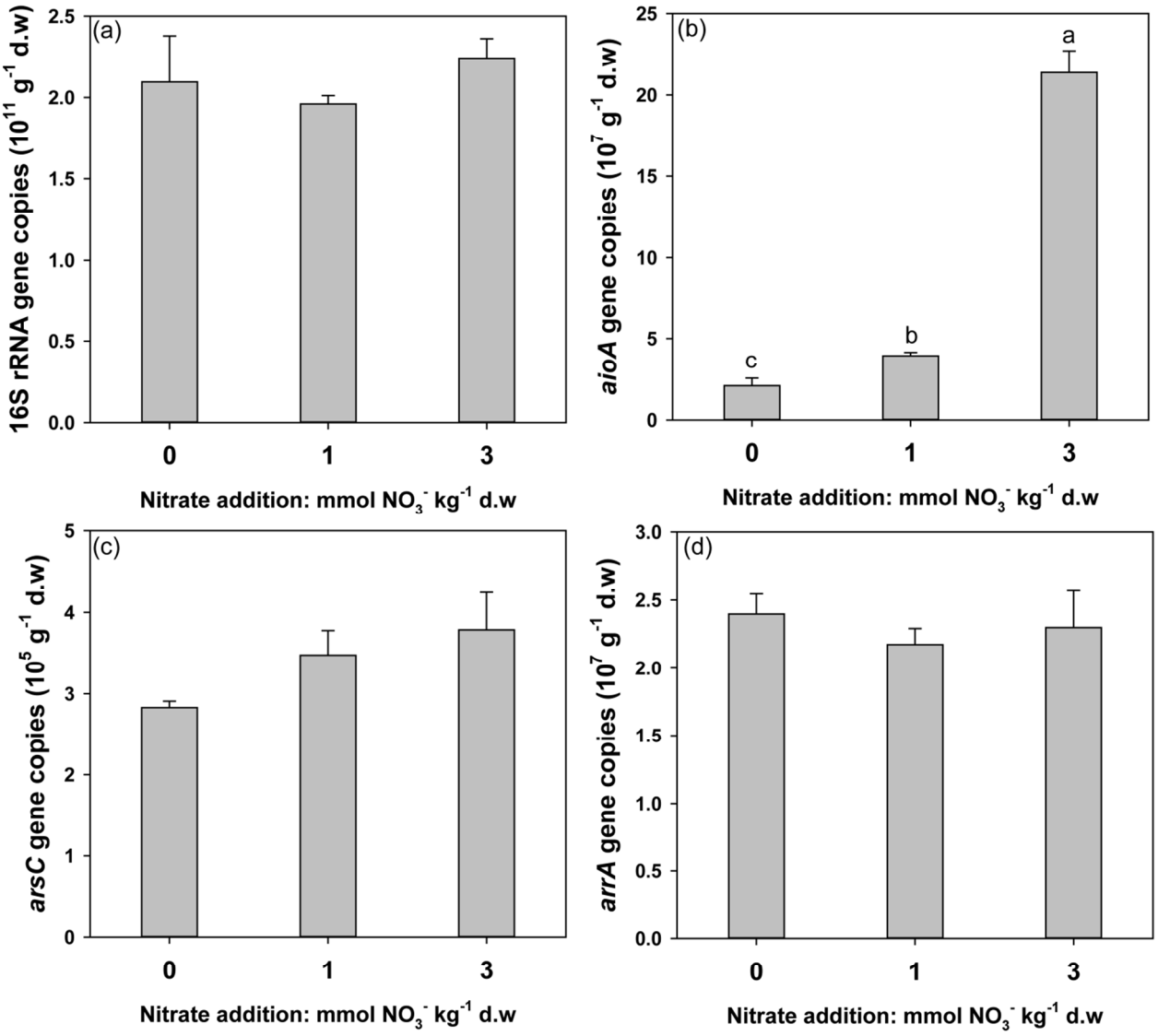

Fig. 3. Effect of nitrate additions on As(III) oxidizing bacterial groups based on the *aioA* gene clone library (a) and the relative abundances of the genus *Acidovorax* in the total bacterial community based on 16S rRNA sequencing (b) in Shantou (ST) soil after anoxic incubation for 28 days.

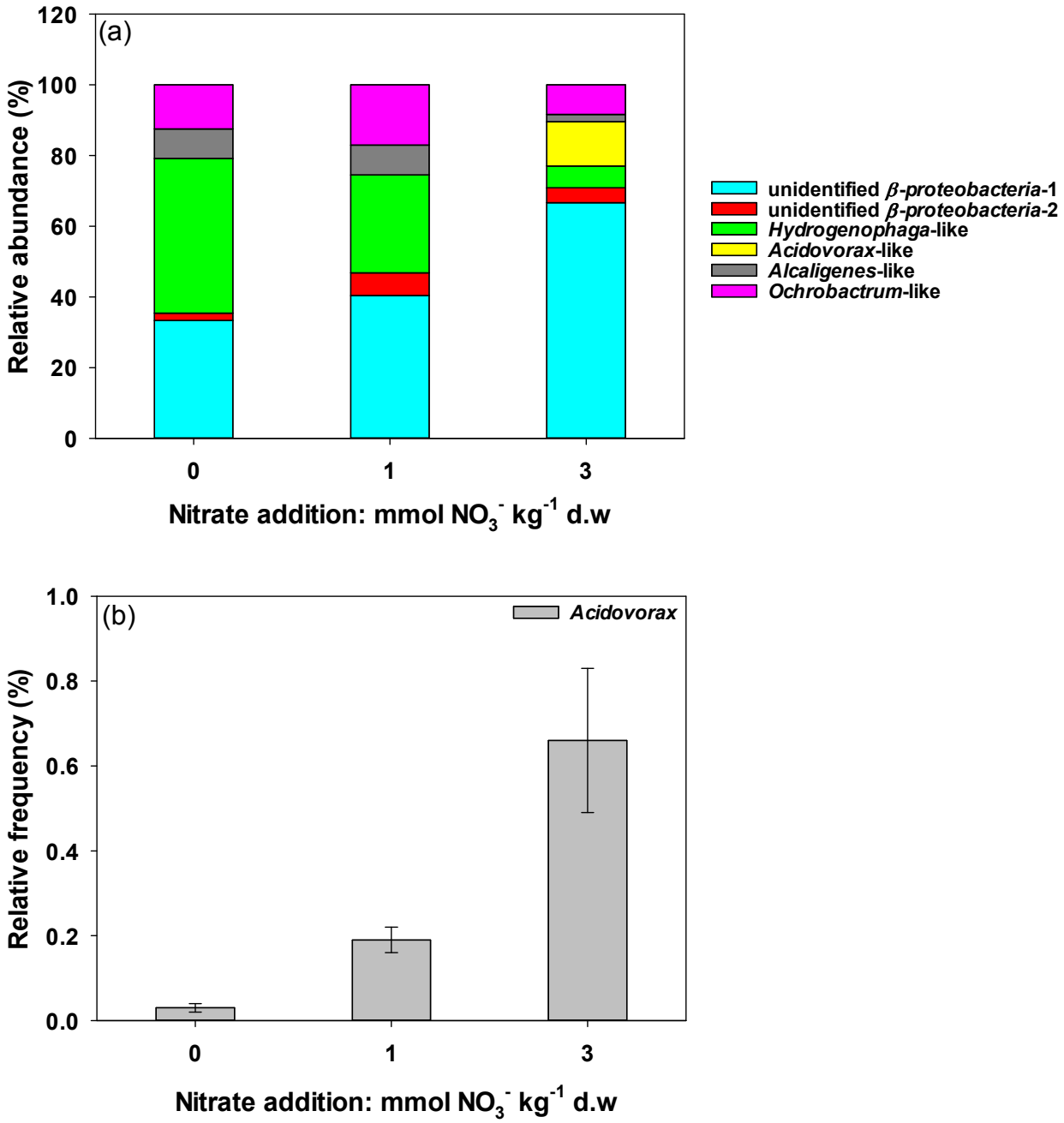

Fig. 4. Oxidation of As(III) (a) and nitrate consumption and nitrite formation (b) by *Acidovorax* strain ST3 under anoxic nitrate-reducing condition. No strain ST3 inoculum was added to the sterile control. Data are mean ± SE (n = 3).

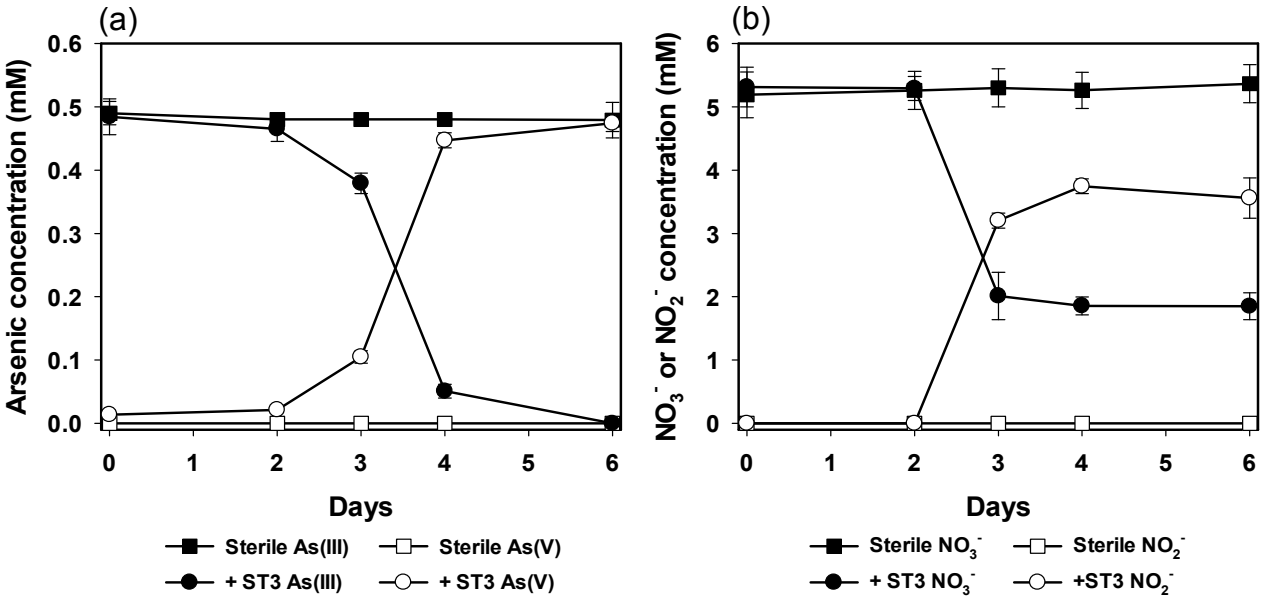

Fig. 5. Oxidation of Fe(II) (a) and nitrate consumption and nitrite formation (b) by *Acidovorax* strain ST3. No strain ST3 inoculum was added to the sterile control. Data are mean ± SE (n = 3).

