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Article

Anaerobic As(III) Oxidation Coupled with Nitrate Reduction and Attenuation of Dissolved Arsenic by *Noviherbaspirillum* Species

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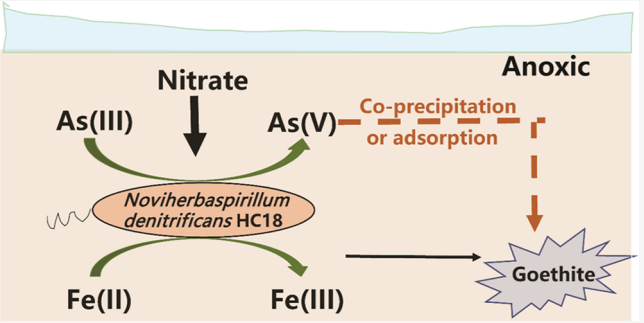
ABSTRACT: Arsenic pollution is a worldwide problem. In flooded arsenic-contaminated paddy soils, arsenic bioavailability to rice plants is elevated due to the reductive dissolution of arsenate (As(V)) containing Fe(III) (oxyhydr)oxides. However, microbial-mediated arsenite (As(III)) and Fe(II) oxidation has great potential for attenuating arsenic mobility in an anoxic environment. In this study, a bacterium strain HC18 belonging to the genus *Noviherbaspirillum* was isolated from arsenic-contaminated paddy soil. This microbe is able to oxidize both As(III) and Fe(II) under anoxic nitrate-reducing conditions. However, strain HC18 was not able to oxidize As(III) to As(V) under oxic conditions, suggesting that the process is nitrate-dependent. Genome mining analysis revealed that the As(III) oxidase *aio* and *arx* gene clusters and denitrification gene clusters are present in *Noviherbaspirillum denitrificans* HC18. Oxidation of aqueous Fe(II) resulted in precipitation of Fe(III)-containing minerals, and As(V) produced by As(III) oxidation was bound to Fe(III) (oxyhydr)oxides such as goethite. The effectiveness of *N. denitrificans* HC18 for As(III) and Fe(II) oxidation under anoxic nitrate-reducing conditions reveals a potential for its use in bioremediation of arsenic-contaminated environments.



KEYWORDS: *anaerobic As(III) oxidation, anaerobic Fe(II) oxidation, denitrification, bacterial diversity, Noviherbaspirillum*

INTRODUCTION

Dietary exposure to inorganic arsenic, a nonthreshold class-1 carcinogen, poses a significant health risk to people who rely on rice as their staple food.[1] Rice grains accumulate 10-fold more arsenic than any other crop, primarily due to its growth under flooded conditions.[2,3] Under flooded conditions, oxygen is depleted, which triggers soil microbial communities to respire alternative terminal electron acceptors such as nitrate, Fe(III) (oxyhydr)oxides, and sulfur compounds.[4] Under flooded conditions, reductive dissolution of Fe(III) (oxyhydr)-oxides, the main sorbents of arsenic in aerobic soils,[5,6] results in excessive arsenic release into paddy soil pore waters predominantly as As(III).[7]

Anaerobic nitrate-reducing As(III) oxidizers are ubiquitous in the environment, including groundwater, salt lakes, sediments, and soils.[8−12] As(III) oxidases Arx and Aio are essential for As(III) oxidation under anoxic nitrate-reducing conditions.[13] The As(III) oxidase Aio is composed of a large (AioA) and a small (AioB) subunit, which catalyze As(III) oxidation under both oxic and anoxic nitrate-reducing conditions.[9,14] The Arx enzyme is an alternative As(III) oxidase that functions during anaerobic respiration of nitrate,[15] in anoxygenic photosynthesis[16] and in autotrophic and heterotrophic As(III) oxidizing bacteria.[17,18] Considering that As(III) is more abundant in anoxic environments, anaerobic As(III) oxidation by Aio or Arx is a major contributor to arsenic biogeochemistry.[13,19] Microbial oxidation of Fe(II) results in the formation of insoluble Fe(III) (hydr)oxide minerals such as ferrihydrite, green rusts, and goethite,[20,21] which have the potential to coprecipitate or adsorb dissolved arsenic and decrease its bioavailability.[5,22] A wide variety of nitrate-reducing organisms have been isolated for their ability to oxidize Fe(II) or As(III) in anaerobic environments.[10,14,23] *Acidovorax* sp. ST3[10] and *Paracoccus* sp. QY30,[23] isolated for their abilities to oxidize As(III), also oxidize Fe(II) heterotrophically under anoxic nitrate-reducing conditions. Based on the results from deletion of the nitrite reductase *nirK* gene and complementation experiments, nitrite accumulation during denitrification was shown to be required for anaerobic Fe(II) oxidation in *Paracoccus* sp. QY30.[23]

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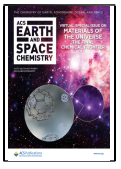

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Because arsenic is usually mobilized in flooded paddy soils, biological oxidation of Fe(II) and As(III) enhances arsenic immobilization under flooded conditions.[24,25] In situ arsenic bioremediation experiments have shown that nitrate and sulfate addition stimulates oxidation and immobilization of As(III) and Fe(II) by indigenous bacterial populations.[10,26,27] However, the optimization of bioremediation strategies is dependent on knowing the indigenous bacterial species available for bioremediation.

The goal of this project was to identify the role of indigenous bacterial communities in arsenic immobilization under anoxic nitrate-reducing conditions. The composition of microbial communities found upon nitrate amendments in anoxic environments was determined using 16S rRNA gene sequencing. Species able to oxidize As(III) and Fe(II) were identified that have the potential to be used to bioremediate arsenic-contaminated environments. The effect of exogenous electron donors and acceptors on anaerobic As(III)/Fe(II) oxidation coupled to denitrification by soil bacterial community was investigated. Our results show that biostimulation of arsenic-containing soils with nitrate and lactate enrich bacterial species belonging to the genus *Noviherbaspirillum*. The knowledge gained from the study of one isolate, *Noviherbaspirillum denitrificans* HC18, contributes to our understanding of the mechanisms of As(III) and Fe(II) oxidation in the arsenic-contaminated paddy soil environment.

MATERIALS AND METHODS

Soil Sampling and Microcosm Setup. An arsenic-contaminated paddy soil from Hechi City, Guangxi Province, was used as inocula for this study. The soil had the following characteristics: pH 6.3, 1.9% organic matter, and loamy clay. The total arsenic concentration of the soil was 400 mg kg^{-1}, and the ammonium oxalate extractable arsenic was 256 mg kg^{-1}. A detailed description of the study area was provided previously.[10] Microcosms were constructed in the Coy anoxic glovebox (N_2, CO_2, and H_2 (90:5:5)). Eight grams of soil was added to 20 mL of sterile deoxygenated MilliQ (Millipore) water in 100 mL sterile serum bottles. Then, the headspace was replaced with N_2/CO_2 (v/v, 80:20) in the anoxic glovebox. The microcosms were cultured in the dark without any amendment for a period of 20 days, to release arsenic from the soil as well as consume readily available carbon and nitrate. Then, three treatments were established involving (i) soil without lactate and nitrate, (ii) soil with 10 mM nitrate, and (iii) soil with 10 mM nitrate and 2 mM lactate. Control microcosms were sterilized with γ ray irradiation at 50 kGy subsequent to the addition of nitrate or lactate. Microcosms were incubated anoxically for 8 days at 28 °C, and the microcosm culture samples were periodically taken (0.5 mL) with sterile needles and syringes and filtered with 0.45 μm membrane filter prior to analyzing changes of Fe(II), total As, As(III), and As(V). All experiments were set up in triplicate.

16S rRNA Gene High Throughput Sequencing. Total genomic DNA was extracted in triplicate from each enrichment sample with each treatment (20 mL) at the end of the experiment using a PowerSoil DNA isolation kit. The purity and the quantity of the extracted DNA were analyzed using a UV−vis spectrophotometer (NanoDrop 2000, Thermo). For 16S rRNA gene sequencing, the V4−V5 regions of the 16S rRNA gene were amplified using the universal primer set 515F and 907R and the sequencing was carried out on the Illumina Miseq PE300 platform at Biozeron Biotechnology Co., Ltd. The Quantitative Insights into Microbial Ecology (QIIME 1.8.0) tool kit was used to analyze the 16S rRNA gene sequences.[28] The details of the 16S rRNA gene polymerase chain reaction (PCR), 16S rRNA gene high throughput sequencing, and bioinformatic analysis are presented in the Supporting Information. Analysis of similarity (ANOSIM) was used to test the differences in the compositions of the bacterial communities.

Isolation of Anaerobic As(III)-Oxidizing Bacteria. Enrichment samples were used for the isolation of anaerobic As(III), as well as Fe(II)-oxidizing strains. One milliliter of cultures was added to 9 mL of anoxic sterilized deionized water under a headspace of N_2/CO_2 (80:20) and then the samples were serially diluted in bicarbonate-buffered minimal freshwater medium (FWM) containing 0.14 g of KH_2PO_4, 0.2 g of NaCl, 0.3 g of NH_4Cl, 0.3g of $MgCl_2{\cdot}6H_2O$, 0.1 g of $CaCl_2{\cdot}2H_2O$, 1 mL of vitamin solution, 1 mL of trace element solution,[29] and 22 mM bicarbonate buffer and pH 7.2/L of medium. Nitrate (10 mM) was spiked as an electron acceptor, while 5 mM lactate was added as a carbon source. The enrichments were transferred onto sterile cultures three times and incubated anaerobically.

Aliquots of the cultures were spread onto FWM agar plates, and then the plates were incubated at 28 °C in an anoxic glovebox. After incubation for 10 days, a number of colonies were picked up and transferred to the FWM medium. After obtaining pure cultures, the As(III) oxidation abilities of the isolates were determined using the high-performance liquid chromatography (HPLC) coupled with inductively coupled plasma mass spectrometry (ICP-MS) method, and anaerobic Fe(II)-oxidation abilities of the isolates were identified by forming brownish-red Fe(III) oxide precipitates in the anoxic FWM medium containing 2.5 mM Fe(II) using the method described previously.[30]

DNA Extraction, PCR Amplification, and Phylogenetic Analysis of Strain HC18. Genomic DNA of the isolate HC18 was extracted using a phenol-chloroform method.[31] 16S rDNA gene of HC18 was PCR amplified using primers 27F and 1492R[32] (Table S1). The sequences for the As(III) oxidase large subunit (*aioA* and *arxA*) genes were amplified using degenerate primers[33,34] (Table S1). The PCR products were sequenced by Sangon Biotech (Shanghai, China). The translated *aioA* and *arxA* sequences were aligned with the reference amino acid sequences using MEGA 6.0.[35] Phylogenetic analysis of 16S rRNA and *aioA* and *arxA* genes was conducted based on the neighbor-joining method with 1200 bootstrap replicates.

Transcription Levels of As(III) Oxidase *aioA* and *arxA* Genes. To investigate the expression of As(III) oxidase *aioA* and *arxA* genes by strain HC18 under anoxic nitrate-reducing conditions, the transcript levels of *aioA* and *arxA* were measured by quantitative real-time PCR (qRT-PCR). Strain HC18 was each inoculated into 20 mL of FWM media with or without 10 μM As(III) or As(V) and incubated under anoxic conditions at 28 °C for 36 h, and then total RNA was isolated using a Trizol reagent. The purified RNA was transcribed into cDNA using Superscript III Reverse Transcriptase (Invitrogen). Real-time qPCR was performed using the SYBR Green kit on a LightCycler real-time thermocycler. The *gyrB* gene was used as an internal control for normalization. The primers used for qRT-PCR are listed in Table S1. The relative expression was quantified according to the method of $2^{-\Delta\Delta CT}$ threshold cycle (CT).[36]

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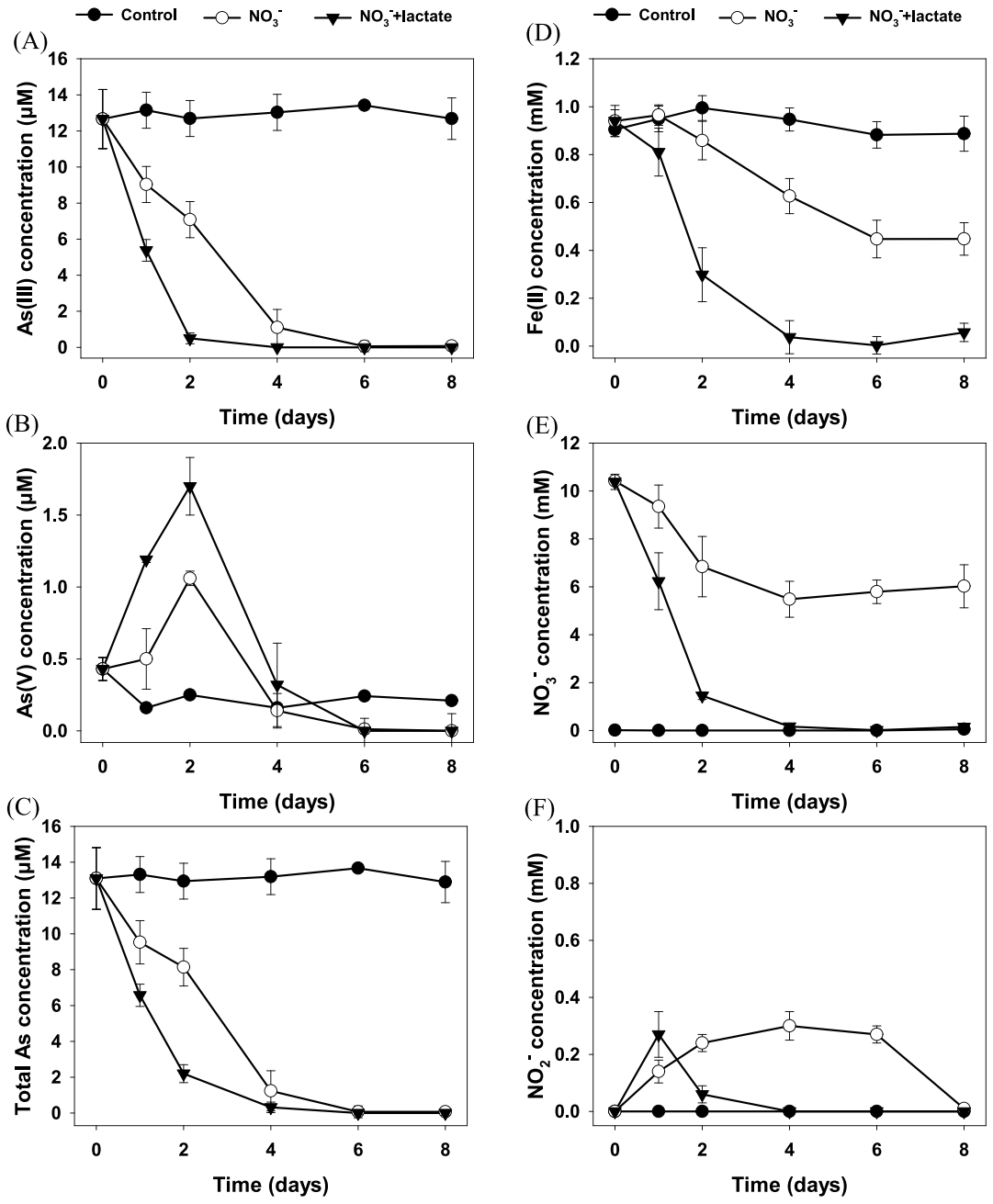

Figure 1. Time course of chemical parameters during anaerobic incubation of the untreated, nitrate-treated, and nitrate-plus-lactate-treated soil microcosms. (A) As(III) concentration, (B) As(V) concentration, (C) total arsenic concentration, (D) Fe(II) concentration, (E) nitrate concentration, and (F) nitrite concentration. Points represent the mean of three replicate samples, and error bars represent ± standard error. The absence of bars indicates that the error was smaller than the symbol.

Anaerobic As(III) and Fe(II) Oxidation Assays. Anaerobic As(III) and Fe(II) oxidation time-course studies were performed in the FWM medium with (i) 2.5 mM Fe(II), (ii) 10 μM As(III), and (iii) 2.5 mM Fe(II) with 10 μM As(III); 10 mM nitrate was spiked as an electron acceptor, while 5 mM lactate was added as a carbon source. In each case, the samples were removed at the indicated time intervals. Abiotic controls (without bacteria) as well as Fe(II)-As(III) abiotic experiments were also carried out for each treatment. All manipulations were conducted under anoxic conditions unless otherwise noted. On day 10, Fe precipitates formed in Fe(II)-treatments were collected by vacuum filtration on a nylon filter (0.45 μm) and air-dried under N_2 protection in anoxic tubes before analysis. The arsenic adsorbed to Fe oxide was separated into two parts, one part was extracted with 0.6 M *ortho*-phosphoric acid and 0.1 M ascorbic acid as described previously.[37] Arsenic species in the supernatant and phosphoric acid extracts were determined by HPLC-ICP-MS. The other solid phases were determined by X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), energy-dispersive X-ray spectrometer (EDS), and scanning electron microscopy (SEM).

To test whether goethite could oxidize As(III) abiotically, 10 μM As(III) and 2.5 mM goethite were amended into the serum bottles containing 50 mL of sterile anoxic FWM medium and incubated for up to 8 days at 28 °C under anoxic

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conditions. The arsenic adsorbed to goethite was extracted as described above.

Analytical Techniques. For arsenic speciation analysis, the pH of the supernatants was acidified with HCl to 2, followed by filtration (0.45 μm filters) in the anoxic chamber, separated by HPLC, and quantified by ICP-MS. For analysis of the concentration of Fe(II), the samples were acidified with sulfamic acid and analyzed using the 1,10-phenanthroline method.[38,39] Nitrate and nitrite concentrations were assayed colorimetrically.[40] The absorbance at 600 nm (A_{600nm}) was used to determine the cell growth. The crystallization products were analyzed by X-ray diffraction (XRD) using a SmartLab diffractometer. Scanning electron microscopy (SEM) image analysis was performed in a FEI Quanta 400 field emission gun (FEG) with an acceleration voltage of 20 kV. Energy-dispersive X-ray spectrometry (EDS) was used for semiquantitative analysis (collecting time: 90 s).

RESULTS

Microbial Oxidation of Fe(II) and As(III) in Enrichment Microcosms. The simultaneous oxidation of As(III) and Fe(II) by paddy soil bacterial communities in the anaerobic denitrifying enrichment microcosms were determined (Figure 1). In the control culture without addition of either lactate or NO_3^-, the concentrations of As(III) or Fe(II) did not change significantly (Figure 1A–D). In cultures incubated with only NO_3^-, NO_3^- was not completely reduced; the concentration of NO_2^- increased over 1 day. During that time period, As(III) almost disappeared from the cultures, with a corresponding increase in As(V). In cultures incubated with NO_3^- plus lactate, NO_3^- was completely reduced within 4 days. Along with the decrease of NO_3^-, the NO_2^- concentrations increased and then decreased after 1 day. As(V) formed after approximately 2 to 4 days, concomitant with the disappearance of total arsenic due to precipitation with Fe(III). These results suggest that the bacterial community anaerobically oxidizes As(III) to As(V) with concomitant oxidation of Fe(II) to Fe(III), which precipitates with As(V).

Microbial Community Characterization in Enrichment Microcosms. With the chemical analysis providing a clear case of microbial oxidation of both As(III) and Fe(II) in the soil microcosms under anoxic denitrifying conditions, the total microbial community was evaluated by high throughput 16S rRNA gene sequencing. The bacterial community structures across all of the samples could be grouped into 1870 unique bacterial operational taxonomic units based on the 97% sequence similarity level. The differences in the microbial community structure were significant based on the analysis of similarities (ANOSIM) ($R = 0.98$, $P < 0.01$). At the phylum level, the sequences for the control microcosms were dominated by *Firmicutes* (29.6%), *Proteobacteria* (19.0%), *Bacteroidetes* (15.9%), and *Actinobacteria* (3.1%) (relative abundance >3% for each phylum). There was a clear shift in the composition of the microbial community in response to the 8-day enrichment with NO_3^- or NO_3^- plus lactate treatment. The relative abundance of *Proteobacteria* increased significantly, representing 40.4 and 47.6% of the total sequences for the NO_3^- or NO_3^- plus lactate treatment, respectively (Figure S1 and Table S2). *Alcaligenaceae* and *Oxalobacteraceae* had the greatest abundance of family level, accounting for 11.8 and 23.7% of the total sequences for NO_3^- plus lactate treatment, respectively (Figure 2A). After NO_3^- or NO_3^- plus lactate stimulation, a few distinct genera emerged as

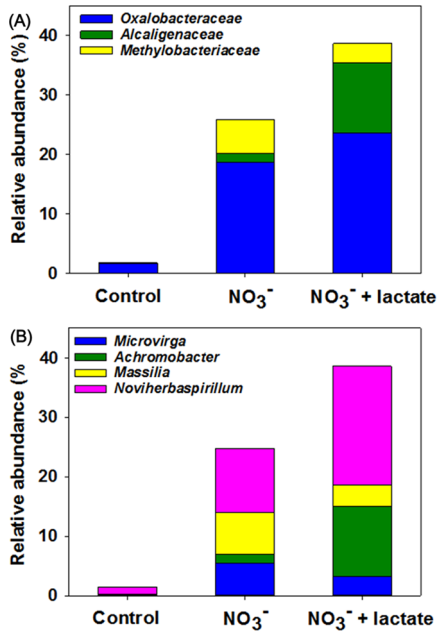

Figure 2. Microbial community structure in different enrichment cultures during 8-day incubation periods as determined by Illumina sequencing of 16S rRNA genes. (A) Relative abundance of the main families (i.e., *Oxalobacteraceae*, *Alcaligenaceae*, and *Methylobacteriaceae*). (B) Relative abundance of the main genera *Microvirga*, *Achromobacter*, *Massilia*, and *Noviherbaspirillum*. Abundance is expressed as the average percentage of targeted sequences out of the total bacterial sequences of samples from each treatment.

the dominant bacterial communities within the enrichment samples. In the NO_3^- treatment group, the majority of identifiable bacterial sequences at the genus level were affiliated with *Noviherbaspirillum* (9.4%), *Achromobacter* (9.1%), *Massilia* (7.1%), and *Microvirga* (5.5%). The lactate plus NO_3^- amendment resulted in a significant shift toward an abundance of *Noviherbaspirillum*-related sequences, represented by 22% of the total sequences (Figure 2B).

Isolation of Anaerobic As(III) and Fe(II) Oxidizers. After three transfers of the enrichment culture, an anaerobic As(III)- and Fe(II)-oxidizing bacterium isolate, termed as HC18, was obtained, and anaerobic As(III) and Fe(II) oxidation by strain HC18 under anoxic nitrate-reducing conditions was observed after several days. Strain HC18 is able to grow anaerobically using nitrate, nitrite, and N_2O as an electron acceptor, and NH_4^+ was not detected during growth with nitrate. Strain HC18 could not use Fe(III) citrate, ferrihydrite, As(V), selenate, selenite, fumarate, malate, sulfate, or thiosulfate as the electron acceptor for growth. In addition to lactate, the organic acids pyruvate, acetate, and succinate could serve as alternate electron donors and carbon sources for nitrate reduction (Table S1). Strain HC18 is defined as heterotrophic since it could not use CO_2 as the sole carbon source, nor could it grow with As(III) or Fe(II) as the sole electron donor (data not shown).

Genomic Analysis. Strain HC18 shares 99% 16S rRNA similarity to the N_2O-reducing bacterial strain *N. denitrificans* TSA40 (GenBank no. AB542402).[41] Phylogenetic analysis based on 16S rRNA sequences confirmed that strain HC18 belongs to the genus *Noviherbaspirillum* (Figure S2). In the genome of strain HC18, both As(III) oxidase *aio* and *arx* gene clusters were identified. The putative amino acid sequence for

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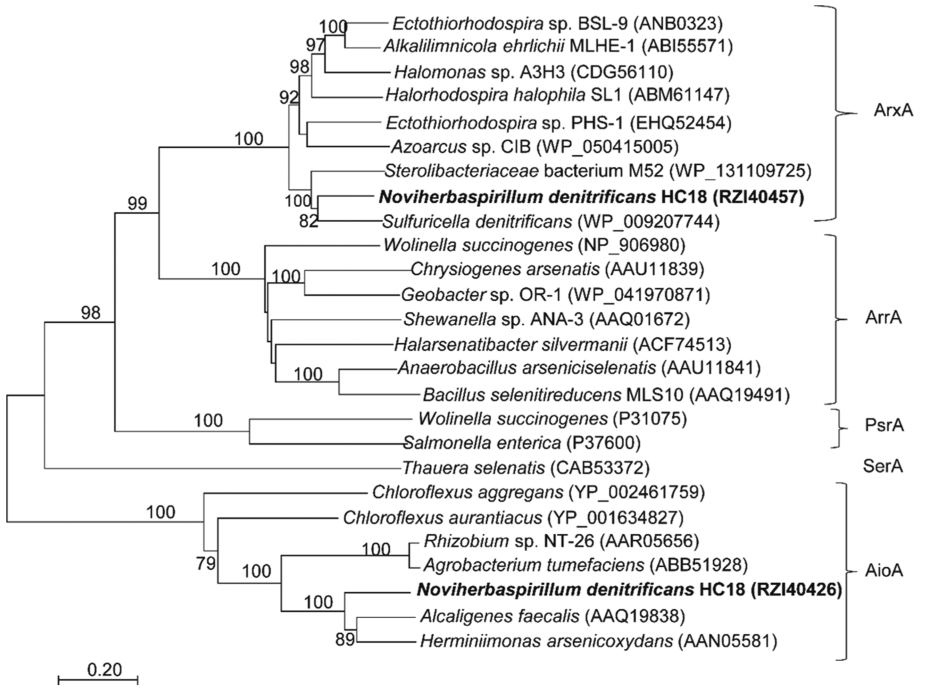

Figure 3. Phylogenetic positions of AioA and ArxA orthologs of *N. denitrificans* HC18, compared with dimethyl sulfoxide (DMSO) reductase family proteins. ArxA, anaerobic arsenite oxidase; AioA, aerobic arsenite oxidase; ArrA, respiratory arsenate reductase; PsrA, polysulfide reductase; and SerA, selenate reductase.

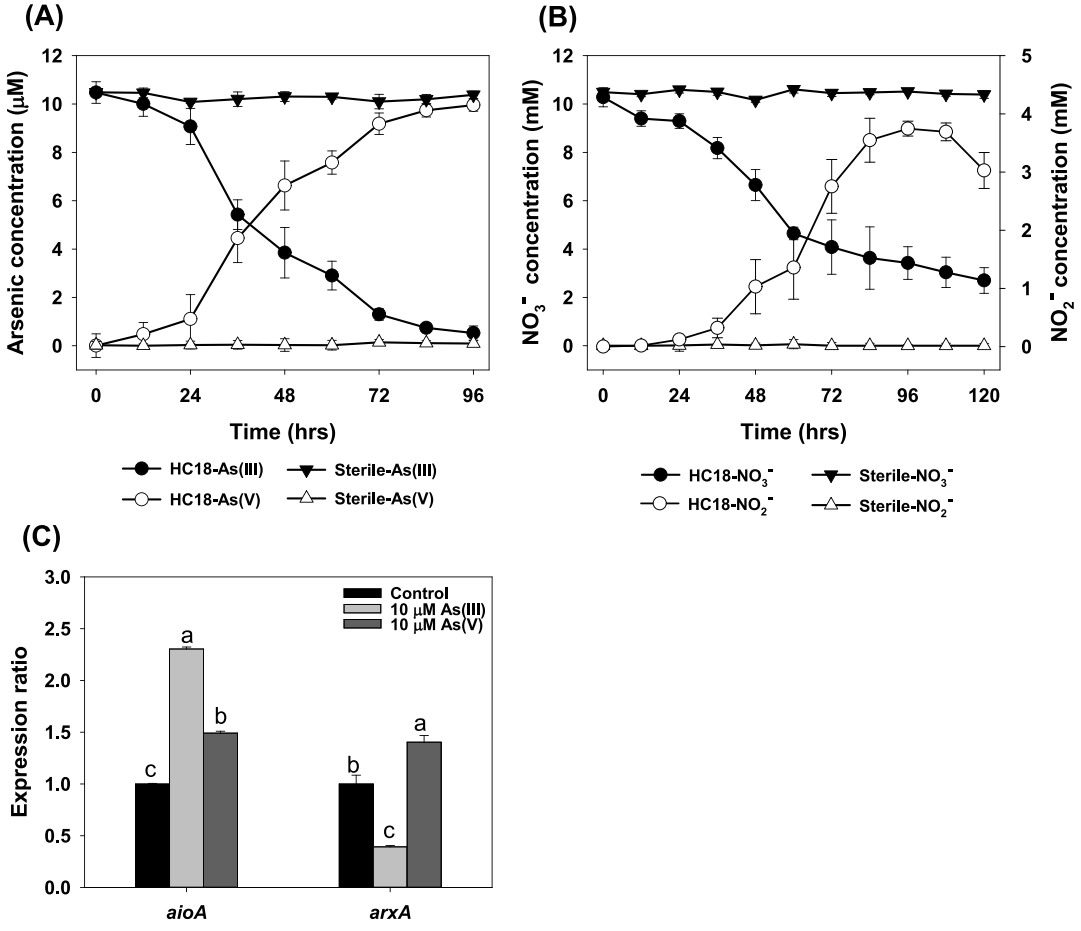

Figure 4. Oxidation of As(III) to As(V) (A) is coupled to nitrate reduction (B) by *N. denitrificans* HC18 under anoxic conditions. Relative expression levels of the arsenite oxidase genes (*aioA* or *arxA*) in strain HC18 (C). Data are shown as the mean of three replicates, with the error bars representing ± standard error.

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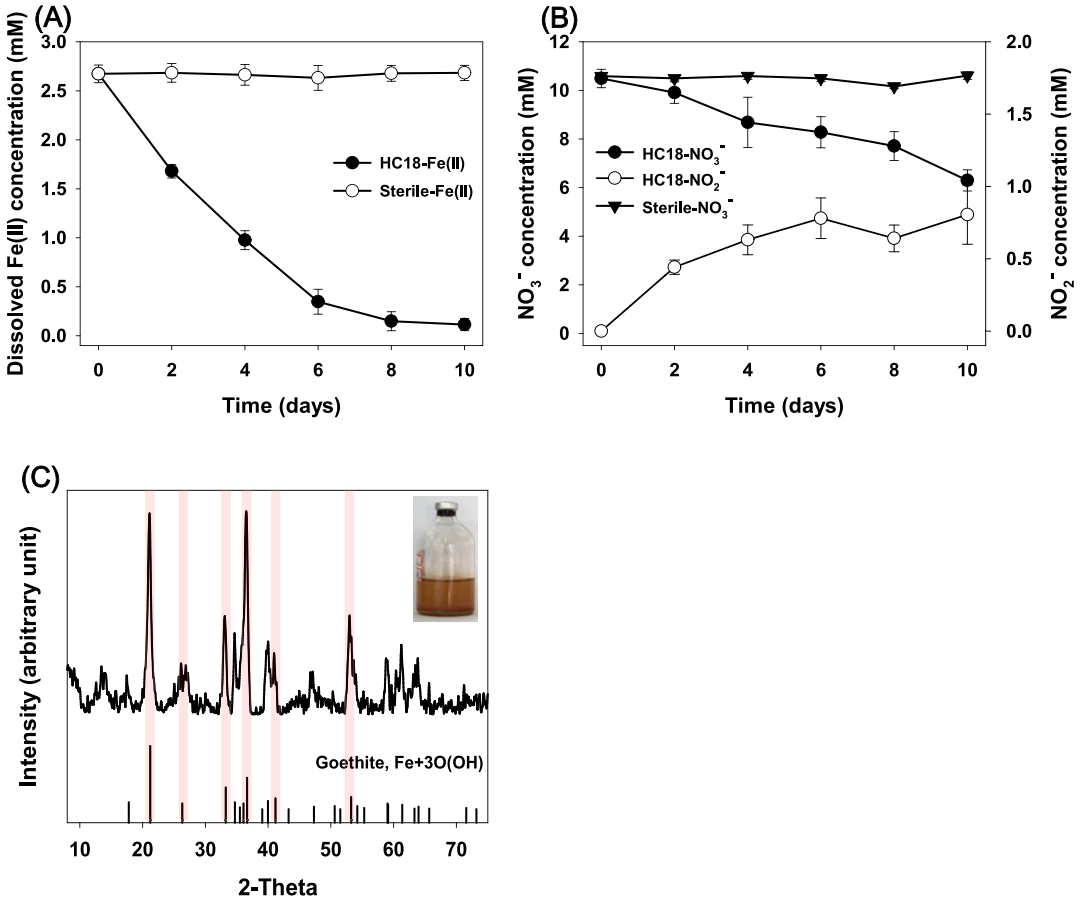

Figure 5. Oxidation of Fe(II) (A) coupled to nitrate reduction (B) by strain HC18 under anoxic nitrate-reducing conditions. XRD spectra of the mineral products formed by strain HC18 (C). The iron oxide samples were taken after 10 days of anoxic incubation. Points represent the mean of three replicate samples, and error bars represent ± standard error.

AioA from HC18 is 84% identical to that of the As(III) oxidizer *Alcaligenes faecalis*[42] (Figure 3). The residues in HC18 AioA are known to play important roles in As(III) oxidase activity and were identified in the HC18 AioA protein (Figure S3). Compared with known ArxA sequences, *arxA* gene of strain HC18 encodes a protein that has three conserved motifs: an iron−sulfur motif ($CX_2CX_3CX_{27}C$); a catalytic binding pocket sequence (NHSSMCSDA); and a twin-arginine signal peptide (Figure S4). The HC18 ArxA has 114 extra amino acid residues, which is similar to its ortholog in *Sterolibacteriaceae* bacterium M52,[18] another member of the β-proteobacteria but in a different family (Figure S4). In addition, gene clusters encoding membrane-bound nitrate reductase (Nar), periplasmic nitrate reductase (Nap), cytochrome cd1-containing nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos) were found in the genome of strain HC18.

Anaerobic As(III) Oxidation by Strain HC18. Under anoxic conditions, 10 μM As(III) was completely oxidized to As(V) by strain HC18 within 96 h. Noticeable As(III) oxidation was first observed after 24 h (Figure 4A), concurrent with the onset of substantial nitrate reduction (Figure 4B). In parallel, NO_2^- began to accumulate, reaching a maximum concentration of 3.8 mM at 96 h, and then slightly decreased toward 108 h. No As(III) oxidation occurred in the sterile control. Neither cell growth nor As(III) oxidation was observed in the FWM medium without NO_3^- (data not shown).

To investigate the As(III) oxidation mechanism in strain HC18, the transcript levels of *aioA* and *arxA* genes were determined using qRT-PCR. The results showed that the transcript level of *aioA* increased significantly upon the addition of As(III) ($p < 0.05$), which was nearly 1.5−2.0-fold higher than that in cells grown without arsenic. In contrast, the expression of *arxA* was downregulated with As(III), while slightly induced with As(V) ($p < 0.05$) (Figure 3C).

Anaerobic Fe(II) Oxidation by Strain HC18. Strain HC18 completely oxidized 2.5 mM Fe(II) within 10 days (Figure 5A), coupled with reduction of approximately 3.0 mM NO_3^-. During cultivation, NO_2^- accumulated steadily in the medium from day 2 to 6 and then stabilized at ∼5 mM, only with a little fluctuation (Figure 5B). Similar to As(III) oxidation, Fe(II) oxidation was not found in the sterile control or in the presence of HC18 without NO_3^- (data not shown). Red precipitates were observed in the medium during cultivation (Figure 5C), indicating the formation of Fe(III)-bearing minerals.

The X-ray diffraction (XRD) analysis indicated that the Fe(II) oxidation mineral precipitate consisted essentially of goethite (α-FeOOH) (Figure 5C). As observed by scanning electron microscopy (SEM), the precipitate particles appeared mostly crystalline. Based on the energy-dispersive X-ray spectroscopy (EDS) data calculated from the atom percentage, the iron precipitate was composed of Fe, O, and P in a ratio of 44.6:51.6:3.7 (Figure S5).

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Immobilization of Arsenic by Strain HC18. Fe(II)-As(III) medium initially contained 10 μM As(III), 2.5 mM Fe(II), and 10 mM NO_3^-. Inoculation of strain HC18 resulted in complete Fe(II) oxidation within 10 days, as well as a 96% removal of As(III) within 2 days (Table 1). The remaining dissolved arsenic (mainly As(V)) in the medium was approximately 2 μM. The concentration of dissolved As(III) was not changed in the sterilized control during 8 days (data not shown). X-ray photoelectron spectroscopy (XPS) analysis was used to analyze the chemical states of various bound elements and surface composition (Figure S6). Fe $2p_{3/2}$ and Fe $2p_{1/2}$ regions showed two peaks at 724.6 and 710.8 eV, with satellite peaks appearing at 732.8 and 718.9 eV (Figure S6A), respectively. The O 1s spectra were fitted with Fe−O at 529.6 and Fe−OH at 531.1 eV (Figure S6B), which were attributed to Fe(III) present in α-FeOOH.[43,44] The As 2p peaks were fitted with As(III) peak at 1326.0 eV and As(V) peak at 1327.0 eV (Figure S6C).[45] These observations indicate that As(III) and Fe(II) were oxidized to As(V) and Fe(III) by strain HC18, respectively. To test whether As(III) could be oxidized abiotically by goethite, which was produced from Fe(II) oxidation by strain HC18, almost 95% of the As(III) was adsorbed by the goethite, less than 5% of As(III) was oxidized to As(V) by goethite under anoxic conditions without strain HC18 (Table S4).

Table 1. Arsenic Removal in the FWM Medium Containing 2.5 mM Fe(II), 10 μM As(III), and 10 mM Nitrate by Strain HC18 under Anoxic Nitrate-Reducing Conditions

	before inoculation (0 days)	after inoculation (10 days)
dissolved Fe(II) (mM)	2.50 ± 0.05	0.09 ± 0.00
dissolved As(III) (μM)	10.62 ± 0.08	0 ± 0.00
dissolved As(V) (μM)	0 ± 0.00	1.98 ± 0.89
total arsenic (μM)	10.62 ± 0.08	1.98 ± 0.89
dissolved total arsenic (μg)	39.83 ± 0.31	7.42 ± 3.35
Phosphate extractable arsenic from Fe oxide		
extractable As(III) (μg)	0 ± 0.00	14.42 ± 1.02
extractable As(V) (μg)	0 ± 0.00	17.62 ± 2.23
extractable total arsenic (μg)		32.04 ± 2.65

DISCUSSION

As(III) and Fe(II) are more abundant in flooded paddy soils compared with dry conditions, so microbial oxidation of As(III) and Fe(II) could be an effective bioremediation strategy to immobilize arsenic in anoxic environments. As(V) produced by As(III) oxidation could be immobilized by complexing with Fe(III) oxides formed by microbial Fe(II) oxidation.[21] In this study, enrichment microcosms were constructed to investigate the effects of electron acceptor (nitrate) and donor (lactate) on As(III) and Fe(II) oxidization under anoxic conditions. Our assumption that a significant decrease of arsenic in the enrichment microcosms was due to the microbial oxidation of As(III) and Fe(II) is based on two observations. First, the addition of nitrate and lactate resulted in enhanced activity of both nitrate-dependent As(III) and Fe(II)-oxidizing microbial communities. Second, NO_2^- produced as the denitrification product participates in anaerobic Fe(II) oxidation.[23,46] *Noviherbaspirillum*-related species in the lactate plus NO_3^- microcosms were higher than those in NO_3^- alone, which indicates that addition of lactate stimulates the activity of native *Noviherbaspirillum* species and results in As(III) oxidation and immobilization. *Noviherbaspirillum* species are often highly represented in bacterial isolates from paddy soils[41,47] and are capable of denitrification. However, neither anaerobic As(III) nor Fe(II)-oxidation by *Noviherbaspirillum* species has been observed previously. Characterization of As(III) and Fe(II)-oxidizing activities of strain HC18 gives an appreciation of the variety of As(III)- and Fe(II)-oxidizing characteristics within this genus. Many bacterial species oxidize As(III).[48] Some of the microbes oxidize As(III) either for detoxification[49] or to generate energy for growth under oxic conditions.[50] Others can grow with As(III) as an electron donor using nitrate as the terminal electron acceptor, reducing it to nitrite[51] or completely denitrifying to N_2.[52,52,52] Strain HC18 cannot oxidize As(III) under oxic conditions but instead couples As(III) oxidation with nitrate reduction.

Genome mining analysis suggests that both the *aio* and *arx* genes encoding As(III) oxidases are present in the genome of strain HC18. AioA functions in As(III) oxidation, and recently Arx from As(III) oxidizing β-proteobacteria has been shown to play a role in anaerobic As(III) oxidation.[17,18] The presence of the *aio* and *arx* gene clusters in the genome of strain HC18 suggests that both genes may be responsible for anaerobic As(III) oxidation, which drives the arsenic cycle in the flooded paddy soils. However, the relative role of Aio and Arx in As(III) oxidation by strain HC18 will need to be further developed and confirmed in further studies.

During arsenic immobilization by strain HC18, both As(III) and As(V) are immobilized by the Fe(III)-containing minerals either through sorption or coprecipitation. The findings suggest that As(III) immobilization occurs in anoxic environments after microbial As(III) oxidation and biogenic Fe(III) mineral formation via the abiotic oxidation of Fe(II). Our observation of the inhibition of nitrate reduction by Fe(II) suggests that Fe(II) oxidation is accomplished via the slower rate of nitrate reduction, which is consistent with the observation that NO_2^- produced by denitrification may be partially related to Fe(II) oxidation.[23,39] Furthermore, the mineral produced by strain HC18 contains Fe(III), which indicates that strain HC18 produces Fe(III)-containing minerals by Fe(II) oxidation. Presumably, when Fe(II) oxidation is faster than As(III) oxidation, some As(III) remains in the culture samples.

This study demonstrates that biological oxidation of both As(III) and Fe(II) coupled to denitrification immobilizes arsenic in anoxic environments. Our demonstration that Arx is involved in As(III) oxidation provides a broader context for the biological transformation of As(III) not only in extreme environments but also in paddy soils. In conclusion, our isolation of the bacterium strain HC18 that oxidizes both As(III) and Fe(II) is the first demonstration that *Noviherbaspirillum* species play a significant role in the cycling of arsenic, Fe, and N in flooded paddy soils.

CONCLUSIONS

In this study, bacterial community analyses suggest that *Noviherbaspirillum* species are the main anoxic As(III) and Fe(II) oxidizers in the nitrate-stimulated microcosms. This conclusion is supported by the fact that *N. denitrificans* HC18 isolated in the microcosms could link anaerobic As(III) oxidation to nitrate reduction. *N. denitrificans* HC18 possesses

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the As(III) oxidase *aio* and *arx* gene clusters as well as the denitrification genes. Furthermore, strain HC18 was able to oxidize Fe(II) and resulted in precipitation of Fe(III)-containing minerals; As(V) produced by As(III) oxidation was bound to Fe(III) (oxyhydr)oxides. The findings revealed that microbial As(III) and Fe(II) oxidation coupled with anaerobic nitrate reduction has potential for its use in bioremediation in anoxic arsenic-contaminated environments.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsearthspacechem.1c00155.

Taxonomic distribution of bacterial phyla and classes; phylogenetic tree; multiple sequence alignments of AioA homologs; SEM and EDS mapping images; details of primer sequences and PCR conditions; growth characteristics of *N. denitrificans* HC18 (PDF)

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Notes

The authors declare no competing financial interest.

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