Environmental Science & Technology

pubs.acs.org/est

Article

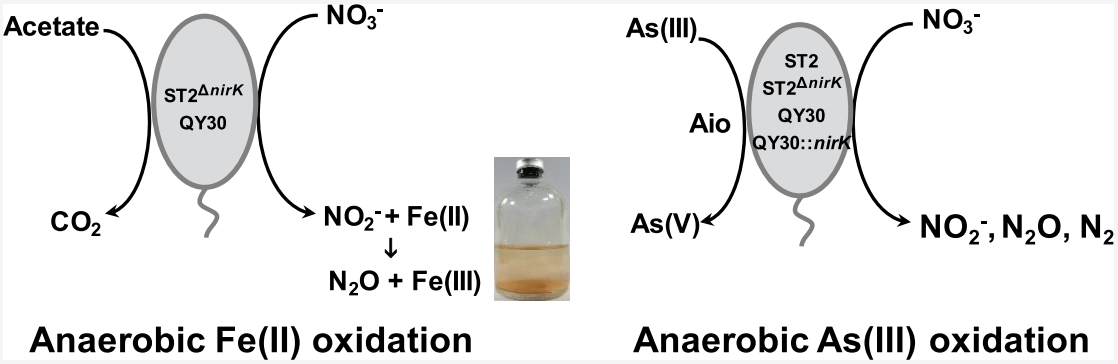
Nitrite Accumulation Is Required for Microbial Anaerobic Iron Oxidation, but Not for Arsenite Oxidation, in Two Heterotrophic Denitrifiers

Jun Zhang, Cheng-Wei Chai, Laurel K. ThomasArrigo, Shi-Chen Zhao, Ruben Kretzschmar, and Fang-Jie Zhao*

Cite This: *Environ. Sci. Technol.* 2020, 54, 4036–4045

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information



ABSTRACT: Phylogenetically diverse species of bacteria can mediate anaerobic oxidation of ferrous iron [Fe(II)] and/or arsenite [As(III)] coupled to nitrate reduction, impacting the biogeochemical cycles of Fe and As. However, the mechanisms for nitrate-dependent anaerobic oxidation of Fe(II) and As(III) remain unclear. In this study, we isolated two bacterial strains from arsenic-contaminated paddy soils, *Ensifer* sp. ST2 and *Paracoccus* sp. QY30. Both strains were capable of anaerobic As(III) oxidation, but only QY30 could oxidize Fe(II) under nitrate-reducing conditions. Both strains contain the As(III) oxidase gene *aioA*, whose expression was induced greatly by As(III) exposure. Both strains contain the whole suite of genes for complete denitrification, but the nitrite reductase gene *nirK* was not expressed in QY30 and nitrite accumulated under nitrate-reducing conditions. When the functional *nirK* gene was knocked out in strain ST2, its nitrite reduction ability was completely abolished and nitrite accumulated in the medium. Moreover, the ST2$^{\Delta nirK}$ mutant gained the ability to oxidize Fe(II). When *nirK* gene from ST2 was introduced to QY30, the recombinant strain QY30::*nirK* gained the ability to reduce nitrite but lost the ability to oxidize Fe(II). These genetic manipulations did not affect the ability of both strains to oxidize As(III). Our results indicate that nitrite accumulation is required for anaerobic oxidation of Fe(II) but not for As(III) oxidation in these strains. The results suggest that anaerobic Fe(II) oxidation in the two bacterial strains is primarily driven by abiotic reaction of Fe(II) with nitrite, while reduction of nitrate to nitrite is sufficient for redox coupling with anaerobic As(III) oxidation catalyzed by Aio. Deletion of *nirK* gene in denitrifiers can enhance anaerobic Fe(II) oxidation.

INTRODUCTION

When paddy soils are submerged, anoxic conditions lead to reductive dissolution of iron oxyhydroxides and reduction of arsenate [As(V)] to arsenite [As(III)]; both processes can result in increased As mobility and availability to rice plants.[1−4] For this reason, arsenic (As) concentrations in rice grain are typically an order of magnitude higher than in other cereal grains.[5] Because rice is a major dietary source of inorganic As for populations consuming rice as a staple food,[6,7] it is imperative to develop strategies to decrease As availability in paddy soil.

Despite the prevailing anoxic conditions in submerged paddy soil, microbial anaerobic oxidation of ferrous iron [Fe(II)] and As(III) can also occur. It has been shown that microbial anaerobic oxidation of Fe(II) and As(III) is coupled to nitrate reduction mediated by diverse denitrifying microbes.[8−17] Nitrate is a thermodynamically favorable electron acceptor for anaerobic respiration in anoxic environments.[18] A number of studies have shown that additions of

Received: November 6, 2019
Revised: March 3, 2020
Accepted: March 4, 2020
Published: March 4, 2020

https://dx.doi.org/10.1021/acs.est.9b06702
Environ. Sci. Technol. 2020, 54, 4036−4045

nitrate into anoxic groundwater or flooded paddy soils stimulate the anaerobic oxidation of Fe(II) and As(III).[8,9] The formation of Fe(III) oxyhydroxides and the oxidation of As(III) to As(V) can increase the sorption and immobilization of As.[9,19,20] Therefore, microbial anaerobic oxidation of Fe(II) and As(III) in paddy soil may be harnessed to decrease As bioavailability to rice plants and to understand As dynamics in the rhizosphere.

Different pathways of microbial anaerobic nitrate-reducing Fe(II) oxidation (NRFeOx) have been proposed.[21,22] First, autotrophic nitrate-reducing Fe(II)-oxidizing bacteria can use Fe(II) as an electron donor and CO_2 as the carbon source for energy generation and growth.[21] To date, only one autotrophic NRFeOx enrichment culture KS has been reported,[23,24] which meets the criteria for autotrophic Fe(II) oxidation proposed by Bryce et al.[21] Second, nitrite, which is the first intermediate of denitrification, can react chemically with dissolved Fe(II) to produce Fe(III) and nitrous oxide (N_2O) under anoxic conditions; this pathway is termed chemodenitrification.[16,25−27] This mechanism is considered to be abiotic because no enzymes are directly involved in the oxidation of Fe(II), although production of nitrite is a biotic process. Third, many NRFeOx bacteria are mixotrophic, which utilize Fe(II) as an electron donor and require organic carbon substrates for growth.[27−29] How Fe(II) is oxidized by mixotrophic NRFeOx bacteria remains unclear. It is possible that both biotic and abiotic reactions are involved in Fe(II) oxidation in mixotrophic NRFeOx bacteria. Recently, it has been suggested that *c*-type cytochromes (*c*-Cyts) in the extracellular polymeric substances (EPSs) of NRFeOx bacteria transfer electrons from Fe(II) to the respiratory chain.[30] Whether *c*-Cyts in the periplasm are involved in Fe(II) oxidation is unknown. For both autotrophic and mixotrophic NRFeOx bacteria, the Fe(II) oxidases remain elusive, and there are debates on the relative contributions of biotic versus abiotic reactions to Fe(II) oxidation.[21,27] During microbial Fe(II) oxidation process, various biogenic Fe(II)- and Fe(III)-bearing minerals (e.g., magnetite) are formed, with the form of the minerals depending on microbial species, medium composition (i.e., phosphate or buffer concentrations), pH, Fe(II) precursor (dissolved or solid), and organic substrate availability.[31] These Fe(III) minerals can serve as strong adsorbents for As species.

In contrast to NRFeOx, anaerobic As(III) oxidation is coupled to nitrate reduction either for respiration or detoxification and is catalyzed by As(III) oxidases Aio and Arx, respectively.[32−34] Aio transfers electrons arising from As(III) oxidation toward a periplasmic soluble electron carrier using O_2 or nitrate as electron acceptor.[32,35,36] Additions of nitrate to submerged paddy soils greatly increased the copy number of *aioA* genes.[9,37] Deletion of *aioA* gene in *Paracoccus* strain SY isolated from a paddy soil abolished its ability to oxidize As(III) under anoxic nitrate-reducing conditions.[34] It has been proposed that Arx transfers electrons arising from As(III) oxidation toward the quinone pool, although the exact mechanism has not been elucidated yet.[32,36]

It has been shown that many bacterial species from diverse genera are able to oxidize Fe(II) and/or As(III) under anoxic nitrate-reducing conditions.[21,32] Although the coupling of nitrate reduction and Fe(II) oxidation has been studied intensively, the underlying mechanisms still remain unclear, especially with regard to the role of biotic versus abiotic processes. It is also unclear why some bacteria are capable of mediating nitrate-dependent anaerobic oxidation of Fe(II) and As(III) simultaneously. We hypothesized that nitrite reduction is the key step in the denitrification process that affects anaerobic oxidation of Fe(II), but not of As(III). To test this hypothesis, we employed a genetic manipulation approach to alter the pathway of nitrate reduction in two nitrate-reducing bacteria isolated from paddy soils and assess the effects of these manipulations on anaerobic oxidations of Fe(II) and As(III).

MATERIALS AND METHODS

Bacterial Strains, Growth Media, and Culture Conditions. Strains *Paracoccus* sp. QY30 and *Ensifer* sp. ST2, two facultative anaerobic As(III)-oxidizing bacteria, were isolated from enrichment cultures of arsenic-contaminated paddy soils ffrom Qiyang, Hunan province and Shantou, Guangdong Province, China, respectively[9] (see Text S1 in the Supporting Information). For routine cultivation of ST2 and QY30 and derivative strains in batch experiments, strains were cultured in PIPES-buffered freshwater basal medium (PFWM) anaerobically containing 5 mM sodium nitrate ($NaNO_3$) and 20 mM sodium acetate as the electron acceptor and carbon source, respectively. For gene deletion and complementation experiments, ST2 and its derivative strains and *Escherichia coli* strains were grown aerobically in Luria−Bertani (LB) medium, while QY30 and its derivative strains were grown aerobically in R2A medium.[38] Where appropriate, kanamycin (50 mg L^{-1}), streptomycin (25 mg L^{-1}), or gentamicin (25 mg L^{-1}) was added. All strains and plasmid constructs used in this study are listed in Table S1.

Molecular Analysis. The phenol−chloroform method was used to extract DNA from QY30 and ST2 cells.[39] The 16S rRNA gene was amplified using universal primers 27F and 1492R as previously described.[40] The complete 16S rRNA gene sequences of strains QY30 and ST2 were compared to the closely related representative strains in the GenBank database using BLAST, and a phylogenetic tree was constructed using the neighbor-joining algorithm as implemented in MEGA 6.0.[41] The As(III) oxidase *aioA* gene was amplified using the primers *aoxB*M1-2F and *aoxB*M3-2R as described by Quemeneur et al.[42] Genome sequencing was performed by Shanghai Biozeron Biotechnology Co. (Shanghai, China) (Text S1). Neighbor-joining phylogenetic trees of genes coding for As(III) oxidase (*aioA*) were generated in MEGA 6.0 based on inferred amino acid sequences obtained from GenBank database and from sequencing analyses performed for this study.[41]

Disruption of *nirK* Gene in *Ensifer* Strain ST2. Standard DNA manipulation techniques were performed as previously described.[39] All primers used in this study are listed in Table S2. Deletion of the *nirK* gene in *Ensifer* sp. ST2 was performed via a two-step homogenetic recombination method using the suicide plasmid pJQ200SK.[43] Two primer pairs (ST2-*nirK*-UF/ST2-*nirK*-UR and ST2-*nirK*-DF/ST2-*nirK*-DR) (Table S2) were used to amplify the homologous recombination-directing sequences. Both polymerase chain reaction (PCR) fragments were cloned into *Sac*I/*Pst*I digested pJQ200SK using the ClonExpress MultiS one-step cloning kit (Vazyme Biotech Co., Nanjing, China). The resulting plasmid, pJQ-Δ*nirK*, was then introduced into ST2 cells by electroporation. Single-crossover mutants were screened on LB plates containing streptomycin and gentamicin. A double-crossover mutant ($ST2^{\Delta nirK}$) was selected on LB plates containing streptomycin and 5% (w/v) sucrose.

https://dx.doi.org/10.1021/acs.est.9b06702
Environ. Sci. Technol. 2020, 54, 4036−4045

Construction of an Anaerobic Nitrite-Reducing Recombinant Strain QY30. Initial experiments in the present study showed that strain QY30 was able to reduce nitrate, but not nitrite. In order to confer strain QY30 with the nitrite-reducing ability, *nirK* gene was cloned from *Ensifer* sp. ST2 and transferred into QY30. For this purpose, a plasmid containing *nirK* (pBAM1-ProKan-ST2-*nirK*) was constructed using the method described by Chen et al.[44] ST2-*nirK* gene fragment (1137 bp) was amplified by PCR with ST2 genomic DNA using primers ST2-NirF and ST2-NirR (Table S2). ST2-*nirK* expression under the control of Kan promoter was placed into the plasmid pBAM1,[45] which formed plasmid pBAM-ProKan-ST2-*nirK*. The plasmid pBAM1-ProKan-ST2-*nirK* was then transferred into the wild-type QY30 by electroporation. The transformants were selected on R2A−kanamycin agar plates, and the complemented strain (QY30::*nirK*) was selected and examined for its ability to utilize nitrite as an electron acceptor.

Anaerobic As(III) and Fe(II) Oxidation. To obtain a preculture, strains *Paracoccus* sp. QY30 (wild type), QY30::-*nirK*, *Ensifer* sp. ST2 (wild type), and ST2$^{\Delta nirK}$ were cultivated in 50 mL of anoxic PFWM medium containing 20 mM acetate and 5 mM nitrate, as described above, and incubated statically in the dark under anoxic conditions at 28 °C. When the optical density at 600 nm (OD_{600nm}) reached 0.1, the preculture was transferred (5%, v/v) into a fresh PFWM medium.

For measurement of As(III) resistance, ST2 and QY30 and derivative strains were grown in the PFWM medium with 50 μM As(III) for 96 h at 28 °C. Growth was estimated by measuring OD_{600nm}. To determine anaerobic As(III) oxidation, an aliquot of the ST2 and QY30 and derivative strain seed culture (5%, v/v) was inoculated into 50 mL of anaerobic PFWM medium containing 50 μM As(III), 5 mM $NaNO_3$, and 20 mM acetate under anoxic conditions for 72 h. Samples were collected during 0−72 h to monitor the changes in As speciation and the concentrations of nitrate and nitrite.

In the anaerobic Fe(II)-oxidation experiments, 4 mM Fe(II) was added from an anoxic sterile $FeCl_2$ stock solution to 50 mL of medium, which contained 1.03 mM phosphate that could form Fe(II) phosphate precipitates overnight. The poorly crystalline Fe(II) phosphate was removed by filtration (0.2 μm pore size) in an anoxic chamber (Coy) according to Kappler and Newman[46] leaving approximately 2 mM dissolved Fe(II) in the medium. Portions (50 mL) of anoxic, sterile medium were added to 100 mL serum bottles, sealed with butyl rubber stoppers, and the headspace was replaced with N_2/CO_2 (80:20 by volume). Five percent of a freshly grown culture was inoculated to the medium. All cultures were incubated at 28 °C in the dark for 2 weeks. Samples were collected to monitor the changes in the concentrations of Fe(II), nitrate, and nitrite.

RNA Isolation, cDNA Preparation, and Reverse Transcription (RT) Analysis. TRIzol reagent (Invitrogen, U.S.A.) was used to isolate total RNA from midexponential-phase cultures of different strains that were grown in PFWM medium under anoxic conditions, according to the manufacturer's instructions. DNA contaminants present in the RNA samples were removed by treatment with RNase-free DNase I (Fermentas) for 1 h at 37 °C, followed by inactivation of the enzyme with EDTA for 10 min at 70 °C. The RNA concentration was first determined using a Nanodrop and was diluted to equal concentrations. RNA integrity was checked by agarose (2.0%) gel electrophoresis, and 1 μg of DNA-free RNA was used for cDNA synthesis. To examine if the expression of *aioA* genes was induced by As(III), ST2 and QY30 cell cultures grown in PFWM media under anoxic conditions were treated with 50 μM As(III), while three other replicates did not receive As(III). Primer sequences of the target genes were designed by Prime 5.0 software (Table S2). Quantification of the transcription of functional genes (*napA*, *narG*, *nirK*, and *aioA*) was performed using the CFX96 real-time PCR detection system (Bio-Rad) using the SYBR Green I detection method. DNA gyrase subunit B (*gyrB*) gene was used as an endogenous control. Melting curve analysis was performed at the end of qPCR runs to check the specificity of the amplification. The relative expression was quantified according to the method of $2^{-\Delta\Delta CT}$ threshold cycle (CT).[47]

Analytical Methods. Arsenic species were quantified using high-performance liquid chromatography−inductively coupled plasma mass spectrometry (HPLC−ICP-MS) (PerkinElmer NexION 300X, U.S.A.) as described previously.[48] For analysis of total Fe(II), 100 μL samples were taken with a syringe at different time points under anoxic conditions and analyzed using the ferrozine assay.[49] Nitrate and nitrite concentrations were measured colorimetrically.[50] The precipitates formed in Fe(II)-oxidation medium by different strains were collected by vacuum filtration on a nylon filter (0.45 μm), washed with deionized oxygen-free water in an anaerobic chamber, and freeze-dried. An amount of 0.05 g of each mineral was digested with 5 mL of HCl/HNO_3 (80:20 v/v) in a heating block. The total Fe and P concentrations of the minerals were determined using ICP-MS.

X-ray Diffraction. Mineral-phase identification was performed by X-ray diffraction (XRD, D8 Advance, Bruker). Sample material (~2 mg) was resuspended in ethanol (~30 μL, Merck) and pipetted onto a polished silicon wafer (Sil'tronix Silicon Technologies, France). Samples were analyzed in Bragg−Brentano geometry using Cu Kα radiation (λ = 1.5418 Å, 40 kV, and 40 mA) and a high-resolution energy-dispersive 1-D detector (LYNXEYE). Diffractograms were recorded from 10° to 70° 2θ with a step size of 0.02° 2θ and 10 s acquisition time per step. Initial XRD patterns indicated that the samples contained significant salts, which largely masked the peaks relating to the Fe mineralogy. To alleviate this, remaining dried sample material was resuspended in 10 mL of doubly deionized (DDI) water and filtered through 0.2 μm cellulose acetate filters. Filters were rinsed with an additional 5 mL of DDI water, and then covered and air-dried in the dark. X-ray diffraction patterns of the filter-dried solid phase (≥0.2-μm) were then collected as previously described.

The relative contributions of identified mineral phases in diffraction patterns were estimated by Rietveld quantitative phase analysis (QPA) with ferrihydrite included as mass-calibrated PONKCS (partial or no known crystal structure) phase.[51] All calculations were performed in TOPAS software (version 5, Bruker AXS). The validity of this method has previously been shown for mixtures of two-line ferrihydrite and goethite with known mass ratios; however, the uncertainty of the estimates increased with decreasing ferrihydrite content.[52] Here, we consider the estimates obtained from Rietveld fits as semiquantitative, because the poorly crystalline mineral fraction in our samples may not only consist of two-line ferrihydrite.

Synchrotron Measurements. To obtain additional information about the iron mineral present in the precipitates,

https://dx.doi.org/10.1021/acs.est.9b06702
Environ. Sci. Technol. 2020, 54, 4036−4045

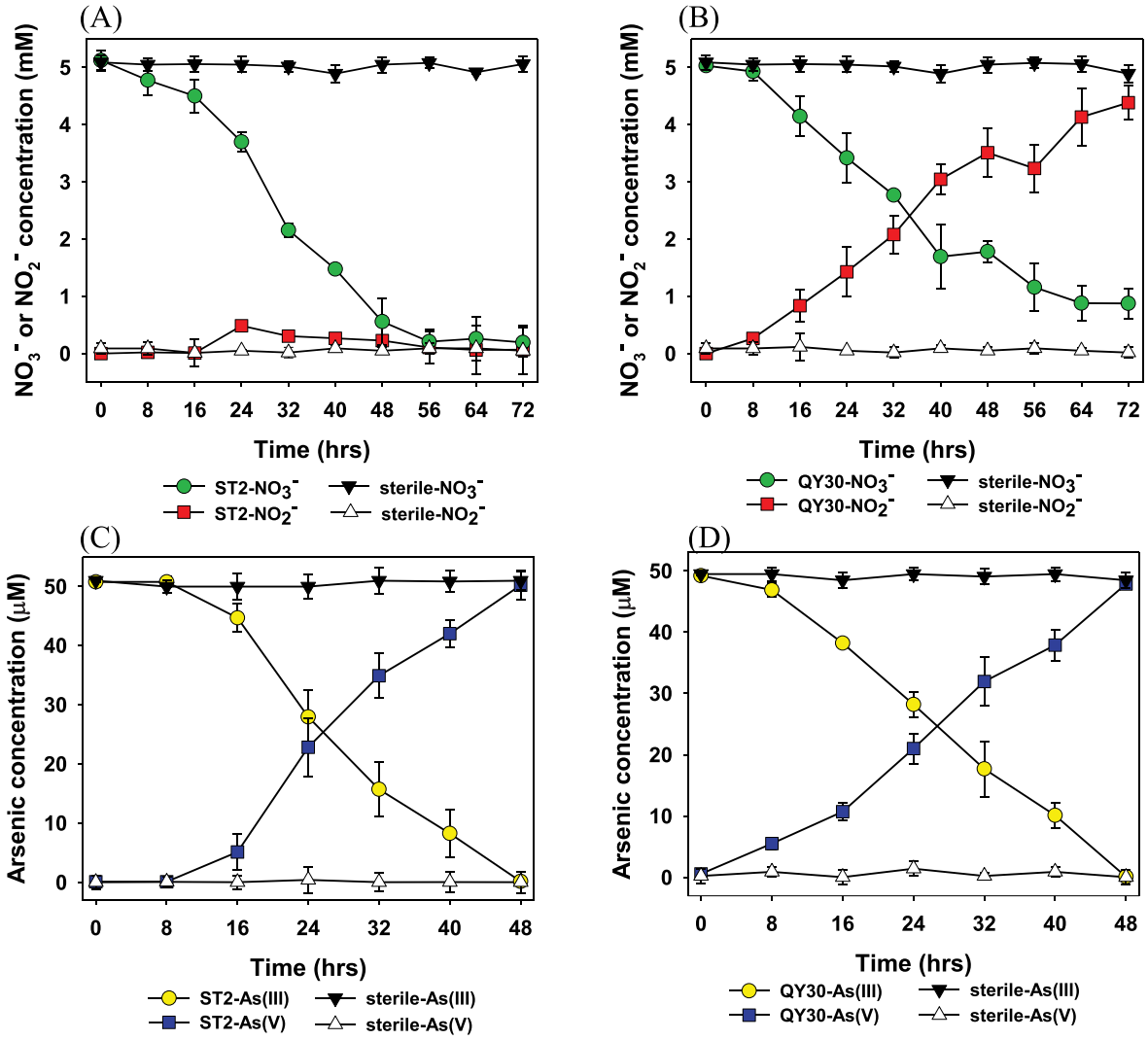


Figure 1. Nitrate consumption and nitrite formation and oxidation of As(III) to As(V) by *Ensifer* strain ST2 (A and C) and *Paracoccus* strain QY30 (B and D) under anoxic nitrate-reducing condition. No strain inoculum was added to the sterile control. Data are mean ± SE (n = 3).

we collected bulk Fe K-edge (7112 eV) X-ray absorption (XAS) spectra. For analysis, sample materials were pressed into 1.3 cm pellets and sealed with Kapton tape. X-ray absorption near-edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) spectra were recorded at the XAFS beamline of ELETTRA (Trieste, Italy) in transmission mode at ∼80 K using a N_2(l) cryostat. Beam energy was selected using a Si(111) monochromator, which was calibrated to the first-derivative maximum of the K-edge absorption spectrum of a metallic Fe foil (7112 eV). The foil was continuously monitored to account for small energy shifts (<1 eV) during the sample measurements. Higher harmonics in the beam were eliminated by detuning the monochromator by 30% of its maximal intensity. Three scans per sample were collected and averaged. All spectra were energy-calibrated, pre-edge subtracted, and postedge normalized in Athena[53] with the edge energy, E_0, defined as the zero-crossing in the second XANES derivative. Linear combination fit (LCF) analyses of k^3-weighted Fe K-edge EXAFS spectra were performed over a k-range of 2−12 Å^{-1} with the E_0 of all spectra and reference compounds set to 7128 eV. Reference compounds offered in linear combination fits included those mineral phases identified with X-ray diffraction as well as plausible amorphous phases based on the determined elemental content of the precipitates and the growth medium. Reference spectra included synthetic ferrihydrite, goethite, lepidocrocite, magnetite, scorodite, vivianite, and Fe(III) citrate (Fluka), Fe(III) phosphate dihydrate (Aldrich), Fe(II) oxalate dehydrate (Alfa Aesar), and Fe(III) oxalate hexahydrate (Alfa Aesar). No constraints were imposed during LCF analyses, and initial fit fractions were recalculated to a compound sum of 100%.

RESULTS

Enrichment and Identification of Anaerobic As(III) Oxidizers. Two anaerobic As(III) oxidizing bacterial strains, ST2 and QY30, were isolated from enrichment cultures of arsenic-contaminated paddy soils. Strain ST2 was able to grow under anoxic nitrate-reducing conditions and completely removed 5 mM NO_3^- in 56 h with transient accumulation of relatively low concentrations of NO_2^- (0.22−0.48 mM) during 24−48 h (Figure 1A). In contrast to ST2, strain QY30 reduced NO_3^- only to NO_2^-, and approximately 4.5 mM NO_2^- accumulated in the culture medium during anoxic nitrate-reducing growth conditions (Figure 1B). Both isolates were able to oxidize As(III) to As(V) and grew optimally under anoxic nitrate-reducing conditions (Figure 1, parts C

https://dx.doi.org/10.1021/acs.est.9b06702
Environ. Sci. Technol. 2020, 54, 4036−4045

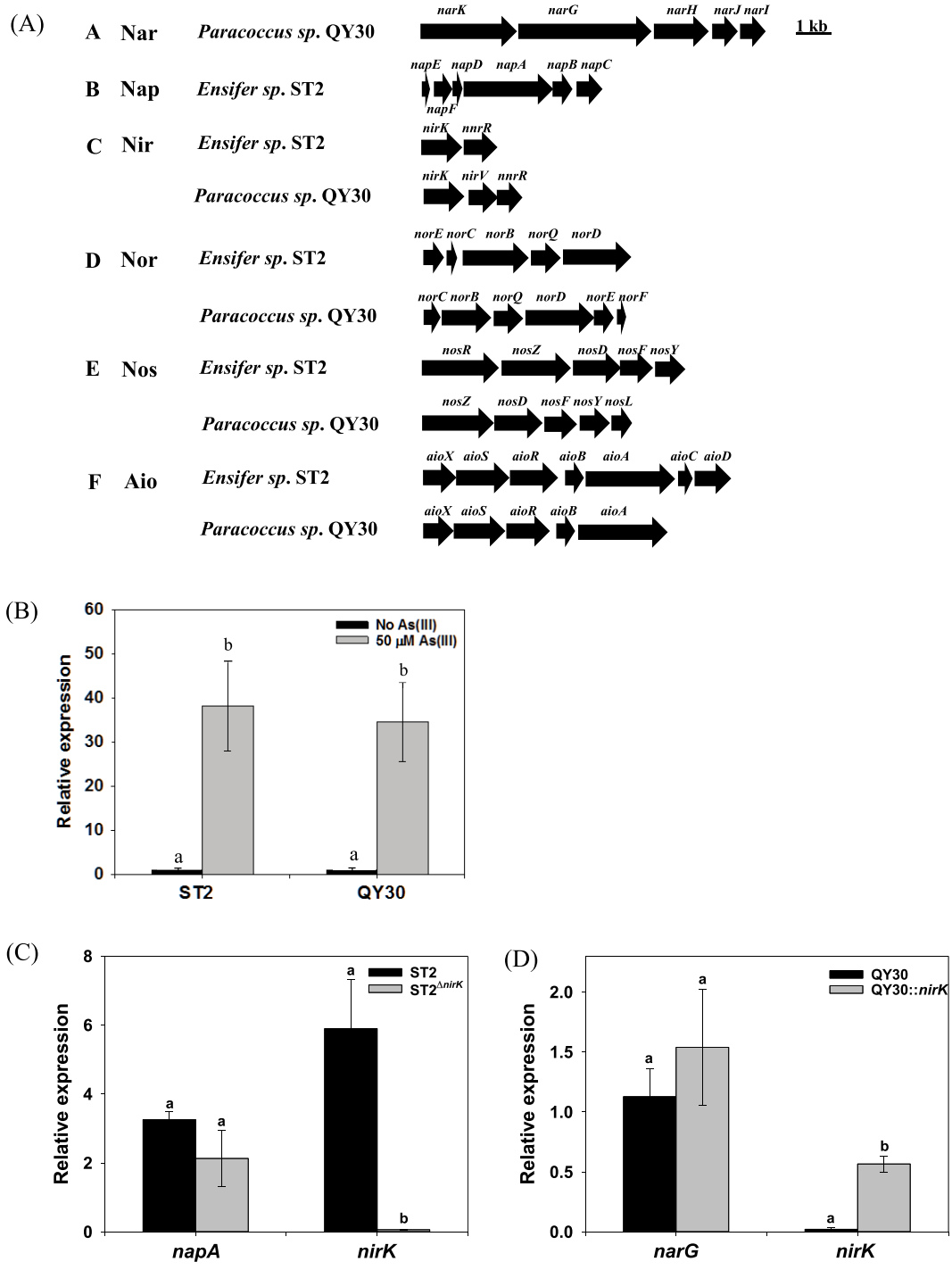


Figure 2. Physical maps of nitrate reductase (Nap or Nar), nitrite reductase (Nir), nitric oxide (Nor), and nitrous oxide (Nos) reductases, and arsenite oxidase (Aio) gene clusters in the genomes of *Ensifer* strain ST2 and *Paracoccus* strain QY30 (A). Effect of As(III) on the relative expression levels of the arsenite oxidase gene *aioA* in strains ST2 and QY30 under anoxic nitrate-reducing conditions, measured after 24 h of incubation (B). Relative expression levels of the nitrate reductase genes (*napA* or *narG*) and nitrite reductase gene (*nirK*) in *Ensifer* strains ST2 and ST2$^{\Delta nirK}$ (C) and *Paracoccus* strains QY30 and QY30::*nirK* (D) under anoxic nitrate-reducing conditions, measured after 48 h of incubation. Data are mean ± SE (n = 3). Different letters indicate significant difference from wild-type strains (P < 0.05).

and D). For both strains, the oxidation of 50 μM As(III) was complete within 48 h. No growth was observed in the absence of NO_3^- (data not shown). Under nitrate-reducing conditions and in the presence of Fe(II) and acetate, strain ST2 was not able to oxidize Fe(II), whereas QY30 was able to oxidize 53% Fe(II) in the medium within 2 weeks (Figure S1). Both strains were able to reduce nitrate in the PFWM medium with acetate as the carbon and energy source without Fe(II) and As(III) (data not shown). Both strains were heterotrophic since they could not use CO_2 as the sole carbon source and also could not grow with As(III) or Fe(II) as the sole electron donor (data not shown). Strain QY30 produced orange-colored precipitates, likely Fe(III) phases, as examined below, whereas strain ST2 did not.

https://dx.doi.org/10.1021/acs.est.9b06702
Environ. Sci. Technol. 2020, 54, 4036−4045

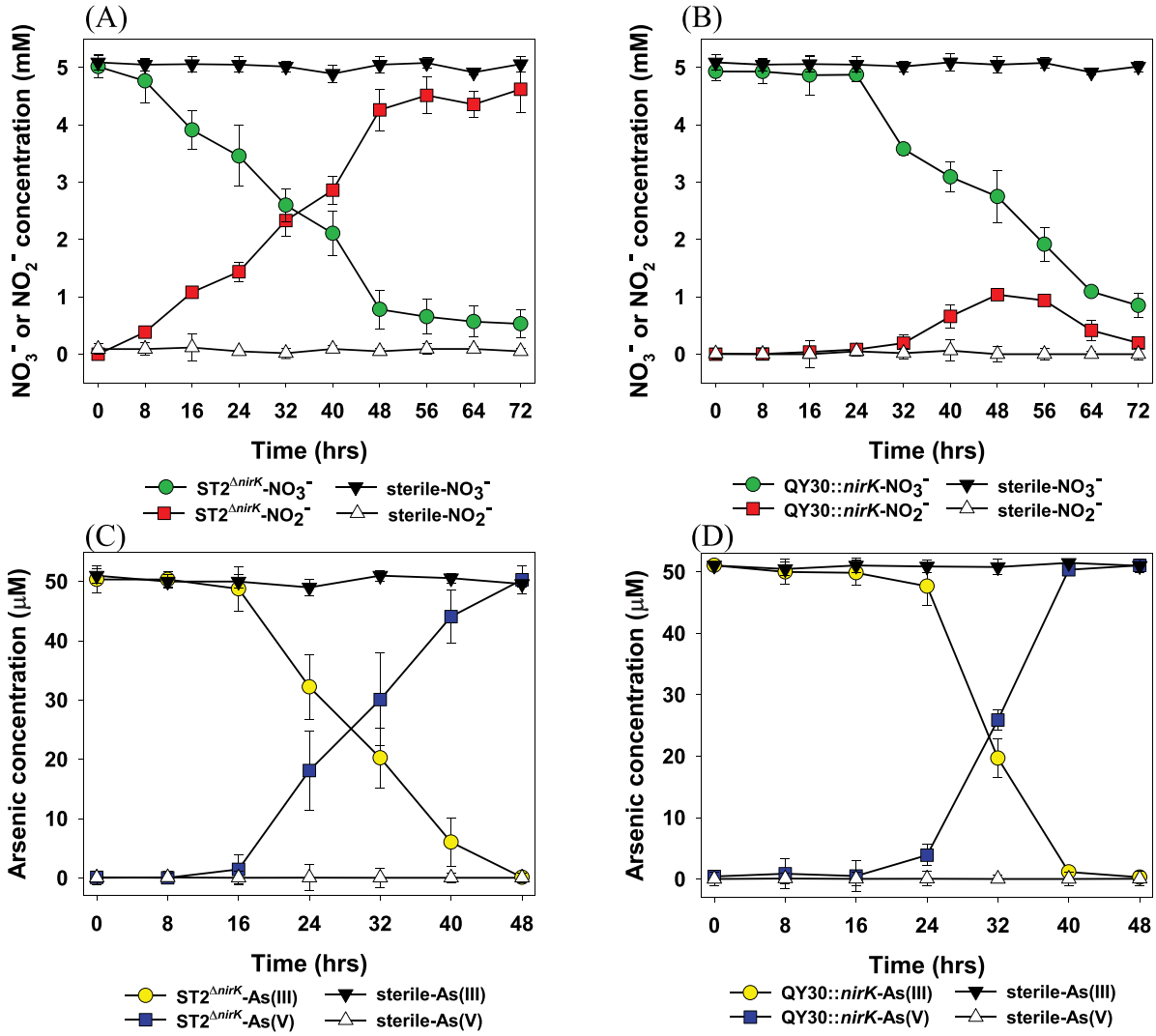


Figure 3. Nitrate consumption and nitrite formation and oxidation of As(III) to As(V) by *Ensifer* strain $ST2^{\Delta nirK}$ (A and C) and *Paracoccus* strain QY30::*nirK* (B and D) under anoxic nitrate-reducing conditions. No strain inoculum was added to the sterile control. Data are mean ± SE (n = 3).

Figure S2 shows the results of the analysis of the 16S rRNA gene from strains ST2 and QY30 in relation to their closest relative strains. The 16S rRNA gene sequences of strains ST2 and QY30 show 99.4% and 96.3% similarity to those of *Ensifer adhaerens* LMG 20216 and *Paracoccus aminovorans* DSM 8537, respectively (Figure S2), establishing ST2 and QY30 as *Ensifer* sp. and *Paracoccus* sp., respectively.

The Presence and Transcription of *aioA* and Denitrification Genes. Both strains contain *aioA* genes (Figure 2A). The AioA sequences of strains ST2 and QY30 show 89.2% and 90.5% similarity to those of *E. adhaerens* As3-1b (CBY79895) and *Paracoccus* sp. SY (AJA37538), respectively (Figure S3). The expression of *aioA* genes was highly induced by As(III) (by 35−38-fold) in both ST2 and QY30 (Figure 2B).

Both strains ST2 and QY30 possess gene clusters encoding a full set of enzymes for complete denitrification, although the two strains differ in their nitrate reductases (Figure 2A, Table S3). The genome of ST2 contains a gene cluster encoding a periplasmic nitrate reductase Nap, whereas the genome of QY30 contains a gene cluster encoding the membrane-bound nitrate reductase Nar. The two strains also contain *nirK* genes encoding the copper-containing nitrite reductase. Gene clusters encoding the nitric oxide (Nor) and nitrous oxide (Nos) reductases were detected in the two genomes and with similar gene organizations (Figure 2A, Table S3). Transcriptions of *narG*, *napA*, and *nirK* in ST2 and QY30 under nitrate-reducing conditions were quantified by qRT-PCR. *narG* and *nirK* were expressed in strain ST2, whereas QY30 expressed *napA* but not *nirK* gene (Figure 2, parts C and D). These results suggest that the lack of *nirK* expression in QY30 was the cause of its inability to reduce nitrite, and hence the accumulation of nitrite in the culture medium.

The Role of *nirK* in Anaerobic Denitrification and Fe(II) and As(III) Oxidation. To investigate the role of *nirK* in denitrification and Fe(II) and As(III) oxidation, we constructed a *nirK* deletion ST2 mutant ($ST2^{\Delta nirK}$) and a *nirK* complementation strain of QY30 expressing the ST2-*nirK* gene (QY30::*nirK*). qRT-PCR analysis showed that *nirK* expression was abolished in $ST2^{\Delta nirK}$ but restored in QY30::*nirK* (Figure 2, parts C and D). These genetic manipulations did not significantly affect the expression of *napA* in ST2 or *narG* in QY30 (p > 0.05). During 48 h of incubation, ST2 and $ST2^{\Delta nirK}$ had a similar rate of nitrate reduction (62 and 59 μM NO_3^- h^{-1}, respectively; p > 0.05), whereas QY30 had a greater rate of nitrate reduction than

https://dx.doi.org/10.1021/acs.est.9b06702
Environ. Sci. Technol. 2020, 54, 4036−4045

QY30::*nirK* (45 and 30 μM NO_3^- h^{-1}, respectively; $p < 0.05$) (Figures 1 and 3). Under anoxic nitrate-reducing conditions, $ST2^{\Delta nirK}$ was able to grow in the presence of 5 mM NO_3^-; however, the mutant lost the ability to reduce NO_2^-, resulting in a quantitative accumulation of NO_2^- in the medium (Figure 3A). In contrast, the introduction of the ST2-*nirK* gene into the chromosome of strain QY30 enabled the engineered strain QY30::*nirK* to reduce NO_2^-, resulting in only a small and transient accumulation of NO_2^- in the medium (Figure 3B). Genetic manipulations of *nirK* gene in both ST2 and QY30 did not affect anaerobic As(III) oxidation. Under anoxic nitrate-reducing conditions, similar rates of As(III) oxidation by the wild-type strains ST2 and QY30 and their *nirK* derivatives were obtained (Figure 3, parts C and D). Under nitrate-reducing conditions and in the absence of As(III), the growth was similar between ST2 and $ST2^{\Delta nirK}$ (Figure S4). Compared with QY30, the growth of QY30::*nirK* was slightly delayed (Figure S4), possibly due to an inhibitory effect from the introduction of a foreign gene. This delay in growth could explain why nitrate reduction and As(III) oxidation were also slightly delayed in QY30::*nirK* compared with QY30 (Figure 1, parts B and D, and Figure 3, parts B and D). In the presence of 50 μM As(III), the growth of all strains was delayed, with $ST2^{\Delta nirK}$ and QY30::*nirK* being slightly more sensitive to As(III) than their respective wild types (Figure S4).

In the Fe(II)-oxidation experiments, wild-type strain ST2 was not able to oxidize Fe(II) under nitrate-reducing conditions. However, deletion of *nirK* enabled $ST2^{\Delta nirK}$ to oxidize 52% of Fe(II) in the medium within 2 weeks, producing orange-brown Fe(III) precipitates (Figure 4, parts A and C). Wild-type QY30 was able to oxidize Fe(II) under nitrate-reducing conditions, but insertion of the *nirK* gene from ST2 into QY30 abolished its ability to oxidize Fe(II) completely (Figure 4, parts A and C). Under the experimental conditions, all four strains reduced similar amounts of NO_3^- (46−53%), but QY30 and $ST2^{\Delta nirK}$ produced NO_2^-, whereas ST2 and QY30::*nirK* produced no or very little NO_2^- in the medium. In the culture medium of QY30 and $ST2^{\Delta nirK}$, the amounts of NO_2^- remaining at the end of the experiment (17−35% of the NO_3^- added) were smaller than of the amounts of NO_3^- reduced (Figure 4B). This difference suggests a consumption of NO_2^- during Fe(II) oxidation.

Characterization of Fe(III) Precipitates by X-ray Diffraction and Synchrotron X-ray Absorption Spectrometry. Three samples of Fe(III) precipitates were obtained from anaerobic Fe(II) oxidation by QY30 and $ST2^{\Delta nirK}$, two under the conditions of Fe(II) oxidation and one in the presence of both Fe(II) and As(III). Two Fe(II) oxidation samples of precipitates formed by QY30 and $ST2^{\Delta nirK}$ and one Fe(II) and As(III) oxidation sample formed by $ST2^{\Delta nirK}$ were analyzed using the XRD method. The two Fe(II) oxidation precipitates were also analyzed for the total Fe and P concentrations. The total Fe concentrations in the minerals formed by QY30 and $ST2^{\Delta nirK}$ were 417.4 and 478.0 g kg^{-1} dw (dry weight), respectively, and the total P concentrations were 19.4 and 10.1 g kg^{-1} dw, respectively.

All three samples were dominated by short-range-ordered (SRO) mineral phases, most likely two-line ferrihydrite (2L-Fh) and amorphous Fe(III) phosphate (Figure S5). Using 2L-Fh fitted as *hkl*-phase and mass-calibrated against an internal standard (corundum, Al_2O_3, CAS no. 1302-74-5), we estimated that the SRO materials amounted to about 90−95% of the total precipitates. Nevertheless, some sharp XRD

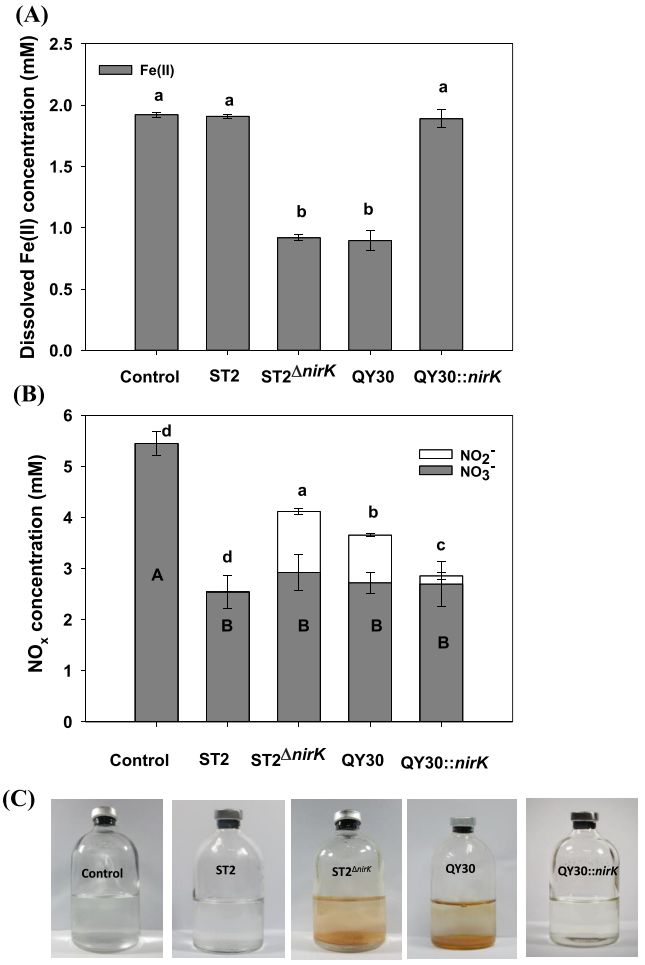


Figure 4. Anaerobic oxidation of Fe(II) (A) and nitrate consumption and nitrite formation (B) by *Ensifer* strains ST2 and $ST2^{\Delta nirK}$ and *Paracoccus* strains QY30 and QY30::*nirK* under anoxic nitrate-reducing conditions, measured after 2 weeks of incubation. Data are mean ± SE ($n = 3$). Different letters indicate significant difference ($P < 0.05$). Bottles containing different inoculants showing the production or not of orange-colored Fe(III) precipitates after 2 weeks of incubation (C).

peaks were also observed (Figure S5). The sample produced by QY30 contained poorly crystalline goethite and traces of vivianite, while the sample produced by $ST2^{\Delta nirK}$ contained lepidocrocite. In the presence of both Fe(II) and As(III), the precipitate produced by $ST2^{\Delta nirK}$ contained vivianite and traces of metavivianite. Additionally, the latter sample showed a small unidentified reflection near 26.7° 2θ. Semiquantitative estimations of these Fe(III) compounds are presented in Table S4.

Synchrotron X-ray absorption spectroscopy at the Fe K-edge was used to provide further evidence of the oxidation state of Fe and its speciation in the Fe(III) precipitates. Linear combination fit analyses of EXAFS spectra confirmed that all three samples of precipitates were dominantly composed of ferrihydrite (Figures S6 and S7 and Table S5). The precipitate produced by $ST2^{\Delta nirK}$ in the presence of Fe(II) and As(III) additionally contained Fe phosphates, both amorphous [Fe(III) phosphate)] and crystalline vivianite, the latter of which was also visible in XRD patterns of this sample.

https://dx.doi.org/10.1021/acs.est.9b06702
Environ. Sci. Technol. 2020, 54, 4036−4045

DISCUSSION

Numerous studies have shown that anaerobic oxidation of Fe(II) and As(III) is coupled to nitrate reduction in many bacteria, e.g., refs 21 and 32. The primary objective of our study was to investigate how anaerobic Fe(II) and As(III) oxidation is coupled to denitrification and the difference between the two processes. We isolated two anaerobic nitrate-dependent As(III)-oxidizing strains, *Ensifer* sp. ST2 and *Paracoccus* sp. QY30, from two arsenic-contaminated paddy soils. Both strains were able to oxidize As(III) to As(V) efficiently under nitrate-reducing conditions (Figure 1). Anaerobic oxidation of As(III) in ST2 and QY30 is likely to be catalyzed by the Aio As(III) oxidase, as has been demonstrated in a previous study with *Paracoccus* sp. strain SY through gene deletion experiments.[34] Neither anaerobic oxidation of As(III) nor cell growth was observed in the absence of nitrate in both strains, suggesting that anaerobic As(III) oxidation is dependent on nitrate reduction.

Although both strains contain the suite of genes for complete denitrification, *nirK* was not expressed in strain QY30 (Figure 2C), which explains why this strain accumulated nitrite in the medium. It is not unusual that some genes present in the genome are not expressed or are expressed only weakly. The expression of genes is controlled by factors such as the promoter sequences and the presence of transcriptional activators or repressors.[54,55] It has been shown that the expression of *nirK* is regulated by NnrR, a transcriptional activator of the CRP/FNR family in many bacterial species.[56] In the genomes of ST2 and QY30, a *nnrR* gene is present downstream of the *nirK* locus (Figure 2A). Further work will be required to elucidate why *nirK* is expressed in ST2 but not in QY30. Introduction of the functional *nirK* gene from ST2 into QY30 conferred onto the latter the ability to reduce nitrite and largely abolished the accumulation of nitrite (Figure 3B). On the other hand, deletion of the *nirK* gene from the ST2 genome stopped the reduction of nitrite, resulting in accumulation of nitrite in the medium (Figure 3A). These results confirm the essential role of NirK in nitrite reduction. However, these genetic manipulations did not affect anaerobic As(III) oxidation (Figure 3, parts C and D), indicating that neither nitrite reduction nor nitrite accumulation is necessary for As(III) oxidation. Reduction of nitrate to nitrite is sufficient to provide a redox couple for anaerobic As(III) oxidation in both strains ST2 and QY30. Nitrite *per se* could not oxidize As(III) chemically.[9]

In contrast to anaerobic As(III) oxidation, only strain QY30 showed the ability to mediate anaerobic Fe(II) oxidation under nitrate-reducing conditions (Figure 4A). Furthermore, this ability may arise because QY30 accumulated nitrite, whereas strain ST2, which lacks the ability to oxidize Fe(II), did not accumulate nitrite (Figure 4B). Previously, it has been suggested that much of the nitrate-dependent anaerobic Fe(II) oxidation is attributed to abiotic reaction of Fe(II) with nitrite, the so-called chemodenitrification.[5,21,26,27] Our genetic manipulations on *nirK* gene in both bacterial strains provide direct evidence for the above hypothesis. Deletion of *nirK* gene in ST2 resulted in nitrite accumulation and conferred onto the mutant strain the ability to mediate anaerobic Fe(II) oxidation (Figure 3A). In contrast, introduction of *nirK* from ST2 into QY30 markedly decreased nitrite accumulation and abolished its ability to oxidize Fe(II) anaerobically (Figure 3A). These results indicate that most, if not all, of nitrate-dependent anaerobic Fe(II) oxidation is via abiotic reaction with nitrite in both strain QY30 and the ST2$^{\Delta nirK}$ mutant. This may also be true for other heterotrophic and mixotrophic nitrate-dependent anaerobic Fe(II) oxidizers present in paddy soils. A possible consequence of Fe(III) mineral formation is the encrustation of bacterial cells, resulting in a harmful effect on bacterial growth.[26] Such a negative effect would not be favorable to the evolution of a biotic mechanism of Fe(II) oxidation. In this regard, nitrate-dependent anaerobic Fe(II) oxidation is totally different from anaerobic As(III) oxidation, which is an enzymatic process leading to detoxification of As(III).[34] Our results highlight the pitfalls associated with linking genomic potential to function, as genes present in the genome may not be expressed. Quantification of gene transcripts would provide a better indicator of potential function than the abundance of genes at the DNA level.

Analyses using XRD and XAS showed that most (82−90%) of the Fe(III) precipitates formed by QY30 and ST2$^{\Delta nirK}$ were poorly crystalline (two-line ferrihydrite, amorphous ferric phosphate) with other more crystalline Fe(III) minerals accounting for only small proportions of the precipitates (Figures S6 and S7). These results are consistent with previous reports on other nitrate-dependent anaerobic Fe(II) oxidizers.[57] When both Fe(II) and As(III) were added to the incubation medium, ST2$^{\Delta nirK}$ appeared to form crystalline vivianite in addition to poorly crystalline ferrihydrite and amorphous ferric phosphates or arsenates (Tables S4 and S5). It is possible that phosphate might not be completely removed by the Fe(II) addition during the preparation of Fe(II) oxidation medium, which would enable the formation of Fe(III) phosphates. Therefore, some of the Fe(II) oxidation products may be a result of the culture medium used.

Environmental Implications. Microbial anaerobic oxidations of As(III) and Fe(II) are important biogeochemical processes occurring widely in anoxic environments, such as paddy soils and sediments.[21,35] These processes can have a strong influence the mobility of As, as well as other nutrients or contaminants that sorb strongly on Fe(III) minerals. In paddy soils, microbial anaerobic oxidation of As(III) and Fe(II) can be promoted by additions of nitrate that increase the population of denitrifiers. Although direct enzymatic oxidation of Fe(II) cannot be ruled out, abiotic oxidation of Fe(II) through reaction with nitrite is likely to be the main pathway. Not all denitrifiers can carry out anaerobic Fe(II) oxidation; those that do not express functional *nirK* genes are likely to possess a strong ability for anaerobic Fe(II) oxidation. Transcript abundance of *nirK* genes may serve as an indicator of the ability of anaerobic Fe(II) oxidation in environmental samples, which warrants further studies. Our study also shows that anaerobic Fe(II) oxidation can be established in denitrifiers by knocking out *nirK* gene. Such genetic engineering approach may be used where anaerobic Fe(II) oxidation is beneficial.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.9b06702.

Supplemental methods describing the isolation and genome sequencing of *Ensifer* sp. ST2 and *Paracoccus* sp. QY30, anaerobic oxidation of Fe(II), phylogenetic trees of *Ensifer* sp. ST2 and *Paracoccus* sp. QY30 and of

https://dx.doi.org/10.1021/acs.est.9b06702
Environ. Sci. Technol. 2020, 54, 4036−4045

arsenite oxidase (AioA), effects of As(III) on the growth of *Ensifer* sp. ST2 and *Paracoccus* sp. QY30 and their derivatives, XRD and normalized Fe K-edge XANES spectra of the biominerals, strains, plasmids, and primers used in this study, gene clusters associated with arsenite oxidation and dissimilatory denitrification, and results of Rietveld fitting of XRD data and linear combination fit analyses of Fe K-edge EXAFS spectra of the biominerals (PDF)

AUTHOR INFORMATION

Corresponding Author

Fang-Jie Zhao − *Jiangsu Key Laboratory for Organic Waste Utilization, Jiangsu Collaborative Innovation Center for Solid Organic Waste Resource Utilization, College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing 210095, China;* orcid.org/0000-0002-0164-169X; Phone: 86 25 84399551; Email: Fangjie.Zhao@njau.edu.cn; Fax: +86 25 84399055

Authors

Jun Zhang − *Jiangsu Key Laboratory for Organic Waste Utilization, Jiangsu Collaborative Innovation Center for Solid Organic Waste Resource Utilization, College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing 210095, China;* orcid.org/0000-0003-1965-7224

Cheng-Wei Chai − *Jiangsu Key Laboratory for Organic Waste Utilization, Jiangsu Collaborative Innovation Center for Solid Organic Waste Resource Utilization, College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing 210095, China*

Laurel K. ThomasArrigo − *Department of Environmental Systems Science, ETH Zurich, CH-8092 Zurich, Switzerland;* orcid.org/0000-0002-6758-3760

Shi-Chen Zhao − *Jiangsu Key Laboratory for Organic Waste Utilization, Jiangsu Collaborative Innovation Center for Solid Organic Waste Resource Utilization, College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing 210095, China*

Ruben Kretzschmar − *Department of Environmental Systems Science, ETH Zurich, CH-8092 Zurich, Switzerland*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.9b06702

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The study was supported by the Natural Science Foundation of China (Grant Nos. 21661132001, 41571312), the Swiss National Science Foundation (Grant No. IZLCZ2_170120), the Innovative Research Team Development Plan of the Ministry of Education of China (Grant No. IRT_17R56), and the fundamental research funds for the Central Universities (Grant Nos. KJJQ201902 and KYT201624). We acknowledge ELETTRA for the provision of synchrotron beamtime (proposal no. 20180024) and thank G. Aquilanti (ELETTRA, XAFS beamline) for support during the measurements.

REFERENCES

(1) Takahashi, Y.; Minamikawa, R.; Hattori, K. H.; Kurishima, K.; Kihou, N.; Yuita, K. Arsenic behavior in paddy fields during the cycle of flooded and non-flooded periods. *Environ. Sci. Technol.* **2004**, *38* (4), 1038−1044.

(2) Weber, F. A.; Hofacker, A. F.; Voegelin, A.; Kretzschmar, R. Temperature dependence and coupling of iron and arsenic reduction and release during flooding of a contaminated soil. *Environ. Sci. Technol.* **2010**, *44* (1), 116−122.

(3) Stroud, J. L.; Norton, G. J.; Islam, M. R.; Dasgupta, T.; White, R. P.; Price, A. H.; Meharg, A. A.; McGrath, S. P.; Zhao, F. J. The dynamics of arsenic in four paddy fields in the Bengal delta. *Environ. Pollut.* **2011**, *159* (4), 947−953.

(4) Yamaguchi, N.; Nakamura, T.; Dong, D.; Takahashi, Y.; Amachi, S.; Makino, T. Arsenic release from flooded paddy soils is influenced by speciation, Eh, pH, and iron dissolution. *Chemosphere* **2011**, *83* (7), 925−932.

(5) Jamieson, J.; Prommer, H.; Kaksonen, A. H.; Sun, J.; Siade, A. J.; Yusov, A.; Bostick, B. Identifying and quantifying the intermediate processes during nitrate-dependent iron(II) oxidation. *Environ. Sci. Technol.* **2018**, *52* (10), 5771−5781.

(6) Zhao, F. J.; McGrath, S. P.; Meharg, A. A. Arsenic as a food chain contaminant: mechanisms of plant uptake and metabolism and mitigation strategies. *Annu. Rev. Plant Biol.* **2010**, *61*, 535−559.

(7) Williams, P. N.; Villada, A.; Deacon, C.; Raab, A.; Figuerola, J.; Green, A. J.; Feldmann, J.; Meharg, A. A. Greatly enhanced arsenic shoot assimilation in rice leads to elevated grain levels compared to wheat and barley. *Environ. Sci. Technol.* **2007**, *41* (19), 6854−6859.

(8) Sun, W.; Sierra-Alvarez, R.; Milner, L.; Oremland, R.; Field, J. A. Arsenite and ferrous iron oxidation linked to chemolithotrophic denitrification for the immobilization of arsenic in anoxic environments. *Environ. Sci. Technol.* **2009**, *43* (17), 6585−6591.

(9) Zhang, J.; Zhao, S.; Xu, Y.; Zhou, W.; Huang, K.; Tang, Z.; Zhao, F. J. Nitrate stimulates anaerobic microbial arsenite oxidation in paddy soils. *Environ. Sci. Technol.* **2017**, *51* (8), 4377−4386.

(10) Xiu, W.; Guo, H.; Shen, J.; Liu, S.; Ding, S.; Hou, W.; Ma, J.; Dong, H. Stimulation of Fe(II) oxidation, biogenic lepidocrocite formation, and arsenic immobilization by *Pseudogulbenkiania* sp strain 2002. *Environ. Sci. Technol.* **2016**, *50* (12), 6449−6458.

(11) Hohmann, C.; Winkler, E.; Morin, G.; Kappler, A. Anaerobic Fe(II)-Oxidizing bacteria show As resistance and immobilize As during Fe(III) mineral precipitation. *Environ. Sci. Technol.* **2010**, *44* (1), 94−101.

(12) Oremland, R. S.; Hoeft, S. E.; Santini, J. A.; Bano, N.; Hollibaugh, R. A.; Hollibaugh, J. T. Anaerobic oxidation of arsenite in Mono Lake water and by facultative, arsenite-oxidizing chemoautotroph, strain MLHE-1. *Appl. Environ. Microbiol.* **2002**, *68* (10), 4795−4802.

(13) Picardal, F. Abiotic and microbial interactions during anaerobic transformations of Fe(II) and NO_X^-. *Front. Microbiol.* **2012**, *3*, 112.

(14) Etique, M.; Jorand, F. P. A.; Zegeye, A.; Gregoire, B.; Despas, C.; Ruby, C. Abiotic process for Fe(II) oxidation and green rust mineralization driven by a heterotrophic nitrate reducing bacteria (*Klebsiella mobilis*). *Environ. Sci. Technol.* **2014**, *48* (7), 3742−3751.

(15) Rhine, E. D.; Phelps, C. D.; Young, L. Y. Anaerobic arsenite oxidation by novel denitrifying isolates. *Environ. Microbiol.* **2006**, *8* (5), 899−908.

(16) Park, S.; Lee, J. H.; Shin, T. J.; Hur, H. G.; Kim, M. G. Adsorption and incorporation of arsenic to biogenic lepidocrocite formed in the presence of ferrous iron during denitrification by *Paracoccus denitrificans*. *Environ. Sci. Technol.* **2018**, *52* (17), 9983−9991.

(17) He, S. M.; Tominski, C.; Kappler, A.; Behrens, S.; Roden, E. E. Metagenomic analyses of the autotrophic Fe(II)-oxidizing, nitrate-reducing enrichment culture KS. *Appl. Environ. Microbiol.* **2016**, *82* (9), 2656−2668.

(18) Borch, T.; Kretzschmar, R.; Kappler, A.; Van Cappellen, P.; Ginder-Vogel, M.; Voegelin, A.; Campbell, K. Biogeochemical redox processes and their impact on contaminant dynamics. *Environ. Sci. Technol.* **2010**, *44* (1), 15−23.

(19) Smith, R. L.; Kent, D. B.; Repert, D. A.; Bohlke, J. K. Anoxic nitrate reduction coupled with iron oxidation and attenuation of

dissolved arsenic and phosphate in a sand and gravel aquifer. *Geochim. Cosmochim. Acta* **2017**, *196*, 102−120.
(20) Harvey, C. F.; Swartz, C. H.; Badruzzaman, A. B.; Keon-Blute, N.; Yu, W.; Ali, M. A.; Jay, J.; Beckie, R.; Niedan, V.; Brabander, D.; Oates, P. M.; Ashfaque, K. N.; Islam, S.; Hemond, H. F.; Ahmed, M. F. Arsenic mobility and groundwater extraction in Bangladesh. *Science* **2002**, *298* (5598), 1602−1606.
(21) Bryce, C.; Blackwell, N.; Schmidt, C.; Otte, J.; Huang, Y. M.; Kleindienst, S.; Tomaszewski, E.; Schad, M.; Warter, V.; Peng, C.; Byrne, J. M.; Kappler, A. Microbial anaerobic Fe(II) oxidation - Ecology, mechanisms and environmental implications. *Environ. Microbiol.* **2018**, *20* (10), 3462−3483.
(22) Liu, T. X.; Chen, D. D.; Li, X. M.; Li, F. B. Microbially mediated coupling of nitrate reduction and Fe(II) oxidation under anoxic conditions. *FEMS. Microbiol. Ecol.* **2019**, *95*, 4.
(23) Blothe, M.; Roden, E. E. Composition and activity of an autotrophic Fe(II)-oxidizing, nitrate-reducing enrichment culture. *Appl. Environ. Microbiol.* **2009**, *75* (21), 6937−6940.
(24) Mobley, H. L.; Rosen, B. P. Energetics of plasmid-mediated arsenate resistance in Escherichia coli. *Proc. Natl. Acad. Sci. U. S. A.* **1982**, *79* (20), 6119−6122.
(25) Klueglein, N.; Kappler, A. Abiotic oxidation of Fe(II) by reactive nitrogen species in cultures of the nitrate-reducing Fe(II) oxidizer *Acidovorax* sp BoFeN1 - questioning the existence of enzymatic Fe(II) oxidation. *Geobiology* **2013**, *11* (2), 180−190.
(26) Klueglein, N.; Zeitvogel, F.; Stierhof, Y. D.; Floetenmeyer, M.; Konhauser, K. O.; Kappler, A.; Obst, M. Potential role of nitrite for abiotic Fe(II) oxidation and cell encrustation during nitrate reduction by denitrifying bacteria. *Appl. Environ. Microbiol.* **2014**, *80* (3), 1051−1061.
(27) Carlson, H. K.; Clark, I. C.; Blazewicz, S. J.; Iavarone, A. T.; Coates, J. D. Fe(II) oxidation is an innate capability of nitrate-reducing bacteria that involves abiotic and biotic reactions. *J. Bacteriol.* **2013**, *195* (14), 3260−3268.
(28) Chakraborty, A.; Roden, E. E.; Schieber, J.; Picardal, F. Enhanced growth of *Acidovorax* sp strain 2AN during nitrate-dependent Fe(II) oxidation in batch and continuous-flow systems. *Appl. Environ. Microbiol.* **2011**, *77* (24), 8548−8556.
(29) Kappler, A.; Schink, B.; Newman, D. K. Fe(III) mineral formation and cell encrustation by the nitrate-dependent Fe(II)-oxidizer strain BoFeN1. *Geobiology* **2005**, *3* (4), 235−245.
(30) Liu, T. X.; Chen, D. D.; Luo, X. B.; Li, X. M.; Li, F. B. Microbially mediated nitrate-reducing Fe(II) oxidation: Quantification of chemodenitrification and biological reactions. *Geochim. Cosmochim. Acta* **2019**, *256*, 97−115.
(31) Rosen, B. P.; Borbolla, M. G. A plasmid-encoded arsenite pump produces arsenite resistance in Escherichia coli. *Biochem. Biophys. Res. Commun.* **1984**, *124* (3), 760−765.
(32) Andres, J.; Bertin, P. N. The microbial genomics of arsenic. *FEMS Microbiol. Rev.* **2016**, *40* (2), 299−322.
(33) Zhu, Y.-G.; Xue, X.-M.; Kappler, A.; Rosen, B. P.; Meharg, A. A. Linking genes to microbial biogeochemical cycling: lessons from arsenic. *Environ. Sci. Technol.* **2017**, *51* (13), 7326−7339.
(34) Zhang, J.; Zhou, W.; Liu, B.; He, J.; Shen, Q.; Zhao, F. J. Anaerobic arsenite oxidation by an autotrophic arsenite-oxidizing bacterium from an arsenic-contaminated paddy soil. *Environ. Sci. Technol.* **2015**, *49* (10), 5956−5964.
(35) Oremland, R. S.; Stolz, J. F. The ecology of arsenic. *Science* **2003**, *300* (5621), 939−944.
(36) van Lis, R.; Nitschke, W.; Duval, S.; Schoepp-Cothenet, B. Arsenic as bioenergetic substrates. *Biochim. Biophys. Acta, Bioenerg.* **2013**, *1827* (2), 176−188.
(37) Li, X.; Qiao, J.; Li, S.; Haggblom, M. M.; Li, F.; Hu, M. Bacterial communities and functional genes stimulated during anaerobic arsenite oxidation and nitrate reduction in a paddy soil. *Environ. Sci. Technol.* **2020**, *54* (4), 2172−2181.
(38) Reasoner, D. J.; Geldreich, E. E. A new medium for the enumeration and subculture of bacteria from potable water. *Appl. Environ. Microbiol.* **1985**, *49* (1), 1−7.
(39) Sambrook, J.; Russell, D. *Molecular Cloning: A Laboratory Manual*, 3rd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2011.
(40) Lane, D. L. 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*; Stackebrandt, E. R., Goodfellow, M., Eds.; Wiley: Chichester, United Kingdom, 1991; pp 115−175.
(41) Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **2013**, *30* (12), 2725−2729.
(42) Quemeneur, M.; Heinrich-Salmeron, A.; Muller, D.; Lievremont, D.; Jauzein, M.; Bertin, P. N.; Garrido, F.; Joulian, C. Diversity surveys and evolutionary relationships of *aoxB* genes in aerobic arsenite-oxidizing bacteria. *Appl. Environ. Microbiol.* **2008**, *74* (14), 4567−4573.
(43) Quandt, J.; Hynes, M. F. Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. *Gene* **1993**, *127* (1), 15−21.
(44) Chen, J.; Qin, J.; Zhu, Y. G.; de Lorenzo, V.; Rosen, B. P. Engineering the soil bacterium *Pseudomonas putida* for arsenic methylation. *Appl. Environ. Microbiol.* **2013**, *79* (14), 4493−4495.
(45) Martinez-Garcia, E.; Calles, B.; Arevalo-Rodriguez, M.; de Lorenzo, V. pBAM1: an all-synthetic genetic tool for analysis and construction of complex bacterial phenotypes. *BMC Microbiol.* **2011**, *11*, 38.
(46) Kappler, A.; Newman, D. K. Formation of Fe(III)-minerals by Fe(II)-oxidizing photoautotrophic bacteria. *Geochim. Cosmochim. Acta* **2004**, *68* (6), 1217−1226.
(47) Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* **2001**, *25* (4), 402−408.
(48) Zhu, Y. G.; Sun, G. X.; Lei, M.; Teng, M.; Liu, Y. X.; Chen, N. C.; Wang, L. H.; Carey, A. M.; Deacon, C.; Raab, A.; Meharg, A. A.; Williams, P. N. High percentage inorganic arsenic content of mining impacted and nonimpacted Chinese rice. *Environ. Sci. Technol.* **2008**, *42* (13), 5008−5013.
(49) Stookey, L. L. Ferrozine-a new spectrophotometric reagent for iron. *Anal. Chem.* **1970**, *42*, 779−781.
(50) Snell, F. D.; Snell, C. T. *Colorimetric Methods of Analysis*, 3rd ed.; D. Van Nostrand Company: Toronto, Canada, 1959; Vol. *2*.
(51) Scarlett, N. V. Y.; Madsen, I. C. Quantification of phases with partial or no known crystal structures. *Powder Diffr.* **2006**, *21*, 278−284.
(52) ThomasArrigo, L. K.; Byrne, J. M.; Kappler, A.; Kretzschmar, R. Impact of organic matter on iron(II)-catalyzed mineral transformations in ferrihydrite-organic matter coprecipitates. *Environ. Sci. Technol.* **2018**, *52* (21), 12316−12326.
(53) Ravel, B.; Newville, M. ATHENA, ARTEMIS, HEPHAESTUS: data analysis for X-ray absorption spectroscopy using IFEFFIT. *J. Synchrotron Radiat.* **2005**, *12*, 537−541.
(54) VanSpanning, R. J. M.; DeBoer, A. P. N.; Reijnders, W. N. M.; Westerhoff, H. V.; Stouthamer, A. H.; VanDerOost, J. FnrP and NNR of *Paracoccus denitrificans* are both members of the FNR family of transcriptional activators but have distinct roles in respiratory adaptation in response to oxygen limitation. *Mol. Microbiol.* **1997**, *23* (5), 893−907.
(55) Tosques, I. E.; Kwiatkowski, A. V.; Shi, J. R.; Shapleigh, J. P. Characterization and regulation of the gene encoding nitrite reductase in *Rhodobacter sphaeroides* 2.4.3. *J. Bacteriol.* **1997**, *179* (4), 1090−1095.
(56) Laratta, W. P.; Shapleigh, J. P. Site-directed mutagenesis of NnrR: a transcriptional regulator of nitrite and nitric oxide reductase in *Rhodobacter sphaeroides*. *FEMS Microbiol. Lett.* **2003**, *229* (2), 173−178.
(57) Fortin, D.; Langley, S. Formation and occurrence of biogenic iron-rich minerals. *Earth-Sci. Rev.* **2005**, *72* (1−2), 1−19.

https://dx.doi.org/10.1021/acs.est.9b06702
Environ. Sci. Technol. 2020, 54, 4036−4045