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Anaerobic Fe(II)-Oxidizing Bacteria Show As Resistance and Immobilize As during Fe(III) Mineral Precipitation

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More than 100 million individuals worldwide are exposed to arsenic-contaminated water, making the investigation of arsenic mobility in aquatic systems of utmost importance. Iron (hydr)oxides play a key role in preventing arsenic release in aquifers and soils due to their strong arsenic sorption and are even used to remove arsenic in water treatment. Neutrophilic Fe(II)-oxidizing bacteria produce Fe(III) minerals and therefore have the potential to affect arsenic mobility. In the present study, we demonstrate that the metabolism of anaerobic nitrate-reducing and phototrophic Fe(II)-oxidizing bacteria is not significantly affected by arsenate concentrations of up to 500 μM (37.5 mg/L). Even in the presence of the more toxic arsenic species, arsenite, cell metabolism was significantly impaired only at the highest arsenite concentration (500 μM) for one of the Fe(II)-oxidizers. All Fe(II)-oxidizing bacteria tested effectively immobilized arsenic during Fe(II) oxidation (>96%), lowering the remaining dissolved arsenic concentrations to values close to or even lower than the current drinking water limit of 10 μg/L. Since the minerals formed by these bacteria included highly crystalline Fe(III) minerals that are hardly reducible by Fe(III)-reducing bacteria, stimulation of arsenic immobilization by Fe(II)-oxidizing bacteria can potentially support water treatment systems or even be applied as an effective remediation strategy.

Introduction

The toxic metalloid arsenic is present in the environment due to geogenic sources, acid mine drainage, and its extensive use as a pesticide and wood preservative (*1*). The two most environmentally relevant oxidation states of arsenic are arsenite [As(III)], prevalent under reducing conditions, and arsenate [As(V)], dominant under oxic conditions, with As(III) being more toxic and mobile than As(V) (*2*). Arsenic speciation and mobility depends on the pH value and the redox potential (E_h), but also on the sorbents (e.g., metal oxides) and sorbates (e.g., phosphate, silicate or carbonate) present in an environment. Ferric iron mineral surfaces are usually positively charged at neutral pH due to their high points of net zero charge (ZPC) and can function as good adsorbents for negatively charged compounds such as arsenate ($H_2AsO_4^-$ and $HAsO_4^{2-}$) or the neutral species arsenite (H_3AsO_3) (*3*).

Various processes have been proposed for arsenic mobilization from geogenic sources in an effort to explain arsenic contamination of groundwaters, e.g., in Southeast Asian aquifers. First, it has been suggested that the oxidation of As-bearing sulfides by oxygen is involved. Due to increased water extraction for irrigation and thus lowering of water levels, O_2 is introduced into the groundwater potentially causing oxidation of arsenic-bearing sulfides (*4*). Second, the simultaneous presence of high arsenic and ferrous iron concentrations in anoxic waters has led to the conclusion that reductive (microbial) dissolution of arsenic-rich Fe(III) oxyhydroxides releases arsenic (*1, 5, 6*). However, it has recently been shown that under certain geochemical conditions, Fe(III) reduction can also lead to arsenic sequestration by adsorption of arsenic onto secondary Fe minerals (*7−9*). This latter process is likely related to Fe-rich media, since long-term reducing conditions in column experiments may lead to progressive Fe depletion and subsequent arsenic release (*8*).

Neutrophilic Fe(II)-oxidizing bacteria form Fe(III) minerals that could coprecipitate and bind arsenic (*10*). Under oxic conditions, several strains of aerobic Fe(II)-oxidizing bacteria were shown to effectively compete with the chemical oxidation of Fe(II) by O_2 (*11*). Under anoxic pH-neutral conditions, dissolved and/or surface-sorbed Fe(II) are only oxidized by manganese oxides, some crystalline iron oxides, and nitrite (*12−14*). Therefore, Fe(II)-oxidizing bacteria are the most important catalysts for the generation of Fe(III) under anoxic conditions. Indeed, Straub et al. (*15*) discovered that some bacteria are able to couple the oxidation of Fe(II) to dissimilatory reduction of nitrate. Another group of Fe(II)-oxidizing organisms, anoxygenic phototrophic Fe(II)-oxidizing bacteria, are able to catalyze anaerobic oxidation of Fe(II) using light energy to fix CO_2 into biomass (*16, 17*). In light of this, it was recently suggested that Fe(II)-oxidizing bacteria could potentially be used for the efficient removal of arsenic in water treatment (*18, 19*). However, arsenic toxicity to Fe(II)-oxidizing bacteria has of yet not been studied. In particular, it is not known whether all or only specific Fe(II)-oxidizing bacteria have the necessary detoxification genes to cope with the toxic As and can still metabolize in the presence of As (*2, 20, 21*).

In addition to the determination of arsenic toxicity to Fe(II)-oxidizers, the mineralogy and stability of biogenic iron minerals formed by Fe(II)-oxidizing bacteria in the presence of As needs to be investigated. Abiotic Fe(II) oxidation typically leads to amorphous or poorly crystalline Fe(III) phases (*22*). In comparison, during the anaerobic oxidation of Fe(II) by microorganisms, the formation of poorly crystalline ferrihydrite-type minerals but also more crystalline minerals such as goethite, lepidocrocite, hematite, magnetite, and green rusts have been observed (*10, 23−25*). It is not yet known how the presence of As changes the identity and crystallinity of Fe(III) minerals formed during microbial Fe(II) oxidation.

Based on these knowledge gaps, the objectives of this study were to (i) determine whether the presence of As(III) and As(V) affects the metabolism of Fe(II)-oxidizing bacteria, (ii) determine whether anaerobic Fe(II)-oxidizing bacteria can oxidize Fe(II) in the presence of As(III) and As(V),

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(iii) quantify the amount of As removed from aqueous solution by iron-mineral forming (Fe(II)-oxidizing) bacteria, and (iv) identify the Fe(III) minerals produced during immobilization of As by biogenic Fe(III) minerals.

Materials and Methods

Sources of the Organisms. Strain BoFeN1 is a chemoorganotrophic, nitrate-reducing β-Proteobacterium closely related to *Acidovorax* sp. and grows mixotrophically oxidizing ferrous iron and acetate as organic cosubstrate (*26*). The enrichment culture strain KS oxidizes Fe(II) autotrophically reducing nitrate (*27*). *Rhodobacter ferrooxidans* strain SW2 oxidizes Fe(II) phototrophically, reduces CO_2 for growth, belongs to the α-Proteobacteria, and is a purple nonsulfur bacterium (*17*).

Microbial Growth Media and Growth Conditions. All strains were cultivated in anoxic mineral medium (0.6 g/L of KH_2PO_4 corresponding to 4.4 mM phosphate, 0.3 g/L of NH_4Cl, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.1 g/L of $CaCl_2 \cdot 2H_2O$, 22 mM bicarbonate buffer, 1 mL/L vitamin solution, 1 mL/L trace element solution, and 1 mL/L selenate-tungstate solution) (*16*). For routine cultivation of strain BoFeN1 and culture KS, 10 mM Na-nitrate and 5 mM Na-acetate were added to the medium. For routine photoautotrophic growth of strain SW2, the headspace was flushed with H_2/CO_2 (v/v, 80/20). For mineral precipitation and As-immobilization experiments, 10 mM Fe(II) from a sterile 1 M $FeCl_2$ stock solution was added to the medium followed by precipitation of whitish Fe(II) carbonate and phosphate minerals since under these conditions the solubility products of these two minerals are exceeded. Medium that contained only dissolved Fe(II) without the presence of Fe(II) precipitates was prepared by removal of the Fe(II) precipitates by filtration according to *24*. After filtration of Fe(II) precipitates, approximately 40 μM phosphate and 3−4 mM dissolved Fe(II) was left in the filtered medium. Using this medium allows identification of the biotically precipitated Fe(III) minerals. For Mössbauer mineral analyses, the medium was prepared with 1 mM phosphate instead of the 4.4 mM phosphate used for the other experiments (see above and refs *16 and 17*) to minimize Fe(II) precipitation and Fe(II) removal during the filtration process. In these experiments, the ionic strength was balanced by addition of NaCl.

As(III) and As(V) solutions (20 mM each) were prepared using a stock solution of 0.05 M sodium arsenite ($NaAsO_2$) and disodium hydrogen arsenate heptahydrate salt ($AsHNa_2O_4 \cdot 7H_2O$) and were sterile-filtered (0.22 μm, MCE, Fisher Scientific).

Experimental Setup. For the growth experiments (As toxicity/resistance experiments), 9.5 mL of mineral medium was filled in 12 mL culture tubes and varying amounts of As(III) and As(V) were added to yield target concentrations of 0, 20, 50, 100, and 500 μM of arsenate or arsenite. Inoculation was carried out with 5% of H_2- or acetate-grown (Fe-free) cultures. BoFeN1 and KS cultures contained 5 mM acetate and 10 mM nitrate as substrates. SW2 cultures were flushed once per day with H_2/CO_2 (v/v, 80/20). Growth was followed by optical density (OD) measurement. For the arsenic immobilization experiments, filtered medium was prepared and filled into sterile serum bottles (50 mL bottles, 25 mL of medium). Different amounts of As(III) and As(V) were added to the bottles to yield target concentrations of 20 and 50 μM. As inoculum for immobilization experiments, 5% Fe(II)-grown culture was used. As inoculum for experiments for Mössbauer and electron microscopical analysis with strain BoFeN1, 5% acetate-grown culture was used. Bottles and tubes were incubated at 22 °C and 400 lx (phototrophic strain SW2) or at 26 °C in the dark (nitrate-reducing bacteria).

Analytical Methods. Optical density (OD) was measured at 600 nm by spectrophotometry to determine growth in the presence of arsenic. For analysis of dissolved iron, 100 μL of culture suspension was withdrawn in an anoxic glovebox with a syringe, filtered with 0.5 mL nylon (0.22 μm) filter tubes (Costar, Corning, NY) and analyzed for dissolved Fe(II) with the ferrozine assay (*28*). For analysis of dissolved arsenic, 990 μL of culture suspension was withdrawn, also filtered with 0.5 mL nylon (0.22 μm) filter tubes in an anoxic glovebox, and immediately acidified with 10 μL of HNO_3 (65%) to preserve the As redox state. Total As was quantified by ICP-MS (ELAN 6000, Perkin-Elmer).

For Mössbauer spectroscopy, biologically formed mineral precipitates were filtered (0.22 μm Millipore Express PLUS (PES) membrane) and dried anoxically at room temperature, ground in an agate mortar, mixed with cellulose, and pressed to pellets. The pellets were sealed between two layers of Kapton tape in the anoxic glovebox. Samples were mounted in a close-cycle exchange-gas cryostat (Janis, USA) that allowed cooling of the sample to 4.2 K. Mössbauer spectra were collected with a constant acceleration drive system in transmission mode and with a ^{57}Co source. Spectra were calibrated against a spectrum of alpha-Fe metal foil collected at room temperature. Spectra calibration and fitting was performed with Recoil software (University of Ottawa, Canada) using Voigt based spectral lines.

The electron microscopy pictures were taken on a Zeiss ULTRA 55 scanning electron microscope equipped with a field emission gun (accelerating voltage of 10 kV; working distance of 4 mm) using the in-lens secondary electron detector. For imaging, the biologically formed mineral precipitates were dried anoxically and mounted on aluminum stubs.

Results

Effect of As(III) and As(V) on Growth of Nitrate-Reducing and Phototrophic Fe(II)-Oxidizing Bacteria. To quantify the toxicity of As(III) and As(V) for different anaerobic Fe(II)-oxidizing bacteria, we quantified their growth in cultures containing either acetate or H_2/CO_2 as electron and carbon source at varying As(III) and As(V) concentrations. In these experiments, we did not add Fe(II) to prevent sorption of the arsenic to precipitated Fe(II) and Fe(III) minerals. This would lead to a decrease of dissolved As concentration and therefore lower the amount of bioavailable As.

The nitrate-reducing strain BoFeN1 showed the highest growth yields in the absence of As(III), but also in the presence of low As(III) concentrations (20−50 μM) (Figure 1A). In comparison, an obvious delay in growth is observed in the presence of high concentrations (500 μM As(III)), although at the end of growth, the same OD value was attained as in the presence of low As(III) concentrations. In contrast, As(V) did not affect growth by strain BoFeN1 (Figure 1B). Even in the presence of 500 μM As(V), similar OD values and growth rates were obtained as in the presence of lower As(V) concentrations (20−100 μM) and in the absence of As(V).

The nitrate-reducing enrichment culture KS generally grows slower than strain BoFeN1, but the influence of arsenic was similar to that observed for strain BoFeN1. As seen with BoFeN1, with increasing As(III) concentrations, an increase in growth lag time was observed for the culture KS (Figure 1C). The enrichment culture KS obviously is slightly more sensitive towards As(III) than strain BoFeN1. As observed for strain BoFeN1, growth of the enrichment culture KS was also not negatively influenced by the presence of As(V) at concentrations of up to 500 μM (Figure 1D). Indeed, OD measured in the tubes containing As(V) were even slightly higher than in tubes without any arsenic present. This suggests that these bacteria are even able to increase growth

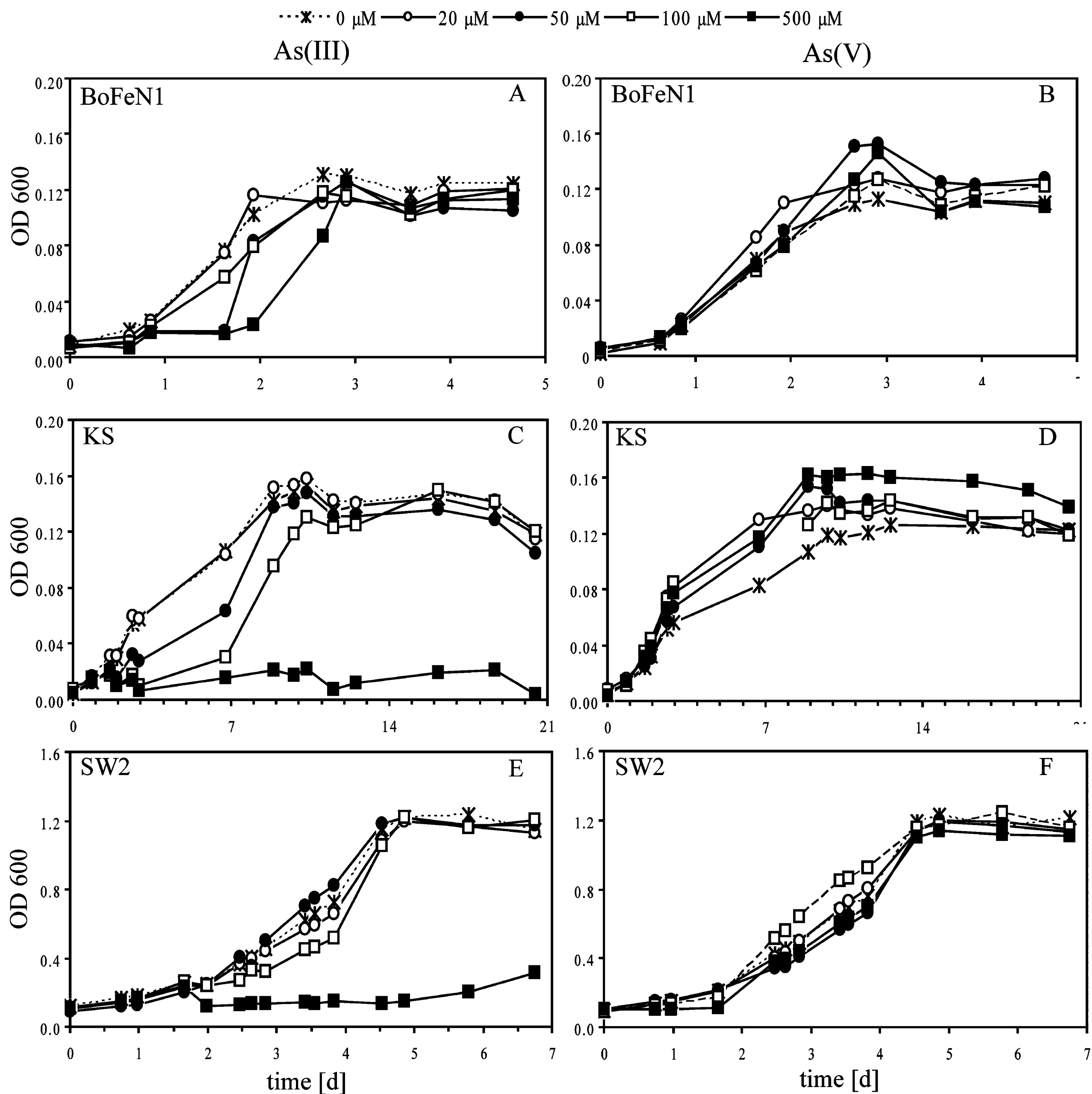

FIGURE 1. Arsenic-affected growth of the nitrate-reducing Fe(II)-oxidizing strains BoFeN1 and KS and the phototrophic Fe(II)-oxidizing strain SW2 grown in the absence of Fe(II) with either acetate (BoFeN1 and KS) or H_2 (SW2) as electron donor. Growth is shown as increase in optical density at 600 nm (OD 600). OD was measured in the absence and presence of different concentrations of As(III) (A, C, E) and As(V) (B, D, F). Please note the different time axis for the various strains. Data shown were selected as representative examples from three independent experiments that showed qualitatively identical results.

yields in the presence of As(V), possibly by using As(V) as electron acceptor.

The phototrophic strain SW2 showed lower growth rates in the presence of 100 μM As(III). The toxic effect of As(III) was obvious in the presence of 500 μM As(III) (Figure 1E) where the lag phase and growth rates as well as the final cell number after 3 weeks was significantly impaired. In contrast to As(III), As(V) did not influence growth of strain SW2 (Figure 1F).

Immobilization of As(III) and As(V) by Nitrate-Reducing and Phototrophic Fe(II)-Oxidizing Bacteria. All strains investigated oxidized 2−4 mM Fe(II) to completion within 4 days (strain BoFeN1), 8 days (strain KS), and 14 days (strain SW2) in the presence of As(III) and As(V) at concentrations of up to 50 μM. During Fe(II) oxidation by these nitrate-reducing and anoxygenic phototrophic Fe(II)-oxidizing bacteria, As(V) and As(III) are removed effectively from solution either through sorption to and/or coprecipitation by the Fe(III) minerals formed (Figure 2 and Table 1). All strains investigated in this study removed more than 96% of the initially added As(V) and As(III) (at initial As target concentrations of both 20 μM and 50 μM) during oxidation of approximately 3.5 mM Fe(II) (Table 1). Depending on the initial target As concentration of either 20 μM (1498 μg/L) or 50 μM (3746 μg/L) (Table 1), these experiments lead to remaining dissolved As(V) and As(III) concentrations of 3.2−13.5 μg/L (for As(V)) and 4.7−113.9 μg/L (for As(III)), and therefore to values mostly below or close to the current WHO drinking water limit of 10 μg/L. The two initial As concentrations were chosen based on a recent study by Berg et al. (*29*). These authors showed that at Fe/As ratios in As-contaminated water >100 (as present in our experiments with an initial target concentration of 20 μM As), the remaining dissolved As concentrations—after As sorption to and im-

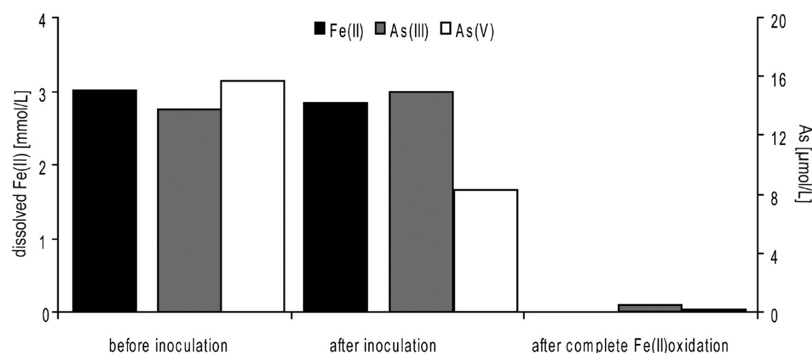

FIGURE 2. Representative data for Fe(II) oxidation by the nitrate-reducing strain BoFeN1 and simultaneous course of concentrations of dissolved As(III) and As(V), respectively. Before the beginning of Fe(II) oxidation, two data points for As(III) and As(V) concentrations are plotted, the first one taken after As addition but before addition of cells (inoculation) and the second one directly after inoculation indicating a significant drop in As(V) but no change in As(III) concentration after addition of cells. The third data point for As(III) and As(V) was measured after complete Fe(II) oxidation.

TABLE 1. Initial As Concentrations, Remaining Dissolved As Concentrations after Fe(II) Oxidation, Amount of As(V) and As(III) Immobilized by Different Fe(II)-Oxidizing Bacteria, and Ratios of As/Fe for As(III) and As(V) Sorbed and/or Coprecipitated to/by Fe(III) (Hydr)oxides Formed during Microbial Fe(II) Oxidation. Initial As Concentrations are Measured Values and given both in µM and µg/L[a]

strain	As target concentration [µM]	initial As(V) (at t_x) [µM]	initial As(V) (at t_0) [µM]	initial As(V) (at t_0) [µg/L]	remaining dissolved As after Fe(II) oxidation (at $t_{removed}$) [µg/L]	As immobilized [%]	molar ratio [As(removed)/ Fe(oxidized)] in precipitates
BoFeN1	20	15.7	8.3	621.8	6.7	98.9	0.003
	50	35.7	24.4	1828.0	13.5	99.3	0.009
KS	20	18.3	7.2	535.7	5.5	98.9	0.002
SW2	20	13.9	3.1	228.5	6.3	98.4	0.001
	50	40.8	12.2	911.0	3.2	99.5	0.004

strain	As target concentration [µM]	initial As(III) (at t_x) [µM]	initial As(III) (at t_0) [µM]	initial As(III) (at t_0) [µg/L]	remaining dissolved As after Fe(II) oxidation (at $t_{removed}$) [µg/L]	As immobilized [%]	molar ratio [As(removed)/ Fe(oxidized)] in precipitates
BoFeN1	20	13.7	14.9	1118.6	33.0	97.1	0.005
	50	42.9	40.0	2996.8	113.9	96.2	0.013
KS	20	20.4	18.9	1418.2	6.1	99.6	0.007
SW2	20	17.3	18.0	1348.6	4.7	99.6	0.006
	50	43.5	39.3	2946.6	5.7	99.9	0.017

[a] As/Fe ratios are given on a per mol basis and were calculated from the amount of As removed by the Fe(III) (hydr)oxides between time points t_0 and $t_{removed}$ with t_0 = time point directly after inoculation and $t_{removed}$ = time point at which no dissolved Fe(II) was detectable anymore. Time point t_x represents the time point after As addition before inoculation. For details of calculations see Supporting Information Table S1.

mobilization with freshly precipitated Fe(III) minerals in sand filters—are below the drinking water limit of 10 µg/L. At ratios of Fe/As <100 (as present in our experiments with an initial target concentration of 50 µM As) the remaining dissolved As concentrations were still above the drinking water limit.

We calculated ratios of arsenic removed to Fe(III) precipitated based on differences in concentrations of dissolved arsenic and Fe(II) determined at different time-points (at t_0 = directly after inoculation and at $t_{removed}$ = when no Fe(II) was no longer detectable). For the different strains, we determined that the ratios were within 1 order of magnitude. In particular, we calculated molar ratios of As:Fe in the precipitate in the range of 0.001−0.009 for As(V) per Fe(III) and 0.005−0.017 for As(III) per Fe(III) for all strains (for details see Table 1 and SI Table S1).

Specifically, in cultures of the nitrate-reducing Fe(II)-oxidizing strain BoFeN1, we followed the As and Fe(II) concentrations in more detail (Figure 2). We observed that in the beginning of the experiment, As(V) behaved differently from As(III); in contrast to As(III) concentrations, As(V) concentrations dropped significantly (up to 50%) in all experiments directly after inoculation (after addition of cells) and even before Fe(II) oxidation had started. Control experiments with growth medium, glassware, filters, and SW2 and BoFeN1 cells were conducted to determine the fate of As(V) and reveal the different behavior as compared to As(III). These controls showed that sorption of arsenic onto glass bottle surfaces, as well as onto filter material was negligible (data not shown). In addition, sorption of As(V) to Fe(III) minerals that were present in the inoculum (pregrown on iron) could not fully explain the initial drop in As(V) concentration (Supporting Information S2). Uptake of arsenic by the bacteria themselves was excluded by iron-free control experiments with strain BoFeN1 (data not shown). Furthermore, control experiments with cells and As(V) compared to an experiment with cells, As(V) and Fe(II) showed that the

initial As(V) drop occurred only in the presence of Fe(II), but not in setups with only cells and As(V). This was not observed for experiments carried out with As(III). We infer from these results that As(V) may be partially bound to the cell surface, possibly by ternary cell−Fe(II)−As complexes, i.e., binding of Fe(II)−As to negatively charged functional groups (carboxyl and phosphate) of the cell surfaces. A plausibility estimation using a cell weight of approximately 1 pg per cell, the cell number of approximately 5×10^7cells/mL present in our experiments and a cell-surface site density (carboxyl and phosphate groups) of approximately 2 μmol sites per mg cell biomass (*30*) shows that theoretically approximately 100 μmol/L As(V) can be bound to the cell surfaces via ternary cell−Fe(II)−As(V) complexes—approximately 3 times more than the up to 28 μM As(V) observed to be removed for example in our SW2 experiments with As(V) (at an initial target concentration of As(V) of 50 μM; see Supporting Information S2).

Characterization of Iron Minerals and Cell-Mineral Aggregates Formed by Strains BoFeN1 and SW2 in the Absence and Presence of As. Scanning electron micrographs of BoFeN1 cells oxidizing Fe(II) in the absence of arsenic showed surface precipitates with crystalline, needle-like structures with only few cells being completely free of surface precipitates (Figure 3A). Cells growing in the presence of 200 μM arsenite (As(III)) were also covered with crystalline needle-like structures, however, the mineral particles generally seemed to be smaller than those in the arsenic-free cultures (Figure 3B). Cultures growing in the presence of 200 μM arsenate (As(V)) show only a few cells covered with crystalline needle-like structures (like the cells in As-free medium), but also many cells covered with iron minerals of smaller particle size (Figure 3C). Mössbauer spectroscopy analysis of these minerals at 77 K showed the presence of goethite in the absence of arsenate/arsenite (see SI Figure S3). In the presence of 200 μM arsenate and 200 μM arsenite, respectively, the analysis showed the presence of goethite and a second Fe(III) mineral phase, most probably superparamagnetic goethite (nanogoethite), ferrihydrite, or lepidocrocite (shown for arsenate in Figure 4). In samples taken from growing cultures of the phototrophic Fe(II)-oxidizer strain SW2, we did not detect any signals for crystalline minerals both in the absence and presence of arsenic by Mössbauer spectroscopy (data not shown), confirming earlier studies (*24*).

Discussion

As Toxicity for Anaerobic Iron-Oxidizing Microorganisms. All anaerobic Fe(II)-oxidizing bacteria tested in this study could grow and metabolize even in the presence of arsenic concentrations (up to 500 μM) that are much higher than typical concentrations observed in the environment (e.g., arsenic concentrations in Bangladesh aquifers were described to range between 10 and 5000 μg/L (0.13−66.73 μM)) (*1*). In rice paddy soil, arsenic concentrations of >30 μg/g have been observed (*31*). This means that Fe(II)-oxidizing bacteria similar to the ones that we have studied here are expected to effectively oxidize Fe(II) even in environments containing high arsenic concentrations. Similar to our experiments, Fe(III)-reducing bacteria were shown to reduce Fe(III) also in the presence of high As concentrations (of up to 10 mM) (*20, 32, 33*). Obviously, various metabolic types of bacteria including the ones used in this study evolved detoxification mechanisms to cope with elevated concentrations of arsenic (*21*). Some organisms can even use As(V) as electron acceptor for respiration (*21*). Based on our data, this may even be the case for the enrichment culture KS that was described to date only as a nitrate-reducing Fe(II)-oxidizing culture.

Detoxification strategies include efflux mechanisms, oxidation of the more toxic species As(III) to the less toxic species As(V) (*34*), minimizing arsenic uptake into the cell (for example by increasing specificity of phosphate uptake), and reduction of As coupled to methylation (*2*). In some bacteria, specific detoxification mechanisms, encoded by *ars* genes, have been detected. Arsenic resistance systems have been found in Gram-positive and Gram-negative bacteria located on plasmids, but also on chromosomal genes (*35*) with the number of genes differing in various strains (*36*). These studies show that although arsenic is highly toxic to many organisms including prokaryotes and eukaryotes, many of these organisms are able to metabolize in the presence of arsenic and overcome its toxicity.

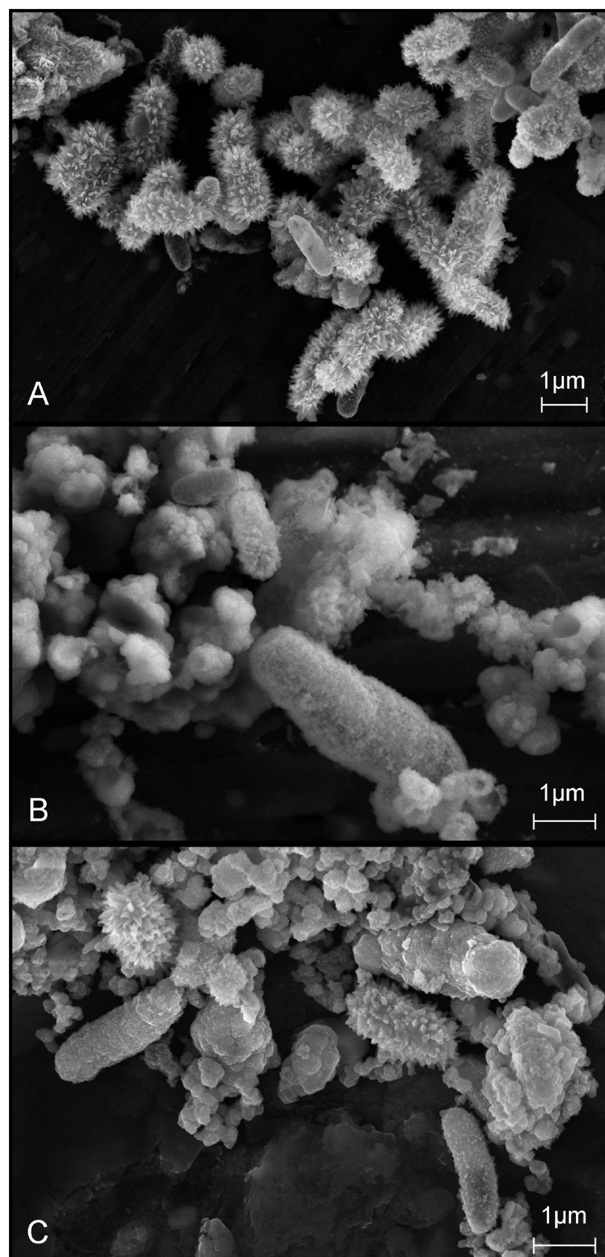

FIGURE 3. Scanning electron micrographs of BoFeN1 cells after complete Fe(II)-oxidation (after 8 days) coupled to nitrate reduction in the presence of 0 μM arsenic (A), 200 μM arsenite As(III) (B), and 200 μM arsenate As(V) (C). The acicular morphology of goethite particles coating bacterial cell is well developed in the absence of arsenic (A), while it tends to be less marked in the presence of As(V) (C) and even less with As(III) (B).

As Immobilization by As Coprecipitation and/or Sorption during Fe(II) Oxidation. It was recently shown that the

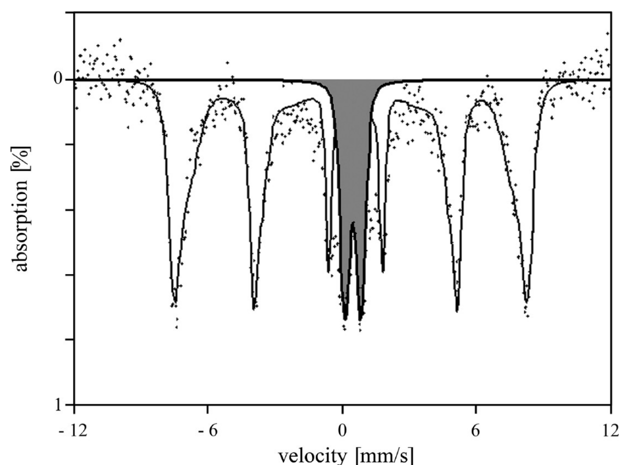

FIGURE 4. Mössbauer spectrum at 77 K of the mineral products formed by the nitrate-reducing Fe(II)-oxidizer strain BoFeN1 in presence of 200 μM As(V). Two distinct iron components were detected: one sextet (line) and one doublet (shaded). Both components have isomer shifts corresponding to Fe(III). The sextet was modeled by a distribution of hyperfine fields with <H> with greatest probability 48.9 T, assigned to goethite. Model candidates for the doublet include ferrihydrite, lepidocrocite, and superparamagnetic goethite.

ratio of Fe/As in As-contaminated water (Fe/As ratio of either >100 or <100) determines whether the remaining dissolved As concentrations after As-sorption to and coprecipitation with freshly precipitated Fe(III) minerals in sand filters reaches values below or above the WHO drinking water limit of 10 μg/L (*29*). Therefore, in biological As immobilization experiments, we tested two concentrations of As (initial target concentrations of 20 and 50 μM) that provided ratios of Fe/As either above (>100) or below (<100) the ratio that was shown to lead to dissolved As concentrations below or above the WHO limit. In these experiments, we found that in all setups with Fe/As ratios >100 the remaining dissolved As concentration was indeed below the WHO limit—with the exception of one experiment (strain BoFeN1, initial target concentration of 20 μM As(III) where 33 μg/L dissolved As remained). Even in most microbiological immobilization experiments with Fe/As ratios <100, the remaining dissolved As concentrations were below or very near the 10 μg/L limit (Table 1). Only in one experiment with Fe/As <100 (strain BoFeN1, initial target concentration of 50 μM As(III)), the remaining As concentration (113.9 μg/L) was significantly above the WHO limit. From these experiments we conclude that microbial immobilization by coprecipitation and/or sorption of As during microbial Fe(II) oxidation removes arsenic from contaminated water at least as efficiently as, if not better than, sorption and/or chemical coprecipitation in sand filters. It is currently unknown, however, whether purely abiotic or perhaps also microbiological processes are involved in these sand filters.

Zoublis and Katsyiannis (*18*) showed that aerobic Fe(II)-oxidizing bacteria, e.g., *Gallionella* and *Leptothrix* species, were also capable of removing more than 90% of As(III) in groundwater by a column-based experiment on an upflow fixbed unit (*18*). Senn and Hemond (*19*) showed that under anoxic conditions, nitrate has a strong influence on As cycling in natural systems, probably by nitrate-dependent Fe(II)-oxidizing bacteria. Thus, biological arsenic coprecipitation may be as important as adsorption to preformed Fe(III) minerals. Generally, it has been found that during chemical coprecipitation, more arsenic can be removed than during adsorption on chemically synthesized Fe(III) minerals (*37*). Fuller and associates (*37*) have determined sorption densities of up to 0.7 mol As(V) per mole Fe in an abiotic coprecipitation experiment with As(V) and ferrihydrite. They also suggested that the presence of adsorbing ions affects crystallite growth. This could lead to a decreased particle size and thus to an increased surface area, precisely what we have shown for the nitrate-dependent Fe(II)-oxidizing strain BoFeN1, where we observed a decreasing iron mineral particle size in the presence of arsenic.

However, not only the As/Fe ratio determined by Fuller et al. in their coprecipitation experiments, but also the As/Fe ratio determined in adsorption experiments (*37*), are 1−2 orders of magnitude higher than the As/Fe ratios we determined in our immobilization experiments, as well as Berg at al. (*29*) determined in their experiments with sand filters. This may be due to different geochemical conditions. For example, our immobilization experiments were conducted at a pH of 7.2 compared to 7.5−9.0 in their experiments. In addition, the presence of 40 μM phosphate in our filtered microbial growth medium possibly had a slight influence on crystallinity and mineral growth. Most importantly, the phosphate present in our medium probably competed with the As for binding/coprecipitation during Fe(III) mineral formation, lowering the amount of As bound to the Fe minerals.

Identity and Crystallinity of Iron Minerals Formed during Fe(II) Oxidation in the Presence of Arsenic and Consequences for As Mobility. The nature and crystal size of the Fe(III) minerals formed during Fe(II) oxidation is expected to influence the amount of arsenic sorbed, since the surface area and thus the number of available surface sites varies for different Fe(III) (hydr)oxides (*22*). Mössbauer analysis showed that strain SW2 forms ferrihydrite in the absence and presence of arsenic whereas strain BoFeN1 produced goethite only in the absence of arsenic and an additional minor Fe(III) phase (nanogoethite, ferrihydrite, or lepidocrocite) in association with goethite in the presence of arsenic. The formation of such additional nanocrystalline Fe(III) phase could explain the overall decrease in iron mineral particle size observed by SEM (Figure 3). Previous studies have reported that the presence of adsorbing ions, especially arsenate and arsenite, affects the rates of crystal growth upon coprecipitation and can lead to a decrease in crystallite size and to a delay in crystal growth (*7, 37, 38*). In our BoFeN1 experiments, such adsorption process could explain the formation of nanocrystalline Fe(III) oxyhydroxides in association with goethite in the presence of arsenic, while only goethite formed in the As-free experiments.

In contrast to ferrihydrite that has a high specific surface area (between 100 and 700 $m^2\ g^{-1}$ determined in different experiments by diverse methods (*22*)), crystalline goethite with larger crystal sizes has generally smaller surface areas (8−200 $m^2\ g^{-1}$) depending on the mineral synthesis procedure, as well as on the analytical method used (*22*). Microbially formed ferrihydrite precipitates, similar to the ones formed by strain SW2, are therefore expected to bind more arsenic to their surface (on a per weight basis) than the goethite precipitates. The low As/Fe molar ratios (0.001−0.017) observed in our biogenic solids are more consistent with As sorption at the surface of goethite and ferrihydrite than with precipitation of a ferric-arsenate phase. Indeed, for As/Fe molar ratios below 0.1, previous studies have shown that the molecular environment of As(V) in the solid phase is the same regardless of whether As(V) is coprecipitated with or adsorbed to ferrihydrite at neutral pH, and corresponds to bidentate inner-sphere complexes at the surface of the ferrihydrite nanoparticles (*38, 39*). Yet, at much higher As/Fe ratios (above 0.5), and at low pH (below 5), incorporation of As in a ferric mineral phase is observed. Under these conditions As(V) forms amorphous mixed Fe(III)−As(V) minerals with a local structure differing from that of

ferrihydrite (*40, 41*) and As(III) forms either amorphous As(III)−Fe(III) mineral phases or crystalline ones as tooeleite (*40*).

However, although our chemistry and SEM results are consistent with As sorption at the surface of the biogenic ferric oxyhydroxides, more detailed investigations, especially X-ray absorption spectroscopy, would be needed to determine the molecular environment of the As in these experiments.

The nature of the mineral forming upon anaerobic microbial Fe(II) oxidation may have important implications for arsenic mobility. Indeed, poorly crystalline Fe(III) minerals, like ferrihydrite, are thermodynamically metastable and have been found to be a good substrate for Fe(III)-reducing bacteria (*42*). In addition, ferrihydrite might transform over time (e.g., into goethite) and release some of the initially bound arsenic (*37, 43*). Although this transformation is strongly decelerated in the presence of arsenate, aging of As(V)-coprecipitated ferrihydrite is known to release As(V) (*38*). In contrast, higher crystalline Fe(III) minerals, such as goethite, have a higher thermodynamic stability and are known to be more difficultly reduced by Fe(III)-reducing bacteria (*44*). Therefore, for efficient immobilization of arsenic, it is more desirable to produce directly crystalline minerals such as goethite via microbial oxidation, since it may retard Fe(II) leaching from the medium even under reducing conditions.

Environmental Implications. Due to As release in anoxic surface water and groundwater, arsenic contamination in water and in the food chain puts millions of people at risk. In this study, we show that anaerobic Fe(II)-oxidizing bacteria can effectively sequester arsenic via the precipitation of ferric oxyhydroxide minerals. Therefore, such microorganisms have the potential to significantly influence the environmental behavior of As, in particular under anoxic conditions in aquifers and rice paddies. However, the presence and involvement of anaerobic Fe(II)-oxidizing bacteria similar to the ones used in the present study (e.g., the nitrate-dependent Fe(II)-oxidizing *Acidovorax* sp. strain BoFeN1) in these systems remains to be determined. Interestingly, a recent report provided evidence for the presence of an *Acidovorax* sp. strain in shallow As-contaminated tube wells in Bangladesh (*45*). Nevertheless, future studies are needed to isolate and identify Fe(II)-oxidizing bacteria from arsenic-contaminated rice paddy fields and/or aquifers.

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Supporting Information Available

Table S1, Text S2, and Figure S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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