Anaerobic Bacterial Immobilization and Removal of Toxic Sb(III) Coupled With Fe(II)/Sb(III) Oxidation and Denitrification

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Antimony (Sb) is a metalloid that is widely used in a variety of industrial products (e.g., flame retardants, small arms ammunition, semiconductors and batteries) and medical treatments (e.g., for leishmaniasis) (Filella et al., 2002a; Vásquez et al., 2006; Wilson et al., 2010; He et al., 2012; Li et al., 2016). The increased mining, smelting and industry have significantly accelerated the

INTRODUCTION

Antimony (Sb) is a metalloid that is widely used in a variety of industrial products (e.g., flame retardants, small arms ammunition, semiconductors and batteries) and medical treatments (e.g., for leishmaniasis) (Filella et al., 2002a; Vásquez et al., 2006; Wilson et al., 2010; He et al., 2012; Li et al., 2016). The increased mining, smelting and industry have significantly accelerated the
emission of Sb into the environment, thereby leading to Sb accumulation in the human food chain (An and Kim, 2009; Hockmann et al., 2014). As suspected carcinogens, antimony species cause damage to many organ systems, such as the lung, heart, liver, and kidney (Sundar and Chakravarty, 2010). The United States Environmental Protection Agency and European Union have listed Sb compounds as priority pollutants (CEC, 1976; USEPA, 1979). Although there is much ongoing work to understand the speciation and relevant solution chemistry of Sb, many basic questions regarding Sb remain unclear, especially how it interacts with co-occurring elements and microbes. In nature, Sb mainly exists in two oxidation states, antimonite [Sb(III)] and antimonate [Sb(V)]. In aqueous environments at a neutral pH, Sb(III) is more prevalent in anoxic environments [as Sb(OH)₃], and Sb(V) is dominated under oxic conditions [as Sb(OH)₅] (Filella et al., 2002b; Hockmann et al., 2014). Sb(III) compounds are much more toxic than those containing Sb(V); thus Sb(III) oxidation, which transforms the toxic Sb(III) to the less toxic Sb(V), has a significant value for bioremediation of Sb-contaminated environments.

In addition to the chemical Sb(III) oxidants (e.g., H₂O₂, iodate, and Fe and Mn oxyhydroxides) (Belzile et al., 2001; Leuz and Johnsona, 2005; Quentel et al., 2006), microorganisms play an important role in Sb(III) oxidation, and this oxidation reaction serves as detoxification process for the microorganisms (Li et al., 2016). Bacteria can oxidize Sb(III) in both oxic and anoxic environments. In recent years, more than 60 aerobic Sb(III) oxidizers have been identified; among them, strains of Pseudomonas, Comamonas, Agrobacterium, and Acinetobacter genera comprise the majority (Li et al., 2013, 2016; Shi et al., 2013). Previously, we clarified the mechanisms of bacterial aerobic Sb(III) oxidation and found that Sb(III) oxidation is a co-metabolism process catalyzed by biotic (the antimonite oxidase AnoA and the arsenite oxidase AioAB) and abiotic (cellular H₂O₂) factors. The antimonite oxidase AnoA was discovered to catalyze Sb(III) oxidation in Agrobacterium tumefaciens GW4 with NADP⁺ as the co-factor (Li et al., 2015). The arsenite oxidase AioAB is composed of a large (AioA) and a small (AioB) subunit. Deletion of aioA partially decreased bacterial Sb(III) oxidation efficiency in A. tumefaciens 5A, while the function of aioA in Sb(III) oxidation in strain GW4 was not obvious (Wang et al., 2015; Li et al., 2017b). In addition, Sb(III) induces bacterial oxidative stress responses and produces substantial H₂O₂, and bacterially produced H₂O₂ acts as an oxidant to oxidize Sb(III) to Sb(V) (Li et al., 2017b). However, the knowledge of bacterial anaerobic Sb(III) oxidation is still limited. So far, only two anaerobic Sb(III) oxidizing strains of Hydrogenophaga and Ensifer have been isolated (Terry et al., 2015; Nguyen et al., 2017); these strains oxidize Sb(III) using nitrate as the electron acceptor, and the detailed mechanisms of these reactions remain unknown.

In mining areas, Sb frequently co-occurs with iron (Fe) and sulfur (S) (Filella et al., 2002a; Shi et al., 2013). Fe is the fourth most abundant element and the most redox-active metal in the Earth’s crust. In nature, Fe exists mainly as particulate ferric [Fe(III)], ferrous [Fe(II)] iron-bearing minerals or dissolved ions (Kappler and Straub, 2005). Fe(III) minerals have a high solubility at strongly alkaline or strongly acidic pH levels, while the solubility of Fe(III) minerals at circumneutral pH are extremely low. In contrast, some Fe(II) minerals are considerably more soluble at neutral pH (Kappler and Straub, 2005). In addition, Fe(II) can be oxidized to Fe(III) minerals by O₂, but it persists in acidic pH even in oxic habitats (Cornell and Schwertmann, 2003). Fe(II) is more stable at neutral or alkaline pH in anoxic environments (Kappler and Straub, 2005). The Fe redox reactions are coupled with carbon, nitrogen, oxygen and sulfur cycles and are involved in important environmental processes such as methane oxidation, aromatic hydrocarbon degradation, heavy metal passivation and immobilization (Vodyanitskii, 2010; Niedźwiecka et al., 2017; Yan et al., 2018; Yuan et al., 2018). Many studies have documented that Fe(II) oxidation is closely associated with the fate of heavy metals in anoxic environments (Vodyanitskii, 2010). It is known that circumneutral pH environments are prevailing on Earth, and the pH of many heavy metal-polluted sites (e.g., mining and shooting areas) are also near neutral (Okkenhaug et al., 2013; Shi et al., 2013). However, in anoxic neutral habitats, only Mn(IV) and nitrite can oxidize Fe(II) chemically (Kappler and Straub, 2005; Melton et al., 2014). It was reported that Fe(II) oxidation is mainly a biotical process mediated by nitrate dependent anaerobic ferrous oxidizing bacteria (NAFOB) under anoxic conditions at neutral pH (Kappler and Straub, 2005; Melton et al., 2014).

NAFOB oxidation of Fe(II) to Fe(III) is coupled with nitrate reduction, which produces Fe(III) minerals and ammonium, nitrogen or denitrification intermediates (Coby et al., 2011; Nordhoff et al., 2017; Schaedler et al., 2018). Thus, NAFOB play an important role in the process of biomineralization and heavy metal passivation, and thereby affect the migration of heavy metals such as chromium (Cr), arsenic (As), and Sb (Senn and Hemond, 2002; Richmond et al., 2004; Vodyanitskii, 2010). Currently, the Fe-N-As coupling mechanism mediated by NAFOB has been found in anoxic environments (Hohmann et al., 2009). Chen et al. (2008) demonstrated that nitrate-dependent Fe(II) oxidation in paddy soil reduced the bioavailability of As through coprecipitation and/or adsorption to Fe(III) minerals. Additionally, the nitrate-dependent, Fe(II)-oxidizing bacteria Acidovorax sp. BoFeN1 isolated from freshwater sediment effectively immobilize more than 96% of soluble As(III) or As(V) in the presence of Fe(II) (Hohmann et al., 2009). However, compared with anaerobic As(III) oxidation, the role of NAFOB in anaerobic Sb(III) oxidation and immobilization remains poorly understood.

The aim of this study was to clarify the coupling mechanism of Fe-N-Sb under anoxic conditions in the facultative-anaerobic, Sb(III)- and Fe(II)-oxidizing strain Sinorhizobium sp. GW3 (Fan et al., 2008). We provide comprehensive evidence showing that Fe(II) and nitrate have a strong influence on the transformation of Sb species and mobility in anoxic environments. This study represents a novel contribution to understanding the Sb biogeochemical cycle and provides a potential bioremediation strategy for anoxic environmental Sb contamination.
MATERIALS AND METHODS

Strains and Genomic Analysis

The facultative anaerobic bacterium Sinorhizobium sp. GW3 (Genome GenBank No. AUSY00000001.1) was mainly used in this study. This strain was isolated by our research group from arsenic contaminated groundwater sediments in Shanyin City, Shanxi Province, China. It could oxidize As(III) aerobically (Fan et al., 2008). The anaerobic As(III) oxidation of strain GW3 was performed using KMnO4 method as described by Cai et al. (2009). For aiaA gene knock-out and complementation study, strain A. tumefaciens GW4 was used (Fan et al., 2008; Wang et al., 2015). Genomic analyses of putative genes involved in denitrification, As(III)/Sb(III) oxidation/resistance, anaerobic selenite-, sulfate-, iron- and chrome- reduction and transportation were conducted through BlastN and BlastP in the genome of Sinorhizobium sp. GW3 on the NCBI website.

Anaerobic Sb(III) Oxidation Using Nitrate as the Electron Acceptor

For aerobic Sb(III) oxidation experiments, bacterial strains were grown in chemically defined medium (CDM) (Weeger et al., 1999) containing 0 or 20 μM K2Sb(C2H5O2)3 [Sb(III)] with shaking at 28°C. Overnight cultures of Sinorhizobium sp. GW3 strain were inoculated into 5 mL of CDM in the presence of 20 μM Sb(III) and incubated at 28°C with 120 rpm shaking. When the OD600 reached 0.5–0.6, the cultures were each inoculated into 100 mL of CDM with 20 μM Sb(III) and different amounts (0, 1, and 5 mM) of KNO3. At designated times, the culture samples were centrifuged (13,400 × g, 5 min) and subsequently filtered (0.22 μm filter) to measure Sb(III) oxidation and denitrification. The Sb(III)/Sb(V) content was measured by HPLC-HG-AFS (Beijing Titan Instruments Co., Ltd., China) (Li et al., 2013), and the concentration of NO3−/NO2− was measured by HPLC (HPLC 2690 series, Waters, MA, United States) according to the method described by Croitoru (2012).

Gene Expression Analyses

To investigate the expression of genes involved in Sb(III) oxidation and resistance under oxic conditions, overnight cultures of strain GW3 were inoculated into 100 mL of CDM with 0 or 100 μM Sb(III) at 28°C with 120 rpm shaking. After 24 h of incubation, bacterial cells were harvested for total RNA extraction. For anaerobic gene expression analysis, bacterial cells were each inoculated into oxygen-free CDM with 0 or 100 μM Sb(III) and incubated at 28°C without shaking. After incubation for 4 days, bacterial cells were harvested and treated with Trizol reagent (Invitrogen).

Total RNA was extracted according to the manufacturer's instructions (Invitrogen). Genomic DNA was removed by treatment with RNase-free DNase I (Takara) at 37°C, and the concentration of RNA was monitored using a spectrophotometer (NanoDrop 2000, Thermo). Reverse transcription was performed with 300 ng of total RNA for each sample using the RevertAid First Strand cDNA Synthesis Kit (Thermo) (Wang et al., 2012). Subsequently, the obtained cDNA was diluted 10-fold and used as a template for qRT-PCR, which was carried out in an ABI VIIA7 system in 0.1 mL Fast Optical 96-well Reaction Plates (ABI). The primers used for qRT-PCR are listed in Supplementary Table S1. Each reaction was replicated three times to estimate error. The 16S rRNA gene of strain GW3 was used as an internal control.

http://www.ncbi.nlm.nih.gov
control. First, standard dilution curves were generated for each pair of primers. Then, qRT-PCR was conducted to obtain Ct values for each sample. The slope (S) of each standard curve was used to calculate the primer amplification efficiency (E) with \[ \log (1 + E) = 1/S. \] The Ct values of target cDNAs were each normalized with the cDNA Ct value of 16S rRNA to generate the ΔCt value of each sample in the control and Sb(III)-treatment groups. Subsequently, the ΔCt values of cDNAs in the control groups were each subtracted from those in the Sb(III) treatment groups. The relative gene expression ratios were analyzed using the \( 2^{-\Delta \Delta CT} \) method (Livak and Schmittgen, 2001). Significance analysis was performed by one-way ANOVA.

To investigate the role of \( aioA \) in anaerobic Sb(III) oxidation, \( \textit{A. tumefaciens} \) GW4 and the corresponding \( aioA \)-deletion and \( aioA \)-complementation strains were used to determine the anaerobic Sb(III) oxidation efficiency. \( \textit{A. tumefaciens} \) strains were each inoculated into 50 mL of CDM containing 1 mM nitrate and 100 \( \mu \)M Sb(III) under anoxic conditions and cultured as described above. At designated times, samples were taken for measuring the Sb(III)/Sb(V) content.

**Effects of Fe(II) on Anaerobic Sb(III) Oxidation and Immobilization in the Presence of Nitrate**

A stock culture of strain GW3 was inoculated into 50 mL of CDM with 1 mM Fe(II), 100 \( \mu \)M Sb(III) and 1 mM nitrate under anoxic conditions. CDM containing 100 \( \mu \)M Sb(III) and 1 mM nitrate was used as a control. All cultures were incubated for up to 10 days at 28°C in the dark without shaking. Changes in Sb(III), Fe(II) and nitrate concentrations were determined as described above. Precipitates from anaerobic Fe(II)- and Sb(III)-oxidizing cultures were collected at the end of the experiment by centrifugation (13,400 \( \times \) g, 5 min) and freeze-dried overnight. The elemental composition of the samples was investigated by energy-dispersive X-ray spectroscopy (EDS) using an Oxford Instruments model Inca X-sight EDS detector, which is coupled with the SEM (FEI Inspect F50). Powder X-ray diffraction (XRD) analysis was conducted using a Bruker D8 Advance X-ray diffractometer with Cu Kα as the X-ray source (40 mA, 20 kV). The elemental chemical status and compositions were analyzed with X-ray photoelectron spectroscopy (XPS) using Al Kα (1486.6 eV) excitation.

**Anaerobic Sb(III) Oxidation by Microbially Produced Fe(III) Minerals**

Bacterial cells were cultured as described above. Stock cultures of strain GW3 were inoculated into 50 mL of CDM containing 1 mM nitrate and 1 mM Fe(II) under anoxic conditions. After 10 days of static cultivation at 28°C in the dark, the orange precipitates produced by strain GW3 were harvested through centrifugation (13,400 \( \times \) g, 5 min); these precipitates included both Fe(III) minerals and bacterial cells. Subsequently, the mixture was resuspended in PBS (pH 7.0) and injected into oxygen-free CDM containing 100 \( \mu \)M Sb(III). Meanwhile, the precipitates were taken immediately, washed three times with PBS, and used for plate counting to detect the bacterial numbers. The bacteria cultured without Fe(II) were used as a control. During cultivation, 100 \( \mu \)L of culture was withdrawn anoxically with a syringe for measuring the Sb(III) content at designated times. To test whether nitrite or Fe(III) could oxidize Sb(III) abiotically, 50 mL of CDM was added to the serum bottles under an atmosphere of \( \text{N}_2 \). After sterilization and sealing, 1 mM \( \text{NaNO}_2 \) or 1 mM FeCl₃ were amended with 100 \( \mu \)M Sb(III) and incubated for up to 10 days at 28°C under anoxic conditions. Samples were regularly taken to monitor the Sb(III)/Sb(V) and \( \text{NO}_3^- \) content.

## RESULTS

### Genomic Analysis of \textit{Sinorhizobium sp. GW3}

Strain GW3 is a facultative anaerobic bacterium which can oxidize As(III) under both oxic and anoxic conditions (Fan et al., 2008; Supplementary Figure S1). The genome of strain GW3 contains putative genes responsible for aerobic As(III) and Sb(III) oxidation [the As(III) oxidase genes \( \textit{aioAB} \), Sb(III) oxidase gene \( \textit{anaA} \) and catalase gene \( \textit{kataA} \) (Supplementary Table S2)]. However, it has no \( \textit{arxA} \) gene, which encodes anaerobic As(III) oxidase (Zargar et al., 2012). The genes involved in As(III) and Sb(III) resistance were also found, such as As(III)/Sb(III) efflux gene \( \textit{acr3} \) and As(V) reductase gene \( \textit{arsC} \) (Rosen, 1995; Supplementary Table S2). These As(III)/Sb(III) oxidation and resistance genes are all single copy. It has been reported that the multicopper oxidase may play a role in bacterial anaerobic Fe(II) oxidation (He et al., 2016). Expectably, we found three multicopper oxidase genes in strain GW3 (Supplementary Table S2). In addition, there are also genes involved in denitrification and sulfate reduction. Interestingly, the strain GW3 genome also harbors genes which might contribute to anaerobic selenite reduction, iron reduction and chromate reduction (Supplementary Table S2), suggesting that strain GW3 may have variable anaerobic functions.

### The Addition of Nitrate Has no Effect on Bacterial Aerobic Sb(III) Oxidation

We first assessed the effect of nitrate addition on bacterial Sb(III) oxidation under oxic conditions. Strain GW3 was inoculated into CDM containing 20 \( \mu \)M Sb(III) and different concentrations (0, 1, and 5 mM) of nitrate, and the culture was aerated by shaking at 28°C. As shown in Figure 1A, strain GW3 could oxidize Sb(III) aerobically; however, there was no significant difference in Sb(III) oxidation rates upon the addition of different concentrations of nitrate (Figure 1A). Nitrate reduction experiments showed that nitrate was only slightly reduced to nitrite regardless of the concentration of nitrate added (Figure 1B). These results suggested that nitrate has no effect on bacterial aerobic Sb(III) oxidation and strain GW3 tends to use \( \text{O}_2 \) as the electron acceptor rather than nitrate under oxic conditions.
Nitrate is the Electron Acceptor in Anaerobic Sb(III) Oxidation

Under anoxic conditions in CDM, strain GW3 could reduce NO$_3^-$ to NO$_2^-$, and the contents of NO$_2^-$ decreased (Supplementary Figure S2A); this suggests that the NO$_2^-$ produced flowed into subsequent denitrification processes to generate N$_2$ or N$_2$O, consistent with the presence of nir, nor, and nos genes in the strain GW3 genome (Supplementary Table S2). Upon addition of Sb(III), the denitrification efficiency increased compared to the efficiency in conditions without Sb(III); this was apparent because the amount of NO$_2^-$ compared to the efficiency in conditions without Sb(III); this was apparent because the amount of NO$_2^-$ was 73% lower than that produced with 1 mM nitrate only (Supplementary Figure S2B). This suggests that the denitrification and Sb(III) oxidation processes enhance electron transfer. Thus, the Sb(III) oxidation rate was significantly increased by the greater amounts of nitrate (Figure 2A). We did not observe any abiotic Sb(III) oxidation by nitrite (Supplementary Figure S3A), and the content of nitrite was constant during 10 days of cultivation (data not shown). Additionally, we tested whether Sb(III) could be oxidized abiotically by FeCl$_3$. The results showed that the Sb(III) content was not decreased and Sb(V) was not observed in the presence of FeCl$_3$ (Supplementary Figure S3B). The results indicated that the intermediate products of denitrification in strain GW3 and FeCl$_3$ could not oxidize Sb(III) abiotically.

Transcription Levels of Genes Involved in Sb(III) Oxidation Under Oxic and Anoxic Conditions

To investigate the potential mechanism of Sb(III) oxidation in strain GW3, the transcript levels of aioA, anoA, and katA were measured under both oxic and anoxic conditions using qRT-PCR. Additionally, we assessed the transcript levels of the As(III)/Sb(III)-resistance genes acr3 and arsC. The original qRT-PCR data and primer efficiency are shown in Supplementary Table S3 and Supplementary Figure S3, respectively. Since the primer amplification efficiencies for 16S rRNA and other genes were similar (∼5%) (Supplementary Figure S4), primer amplification efficiencies were not included in the calculation of the relative gene expression ratios.

The results showed that the transcript levels of anoA, katA, acr3, and arsC all significantly increased upon the addition of Sb(III), while the transcript level of aioA was not increased by Sb(III) under oxic conditions (Figure 3A). These findings hint that the gene products of anoA and the cellular oxidative stress response may substantially contribute to aerobic Sb(III) oxidation in strain GW3, which is consistent with our previous observations in A. tumefaciens GW4 (Li et al., 2015, 2017a,b). In contrast, under anoxic conditions, the addition of Sb(III) had no significant effect on the transcript levels of anoA and katA, while aioA, acr3, and arsC levels were promoted by Sb(III) (Figure 3B). The results suggested that AioA may play a role in bacterial anaerobic Sb(III) oxidation, and Acr3 and ArsC may be involved in Sb(III) resistance under both oxic and anoxic conditions.

To further analyze the role of aioA in anaerobic Sb(III) oxidation, strain A. tumefaciens GW4 and the corresponding aioA-deletion and aioA-complementation strains were used in the anaerobic Sb(III) oxidation experiments. Strain GW4 is closely related to strain GW3 based on the phylogenetic analysis of the 16S rRNA genes (Fan et al., 2008); furthermore, the amino acid sequence similarity of AioA in these two strains is 77%. We found that the oxidation efficiency of GW4-∆aioA was 73% lower than that of wild-type strain GW4, and the complementary strain rescued the oxidation efficiency phenotype (Supplementary Figure S5). This suggests that AioA contributes to bacterial anaerobic Sb(III) oxidation in strain GW4 and may also contribute in the closely related strain GW3.
The Addition of Nitrate Enhanced Anaerobic Fe(II) Oxidation

The ability of strain GW3 to oxidize Fe(II) under anaerobic nitrate-reducing conditions was also tested. The Fe(II) stock culture was prepared anaerobically in an anaerobic chamber and then injected into the culture. Compared with the sterile control, the bacterial Fe(II) oxidation rate increased as the concentration of added nitrate increased (Figure 4A). At the highest concentration of nitrate tested (5 mM nitrate), all of the Fe(II) was consumed within 10 days (Figure 4A), indicating that 5 mM nitrate could accept all electrons from 100 µM Fe(II) oxidation. Correspondingly, the NO$_3^-$ content increased first and subsequently decreased (Figure 4B), consistent with the trend of nitrate reduction during anaerobic Sb(III) oxidation (Figure 2B). The addition of nitrate significantly stimulates bacterial Fe(II) oxidation, indicating that strain GW3 is also a nitrate-dependent, anaerobic ferrous-oxidizing bacterium. Additionally, orange precipitates were observed during cultivation (Figure 4B), indicating that Fe-containing minerals were formed during the Fe(II) oxidation process.

Fe(II) Stimulated Anaerobic Sb(III) Oxidation and Immobilization in the Presence of Nitrate

We assessed the effects of Fe(II) on bacterial anaerobic Sb(III) oxidation in CDM containing 100 µM Sb(III) and 1 mM nitrate with or without 1 mM Fe(II) under anoxic conditions. Sb(III) oxidation experiments showed that up to approximately 15.9 µM
Sb(III) was reduced by the end of 10 days of cultivation in the presence of Sb(III) and nitrate, and 82.4 µM Sb(III) was reduced when Fe(II) was added (Figure 5A); Fe(II) significantly increased bacterial Sb(III) oxidation efficiency by 66.5%. Interestingly, the Sb(V) content increased first and subsequently decreased, and orange precipitates were observed in the culture with Fe(II). The increase in Sb(V) concentration did not compensate for the decrease in Sb(III) concentration, suggesting that Sb may adsorb or coprecipitate on the Fe-containing minerals. The Fe(II) oxidation experiment showed that up to approximately 0.75 mM Fe(II) was consumed by the end of 10 days of cultivation, and this value was significantly lower than the sterile control (Figure 5B).

As the electron acceptor, nitrate was reduced to nitrite and other denitrification intermediates. Fe(II) addition accelerated the consumption of nitrate, since Fe(II) oxidation also needs nitrate as an electron acceptor (Figure 5C).

To investigate the chemical components of the precipitate, XRD analysis was conducted. However, we did not identify any characteristic peaks, indicating that the secondary mineral formed by strain GW3 was poorly crystalline (Supplementary Figure S6A). Thus, XPS analysis was further employed to determine the valence state of elements. The results showed that the valence state of iron mineral produced by strain GW3 in the presence of Fe(II) and nitrate was positive trivalent (Fe2p peak at 711.25 eV; Supplementary Figure S6B), indicating that strain GW3 could produce Fe(III)-containing minerals during anaerobic Fe(II) oxidation. In the presence of Fe(II), Sb(III) and nitrate together, the XPS spectra showed Fe2p and Sb3p peaks at 711.05 (Supplementary Figure S6C) and 531.71 eV (Supplementary Figure S6D), respectively, which were attributed to Fe(III) and Sb(V), respectively (Wu et al., 2018). These observations indicated that the removed Sb(III) was its oxidized product Sb(V) on the Fe(III)-containing minerals.

Microbiologically Produced Fe(III)-Containing Minerals Efficiently Oxidized and Immobilized Sb(III)

To identify the role of microbially produced Fe(III)-containing minerals in anoxic Sb(III) oxidation, the orange precipitates were harvested after 10 days of cultivation and then added to CDM in the presence of 100 µM Sb(III) under anoxic conditions. Since the precipitates need to be maintained in their original state with their original chemical properties, no extra steps were taken to remove cells. To determine whether there were living bacteria in the Fe(III)-containing mineral precipitates, a plate counting assay was employed to compare the number of bacteria in the precipitates and the control cells after 10 days of cultivation. The results showed that there was no difference in the cell counts between the precipitates and the cultures with only cells, and no Sb(III) oxidation was observed in the supernatant of the culture (data not shown).

To compare the contribution of biotic and abiotic Sb(III) oxidation, bacterial cells cultured without Fe(II) were also collected as a control. Compared with the control cells without precipitates, the amount of Sb(III) significantly decreased in the culture with microbially produced Fe(III)-containing precipitates (Figure 6A). However, the increased Sb(III) oxidation efficiency is due to the function of both Fe(III)-containing minerals (abiotic) and bacterial cells (biotic). As shown in Figure 6A, after 10 days of cultivation, the Sb(III) oxidation efficiency of bacterial cells was 18% (the biotic effort), while the Sb(III) oxidation efficiency of the precipitates which included Fe(III)-containing minerals and bacterial cells was 89%. Thus, the contribution of abiotic Sb(III) oxidation in this system was approximately 71%.

The precipitates were harvested for SEM and EDS analyses. Consistent with the previous results, the precipitates contained Fe and Sb (Figure 6B), indicating that the microbially produced Fe(III)-containing minerals greatly contributed to the increased oxidation of Sb(III).
FIGURE 5 | The addition of FeCl$_2$ enhanced bacterial anaerobic Sb(III) oxidation and immobilization in the present of nitrate. (A) Sb(III) oxidation curves. (B) Fe(II) oxidation curves. A sterile culture without inoculation of strain GW3 was used as a control. (C) Nitrate consumption curves. Error bars represent standard deviations of the means from three independent experiments.

anaerobic Sb(III) oxidation and the immobilization of Sb(V). As shown in Figure 6C, the surface of the precipitate was porous, which provided a sorption phase for Sb(V) immobilization. Additionally, Sb(III) could not be oxidized abiotically by FeCl$_3$, indicating that the artificial Fe(III) solution without secondary mineral formation from microbes has no functional roles in Sb(III) oxidation.

DISCUSSION

Sb(III) oxidation by oxygen is naturally very slow (Leuz and Johnsona, 2005), and Sb(III) can readily precipitate with sulfide or be strongly adsorbed by Fe(III) hydroxides at neutral pH under anoxic environments (Lai et al., 2016). Thus, microorganisms play an important role in Sb(III) oxidation (Li et al., 2016). Compared to the increasing knowledge of bacterial aerobic Sb(III) oxidation, there are only two known bacteria capable of anaerobic Sb(III) oxidation to date (Terry et al., 2015; Nguyen et al., 2017). We observed that during anaerobic cultivation, the bacterial denitrification efficiency was enhanced by Sb(III) since Sb(III) provides more electrons besides the electron donors in the CDM (such as the sodium lactate); meanwhile, the anaerobic Sb(III) oxidation efficiency also increased significantly as the concentration of nitrate increased. Thus, in the presence of nitrate and Sb(III) oxidizing bacteria, the rate of Sb(III) oxidation is enhanced greatly under anoxic environments and thereby accelerates the biogeochemical cycle of Sb.

We previously elucidated the co-metabolism mechanism of bacterial aerobic Sb(III) oxidation (Wang et al., 2015; Li et al., 2016, 2017a,b). However, there is no evidence describing the anaerobic Sb(III) oxidation mechanism. In this study, based on the qRT-PCR and gene knock-out and complementation analyses, we show that the bacterial Sb(III) oxidation mechanisms differ in oxic and anoxic conditions. The AnoA catalysis and active oxygen generation tend to occur under oxic conditions. Under anoxic conditions, AioA plays a role in anaerobic bacterial Sb(III) oxidation. Additionally, Acr3 and ArsC are involved in Sb(III) resistance under both oxic and anoxic conditions.

Interestingly, Sinorhizobium sp. GW3 is also a nitrate-dependent Fe(II)-oxidizing strain. The addition of nitrate significantly increased bacterial anaerobic Fe(II) oxidation and produced Fe(III)-containing precipitate, which would provide a sorption phase for metal(loid)s. These findings are consistent with previous studies of Acidovorax sp. ST3 isolated from As-contaminated paddy soils (Zhang et al., 2017). As expected, we confirmed that Sb(III) could be efficiently oxidized and immobilized by the microbially produced Fe(III)-containing precipitate. Thus, we propose that the coupling mechanism of Fe-N-Sb driven by strain GW3 under anoxic conditions is a combination of biotic and abiotic redox reactions, and abiotic Sb(III) oxidation is more efficient than biotic Sb(III) oxidation (Figure 6D). This is the first demonstration of the coupling mechanism of Fe-N-Sb driven by a nitrate-dependent Fe(II)- and Sb(III)-oxidizing bacterium. Such processes explain why the anaerobic Sb(III) oxidation with Fe(II), Sb(III) and nitrate was more efficient than that with Sb(III) and nitrate only. Furthermore, the secondary mineral formed by strain GW3 was poorly crystalline, which is consistent with the Fe(III)- and As(V)-containing minerals produced by the thermoacidophilic Fe(II)-oxidizing archaeon Acidianus brierleyi (Okibe et al., 2013). The chemical composition and the adsorption efficiency of Fe(III)- and Sb(V)-containing minerals produced by strain GW3 may be further studied by changing the initial concentration of Fe(II), Sb(III) and nitrate.

Analysis of the facultative anaerobic, nitrate-dependent, Sb(III)-oxidizing strain provides an important environmental perspective for understanding the global Sb biogeochemical cycle under both oxic and anoxic environments. It may be a useful way to decrease Sb toxicity in anoxic soils and
FIGURE 6 | Microbially produced Fe(III)-containing minerals enhanced Sb(III) oxidation and immobilization (A–C), and a proposed model of the Fe-N-Sb coupling mechanism mediated by strain GW3 (D). (A) Sb(III) oxidation curves of the microbially produced Fe(III)-containing minerals. Strain GW3 was anaerobically inoculated into CDM containing 1 mM KNO$_3$ with or without 1 mM Fe(II). After 10 days of cultivation, the precipitate was harvested and added to fresh CDM in the presence of 100 µM Sb(III). Meanwhile, the precipitate was washed three times with PBS and then used for plate counting to detect the number of bacteria. Error bars represent standard deviations of the means from three independent experiments. (B,C) EDS analysis and SEM image of the Fe(III)-containing minerals after 10 days of cultivation under the conditions described in (A). The EDS peaks for Al, Mg, and Pt may come from aluminum alloy on the sample table and the spraying Pt treatment, which is used to increase electrical conductivity.

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**SUPPLEMENTARY MATERIAL**

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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