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Proteomics and Genetics for Identification of a Bacterial Antimonite Oxidase in *Agrobacterium tumefaciens*

Jingxin Li,[†,∥] Qian Wang,[†,∥] Mingshun Li,[†] Birong Yang,[†] Manman Shi,[†] Wei Guo,[†] Timothy R. McDermott,[‡] Christopher Rensing,[§] and Gejiao Wang*[,†]

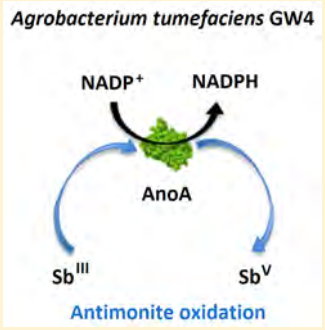
[†]State Key Laboratory of Agricultural Microbiology, College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, P. R. China

[‡]Department of Land Resources and Environmental Sciences, Montana State University, Bozeman, Montana 59717, United States

[§]Department of Plant and Environmental Sciences, University of Copenhagen, Frederiksberg, DK-1871, Denmark

Supporting Information

ABSTRACT: Antimony (Sb) and its compounds are listed by the United States Environmental Protection Agency (USEPA, 1979) and the European Union (CEC, 1976) as a priority pollutant. Microbial redox transformations are presumed to be an important part of antimony cycling in nature; however, regulation of these processes and the enzymology involved are unknown. In this study, comparative proteomics and reverse transcriptase-PCR analysis of Sb(III)-oxidizing bacterium *Agrobacterium tumefaciens* GW4 revealed an oxidoreductase (*anoA*) is widely distributed in microorganisms, including at least some documented to be able to oxidize Sb(III). Deletion of the *anoA* gene reduced Sb(III) resistance and decreased Sb(III) oxidation by ∼27%, whereas the *anoA* complemented strain was similar to the wild type GW4 and a GW4 *anoA* overexpressing strain increased Sb(III) oxidation by ∼34%. Addition of Sb(III) up-regulated *anoA* expression and cloning *anoA* to *Escherichia coli* demonstrated direct transferability of this activity. A His-tag purified AnoA was found to require $NADP^+$ as cofactor, and exhibited a K_m for Sb(III) of 64 ± 10 μM and a V_{max} of 150 ± 7 nmol min^{-1} mg^{-1}. This study contributes important initial steps toward a mechanistic understanding of microbe-antimony interactions and enhances our understanding of how microorganisms participate in antimony biogeochemical cycling in nature.



INTRODUCTION

Antimony (Sb) belongs to Group 15 of the Periodic Table of the Elements and shares some chemical properties with arsenic (As).[1] Sb is recognized as a priority pollutant by United States Environmental Protection Agency and the European Union.[2,3] It is prevalent in the environment at low levels but in contaminated sites (e.g., mining) it raises significant toxicology issues (drinking water standard 6 ppb).[2,4] Consequently, there is growing interest in the ecology of antimony-metabolizing microbes. In nature, Sb exists in four oxidation states: (−III, 0, III, and V), with antimonite (Sb(III)) and antimonate (Sb(V)) being the most common in environmental and biological samples.[4] Sb(III) compounds are much more toxic than Sb(V) and so consequently transformation of Sb(III) to Sb(V) is an important environmental Sb bioremediation strategy to consider. Since abiotic Sb(III) oxidation is relatively slow,[5] a better understanding of potential biological oxidants could be of significant value in this regard. At present, little is known about the mechanisms and minimum genetic requirements that control or contribute to microbial Sb redox reactions, and thus there is a need to identify, characterize, and understand enzymes that control microbial Sb(III) oxidation.

Regarding microbe−Sb interactions, it is known that the glycerol transporter GlpF is involved in Sb(III) uptake in *Escherichia coli* and the GlpF homologue, Fps1p, plays a similar role in *Saccharomyces cerevisiae*.[6,7] Because of its molecular similarity to arsenite (As(III)), Sb(III) extrusion occurs via the As(III) efflux proteins ArsB or Acr3,[7] and Sb(III) methylation can produce organoantimonials in *Clostridium* sp., *Methanobacterium* sp., and *Flavobacterium* sp.[8−10] Potential Sb(V) uptake mechanisms remain unknown, but recently it was reported that microbiological reduction of Sb(V) in anoxic freshwater sediments is enhanced by acetate or lactate, though the organism(s) involved were not identified.[11] Also recently, a Sb(V) reducing bacterium, *Bacillus* sp. MLFW-2, was isolated and shown to grow using the energy generated from anaerobic Sb(V) reduction.[12]

At present, nearly 50 Sb(III)-oxidizing isolates have been reported, primarily being isolated from heavy metal rich mining environments.[13−18] Some of these Sb(III)-oxidizing bacteria were found to also oxidize As(III) (*Agrobacterium tumefaciens* 5A[15]), while some were not.[16,18] Recently, the arsenite oxidase AioBA responsible for As(III) oxidation in *A. tumefaciens* 5A was reported to also function as a Sb(III) oxidase.[19] However, since the disruption of *aioA* caused only about 25% decrease of

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the Sb(III) oxidation rate, presumably there are other enzymes involved in bacterial Sb(III) oxidation.

Here we identify and characterize a gene cloned from *Agrobacterium tumefaciens* GW4 that we refer to *anoA* (**an**timonite **o**xidase). Based on proteomics-based gene identification, gene mutation, expression data and kinetics, we proposed AnoA as a Sb(III) oxidase. The deletion of *anoA* reduced both Sb(III) resistance and the rate of Sb(III) oxidation. Sb(III) induced the expression of *anoA* and increased AnoA activity, and overexpression of *anoA* in the wild type strain increased the rate of Sb(III) oxidation. Kinetics studies showed AnoA could oxidize Sb(III) in vitro using $NADP^+$ as cofactor, and transfer of *anoA* to *Escherichia coli* corresponded to increased Sb(III) oxidation. This study represents a substantial and novel contribution to our understanding of the genetics and enzymology of microbial Sb(III) oxidase, providing a potential remediation tool for environmental antimony contamination and an important step toward understanding microbe-Sb interactions in general.

MATERIALS AND MMETHODS

Strains and Culture Conditions. Bacterial strains and plasmids used in this study are listed in Supporting Information Table S1. *A. tumefaciens* strains were grown in CDM medium at 28 °C with 0 or 50 μM $K_2Sb_2(C_4H_2O_6)_2$ (Sb(III)).[20] *E. coli* strains were cultured at 37 °C in Luria−Bertani medium.[21] When needed, ampicillin (Amp, 100 mg/mL), kanamycin (Kan, 50 mg/mL), tetracycline (Tet, 5 mg/mL), or gentamicin (Gen, 50 mg/mL) were added.

Proteomics. Mid exponential phase cells (32 h, Figure 2), grown in CDM medium with or without addition of 50 μM Sb(III), were collected by centrifugation (13 400*g*, 10 min, at 4 °C) and washed three times with ice-cold PBS buffer (pH 7.4). The cells were resuspended in 0.5 mL lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 65 mM DTT) with addition of a protease inhibitor cocktail (Amerso, U.S.), then treated by ultrasonication for 5 min. DNase I and RNaseA were added to the lysate at a final concentration of 1 and 0.25 mg/mL, respectively. After incubating for 1 h at 28 °C, protein extracts were obtained by centrifugation (13 400*g*, 10 min, 4 °C) to remove cell debris. Protein concentrations were determined by the Coomassie Brilliant Blue method using BSA as standard.[22] A volume equivalent to 500 μg of protein extracts were further purified using the ReadyPrep 2-D Clean-up Kit (Bio-Rad Laboratories, Philadelphia, PA). Then the concentration of the purified protein samples were determined again and the samples were used for two-dimensional gel electrophoresis.

Purified protein samples (100 μg) were mixed with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT, 0.5% IPG buffer pH 4−7 and 0.002% bromophenol blue) to a total volume of 300 μL per immobiline dry strip. The samples were run on isoelectric focusing (IEF) IPG-strip gels (pH 4−7, 18 cm, GE), which were first loaded by passive rehydration for 4 h and then by active rehydration at 50 V for 12 h. After IEF, the IPG strips were first balanced in an equilibration buffer (6 M urea, 2% SDS, and 50 mM Tris-HCl, pH 8.8, 20% glycerol) with the addition of 1% DTT (w/v) for 15 min. Then the strips were balanced again using 4% iodacetamide (IAA) with the same equilibration buffer for 15 min. The equilibrated strips were inserted on the top of a 12% SDS separating polyacrylamide gel and covered with 0.5% (w/v) low melting point agarose prepared with the same SDS separation buffer but containing trace amount of bromophenol blue. The gels were run in a Dodeca Cell system (Bio-Rad) at a constant current of 20 mA for 1 h, and then transferred to 40 mA until the marker dye reached the bottom of the gels. After electrophoresis, the gels were stained with silver nitrate according to the protocol of Yan et al.[23] The images of the gels were scanned by a GS-800 calibrated densitometer (Bio-Rad) and analyzed with PDQuest software (version 8.0, Bio-Rad). All of the gels in the MatchSet were normalized by total quantity in valid spots. Proteins were considered differentially expressed when the average values exceeded the 2-fold threshold with a p-value <0.05. Three replicate gels were analyzed for each sample.

Protein spots of interest were manually excised from gels, washed twice with deionized water and destained three times each in 50 μL destaining solution (30 mmol/L $K_3Fe(CN)_6$ and 100 mmol/L $Na_2S_2O_3$). The distained gel fragments were dehydrated with ACN and vacuum-dried for 10 min. Gel pieces were digested with 2.5−10 ng/μL trypsin (Promega, sequencing grade) and incubated at 37 °C overnight. The enzymatic hydrolysates were transferred to new 1.5 mL tubes, while the residues were sonicated on ice, with 100 μL 60% ACN added and incubated for 15 min, and then mixed with the enzymatic hydrolysates.[24] The mixtures were vacuum-dried for MALDI-TOF-MS analysis.

All MALDI-TOF-MS measurements were performed on a Bruker traflex III TOF/TOF (Bruker Daltonik GmbH, Bremen, Germany). The vacuum-dried protein samples redissolved by 20% acetonitrile were deposited on the SCOUTMTP MALDI target chip and 0.5 μL of the matrix solution (4 mg/mL CHCA in 70% ACN and 0.1% TFA).[25] Proteins were identified by peptide mass fingerprinting (PMF) through a Bruker Ultraflex automated data analysis software and searched with a local MASCOT search engine (Matrix Science, London, UK).

Quantitative RT-PCR Analysis. Bacterial cells used for RNA isolation were harvested at the same time as for the protein extracting preparation described above. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions (Invitrogen, Grand Island, NY). The RNA samples were treated with RNase-free DNase I (Takara) to remove genomic DNA contamination and then reactions were terminated by addition of 50 mM EDTA at 80 °C for 5 min. The quality and quantity of the RNA were assessed by spectrophotometer (NanoDrop 2000, Thermo). Reverse transcription was performed by RevertAid First Strand cDNA Synthesis Kit (Thermo) with 300 ng total RNA for each sample. In addition, the obtained cDNA was diluted 10-fold for further real-time RT-PCR analysis with SYBR Green Realtime PCR Master Mix (Toyobo).[26] Primers used for the reactions are listed in Supporting Information Table S2. Quantitative RT-PCR was performed by ABI VIIA7 in 0.1 mL Fast Optical 96-well Reaction Plate (ABI) and three technical and biological replicates were established for each reaction. Gene expression was normalized by ΔΔCT analysis with an iQ5 Real-Time PCR Detection System (Bio-Rad, U.S.). The 16S rRNA gene was used as reference in the calculations. In addition, the phylogenetic tree was constructed based on the amino acid sequence of AnoA using the neighbor-joining distance method with the mega 6.0 software,[27] and the reliability of the inferred tree was tested by 500 bootstrap.

Construction of Δ*anoA* Mutant, Complementation and Overexpression in GW4. An in-frame deletion in *anoA* was constructed using crossover PCR.[28] The primers used for the construction of the deletion are listed in Supporting

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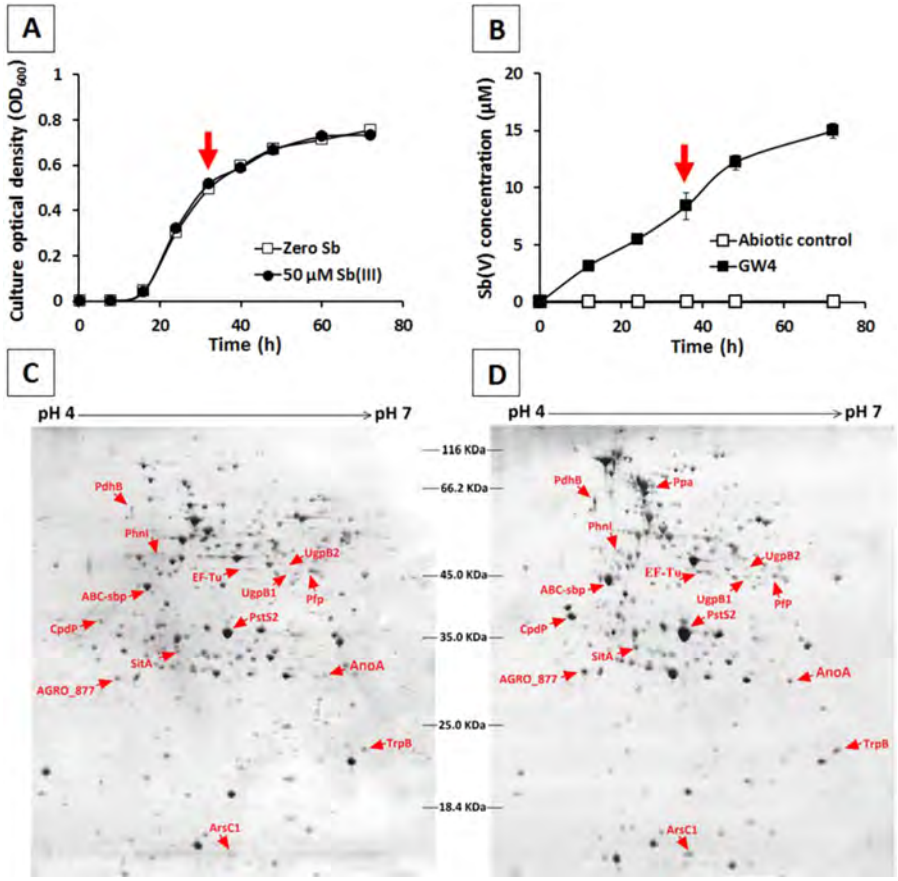

Figure 1. GW4 growth, Sb(III) oxidation and proteomics. (A) Growth profile in the presence and absence of 50 mM Sb(III). (B) Sb(III) oxidation profile; arrow indicates sampling time for proteins used for 2-D electrophoresis detection. (C) and (D) gel images 2-D electrophoresis of total proteins in GW4 grown without (C) or with (D) Sb(III), respectively. Arrow heads in (C) and (D) indicate proteins with differential expression.

Information Table S2. The PCR products were cloned into *BamH*I and *Xba*I double digested pJQ200SK resulting in pJQ-*anoA*, which was then mobilized into strain GW4 via conjugation with *E. coli* S17-1. Single-crossover mutants were screened on CDM agar containing 20% sucrose, selecting for sucrose resistance resulting from resolution of the single crossover.[29] Sucrose^{R} and Gen^{Sen} transconjugants were then screened using PCR to verify the *anoA* deletion.

For Δ*anoA* complementation, the complete *anoA* coding region was PCR-cloned as a *Pst*I-*BamH*I fragment into pCPP30 and then conjugated into GW4-Δ*anoA* via *E. coli* S17-1 and named GW4-Δ*anoA*-C. An *anoA* overexpressing strain GW4 (GW4::*anoA*) was constructed by transformation with plasmid pCPP30-*anoA* into strain GW4. The mutant strain and the complementary strain were further verified by PCR using primers ManoA-1/ManoA-4 and ManoA-F/ManoA-R (Supporting Information Table S2). The *anoA* overexpressing strain was verified by a double digest of the plasmid pCPP30-*anoA*.

Growth, Sb(III) Oxidation and Sensitivity Assays. Strains GW4, GW4-Δ*anoA*, GW4-Δ*anoA*-C and GW4::*anoA* were each inoculated into 5 mL CDM with the addition of 50 μM Sb(III) and incubated at 28 °C with 120 rpm shaking. When OD_{600} reached 0.5−0.6, the strains were each inoculated into 100 mL CDM with presence or absence of 50 μM Sb(III), respectively. At designated times, culture samples were taken for measuring OD_{600} by spectrophotometry (DU800, Beckman). At the same time, the samples were centrifuged (13 400*g*), and the supernatants were used to test the Sb(III)/Sb(V) content by HPLC-HG-AFS (Beijing Titan Instruments Co., Ltd.). For the Sb(III) sensitivity assays, overnight cultures of GW4, GW4-Δ*anoA* and GW4-Δ*anoA*-C were inoculated into 5 mL CDM medium (same starting OD_{600} value) containing various concentrations of Sb(III). Cultures were incubated for 3 days, during which time culture samples were periodically taken for viable plate counts.

***anoA*::*lacZ* Reporter Assays.** The *anoA* promoter region was amplified by PCR using primers PRanoA-F/PRanoA-R (Supporting Informtion Table S2). The PCR amplicon was then directionally cloned into *lacZ* reporter plasmid pLSPkt2lacZ (Supporting Information Table S1) after double digestion by *Eco*RI and *BamH*I and subsequent ligation. The resulting plasmid pLSP-P*anoA* was introduced in *A. tumefaciens* GW4 by conjugation via *E. coli* S17-1. After 24 h of cultivation in 50 mL CDM at 28 °C, 0, 50, 150, or 200 μM Sb(III) or As(III) were each added to the cultures, respectively. In another batch of cultures with the same cultivation condition, 0, 0.5%, 1% or 2% (v/v) H_2O_2 were each added to the cultures, respectively. In both treatments, cells were harvested after 2 h cultivation and β-galactosidase assays were performed as described by Miller.[30]

Overexpression and Purification of AnoA. The complete *anoA* coding region, including the ATG start was PCR-cloned (primers listed in Supporting Information Table S2) as a *Eco*RI-*Hind*III fragment into double digested pET-28a(+) (Novegen) and then transformed into *E. coli* BL21 Star (DE3) pLysS (Invitrogen, Supporting Information Table S1).

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Table 1. Message of the Proteins with Different Expression Ratio in Proteomics[a]

gene name	protein name	PI	MW	C.I%	up-regulated ratio zero Sb(III):50 μM Sb(III)
Cellular Processes and Signaling					
arsC1	arsenate reductase	5.2	16 111.4	100	4.7:13.8
arsC2	arsenate reductase	5.4	18 903.3	100	1.7:8.0
ohr	organic hydroperoxide resistance protein	4.93	14 770.7	96.21	1.8:5.8
EF-Tu	dehydrogenase	5.09	41 769.3	100	0:6.2
Energy Production and Conversion					
pdhe1-b	pyruvate dehydrogenase E1 component beta subunit	4.75	50 676.9	100	6.3:16.9
Carbohydrate Transport and Metabolism					
pfp	pyrophosphate fructose 6-phosphate 1-phosphotransferase	5.57	43474	100	5.1:14.3
ugpB1	periplasmic glycerol-3-phosphate-binding protein	6.79	47 461.8	100	0:5.3
ugpB2	periplasmic glycerol-3-phosphate-binding protein	6.49	49 967.8	100	0:8.7
Inorganic Ion Transport and Metabolism					
anoA	oxidoreductase	5.62	30469.7	100	4.2:17.1
pstS2	Phosphate binding protein	5.38	36341.7	100	194.0:392.6
Lipid Transport and Metabolism					
ABC-sbp	ABC transporter, substrate binding protein	5.17	39193	99.61	58.6:155.6
phnI	Putative enzyme of phosphonate metabolism	4.84	40564.4	100	1:4.1
sitA	Manganese ABC transporter, periplasmic-binding protein	5.57	32262.6	100	6.9:14.7
Ge Neral Function Prediction Only					
ppa	Putative phosphatase	5	69785.1	100	0:11.2
phnM	Metal-dependent hydrolase involved in phosphonate metabolism	5.32	41455	100	1.5:4.2
trpB	Trp repressor binding protein	5.84	20985.6	100	5.3:20.7
cpdP	3′,5′-cyclic-nucleotide phosphodiesterase	4.56	32433.1	99.76	1.4:36.3
Function Unknown					
AGRO_877	hypothetical protein AGRO_4746	4.61	14856.5	100	10.8:26.9
AGRO_5099	hypothetical protein AGRO_5099	4.98	49046.9	99.44	8.8:26.8
AGRO_4594	hypothetical protein AGRO_4594	4.91	69684.9	100	10.3:76.8
AGRO_2314	hypothetical protein AGRO_2314	5.46	15989.5	100	11.7:44.9

[a]The up-regulated ratio was calculated with expression of the proteins, and the expression of PhnI with zero-Sb condition was used as standard.

AnoA was overexpressed by adding 0.2 mM IPTG at an OD_{600} of 0.4 and further culturing for 2 h at 28 °C, then cells were harvested by centrifugation (13,400g for 10 min at 4 °C). After washing twice by 50 mM Tris-HCl (pH 8.0), the pellets were lysed via sonication on ice for 5 min. The soluble supernatant was mixed with 1 mL pre-equilibrated Ni-NTA His Bind Resins (7sea Biotech) and gently agitated at 4 °C for 2 h. The resin was transferred into a 10 mL gravity-flow column and washed by 3 mL Tris-HCl with 60 mM imidazole to elute the miscellaneous proteins. The protein was eluted in 1 mL Tris-HCl with 100 mmol/L imidazole and the eluted fractions were detected with SDS-PAGE.[31] The quality and quantity of the protein were assessed by the spectrophotometer (NanoDrop 2000, Thermo) and SDS-PAGE.

Sb(III) Kinetics. To measure AnoA Sb(III) oxidation activity in vitro, the coenzyme requirements were determined first. No AnoA activity was detected with NAD^+, whereas $NADP^+$ was actively reduced when Sb(III) was added to the reaction. $NADP^+$ was used along to test the oxidize Sb(III) and with the addition of AnoA. For kinetics studies, 3 μmol AnoA was incubated with excessive Sb(III) (5 mM) in Tris-HCl (pH 7.5) and then $NADP^+$ added at 0, 1, 5, 10, 20, 50, 100, or 200 μM to initiate the reaction. Change in absorbance (340 nm) was tracked during the 20 s reactions to estimate enzyme velocity, with the catalytic activity then expressed as nmol min^{-1} mg^{-1}.[32] For estimating Sb(III) kinetics, reactions were oversaturated for $NADP^+$ (200 μM), with 3 μmol AnoA incubated with 0, 50, 100, 150, 200, or 250 μM Sb(III) in Tris-HCl (pH 7.5) at 28 °C for 20s, at which time samples were injected immediately into a HPLC-HG-AFS (Beijing Titan Instruments Co., Ltd.) to quantify Sb(V). The AnoA activity was expressed as nmol min^{-1} mg^{-1}. Michaelis constants K_m and V_{max} were determined using the GraphPad Prism 5 graphing package.

Sb(III) Oxidation in *E. coli*. The complete *anoA* coding region was PCR-cloned as an *EcoRI-Hind*III fragment into pCPP30 and then transformed into *E. coli* S17-1. Strain *E. coli* S17-1 (pCPP30-*anoA*) was inoculated in LB medium and incubated at 37 °C with 120 rpm shaking. When the OD_{600} reached 0.4−0.5, cells were harvested by centrifugation (5000*g*, 10 min, room temperature). After washing twice in PBS buffer (pH 7.4), cells with a final OD_{600} of 0.4 were inoculated into 100 mL CDM with the addition of 10 μM Sb(III) and 0.02% yeast extract.[33] *E. coli* growth in CDM minimal medium is slow and so for convenience, assays were conducted at a high beginning cell density (OD_{600} = 0.4) to achieve reasonable whole cell activity spanning a few hours. Culture samples were taken at 0.5 h intervals for testing of both OD_{600} and Sb species in the culture fluids. Strains *E. coli* S17-1 (pCPP30) and *E. coli* S17-1 were used as negative controls.

RESULTS

Proteomic Analysis of *A. tumefaciens* Strain GW4. *Agrobacterium tumefaciens* GW4 is an Sb(III)-oxidizing strain.[34,35] To investigate the suitable time point to study the proteins potentially involved with the Sb(III) oxidation, GW4 was inoculated into CDM medium with or without the addition of 50 μM Sb(III). At this concentration, Sb(III) had no effect on growth (Figure 1A) and was oxidized in a linear fashion

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(Figure 1B). At the midexponential phase (32 h), Sb(III) oxidation was continuous and the protein concentration was sufficient for further analysis (Figure 1A,B). Thus, 32 h was determined to be an appropriate time point for proteomic analysis.

Based on a genomic analysis of strain GW4, most of the proteins were expected in the pI range of 4−7 and molecular weight range of 18.4−116 kDa (data not shown). Thus, we focused analysis on proteins in this pI range in 12% PAGE gels. Example 2-DE gel patterns are provided in Figure 1 C−D. The correlation coefficients of triplicate gels Sb(III)-treated and control cells were 0.8 and 0.76, respectively, indicating good reproducibility. A total of 228 protein spots were identified in the gels comparing the proteomes of ± Sb(III) cells (Figure 1 C−D). Thirty-eight stable spots exhibiting at least 2-fold change were chosen for MALDI-TOF-MS analysis, 21 of which were successfully identified by the MASCOT Peptide Mass Fingerprinting (PMF) search (Table 1). All 21 proteins were up-regulated in response to Sb(III) (Table 1). As analyzed by KEGG pathway assignments, they were distributed in cellular processes and signaling, energy production and conversion, carbohydrate transport and metabolism, inorganic ion transport and metabolism, lipid transport and metabolism, and general function predictions. An additional four proteins were of unknown function (Table 1).

Expression of Microbe-Sb Interaction Related Genes. Among the 21 proteins up-regulated by Sb(III), we focused additional attention on a subset of proteins: (i) the ArsC proteins are known to be responsive to As(III) and Sb(III) and thus serve as positive controls;[36] (ii) PstS2 is involved in phosphate metabolism and is linked to the regulation of As(III) oxidation;[37] and (iii) the UgpB1, UgpB2, and PdhB are associated with carbon and energy metabolism (Table 1). We were also interested in AnoA because it was the only protein in this group that was annotated as an oxidoreductase having a wide range of substrates and thus potentially involved in Sb(III) oxidation.[38,39] The BlastP results indicated that the protein was a member of the short-chain dehydrogenase/reductase (SDR), and also predicted that the protein contained an $NADP^+$ binding site.

In the 2-D gels, with the addition of Sb(III), ArsC1, ArsC2, AnoA, PstS2, UgpB1, UgpB2, and PdhB showed 2.9, 4.7, 4.1, 2.0, 5.3, 8.7, and 2.7 folds up-regulation, respectively (Figure 1, Table 1). The Reverse transcriptase assays verified that expression of the encoding genes was almost consistent with the proteomic data. Compared to the zero-Sb control growth condition, *arsC1* and *arsC2* were up-regulated by more than 40-fold, whereas *anoA*, *pstS2*, *ugb1*, *ugb2*, and *pdhB* were all up-regulated by about 2-fold (Figure 2). Though the response of these genes to Sb(III) differed, their positive transcriptional response was consistent with their proteomic representation.

Identifying a Candidate Sb(III) Oxidase. Subsequent efforts focused on *anoA*. This gene can be found in four different *Agrobacterium* genomes (Figure 3). In addition, *anoA* occurs in the genomes of three *Sinorhizobium* and four *Rhizobium* strains (Figure 3), illustrating that the *anoA* gene is conserved in all three genera. The gene arrangement in the *Agrobacterium* genome is consistent with *Rhizobium gallicum bv. gallicum* R602sp^T, *Rhizobium grahamii* CCGE502^T, and *Rhizobium etli* CFN42^T (Figure 3). In addition, gene synteny in the *Sinorhizobium* genomes are almost the same and consistent with *R. giardinii bv. giardinii* H152, but quite different with that in *Agrobacterium* (Figure 3). Phylogenetic

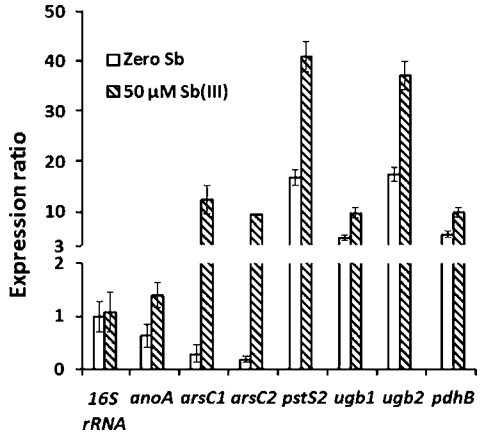

Figure 2. Quantitative reverse transcriptase-PCR analysis of representative genes encoding proteins enhanced by Sb(III). Total RNA was isolated from strain GW4 cultured with or without 50 μM Sb(III). The 16S rRNA gene was used as a reference. Data are shown as the mean of three replicates, with the error bars representing ±1 SD.

analysis of AnoA amino acid sequences illustrates how the AnoA separates and clusters largely according taxonomic designation (Figure 3). *A. tumefaciens* GW4, *Agrobacterium tumefaciens* 5A, and *Sinorhizobium* sp. Sb3 have all been documented to oxidize Sb(III),[15,18] however there is no information available for the other bacteria represented in Figure 3.

AnoA Affected Sb(III) Resistance and Oxidation in *A. tumefaciens* GW4. To investigate the relationship between AnoA and Sb(III) oxidation, a *anoA* deletion strain (GW4-Δ*anoA*) and complemented strain (GW4-Δ*anoA*-C) were constructed. Diagnostic PCRs were used to confirm the successful deletion and complementation (Figure 4). In addition, the same pCPP30::*anoA* construct was also conjugated into the wild type strain GW4 (GW4::*anoA*) to provide additional copies of *anoA in trans* (pCPP30 is a low copy plasmid) (Supporting Information Table S1). Stable maintenance of pCPP30::*anoA* in GW4-Δ*anoA*-C and GW4::*anoA* was confirmed by antibiotic resistance, and by reisolating the plasmid pCPP30-*anoA* (Supporting Information Table S1) and enzymatic digestion (data not shown). In growth tests, the wild type strain, GW4-Δ*anoA* mutant, and GW4-Δ*anoA*-C showed consistent growth profiles in CDM medium containing 50 μM Sb(III) (Figure 5 A), although at higher Sb(III) levels, growth of the mutant was constrained relative to all other strains (Figure 5B). Thus, the Sb(III) oxidation was tested with 50 μM Sb(III), and the disruption of *anoA* reduced the Sb(III) oxidation rate by ∼27% (Figure 5 C), while the *anoA* complemented strain showed a similar Sb(III) oxidation rate as that of the wild type GW4. Additional *anoA* copy number in GW4::*anoA* coincided with an increase in Sb(III) oxidation by ∼34% (Figure 5 C). Sb(III)/(V) levels in media taken from uninoculated control flasks did not change during the course of these experiments (i.e., remained roughly 50 μM throughout), indicating that the Sb(III) did not bind to the flack glass walls nor precipitate (Supporting Information Figure S1). Thus, Sb speciation and culture sensitivity were not influenced by the media nor culture methods.

Sb(III) Induces *anoA* Expression. To understand how AnoA responds to Sb(III) in *A. tumefaciens* GW4, we examined the *anoA* expression (monitored as an *anoA*::*lacZ* reporter)

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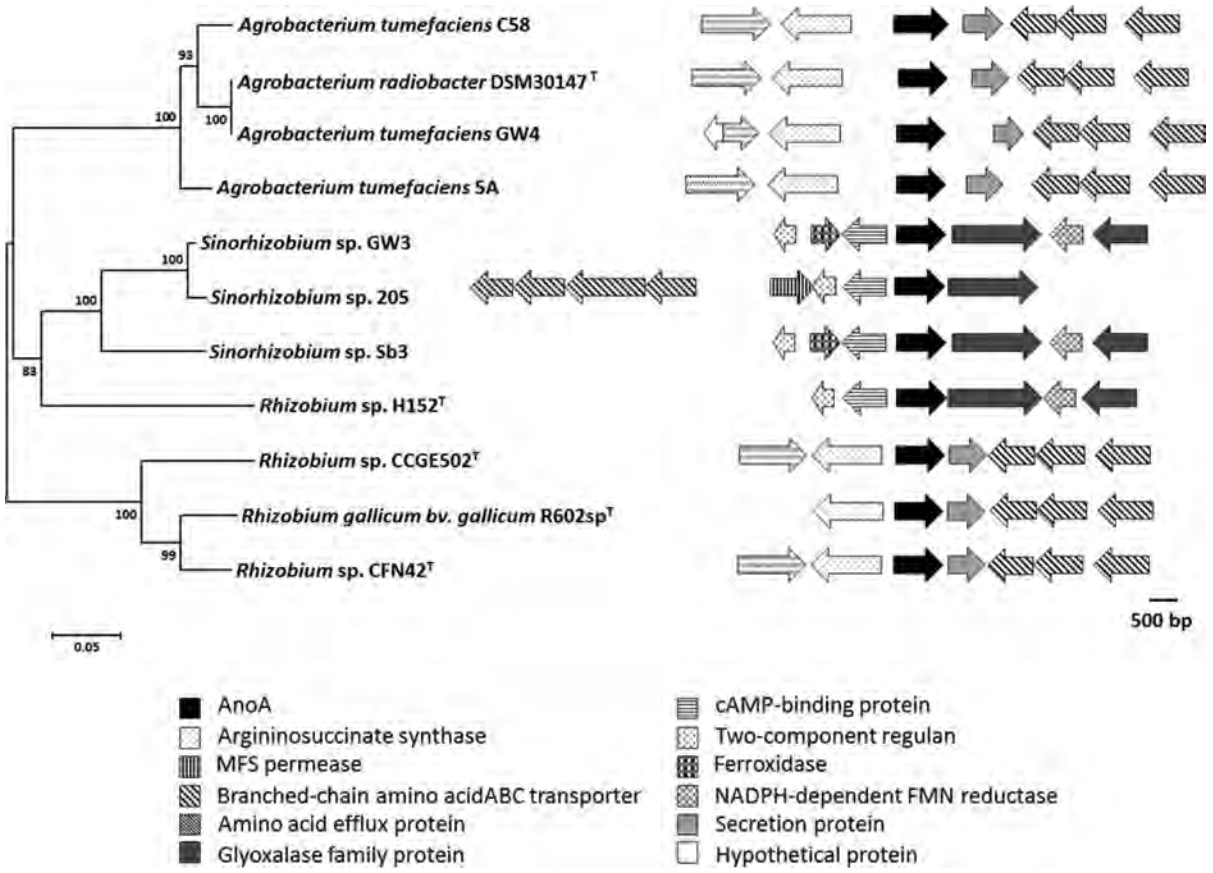

Figure 3. The amino acid–based phylogenetic relationship of AnoA relative to homologues in other members of the *Rhizobiaceae* and the gene arrangement surrounding the *anoA* gene in different strains of *Agrobacterium, Sinorhizobium* and *Rhizobium*. AnoA represented in the different tree branches align with the corresponding gene shown in the gene arrangement diagram.

with different concentrations of Sb(III)/As(III), since they were with the similar chemical characters (Figure 5D). When the culture optical density (OD_{600}) reached about 0.5, Sb(III) or As(III) were added separately at 0, 50, 150, or 200 μM and then growth and reporter enzyme activity monitored, respectively. GW4 growth was not affected and with the addition of up to 200 μM Sb(III) (Figure 5B) and As(III) thus consistent with the high MICs.[34] After 2 h incubation, *anoA*::*lacZ* expression was not statistically different between the controls and the As(III) spiked cultures (Figure 5 D). However, with different concentrations of Sb(III), the *anoA*::*lacZ* expression increased along with the increasing Sb(III) concentration (Figure 5 D). To determine whether up-regulation of *anoA* might be linked to a Sb(III)-based oxidative stress-like response, we also examined *anoA*::*lacZ* expression in reaction to added H_2O_2. There was no apparent induction affect, and at H_2O_2 concentrations above 0.5%, *anoA*::*lacZ* reporter activity decreased approximately 60% (Supporting Information Figure S2), suggesting oxidative stress *per se* was not likely involved in up-regulating *anoA*.

AnoA Kinetics analysis. To further investigate AnoA, Sb(III) oxidation with His-tag purified AnoA was tested in vitro. NAD^+ was without effect, but $NADP^+$ was found to be an essential cofactor and thus consistent with the BlastP prediction, but $NADP^+$ would not oxidize Sb(III) in the absence of AnoA (Supporting Information Figure S3). A kinetics analysis of AnoA (with 5 mM Sb(III)) yielded K_m and V_{max} estimates for $NADP^+$ of 5.9 ± 0.7 μM and 7.9 ± 0.2 nmol min^{-1} mg^{-1}, respectively (Figure 6A). In the presence of over saturating 200 μM $NADP^+$ as coenzyme, the Sb(III) K_m was 64 ± 10 μM and V_{max} was 150 ± 7 nmol min^{-1} mg^{-1} (Figure 6B). The results indicated that AnoA is able to oxidize Sb(III) using $NADP^+$ as coenzyme.

anoA Expression in *Escherichia coli*. To further study AnoA function, we transformed pCPP30-*anoA* into the *Escherichia coli* S17-1. Washed cells of *E. coli* strains S17-1, S17-1 (pCPP30), and S17-1 (pCPP30-*anoA*) were inoculated into CDM liquid media containing 0.02% yeast extract and 1.0 μM Sb(III) (OD_{600} of ~0.4). During the 2 h incubation, Sb(III) oxidase activities were not significantly different between strains S17-1 and S17-1(pCPP30) (both oxidized ~0.23 μmol Sb(III)). However, Sb(III) oxidation in strain S17-1(pCPP30-*anoA*) was approximately 70% higher (roughly 0.39 μmol Sb(III)) and thus consistent with the interpretation that increased Sb(III) oxidation was attributable to the cloned *anoA* from strain GW4 (Figure 7).

■ DISCUSSION

The first strategy of this study was to use proteomics as a tool for examining bacterial response to Sb(III), and in particular to search for a potential Sb(III) oxidase. To our knowledge, Sb(III) comparative proteomics has not been attempted and so this study introduces a novel element to the microbe-metalloid interaction literature. The proteomics component was not a comprehensive effort, but as a first generation study it was nevertheless revealing. Functions of the up-regulated proteins included several different categories of metabolism and cell activities (Table 1). The linkage between Sb(III) and enhanced

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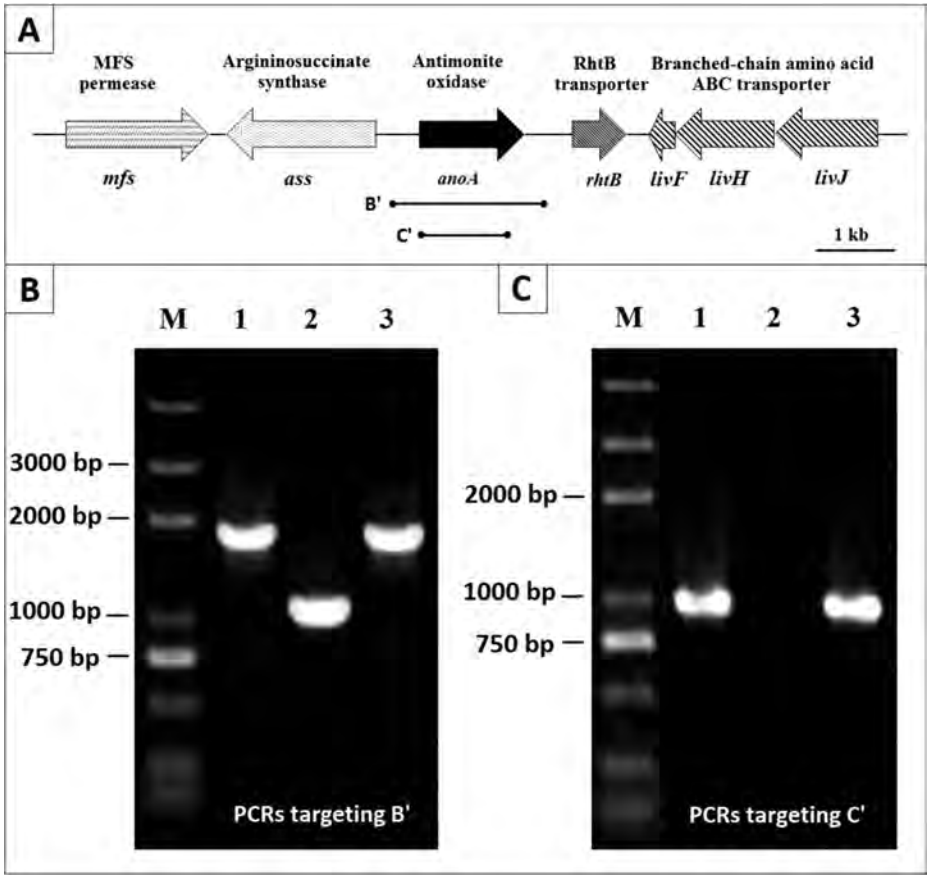

Figure 4. Physical evidence of *anoA* deletion and complementation relevant to Sb(III) oxidation phenotypes characterized in this study. (A) Physical map of the *anoA* gene in *A. tumefaciens* GW4. B′ designates a 1813 bp DNA fragment targeted in diagnostic PCR with primers PManoA-1F/PManoA-1R (Supporting Information Table S2) and depicted in panel B; C′ designates a 973 bp DNA fragment targeted in diagnostic PCR with primers PManoA-2F/PManoA-2R (Supporting Infomation Table S2) and depicted in panel C. For panels B and C: Lane 1, WT strain, lane 2, *anoA* deletion strain GW4-Δ*anoA*, and lane 3, the complemented strain GW4-Δ*anoA*-C. M, the molecular weight marker (DL 2000 plus). Amplicon identities were confirmed by DNA sequencing.

levels of specific proteins was verified by reverse transcriptase PCR (Figure 2). Expression of *arsC1* and *arsC2* (encode arsenate reductases) served as internal positive controls because prior work from the Rosen group with *E. coli* and our efforts with *A. tumefaciens* strain 5A have illustrated that *arsC* genes are inducible by As(III) and/or Sb(III) and as part of the *ars* operons are cotranscribed with *acr3* that contributes directly to As(III) and Sb(III) resistance.[40,41] At this stage, the exact mechanism by which Sb(III) exerts its regulatory effect on *anoA* expression (or that of other proteins/genes described herein) is not clear. It may be direct or indirect. We tested As(III) (potentially direct), H_2O_2 (potentially indirect) and Sb(III) (potentially direct). Of these, only Sb(III) resulted in increased *anoA* expression (Figure 5D and Supporting Information Figure S2).

Bacterial Sb(III) oxidation has been reported in several genera, including *Agrobacterium*, *Sinorhizobium*, *Comamonas*, *Acinetobacter*, *Stenotrophomonas*, *Pseudomonas*, *Sphingopyxis*, *Arthrobacter*, *Bacillus*, and *Variovorax*.[13−18] Li et al.[16] isolated a Sb(III)-oxidizing *Comamonas testosteronis* strain whose genome sequence lacks identifiable *aioBA* genes and is negative for As(III) oxidation. Further, there are As(III)-oxidizing strains which failed to exhibit Sb(III) oxidation.[17] However, in recent work we found the arsenite oxidase AioBA could also function as a Sb(III) oxidase.[19] In vitro assays with a his-tag purified AioBA from *Rhizobium* strain NT-26 unequivocally demonstrated Sb(III) oxidation, however, the Sb(III) oxidation activity in the 5A *aioA* mutant was decreased by only about 25% relative to the wild type strain, indicating the presence of (an)other Sb(III) oxidase. Since the *A. tumefaciens* 5A strain also carries *anoA* (Figure 3), it is highly likely that AnoA accounted for at least some of the residual Sb(III) oxidation activity in the *aioA* mutant.

The results of this study are to demonstrate direct evidence of an enzyme, in this case a gene belonging to the SDR family that we coined *anoA*. The SDR superfamily is an ancient and ubiquitous group of enzymes[42] that can catalyze NAD(P)H/NAD(P)$^+$ dependent reduction/oxidation reactions with a wide range of substrates,[43] including polyols, retinoids, steroids,[38] and fatty acid derivatives.[39] Given example structures of such compounds and the pH of culture conditions, it would seem reasonable to predict that the antimony provided as $K_2Sb_2(C_4H_2O_6)_2$ in this study would be in equilibrium with a polyol type structure of $Sb(OH)_3$ that would then be the substrate for AnoA. Whether $Sb(OH)_3$ is monomeric or polymeric, and whether the latter is cyclic in nature is a topic for further investigation. See Meng et al.[7] and Palenik et al.[44] for additional discussion of substrate structural possibilities.

While the exact structural nature of the Sb(III) substrate requires additional effort to resolve, we nevertheless conclude that AnoA is a Sb(III) oxidase in *A. tumefaciens* GW4 and almost certainly in highly related bacteria such as *A. tumefaciens*

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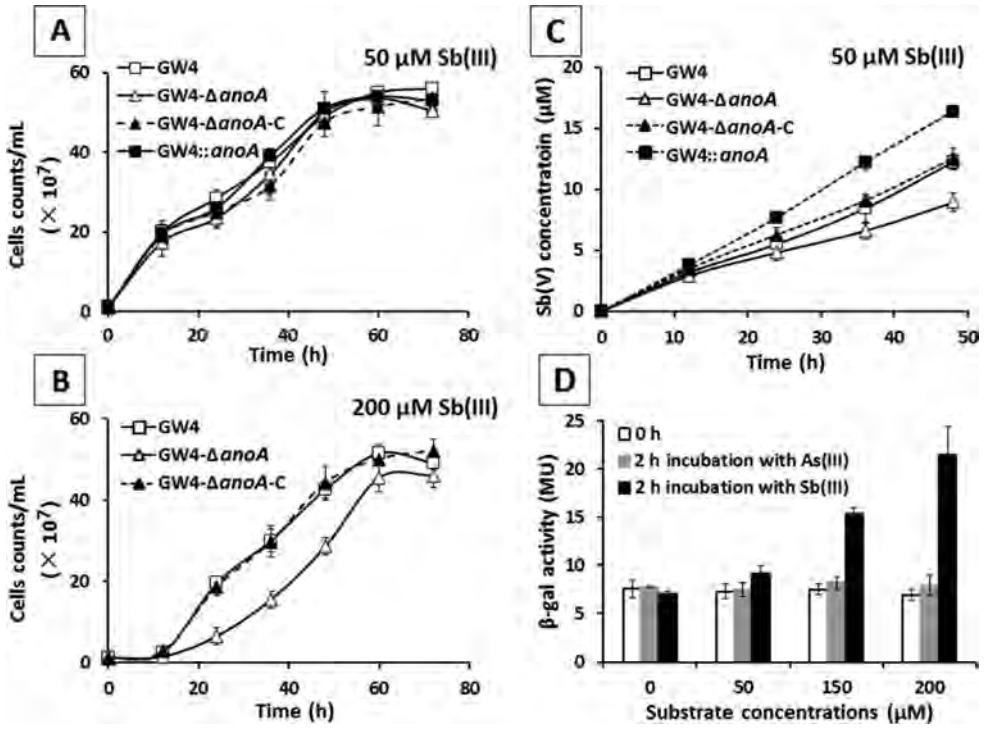

Figure 5. The *anoA* genotype influenced Sb(III) resistance and oxidation in *A. tumefaciens* GW4, and the expression of *anoA* is induced by Sb(III). (A) and (B) Growth curves of *A. tumefaciens* strains with the addition of 50 and 200 μM Sb(III), respectively. (C) Sb(III) oxidation profiles of the same strains with the same symbols as in panel A. (D) Reporter gene activity of *anoA::lacZ* in strain GW4 inoculated into CDM medium containing Sb(III) or As(III). Assays were conducted at t = 0 h and t = 2 h relative to inoculation. Data are shown as the mean of three replicates, with the error bars represents ±1 SD.

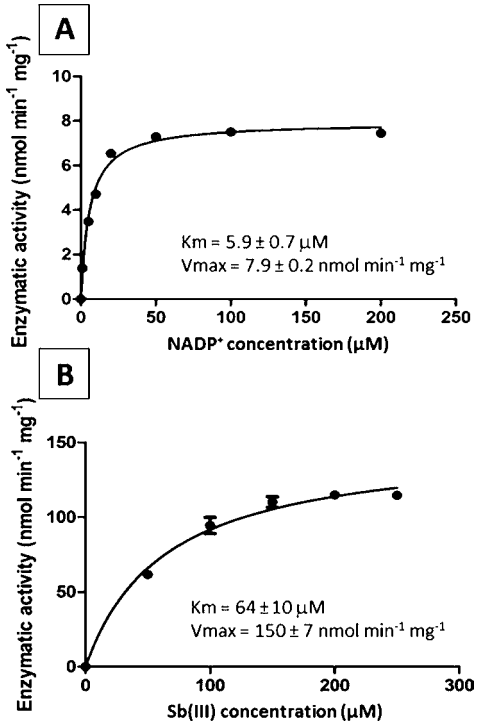

Figure 6. Kinetic analysis of AnoA. (A) $NADP^+$. (B) Sb(III). For Sb(III), data points and error bars represent as average plus or minus range of two replicates.

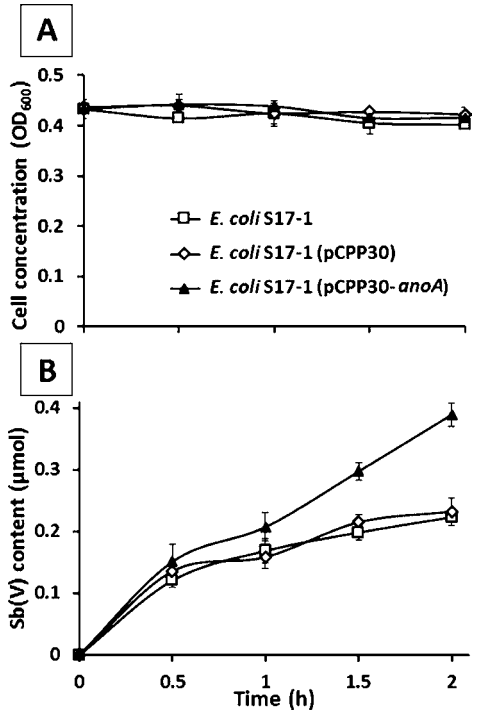

Figure 7. Sb(III) oxidation by AnoA in *E. coli* S17-1. (A) Growth profile of *E. coli* S17-1, *E. coli* S17-1 (pCPP30) and *E. coli* S17-1 (pCPP30-*anoA*) in CDM medium with the addition of 10 μM Sb(III). (B) Sb(V) content in spent culture fluids generated by Sb(III) oxidation in the same strains (symbol assignments are the same in both panels and are as shown in panel A). Data are shown as the mean of three replicates, with the error bars representing ±1 SD.

5A (compare relevant genome structure and synteny, Figure 3). The evidence is substantial and internally consistent: (i) *anoA* transcription measured by three independent techniques

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(AnoA protein levels, Figure 1; reverse transcriptase, Figure 2 and reporter construct, Figure 5D;) are enhanced by the addition of Sb(III) to the culture medium; (ii) creation of a Δ*anoA* mutant resulted in decreased Sb(III) resistance and Sb(III) oxidation rate (Figure 5A−C); (iii) increased *anoA* transcription correlated with increased Sb(III) oxidation activity (Figure 5C); (iv) purified AnoA could oxidize Sb(III) in vitro using $NADP^+$ as cofactor (Figure 6); and (v) providing extra copies of *anoA in trans* complemented the Δ*anoA* mutant back to wild type status (Figure 5A−C) and indeed this also increased activity in the wild type strain GW4 (Figure 5C) as well as in *E. coli* (Figure 7). We find the last observation as being potentially important in the context of bioremediation technologies (patent pending, #201410538034.6); i.e. being able to transfer this activity via a single gene and acquire heterologous expression and activity holds promise for custom designing Sb(III)-relevant bacteria for the purpose of altering or controlling Sb behavior and toxicity in anthropogenically impacted environments (e.g., mining). As of yet, we have been unable to master such a simple transfer of As(III) oxidation activity, even among closely related *A. tumefaciens* strains (unpublished data).

Among *anoA* harboring bacteria, the ability to establish a relationship between the presence of a *anoA* gene and Sb(III) oxidation is constrained by the paucity of genome sequenced organisms that have also been directly tested for Sb(III) oxidation. *A. tumefaciens* strain GW4 (this study), *A. tumefaciens* strain 5A[15] and *Sinorhizobium* sp. Sb3[18] are all Sb(III)-oxidizing, and are highly related phylogenetically (Figure 3).[45] Additional related studies are underway to assess the relative environmental abundance and activities of *anoA* as one way of determining linkages between this gene and the antimony biogeochemical cycle. We draw attention to the obvious presence of additional, AnoA-independent, Sb(III) oxidation activity. This study offers up the gene, proteomics, expression data and kinetics for an enzyme that catalyzes Sb(III) oxidation that has yet remained undiscovered/uncharacterized for decades. The activity was observed in the Δ*anoA* mutant (Figure 5) as well as in *E. coli* strain S17-1 (Figure 7). Consequently, this implies there are other enzymes capable of influencing antimony oxidation and biogeocycling in nature. It remains to be determined whether Sb(III) is the primary substrate for such enzymes or a cometabolite.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary Figures 1, 2, and 3 and Supplementary Table 1 and Table 2. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/es506318b.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 86-27-87281261; fax: 86-27-87280670; e-mail: gejiao@mail.hzau.edu.cn.

Author Contributions

‖J.L. and Q.W. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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