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# Regulation of antimonite oxidation and resistance by the phosphate regulator PhoB in *Agrobacterium tumefaciens* GW4

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ABSTRACT

Microbial oxidation of antimonite [Sb(III)] to antimonate [Sb(V)] is a detoxification process which contributes to Sb(III) resistance. Antimonite oxidase AnoA is essential for Sb(III) oxidation, however, the regulation mechanism is still unknown. Recently, we found that the expressions of phosphate transporters were induced by Sb(III) using proteomics analysis in *Agrobacterium tumefaciens* GW4, thus, we predicted that the phosphate regulator PhoB may regulate bacterial Sb(III) oxidation and resistance. In this study, comprehensive analyses were performed and the results showed that (1) Genomic analysis revealed two *phoB* (named as *phoB1* and *phoB2*) and one *phoR* gene in strain GW4; (2) Reporter gene assay showed that both *phoB1* and *phoB2* were induced in low phosphate condition (50 μM), but only *phoB2* was induced by Sb(III); (3) Genes knock-out/complementation, Sb(III) oxidation and Sb(III) resistance tests showed that deletion of *phoB2* significantly inhibited the expression of *anoA* and decreased bacterial Sb(III) oxidation efficiency and Sb(III) resistant. In contrast, deletion of *phoB1* did not obviously affect *anoA*'s expression level and Sb(III) oxidation/resistance; (4) A putative Pho motif was predicted in several *A. tumefaciens* strains and electrophoretic mobility shift assay (EMSA) showed that PhoB2 could bind with the promoter sequence of *anoA*; (5) Site-directed mutagenesis and short fragment EMSA revealed the exact DNA binding sequence for the protein-DNA interaction. These results showed that PhoB2 positively regulates Sb(III) oxidation and PhoB2 is also associated with Sb(III) resistance. Such regulation mechanism may provide a great contribution for bacterial survival in the environment with Sb and for bioremediation application.

## 1. Introduction

Antimony (Sb) is a metalloid that is widely distributed in natural environment, especially in the mining and smelting areas (Filella et al., 2002a; He et al., 2012). Sb mainly exists in the inorganic forms of antimonite [Sb(III)] and antimonate [Sb(V)] (Filella et al., 2002b; Hockmann et al., 2014), and those compounds are considered as a kind of the priority pollutants by the United States Environmental Protection Agency and the European Union (CEC, 1976; USEPA, 1979). During evaluation, microorganisms have developed diverse mechanisms to resistant the toxicity of Sb compounds mediating efflux and redox transformation of Sb (Li et al., 2016). Bacterial Sb(III) oxidation transforms the more toxic Sb(III) to the less toxic Sb(V). In some autotrophic bacteria, Sb(III) oxidation can provide energy to support the chemoautotrophic growth (Lialikova, 1974; Terry et al., 2015). For heterotrophic bacteria, Sb(III) oxidation is an important detoxification mechanism, which enables bacteria to resist high concentration of Sb (III) (Li et al., 2015, 2016).

Different from bacterial arsenite [As(III)] oxidation, which is catalyzed by arsenite oxidase AioAB (Wang et al., 2015a), bacterial Sb (III) oxidation is a multi-factor oxidation process (Li et al., 2017a). Previously, based on comparative proteomics study, we identified a Sb (III) oxidase AnoA, which was induced by Sb(III) and significantly affected Sb(III) oxidation and resistance. Heterologous expression *anoA* in *E. coli* remarkably increased bacterial Sb(III) oxidation rate. Furthermore, AnoA could catalyze Sb(III) oxidation with $NADP^+$ as a **co**-factor *in vitro*. In addition to the enzymatic Sb(III) oxidation catalyzed by AnoA, cellular $H_2O_2$ generated from oxidative stress could also oxidize Sb(III) to Sb(V) as an abiotic factor (Li et al., 2015). Due to the chemical similarities between As(III) and Sb(III), AioAB could also catalyze Sb (III) oxidation, however, the expression of *aioA* was not induced by Sb (III) (Wang et al., 2015b).

The regulation of As(III) oxidation is controlled by the three-component system AioXSR in most of the As(III)-oxidizing bacteria, and the correlation of arsenic and phosphorus metabolism has been well documented (Liu et al., 2012; Kang et al., 2012; Wang et al., 2015a). Wang et al., demonstrated that the high affinity phosphate (Pi) binding protein PstS could bind with As(V) and transport As(V) into bacterial

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cells. The cellular As(V) was found in proteins and lipids under low-Pi condition (Wang et al., 2015a). Currently, it has been reported that bacterial As(III) oxidation is ultimately regulated by the Pi starvation response (PSR), and the Pi two-component system PhoBR and AioSR could cross-talk by transphosphorylation (Wang et al., 2018).

As a necessary element for organic activities, phosphate exists in aquatic and terrestrial ecosystems at low concentrations (Vieira et al., 2008). For many bacterial species, inorganic Pi uptake and metabolism are associated with environmental Pi concentration. The high affinity Pi transport system Pst (PstSCAB-PhoU) was reported to be responsible for Pi uptake and assimilation under low-Pi condition (Lamarche et al., 2008; Santos-Beneit, 2015). As a member of the Pho regulon, Pst is regulated by the Pi two-component system PhoBR (Hsieh and Wanner, 2010). Recent studies have shown that under low-Pi condition, the Pi-bound PstS interacts with the PstC/PstA transmembrane proteins and resulting in the hydrolysis of ATP in the presence of PstB dimer (Vuppada et al., 2018). This conformational change could be recognized by PhoU, which subsequently bind with PhoR to activate its kinase function (diCenzo et al., 2017; Vuppada et al., 2018). The activated PhoR autophosphrylates and transfers the phosphoryl moiety to response regulator PhoB. The phosphorylated PhoB forms a head-to tail dimer and binds to the Pho box, which is known as an 18-bp specific DNA sequence and located in the promoter region of the Pho genes (Hsieh and Wanner, 2010). The *E. coli* consensus Pho box (CT G/T TCAT A A/T A A/T CTGTCA T/C) consists of two 7-bp direct repeats separated by a conserved 4-bp AT-rich spacer (Yuan et al., 2006). After binding to the Pho box, PhoB interacts with the RNA polymerase $\sigma^{70}$ submit to activate the transcription of *pstS* and itself (Makino et al., 1993).

It has been found that about 40 genes are regulated by PhoBR in *E. coli*, indicating the widespread distribution of the Pho box and the global regulation of PhoBR (Lubin et al., 2015). Currently, PhoBR was proved to regulate the genes involved in bacterial mobility, biofilm formation, oxidative stress response, nutritional deprivation, acid shock resistance, antibiotic resistance and virulence attenuation (Monds et al., 2001; Lamarche et al., 2005, 2008; Pratt et al., 2009; Crépin et al., 2011; Srinivasan et al., 2012; Santos-Beneit, 2015). In addition, a previous study demonstrated that PhoBR regulates bacterial As(III) oxidation in *Halomonas* sp. HAL1 under low-Pi condition (Chen et al., 2015). So far, the regulatory mechanism of Sb(III) oxidase AnoA remains known. In the previous study, we found that Sb(III) could induce the transcription of *pstS2* and some other genes (e.g. methylphosphonate 5-triphosphate synthase subunit gene *phnI* and methylphosphonate 5-triphosphate diphosphatase gene *phnM*) involved in phosphate and phosphonate metabolisms in strain GW4, suggesting that Sb(III) oxidation may also associate with phosphorus metabolism (Li et al., 2015, 2017b). Thus, based on our proteomics analysis and literatures, it is rational to assume that PhoBR may also involve in the regulation of bacterial Sb(III) oxidation.

In this study we used *A. tumefaciens* GW4 as a model to further investigate the regulation of Sb(III) oxidation (Fan et al., 2008). There are two *phoB* genes (named as *phoB1* and *phoB2*) in the genome of strain GW4 and they share one *phoR* gene. Thus, the objective of this study was to identify the roles of the two *phoB* genes in the regulation of Sb(III) oxidation in strain GW4. The present study represents a novel contribution and demonstrated that the phosphate regulator PhoB2 could regulate the expression of Sb(III) oxidase gene *anoA* under low-Pi condition. The results provide an important step for better understanding the regulation mechanisms of microbial Sb(III) oxidation.

## 2. Materials and methods

### 2.1. Strains and genomic analysis

Bacterial strains and plasmids used in the present study are listed in Table 1. *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) medium. *A. tumefaciens* strains were cultured at 28 °C with aeration in the chemically defined medium (CDM) (Weeger et al., 1999), which contains ~ 50 μM Pi. When required, $K_2Sb_2(C_4H_2O_6)_2$ [Sb(III)] and phosphate buffer solution ($K_2HPO_4{\cdot}3H_2O$:$KH_2PO_4$ = 4:1, pH = 7.2) were added to the culture at final concentrations of 50 μM and 2 mM, respectively. When needed, stock solutions of ampicillin (Amp, 100 mg/mL), kanamycin (Kan, 50 mg/mL), chloromycetin (Cm, 50 mg/mL), gentamicin (Gen, 50 mg/mL) or tetracycline (Tet, 5 mg/mL) were added to the medium with 1‰ (v:v) addition. The blastN and blastP analyses of *phoB1* and *phoB2* were conducted in the genome of *A. tumefaciens* GW4 (CCTCC AB 2018213) on the NCBI website (http://www.ncbi.nlm.nih.gov).

### 2.2. Reporter gene assays of phoB1 and phoB2

The reporter gene assays were tested by β-galactosidase activity using *lacZ* reporter plasmid pLSPkt2lacZ (Li et al., 2015). The promoter regions of *phoB1* and *phoB2* were PCR amplified with primers PRphoB1-F/PRphoB1-R and PRphoB2-F/PRphoB2-R, respectively (Table S1). The *phoB2* was co-transcribed with genes *pstCAB2-phoU2* (Qiao et al., in preparation), thus, the promoter region of *phoB2* was located upstream of *pstC2*. The PCR amplicons were directionally cloned into *EcoR*I and *BamH*I double-digested pLSPkt2lacZ. The resulting plasmids pLSP-PphoB1 and pLSP-PphoB2 were each transformed into strain GW4 through conjugation via *E. coli* S17-1. All strains were inoculated into CDM medium with or without 50 μM Sb(III). For normal-Pi condition, the phosphate buffer solution was added to the culture at a final concentration of 2 mM Pi. After being incubated at 28 °C for 24 h, samples were taken for testing the β-galactosidase activities using the method described previously (Miller, 1972; Kang et al., 2012).

### 2.3. Gene knock out and complementation of phoB1, phoB2, pstS1 and pstS2

Crossover PCR was used for the in-frame deletion (Link et al., 1997). The PCR products were both cloned into the gene knock-out plasmid pJQ200SK double digested by *BamH*I and *Xba*I, and then the generated pJQ-phoB1 and pJQ-phoB2 were mobilized into the wild type strain GW4 respectively. The single crossover mutants of *phoB1* or *phoB2* were screened on LB-Gen agar plate, which were then spread on CDM medium with 20% sucrose, selecting for the double crossover mutants (Pelicic et al., 1996). The successful construction of mutant strains GW4-Δ*phoB1* and GW4-Δ*phoB2* were verified by diagnostic PCR and DNA sequencing, respectively. For complementation, the full length of *phoB1* or *pstC2A2U2-phoB2* coding region was PCR amplified and cloned into plasmid pCPP30 double-digested by *BamH*I-*Xba*I, which was subsequently transferred into the corresponding mutant strains, resulting in complemented strains GW4-Δ*phoB1*-C and GW4-Δ*phoB2*-C, respectively. The primers used for construction of gene deletion and complementation of *phoB1* and *phoB2* are listed in Table S1.

### 2.4. Growth, Sb(III) oxidation and Sb(III) resistance tests

The CDM broth contains ~50 μM Pi, which is a low-Pi condition for bacterial growth. To eliminate the growth difference caused by the deletion of *phoB* genes, *A. tumefaciens* strains were each inoculated into 100 mL of CDM medium and incubated at 28 °C for 36 h–84 h. When the $OD_{600}$ reached 0.5 − 0.6, cells were harvested by centrifugation at room temperature (5000 × g for 5 min) and washed twice using physiological saline (0.85% NaCl). Subsequently, cells with a final $OD_{600}$ of ~0.35 were each inoculated into 100 mL of CDM amended with 50 μM Sb(III) and incubated at 28 °C with aeration. Bacterial growth was measured regularly with spectrophotometry (DU800, Beckman), and determination of Sb(III)/Sb(V) concentrations was accomplished by HPLC-HG-AFS (Beijing Titan Instruments Co., Ltd., China) according to Li et al. (2013). For Sb(III) resistance assays, *A. tumefaciens* strains were

**Table 1**
Strains and plasmids used in this study.

| Strain/plasmid | Relevant properties or derivation | Source or reference |
|---|---|---|
| **Strains** | | |
| *Agrobacterium tumefaciens* | | |
| GW4 | Wild type, As(III) oxidizing, Sb(III) oxidizing | Fan et al., 2008 |
| GW4-Δ*phoB1* | *phoB1* gene deleted | This study |
| GW4-Δ*phoB1*-C | Δ*phoB1* (pCPP30::*phoB1*) Complementation of Δ*phoB1* | This study |
| GW4-Δ*phoB2* | *phoB2* gene deleted | This study |
| GW4-Δ*phoB2*-C | Δ*phoB2* (pCPP30::*phoB2*) Complementation of Δ*phoB2* | This study |
| *Escherichia coli* | | |
| DH5α | *supE*44 *lacU*169(*φ80lacZM15*) *hRDR*17 *recA*1 *endA*1 *gyrA96 thi-1 relA*1 | Hanahan, 1983 |
| S17-1 λ*pir* | $F^-$ RP4-2-Tc::Mu *aphA*::Tn7 *recA* λ*pir* lysogen; $Sm^R$ $Tp^R$ | Simon et al., 1983 |
| BL21StarTM(DE3)pLysS | *F- ompT hsdSB (rB- mB-) gal dcm me*131 (DE3) pLysS (CmR) | Invitrogen |
| **Plasmids** | | |
| pGEM-T | TA cloning vector; $Amp^R$ | Promega |
| pJQ200SK | *sacB sacR* $Suc^S$; $Gen^R$ | Quandt and Hynes, 1993 |
| pJQ-phoB1 | Gene mutation plasmid to create Δ*phoB1*; *sacB sacR* $Suc^S$; $Gen^R$ | This study |
| pJQ-phoB2 | Gene mutation plasmid to create Δ*phoB2*; *sacB sacR* $Suc^S$; $Gen^R$ | This study |
| pCPP30 | Broad host range; $Tet^R$ | Liu et al., 2012 |
| pCPP30-phoB1 | Complemented plasmid for Δ*phoB1*; $Tet^R$ | This study |
| pCPP30-phoB2 | Complemented plasmid for Δ*phoB2*; $Tet^R$ | This study |
| pLSP-kt2lacZ | $Kan^R$ *oriV*; *lacZ* fusion vector used for *lacZ* fusion constructs | T.R. McDermott, MSU |
| pLSP-PanoA | pLSP-kt2lacZ containing *anoA* promoter region | This study |
| pLSP-MPanoA | pLSP-kt2lacZ containing site mutated *anoA* promoter region | |
| pLSP-PphoB1 | pLSP-kt2lacZ containing *phoB1* promoter region | This study |
| pLSP-PphoB2 | pLSP-kt2lacZ containing *phoB2* promoter region | This study |
| pET-28a(+) | T7 RNA polymerase-based expression vector; $Kan^R$ | Novagen |
| pET-phoB2 | phoB2 gene cloned into pET-28a(+) for expression of PhoB2; $Kan^R$ | This study |

cultivated as described above, cells were each inoculated into 100 mL of CDM in the presence of 0 or 200 μM Sb(III) with an initial $OD_{600}$ of ~ 0.3. Samples were taken after 24 h cultivation for measuring $OD_{600}$.

### 2.5. Reporter gene assays of anoA and $H_2O_2$ content assays

The promoter region of *anoA* (Accession No. KDR88348) was amplified by PCR with primers listed in Table S1 (PRanoA-F/PRanoA-R), and subsequently cloned into *Eco*RI and *BamH*I double-digested pLSPkt2lacZ. The resulting plasmid pLSP-PanoA was introduced into strains GW4, GW4-Δ*phoB1* and GW4-Δ*phoB2*, respectively. All strains were cultured as described above. The cotransformed cells were grown in 100 mL of CDM medium with or without the addition of 50 μM Sb (III) with an initial $OD_{600}$ of ~0.3. Samples were taken at regular intervals for testing the β-galactosidase activities (Miller, 1972; Kang et al., 2012). For $H_2O_2$ content assays, *A. tumefaciens* strains were cultivated in CDM medium with or without the addition of 50 μM Sb(III) for 36 h. Bacterial cells were harvested (13,400 × g for 5 min at 4 °C) and washed twice with 50 mmol/L $K_3PO_4$ (pH 7.8). After sonication on ice for 5 min, the supernatant was used for measuring the cellular $H_2O_2$ content with the spectrofluorometry method described by Li et al. (2017a).

### 2.6. Electrophoretic mobility shift assay (EMSA)

The full length of *phoB2* coding region was PCR amplified from ATG start codon using high-fidelity DNA polymerase (TransGen Biotech) with primers listed in Table S1. The fragment was cloned into *Eco*RI-*Hind*III double digested pET-28a(+) (Novegen) and subsequently introduced into *E. coli* BL21 Star (DE3) pLysS (Invitrogen, Table S1). The recombinant was screen on LB-Kan agar plate and further confirmed by DNA sequencing. Strain *E. coli* BL21 (pET28a-phoB2) was inoculated into 100 mL LB-Kan medium with 120 rpm shaking at 37 °C until $OD_{600}$ reached ~0.3. Then, 0.1 mM IPTG was added to the culture for another 4 h shaking at 25 °C. Cells were harvested by centrifugation (13,400 g for 10 min at 4 °C), and washed twice with 50 mM Tris − HCl (pH 8.0). The pellets was resuspended in 10 mL 50 mM Tris − HCl (pH 8.0) and lysed via pressure cell lysis. After binding with 1 mL pre-equilibrated Profinity™ IMAC Resins (Bio-RAD) at 4 °C for 2 h, the His-tag PhoB2 was eluted in 1 mL 50 mM Tris-HCl (pH 8.0) with 100 mmol/L imidazole. The eluted protein was dialyzed and then detected with SDS-PAGE.

To investigate the interactions between PhoB2 and *anoA* promoter *in vitro*, a 224 bp fragment of *anoA* regulatory region was amplified using the fluorophore FAM labeled primers PanoA-F/PanoA-R (Table S1). The PCR product was purified with the high pure PCR product purification kit (Roche). EMSA was performed with a 1.6 pmol labeled probe and different concentrations of PhoB2 (0–5 μM). The heat-denatured protein and unspecific DNA were used as negative controls. In addition, 1.6, 3.2, and 6.4 pmol unlabeled probes were mixed with 4 μM PhoB2 and 1.6 pmol labeled probe respectively for the competition experiments. The DNA-protein complexes were loaded onto a 4% native PAGE in 0.5 × TGE buffer for 2 h. The gels were imaged by exposing to a phosphor imaging system (Fujifilm FLA-5100). Meanwhile, the promoter region of *pstS2* was amplified using the fluorophore FAM labeled primers PpstS2-F/PpstS2-R (Table S1), which was used as a positive control.

### 2.7. Identification of the putative PhoB box and conserved binding sites

To identify the putative Pho box in *anoA* promoter region, we predicted a Pho motif using 25 putative Pho box in *Agrobacterium* sp. strains through the MEME on-line program (http://meme.nbcr.net/meme/cgi-bin/meme.cgi; Bailey and Elkan, 1994). Based on the Pho motif, an 18 bp putative Pho box was found in *anoA* promoter region. Thus, a fluorophore FAM labeled DNA fragments containing the putative Pho box and its bilateral 10 bp protection bases, was synthesized by Tsingke Biological Technology Company, Beijing, China. Subsequently, short fragment EMSA was carried out for verification the predicted Pho box using 10 pmol probes and different concentrations of PhoB2 (0–10 μM), respectively. Meanwhile, the predicted Pho box in *pstS* promoter region was used as a positive control.

To verify the putative binding sites, two unlabeled oligonucleotides containing four conserved sites substitution of the Pho box in *anoA* and *pstS* promoter regions were synthesized for short fragment EMSA, respectively. The gels with unlabeled probe were dyed with ethidium bromide (EB) and then imaged with a gel imaging system (AlphaImager

Mini, ProteinSimple). In addition, gene reporter assay was employed to verify the conserved binding sites *in vivo*. The oligonucleotide fragment containing four mutated putative binding sites of *anoA* promoter region was synthesized and then cloned into plasmid pLSPkt2lacZ. The final construct GW4-pLSP-MPanoA (Table 1) was inoculated into 100 mL CDM medium with or without the addition of 50 μM Sb(III) with an initial $OD_{600}$ of ~ 0.3. Samples were taken regularly for measuring the β-galactosidase activities as described above.

### 2.8. Putative roles of PstS1 and PstS2 in Sb sensing

To investigate the roles of *pstS1* and *pstS2* in Sb sensing and Sb(III) oxidation, the in-frame deletion of *pstS1* and *pstS2* were also performed using plasmid pJQ200SK. The methods of gene knock-out and culture conditions for Sb(III) oxidation were the same as described above and the primers are shown in Table S1. For tryptophan fluorescence spectrometry assay, the complete gene sequence of *pstS1* or *pstS2* was PCR-cloned, overexpressed and His-tag purified as described above. Subsequently, the purified PstS1 or PstS2 was incubated with increasing concentrations of Sb(III) and Sb(V) at room temperature for 1 h. The tryptophan fluorescence quenching was monitored with an excitation wavelength of 280 nm (Liu et al., 2012) using a fluorescence spectrophotometer (PerkinElmer, Massachusetts, USA).

## 3. Results

### 3.1. Genomic information of two phoB genes in A. tumefaciens GW4

The draft genome sequencing of *A. tumefaciens* GW4 (AWGV00000000) revealed two high affinity phosphate regulator genes (*phoB1* and *phoB2*). The *phoB1* has opposite transcription direction with *pstSCAB1-phoU1* (phosphate transport system 1), and was located immediately upstream of the arsenite oxidation gene cluster *aioXSRBA* in the arsenic island (contig 215) (Fig. 1a). The *phoB2* was located immediately downstream of *phoR-pstSCAB2-phoU2* in contig 449 (phosphate transport system 2) (Fig. 1a). BlastP analysis showed that the protein sequence identity between PhoB1 and PhoB2 was 80%, indicating that they are not different copies of the same protein.

### 3.2. Sb(III) induced the expression of phoB2 but not phoB1 under low-Pi conditions

To understand how *phoB1* and *phoB2* respond to Sb(III) in strain GW4, *lacZ* reporter gene analyses were conducted under both low-Pi (50 μM Pi) and normal-Pi (2 mM Pi) conditions. Bacterial cells were each inoculated in CDM medium with or without the addition of 50 μM Sb(III) and incubated for 24 h (exponential phase). Under low Pi conditions, the addition of Sb(III) had no effect on the expression of *phoB1*::*lacZ* (Fig. 1 b), whereas the expression of *phoB2*::*lacZ* was significantly induced by Sb(III) (Fig. 1c), indicating that PhoB2 may involve in bacterial Sb(III) oxidation. However, under normal-Pi conditions, the expressions of *phoB1*::*lacZ* and *phoB2*::*lacZ* were not induced by Sb(III), and the β-galactosidase activities of these two reporter genes were extremely low (Fig. 1b and c), which is consistent with the properties of PhoB since it is the regulator under Pi limited condition. These results suggested that *phoB2* may be essential in bacterial Sb(III) oxidation under low-Pi condition.

### 3.3. Deletion of phoB2 decreased Sb(III) oxidation rate

To investigate the effects of *phoB1* and *phoB2* on bacterial Sb(III) oxidation and resistance, subsequent experiments were conducted under low-Pi condition. The mutant strains GW4-Δ*phoB1* and GW4-Δ*phoB2* and their complemented strains were constructed. Diagnostic PCR and DNA sequencing were employed to verify the successful deletion and complementation (data not shown). The seed cultures of the *A. tumefaciens* strains were cultivated in CDM medium until $OD_{600}$ reached 0.5 ~ 0.6, then the washed cells were each inoculated into fresh CDM medium with the addition of 50 μM Sb(III) and 50 μM Pi with a final $OD_{600}$ of ~ 0.35. As shown in Fig. 2a and 2c, the deletion of *phoB1* and *phoB2* did not influence bacterial growth. Sb(III) oxidation experiments showed that deletion of *phoB2* decreased Sb(III) oxidation efficiency by ~ 35%, and the phenotype of the complemented strain GW4-Δ*phoB2*-C was recovered (Fig. 2b). While the Sb(III) oxidation rate of strain GW4-Δ*phoB1* showed no significant differences compared with that of the wild type strain (Fig. 2d). The Sb(III) oxidation of the mutant strains GW4-Δ*phoB1* and GW4-Δ*phoB2* and their complemented strains were the same with those of wild-type strain GW4 in normal-Pi

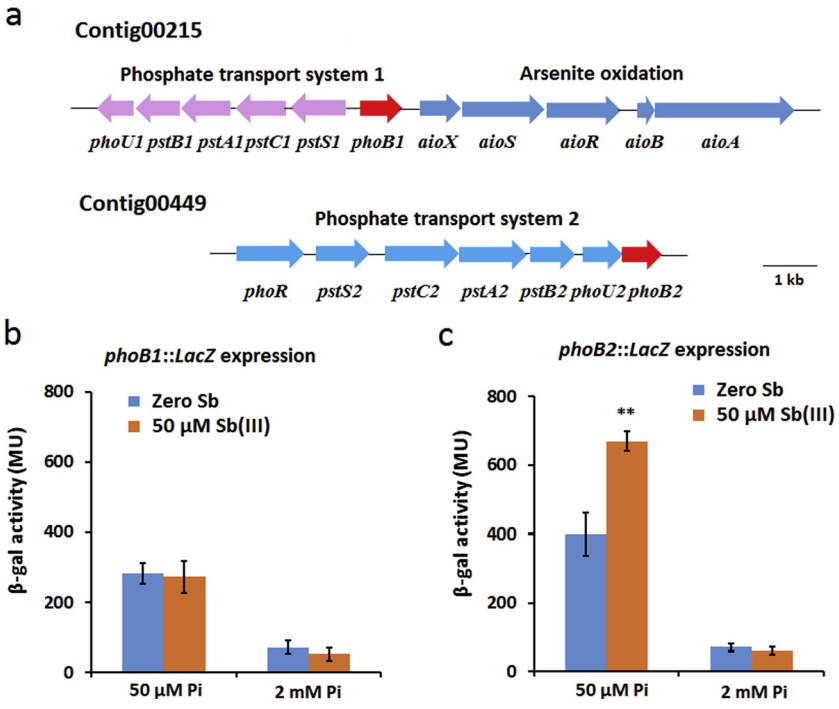


**Fig. 1.** Gene arrangement and reporter gene assays of *phoB1* and *phoB2* in *A. tumefaciens* GW4. (a) Physical map of *phoB1* and *phoB2* in strain GW4. (b–c) Reporter gene activities of *phoB1*::*lacZ* and *phoB2*::*lacZ* under both low-Pi and normal-Pi conditions. For low-Pi conditions, bacterial cells were each inoculated into CDM medium (50 μM Pi) with or without the addition of 50 μM Sb(III). For normal-Pi conditions, the phosphate buffer solution was added to the culture at a final concentration of 2 mM Pi and the culture conditions were the same as described above. Samples were taken after 24 h cultivation for testing the β-galactosidase activities. Values are mean ± SD; n = 3. Significance analysis was performed by one-way ANOVA. ** Represents $p < 0.01$.

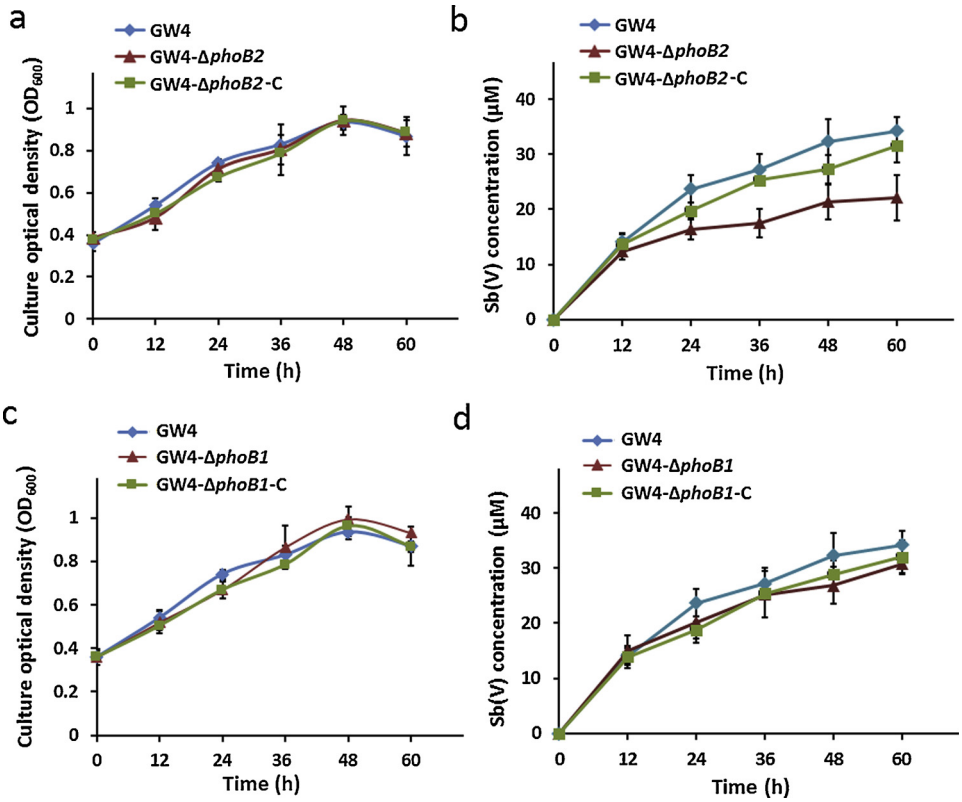


**Fig. 2.** Bacterial growth and Sb(III) oxidation curves of *A. tumefaciens* strains. (a, c) The growth curves of *A. tumefaciens* strains cultured in CDM medium in the presence of 50 μM Sb (III). (b, d) Sb(III) oxidation profiles of the *A. tumefaciens* strains under the same culture conditions. Sb(V) concentrations in the medium were measured using HPLC-HG-AFS. Values are mean ± SD; n = 3.

condition (2 mM Pi, data not shown).

### 3.4. Deletion of phoB2 decreased bacterial Sb(III) resistant level

For Sb(III) resistance assays, *A. tumefaciens* strains were cultured as described before. Cells were each inoculated into CDM medium with or without the addition of 200 μM Sb(III) with a final $OD_{600}$ of ~ 0.3. After 24 h cultivation, all the strains showed a similar growth profile without Sb(III) addition (Fig. 3). However, with the addition of 200 μM Sb(III), the growth of strain GW4-Δ*phoB2* was significantly inhibited (Fig. 3), while growth of strain GW4-Δ*phoB1* only slightly decreased compared with that of wild type strain (Fig. 3). The phenotype of the complemented strains were recovered. These results indicated that *phoB2* plays an important role in bacterial Sb(III) oxidation and resistance under low-Pi condition and the function of *phoB1* could be neglected.

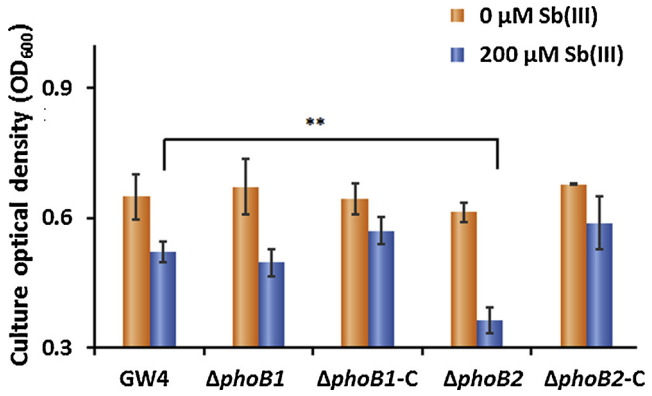


**Fig. 3.** Sb(III) resistance of *A. tumefaciens* strains under low-Pi conditions. Bacterial cells were inoculated into CDM medium (50 μM Pi) with the addition of 0 or 200 μM Sb(III) with an initial $OD_{600}$ of ~ 0.3. After 24 h cultivation, samples were taken for measuring $OD_{600}$. Data are shown as the mean of three replicates, with the error bars represents ± SD. ** Represents $p < 0.01$.

### 3.5. PhoB2 influenced the expression of antimonite oxidase gene anoA

To understand how *phoB1* and *phoB2* influenced the expression of Sb(III) oxidase gene *anoA*, *lacZ* reporter gene assays were performed in CDM broth with or without the addition of 50 μM Sb(III) during the exponential phase of bacterial growth. As shown in Fig. 4 the expression of *anoA*::*lacZ* was induced by Sb(III) in strain GW4, consistent with our previous observations (Li et al., 2015). Deletion of *phoB2* significantly decreased the *anoA*::*lacZ* expression in the presence of Sb(III) (Fig. 4a). In contrast, the expression of *anoA*::*lacZ* in strain GW4-Δ*phoB1* was slightly decreased but had no statistics significant difference compared with that of wild-type strain GW4 (Fig. 4b). These observations demonstrated that *phoB2* is essential for AnoA catalyzed enzymatic Sb(III) oxidation and *phoB1* is not responsible for the regulation of *anoA*.

### 3.6. PhoB1 and PhoB2 did not influence the non-enzymatic bacterial Sb (III) oxidation mediated by $H_2O_2$

Our previous study demonstrated that bacterial Sb(III) oxidation also include $H_2O_2$ mediated abiotic Sb(III) oxidation. The cellular $H_2O_2$ generated from oxidative stress could oxidize Sb(III) to Sb(V) in cytoplasm (Li et al., 2017a). Therefore, we also tested the cellular $H_2O_2$ contents of the *A. tumefaciens* strains. The results showed that Sb(III) addition could increase the cellular $H_2O_2$ contents in all of the *A. tumefaciens* strains (Fig. 5), which is consistent with the previous observation (Li et al., 2017a). However, statistical analysis showed that the contents of cellular $H_2O_2$ had no significant difference among strains GW4, GW4-Δ*phoB1*, GW4-Δ*phoB2*, GW4-Δ*phoB1*-C and GW4-Δ*phoB2*-C (Fig. 5), suggesting that the *phoB* genes are most likely not associated in abiotic Sb(III) oxidation.

### 3.7. PhoB2 could bind with the promoter region of anoA

BlastP analysis showed that PhoB1 and PhoB2 all contain a HTH

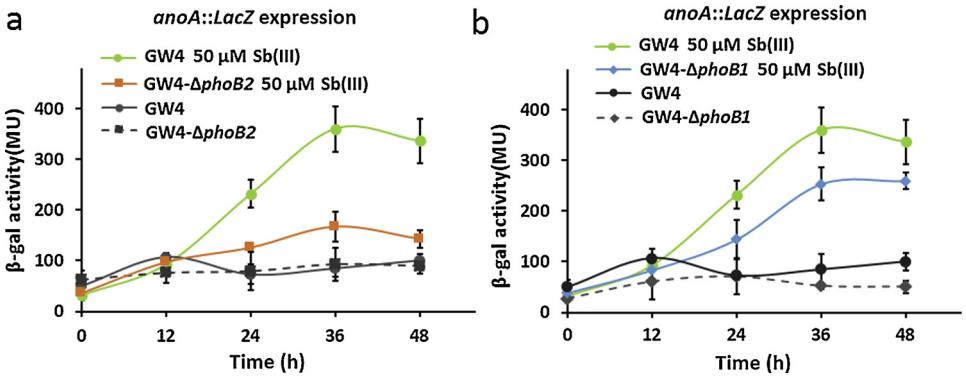


**Fig. 4.** Influences of *phoB2* (a) and *phoB1* (b) on the expression of *anoA*::*lacZ*, respectively. Bacterial cells were inoculated with an initial $OD_{600}$ of ˜0.3 into 100 mL of CDM medium with or without the addition of 50 μM Sb(III) under low-Pi (50 μM Pi) conditions. Samples were taken every 12 h for testing the β-galactosidase activities. Panels A and B share the same data of strain GW4. Values are mean ± SD; n = 3.

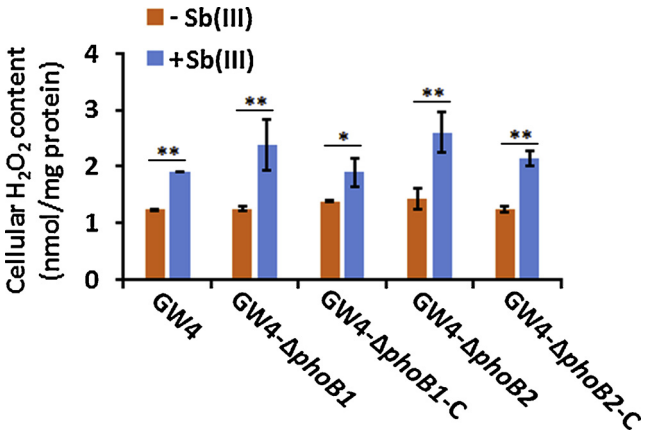


**Fig. 5.** The two *phoB* genes did not affect cellular $H_2O_2$ contents. *A. tumefaciens* strains were cultured as described above. After 36 h of incubation, samples were taken for measuring the cellular $H_2O_2$ contents. There were no significant differences on the cellular $H_2O_2$ contents among the two *phoB* mutant strains and wild type strain GW4 with the addition of Sb(III) analyzed by one-way ANOVA. Data are shown as the mean of three replicates, with the error bars represents ± SD. **Represents $p < 0.01$; *represents $p < 0.05$.

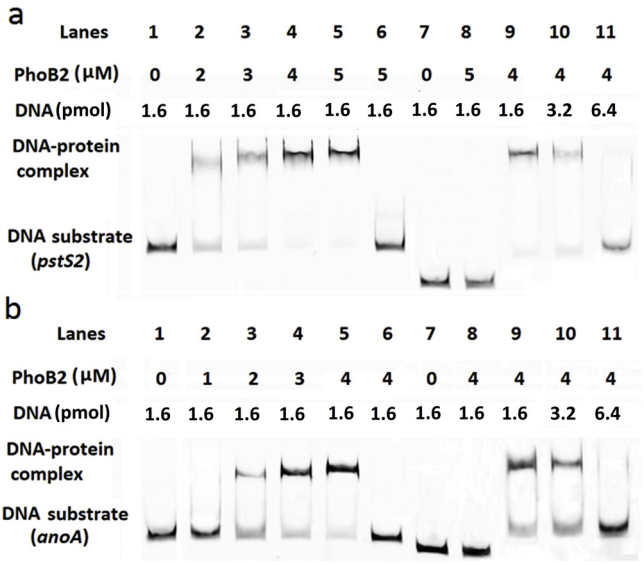


**Fig. 6.** EMSA analyses for the interaction between PhoB2 and the promoter sequences of the positive control *pstS2* (a) and *anoA* (b), respectively. Lanes 1–5, FAM-labeled DNA were co-incubated with various concentrations of PhoB2; Lane 6, FAM-labeled DNA were co-incubated with heat-denatured PhoB2; Lanes 7–8, FAM-labeled unspecific DNA (without putative Pho box) were co-incubated with PhoB; Lanes 9–11, the competition assay using FAM-labeled DNA and PhoB against different concentrations of unlabeled DNA.

domain, such domain may responsible for the binding with the promoter region of genes. However, the above experiments revealed that *phoB2* was induced by Sb(III) and was involved in bacterial Sb(III) oxidation and resistance through activating the expression of *anoA* (Fig. 1c; Fig. 2b; Fig. 3; Fig. 4a). While *phoB1* seems had no effects on Sb (III) oxidation and resistance (Fig. 1b; Fig. 2d; Fig. 3; Fig. 4b), suggesting that the function of PhoB1 and PhoB2 are different *in vivo*, and PhoB2 is more likely to responsible for regulation of Sb(III) oxidation. Thus, the subsequent regulatory experiments *in vitro* were mainly focused on PhoB2.

EMSA analysis was employed to investigate the interaction between PhoB2 and the promoter region of *anoA*. The His-tag PhoB2 was purified and detected by SDS-PAGE (Fig. S1), and a 224 bp DNA fragment was amplified using the fluorophore FAM labeled primers (Table S1). As shown in Fig. 6b, the free DNA substrates gradually disappeared with the increasing PhoB2 concentration, whereas the shifted DNA band increased. The heat-denatured PhoB2 and a non-specific DNA were used as negative controls. In addition, an unlabeled fragment as the competitor DNA inhibited the labeled probe binding to PhoB2, indicating that PhoB2 could bind specifically to the *anoA* regulatory region. As a positive control, EMSA analysis showed similar binding profile with PhoB2 and the promoter region of PstS2 (Fig. 6a).

### 3.8. The conserved sites of the putative Pho box are essential for binding

To find the putative Pho box in the promoter region of *anoA*, a Pho motif was predicted on the MEME on-line program using 25 promoter regions of *pstS* or *phoB* in *Agrobacterium* spp. strains (Fig. 7a). The 18 nt length motif contains two 7 bp direct repeats separated by a 4 bp AT rich spacer, however, it is not as conserved as the *E. coli* consensus Pho box (Diniz et al., 2011). Based on this predicted Pho motif in *Agrobacterium* spp., a putative Pho box (TTGTCACATGCATTATGA) was found located in the sense strand within 119–136 bp upstream from the *anoA* start codon (Fig. 7b). In addition, a putative Pho box (TTGTCAC AAATCTTTCGT) within 117–134 bp upstream from the *pstS2* start codon was used as a positive control (Fig. 7c). The putative PhoB binding sites of *pstS2* and *anoA* are 82% and 59% identical to the *E. coli* consensus Pho box, respectively.

Short fragment EMSA was conducted with a 38 bp DNA fragment containing the putative Pho box sequences in the promoter region of *anoA*. The putative Pho box in the promoter region of *pstS2* was used as a positive control. As shown in Fig. 7b and 7c, PhoB2 could bind with the DNA substrates containing putative Pho boxes of *pstS2* and *anoA*, respectively. Furthermore, the conserved base pairs in the Pho boxes of *pstS2* and *anoA* were found and subsequently mutated, respectively. Expectedly, there was no band shift with the site mutated Pho boxes of *pstS2* and *anoA* (Fig. 7d and 7e). In addition, the reporter gene assay showed that the expression of *anoA*::*lacZ* with the PhoB binding sites mutated promoter sequence was not induced by Sb(III) (Fig. S2). These

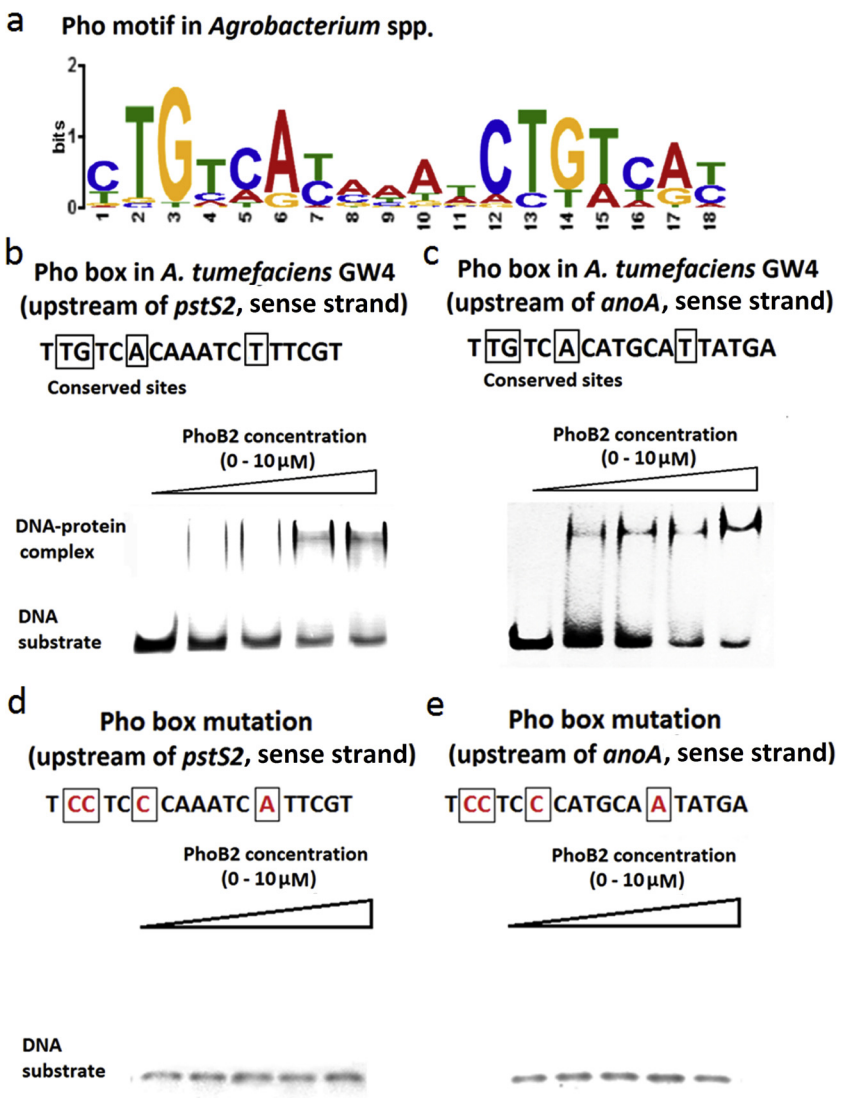


**Fig. 7.** Short fragment EMSA for identifying the putative Pho box and the conserved binding sites in promoter regions of *pstS2* and *anoA*. (a) The putative Pho motif in *A. tumefaciens* strains. The Pho motif was predicted using the MEME on-line program based on 25 promoter regions of *pstS* or *phoB* in *Agrobacterium* strains. (b–c) The putative Pho box in promoter regions of *pstS2* and *anoA*. To verification the function of the putative Pho box, 10 pmol unlabeled DNA (18 bp putative Pho box and bilateral 10 bp protection bases) was co-incubated with increasing concentrations of PhoB2 (0, 2.4, 4.8, 9.6 μM, respectively). (d–e) The site mutated Pho box in promoter regions of *pstS2* and *anoA*. To identify the conserved binding sites, the DNA sequence of the putative Pho box was site mutated and subsequently co-incubated with increasing concentrations of PhoB2. The concentrations of PhoB2 and target DNA were the same as panels b and c.

results indicated that the putative Pho boxes contain the binding sequences of PhoB2 and the conserved sites are essential for the protein-DNA interactions both *in vivo* and *in vitro*.

### 3.9. The putative role of pstS1 and pstS2 in Sb sensing

Using proteomics analysis, we observed that PstS2 was highly expressed under low Pi environment containing Sb(III), but PstS1 was not (Li et al., 2015). In this study, to investigate the potential role of *pstS1* and *pstS2* in Sb(III) sensing, the purified His-tag PstS1 and PstS2 were employed for tryptophan fluorescence spectrometry assay to examine their binding affinity with Sb(III) and Sb(V) (Fig. S3a and S3b). The results showed that there was no fluorescence quenching of PstS2 with the addition of increasing concentrations of Sb(III) (Fig. S3c), while obvious fluorescence quenching was observed with the addition of increasing concentrations of Sb(V) (Fig. S3d), indicating that PstS2 could bind with Sb(V) but not Sb(III). Furthermore, there was no fluorescence quenching of PstS1 with the addition of Sb(III) or Sb(V) (Fig. S3e and S3f), indicating that PstS1 could not bind with Sb(III) or Sb(V). These observations suggested that PstS2 may play a role in sensing Sb(V) - the product of Sb(III) oxidation. In addition, the mutant strains GW4-Δ*pstS1* and GW4-Δ*pstS2* were successfully constructed. Sb(III) oxidation experiment showed that deletion of *pstS1* or *pstS2* could not affect bacterial Sb(III) oxidation efficiency (Fig. S4), suggesting that *pstS1* or *pstS2* may not affect the expression of Sb(III) oxidase gene *anoA*.

## 4. Discussion

Previously, we identified a bacterial Sb(III) oxidase gene *anoA* in *A. tumefaciens* GW4 (Li et al., 2015). However, the regulation of *anoA* remains unknown. Proteomics and gene transcription analysis showed that the transcription of *pstS2*, which is regulated by PhoB2 was induced by Sb(III) in strain GW4 (Li et al., 2017b). As a global regulator, PhoB was reported to have many additional functions in bacteria growing under low-Pi conditions, including regulating As(III) oxidation in *Halomonas* sp. HAL1 (Chen et al., 2015). Thus, we predicted that PhoBR may also regulate Sb(III) oxidation under low-Pi conditions. Different from *Halomonas* sp. HAL1 and *E. coli*, which having only one PhoBR system (Yuan et al., 2006; Chen et al., 2015), *A. tumefaicens* GW4 has two *phoB* genes in the genome and they share the same histidine kinase gene *phoR*. To understand whether the two *phoB* genes in *A. tumefaciens* GW4 are both involved in Sb(III) oxidation, in this study, we conducted a comprehensive analysis and concluded that only PhoB2 could regulate the expression of *anoA* under low-Pi condition based on the following observations: i) The expression of *phoB2* was induced by Sb(III); ii) deletion of *phoB2* decreased bacterial Sb(III) oxidation efficiency and Sb(III) resistance; iii) deletion of *phoB2* significantly inhibited the expression of *anoA*; iv) PhoB2 could directly bind with the putative Pho box located in the regulation region of *anoA*, and the conserved sites of the Pho box were essential for binding both *in vivo* and *in vitro*.

In contrast, the expression of *phoB1* was not induced by Sb(III), and deletion of *phoB1* had no effect on Sb(III) oxidation and resistance. Moreover, the expression of *anoA* was not significantly inhibited in mutant strain GW4-Δ*phoB1*, indicating that *phoB1* is not involve in the regulation of Sb(III) oxidation. Genomic analysis showed that *phoB1* is located immediately upstream of the arsenite oxidation gene cluster *aioXSRBA*. It seems that *phoB1* may evolve in the genome through horizontal gene transfer with the arsenic gene island. Interestingly, we revealed that PhoB1 is more relevant to As(III) oxidation and regulation in strain GW4 (Qiao et al., in preparation). It is very different from *A. tumefaciens* 5 A, another model strain for study of As(III)/Sb(III) oxidation. In *A. tumefaicens* strain 5 A, the two *phoB* genes play the same role in As(III) oxidation and transphosphorylation cross-talk with AioSR (Wang et al., 2018). Compared with strain 5 A, strain GW4 has considerably higher As(III)/Sb(III) oxidation efficiency and resistance (Li et al., 2015; Wang et al., 2015). The intricate division of function of the two *phoB* genes may partially contribute to the high As(III)/Sb(III) resistance in strain GW4.

In addition, we predicted a Pho motif based on 25 putative Pho box in *A. tumefaicens* strains. The DNA sequence also contains two classical direct repeats, however, it is not as conserved as the consensus Pho motif predicted by the known Pho box in *E. coli* (Yuan et al., 2006). Furthermore, compared with the *E. coli* consensus Pho box, the Pho box in the promoter region of *pstS2* has a higher DNA sequence identity than that of *anoA* in strain GW4. The non-conservative Pho box in *A. tumefaciens* strains may enrich the diversity of genes regulated by PhoBR. Using MEME online website, about 110 putative Pho boxes were predicted (p-value < 0.0001) in the genome of strain GW4 (data not shown), which is significantly more than the number of Pho boxes in *E. coli* (Lubin et al., 2015). Thus, it seems that *phoBR* may participate in a variety of metabolic processes in strain GW4, which needs to be further investigated.

Currently, the correlation of arsenic and phosphorus metabolism has been well documented. Our previous proteomics analysis showed that the expression of proteins involved in phosphonate and phosphinate metabolisms (e.g. PhnI, PhnM, UgpB1 and UgpB2) were induced by Sb(III) in strain GW4 under low-Pi condition (CDM medium) (Li et al., 2017b), suggesting that Sb(III) oxidation and resistance may also associate with Pi metabolism. In this study, bacterial Sb(III) oxidation efficiency and resistance were not affected by the deletion of *phoB1* or *phoB2*, and the expression of *anoA* was not induced by Sb(III), under normal-Pi condition (data not shown). These observations are consistent with the function of PhoBR, which is responsible for the regulation of Pho genes under low-Pi condition (Hsieh and Wanner, 2010). It is well known that Pi exists in aquatic and terrestrial ecosystems at low concentrations (Vieira et al., 2008), hinting that the regulation of bacterial Sb(III) oxidation by PhoB may be widespread in the environment. The results has a significant value for understanding Sb biogeological cycle.

It has been demonstrated that PstS plays an important role in the signal sensing of Pi uptake under low-Pi conditions (Vuppada et al., 2018). Using proteomics analysis, we found that the expression of PstS2 was up-regulated by the addition of Sb(III) (Li et al., 2015), however, in this study, we found that PstS2 could bind with Sb(V). Since the protein samples for proteomics analysis was taken in 36 h of cultivation (Li et al., 2015), in such time point, some Sb(III) had already been oxidized to Sb(V). In combination with the results of proteomics and tryptophan fluorescence spectrometry assay (this study), we think that the real sensing signal of PstS2 is Sb(V).

In addition, the PhoBR has been found be capable of transphosphorylation cross-talk with arsenite two-component system AioSR in *A. tumefaicens* strains 5 A. The amino acid sequences of PhoR, PhoB1, PhoB2 and AioS between strain 5 A and GW4 are highly homologous (91%, 80%, 100% and 84%, respectively). Furthermore, As(V) could promote bacterial growth and may replace Pi in arsenolipids in both strain 5 A and GW4 under low-Pi conditions (Wang et al., 2018). As a global regulator, PhoB may receive the phosphorylation signal from different sensor proteins. Thus, we cannot exclude the possibility that PhoB may work in a two-component system to receive the phosphoryl moiety from other kinases for the regulation of AnoA's expression in strain GW4 (e.g. AioS). The signal sensing and transmission mechanism of PhoB2-dependent regulation of Sb(III) oxidation still needs to be further studied. Understanding of the regulation mechanism of Sb(III) oxidation will provide basis for bioremediation application of environmental Sb contamination.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.04.008.

## References

CEC, Council of the European Communities, 1976. Council Directive 76/464/EEC of 4 May 1976 on Pollution Caused by Certain Dangerous Substances Discharged Into the Aquatic Environment of the Community. Belgium OJEC L-129, Brussels, pp. 23–29.
Chen, F., Cao, Y., Wei, S., Li, Y., Li, X., Wang, Q., Wang, G., 2015. Regulation of arsenite oxidation by the phosphate two-component system PhoBR in *Halomonas* sp. HAL1. Front. Microbiol. 6, 923.
Crépin, S., Chekabab, S.M., Le Bihan, G., Bertrand, N., Dozois, C.M., Harel, J., 2011. The Pho regulon and the pathogenesis of Escherichia coli. Vet. Microbiol. 153, 82–88.
diCenzo, G.C., Sharthiya, H., Nanda, A., Zamani, M., Finan, T.M., 2017. PhoU allows rapid adaptation to high phosphate concentrations by modulating PstSCAB transport rate in Sinorhizobium meliloti. J. Bacteriol. 199 e00143–17.
Diniz, M.M., Goular, C.L., Barbosa, L.C., Farache, J., Lery, L.M., Pacheco, A.B., Bisch, P.M., von Krüger, W.M., 2011. Fine-tuning control of *phoBR* expression in *Vibrio cholera* by binding of PhoB to multiple Pho boxes. J. Bacteriol. 193, 6929–6938.
Fan, H., Su, C., Wang, Y., Yao, J., Zhao, K., Wang, Y., Wang, G., 2008. Sedimentary arsenite-oxidizing and arsenate-reducing bacteria associated with high arsenic groundwater from Shanyin, Northwestern China. J. Appl. Microbiol. 105, 529–539.
Filella, M., Belzile, N., Chen, Y.W., 2002a. Antimony in the environment: a review focused on natural waters: I. Occurrence. Earth-Sci.. Rev. 57 (1), 125–176.
Filella, M., Belzile, N., Chen, Y.W., 2002b. Antimony in the environment: a review focused on natural waters: II. Relevant solution chemistry. Earth-Sci. Rev. 59 (1), 265–285.
He, M., Wang, X., Wu, F., Fu, Z., 2012. Antimony pollution in China. Sci. Total Environ. 421, 41–50.
Hockmann, K., Lenz, M., Tandy, S., Nachtegaal, M., Janousch, M., Schulin, R., 2014. Release of antimony from contaminated soil induced by redox changes. J. Hazard. Mater. 275, 215–221.
Hsieh, Y.J., Wanner, B.L., 2010. Global regulation by the seven component Pi signaling system. Curr. Opin. Microbiol. 13, 198–203.
Kang, Y.S., Heinemann, J., Bothner, B., Rensing, C., McDermott, T.R., 2012. Integrated co-regulation of bacterial arsenic and phosphorus metabolisms. Environ. Microbiol. 14, 3097e3109.
Lamarche, M.G., Dozois, C.M., Daigle, F., Caza, M., Curtiss 3rd, R., Dubreuil, J.D., Harel, J., 2005. Inactivation of the *pst* system reduces the virulence of an avian pathogenic *Escherichia coli* O78 strain. Infect. Immun. 73, 4138–4145.
Lamarche, M.G., Wanner, B.L., Crepin, S., Harel, J., 2008. The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. FEMS Microbiol. Rev. 32, 461–473.
Li, J., Wang, Q., Zhang, S., Qin, D., Wang, G., 2013. Phylogenetic and genome analyses of antimony-oxidizing bacteria isolated from antimony mined soil. Int. Biodet. Biodegr. 76, 76–80.
Li, J., Wang, Q., Li, M., Yang, B., Shi, M., Guo, W., McDermott, T.R., Rensing, C., Wang, G., 2015. Proteomics and genetics for identification of a bacterial antimonite oxidase in Agrobacterium tumefaciens. Environ. Sci. Technol. 49 (10), 5980–5989.

Li, J., Wang, Q., Oremland, R.S., Kulp, T.R., Rensing, C., Wang, G., 2016. Microbial antimony biogeochemistry: enzymes, regulation and related metabolic pathways. Appl. Environ. Microbiol. 82 (18), 5482–5495.
Li, J., Yang, B., Shi, M., Yuan, K., Guo, W., Wang, Q., Wang, G., 2017a. Abiotic and biotic factors responsible for antimonite oxidation in *Agrobacterium tumefaciens* GW4. Sci. Rep. 74, 3225.
Li, J., Yang, B., Shi, M., Yuan, K., Guo, W., Wang, Q., Wang, G., 2017b. Effects upon metabolic pathways and energy production by Sb(III) and As(III)/Sb(III)-oxidase gene *aioA* in *Agrobacterium tumefaciens* GW4. PLoS One 12 (2), e0172823.
Lialikova, N.N., 1974. *Stibiobacter senarmontii*: a new microorganism oxidizing antimony. Mikrobiologiian. 43, 941–943.
Link, A.J., Phillips, D., Church, G.M., 1997. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. J. Bacteriol. 179, 6228–6237.
Liu, G., Liu, M., Kim, E.H., Maaty, W.S., Bothner, B., Lei, B., Rensing, C., Wang, G., McDermott, T.R., 2012. A periplasmic arsenite-binding protein involved in regulating arsenite oxidation. Environ. Microbiol. 14, 1624–1634.
Lubin, E.A., Henry, J.T., Fiebig, A., Crosson, S., Laub, M.T., 2015. Identification of the PhoB regulon and role of PhoU in the phosphate starvation response of Caulobacter crescentus. J. Bacteriol. 198 (1), 187–200.
Makino, K., Amemura, M., Kim, S.K., Nakata, A., Shinagawa, H., 1993. Role of the sigma 70 subunit of RNA polymerase in transcriptional activation by activator protein PhoB in *Escherichia coli*. Genes Dev. 7, 149–160.
Miller, J.H., 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 431.
Monds, R.D., Silby, M.W., Mahanty, H.K., 2001. Expression of the Pho regulon negatively regulates biofilm formation by *Pseudomonas aureofaciens* PA147-2. Mol. Microbiol. 42, 415–426.
Pelicic, V., Reyrat, J.M., Gicquel, B., 1996. Expression of the *Bacillus subtilis sacB* gene confers sucrose sensitivity on mycobacteria. J. Bacteriol. 17, 1197–1199.
Pratt, J.T., McDonough, E., Camilli, A., 2009. PhoB regulates motility, biofilms, and Cyclic di-GMP in Vibrio cholerae. J. Bacteriol. 191, 6632–6642.
Santos-Beneit, F., 2015. The Pho regulon: a huge regulatory network in bacteria. Front. Microbiol. 6, 402.
Srinivasan, V.B., Venkataramaiah, M., Mondal, A., Vaidyanathan, V., Govil, T., Rajamohan, G., 2012. Functional characterization of a novel outer membrane porin KpnO, regulated by PhoBR two-component system in *Klebsiella pneumoniae* NTUH-K2044. PLoS One 7, 18.
Terry, L.R., Kulp, T.R., Wiatrowski, H., Miller, L.G., Oremland, R.S., 2015. Microbiological oxidation of antimony(III) with oxygen or nitrate by bacteria isolated from contaminated mine sediments. Appl. Environ. Microbiol. 81, 8478–8488.
USEPA, United States Environmental Protection Agency, 1979. Water Related Fate of the 129 Priority pollutants; EP-440/4-79-029A. Washington, DC. .
Vieira, R.P., Gonzalez, A.M., Cardoso, A.M., Oliveira, D.N., Albano, R.M., Clementino, M.M., Martins, O.B., Paranhos, R., 2008. Relationships between bacterial diversity and environmental variables in a tropical marine environment, Rio de Janeiro. Environ. Microbiol. 10, 189–199.
Vuppada, R.K., Hansen, C.R., Strickland, K.A.P., Kelly, K.M., McCleary, W.R., 2018. Phosphate signaling through alternate conformations of the PstSCAB phosphate transporter. BMC Microbiol. 18 (1), 8.
Wang, Q., Qin, D., Zhang, S., Wang, L., Li, J., Rensing, C., McDermott, T.R., Wang, G., 2015a. Fate of arsenate following arsenite oxidation in *Agrobacterium tumefaciens* GW4. Environ. Microbiol. 17, 1926–1940.
Wang, Q., Warelow, T.P., Kang, Y.S., Romano, C., Osborne, T.H., Lehr, C.R., Bothner, B., McDermott, T.R., Santini, J.M., Wang, G., 2015b. Arsenite oxidase also functions as an antimonite oxidase. Appl. Environ. Microbiol. 81 (6), 1959–1965.
Wang, Q., Kang, Y.S., Alowaifeer, A., Shi, K., Fan, X., Wang, L., Jetter, J., Bothner, B., Wang, G., McDermott, T.R., 2018. Phosphate starvation response controls genes required to synthesize the phosphate analog arsenate. Environ. Microbiol. 20 (5), 1782–1793.
Weeger, W., Lièvremont, D., Perret, M., Lagarde, F., Hubert, J.C., Leroy, M., Lett, M.C., 1999. Oxidation of arsenite to arsenate by a bacterium isolated from an aquatic environment. Biometals. 12, 141–149.
Yuan, Z.C., Zaheer, R., Morton, R., Finan, T.M., 2006. Genome prediction of PhoB regulated promoters in *Sinorhizobium meliloti* and twelve proteobacteria. Nucleic Acids Res. 34, 2686–2697.