Environmental Pollution 235 (2018) 700–709

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Proteomics and genetic analyses reveal the effects of arsenite oxidation on metabolic pathways and the roles of AioR in *Agrobacterium tumefaciens* GW4☆

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ARTICLE INFO

Article history:
Received 27 July 2017
Received in revised form
2 January 2018
Accepted 2 January 2018

Keywords:
Arsenite oxidation regulator
Comparative proteomics
Phosphate
Arsenite resistance
Energy

ABSTRACT

A heterotrophic arsenite [As(III)]-oxidizing bacterium *Agrobacterium tumefaciens* GW4 isolated from As(III)-rich groundwater sediment showed high As(III) resistance and could oxidize As(III) to As(V). The As(III) oxidation could generate energy and enhance growth, and AioR was the regulator for As(III) oxidase. To determine the related metabolic pathways mediated by As(III) oxidation and whether AioR regulated other cellular responses to As(III), isobaric tags for relative and absolute quantitation (iTRAQ) was performed in four treatments, GW4 (+AsIII)/GW4 (-AsIII), GW4-Δ*aioR* (+AsIII)/GW4-Δ*aioR* (-AsIII), GW4-Δ*aioR* (-AsIII)/GW4 (-AsIII) and GW4-Δ*aioR* (+AsIII)/GW4 (+AsIII). A total of 41, 71, 82 and 168 differentially expressed proteins were identified, respectively. Using electrophoretic mobility shift assay (EMSA) and qRT-PCR, 12 genes/operons were found to interact with AioR. These results indicate that As(III) oxidation alters several cellular processes related to arsenite, such as As resistance (*ars* operon), phosphate (Pi) metabolism (*pst/pho* system), TCA cycle, cell wall/membrane, amino acid metabolism and motility/chemotaxis. In the wild type with As(III), TCA cycle flow is perturbed, and As(III) oxidation and fermentation are the main energy resources. However, when strain GW4-Δ*aioR* lost the ability of As(III) oxidation, the TCA cycle is the main way to generate energy. A regulatory cellular network controlled by AioR is constructed and shows that AioR is the main regulator for As(III) oxidation, besides, several other functions related to As(III) are regulated by AioR in parallel.

1. Introduction

Arsenic (As) is widespread in the environment and is related to human health. The most common As species are arsenite [As(III)] and arsenate [As(V)] (Cai et al., 2009). Of these two species, As(III) is more toxic than As(V) (Zhu et al., 2014). Microbes are the principal drivers of arsenic transformation, and the oxidation of As(III) to As(V) results in less toxic and less mobile species (Stolz et al., 2006). Some As(III)-oxidizing bacteria have evolved to use the energy generated from As(III) oxidation in nature, especially chemoautotrophic As(III)-oxidizing bacteria that assimilate inorganic carbon using As(III) as the electron donor (Rhine et al., 2007; Santini et al., 2000). In addition, some heterotrophic As(III)-oxidizing strains, such as *Hydrogenophaga* sp. NT-14 and *A. tumefaciens* GW4 (vanden Hoven and Santini, 2004; Wang et al., 2015), could also gain energy from the As(III) oxidation reaction, suggesting that some heterotrophic As(III) oxidizers could generate energy from both carbohydrate metabolism and As(III) oxidation; however, the mechanisms of energy generation for heterotrophic As(III) oxidizers remain unknown.

As and phosphorus are both members of Group 15 on the periodic table, indicating they are structural analogs and that As(V) and phosphate may be co-metabolized (Chen et al., 2015). In *H. arsenicoxydans* ULPAs1, phosphate import ATP-binding protein (PstB1) and putative phosphate uptake regulator (PhoU1) were induced by 3 or 4 fold in the presence of As(III) (Weiss et al., 2009). Of 54 organisms containing the As(III) oxidase *aioBA* genes, 11 genes encoding proteins related to various functions associated with phosphorus acquisition were located directly adjacent to, or very nearby, the *aio* genes (Kang et al., 2012). These genes were expressed in response to phosphate starvation and included *pst* and *pho* genes encoding proteins for high-affinity Pi transport or

☆ This paper has been recommended for acceptance by Dr. Jorg Rinklebe.

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https://doi.org/10.1016/j.envpol.2018.01.006
0269-7491/

regulatory functions (Li et al., 2013). The *pst1/pho1* system was located within As islands, while the *pst2/pho2* system was localized distantly on the respective chromosomes (Li et al., 2013). In *A. tumefaciens* 5A, phosphate starvation regulators PhoB1 and PhoB2 were involved in the expression of three-component signal transduction system including the periplasmic As(III)-binding protein AioX, the sensor kinase AioS, its cognate response regulator AioR and AioBA (Kang et al., 2012), and the expression of *aioBA* was proven to be regulated by PhoB in *Halomonas* sp. HAL1 (Chen et al., 2015). These observations revealed that there was a link between As(III) oxidation and the *pst/pho* system.

Previously, cellular global responses to As(III) have been analyzed by proteomic and transcriptomic studies. In chemoautotrophic As(III)-oxidizing strains, the ribulose-1,5-biphosphate carboxylase (RuBisCo) and fructose-1,6-biphosphatase involved with CO_2 fixation were up-regulated in the presence of As(III), indicating that As metabolism, carbon assimilation, and energy acquisition were linked in chemoautotrophic As(III)-oxidizing bacteria, and As(III) oxidation was a mechanism for generating bioenergy (Bryan et al., 2009; Andres et al., 2013). In the heterotrophic As(III)-oxidizing strains *Herminiimonas arsenicoxydans* ULPAs1 and *Pseudomonas aeruginosa* PAO1, the proteins involved in carbohydrate metabolism were all up-regulated (Bryan et al., 2009; Andres et al., 2013). The increased expression of enzymes involved in carbohydrate metabolism represent an important means of adaptation during stress such as that resulting from the exposure of bacteria to heavy metals (Mallik et al., 2012). In addition, the differentially expressed proteins and genes were mainly involved with As(III) oxidation, As resistance and efflux, stress response, exopolysaccharide synthesis and phosphate mechanism (Bryan et al., 2009; Andres et al., 2013). Another common defense mechanism against As(III) exposure results from the induction of the As resistance and efflux (*ars* operon) (Kashyap et al., 2006). As(V) was reduced by a cytoplasmic As(V) reductase (ArsC) to the more toxic As(III), and then As(III) was extruded by a membrane-associated ArsB or Acr3 efflux pump (Kashyap et al., 2006). In addition, the alteration of membrane permeability has been proven to be another As(III) resistance mechanism of Gram-negative bacteria (Weiss et al., 2009). It leads to the adsorption of As(III) into the lipopolysaccharide layer of the outer membrane of these bacteria, and strains produce a thick capsule of exopolysaccharides, which has been shown to scavenge arsenic as granules (Weiss et al., 2009). Overall, As(III) oxidation in heterotrophic As(III) oxidizers was mainly detoxification and stress response mechanisms.

In a previous study, we isolated the highly As(III) resistant [minimal inhibitory concentration (MIC) = 25 mM] and As(III)-oxidizing bacterium *A. tumefaciens* GW4 from As-enriched groundwater sediments (Fan et al., 2008). Unlike most of the heterotrophic As(III)-oxidizing bacteria using As(III) oxidation as a detoxification process, the As(III) oxidation of strain GW4 enhanced the bacterial growth, and the strain showed positive chemotaxis toward As(III) (Wang et al., 2015; Shi et al., 2017). However, the mutant strain GW4-Δ*aioR* failed to demonstrate increased growth, and its As(III) oxidation and As(III) chemotaxis phenotypes were both disrupted (Shi et al., 2017). It appeared that the effect of As(III) oxidation in strain GW4 was different from that in all the well-recognized heterotrophic and chemoautotrophic As(III)-oxidizing strains. Genes *aioBA* are regulated by AioXSR (Liu et al., 2012; Li et al., 2013). It has been revealed that AioR was the key factor to regulate bacterial As(III)-oxidation in *A. tumefaciens* 5A (Liu et al., 2012). However, AioR was also expressed at a low level without As(III) in *A. tumefaciens* GW4 (Fig. S1). The consensus DNA-binding sequence GT[TC][AC][CG][GCT][AG][AG]A[ACT][CGA][GCT][GTA]AAC has been documented for the regulator AioR (Shi et al., 2017), and the AioR putative binding sites have been found at 49 locations on the *Rhizobium* sp. NT-26 chromosome (Andres et al., 2013). Based on the phenotypic characteristics of strain GW4, we speculated that As(III) may be involved with several different metabolism pathways, and AioR may regulate other cellular functions besides As(III) oxidation.

Thus, in this study, we developed *A. tumefaciens* GW4 as a model to understand the alteration of global metabolism pathways with As(III) oxidation, and the regulatory roles of AioR. Using isobaric tags for relative and absolute quantitation (iTRAQ) proteomics combined with the electrophoretic mobility shift assay (EMSA) and gene transcription analyses, we found that As(III) oxidation is related to several metabolic pathways, and AioR is the main regulator of As(III) oxidation; however, it is also related to several cellular processes, especially with phosphate metabolism and As(III) resistance.

2. Materials and methods

2.1. Strain and culture condition

A. tumefaciens GW4 was grown at 28 °C in a defined minimal mannitol medium $MMNH_4$ containing 55 mM mannitol as the primary carbon source and modified to contain 0.1 mM phosphate. As noted, 1.0 mM $NaAsO_2$ [As(III)] was added to the medium. When required, 50 μg mL^{-1} of kanamycin (Kan), 50 μg mL^{-1} chloramphenicol of (Cm) or 100 μg mL^{-1} of ampicillin (Amp) was added (Shi et al., 2017; Somerville and Kahn, 1983). Strains GW4-Δ*aioR* and GW4-Δ*aioR*-C have been constructed in our previous study (Shi et al., 2017).

2.2. Protein preparation

Four experimental groups GW4 (+AsIII)/GW4 (-AsIII), GW4-Δ*aioR* (+AsIII)/GW4-Δ*aioR* (-AsIII), GW4-Δ*aioR* (-AsIII)/GW4 (-AsIII) and GW4-Δ*aioR* (+AsIII)/GW4 (+AsIII) were designed. Total protein was extracted from the controls, the As(III) treated strain GW4 and GW4-Δ*aioR* cells. The cells were collected by centrifugation and freeze-dried, and then 200 μL of L3 buffer (the composition was undisclosed by Gene-create Company) and 800 μL pre-cooling acetone (containing 10 mM final concentration of dithiothreitol) were added. Then, samples were centrifuged at 13,000 rpm for 20 min to remove supernatant. The same step was performed one more time, and the sediment was dissolved into 100 μL L3 buffer. The concentration of protein was measured using the Bradford method (Martinez-Esteso et al., 2014).

2.3. iTRAQ labeling and strong cation exchange

Proteins (100 mg) from each sample were blended with 500 μL 50 mM NH_4HCO_3 and 2 μg Trypsin for 8–16 h at 37 °C. Tryptic peptides were then added with isometric 0.1% formic acid to acidification dispose. Strata −X C18 column was activated by 1 mL methanol and balanced by 1 mL 0.1% formic acid. The acidulating Tryptic peptides was added into the strata −X C18 column thrice, and then 0.1% formic acid +5% acetonitrile was used to clean the column twice. At last, 1 mL 0.1% formic acid and 80% acetonitrile were added into the column to collect the solution, and then the sample was performed by vacuum drying treatment. Dry powder peptides was diluted with 0.5 M TEAB to 20 μL, and sample was labeled with 8-plex (Martinez-Esteso et al., 2014).

2.4. Protein identification by MS/MS

The instrument was AB SCIEX nanoLC-MS/MS (Triple TOF 5600 plus). The analytical column was AB SCIEX column (inside radius