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Anoxygenic phototrophic arsenite oxidation by a *Rhodobacter* strain

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Abstract

Microbially mediated arsenic redox transformations are key for arsenic speciation and mobility in rice paddies. Whereas anaerobic anoxygenic photosynthesis coupled to arsenite (As(III)) oxidation has been widely examined in arsenic-replete ecosystems, it remains unknown whether this light-dependent process exists in paddy soils. Here, we isolated a phototrophic purple bacteria, *Rhodobacter* strain CZR27, from an arsenic-contaminated paddy soil and demonstrated its capacity to oxidize As(III) to arsenate (As(V)) using malate as a carbon source photosynthetically. Genome sequencing revealed an As(III)-oxidizing gene cluster (*aioXSRBA*) encoding an As(III) oxidase. Functional analyses showed that As(III) oxidation under anoxic phototrophic conditions correlated with transcription of the large subunit of the As(III) oxidase *aioA* gene. Furthermore, the non-As(III) oxidizer *Rhodobacter capsulatus* SB1003 heterologously expressing *aioBA* from strain CZR27 was able to oxidize As(III), indicating that *aioBA* was responsible for the observed As(III) oxidation in strain CZR27. Our study provides evidence for the presence of anaerobic photosynthesis-coupled As(III) oxidation in paddy soils, highlighting the importance of light-dependent, microbe-mediated arsenic redox changes in paddy arsenic biogeochemistry.

INTRODUCTION

Arsenic, a toxic metalloid and a non-threshold carcinogen, is widespread in the environment (Nordstrom, 2002). Large areas of paddy soils in South and Southeast Asia are contaminated with arsenic from geogenic sources, and anthropogenic activities such as irrigation with arsenic-tainted groundwater, mining and usage of arsenic-contained herbicides (Harvey et al., 2002; Islam et al., 2004; Meharg, 2004). Rice is particularly efficient in arsenic accumulation in comparison with other cereal crops (Su et al., 2010; Zhao et al., 2010). This poses a health risk to millions of people, particularly those in South and Southeast Asia who depend on rice as the main staple food (Zhao et al., 2010). The biogeochemical cycle of arsenic depends on microbial transformations, which affect the mobility and distribution of arsenic species in the environment (Oremland & Stolz, 2003; Zhu et al., 2014). The oxidation state of inorganic arsenic governs the toxic effects of arsenic (Cullen & Reimer, 1989).

Trivalent inorganic arsenite (As(III)) is more mobile and toxic than pentavalent arsenate (As(V)). Environmental As(III) is oxidized to As(V) both biologically and abiotically (Ehrlich, 2002). In Bangladesh and India, where the paddy soils are irrigated with As(III)-tainted groundwater, about 80%–100% of the arsenic in standing water is in the form of arsenate (As(V)) (Stroud et al., 2011). This indicates that As(III) oxidation occurs in the surface water of paddy soils as a result of microbial, photochemical or other oxidation pathways (Roberts et al., 2007). The oxidation of As(III) by microorganisms has promising implications in the biogeochemical cycling of arsenic, in part due to the lower toxicity and mobility of As(V) compared with As(III). Numerous phylogenetically diverse As(III)-oxidizers have been isolated from various habitats (Inskeep et al., 2007; Quemeneur et al., 2010). In some gram-negative bacteria, As(III) is oxidized by the *aio* and *arx* systems (Andres & Bertin, 2016). Bacterial oxidation of As(III) may serve as a source of electrons for the respiration of oxygen, nitrate and chlorate (Santini et al., 2000; Sun et al., 2009, 2010). Microbially mediated As(III) oxidation can also occur under anoxic conditions in light (Budinoff & Hollibaugh, 2008; Kulp et al., 2008). Expression of anaerobic As(III) oxidizing gene *arxA* was recently shown to be involved in light-dependent As(III) oxidation (Hernandez-Maldonado et al., 2017).

Rhodobacter species are facultative purple non-sulfur bacteria that use either organic substrates or CO_2 as carbon sources under anoxic phototrophic conditions (Pfennig & Trüper, 1989). These species are frequently detected in soil and aquatic environments and shown to mediate oxidation of metals (such as Mn(II) and Fe(II)) (Anderson et al., 2011; Braeuer et al., 2011; Ehrenreich & Widdel, 1994). Duckweeds are a common macrophyte in paddy environments (Zhang et al., 2009). At the duckweed–water interface, As(III) is produced by duckweed via reduction from As(V) to As(III) and then is quickly re-oxidized to As(V) by bacterial communities in the phyllosphere, suggesting that the prevalence of As(V) in the surface water is due to microbial As(III) oxidation (Xie et al., 2014). The As(III) oxidase large subunit AioA, which belongs to the DMSO reductase family, is a molybdopterin reductase (Santini & vanden Hoven, 2004). It has been widely used as a biomarker for reflecting As(III)-oxidizing communities in the environment (Quemeneur et al., 2008). Based on analysis of an *aioA* gene library, sequences similar to the *aioA* gene of *Rhodobacter* species were detected in the duckweed phyllosphere (Xie et al., 2014). This finding prompted us to examine several *Rhodobacter* strains for arsenic biotransformation. Previous studies on As(III) oxidation in paddy soils focused primarily on the sequencing-derived diversity of As(III) oxidizers (Jia et al., 2014; Xie et al., 2014). Those studies largely ignored the actual involvement of anoxygenic photosynthetic bacteria in As(III) oxidation. Thus, it is not clear whether *Rhodobacter* species participate directly in phototrophic As(III) oxidation.

In this study, we isolated an anaerobic As(III)-oxidizer (*Rhodobacter* strain CZR27) from the duckweed phyllosphere. This bacterium shows efficient As(III) oxidation activity. To elucidate the mechanism of As(III) oxidation in the photosynthetic bacterium *Rhodobacter* sp. strain CZR27, heterologous complementation was used to identify the genes involved in As(III) oxidation. We demonstrated that the *aioXSRBA* operon from CZR27 confers light-dependent As(III) oxidation activity in *R. capsulatus* strain SB1003. Our discovery of a phototrophic As(III)-oxidizer in paddy soil also provide basis and incentive to future investigate its ecological distribution and geochemical significance in analogous systems such wetland, peatland and floodplain.

EXPERIMENTAL PROCEDURES

Bacterial strains, growth media, and culture conditions

All bacterial strains and plasmids used in this study are listed in Table S1. All bacteria employed in this study were strains of the phototrophic purple non-sulfur bacterium *Rhodobacter* species; strains and their origins were as follows: The anaerobic phototrophic As(III)-oxidizing bacterium strain *Rhodobacter* sp. CZR27 was isolated from enrichment cultures of arsenic-contaminated paddy soils from Chenzhou, Hunan province, China (see Text S1 in the Supporting Information). Chenzhou paddy soil had a relatively high arsenic concentration in the standing water was (70 μg As L^{-1}), with As(V) accounting for 85% of the total concentration. *Rhodobacter* strain SW2 (Ehrenreich & Widdel, 1994) was kindly provided by Prof. Andreas Kappler, and *R. capsulatus* strain SB1003 was obtained from the Biological Resource Center, NITE (NBRC), Japan.

For anaerobic phototrophic growth of *Rhodobacter* strains SB1003 and CZR27, strains were cultured in an anoxic minimal-salts medium (RCV) (pH 6.8) (Weaver et al., 1975), supplemented with 22 mM $NaHCO_3$ per litre, and 10 mM malate as electron donor. *Rhodobacter* strain SW2 was grown in RCV medium, with H_2 as an electron donor. Incubation was illuminated (200 μmol m^{-2} s^{-1}) from a 40 W incandescent light bulb installed above the culture bottles at a distance of 20 cm at 30°C, except for strain SW2, which was incubated at 20°C. All cultures were cultivated phototrophically (illuminated anoxic conditions) in 25 mL anoxic tubes or 120 mL serum bottles.

For gene complementation experiments, *Rhodobacter* strain SB1003 and its derivative strains were grown aerobically in Luria-Bertani (LB) medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl) at 30°C. *Escherichia coli* strain WM3064 (Saltikov & Newman, 2003) was cultivated aerobically in LB medium at 37°C with the addition of 50 mg L^{-1} D,L-α,ε-diaminopimelic acid. Where appropriate, rifampicin (50 mg L^{-1}) and kanamycin (50 mg L^{-1}) were added for the selection of transformants when mobilizing plasmids into strain SB1003.

As(III) resistance and biotransformation in strain CZR27

CZR27 cultures were grown to mid-logarithmic phase in RCV medium anaerobically in the light, and then 1% of the cell cultures were inoculated into fresh RCV medium supplement with 10 mM malate. As(III) was spiked into the medium at indicated concentrations (0, 50, 100, 150, and 200 μM) to investigate cell growth and As(III) transformation by strain CZR27 under anoxic phototrophic conditions. Aliquots of 1 mL culture were taken at different time points after the addition of As(III). Arsenic species in the filtered samples were analysed by HPLC-ICP-MS. The cell concentration was determined by the measurement of protein concentration as described previously (Ehrenreich & Widdel, 1994). The experiment was conducted in triplicate. Sterile controls were prepared by autoclaving (121°C at 250 kPa for 25 min).

Assay of As(III) oxidation by *Rhodobacter* strains

Although no significant As(III) oxidation was observed in the uninoculated control, it was not clear whether the intermediate metabolite products generated by phototrophic strains were directly involved in the abiotic oxidation of As(III). We tested this with cell-free supernatant. *Rhodobacter* strains were grown in RCV medium containing malate or H_2. At mid-log phase, the cells were harvested and separated from the culture medium by filtration through a 0.22 μm filter inside an anoxic glovebox (UNIlab Plus; MBraun, Germany) containing 100% N_2 atmosphere. The cell-free supernatant was divided into two aliquots. The first aliquot was immediately tested for its ability to oxidize As(III). To determine chemical (nonenzymatic) oxidation of As(III), the second aliquot of cell-free supernatant was autoclaved to inactivate the enzymes. Both samples spiked with 50 μM As(III) were incubated in serum bottles for 48 h at 30°C under anoxic conditions in the light. At the end of the incubation period, the samples were collected, and oxidation of As(III) was tested as described before. All manipulations were performed inside the anaerobic glove box. The filtered cell-free supernatant and the heat-treated supernatant were checked for viable cells by incubating an aliquot in a fresh RCV medium.

Genome sequencing and phylogenetic analyses

Genomic DNA of strain CZR27 was extracted by the phenol-chloroform method (Sambrook & Russel, 2011). The 16S rRNA gene was amplified by PCR using the universal primer 27F and 1492R as described previously (Lane, 1991). The CZR27 genome was sequenced using Pacific Biosciences (PacBio) technology by Biozeron Biotechnology Co., Ltd. (Shanghai, China). BLASTn and BLASTp were used for identity searches of the nucleotide sequence and deduced amino acid, respectively. The phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) with MEGA version 6.0 (Tamura et al., 2013).

Heterologous expression of *aio* genes in non-As(III) oxidizer SB1003

Considering the key role of *aioXSRBA* in As(III) oxidation that has been reported in a variety of bacteria strains (Branco et al., 2009; Kashyap et al., 2006), and the transcriptional response of *aioA* to As(III), we hypothesized that that the *aioXSRBA* gene cluster in CZR27 is involved in anaerobic phototrophic As(III) oxidation. To test this hypothesis, we made numerous attempts to disrupt the *aioA* or *aioB* gene in strain CZR27. Unfortunately, all our attempts failed. Alternatively, to evaluate the role of these genes in the As(III) oxidation process, we constructed plasmids with either *aioAB* or *aioXSRBA* genes and expressed them in the non-As(III)-oxidizer SB1003 (Yen & Marrs, 1976)

For the pBBR-*aioXSRBA* construct, the *aioXSRBA* genes were amplified from strain CZR27 by primers *aio1*-F/*aio1*-R, *aio2*-F/*aio2*-R and *aio3*-F/*aio3*-R (Table S2). The fragments were combined by using recombinase (Vazyme) and ligated into the *EcoR*I/*BamH*I-digested plasmid pBBR1MCS-2 (Kovach et al., 1995). For the pBBR-*aioBA* construct, a 3538-bp fragment including the *aio* promoter and *aioBA* genes was PCR amplified from strain CZR27 using primers *aio4*-F/*aio4*-R (Table S2). The PCR product was cloned into *EcoR*I and *BamH*I sites of the broad-host-range plasmid pBBR1MCS-2 using the ClonExpress II one-step cloning kit (Vazyme, Nanjing, China) to generate pBBR-*aioBA*. Then, transfer of each construct to wild-type strain SB1003 was performed with the donor *E. coli* strain WM3064 by biparental conjugation

according to the filter mating method (Saltikov & Newman, 2003). *R. capsulatus* strain SB1003 bearing vector plasmids pBBR1MCS-2, pBBR-*aioBA*, or pBBR-*aioXSRBA* were analysed for their As(III) oxidation abilities as for strain CZR27 as described earlier.

Quantitative reverse transcriptase PCR

To determine the transcription of *aioA* gene induced by As(III) during the incubation period under anoxic photoheterotrophic conditions, the cell cultures grown with and without 50 μM As(III) were collected at different time points (24, 36, 48, 60, and 72 h) for anaerobic treatment and tested for *aioA* gene transcription. The methods for RNA extraction, cDNA synthesis, and Quantitative reverse transcriptase PCR (qRT-PCR) were used as described elsewhere (Zhang et al., 2020). Primer sequences of the *aioA* and DNA gyrase *gyrB* genes were designed by Prime 5.0 Software (Table S2). Quantification of the transcription of *aioA* gene was performed on a CFX96 thermocycler (BioRad, USA) using the SYBR Green I detection method. qRT-PCR reaction (20 μL) contained 10 μL of 2 ×AceQ qPCR SYBR Green Master Mix (Vazyme, China), 0.4 μM of each primer, and 2 μL of cDNA (10 ng). The transcription of the *gyrB* gene was used as an internal standard. To determine the specificity of the reaction, a melting curve analysis was performed using the following: 65.0–95.0°C, in increments 0.5°C, for 5 s (Bio-Rad CFX96 Touch). The relative transcription level was quantified according to the method of $2^{-\Delta\Delta CT}$ threshold cycle (*CT*) (Livak & Schmittgen, 2001), and the variation in expression was estimated using three biological replicates. Negative controls for cDNA synthesis were reactions with no template, and no reverse transcriptase enzyme.

Arsenic and malate analysis in culture medium

The concentrations of arsenic species in the culture samples were determined using HPLC-ICP-MS (Perkin Elmer NexION 300X, USA). Arsenic species were separated using an anion exchange column (Hamilton PRP X-100, 250 mm length). A solution containing 8.5 mM $NH_4H_2PO_4$ and 8.5 mM NH_4NO_3 (pH 6.0) was used as the mobile phase, which was pumped through the column at a flow rate of 1 mL min^{-1}.

To analyse the concentrations of malate in the culture medium, liquid chromatography was performed on a Thermo 1200 series liquid chromatography (USA). The separation was carried on a Phenomenex Kinetex C18 column (2.6 μm; 4.6 mm × 100 mm). The mobile phase composition was 2% of methanol and 98% of water (with 0.1% formic acid) in isocratic mode at a flow rate of 0.3 mL min^{-1}.

RESULTS

Isolation of *Rhodobacter* bacterial strain CZR27 from paddy soil

To date, at least 45 complete or draft *Rhodobacter* genomes are publicly available (www.ncbi.nlm.nih.gov/genome). From analysis of an AioA clone library, we suggested that *Rhodobacter* strains are predominant As(III)-oxidizers in paddy soils (Xie et al., 2014). However, the ability of As(III) oxidation by a pure *Rhodobacter* strain was not investigated. To obtain such information, one bacterial strain (designated as CZR27) that is capable of oxidizing As(III) anaerobically in the presence of light was isolated. As shown in Figure 1A, strain CZR27 was capable of oxidizing 50 μM of As(III) within 72 h when growing with malate under anoxic phototrophic conditions. However, under these

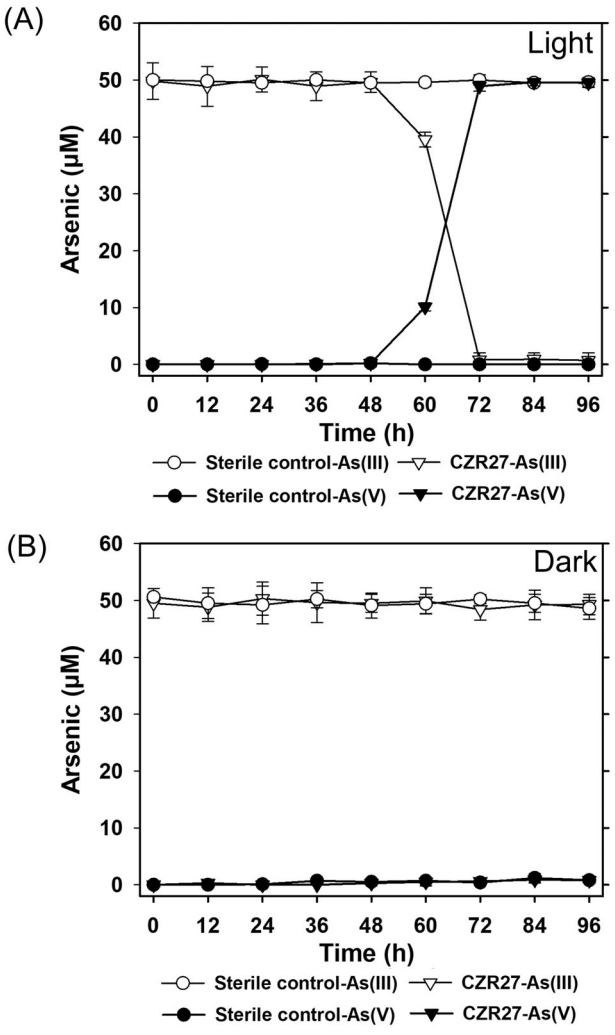

FIGURE 1 Time course of As(III) oxidation by *Rhodobacter* sp. CZR27 in the RCV medium containing 50 μM As(III) under anoxic conditions in the light (A), or in the dark (B). As(III) was added at 0 h. The symbols show the averages of triplicate cultures, and the error bars represent the standard errors.

conditions, As(III) oxidation did not closely mirror malate consumption; strain CZR27 could oxidize As(III) to As(V) only after 60 h, when all the malate has been consumed. The most rapid malate consumption occurred between 12 and 48 h (Figure S1), whereas the most rapid As(III) oxidation occurred between 48 and 72 h. Neither cell growth nor As(III) oxidation was observed in RCV medium (Weaver et al., 1975) without the addition of malate. No As(III) oxidation occurred in sterile controls with heat-killed cells in the presence of malate during incubation for 96 h (Figure 1A). In comparison, no oxidation of As(III) was observed when strain CZR27 was incubated in the dark (Figure 1B), demonstrating the light-dependence of this reaction. In addition to malate, strain CZR27 could also use or H_2, acetate, pyruvate, butyrate, malate, glucose, lactate as electron donors for growth. Cell growth was never observed in RCV media without the addition of H_2, or organic substrates.

The phylogenetic relationship among strain CZR27 and other representatives of heterotrophic/autotrophic As(III)-oxidizers is illustrated in Figure S2. Strain CZR27 is most closely related to members of the *Rhodobacter* genus in the *Rhodobacteraceae* family of the α-*Proteobacteria*. The strain forms a tight phylogenetic cluster with *R. johrii* strain JA192. These results confirm that strain CZR27 belongs to the genus *Rhodobacter*.

As(III) resistance and oxidation by strain CZR27

Growth of strain CZR27 was assayed in malate-containing RCV medium at different concentrations of As(III), which showed that strain CZR27 was not significantly inhibited by 50 μM As(III) (Figure 2A). However, severe growth inhibition was found at 150 and 200 μM As(III). The cell concentration in the 150 μM As(III) treatment was only about 50% of the control in the mid-exponential phase (48 h). Cell growth was almost completely inhibited at 200 μM As(III).

The rate of cell growth did not correspond with the rate of As(III) oxidation (Figure 2B). The apparent acceleration of As(III) oxidation may be primarily due to more cells being available for As(III) oxidation. In the treatments of 50 and 100 μM As(III), As(III) was completely oxidized during the transition from exponential phase to stationary phase (Figure 2B).

Comparison of As(III) oxidation in two phototrophic bacteria

To verify whether phototrophic As(III)-oxidation also occurs in other *Rhodobacter* strains, *Rhodobacter* sp. SW2 (Ehrenreich & Widdel, 1994) and *R. capsulatus* SB1003 (Yen & Marrs, 1976) were

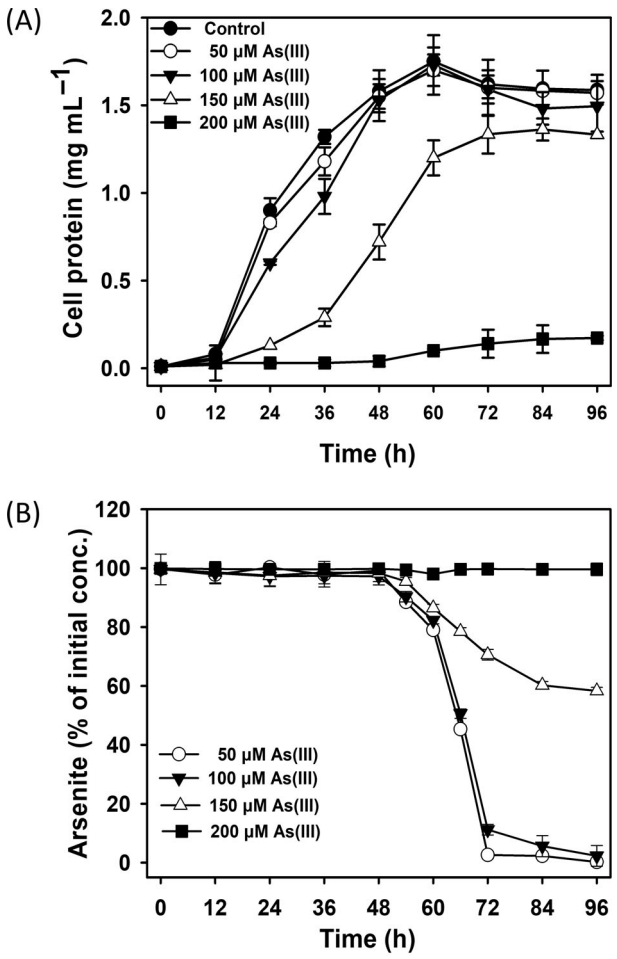

FIGURE 2 Time course of anoxic phototrophic growth (A) and As(III) oxidation (B) by *Rhodobacter* sp. CZR27 in the presence of different As(III) concentrations. As(III) was added at 0 h. The symbols show the averages of triplicate cultures, and the error bars represent the standard errors.

investigated. *R. capsulatus* SB1003 did not oxidize As(III), whereas *Rhodobacter* sp. SW2 oxidized >60% of the As(III) within 2 weeks when H_2 was present as an additional electron donor (Table 1).

To determine whether As(III) was oxidized biologically, we used the filtered cell-free supernatant to examine the possibility of abiotic reactions. No As(III) oxidation was observed in the uninoculated medium, cell-free supernatant, or autoclaved cell-free supernatant (Table 1). This result indicates that the As(III)-oxidizing activity was due to biologically mediated reactions.

The cluster of As(III) oxidase genes in *Rhodobacter* strain CZR27

The complete genome sequence of strain CZR27 was determined. The genome contains one circular chromosome (3,335,106 bp) and three circular plasmids:

TABLE 1 Oxidation of As(III) by *Rhodobacter* strains.

Rhodobacter strains	*aio* genes	% As(III) oxidized in solution (±SE) with the following treatment[a]			
		Control (uninoculated)	Inoculated	Cell-free supernatant	Cell-free supernatant (autoclaved)
CZR27	*aioXSRBA*	ND	98.8 ± 1.5	ND	ND
SW2	*aioXSRBA*	ND	62.2 ± 1.9	ND	ND
SB1003	–	ND	ND	ND	ND

Abbreviation: ND, not detectable.
[a]Values are from triplicate HPLC-ICP-MS measurements and expressed as a percentage of As(III) oxidized after 2 weeks relative to uninoculated controls.

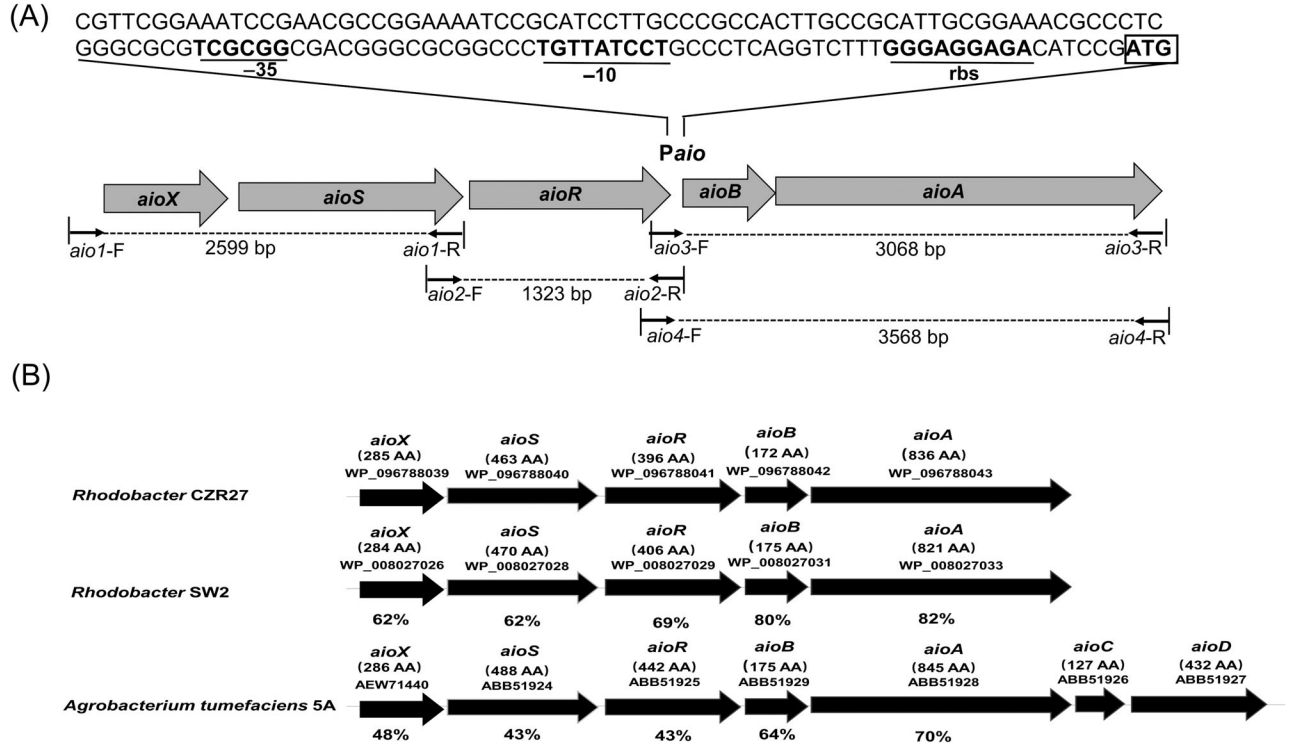

FIGURE 3 (A) Genetic organization of the *aio* gene cluster in *Rhodobacter* strain CZR27. Upstream of *aioB* is a putative promoter sequence (P*aio*). The −10 and −35 consensus sequences, and the ribosome-binding site are indicated in bold and underlined, and the start codon is in a box. Predicted PCR products are represented by dotted lines under the genes and are labelled with predicted product sizes. (B) Organization of the *aio* clusters in *Rhodobacter* sp. CZR27 (accession no. NZ_CP023550.1), *Rhodobacter* sp. SW2 (accession no. NZ_ACYY00000000.1), and *Agrobacterium tumefaciens* 5A (accession no. NZ_AGVZ00000000.1). Genes are represented by arrows as follows: *aioX*, periplasmic As(III)-binding protein gene; *aioS*, arsenic sensor histidine kinase gene; *aioR*, arsenic response regulator transcription factor gene; *aioB*, the small subunit of the As(III) oxidase gene; *aioA*, the large subunit of the As(III) oxidase gene; *aioC*, cytochrome c family protein gene; and *aioD*, molybdopterin molybdotransferase gene. At the bottom of each gene is indicated its percentage of amino acid sequence identity to the corresponding *Rhodobacter* sp. CZR27 ortholog.

pCZR27-1 (860,520 bp), pCZR27-2 (193,105 bp) and pCZR27-3 (48,382 bp). Only one putative As(III) oxidase gene cluster, designated *aioXSRBA* located on plasmid 2, was found in the genome of strain CZR27 (Table S3). The *aioXSR* genes in CZR27 encoding regulators displayed the same transcriptional orientation as *aioBA*. A putative promoter was also found upstream of the *aioB* gene in strain CZR27 (Figure 3A). The genetic organization of the As(III) oxidation cluster, and the encoded amino acid sequences are highly conserved in the *aio* clusters identified in the genus *Rhodobacter* (Figure 3B). The *aioA* gene encodes the large subunit of the As(III) oxidase protein (AioA), with 836 amino acids, and showed 82% identity and 70% identity with the AioAs from *Rhodobacter* sp. strain SW2 and *A. tumefaciens* 5A, respectively. Upstream from *aioA* is the *aioB* gene, which encodes the As(III) oxidase small subunit. The predicted protein AioB (172 amino acids) displayed 80% identity and 64% identity with the AioBs from strain SW2 and *A. tumefaciens* 5A, respectively. However, two additional *aio* genes termed *aioC* and *aioD*, that are cotranscribed with the upstream genes were not found in strain CZR27.

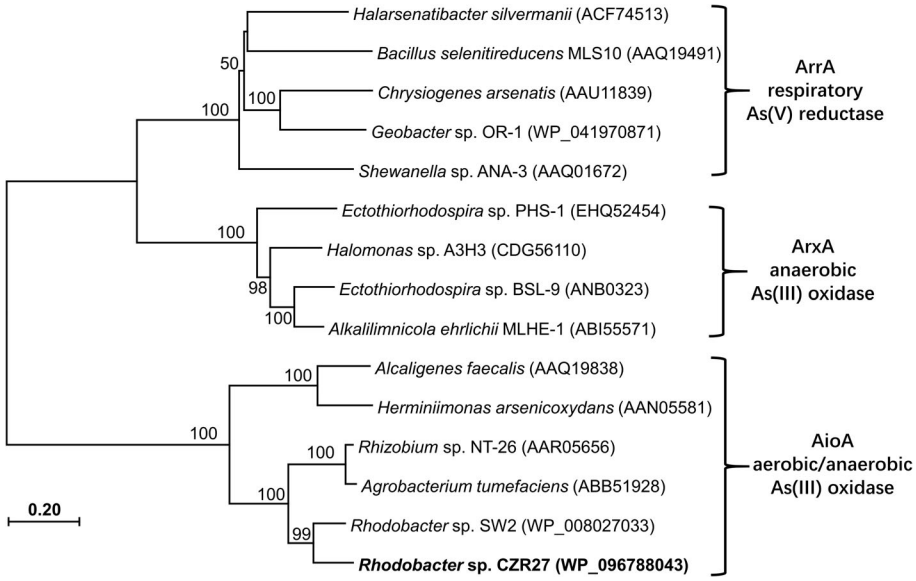

FIGURE 4 Phylogenetic tree built from the multiple amino acid sequence alignment of the ArxA, ArrA, and AioA proteins. Bootstrap values >50% are shown. Scale bar shows changes per base position.

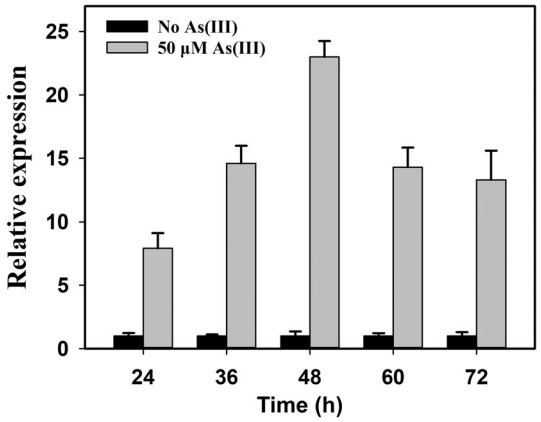

FIGURE 5 Transcriptional analysis of the *aioA* gene. Dynamics of *aioA* gene expression induced by 50 μM As(III) in strain CZR27 under anoxic phototrophic conditions. Expression was determined by real-time PCR and normalized to *gyrB* gene expression. The relative expression of *aioA* in the absence of As(III) treatment was converted to a value of 1 as a reference. The *aioA* gene expression data points and error bars represent the averages and standard error from triplicate cultures.

Phylogenetic analysis of the predicted AioA amino acid sequence of CZR27 revealed that it was most similar to the AioA-like sequence from *Rhodobacter* sp. SW2, an anaerobic phototrophic Fe(II)-oxidizer isolated from a freshwater lake (Figure 4). From this analysis, it became obvious that the CZR27 AioA sequence is very distantly related to the ArxA sequences of haloalkaliphile *Alkalilimnicola ehrlichii* MLHE-1 (Zargar et al., 2010) and to the anoxygenic photoautrophic anaerobe *Ectothiorhodospira* sp. strain BSL-9 (Hernandez-Maldonado et al., 2016). The amino acid sequence of CZR27 AioA shows a low similarity (<23%) to those of ArrA- and ArxA-like proteins.

The transcription of As(III) oxidase gene is upregulated by As(III)

We examined induction of transcription of the *aioA* gene in strain CZR27 by As(III) under anoxic photoheterotrophic conditions (Figure 5). The dynamics of *aioA* transcription were investigated in strain CZR27 at different growth phases. The transcription of *aioA* gene increased 20-fold with 50 μM As(III) after 48 h, followed by a decrease in transcription levels when cells entered the stationary phase (60–72 h).

Aio genes from strain CZR27 confer As(III) oxidation

As shown in Figure 6A, As(III) oxidation was not observed in the medium without an inoculum (abiotic control). Strain SB1003 was not able to oxidize As(III) to As(V) (Figure 6A). As expected, under anoxic phototrophic conditions in the presence of 50–150 μm As(III), strain SB1003 carrying constructs *aioXSRBA* or *aioBA* could oxidize As(III) to As(V). SB1003/pBBR-*aioBA* showed significant light-dependent As(III) oxidation activity, at rates of 20%–50% of that in SB1003/pBBR-*aioXSRBA*. In contrast, As(III) oxidation was not observed in strain SB1003 harbouring the vector control (SB1003/pBBR) (Figure 6B). There was also time-lagged As(III) oxidation in strain SB1003/pBBR-*aioBA*,

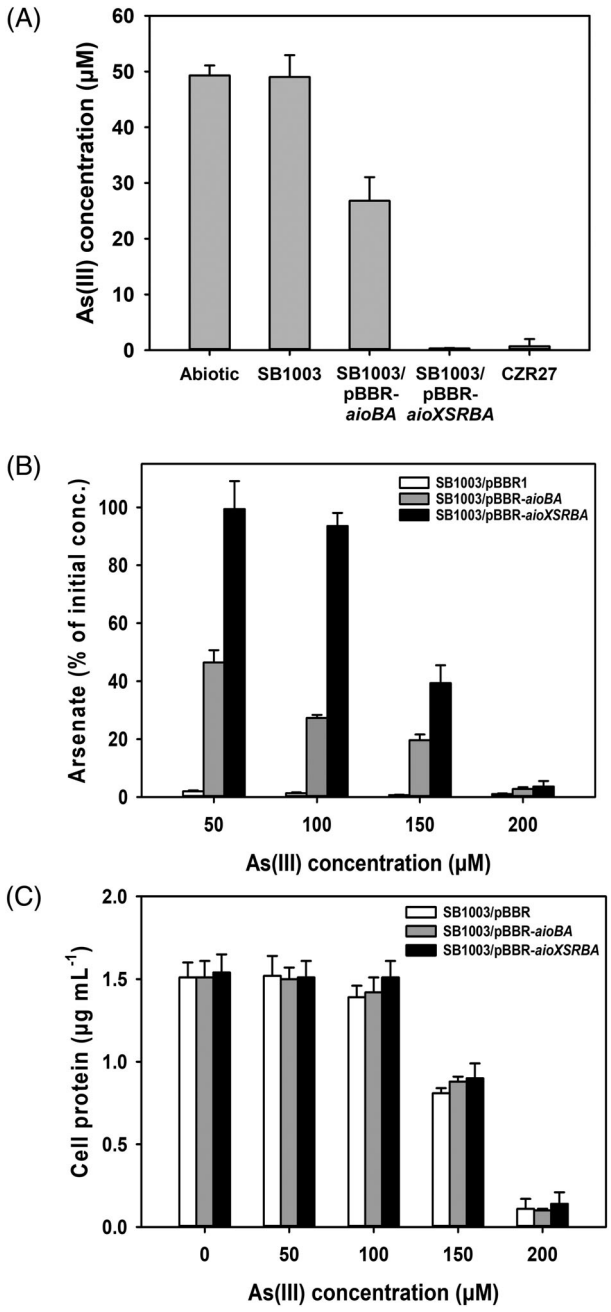

FIGURE 6 Heterologous expression of *aio* genes in *Rhodobacter* sp. SB1003. Comparison of the As(III) oxidation phenotypes of strain CZR27 and strain SB1003 carrying the different clones (empty vector pBBR1MCS2, pBBR-*aioXSRBA*, and pBBR-*aioBA*) (A, B). Growth of several constructs in malate-containing RCV medium amended with 50–200 μM As(III) (C). Error bars represent standard errors of the means calculated from triplicate experiments.

compared to strain SB1003/pBBR-*aioXSRBA* (Figure 6B). These results clearly demonstrate that *aio* genes from strain CZR27 contribute to As(III) oxidation in vivo.

We also examined whether *aio* genes increase strain SB1003 resistance to As(III). When treated with 50–200 μM As(III), there was no difference in the growth between wild-type strain SB1003 and strain SB1003 harbouring either pBBR-*aioBA* or pBBR-*aioXSRBA* constructs (Figure 6C). These results indicate that, at least in *R. capsulatus* strain SB1003, *aio* genes do not seem to play a significant role in As(III) resistance.

DISCUSSION

The aim of this study was to examine the connection of anoxygenic photosynthetic bacteria in the phyllosphere of duckweeds with the observed As(III) oxidation under anoxic photoheterotrophic conditions. Duckweeds have been suggested as a potential candidate for arsenic phytoremediation (Rahman et al., 2007). The tissue of duckweeds itself does not oxidize As(III), whereas the bacterial communities inhabiting in its phyllosphere displayed substantial As(III) oxidation. Based on analysis of *aioA* genes in the phyllosphere, genes closely related to *Rhodobacter* species account for 53% of all sequences detected (Xie et al., 2014). However, it has been unclear whether the genus *Rhodobacter* is involved in phototrophic As(III) oxidation in paddy soils, mainly due to the lack of pure cultures. Some purple sulfur bacteria in the genus of *Ectothiorhodospira* gain energy from As(III) oxidation during anoxygenic photosynthesis, illustrating a microbial mechanism, whereby As(III) is anaerobically oxidized to As(V) in the light (Hoeft et al., 2010; Kulp et al., 2008). In the present study, we isolated a novel bacterial strain, CZR27 in the genus of *Rhodobacter*, from arsenic-contaminated paddy soil and showed that it oxidizes As(III) anaerobically under photoheterotrophic conditions. Previously only a few anaerobic photoautotrophic As(III)-oxidizers that belong to the genus *Ectothiorhodospira* have been isolated from hypersaline, alkaline lakes (Budinoff & Hollibaugh, 2008; Kulp et al., 2008). *Rhodobacter* sp. strain CZR27 is a purple non-sulfur bacterium obtained from paddy soil that is capable of photoheterotrophic anaerobic As(III) oxidation. This property suggests that in the paddy environment, *Rhodobacter* species carrying As(III) oxidase genes play a major role in anaerobic phototrophic oxidation of As(III).

We characterized strain CZR27 to examine its role in the arsenic biogeochemical cycle. It can grow in the presence of up to 150 μM As(III). This level of tolerance is higher than the typical As(III) concentrations found in paddy soil pore water (Zhang et al., 2017) or in the paddy standing water (Stroud et al., 2011). Under anoxic photoheterotrophic conditions, strain CZR27 oxidized As(III) only after malate was nearly exhausted. The metabolites produced by CZR27 in the culture medium did not affect As(III) oxidation. These results support the findings that oxidation of As(III) by phototrophic bacterium strain CZR27 is due to biological oxidation. As(III) oxidation is closely related to the growth

kinetics of cultures and occurs only when the cells reach the transition between the exponential and stationary phases. This finding is consistent with the results of Ehrenreich and Widdel (1994), who reported that maximum Fe(II) oxidation occurs during late exponential phase. This suggests that As(III) oxidation maybe regulated by metabolites produced in stationary phase.

The identification of *aio*-related genes and the absence of an *arx*-type gene cluster in the genome of CZR27 supports our hypothesis that the mechanism of phototrophic As(III) oxidation is associated with the As(III) oxidase AioAB enzyme. However, the molecular basis of the light-dependent As(III) oxidation process in the *Rhodobacter* species was largely unknown, because phototrophic bacteria harbouring *aio* genes (such as *Rhodobacter* strains CZR27 and SW2) remain genetically intractable (Croal et al., 2007). In this study, we used a heterologous complementation approach to identify the functional genes involved in As(III) oxidation. We showed that the five-gene *aioXSRBA* cluster from strain CZR27 confers light-dependent As(III) oxidation activity when expressed in *R. capsulatus* strain SB1003. The As(III) oxidase system usually comprises a three-component system containing AioXSR and the As(III) oxidase AioAB (Kashyap et al., 2006; Liu et al., 2012). Surprisingly, *R. capsulatus* strain SB1003 expressing only the *aioBA* genes also exhibited the ability to oxidize As(III), although the maximal activity was achieved when *aioBA* was co-expressed with the upstream *aioXSR* genes. How can *R. capsulatus* strain SB1003 heterologously expressing *aioBA* oxidize As(III)? The *aioX* gene has been shown to encode an As(III)-binding protein responsible for transferring the As(III) signal to AioS (Liu et al., 2012), and AioSR regulates expression of the As(III) oxidase genes *aioBA* (Kashyap et al., 2006). We speculate that genes encoding proteins with similar functions to AioX, AioS or AioR in the genome of *R. capsulatus* strain SB1003 play an auxiliary role in As(III) oxidation. This is supported by the fact that the heterologous expression of *aio* genes in strain SB1003 does not seem to confer an enhanced As(III) resistance capability.

The AioAB enzyme is responsible for both aerobic and anaerobic As(III) oxidation (Andres & Bertin, 2016), with the latter often being coupled with nitrate reduction (Zhang et al., 2015). We show that AioAB catalyses anaerobic As(III) oxidation by coupling anoxygenic photosynthesis in phototrophic bacteria containing *aioAB* genes. In the case of *Rhodobacter* strain CZR27, photosynthetic light reactions may provide electron acceptors that couples with As(III) oxidation (Kulp et al., 2008). Due to thermodynamic constraints, electron flow from As(III) oxidation to the photosynthetic reaction centre may be mediated by a quinone (Q) or cyt-*c2* cytochrome (Figure S3).

In this study, we provided the first experimental evidence for the existence of phototrophic anaerobic As(III) oxidation in the paddy environment mediated by indigenous *Rhodobacter* sp. strain CZR27. Also, the discovery of *aioXSRBA* and its involvement in phototrophic As(III) oxidation expands current understanding of the diversity of As(III) oxidizing gene clusters in phototrophic purple bacteria. In addition to recently reported photochemical As(III) oxidation in paddy waters (Zeng et al., 2021), our study highlights the importance of considering light as an impacting factor in paddy arsenic geochemistry.

AUTHOR CONTRIBUTIONS

Yi-Fei Wu: Conceptualization (equal); data curation (equal); writing – original draft (equal). **Jian Chen:** Methodology (equal). **Wan-Ying Xie:** Data curation (equal); writing – review and editing (equal). **Chao Peng:** Methodology (equal); resources (equal). **Shi-Tong Tang:** Investigation (equal); methodology (equal). **Barry P. Rosen:** Conceptualization (equal); data curation (equal); funding acquisition (equal); writing – review and editing (equal). **Andreas Kappler:** Resources (equal); writing – review and editing (equal). **Jun Zhang:** Methodology (equal); resources (equal). **Fangjie Zhao:** Resources (equal); supervision (equal); validation (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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