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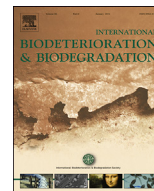

Oxidation of arsenite to arsenate in growth medium and groundwater using a novel arsenite-oxidizing diazotrophic bacterium isolated from soil

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ABSTRACT

An arsenic hyper-tolerant diazotrophic bacterium was isolated from a heavy metal contaminated soil. The pure isolate MM-17 was identified as *Azospirillum* sp. based on phylogenetic analysis of 16S rRNA. The strain oxidized 100 μM As(III) to As(V) in both culture media (minimal salts) and real groundwater within 8 and 10 h, respectively. The oxidation of As(III) by this strain was observed within the pH range 5–10 with the best performance at pH 7–8. As(III) oxidation was found to be independent of cell growth which implies the oxidation enzymes are constitutively expressed. The whole cell kinetic study highlighted a lower value of kinetic constant, K_s as 32.9 μM As(III), which indicates that the strain MM-17 has greater affinity for As(III). The gene sequence of the large subunit of arsenite oxidase of MM-17 showed 99 and 72% similarity to the large subunit of arsenite oxidase of *Stenotrophomonas* sp. MM-7 and *Sinorhizobium* sp. M14, respectively. Sphaeroplasts experiments suggest that arsenite oxidase is a membrane associated protein in MM-17.

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1. Introduction

Arsenic is a naturally occurring element distributed widely but unevenly in the environment. This toxic metalloid can also enter the environment from various anthropogenic activities. The advent of higher concentrations of arsenic in numerous aquifers results mostly from geological processes. Furthermore the chronic exposure to humans via drinking water or the food chain has become a global concern due to the risks to health (Smedley and Kinniburgh, 2002). To this end, the World Health Organization (WHO) has revised the guideline value of arsenic in drinking water from 50 μg l^{-1} to 10 μg l^{-1} (WHO, 1993). However, it has retained the previous guideline value for some developing countries where high concentrations of arsenic have been recorded. It is, therefore, very important to develop a cost-effective arsenic remediation technology for those countries' low income communities.

Inorganic arsenic mainly exists in the natural environment as two stable oxidation states, i.e. arsenite [As(III)] and arsenate [As(V)]. The toxicity of As(III) has been reported to be much higher than that of As(V) since As(III) binds the sulfhydryl groups of enzymes and thereby inactivates them. Due to the pH dependent ionization of As(III) (zero charge up to pH 9.2) (Meng et al., 2000) it attains a high degree of solubility and remains more mobile in aqueous solution, which in turn makes it difficult to remove it from contaminated water. Therefore, in conventional treatment processes an oxidation stage is often required to oxidize As(III) to easily removable As(V) by using chemical oxidants. However, the chemical oxidants may react with other substances in water and produce harmful substances, and excess amounts of chemicals result in high costs (Jekel and Amy, 2006). As an alternative, biological oxidation of As(III) has gained significant attention in the last few years because it is perceived to be environmentally friendly.

Although arsenic is highly toxic, microorganisms have developed mechanisms to tolerate arsenic and/or utilize the element for respiratory metabolism (Hamamura et al., 2014). Microbial As(III) oxidation has been described either as a detoxification mechanism (Muller et al., 2003) or energy source for chemolithoauthotrophic

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bacteria (Santini et al., 2000; Hoeft et al., 2007; Garcia-Dominguez et al., 2008). So far a number of phylogenetically diverse As(III)-oxidizing bacteria have been identified (Stolz et al., 2002) and the genetic mechanism of As(III) oxidation has been elucidated (Anderson et al., 1992; Zargar et al., 2010). As(III) oxidation by prokaryotes is catabolized by an enzyme called arsenite oxidase. The crystal structure of the arsenite oxidase revealed that it is composed of a small subunit containing a Rieske [2Fe–2S] cluster and a large subunit harboring molybdopterin guanosine dinucleotide at the active site and a [3Fe–4S] cluster (Ellis et al., 2001; Slyemi and Bonnefoy, 2012; Lett et al., 2012). To our knowledge there is no report on a pure diazotrophic bacterium able to oxidize As(III). In this study, we have: (a) isolated and characterized a novel As(III)-oxidizing diazotroph, (b) conducted As(III) oxidation kinetics study, and (c) identified the arsenite oxidase gene in the isolated strain.

2. Materials and methods

2.1. Strain isolation and cultivation

The As(III)-oxidizing strain was isolated from a soil collected from the Port Pirie area of South Australia. Initial enrichments were made in a modified minimal salt medium (MMSM) amended with 1 mM (75 mg l^{-1}) As(III) and glucose (0.5% w/v) as the sole carbon source (Bahar et al., 2012). The enrichments were subcultured twice and after the second transfer, the resulting enriched cultures were serially diluted and plated onto an MS agar (1.5%) medium containing 1 mM of As(III). Single colonies were selected and re-streaked several times onto the same medium to obtain pure isolates. As(III)-oxidizing bacteria were screened from the arsenite-resistant colonies using the $KMnO_4$ assay (Salmassi et al., 2002; Bahar et al., 2012). After confirmation of pure arsenite-oxidizing strains, they were preserved in 20% glycerol medium at −80 °C and MS agar medium was used for routine maintenance.

2.2. Batch oxidation experiment

As(III) oxidation ability of strain MM-7 was determined in a batch test. Cells grown in MMSM containing 500 μM As(III) were harvested by centrifugation at 4000 rpm and washed with 0.85% NaCl. The cells were then inoculated into the MMSM to obtain the cell density of 1.5×10^7 cell ml^{-1} in sterile Erlenmeyer flasks containing As(III) at concentrations ranging from 10 to 1000 μM. Uninoculated As(III) containing medium served as control. The pH value of the medium was adjusted to 7.2 and the flasks were kept on a rotary shaker at a speed of 130 rpm in a temperature controlled room (25 °C). Samples were taken at different time intervals to quantify the As(III) and As(V) concentration in solution as well as to determine the bacterial growth in terms of optical density (600 nm). All experiments were done in triplicate, including controls and the mean values were taken into account. Another batch test was conducted with natural groundwater (pH, 6.30; EC, 267 μs cm^{-1}; Dissolved organic carbon, 5.3 mg l^{-1}; NO_3^-, 0.61 mg l^{-1}; SO_4^{2-}, 20.0 mg l^{-1}; Fe, 0.12 mg l^{-1}; Al, 1.46 mg l^{-1}; Mg, 3.19 mg l^{-1}; K, 1.95 mg l^{-1}; Ca, 14.8 mg l^{-1} Mn, 0.013 mg l^{-1}; Zn, 0.004 mg l^{-1}) spiked with 100 μM As(III) to assess the potential applicability of this strain in a real environmental scenario. The effect of pH on bacterial oxidation of As(III) was assessed over the pH range 4–9 with the same conditions used in the batch oxidation experiment. The pH of the medium was adjusted to the desired level with a predetermined amount of filter-sterilized NaOH and HCl.

Whole cell kinetic experiments were initiated by adding As(III) (10–2500 μM) to the Tris–HCl buffer at pH 7.0. The buffer with no addition of As(III) served as the control. Samples were taken at 15–30 min intervals to determine the change in As(III) concentration until complete oxidation had occurred in all the flasks.

2.3. Isolation, sequencing and phylogenetic analysis of 16S rRNA and aioA gene

Genomic DNA isolation and PCR amplification were performed according to the methods and protocols described in Bahar et al. (2012). The 16S rRNA was amplified with eubacterial primers (E8F: 5′-AGAGTTTGATCCTGGCTCAG-3'; 1541R: 5′-AAGGAGGT-GATCCANCCRCA-3′) (Lane, 1991) and the *aioA* gene with degenerate primers (69F: 5′-TGYATYGTNGGNTGYGGNTAYMA-3'; 1374R: 5′-TANCCYTCYTGRTGNCCNCC-3′) (Rhine et al., 2007). The yield of genomic DNA and all PCR products were checked by gel electrophoresis using 1% agarose gel with ethidium bromide at 80 V for 35 min and the specific bands were visualized with a UV trans-illuminator (Bio-Rad, USA). The PCR products were purified using an UltraClean PCR clean-up kit (Mo-Bio Laboratories Inc, CA, USA). The purified PCR product of *aioA* gene was ligated into pGEM-T Easy vector (Promega, Madison, WI, USA) and the ligation products were transformed into *E. coli* DH5α competent cells. The transformants were grown on LB agar containing ampicillin, X-Gal and IPTG at 37 °C for 16 h according to the manufacturer's recommendations, and the plasmid DNA was extracted using an ultraclean standard plasmid mini prep kit (Mo-Bio Laboratories Inc, CA, USA). The clones were then sequenced using the T7 and SP6 primers from the vector.

DNA sequencing was performed using ABI 3130 Sequencer (Applied Biosystems, USA) at Southpath Flinders Sequencing Facility, Flinders Medical Centre, Adelaide. The initial sequence analysis was done using BlastN (for 16S rRNA) and BlastX (for *aioA*). Phylogenetic analyses were carried out with MEGA 5.0 software using a neighbour-joining method.

2.4. Preparation of sphaeroplast and periplasmic fraction

Sphaeroplasts were prepared as described by Prasad et al. (2009) and Lieutaud et al. (2010). MM-7 cells were grown in LB medium containing 500 μM As(III) and harvested during the exponential growth phase by centrifugation (4000 rpm, 35 min). After being washed twice with 20 mM Tris–HCl, the pellet was suspended in 30 ml suspension buffer containing 20 mM Tris–HCl, 0.1 mM PMSF and 10 mM EDTA at pH 8.4 with 20% sucrose. The cells were then incubated for 40 min at 25 °C with 0.5 mg l^{-1} lysozyme. After incubation, the cells were centrifuged at 4000 rpm for 35 min and sphaeroplasts were retrieved in the pellet whereas the supernatant constitutes the periplasm. The sphaeroplasts were resuspended in 10 mM Tris–HCl (pH 7.5) for As(III) oxidation assay after being washed with the same buffer.

2.5. Chemicals and analytical methods

All chemicals used in this study were of analytical grade and obtained from Sigma–Aldrich. All solutions were prepared in Milli-Q water (18Ωcm^{-1}, ELGA Labwater, UK) and were sterilized by filtration or by autoclaving. The As(III) and As(V) stock solutions were prepared from sodium arsenite ($NaAsO_2$) and sodium arsenate ($Na_2HAsO_4.7H_2O$), and stored at 4 °C in the dark.

Arsenic species were separated by high-performance liquid chromatography (Agilent 1100, Japan) equipped with a guard column and separation column (Hamilton PRP-X100) and quantified by inductively coupled plasma-mass spectrometry (Agilent 7500C, Japan). Prior to arsenic speciation analysis, samples taken from the experimental culture medium were centrifuged at 10,000 rpm for

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