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Arsenic redox transformation by *Pseudomonas* sp. HN-2 isolated from arsenic-contaminated soil in Hunan, China

Zhennan Zhang, Naiyi Yin, Xiaolin Cai, Zhenzhou Wang, Yanshan Cui*

1. College of Resources and Environment, University of Chinese Academy of Sciences, Beijing 101408, China. E-mail: zhangzhennan10@mails.ucas.ac.cn

2. Research Center for Eco-environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

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ABSTRACT

A mesophilic, Gram-negative, arsenite[As(III)]-oxidizing and arsenate[As(V)]-reducing bacterial strain, *Pseudomonas* sp. HN-2, was isolated from an As-contaminated soil. Phylogenetic analysis based on 16S rRNA gene sequencing indicated that the strain was closely related to *Pseudomonas stutzeri*. Under aerobic conditions, this strain oxidized 92.0% (61.4 $\mu\text{mol/L}$) of arsenite to arsenate within 3 hr of incubation. Reduction of As(V) to As(III) occurred in anoxic conditions. *Pseudomonas* sp. HN-2 is among the first soil bacteria shown to be capable of both aerobic As(III) oxidation and anoxic As(V) reduction. The strain, as an efficient As(III) oxidizer and As(V) reducer in *Pseudomonas*, has the potential to impact arsenic mobility in both anoxic and aerobic environments, and has potential application in As remediation processes.

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Introduction

Arsenic(As) is a natural and ubiquitous metalloid that ranks the 20th most abundant in the earth's crust (Cullen and Reimer, 1989). Many people around the world are exposed to As-polluted underground water and soil and suffer from chronic As poisoning, which causes hyperkeratosis, cancer, diabetes, cardiovascular disease, and other diseases (Sumi and Himeno, 2012).

Arsenic occurs in several oxidation states including arsenate (As(V)), arsenite (As(III)), elemental As (As⁰) and arsenide (As(–III)). Arsenic is mostly found in inorganic forms as trivalent arsenite (As(III)) or pentavalent arsenate (As(V)) (Cullen and Reimer, 1989). Among them, As(III) is generally regarded as more mobile and more toxic than As(V) (Liu et al., 2001). The affinity of As(III) for protein thiols or vicinal

sulfhydryl groups makes it highly toxic. As(III) also acts as an endocrine disruptor by binding to hormone receptors and interfering with normal cell signaling (Kaltreider et al., 2001). As(V) is a chemical analog of phosphate with subsequent inhibition of oxidative phosphorylation (Goyer and Clarkson, 1996).

The contribution made by microorganisms to the biogeochemistry of As in the environment is extensive and detailed. Microbial activity strongly influences As speciation. Various bacteria are known for their ability to transform inorganic As species through oxidation or reduction processes (Jones et al., 2012; Oremland and Stolz, 2003). For chemoautotrophic As(III) oxidizers, the oxidation of As(III) generates energy, which promotes their growth and proliferation (Santini et al., 2000); whereas for heterotrophic As(III) oxidizers, the oxidation of As(III) is described as a detoxification mechanism catalyzed

* Corresponding author. E-mails: zhangzhennan10@mails.ucas.ac.cn (Zhennan Zhang), cuiyanshan@ucas.ac.cn (Yanshan Cui).

by the periplasmic enzyme arsenite oxidase (Muller et al., 2003). Dissimilatory arsenate-reducing prokaryotes conserve energy by linking the oxidation of lactate to the reduction of As(V) to As(III). Reduction of As is also carried out by resistant microbes utilizing an As(V)-reductase and As(III)-expulsion pump (Tsai et al., 2009). Biotic arsenic transformation could cause either arsenic release or immobilization in soil. It was found that bacterial activity was responsible for the reduction of arsenic, causing the solubilization of the metalloid from pyrite cinders to the aqueous phase (Corsini et al., 2010). Oxidation of As(III) to As(V) contributes to improved immobilization of As and thus helps to mitigate As contamination (Sun et al., 2010).

In order to better understand the mechanism of biotic As transformation in the environment and further understand the combined effect of abiotic (e.g., mineral oxidants and humus) and biotic factors on As transformation in soil, isolation of As-transformation bacteria is necessary. Furthermore, the microbial transformation of As(III) to As(V) might represent an eco-friendly, cost-effective alternative approach to conventional remediation processes (Bahar et al., 2012). Several bacterial strains have been isolated and identified as As(III) oxidizers since the first arsenite-oxidizing bacterium was described in 1918 (Green, 1918), including *Stenotrophomonas* (Bahar et al., 2012), *Polaromonas* (Osborne et al., 2010), *Bacillus* (Shivaji et al., 2005), *Alcaligenes* (Osborne and Ehrlich, 1976), *Agarobacterium* (Salmassi et al., 2002), *Thiomonas* (Bruneel et al., 2003), *Microbacterium lacticum* (Mokashi and Paknikar, 2002) and *Bosea* (Liao et al., 2011). Dissimilatory reduction of As(V) has been shown to occur in at least nine different genera scattered throughout the domain Bacteria and has also been observed in two hyperthermophilic Archaea (Macur et al., 2004). The As(V)-reducing bacteria utilizing a detoxification or resistance mechanism have also been identified and classified as *Pseudomonas*, *Psychrobacter*, *Vibrio*, *Citrobacter*, *Enterobacter*, *Bacillus* (Liao et al., 2011), *Alcaligenes* (Cavalca et al., 2013), *Pantoea* (Wu et al., 2013), *Agrobacterium*, *Flavobacterium* and *Microbacterium* (Macur et al., 2004). These bacteria have been generally isolated from soils (Bahar et al., 2012; Majumder et al., 2013), groundwater (Liao et al., 2011; Mokashi and Paknikar, 2002), Hot Creek (Salmassi et al., 2002), mines (Santini et al., 2000), the Arctic Circle (Osborne et al., 2010), sediments (Valenzuela et al., 2009) and plant effluents (Bachate et al., 2013). Although various kinds of As-transformation bacteria have been found, the expression by a single organism of both reductive and oxidative metabolic functions, has, to our knowledge, only been observed so far in members of *Thermus*, *Bacillus*, *Marinobacter*, *Comamonas*, *Flavobacterium*, *Staphylococcus*, and *Pseudomonas* genera (Fisher and Hollibaugh, 2008; Gihring and Banfield, 2001; Handley et al., 2009). These bacteria mentioned above were isolated from hot springs, lakes, marine hydrothermal sediments and plants. No soil bacterial strain with both As(III)-oxidizing and As(V)-reducing ability has been reported before.

In this article, we presented (i) the isolation of a strain with the ability of both heterotrophic As(III)-oxidation and As(V)-reduction from an As-contaminated soil, (ii) phylogenetic analysis of the strain, (iii) the identification and characterization of arsenite oxidase and arsenate reductase genes, (iv) As oxidation and reduction assays.

1. Materials and methods

1.1. Isolation and growth of As-resistant bacteria

A soil sample ([As] = 284 mg/kg) collected from a mine in Zhuzhou, Hunan, China, was used as an inoculum for enrichment culture. The isolation and subsequent growth experiments were carried out with a modified chemically defined medium (CDM) (8.12 mmol/L MgSO_4 , 18.7 mmol/L NH_4Cl , 7 mmol/L Na_2SO_4 , 0.0574 mmol/L K_2HPO_4 , 0.457 mmol/L CaCl_2 , 44.6 mmol/L Na-lactate and 9.5 mmol/L NaHCO_3 , with the pH adjusted to 7.0) as described by Weeger et al. (1999). As-resistant bacteria were enriched with 1.33 mmol/L As(III) under oxic incubation. 1 g soil samples were placed in sterile flasks containing CDM supplemented with 1.33 mmol/L As(III), and incubated at 25°C on a rotary shaker (170 r/min) in the dark for 48 hr. After incubation, samples were serially diluted, and a cell suspension was spread onto CDM plates with 1.33 mmol/L As(III) at 25°C for 48 hr to obtain single colonies. A pure culture was obtained by successive isolation of colonies at 25°C in As(III)-supplemented medium.

1.2. Selection of as(III)-oxidizing strain

The ability of the obtained As-resistant bacteria to oxidize As(III) was tested using a qualitative AgNO_3 screening method as described by Liao et al. (2011). Briefly, exponential-phase culture was centrifuged and then resuspended in CDM containing 1 mmol/L As(III). The bacterial suspensions were incubated at 30°C for 72 hr. Subsequently, the bacterial cultures were centrifuged, and 100 μL of the liquid phase was mixed with 100 μL of 0.1 mol/L AgNO_3 solution. The resulting precipitates containing As showed colors ranging from the light yellow of Ag_3AsO_3 (silver orthoarsenite) due to As(III) to the light brown-red of Ag_3AsO_4 (silver orthoarsenate) due to As(V). The observation of a light brown-red precipitate was regarded as indicating an As(III)-oxidizing strain. After confirmation of a pure As(III)-oxidizing strain, bacteria were preserved in 30% glycerol at –80°C.

1.3. Bacterial As(III) oxidation experiment

The ability of the bacterial isolate to oxidize As(III) was further quantitatively determined by batch tests. The cells were grown in CDM. Once the cells reached the exponential phase, they were harvested by centrifugation at 5000 r/min for 20 min at 4°C and cell pellets were resuspended in the required volume of CDM to obtain a cell density of about 10^7 CFU/mL. The test medium was supplemented with 5 mg/L (66.7 $\mu\text{mol/L}$) of As(III) and incubated on a rotary shaker (150 r/min) at 30°C. The pH value of the medium was adjusted to 7.0. Controls without inoculation were also incubated under the same conditions. Samples were taken over time for measurement of cell density and for determination of As speciation. Samples were centrifuged, decanted and stored at –20°C prior to As analyses. Dissolved oxygen (DO), oxidation reduction potential (ORP) and pH were also monitored. All experiments were done in triplicate, and the mean values were taken into account.

1.4. Effect of pH on As(III) oxidation

The effect of pH on bacterial oxidation of As(III) was assessed following the same procedure used in the batch oxidation experiment. The pH values of the culture medium were adjusted to 4, 5, 6, 7, 8, 9 and 10 with predetermined amounts of filter-sterilized NaOH (1 mmol/L) and HCl (1 mmol/L). Samples were taken over time to determine the As(III) and As(V) concentrations as well as the bacterial growth in terms of optical density (600 nm).

1.5. Bacterial As(V) reduction experiment

A batch test was also done to determine the As(V) reduction ability of *Pseudomonas* sp. HN-2. The test medium was initially supplemented with 5 mg/L of As(V) under the same conditions as in Section 2.4.

To test the strain for its ability to grow by arsenate respiration, anaerobic growth experiments were conducted. The basal growth medium for anaerobic culturing was a CDM that consisted of resazurin dye (1 mg/L) and cysteine-HCl (1 mmol/L). The medium was first boiled for 30 min, then cooled on ice while purging with N₂, and then autoclaved. After autoclaving, cysteine-HCl was added separately from sterile anaerobic stock solutions. Three sets of anaerobic experiments were carried out in serum bottles: (i) inoculated medium containing 5 mg/L As(V), (ii) inoculated medium with no As added, and (iii) non-inoculated controls containing 5 mg/L As(V) (three replicates). The cell density was 10⁷ CFU/mL at the beginning. The experiments were incubated at 30°C.

1.6. Effect of As(III) on bacterial growth

The effect of As(III) on growth of the bacterium was evaluated to determine the strain's tolerance to As(III) using 48 hr growth-inhibition bioassays. Chemically defined medium containing As(III) (0.5–20 mmol/L) was inoculated with cell suspensions to a final density of 10⁷ cells/mL. A no-As(III) medium was served as the control. The 50% effective concentration (EC₅₀) was defined as the initial As(III) concentration resulting in a 50% decrease in growth when compared with no-As(III) control culture (Bahar et al., 2012). It was determined by applying a non-linear regression model with a Four Parameter Logistic Curve, using the software SigmaPlot (Version 10.0). The minimum inhibitory concentration (MIC) represented the lowest concentration that completely inhibited the growth.

1.7. Analysis methods

Total As (As(III) + As(V)) concentration was determined using inductively coupled plasma optical emission spectroscopy (ICP-OES) (OPTIMA 8300, USA). Prior to the analysis, the aqueous samples were acidified with concentrated HCl, and stored in acid-washed glass vessels. Selective As(III) analysis was performed using hydride generation-atomic fluorescence spectroscopy (HG-AFS) (Haiguang, China) (Shi et al., 2003). 1 mL of the test sample was transferred into a 10 mL test tube and 2 mL of 0.5 mol/L citric acid solution was added. The solution was diluted to 10 mL with deionized water and then was analyzed. Citric acid (0.1 mol/L) was used as the carrying fluid (Zhang et al., 2007).

Cell densities were determined by measuring the optical density at 600 nm using a UV/VIS spectrophotometer (UNICO, China). DO and pH were measured using a portable Hach (Sens-Ion) Multi meter (USA); ORP was measured using a portable ORP meter (METTLER TOLEDO SG, China).

1.8. Scanning electronic microscopy analysis

The isolated As(III)-oxidizing bacteria was investigated by scanning electronic microscopy (SEM). Exponential-phase culture was centrifuged and fixed with 2.5% glutaraldehyde in 4°C. Then, cells were washed with phosphate buffer three times. Samples were dehydrated with alcohol, and dried with liquid CO₂ using the critical point method. Finally, samples were gold coated in a sputter coater and examined in the SEM (FEI Quanta 200, Czech).

1.9. PCR amplification of 16S rDNA, *aroA* gene and *arsC* gene

Bacterial genomic DNA was extracted using TIANamp Bacteria DNA Kit (TIANGEN, China) following the manufacturer's instructions. DNA was dissolved in Tris-EDTA buffer and stored at –20°C until use. The extracted DNA was used in PCR amplification as DNA template. The PCR amplification was carried out in a 50 µL PCR mixture. The mixture contained 5 µL 10 × PCR buffer, 4 µL dNTP (1.5 mmol/L, final concentration), 1 µL of each primer (0.4 mmol/L, final concentration), 0.2 µL BSA (10 mg/mL), 2 µL template DNA, 0.4 µL Taq DNA polymerase (5 U/µL, Takara, China) and addition of RNase free ddH₂O (Takara, China) to a final volume of 50 µL. Universal eubacterial primers (27F: 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R: 5'-GGYTACCTTGTTCAGACTT-3') (Lane, 1991) targeting 16S rRNA gene and primers (F: 5'-GTSGGBTGYGGMTAYCABGYCTA-3'; R: 5'-TTGTASGCBGGNCGRTTTRTGRAT-3') targeting *aroA* gene (Inskeep et al., 2007) were used for PCR. The occurrence of arsenate reductase gene *arsC* in the isolated strain was investigated using five reported primers (Bachate et al., 2009; Chang et al., 2008; Macur et al., 2004; Macy et al., 2000; Sun et al., 2004). The PCR products were checked using 1.0% agarose gel electrophoresis and sequenced.

1.10. Phylogenetic analysis

The initial sequences were searched against the GenBank database by using BLASTn (for 16S rDNA) and BLASTx (for *aroA* gene). Phylogenetic analysis was carried out with MEGA 5.0 using a neighbor-joining method (Tamura et al., 2007).

1.11. GenBank accession numbers

The nucleotide sequences of 16S rDNA and *aroA* gene of *Pseudomonas* sp. HN-2 have been deposited in GenBank under the accession numbers KJ452338 and KJ452340.

2. Results

2.1. Isolation and morphology of *Pseudomonas* sp. HN-2

Eight indigenous bacteria strains were isolated from the As-contaminated soil (data not shown), among which one

strain capable of As(III) oxidation was isolated, and its oxidizing ability was evaluated in additional experiments. Cells of strain HN-2 were rods, 1–1.4 μm in length and 0.5–0.6 μm in diameter (Fig. 1). The strain was characterized as aerobic and gram-negative.

2.2. Phylogenetic identification

BLAST and similarity analysis of the near full length 16S rRNA gene sequences (1465 bp) of strain HN-2 showed that it belonged to *Pseudomonas* genera and was most similar to *Pseudomonas stutzeri*, with sequence similarities of 99.2%. The phylogenetic relationships among the non-As(III)-oxidizing bacteria of *Pseudomonas* and selected representatives of As(III)-oxidizing bacteria and As(V)-reducing bacteria within α -, β - γ -Proteobacteria and Firmicutes are shown in Fig. 2. The As(III)-oxidizing bacteria and As(V)-reducing bacteria are phylogenetically diverse. *Pseudomonas* sp. HN-2 had more than 98% 16S rDNA sequence identity with *P. stutzeri*, suggesting it may be a strain of the same species. Both As(III)-oxidizers and As(V)-reducers have been found in *Pseudomonas*.

2.3. Sequencing and characterization of arsenite oxidase gene *aroA* and arsenate reductase gene *arsC*

Fragments (500 bp) of the *aroA* gene (synonymous with *aoxB* and *asoA*) that encode the Mo-pter subunit of arsenite oxidase were amplified from the genomic DNA of *Pseudomonas* sp. HN-2. Nucleotide sequences of the PCR product obtained from the strain were translated and the deduced amino acid sequences (164 aa) showed clear homology to the arsenite oxidase large subunit from different organisms. Alignment with different proteins showed the highest identity (100%) with the arsenite oxidase large subunit from *P. stutzeri* (Genebank accession no. WP_003291052.1).

Although growth studies confirmed the strain as an As(V)-reducing bacterium, amplification of arsenate reductase gene

arsC was not successful using a range of primers and PCR conditions.

2.4. As(III) oxidation by the isolated strain in batch experiments and the effect of pH

Strain HN-2 was able to grow in CDM, containing 5 mg/L As(III), by oxidizing As(III). The optimum pH and temperature for the isolate growth were found to be 7 and 30°C. In aerobic conditions, with initial cell density of 10^7 CFU/mL, strain HN-2 was able to oxidize 92.0% (61.4 $\mu\text{mol/L}$) of As(III) to As(V) (Fig. 3). However, after 5 hr of incubation, reduction of As(V) to As(III) was observed. It was suspected to be caused by the decline of dissolved oxygen (Table 1), which might be expended by the microorganisms.

No growth was observed when cells were cultured with As(III) as a sole energy source and growth was not enhanced in the presence of arsenic, indicating that the strain is a heterotrophic oxidizer (data not shown). No oxidation of As(III) was observed in sterile controls, indicating that As(III) oxidation required the presence of microbial cells.

Optimum growth of the strain occurred at pH between 6 and 9. Transformation of As(III) to As(V) was 86.0%, 59.6%, 34.1% at pH 6, 8, 9, respectively. No growth or oxidation of As(III) was observed at pH 4, 5 and 10 even after 72 hr of incubation.

2.5. Arsenate reduction by strain HN-2

The reduction ability of HN-2 was also tested under aerobic and anoxic conditions. During the first 5 hr, microbes grew rapidly, but the concentration of As(V) remained relatively steady. After 5 hr of incubation, dissolved oxygen fell to around 2 mg/L (Table 1), and reduction took place (Fig. 4). After 10 hr incubation, dissolved oxygen dropped below 1 mg/L, thus creating an anoxic environment. Transformation of As(V) to As(III) by HN-2 was 94.4% (63.0 $\mu\text{mol/L}$) within 96 hr. The As(V) reduction rate was 36.5% at 24 hr.

No growth of the strain was observed when cultured with As(V) as the sole electron acceptor under anaerobic conditions. Therefore, strain HN-2 cannot grow by arsenate respiration.

2.6. Effect of As(III) on bacterial growth

The strain HN-2 was exposed to different concentrations of As(III) in CDM and the growth in terms of cell density (OD_{600}) was measured at different time intervals. A decrease in growth was observed with increasing As(III) concentration at any given time interval compared to the control (Fig. 5). There was 65.5%, 42.8% and 22.5% decrease over control in the growth of the strain at 48 hr incubation with 6, 7 and 8 mmol/L As(III), respectively. At 9, 10 and 12 mmol/L concentration of As(III), although little growth was observed initially, growth appeared after 48 hr of incubation. This could be due to the very high toxicity at higher concentrations, requiring an adaptive phase in the initial hours, and subsequently entering a growth phase after 48 hr. No growth was observed at 14 mmol/L concentration of As(III). Thus, the MIC would lie between 12 and 14 mmol/L. The isolate HN-2 was found to be tolerant to As(III) with a 48 hr EC_{50} value of 7.134 ± 0.249 mmol/L (99% confidence limits).

Fig. 1 – Electron micrographs of strain HN-2 grown in CDM. CDM: chemically defined medium.

Fig. 2 – Neighbor-joining tree showing the phylogenetic relationship of the isolated strain HN-2 compared with species of non-As(III)-oxidizing bacteria of *Pseudomonas*, As(III)-oxidizers and As(V)-reducers of *Pseudomonas* isolated so far and other main As(III)-oxidizing bacteria and As(V)-reducing bacteria within α -, β -, γ -Proteobacteria and Firmicutes (the scale bar represents 2 nucleotide substitution per 100 nucleotides of 16S rDNA sequence).

3. Discussion

Microbial oxidation of toxic As(III) is indispensable in biogeochemical cycling of As and has a great potential as a preoxidation strategy in the treatment process of As-contaminated water and soil. Abiotic reactions are comparatively slow and may

Fig. 3 – Growth (expressed as OD₆₀₀) and As(III) oxidation by strain HN-2 in CDM containing 5 mg/L As(III). CDM: chemically defined medium; As: arsenic.

take months or years to reach equilibrium (Cherry et al., 1979). As(III)-oxidizing bacteria are widely distributed in the environment. Some strains can be tolerant to extreme environments, such as *Polaromonas* GM1 isolated from the Arctic Circle (Osborne et al., 2010) and *Thermus* spp. isolated from a hot spring (Gihring et al., 2001). The ability of both As(III) oxidation and As(V) reduction has been shown for a thermophilic terrestrial hot spring bacterium, *Thermus* HR13 (Gihring and Banfield, 2001), halophilic Mono Lake bacterium, strain *Bacillus* sp. ML-SRAO (Fisher and Hollibaugh, 2008) and mesophilic hydrothermal marine bacterium *Marinobacter santoriniensis* NKSG1 (Handley et al., 2009). *Thermus* HR13 was isolated aerobically, while NKSG1 and ML-SRAO were isolated anaerobically or from reducing enrichment cultures. In this study, the results illustrate the capacity of an isolated soil bacterium, *Pseudomonas* sp. HN-2, to undertake both As(III) oxidation and As(V) reduction.

Pseudomonas is broadly represented among As-resistant bacterial strains isolated from other As-contaminated sites (Anderson and Cook, 2004). Both As(III) oxidizers and As(V) reducers in *Pseudomonas* have been isolated before. *P. stutzeri* GIST-BDan2 (EF429003) is an As(III)-oxidizing strain isolated from constructed wetlands (Chang et al., 2010). Three arsenate reducers of *Pseudomonas* were isolated in As-rich groundwater (Liao et al., 2011). *Pseudomonas* sp. C-4 (Wang et al., 2012) was found to have dual capacity, but only 70% of As(III) was oxidized and 1.6% of As(V) was reduced. With its dual capacity, strain HN-2 in this study is more efficient than *Pseudomonas* sp. C-4 in both As(III) oxidation and As(V) reduction.

Table 1 – Dissolved oxygen (DO), oxidation reduction potential(ORP) and pH of samples taken at different time intervals ^a.

Time(hr)	0			2			5			10			24			48		
	DO (mg/L)	ORP (mV)	pH	DO	ORP	pH	DO	ORP	pH	DO	ORP	pH	DO	ORP	pH	DO	ORP	pH
OE ^b	7.94	–52	7.5	4.38	–57	7.2	3.39	–60	7.2	0.81	–72	7.3	0.81	–47	7.7	0.45	–44	8.0
RE ^c	7.93	–52	7.5	4.45	–57	7.2	2.56	–60	7.2	0.92	–72	7.3	0.54	–47	7.7	0.64	–47	8.0

^a Values in the table are mean values of three samplings.

^b “OE” stands for “oxidation experiment”.

^c “RE” stands for “reduction experiment”.

Previous studies reported some highly efficient As(III) oxidizers: *Agrobacterium albertimagni* AOL15 completely oxidized 585 $\mu\text{mol/L}$ As(III) within 24 hr (24.38 $\mu\text{mol/L}\cdot\text{hr}$) with initial cell density of 10^6 – 10^7 CFU/mL (Salmassi et al., 2002); *Bacillus megaterium* AMO-10 oxidized 30 mmol/L of As(III) within 24 hr (1.25 mmol/L·hr) (Majumder et al., 2013); *Stenotrophomonas* sp. MM-7 oxidized 500 $\mu\text{mol/L}$ As(III) within 12 hr (41.67 $\mu\text{mol/L}\cdot\text{hr}$) with initial cell density of 10^6 – 10^7 CFU/mL (Bahar et al., 2012). However, some isolated strains tend to be low efficiency oxidizers, or even unable to completely oxidize As(III): *Bacillus* sp. AGH-02, AGO-S5, ADP-25 could only oxidize 83%, 74%, 80% of As(III) (Majumder et al., 2013); *Klebsiella pneumoniae* and *Klebsiella variicola* could only oxidize 87% and 83% of As(III) after 72 hr (Butt and Rehman, 2011); it took 48 hr for *Pseudomonas arsenicoxydans* sp. to finish oxidizing As(III) (0.138 $\mu\text{mol/L}\cdot\text{hr}$) (Campos et al., 2010). In this study, strain HN-2 oxidized 61.4 $\mu\text{mol/L}$ (92.0%) of As(III) to As(V) (20.47 $\mu\text{mol/L}\cdot\text{hr}$) with initial cell density of 10^7 CFU/mL within 3 hr.

Microbial oxidation of As(III) occurs over a wide range of pH. Some As(III)-oxidizing bacteria are able to oxidize As(III) at low pH (≤ 4), e.g., *Sulfolobus acidocaldarius* (Sehlin and Lindstrom, 1992) and *Thiomonas arsenivorans* (Dastidar and Wang, 2009). An autotrophic As(III)-oxidizing population (CASO1) was

isolated that exhibited As(III) oxidation activity between pH 3–8 (Battaglia-Brunet et al., 2002). However, most of the known As(III)-oxidizing bacteria demonstrated optimum oxidation at near-neutral pH. The pH for growth of strain HN-2 ranged from 6 to 9, while the optimum was 7. Bahar et al. (2012) reported that the optimum pH for As(III) oxidation was 5–7 for *Stenotrophomonas* sp. MM-7 and 6–8 for *Variovorax* sp. MM-1. Thus, pH modification is important for different cultures to maximize As(III) oxidation in the bioremediation of As(III). The ability of HN-2 to oxidize As(III) over a wide range of pH reveals its potential in remediation of As-contaminated water and soil.

The reduction rate for heterotrophic As(V) reduction by different strains varies considerably. Previous studies showed that the As(V)-reducing bacterium, *Citrobacter* sp. AR-7, almost completely reduced As(V) to As(III) with 3 hr incubation (833.33 $\mu\text{mol/L}\cdot\text{hr}$) (Liao et al., 2011), *Bacillus* sp. SXB reduced 95% As(V) of 1 mmol/L in 22 hr (43.18 $\mu\text{mol/L}\cdot\text{hr}$), and *Pantoea* sp. IMH reduced 97% As(V) of 1 mmol/L in 60 hr (16.17 $\mu\text{mol/L}\cdot\text{hr}$) (Wu et al., 2013). Some isolated strains reduced As relatively slowly. *Agrobacterium tumefaciens*, *Flavobacterium* sp., *Microbacterium* sp. and *Arthrobacter* sp. were As(V) reducing bacteria isolated in aerobic conditions. Rates of As(V) reduction by those strains ranged from 0.5 to 3.3 $\mu\text{mol/L}\cdot\text{day}$ per 10^9 cells

Fig. 4 – Growth (expressed as OD₆₀₀) and As(V) reduction by strain HN-2 in CDM containing 5 mg/L As(V).

Fig. 5 – Growth curves of strain HN-2 exposed to different concentrations of As(III) in CDM.

(Macur et al., 2004). In our study, the reduction rate for HN-2 was $0.66 \mu\text{mol}/(\text{L}\cdot\text{hr})$ with initial cell density of 10^7 CFU/mL.

To date, all known aerobic arsenite oxidases exhibit a heterodimeric structure with molybdopterin (Mo-pterin) and Rieske-like subunits (Inskeep et al., 2007). The large subunit (AroA ~90 kDa) of the arsenite oxidase is the first example of a new subgroup of the dimethylsulfoxide (DMSO) reductase family of molybdoenzymes (Ellis et al., 2001). It is reported that this heterodimeric enzyme is located on the outer surface of the inner membrane, and is transported to the cytoplasmic membrane (Lebrun et al., 2003). The arsenite oxidase gene has been used as genetic marker of As(III) oxidation (Hamamura et al., 2009). The large subunits of the arsenite oxidase gene of strain HN-2 were identified by PCR amplification using degenerate primers. The PCR products were later sequenced and confirmed as a part of the *aroA* gene. No oxidation was observed in the sterile controls, which indicated that As transformation required the presence of microbial cells. In addition, the strain could not grow in media designed for chemolithotrophic metabolism using As(III) as the sole electron donor. With the result that As(III) oxidation corresponded with microbial growth (Fig. 3), it is suggested that the mechanism of As(III) oxidation by the isolate was related to As detoxification rather than energy generation. Heterotrophic metabolic conversion of As(III) to As(V) is considered an As tolerance strategy, as As(V) is generally less toxic than As(III) (Cervantes et al., 1994; Cullen and Reimer, 1989).

Likewise, strain HN-2 could not grow in media designed for dissimilatory As(V) reduction, in which lactate served as the primary carbon and energy source and As(V) as the primary electron acceptor. This result was reasonable since the isolated organism was enriched under aerobic conditions. Thus, the reduction of As(V) by the isolated strain was also for detoxification purposes. Although growth studies confirmed the strain as an As(V)-reducing bacteria, amplification of the *arsC* arsenate reductase gene was not successful using a range of primers and PCR conditions. This result may suggest a significant degree of novelty in the genes encoding enzymatic As(V) reduction in HN-2. Several studies observed that despite clear evidence of As-transforming activity by microorganisms, no amplicon for arsenite oxidase (*aoxB*), As(V) respiratory reductase (*arrA*) or arsenate reductase (*arsC*) was attained using the reported PCR primers and protocols (Handley et al., 2009; Liao et al., 2011). Liao et al. isolated three arsenate-reducing strains of *Pseudomonas*, and PCR failed to amplify any As marker genes for the two *Pseudomonas* sp. strains AR-2 and AR-3. Several studies also showed that no *ars* gene was found in *Pseudomonas* sp. (Cai et al., 1998; Chang et al., 2007), suggesting that the *ars* operon is not always the same, even though the strains are categorized into the same genera. Therefore, this type of diversity should be considered when *ars* systems are examined using PCR-based techniques.

The MIC of As(III) for strain HN-2 lies between 12 and 14 mmol/L. Bacterial tolerance to metal can be higher in complex organic growth media due to the reduced availability of metal ions that are chelated by organic ligands, when compared to the values found in minimum medium (Weeger et al., 1999). Therefore, the values could be higher in real soil environments. The As(III) tolerance level of HN-2 is significantly higher than the previously reported arsenic-tolerant *Pseudomonas* sp.

(2–2.5 mmol/L) (Liao et al., 2011) and *P. arsenicoxydans* VC-1 (5 mmol/L) (Campos et al., 2010).

In our study, the DO, ORP and pH of the samples at different time intervals were monitored. Conditions within the culture were altered from aerobic to anoxic during the experiments. The change of pH from 7 to 8 has little effect on As transformation (Jing et al., 2008). Results indicated that the isolated strain behaved differently at different levels of DO, while pH and ORP kept relatively steady. It was reported by Liao et al. (2011) that an isolated As-resistant bacteria showed different As tolerance and that As(V)-reducing bacteria showed different As(V) reduction rates under aerobic and anoxic conditions. Thus, it is possible that bacteria behave distinctly at different DO because of diverse metabolic mechanisms in different growth conditions.

This study provides the first example of a soil As(III)-oxidizing and As(V)-reducing bacterium, and illustrates the potential impact on arsenic redox cycling within soil. Based on the results, *Pseudomonas* sp. HN-2 has the potential to impact arsenic mobility in both anoxic and aerobic environments. Under anoxic conditions, strain HN-2 may promote arsenic mobilization, as seen with other As(V)-reducing microorganisms. However, if conditions favor the formation of arsenic sulfides, As(V) reduction may lead to arsenic immobilization (Gihring and Banfield, 2001). In aerobic conditions, by rapid conversion of As(III) to a more readily adsorbed species, As(III) oxidation may result in diminished transport of As.

For the As remediation process in the environment, the best approach to remove As(III) is to oxidize it into As(V) first, which is less soluble and much easier to remove. The isolated strain, with oxidizing ability over a range of pH, has potential application in As remediation processes.

Due to the ability of strain HN-2 to grow under a variety of conditions and environments while reducing or oxidizing As, further studies are necessary to explore the impact of this organism on As cycling in nature.

4. Conclusions

Pseudomonas sp. HN-2 isolated from an As-contaminated soil was found to contain an arsenite oxidase gene catalyzing oxidation of As(III) to As(V). Optimum growth of the strain occurred at pH ranging from 6 to 9. The oxidation of As(III) to As(V) was 92.0% ($61.4 \mu\text{mol}/\text{L}$) within 3 hr of incubation. Reduction of As(V) to As(III) occurred in anoxic conditions. Our study provides the first example of a soil As(III)-oxidizing and As(V)-reducing bacterium, and illustrates the potential impact on arsenic redox cycling within soil. Therefore, strain HN-2 has the potential to impact arsenic mobility in both anoxic and aerobic environments, and has potential application in As remediation processes.

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