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Isolation and identification of indigenous prokaryotic bacteria from arsenic-contaminated water resources and their impact on arsenic transformation

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ABSTRACT

Arsenic is a known human carcinogen. Arsenite [As(III), H_3AsO_3] and arsenate [As(V), $H_2AsO_4^-$ and $HAsO_4^{2-}$] are the two predominant compounds of As found in surface water and groundwater. The aim of this study was to explore a bioremediation strategy for biotransformation of arsenite to arsenate by microorganisms. In this study, Babagorgor Spring, located west of Iran, was selected as the arsenic-contaminated source and its physicochemical characteristics and in situ microbiological composition were analyzed. Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) analysis indicated that the arsenic level was 614 µg/l. Fourteen arsenic tolerant indigenous bacteria were isolated from arsenic-contaminated water using chemically defined medium (CDM), supplemented with 260–3900 mg/l arsenite and 1560–21800 mg/l arsenate. Among the isolates, a strain As-11 exhibited high ability of arsenic transformation. Biochemical tests were used for bacterial identification and confirmation was conducted by 16 S rRNA sequence analysis. Results confirmed that As-11 was related to the genus *Pseudomonas*. This bacterium showed maximum tolerable concentration to arsenite up to 3250 mg/l and arsenate up to 20280 mg/l. Under heterotrophic conditions, the bacterium exhibited 48% of As(III) and 78% of As(V) transformation from the medium amended with 130 and 312 mg/l of sodium arsenite and sodium arsenate, respectively. Moreover, under chemolithotrophic conditions, bacterium was able to transform 41% of 130 mg/l of As(III) from the medium amended with nitrate as the terminal electron acceptor. *Pseudomonas* strain As-11 was reported as an arsenic transformer, for the first time.

1. Introduction

Heavy metals (HMs) are usually found in natural water, soils, and sediments (Ali et al., 2016; Zazouli et al., 2010). An important consideration in metal remediation is that they are generally non-biodegradable but can be transformed via several processes such as sorption, methylation, complexation, and/or change in valence oxidation states, affecting their mobilization and bioavailability (Adeniji, 2004). At low concentrations, metal ions are essential components in various life processes, and often play a crucial role in enzyme production (Akoto et al., 2014). However, at sufficiently high concentrations, HMs are known to be toxic for many species (Sundararajan et al., 2015). Fortunately, some microorganisms can induce the speciation and mobility of HMs; therefore, they can be used for the detoxification of HMs and as environmental clean-up agents (Tchounwou et al., 2012).

Arsenic (As) is one of the most abundant toxic HMs, and it occurs primarily in the form of the inorganic oxyanions arsenate (H_3AsO_4) [As(V)] and arsenite (H_3AsO_3) [As(III)]. In nature, the most common oxidation states of As are the trivalent (arsenite, As(III)) and pentavalent (arsenate, As(V)) forms. As(V) is often found co-precipitated with iron oxy-hydroxide (FeOOH), which may be immobilized under acidic and moderately reduced conditions. Under reducing conditions, As occurs in the form of As(III), which can cryoprecipitate with metal sulfides (Niggemyer et al., 2001). In addition, numerous environmental factors such as pH, redox potential, presence of other ions, organic matter content, soil texture, and fungal or bacterial activities also influence As speciation in soil (Turpeinen et al., 1999). Conventional remediation techniques for removing As include chemical precipitation, chemical oxidation or reduction, ion exchange, filtration, electrochemical treatment, reverse osmosis, membrane technologies, evaporation recovery, and *ex-situ* treatment systems based on metallic iron (Ahluwalia and Goyal, 2007; Casentini et al., 2016; Ebrahimi et al., 2013; Maleki et al., 2015). These techniques may require expensive

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equipment or lots of chemical reagents. For example, reverse osmosis and ion exchange are expensive methods, especially when used for high concentrations of HM ions (Saeed and Shaker, 2008). In addition, the by-products of chemical reagents may produce secondary environmental pollutants. Thus, to overcome the above-mentioned drawbacks, novel low-cost techniques for reducing the toxicity of contaminants have been proposed.

Although As is toxic to most organisms, some prokaryotes have evolved mechanisms to gain energy by oxidizing or reducing it (Oremland and Stolz, 2003; Stolz et al., 2006). Microorganisms are ubiquitous in arsenical geochemical environments and influence the biochemical cycle of As through conversion to As forms with different solubility, mobility, bioavailability, and toxicity (Silver and Phung, 2005). Several bacteria of different phylogenetic groups have been reported to be involved in As transformation processes through reduction, oxidation, and methylation mechanisms (Stolz and Oremland, 1999).

Bacterial oxidation of As(III) to As(V) has long been recognized (Tsai et al., 2009). Bacteria were isolated from As-rich environments, and some were identified as belonging to *Achromobacter*, *Pseudomonas*, *Alcaligenes*, *Thiobacillus,* and *Agrobacterium* genera. Although chemolithotrophic bacteria have been previously isolated (Santini et al., 2000), most of the As(III)-oxidizing bacteria are heterotrophs; they do not gain energy through As(III) oxidation (Lloyd and Oremland, 2006).

Arsenic speciation can affect detoxification reactions in aqueous media, focusing mainly on redox changes between the As(III) and As(V) oxidation states. Other microbial defense mechanisms include a variety of methylation reactions that produce methylated oxyanions of both As (III) and As(V) or form highly toxic methylated arsine gases, where the arsenic end product is in its most chemically reduced form (Welch et al., 2006).

Arsenic contamination is a big health concern in some areas in Iran. Babagorgor Spring, located southeast of Ghorveh, Kurdistan Province, in the west of Iran is contaminated with high levels of toxic oxyanion forms of As. In this region, no scientific and research-based study has been conducted on indigenous bacteria and their impact on arsenic transformation. The present study aimed to isolate and identify the native prokaryotic bacteria from As-contaminated water resources of Babagorgor Spring and to evaluate their ability to enhance As bioremediation.

2. Materials and methods

2.1. Sample collection

Water samples were collected from Babagorgor Spring, Iran, which has an As content of 614 µg/L. The geographic coordinates of the spring are between 35°17′22″N latitude and 47°54′14″E longitude. Then, 250 mL water samples were aseptically collected in sterile screw-capped bottles and immediately transferred to the laboratory for analysis. Samples were processed within 5 h to isolate As-resistant bacteria. Physicochemical parameters such as temperature, pH, dissolved oxygen, and As concentration were measured according to the Standard Methods for the Examination of Water and Wastewater (Eaton et al., 1998).

2.2. Isolation of arsenic resistant bacteria

For isolation of heterotrophic bacteria, the water samples were inoculated into a chemically defined medium (CDM) described previously (Weeger et al., 1999) containing 260 mg/L As(III) or 3120 mg/L of As(V) to enrich bacterial populations, and the medium was incubated in oxic conditions. Briefly, CDM was prepared as follows: 100 mL of solution A (81 mM $MgSO_4{\cdot}7H_2O$, 187 mM NH_4Cl, 70 mM Na_2SO_4, 0.574 mM K_2HPO_4, 4.57 mM $CaCl_2{\cdot}2H_2O$, 446 mM sodium lactate), 2.5 mL of solution B (4.8 mM $Fe_2SO_4{\cdot}7H_2O$), and 10 mL of solution C (950 mM $NaHCO_3$) were mixed. Enough water was added to this solution to obtain a volume of 1 L. The final pH of the medium was approximately 7.2. All the solutions were prepared using purified water (Milli-Q system; Millipore). Solution A was sterilized by autoclaving (at 120 °C for 20 min), and solutions B and C were sterilized by filtration (0.45-µm-pore-size filter; Millipore). Each sample was then incubated on a rotary shaker at 25 °C. Then, at 48-h intervals, a subculture was prepared on CDM agar plates containing 260 mg/L of As(III) or 3120 mg/L of As(V) at 25 °C, and the plates were incubated for up to 10 days to obtain single colonies. A pure culture was obtained by successive isolation of colonies at 25 °C in As(III)- or As(V)-supplemented medium. Resistance to As was defined as the ability of bacteria to grow on CDM agar plates containing either 260 mg/L of As(III) or 3120 mg/L of As(V) at 25 °C.

Chemolithotrophic bacteria in water samples were enriched by inoculation into a CDM without sodium lactate and containing 260 mg/L As(III) or 3120 mg/L As(V) as the only energy source, and the plates were incubated in oxic conditions. Samples were then incubated on a rotary shaker at 25 °C for 2 months. Then, at intervals of seven days, a subculture was made on CDM agar plates containing either 260 mg/L of As(III) or 3120 mg/L of As(V) at 25 °C to obtain single colonies.

Pure culture was obtained by successive isolation of colonies at 25 °C in As(III) or As(V)-supplemented medium. Resistance to As was defined as the ability of bacteria to grow on CDM agar plates containing 260 mg/L of As(III) or 3120 mg/L of As(V) at 25 °C.

2.3. Evaluation of Maximum Tolerable Concentration (MTC)

We determined the arsenic tolerance property in terms of maximum tolerable concentration (Schmidt and Schlegel, 1994). The MTCs of 260–3900 mg/L sodium arsenite ($NaAsO_2$) and 1560–21800 mg/L sodium arsenate ($Na_2HAsO_4{\cdot}7H_2O$) were defined as the highest concentration of As salt that allows microbial growth after incubation for 2 days at 25 °C (Srivastava et al., 2012).

2.4. Resistance to HMs ions

Resistance of isolated bacteria to some HMs were tested using 1 M stock solutions of different metals (Ni, Cu, Co, Zn, Hg, Pb, and Ag). The minimum inhibitory concentrations (MICs) of the metals were determined by broth dilution method using flasks containing 50 mL of Luria-Bertani broth supplemented with various concentrations of $NiCl_2$, $CuSO_4$, $CoCl_2$, $ZnSO_4$, $HgCl_2$, $Pb(NO_3)_2$, and $AgNO_3$. The flasks were then inoculated with overnight bacterial cultures and incubated at 25 °C for 72 h. Broth tubes containing no added HMs were used as the control. After incubation, growth was confirmed by measuring the optical density at 600 nm (Raja et al., 2006). The MIC was defined as the minimum metal concentrations that inhibited the confluent growth of bacteria (Wiegand et al., 2008). Each experimental set up was prepared in duplicate.

2.5. Identification and characterization of bacterial strains

Morphological and physiological characteristics of bacteria isolated on CDM medium were determined. The bacterial isolates were obtained from growth cultures with the optimum experimental conditions of pH, temperature, and contact time, and characterized for different biochemical tests, including catalase, oxidase, urease, Voges Proskauer, citrate utilization, and oxidation/fermentation (O/F) tests. These tests were then used to identify the isolates with reference to the Bergey's Manual of Systematic Bacteriology (David et al., 2001), determinative bacteriology, and probabilistic identification matrix. For 16 S rRNA sequence determination, total DNA was extracted according to the modified Marmur method (Marmur, 1961). Bacterial 16 S rRNA was amplified from the extracted genomic DNA using the universal bacterial