Detection of arsenite-oxidizing bacteria in groundwater with low arsenic concentration in Rayong province, Thailand

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Abstract. As contamination in groundwater has become one of the global problems. It has been reported that million people around the world get adverse effects on their health by direct and indirect As-contaminated groundwater consumption. In groundwater, the most abundant As species are arsenite (As^{3+}) and arsenate (As^{5+}). Arsenite is more toxic and mobile than arsenate. Consequently, arsenite oxidation is considered as an important process in groundwater As bioremediation. It has been reported that arsenite-oxidizing bacteria play an important role in reducing As toxicity in contaminated groundwater environment. A functional gene involved in arsenite oxidation is *aoxA* gene. The key objective of this study was to investigate the arsenite-oxidizing bacteria in groundwater with low arsenic concentration in Rayong province, Thailand. The results showed that the arsenite-oxidizing bacteria were detected in groundwater with low arsenic concentration. Phylogenetic analysis revealed that they were closely related to *Rhizobium* sp., *Bradyrhizobiaceae* sp., *Hydrogenophaga* sp., and *Stenotrophomonas* sp. The knowledge gained from this study will help better understand the distribution of arsenite-oxidizing bacteria in groundwater with low As concentration.

1 Introduction

Arsenic (As) is considered as toxic metalloids that widely distributes on the earth's surface. It can cause both acute and chronic effects to human health [1]. It has been reported that there are several countries around the world such as Vietnam, China, Taiwan, Mexico, Argentina, India, Bangladesh and Thailand have As contamination in groundwater problems and millions of people get effects from As by the consumption of this As-contaminated groundwater. Due to As toxicity, the World Health Organization has set the standard of As concentration in drinking water to be $<10 \mu\text{g/l}$. In groundwater, the most abundant As species are arsenite and arsenate. It is found that arsenite has more toxicity and mobility than arsenate [2]. Both geogenic and anthropogenic origins are considered as sources of As in environment. According to the geological information, As can be released to an environment by the weathering of As-bearing rocks such as granite or the oxidation and dissolution of As-bearing minerals for instance, sulfide minerals and oxide minerals. Furthermore, As can be discharged to an environment by human activities such

as agriculture, industry and mining operations which can generate high As concentration. [2, 3]. There are many techniques which are used to remove As from groundwater for example, physiochemical techniques such as chemical oxidation, coagulation, reverse osmosis and ion-exchange. It has been reported that these techniques are supposed to be very expensive, inefficient, particularly when As concentration in groundwater is very low [4]. In the present, As bioremediation is considered as an alternative choice to treat As contaminated groundwater. It is found that several microorganisms can survive in high As concentration environments by relying on their cellular mechanisms [5]. In oxidizing environments which arsenate is a predominant As specie. Arsenate can be converted to arsenite by the mechanism of arsenate-reducing bacteria. Heterotrophic cytoplasmic arsenate-reducing bacteria can reduce arsenate to arsenite by using their cytoplasmic arsenate reductase (*arcC*) and this activity is considered as a detoxification process [6]. Conversely, dissimilatory arsenate reducing bacteria is considered as an arsenate respirer. These microbes enable to transform arsenate to arsenite by their

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respiration process and gain energy for growth [7]. It has been reported that this mechanism is driven by their respiratory arsenate reductases (*arr*) [8]. In reducing environments, arsenite is dominant. Arsenite-oxidizing bacteria have an important role in controlling this toxic arsenite [9]. Arsenite is oxidized to arsenate by mechanism of heterotrophic and chemoautotrophic arsenite-oxidizing bacteria by using their specific enzyme called respiratory arsenite oxidase (*aio/aox*) [10]. The arsenite oxidation of heterotrophic arsenite-oxidizing bacteria (HAOs) is considered as a detoxification process because these microbes unable to gain any energy from this activity to produce their cell materials [11]. While chemoautotrophic arsenite oxidizing bacteria (CAOs) are regarded as an arsenite eater. They can conserve energy for growth through their oxidation process [12]. It has been reported that *Bordetella* sp. strain SPB-24 and *Achromobacter xylosoxidans* strain SPB-31, could survive in high As contaminated garden soil by their As detoxification process [13]. It has also been reported that *Herminiimonas arsenicoxydans* strain UPLAs could chemoautotrophically grow in arsenite concentration up to 500 mg/l [14]. Since several arsenite oxidizing bacteria were found in various rich As concentration environments, the purpose of this study was to investigate the communities of arsenite-oxidizing bacteria in groundwater with low As concentration to better understand their inherent distributions in this natural environment.

2 Material and methods

2.1 Site description and sample collection

The groundwater sampling point (GW1) located in Muang district, Rayong province, Thailand with the coordinate of 12° 44' 2" N and 101° 9' 51" E. This area was considered as a dense residential area. The groundwater samples were collected from the groundwater well in the volume approximately 3 liters and stored on ice during transportation.

2.2 Geochemical analysis

Geochemical parameters such as pH, electrical conductivity (EC), oxidation-reduction potential (ORP), dissolved oxygen (DO) and temperature were measured on site. Total organic carbon was analyzed using a total organic carbon analyser (TOC-VCPH). Total As was analyzed using inductively coupled plasma mass spectrometry (ICP-MS) at the Central Laboratory (Thailand) Co., Ltd. Chachoengsao province.

2.3 DNA extraction

The genomic DNA was extracted by using the FastDNATM SPIN Kit for Soil and the FastPreo® instrument (MP Biomedicals, Santa Ana, CA). DNA quality was determined by using agarose gel

electrophoresis, while DNA quantity was evaluated by using spectrophotometer nanodrop, respectively. Then, extracted DNA was diluted for the final concentration of 5 ng/μl for using as a DNA template in PCR reaction.

2.4 Polymerase chain reaction (PCR)

A DNA template was used to amplify an arsenite oxidase gene (*aioA* gene) by following conditions of the manufacturer protocol of BioLabs (Taq DNA Polymerase with Thermopol® Buffer: M0267S). The primers: Aox BM1-2F ND and Aox BM2-1R ND were used to amplified *aio* gene [15]. PCR profile was performed as follow: initial denaturation at 95°C for 30 seconds followed by 35 cycles of denaturation 95°C for 30 seconds, annealing 55°C for 30 seconds, extension 68°C for 30 seconds, and final extension at 68°C for 5 minutes.

2.5 DNA purification

DNA purification was performed by following NucleoSpin® Gel and PCR Clean-up [16]. The PCR product was mixed with Buffer NT1 in NucleoSpin® Gel and PCR Clean-up column within 2ml. collection tube, after that the centrifugation was done for 30 seconds at 11,000 x g. Then, 700 μl. of Buffer NT3 was added, and the centrifugation was performed again at the same condition. After that, the column was put into 1.5 ml. Microcentrifuge-tube. The 30 of Buffer NE was added, then the microcentrifuge tube was centrifuged for 1 minute at 11,000 x g. Then, incubation at room temperature was done for 1 minute. Finally, purified DNA in the microcentrifuge tube was kept for cloning.

2.6 Clone library

The pGEM®-T and pGEM®-T Easy Vector Systems' protocol was used as a ligation and transformation guideline. The ligation product was incubated overnight at 4°C. After that, transformation was conducted by using XL1-blue super competent cells. The heat shocking was performed at 42°C for 45 seconds following by incubation in shaker at 37°C for 1 hour with shaking at 250 rpm. After that, the transformed product was spread on an LB agar plate which contained 100 mg/ml. of Ampicillin, 50mg/ml. of X-gal and 1mM. of IPTG. The incubation was performed overnight at 37°C. Finally, 20-25 white colonies which contain arsenite oxidase gene were selected for sequencing.

2.7 DNA sequencing and phylogenetic analysis

The cloning samples were sent to Macrogen company in South Korea for bacterial DNA sequences analysis. The obtained sequences were compared with database of Genbank. After that the neighbor-joining tree was constructed with 1,000 bootstrap tests by using MEGA 7.021 [17].

3 Result and discussion

3.1 Geochemical properties of groundwater

The geochemical properties of groundwater sample could be briefly described. The pH of the groundwater sample was 7.1, whereas the dissolved oxygen (DO) of the groundwater was unexpectedly high showing 8.90 mg/l. Total dissolved solid (TDS) and total organic carbon (TOC) were found at 306.56 mg/l and 5.10 mg/l, respectively. Furthermore, the value of oxidation-reduction potential was 182.80 mV and total As concentration in groundwater was 0.64 µg/l. From the geochemical properties, it could be described that the groundwater was relatively neutral and moderately oxidizing condition. With this condition, As could be appeared in both arsenite and arsenate species [18]. The TDS was fairly high representing the salinity of groundwater. Moreover, this elevated TDS corresponds to the location of the study area which located near the Gulf of Thailand. The high TDS might result from the influence of saltwater intrusion [19]. A very high DO might be affected from the shallow depth of groundwater level and the continuous utilization by dense population. In the part of As concentration in groundwater, it was still in the range of the standard of 10 µg/l, reflecting the high-quality management in protecting the toxic substance releasing to groundwater resources. However, the existence of As and other geochemical physiochemical parameters could be supportive factors for the arsenite-oxidizing bacteria to survive.

3.2 Detection of arsenite-oxidizing bacteria

The arsenite-oxidizing bacteria in groundwater with low As concentration was detected by specific primers targeting *aioA* gene using the polymerase chain reaction (PCR). The size of *aioA* gene PCR product is 550 base pairs which was verified using an agarose gel electrophoresis. The groundwater sample analyzed in this study showed a positive result. This result implied that arsenite-oxidizing bacteria could be found in the environment which low As concentration [20]. Then this sample was analyzed to find the diversity of arsenite-oxidizing bacterial species by performing the clone library. Five (GW1-1, GW1-2, GW1-3, GW1-4 and GW1-5) analyzed sequences were blasted against the Genbank database using the BLASTn program. The results demonstrated that all analyzed sequences showed 76 – 84 % identity to previously reported arsenite-oxidizing bacteria (Table 1).

Table.1 The identification of *aioA* gene sequences analyzed by the BLASTⁿ program.

Sequence ID	Bacterial species	Genbank acc. No.
GW1-1	<i>Stenotrophomonas</i> sp.	JQ739209.1
GW1-2	<i>Hydrogenophaga</i> sp.	KM884951.1
GW1-3	<i>Rhizobium</i> sp.	KT992344.1
GW1-4	<i>Stenotrophomonas</i> sp.	JQ739209.1
GW1-5	<i>Bradyrhizobiaceae</i> sp	AB974343.1

Phylogenetic analysis showed that all arsenite-oxidizing bacteria detected in this study were closely related to α , β and γ -Proteobacteria (Fig 1). The bacterial clone GW1-3 and GW1-5 were closely related to *Rhizobium* sp. and *Bradyrhizobiaceae* sp., respectively. These two bacterial species were a member of α -Proteobacteria. The bacterial clone GW1-2 was highly similar to *Hydrogenophaga* sp. which belongs to β -Proteobacteria. Furthermore, the result also demonstrated that the bacterial clone GW1-1 and GW1-4 were closely related to *Stenotrophomonas* sp. which were a member of γ -Proteobacteria (Fig. 1). It has been reported that the closely related bacterial species such as *Rhizobium* sp., *Bradyrhizobiaceae* sp., *Hydrogenophaga* sp. and *Stenotrophomonas* sp. carried the *aioA* (*aoxB*) gene which was considered as a genetic marker of arsenite-oxidizing bacteria [15]. *Rhizobium* sp. NT-26 was found on arsenopyrite rocks in gold mining where As concentration was in a range of 2,000-13,000 µg/l [21]. This bacteria showed chemolithoautotrophic growth which used arsenite as an electron donor, oxygen as an electron acceptor and carbondioxide or bicarbonate as carbon source [21, 22]. *Hydrogenophaga* sp. was detected in the shallow groundwater aquifer with various As concentrations (1-763 µg/l.) [23]. In addition, *Stenotrophomonas* sp. isolated from soil with low As concentration of 8.8 mg/kg. demonstrated the heterotrophic arsenite-oxidation by using organic substances such as glucose and maltose as a carbon source, but this bacteria cannot gain energy for its growth [24]. This information indicated that arsenite-oxidizing bacteria could be found in several environments for example, mineralized area, soil, and groundwater which various As concentrations. Furthermore, all of these bacteria for instance, *Rhizobium* sp., *Bradyrhizobiaceae* sp., *Hydrogenophaga* sp. and *Stenotrophomonas* sp. could relieve the toxicity of As by transforming to its less toxic form. The knowledge from this study will be useful for better understanding the distribution of arsenite-oxidizing bacteria in groundwater found in low As concentration. The knowledge gain from this study is the first important step which can be built on for further development of As bioremediation.

Fig. 1. *aioA* gene based phylogenetic tree constructed by using the neighbor-joining model with 1000 bootstrap value.

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