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# Arsenite Oxidation and Arsenite Resistance by *Bacillus* sp. PNKP-S2

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**Abstract**

Arsenic causes human health problems after accumulate in the body for 10-15 years and arsenite [As(III)] is generally regarded as being more mobile and toxic than other oxidation states. In this study, two-hundred and three bacterial strains were isolated from groundwater and soil samples collecting in Ubon Ratchathani Province, Thailand. All strains were screened for arsenic tolerant efficiency at 1-10 mM of sodium arsenite. Eighteen selected strains which had the highest resistance to 10 mM of As(III) were further studied for their As(III)-oxidizing activity and growth in enrichment and growth medium (EG medium) supplemented with 0.58 mM of As(III). It was found that strain PNKP-S2 was able to grow in the medium with As (III) as a sole energy source and had 89.11% As(III) removal within 48 h. The PCR-based 16S rDNA sequencing analysis revealed that the strain PNKP-S2 was closed relative to *Bacillus* sp. This is the first report on *Bacillus* sp. chemolithoautotrophic As(III)-oxidizer and this strain could be a potential candidate for application in arsenic remediation of contaminated water.

***Keywords:*** arsenic; arsenite; arsenite-oxidizing bacterium; arsenic-tolerant bacteria; *Bacillus* sp.

## 1. Introduction

Arsenic (As) is a semimetal or metalloid which is the twentieth most abundant element in the earth's crust and ubiquitous in the environment. It is mobilized through natural process such as weathering reaction, volcanic emissions and biological activities as well as through anthropogenic activities including mining activity, herbicide use, and livestock feeding (Smedley and Kinniburgh, 2002). Thus it is often responsible for contaminating in soil, ground and surface water and subsequent serious environment hazard and public health concern due to chronic arsenic poisoning (arsenicosis) in many part of the world, mainly in Bangladesh and India (West Bengal) (Nickson *et al*., 2000). Arsenic contamination of groundwater is also an emerging issue in Mekong Basin including Cambodia, Vietnam, and Thailand. In some area of Northeastern part of Thailand, few parameters like Cl, Fe, Mn, and As exceeded the World Health Organization (WHO) guideline limits (Pattanapipitpaisal and Suraruk, 2012). Arsenic is stable in several oxidation states: arsine (-III), elemental arsenic (0), arsenite (+III), and arsenate (+V), but the most common observed in the environment are the trivalent form arsenite [$H_3AsO_3$;As(III)] and pentavalent form arsenate [$HAsO_4^{2-}$; As(V)] (Smedley and Kinniburgh, 2002). The As(III) is hundred times more toxic than As(V). Further, it is more difficult to remove from water due to its high solubility. It is most common in the aqueous phase, where it is more mobile and can entry into food chain under environmental condition (Kingegam *et al*., 2008). As(III) could bind sulfhydryl groups of cysteine residues in protein, thereby inactivating them. In contrast, As(V) is poorly soluble in water and, typically bound to minerals in the solid phase and thus is less available. As(V) is a chemical analogue of phosphate which can interfere with the normal oxidative phosphorylation (Mandal and Suzuki, 2002; Ordonez *et al*., 2005). Arsenic remediation techniques could be applied via physical and chemical method including coagulation with ferric chloride or alum, sorption on activated alumina, activated carbon, and iron oxide-coated sand particles; hybrid cation-exchange resins; hybrid anion-exchange resins; polymeric anion exchange; membrane filtration and reverse osmosis (Ahuja, 2008). However, these methods generally require an oxidation step to transform As(III) to As(V) by using chemical oxidants such as ozone, chlorine and hydrogen peroxide which may produce harmful by-products (Jekel and Amy, 2006). Biological treatment could, therefore, provide a useful alternative economical process and environment-friendly. Many microorganisms have been reported to oxidize As(III) to As(V) and could be divided into two groups. For chemolithoautotrophs, As(III) act as electron donor, whereas $CO_2/HCO^-_3$ is used as the sole carbon source. As(III) oxidation is couple to oxygen or nitrate reduction such as the aerobe NT-26 which belongs to *Agrobacterium/Rhizobium* branch of the

α-*Proteobacteria* used oxygen as the terminal electron acceptor (Santini *et al*., 2000). Oremland *et al*. (2002) reported that 16S ribosomal DNA sequence placed strain MLHE-1 within the haloalkaliphilic Ectothiorhodospira of the γ-Proteobacteria. This strain used nitrate as the terminal electron acceptor. In the case of heterotrophs, the As(III) oxidation process is described as a detoxification mechanism catalyzed by the enzyme-arsenite oxidase (Muller *et al*., 2003). Several heterotrophic arsenite-oxidizing bacteria have been isolated such as *Alcaligenes faecalis* (Phillips and Taylor, 1976); *Agrobacterium albertimagni* AOL15 (Salmassi *et al*., 2002); *Thermus aquaticus* and *Thermus thermophilus* (Gihring *et al*., 2001); *Hydrogenophaga* sp. str. NT-14 (Hoven and Santini, 2004); *Bordetella* sp. SPB-24 and *Achromobacter* sp. SPB-31 (Bachet *et al*., 2012); *Variovorax* sp. MM-1 (Bahar *et al*., 2013). In present study, we collected samples in Ubon Ratchathani Province according to the groundwater in some areas of the Amphoe Khemmarat had arsenic concentration exceeding the WHO guideline limit of 10 µg/l (Pattanapipitpaisal and Suraruk, 2012). We then isolated, screened for As(III)-oxidizing bacteria and characterized of its potential for arsenite detoxification by the selected strains.

## 2. Materials and Methods

### *2.1. Sampling and strain isolation*

Ground water and soil samples were collected from Warin Chamrap district, Khong Chiam district and Khenmarat district in Ubon Ratchathani Province. The enrichment and growth medium (EG medium) was used as described by Gihring and Banfield (2001) with a little modified. The medium contained 0.2% (w/v) yeast extract, 0.8 g/l $(NH_4)_2SO_4$, 0.4 g/l $KH_2PO_4$, 0.18 g/l $MgSO_4 \bullet 7H_2O$, and 1.75 g/l NaCl and adjust pH to 7.0 with NaOH. The samples were inoculated to EG medium supplemented with 0.38 mM of As(III) (as $NaAsO_2$). Flasks were incubated at 30°C on a rotary shaker (150 rpm) for 5 days. Serial dilution of the cultures was spread on the same medium. Different colonies were purified using cross-streak plate technique. The stock culture was kept in Luria-Bertani broth (LB broth) with 15% glycerol at -70°C for further uses.

### *2.2. Screening of As(III)-resistant bacteria*

The isolated strains were inoculated in EG medium, and incubated with shaking at 30°C for 24 h. The culture (20 µl) was dropped on EG agar plate supplement with As(III) at concentration of 1.0, 5.0, and 10.0 mM and incubated at 30°C for 72 h. The arsenite-resistant level was defined as the ability of bacteria to grow on EG agar plate containing various concentration of As(III). Minimum inhibitory concentration (MIC) was defined as the lowest concentration of arsenite added which completely inhibited growth. Triplicate measurements were conducted for each isolates. Bacterial isolates that could resist to the highest As(III) concentration were selected for further study.

### *2.3. Assay of bacterial growth and arsenite removal*

The selected arsenite-resistant strains were screened for growth and arsenite-oxidizing activity. A single colony was grown in EG medium (pH 7.0) supplemented with 0.58 mM of As(III) at 30°C on a rotary shaker (150 rpm) for 48 h. The cultures were withdrawn and centrifuged at 5,500 *x*g at 4°C for 10 min. The cell pellets were resuspended with normal saline and were serially diluted and plated on EG medium and the number forming units per ml (cfu/ml) was calculated after incubating at 30°C for 48 h. The supernatant was determined for residual of As(III) using silver diethyl dithiocarbamate assay (APHA, 1998). Controls without inoculation were also incubated under the same condition.

### *2.4. Arsenic-transformation by PNKP-S2*

The selected strain, PNKP-S2, was test for arsenic-transformation by using a qualitative $AgNO_3$ as described by Simeonava *et al*. (2004) and Liao *et al*. (2011) with slightly modified. Briefly, the overnight culture was centrifuged and then washed twice with normal saline solution. The bacterial pellets were resuspended in EG medium (pH 7.0) supplemented with 0.58 mM of As(III). The flask was incubated at 30°C on a rotary shaker (150 rpm) for 48 h. Subsequently, the bacterial culture was centrifuged, and 100 µl of supernatant was mixed with 100 µl of a 0.1 M $AgNO_3$ solution. The result precipitates containing arsenic were colored from light yellow of $Ag_3AsO_3$ (silver orthoarsenite) due to As(III) to light brown-red of $Ag_3AsO_4$ (silver orthoarsenate) due to As(V).

### *2.5. Identification of As(III)-oxidizing bacterium*

For PCR amplification, a small amount of a bacterial colony was resuspended in 100 µl of sterile deionised water (SDW), mixed and lysed at 70°C (10 min). Crude lysate (0.2 µl) was added to 19.8 µl SDW and used as a PCR template. Universal bacterial 16S

rRNA gene primers pA (5′-AGAGTTTGATCCTG-GCTCAG-3′) and pH′ (5′AAGGAG GTGATCCAGC-CGCA-3′) were used to amplify the ~1.5 kb 16S rRNA gene fragment (Edwards *et al*., 1989). The following was added to each PCR template: 20 pmol of each primer, 50 µmol of each deoxynucleoside triphosphate, 2.5 unit of *Taq* DNA polymerase (Bioline) and 10 µl of 10× *Taq* DNA polymerase buffer (Bioline); reaction volumes were made up to 100 µl with SDW. Lysed *Escherichia coli* cells and 20 µl of SDW were used as positive and negative controls, respectively. Temperature cycling comprised 35 cycles of 94°C for 40 s, 55°C for 1 min, and 72°C for 2 min, followed by an additional 10 min at 72°C. Purified PCR products were sequenced by SolGent (Korea) using 16S sequencing primer 943 reverse (Lane *et al*., 1985). The 16S rRNA gene sequences were compared with known sequences in the European Bioinformatics Institute (EMBL) database using ADVANCED BLAST [BLASTN 2.1.1 (Altschul *et al*., 1997) to identify the most similar sequence alignment.

### *2.6. Statistical analysis*

The experiments were carried out at least in duplicate, and in triplicate in some cases. The results represent the means of the three separate experiments. Standard deviations and 95% confidence intervals were calculated using Microsoft Excel™.

## 3. Results and Discussion

### *3.1. Strain isolation*

Twenty-four ground water samples and twenty-five soil samples were collected from Warin Chamrap district, Khong Chiam district and Khenmarat district in Ubon Ratchathani Province, Northeastern part of Thailand. The samples were enriched in EG medium supplemented with 0.38 mM of As(III), and different 203 colonies which grew on the media were picked up and purified. Most of them were Gram-positive bacilli (124 strains), short rod (29 strains), and cocci (19 strains). Some are Gram-negative bacilli (21 strains), short rod (9 strains). Only one strain was cocci. These bacterial strains were isolated on the basis of their ability to grow in the presence of 0.38 mM of As(III). To date, most As(III)-resistant and -oxidizing bacteria have been reported and are isolated from high levels of arsenic-contaminated environment such as gold and sulphur pyrite mine wastewater (Ilyaletdinov and Abdrashitova, 1981), gold mine (Santini *et al*., 2000), hot spring (Gihring and Banfield, 2001), hot creeks (Salmassi *et al*., 2002), abandoned mines (Yoon *et al*., 2009), arsenic-rich groundwater (Liao *et al*., 2011), arsenic contaminated soil (Kinegam *et al*., 2008; Bahar *et al*., 2013) and from low levels of arsenic and uncontaminated sites such as garden soil (Bachate *et al*., 2012), metal industrial soil (Bahar *et al*., 2012). In this study, the bacteria were isolated from ground water and soil which contaminated with arsenic at low level concentrations ranging from 0.07 to 20.19 µg/l (Pattanapipitpaisal and Suraruk, 2012). This suggests a wide distribution of arsenic-resistant and -oxidizing bacteria in the natural environments.

### *3.2. Screening of As(III)-resistant bacteria*

Two hundred and three bacterial strains were then screened for their resistance in EG medium supplemented with 1.0, 5.0, and 10.0 mM of sodium arsenite. It was found that the bacterial strains were able to grow at different As(III) concentrations. Twenty-one strains had a MIC of arsenite of 1.0 mM, whist eighteen strains had a MIC of arsenite of 5.0 mM and fifty-six strains had a highest MIC of arsenite of 10.0 mM. Yoon *et al*. (2009) isolated As-resistant bacteria from soil samples in abandoned mine and found that *Alcaligenes* sp. RS-19 showed relatively high resistance to As(III) up to 26 mM. As-reducing bacteria exhibited resistance to As(III) ranging from 2.0 to 5.0 mM (*Pseudomonas* sp., *Psychobacter* sp., *Vibrio* sp., *Citrobacter* sp., *Enterobacter* sp., and *Bacillus* sp.) while As-oxidizing bacteria, *Bosea* sp. AR-11 was resistant to As(III) at 2.0 mM (Liao *et al*., 2011). Two heterotrophic As(III)-oxidizing bacteria, isolating from garden soil, *Bordetella* sp. SPB-24 and *Achromobacter* sp. SPB-31 exhibited high As(III) resistance at 15 mM and 40 mM, respectively (Bachate *et al*., 2012). A Gram-negative, arsenite-oxidizing bacteria, MM-1 tolerant to As(III) at 20 mM (Bahar *et al*., 2013). However, several factors such as the method of resistant determination and the medium composition, can affect arsenic bioavailability and toxicity, resulting in discrepancies of MIC values in microorganisms (Achour *et al*., 2007). Bacterial resistance to As has been understood through detoxification process (Silver and Phung, 2005), which could be divide into two systems include 1) an As resistance (as *ars* genes) (Rosen, 2002) and 2) As(III) oxidation (Muller *et al*., 2003; Kashyap *et al*., 2006)

### *3.3. Growth and arsenite oxidation*

Eighteen arsenite-resistant strains were selected for arsenite-oxidizing activity and growth in EG medium supplemented with 0.58 mM of As(III) for 48 h. There were seven strains (PRJK-W1, PRJK-W11, PRJK-W19, PRJK-W26, PRJK-W28, PRJK-W31,

PRJK-W43) and four strains (PRJK-S25, PRJK-S26, PRJK-S34, PRJK-S44) from groundwater and soil in Khong Chiam district, respectively. One strains (PNKR-W2) and two strains (PNKR-S7 and PNKR-S30) were isolated from groundwater and soil in Khenmarat district, respectively. While four strains (PNKP-S2, PNKP-S4, PNKP-S6, PNKP-S7) were obtained from soil in Warin Chamrap district. Morphological and colony characteristics of selected bacterial strains are shown in Table 1. The result showed that eighteen strains could grow and remove arsenite, but the strain PNKP-S2 showed the highest cell concentration and arsenite removal at 1.8 x $10^{11}$ cfu/ml and 89.11%, respectively within 48 h as showed in Figs. 1 and 2. Abiotic controls showed little change in As(III) concentrations. As seen in Figs. 1 and 2, almost in PRJK-strains, PNKR-W2 and PNKR-S7 had high properties in As(III) removal (Fig. 2) even they had less cell mass (Fig. 1), it is probably due to resting cell mechanism. Of the eighteen selected bacteria, the strain PNKP-S2 was selected for the further study of arsenic-transformation activity due to the highest As(III) removal activity in batch test.

The result showed a light brown-red precipitate revealed the presence of arsenate in the medium. It was concluded that strain PNKP-S2 was arsenite-oxidizing bacteria and bacterial oxidation of arsenite to the less mobile arsenate represents a potential detoxification mechanism by this strain. In addition, strain PNKP-S2 was able to grow in the medium with As(III) as a sole energy source, indicating that it is a chemolithoautotrophic As(III) oxidizer. There are other chemolithoautotrophs that have been reported. *Agrobacterium/Rhizobium-like* bacteria, NT-25 and NT-26, could promote growth by deriving energy from the oxidation of As(III) to As(V) using oxygen as the terminal electron acceptor, As(III) as the electron donor and carbon dioxide as the carbon source (Santini *et al*., 2000). MLHE1, a number of the γ- *Proteobacteria* oxidizes As(III) to As(V) using nitrate as the terminal electron acceptor (Oremland *et al*., 2002). *Bosea* sp. AR-11 was able to oxidize As(III) to As(V) under aerobic condition without the addition of any electron donors or acceptors (Liao *et al*., 2011). Therefore, several heterotrophic arsenite-oxidizing bacteria have been isolated including: *Alcaligenes faecalis* (Phillips and Taylor, 1976), *Thermus* sp. HR-13 (Gihring and Banfield, 2001), *Agrobacterium albertimagni* AOL15 (Salmassi *et al*., 2002) *Hydrogenophaga* sp. NT-14 (Hoven and Santini, 2004), *Alcaligenes* sp. RS-19 (Yoon *et al*., 2009), *Stenotrophomonas* sp. MM-7 (Bahar *et al*., 2012), *Bordetella* sp. SPB-24 and *Achromobacter* sp. SPB-31 (Bachate *et al*., 2012). It is concluded that heterophic metabolic conversion of As(III) to As(V) is a detoxification mechanism catalyzed by the enzyme-arsenite oxidase, rather than growth-supporting process (Muller *et al*., 2003; Santini *et al*., 2000)

### *3.4. Identification of As(III)-oxidizing bacterium*

DNA fragments of 1.5 kb of strain PNKP-S2 were amplified using pA and pH′ primers. The nucleotide sequence of approximately 1,090 bp of the 16S rRNA

Table 1 Morphological and colony characteristics of selected bacterial strains

| Strain no. | Colony characteristics | Gram stain and cell shape |
|---|---|---|
| PRJK-W1 | Circular, entire, raised, off-white color, moist | Gm -, short rod |
| PRJK-W11, PRJK-W28 | Irregular, undulate, convex, off-white color, moist | Gm +, bacilli |
| PRJK-W19, PRJK-W26, PRJK-S25, PRJK-S26 | Circular, entire, raised, off-white color, moist | Gm +, bacilli |
| PRJK-W31, PRJK-W43 | Circular, undulate, convex, transparent, moist | Gm +, bacilli |
| PRJK-S34 | Irregular, undulate, convex, off-white color, moist | Gm +, cocci |
| PRJK-S44 | Circular, entire, convex, off-white color, moist | Gm +, bacilli |
| PNKR-W2 | Circular, raised, smooth, entire, off-white color, translucent | Gm +, bacilli |
| PNKR-S7 | Circular, convex, smooth, entire, off-white color, moist | Gm +, bacilli |
| PNKR-S30 | Circular, raised, smooth, undulate, off-white color, translucent | Gm -, bacilli |
| PNKP-S2 | Circular, pulvinate, smooth, entire, off-white color, moist | Gm +, bacilli |
| PNKP-S4 | Circular, flat, smooth, entire, off-white color, moist | Gm +, bacilli |
| PNKP-S6 | Irregular, convex, smooth, entire, off-white color, moist | Gm +, bacilli |
| PNKP-S7 | Punctiform, pulvinate, smooth, entire, off-white color, translucent | Gm -, bacilli |

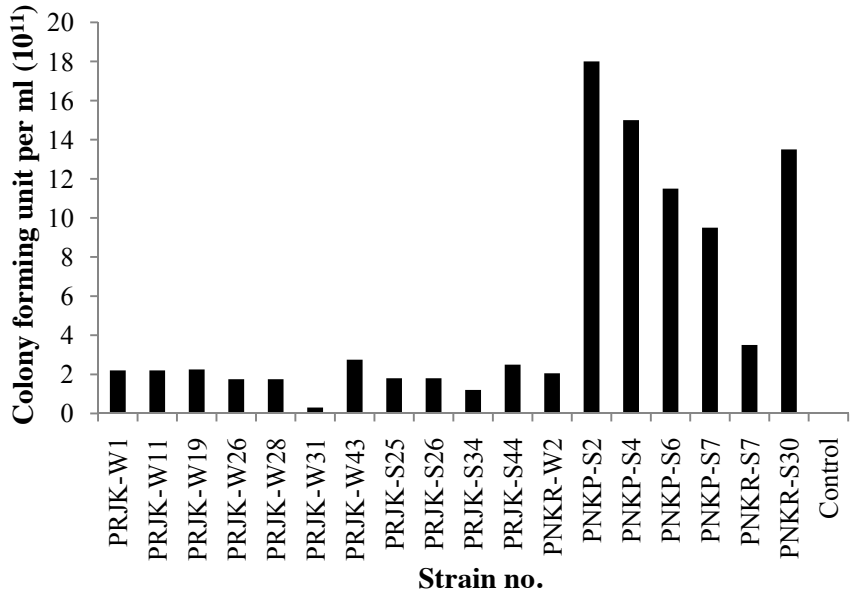


Figure 1. Cell concentration (cfu/ml) of eighteen strains in EG medium supplemented with 0.58 mM of As(III) for 48 h.

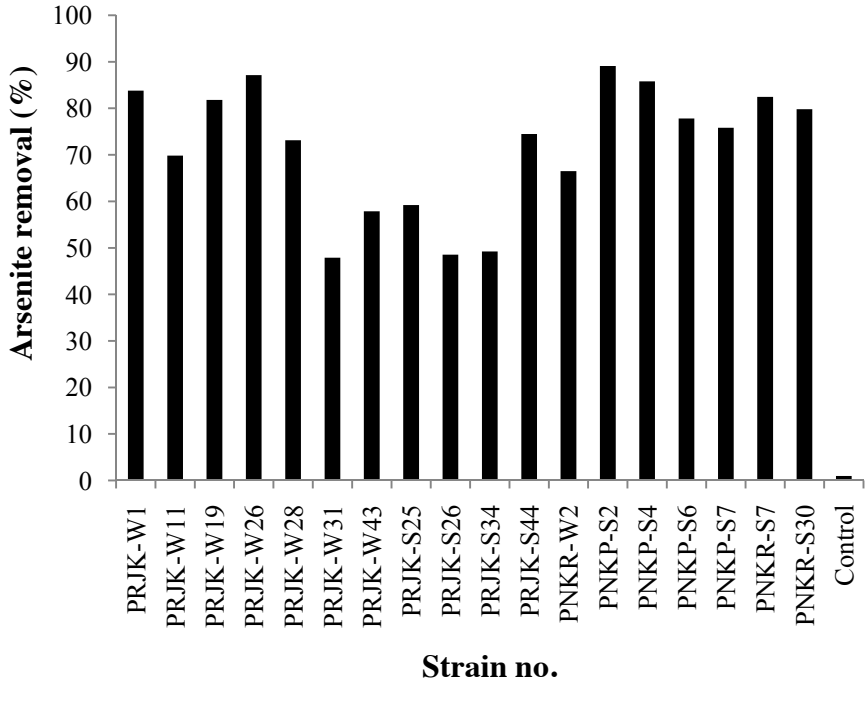


Figure 2. Arsenite removal of eighteen strains in EG medium supplemented with 0.58 mM of As(III) for 48 h.

was obtained, which aligned with the equivalent sequences of *Bacillus megaterium* strain TOBCMDU-1 16S rRNA gene with 97% identity. Based on the comparisons, we conclude that strain PNKP-S2 belonged to *Bacillus* sp. PNKP-S2.

## 4. Conclusion

Two-hundred and three bacterial strains were isolated from groundwater and soil samples collecting in Ubon Ratchathani Province, Thailand. Eighteen selected strains showed a resistance to high concentration of As(III). Among of them, *Bacillus* sp. PNKP-S2 was the most effective bacterium. This strain oxidized 0.58 mM of As(III) nearly completely within 48 h and it was suggested that *Bacillus* sp. PNKP-S2 was a chemo-lithoautotrophic As(III) oxidizer. Further studies are necessary to understand the factors effecting to As(III) oxidation by this strain as well as feasibility to use in arsenic remediation of contaminated wastewater.

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