Screening and Evaluation of Arsenite Transformation of Arsenic-resistant Bacteria Isolated from Cultivated Soil in Northern Thailand

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Abstract

A total of 47 isolates were isolated from cultivated soil of seven areas of northern Thailand. The results indicated that, with arsenic addition, only nine isolates (isolate 7, 8, 11, 19, 20, 22, 29, 30 and 36) showed a potential to tolerate high level of arsenic at low pH levels. When a concentration of arsenic was adjusted to the analyzed level found in the soil, only four isolates (isolate 8, 11, 19, and 29) were considered and selected as arsenic tolerant isolates. The results of incubation of the selected isolates arsenic-containing medium at pH 4.7 indicated that isolate 8, 11, 19 and 29 showed a potential to increase the medium pH to a much higher value with a good growth. Furthermore, the four isolates, particularly isolate 29, showed certain ability in reducing arsenic toxicity. These isolates could oxidized highly toxic form of arsenic; arsenite [As (III)] into less toxic form, arsenate [As (V)] (11.29 to 47.07%). Mechanisms involves in toxification of arsenic might be involved in the pH values change and arsenic reduction. Further investigations of the uptake of arsenic and arsenic species by some economic crops cultivated in contaminated soils are required because of their relevance to human health risk.

Keywords: Arsenic-resistant bacteria Northern Thailand

Introduction

In Thailand, concentrations of arsenic in soils that exceed the national environment standard have been found in many cultivated soils including on highland areas of northern Thailand (Shutsrirung, 2012). In these contaminated areas, extremely

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high input of agro-chemical is a common practice in farms and seemed to be a major source of the high arsenic contamination. In addition, some places were claimed to be former mining areas. High arsenic level in cultivated soil can lead to high arsenic accumulation in food thus an increased risk of human health.

Arsenic, a metalloid widely distributed in soil, waters, air and all living matter, released both from natural and anthropogenic sources, from the weathering of rocks or by mining industries and agricultural practices (Pepi et al., 2006). It is found in the oxidation states +5 (arsenate), +3 (arsenite), 0 (elemental arsenic) and -3 (arsine). The most common oxidation states of arsenic in the environment are the pentavalent arsenate (As (V)) and trivalent arsenite (As (III)) forms (Cullen and Reimer, 1989). Generally, arsenite is more broadly toxic than arsenate, because of the formation of strong bond with functional group, such as the thiol group of cysteine residues and the imidazolium nitrogen of histidine residues, of cellular proteins and thus the functional inactivation of many cellular proteins including enzymes (NRC, 1999). Arsenic is toxic to humans, animal and plants. It is a strong carcinogen to humans which can cause skin and internal organ cancers as well as other diseases such as skin lesion, hyperkeratosis, melanosis (Xuexia et al., 2008). Arsenic may accumulate in agricultural soil due to agricultural practices such as the application of As-containing pesticides and herbicides, pig manure, poultry farming, and phosphorus fertilizers (Li and Chen, 2005). The arsenic cycle involves both biotic and abiotic reaction. Various plants, invertebrates and microorganisms play a major role in the transformation and movement of arsenical in soil and water (Tamaki and Frankenberger, 1989).

Bacteria have evolved a number of mechanisms to cope with, or even benefit from, arsenic exposure (Jackson et al., 2003). A number of microorganisms that use arsenic in their metabolism have been isolated, either using arsenate as a terminal electron acceptor in anaerobic respiration (Kuhn and Sigg, 1993). Other bacteria show resistance to arsenite through the activity of an arsenite oxidase or resistance to both arsenite and arsenate through the genes of the ars operon. This study, therefore, aims to

screen and evaluation of arsenite transformation of arsenic resistant bacteria from cultivated soil of northern Thailand.

Materials and Methods

Soil sampling and isolation of bacteria

Soil samples were collected from seven cultivated soil contaminated with arsenic on highland areas of Chiang Mai Province, northern Thailand and analysed for arsenic content at the Central Laboratory (Thailand) Co. Ltd. Serial dilution and plate count technique was used to isolate bacteria from all the soil samples. Zero point one milliliter of each dilution of each soil samples was spread on nutrient agar (NA) plate (pH 6.5). Colonies which grown on the medium were purified and suspended in 10% glycerol solution with DMSO 10% for preservation. All isolates preserved in this solution were maintained at -20ºC until use.

Screening of bacterial isolate for arsenic tolerance

All the isolates were used to evaluate their resistance under high concentration of arsenic. Each pure isolate was first multiply in nutrient broth (NB) until reaching maximum growth. After that 0.01 ml of the culture solution of each isolate was dropped on the nutrient agar plate (NA) containing aluminum (Al) and arsenic (As). The concentration of Al in all plates was 50 µM. Three levels of arsenic concentration were applied; 0, 5 and 10 mM. The initial pH of the medium was adjusted to 4.5, 4.7, 5.0 and 6.0. The tolerant isolates grown on nutrient agar containing sodium arsenite (Na-As (III)) at 0, 5 and 10 mMAs with 50 µM Al were selected for further investigation.

Selected isolates from the above experiment were further tested in another experiment. In which, a concentration of arsenic was adjusted close to the analyzed level found in the soil. Culture solution of each isolate was dropped (0.01 ml) on the nutrient agar plate (NA) containing indicator (0.05 g/L bromocersol green), aluminum (Al)(50 µM) and various levels of sodium arsenite (Na-As (III); 0, 10, 15, 25, 50, 100, 250 and 650 mg/L (which equal to 0, 0.08, 0.12, 0.19, 0.38, 0.77, 1.9 and 5.0 mM), adjusted pH of the media to 4.5 and 4.7. Growth observation was made after 7 days of incubation.

Evaluation of arsenite transformation and selection of high efficient isolates

Selected isolates from the above experiment were inoculated into nutrient broth (NB) and incubated for 3 days to obtain a standard inoculum for each experiment. This experiment was carried out in 125 ml Erlenmeyer flasks containing 50 ml of nutrient broth containing 100 mg/L of sodium arsenite (Na-As (III)) and 50 µM aluminum, the initial pH of the medium was adjusted to 4.7. The inoculum of 0.5 ml of each isolate was added into each flask and incubated at room temperature for 7 days. At the end of the incubation period, each sample solution was determined for growth and pH change, respectively. The culture broths of all the isolates were centrifuged. The supernatant of each isolate was further analyzed for the remaining arsenite (As (III) and the forming arsenate(As(V)) by high-pressure liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC-ICP-MS).

Results and Discussion

Soil analysis and bacterial isolation

Arsenic concentration of the seven cultivated soil in northern Thailand ranged from 5.45-39.48 mg/kg (3.93-28.46 mg/L) and the values were higher than that of standard arsenic value (The standard background level for arsenic in soil is set at 3.9 mg/Kg (National Environment Board No.8, 1994)) (Table 1). Kitja et al. (2009) analysed arsenic concentration of the nine agricultural soil samples from central Thailand, the concentration were 4.11-4.35 mg/kg, which were approximately close to the standard arsenic value. Majumder et al., (2013) analysed arsenic in the contaminated soil of West Bengal, India, and found that arsenic concentrations of soils varied from 7.4-13.4 mg/kg. The typical range for arsenic contamination in soil is 0.2-40 mg/kg (Huysman and Frankenberger, 1990). These reports indicated high arasenic contamination in cultivated soil of northern Thailand. Therefore, in contaminated soil, arsenic in the soil solution is readily available for uptake by plant root or submerged shoots. Elevated levels of arsenic in soils may lead to increased concentration of arsenic in plants. Humans are then exposed to this arsenic through contact with contaminated soil, affected drinking water, and by eating crops grown on contaminated sites. In order to evaluate the

possible health risk to humans consuming crops cultivated in the contaminate soil; information is needed regarding the soil-to-plant uptake rate (bioavailability) of the crop plant.

Fourty seven bacterial isolates were obtained from the seven cultivated soil in northern Thailand. The number of isolates obtained from each area was shown in Table 1. However, some isolates were contaminated and/or died during preservation. Therefore, only 40 isolates were usable for further investigation.

Table1 Arsenic concentrations and bacterial isolates obtained from cultivated soil

areas	pH	Arsenic[1] (mg/kg)	No. of isolates
1. Prabathhuaytom	5.63	5.61	6
2. Pangda	6.45	8.83	5
3. Tungroeng	6.62	39.48	7
4. Maehae	5.09	5.45	8
5. Angkhang	4.85	6.66	10
6. Vavee	4.67	30.52	5
7. Sobkhong	6.39	16.06	6
		mean 16.08	**Total** 47

The standard background level for arsenic in soil is set at 3.9 mg/Kg, National Environment Board No.8, 1994)

Screening of bacterial isolate for arsenic tolerance

Fourty isolates were tested for the arsenic tolerant ability at the concentration of 0, 5 and 10 mM and all levels of the tested pH (4.5, 4.7, 5.0 and 6.0). At high pH (5.0 and 6.0), all isolates could grow well in all levels of arsenic concentration (data not shown). However at low pH values (4.5 and 4.7), the results indicated that only nine isolates showed fair or good growth at arsenic concentration of 0, 5 and 10 mM (isolate 7, 8, 11, 19, 20, 22, 29, 30 and 36) (Table 2 and 3) which suggested that there might be some mechanisms of these isolates to reduce toxicity of acidity (aluminum) and arsenic.

From the results of the above experiment, nine isolates (isolate 7, 8, 11, 19, 20, 22, 29, 30 and 36) were selected for screening the bacterial isolates in another set of arsenic concentration (Na-As (III)) 0, 0.08, 0.12, 0.19, 0.38, 0.77, 1.9 and 5.0 mM. Our results indicated that good growth of all isolate was obtained without arsenic addition (0 mM) at low pH (4.5 and 4.7). At these low pH values, only isolate 8, 11, 19 and 29 could grow at the concentration of 10 to 100 mg/L. However, at higher concentrations (250 and 650 mg/L), no growth of all isolates were observed. Pepi et al. (2007) concluded that the minimum inhibitory concentration (MIC) values of the isolates varied widely in the range 50-125 mM (As) as arsenate and 10-100 mM (As) as arsenite. Four isolates show minimum inhibitory concentration values equal or superior to 16.68 mM and 133.47mM in the presence of As(III) and As(V), respectively (Pepi et al., 2007). Some isolates obtained from the study of Majumder et al. (2013) found to be hyper-resistant to both As(V) (167–400 mM) and As(III) (16–47 mM). In contrast to these reports, our isolates seemed to be less resistant to arsenite. This might due to lower arsenic contamination in the soils from which bacteria were isolated.

Table2 Growth of bacterial isolate under different levels of arsenic at pH 4.5

isolate	areas	Sodium arsenic concentration (mM)			isolate	areas	Sodium arsenic concentration (mM)		
		0	5	10			0	5	10
1	Vavee	NG	NG	NG	21	Tungroeng	+	NG	NG
2	Vavee	+	NG	NG	22	Prabathhuaytom	++++	NG	NG
3	Vavee	+	NG	NG	23	Prabathhuaytom	+	+	NG
4	Vavee	+++	NG	NG	24	Prabathhuaytom	++	NG	NG
5	Pangda	++	+	NG	25	Prabathhuaytom	+	NG	NG
6	Pangda	++	+	NG	26	Prabathhuaytom	+	NG	NG
7	Pangda	+++	+	NG	27	Prabathhuaytom	++	+	NG
8	Sobkhong	+	NG	NG	28	Angkhang	++	NG	NG
9	Sobkhong	++	NG	NG	29	Angkhang	++++	++++	+++
10	Maehae	NG	NG	NG	30	Angkhang	++++	++++	+++
11	Maehae	++++	+++	NG	31	Angkhang	+	NG	NG
12	Maehae	NG	NG	NG	32	Angkhang	++	NG	NG
13	Maehae	++	NG	NG	33	Angkhang	+	NG	NG

14	Maehae	++	+	NG	34	Angkhang	+	NG	NG
15	Tungroeng	++	+	NG	35	Angkhang	+	NG	NG
16	Tungroeng	NG	NG	NG	36	Angkhang	++++	+++	+++
17	Tungroeng	++	NG	NG	37	Angkhang	++	NG	NG
18	Tungroeng	++	NG	NG	38	Maehae	+	NG	NG
19	Tungroeng	+++	++	NG	39	Maehae	++	NG	NG
20	Tungroeng	+++	++	++	40	Maehae	++	NG	NG

Growth ++++; very good, +++; good, ++; fair; +; little, NG; No Growth

Table3 Growth of bacterial isolate under different levels of arsenic at pH 4.7

isolate	areas	Sodium arsenic concentration (mM)			isolate	areas	Sodium arsenic concentration (mM)		
		0	5	10			0	5	10
1	Vavee	NG	NG	NG	21	Tungroeng	++	+	NG
2	Vavee	+	NG	NG	22	Prabathhuaytom	++++	+	NG
3	Vavee	+	NG	NG	23	Prabathhuaytom	++	NG	NG
4	Vavee	+++	NG	NG	24	Prabathhuaytom	+	NG	NG
5	Pangda	++	+	NG	25	Prabathhuaytom	++	NG	NG
6	Pangda	++	+	NG	26	Prabathhuaytom	++	NG	NG
7	Pangda	+++	+	NG	27	Prabathhuaytom	+	NG	NG
8	Sobkhong	+++	+	NG	28	Angkhang	++	NG	NG
9	Sobkhong	++	NG	NG	29	Angkhang	++++	++++	+++
10	Maehae	NG	NG	NG	30	Angkhang	++++	++++	+++
11	Maehae	++++	+++	NG	31	Angkhang	++	NG	NG
12	Maehae	NG	NG	NG	32	Angkhang	++	NG	NG
13	Maehae	++	NG	NG	33	Angkhang	+	NG	NG
14	Maehae	++++	+	NG	34	Angkhang	+	NG	NG
15	Tungroeng	++++	+	NG	35	Angkhang	+	NG	NG
16	Tungroeng	NG	NG	NG	36	Angkhang	++++	+++	+++
17	Tungroeng	++	NG	NG	37	Angkhang	+++	NG	NG
18	Tungroeng	++	NG	NG	38	Maehae	+++	NG	NG
19	Tungroeng	++++	++	NG	39	Maehae	+++	NG	NG
20	Tungroeng	++++	++	++	40	Maehae	+++	NG	NG

Growth ++++; very good, +++; good, ++; fair; +; little, NG; No Growth

Evaluation of arsenite transformation and selection of high efficient isolates

After the screening, we selected only four effective isolates (8, 11, 19 and 29) (arsenic tolerant isolate) for this experiment. Isolate 7 was selected as an ineffective isolate (arsenic sensitive isolate). The results of incubation of isolate 8, 11, 19 and 29 in acidic broth media (pH 4.7), high aluminum (50 µM) and arsenic (100 mg/L), indicated that isolate 7 (ineffective isolate) did not change the pH of the media when compared with the control (pH 4.74 and 4.75, respectively). In Table 4, effective isolate; 8, 11, 19 and 29 increased the pH of the broth to much higher value; 8.12, 6.79, 8.34 and 8.23, respectively. In these high pH values, isolate 11 performed maximum growth followed by 29, 19 and 8 respectively. No growth was observed for isolate 7. The results of this study suggested that altering the medium pH might be a mechanism to survive under stress environment of the isolates. In addition to pH measurement, the supernatant of the culture solution was analyzed for arsenite [As (III)] and arsenate [As (V)]. The results demonstrated that isolate 29 showed most effective in transforming arsenite to arsenate. This isolate could oxidize highly toxic form of arsenic, arsenite [As (III)], into less toxic form, up to 47.07% of arsenate [As (V)]; followed by isolate 8, 19, and 11 with 41.74%, 35.67% and 11.29%, respectively. Isolate 7 gave similar value to the control, 4.73% and 4.63%, respectively. The process of arsenic oxidation catalyzed by bacteria is much faster than chemical oxidation (in the presence of O_2) and has been observed in many environments (Osborne et al., 2010). Several pure cultures of bacterial strains (Rhine et al., 2006), as well as microbial consortia (Battaglia-Brunet et al., 2002) were able to transform As(III) to As(V). The transformation was affected by the As(III) oxidation rate, and only 40% of the arsenite was oxidized to arsenate.

Table4 Effectiveness of arsenic resistant bacteria in arsenite transformation

Isolate	$NaAsO_2$ (mg/kg)	pH begin	pH final	OD (600 nm)	Total As (mg/kg)[1]	As(III) (mg/kg)[2]	As(V) (mg/kg)[2]
Control (no isolate)	100	4.70	4.74	0.000±0.00	139.16 (100%)	132.72 (95.37%)	6.44 (4.63%)
7			4.75	0.005±0.00	141.23 (100%)	134.55 (95.27%)	6.68 (4.73%)
8			8.13	0.741±0.06	138.77 (100%)	80.86 (58.26%)	57.91 (41.74%)
11			6.79	1.341±0.01	140.87 (100%)	124.96 (88.71%)	15.91 (11.29%)
19			8.34	1.117±0.01	137.27 (100%)	88.30 (64.33%)	48.62 (35.67%)
29			8.23	1.300±0.01	143.28 (100%)	75.84 (52.93%)	61.43 (47.07%)

Reference Methods, In house method based on EPA 3052, by ICP-OES technique.

Reference Methods, by HPLC-ICP-MS

Conclusion

Fourty seven bacterial isolates were obtained from the seven cultivated soil in northern Thailand. Only nine isolates (isolate 7, 8, 11, 19, 20, 22, 29, 30 and 36) showed a potential to tolerate high level of arsenic at low pH levels. Isolate 8, 11, 19 and 29 showed a potential to tolerate high level of arsenic at low pH levels when adjusting arsenic concentration to the analyzed level found in the soil. In the media with 100 mg/L arsenic at pH 4.7, these isolates increased the pH of the broth to much higher value. Isolate 11 performed maximum growth followed by 29, 19 and 8 respectively. The isolates could also oxidize highly toxic form of arsenic, arsenite [As (III)], into less toxic form of arsenate [As (V)].

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