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Arsenic-methylation and arsenic-removal using *Bordetella petrii* strain KC42

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Abstract

Bordetella petrii strain KC42 isolated from soil in Miyazaki prefecture was shown to be a microorganism with methylation and removal activities of inorganic arsenic. Arsenic methylation using intracellular extracts from strain KC42 at pH 6.5 and 35°C for 2 h yielded 35.9% methylated organic arsenic compound and 10.8% trimethylarsenic compound (TMAC). As a result of the analysis of TMAC produced under these conditions, TMAC was identified by trimethylarsine oxide and arsenobetaine. In experiments of arsenic adsorption by dried cells of strain KC42, the ratio of As(III) adsorption by strain KC42 was 99.4% in solutions containing 0.2 mg As/L at pH 7.0 and 30°C for 2 h. The maximum adsorption value was 0.179 mg As/g dw at an initial concentration of 10.0 mg As/L As(III). These results suggest that strain KC42 may be utilized for bioremediation.

Keywords: *Bordetella petrii* strain KC42, Bioremediation, Arsenic methylation, Methylated organic arsenic, Arsenic removal

1. INTRODUCTION

The need for low-cost and environmentally friendly technologies for removing arsenic from the environment has recently become all the more pressing, and there is now great interest in the use of bioremediation in order to counter contamination by arsenic and other metals, with various studies being carried out in this field. Microorganisms that possess mechanisms for insolubilizing and reducing the toxicity of various harmful metals have been reported, and adsorption techniques using microbial biomass are being investigated. Beginning in the late 1970s, studies reported that the toxicity of arsenic compounds vary according to differences in their chemical structure or chemical form. The toxicity of most organic methylated arsenic is much less than that of inorganic arsenic. In particular, the toxicity of arsenobetaine (AB) (half lethal dose of 10 g/kg) is relatively low in organic methylated arsenic, and is 1/300 times that of inorganic arsenic (0.03 g/kg). Therefore, biomethylation of arsenic is usually considered as a detoxification of arsenic. There is every possibility that microorganisms with the ability to remove and methylate environmental arsenic can be used for bioremediation, and there are hopes that this approach could replace or supplement existing physico-chemical methods. However, no bacteria have been isolated which can be effectively used for the bioremediation of arsenic.

The aim of the present study was to therefore discover a bacterium capable of arsenic mathylation and removal from the environment to levels suitable for bioremediation applications. As a result, we have successfully isolated a strain, *Bordetella petrii* strain KC42 that has arsenic methylation and removal ability, from the soil in Miyazaki prefecture (Miyatake 2016). In this study, arsenic methylation using intracellular extracts and arsenic removal using dried cells from this bacterial strain were examined, and are reported.

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2. MATERIALS AND METHODS

2.1 Bacterial strain

Bordetella petrii strain KC42 was isolated from soil that was not polluted with arsenic in Miyazaki prefecture.

2.2 Experiments using intracellular extracts

Sodium arsenite (Wako Pure Chemical Industries, Ltd.) was used as trivalent arsenic (As(III)) and disodium arsenate (Wako Pure Chemical Industries, Ltd.) was used as pentavalent arsenic (As(V)), at various concentrations. Strain KC42 was aerobically cultured in R2A medium (Nippon Seiyaku Co., Ltd.) supplemented with 1.0 g/L glucose and 10.0 mg As/L of As(V) at 30°C for 3 days with shaking at 100 strokes/min, and the bacterial cells were harvested by centrifugation (10,000×g, 15 min) and lyophilized. The bacterial cells were suspended in purified water and disrupted by ultrasonication. Disrupted cells were separated from the supernatant by centrifugation (10,000×g, 15 min). The supernatant was used as an intracellular extract. Intracellular extracts from *Bacillus megaterium* strain UM-123 was prepared as described above after aerobic culture for 1 day at 30°C in Nutrient broth (Nissui Pharmaceutical Co., Ltd.). Intracellular extracts from *Cellulomonas* sp. strain K63 was prepared as described above after aerobic culture for 2 day at 30°C in R2A medium supplemented with 1.0 g/L glucose and 10.0 mg As/L of As(III).

Arsenic methylation was carried out in 0.50 mL reactions with 0.10 mL of intracellular extract, 0.05 mL of 1.0 mg As/L solution of As(III) or As(V), and 0.05 mL of 20 mM S-adenosyl methionine (SAM) in 25 mM phosphoric acid buffer solution. To evaluate the influence of pH on the arsenic methylation reaction, the concentration of arsenic in the reaction was measured after incubation in phosphate-buffered solutions (pH 5.5-8.0) at 35°C for 2 h. To evaluate the influence of temperature on the arsenic methylation reaction, the arsenic concentration in the reaction solution was measured after incubation in phosphate-buffered solution (pH 6.5) at 25°C-50°C for 2 h.

2.3 Experiments using dried cells

After culturing strain KC42 in R2A medium supplemented with 1.0 g/L glucose at 30°C for 2 days with shaking at 100 strokes/min, the bacterial cells were harvested by centrifugation (10,000×g, 15 min) and lyophilized. The bacterial cells were suspended in purified water and disrupted by ultrasonication. Disrupted cells were separated from the supernatant by centrifugation (10,000×g, 15 min) and lyophilized. The disrupted cells were used as a dried cell. The amount of arsenic adsorbed to cells was calculated based on the decrease of arsenic in the supernatant and the weight of dried cells (dw) added to the solution.

The effects of pH on arsenic removal were investigated by adding 20 g dw/L of dried cells to 1.0 mg As/L of As(III) solution adjusted to pHs ranging from 2.0-12.0, and the resulting suspensions were stirred at 30°C for 2 h. Cells were removed by centrifugation (10,000×g, 15 min) and the arsenic concentration in the supernatant was measured. In order to examine the effects of temperature, 20 g dw/L of dried cells were added to 1.0 mg As/L of As(III) solutions adjusted to pH 7.0. The solutions were incubated at temperatures ranging from 20-60°C with stirring for 2 h. The arsenic concentration in the supernatant was then measured.

2.4 Analyses

Quantitative analysis of arsenic speciation was carried out using an atomic absorption spectrophotometer (Shimadzu AA6650) with an arsenic speciation pretreatment system (Shimadzu ASA-2sp). Trimethylarsine oxide (TMAO) and AB were measured using a liquid chromatography-mass spectrometry (Waters QuattroMicro API). The column of Shodex RSpak NN-614 (150 mm×6.0 mm) was used for chromatographic separation. The mobile phase consisted of 8.0 mmol/L formic acid and 5.0 mmol/L ammonium formate. Every experiment was repeated three times, and each measurement was repeated twice. The results shown in this report are the means of the obtained values, and all these values were within 3.0% of the mean.

3. RESULTS AND DISCUSSION

3.1 Methylation of arsenic using intracellular extracts

An intracellular extract from *Bordetella petrii* strain KC42 cells was prepared, and arsenic methylation reactions were performed.

The results of a time course experiment for arsenic methylation are shown in Fig. 1. When As(III) was added to the reaction solution, inorganic arsenic decreased for the first 2 h, and methylated organic arsenic compounds, including dimethylarsinic acid (DMAA) as a major component, were produced; however, almost no change was observed after the first 2 h. Inorganic arsenic was decreased to 64.1%. In As(V)-added reaction solution, inorganic arsenic decreased for the first 3 h, and methylated organic arsenic compounds were produced. The ratios of inorganic arsenic and methylated organic arsenic compounds after the first 4 h were similar for the two arsenic forms. The tendency to increase in DMAA and trimethylarsenic compound (TMAC) rapidly, when monomethylarsonic acid

(MMAA) turned from increase to decrease, was admitted irrespective of As(III) and As(V) like arsenic methylating bacterium *Cellulomonas* sp. strain K63 (Miyatake 2014). This result suggests a possibility that MMAA production was rate-limiting step of DMAA and TMAC of end product.

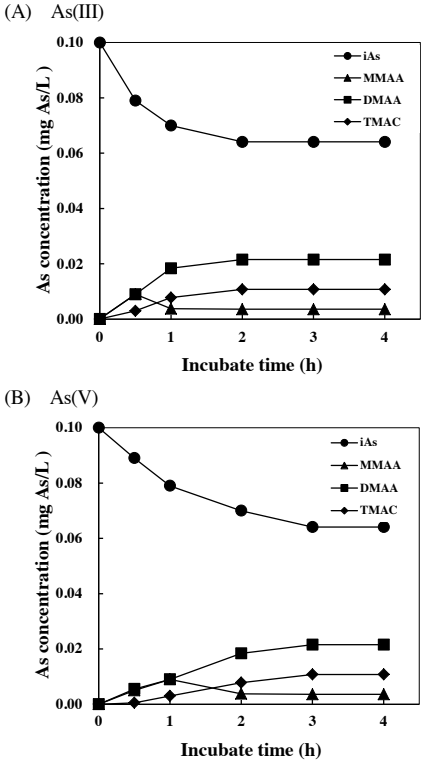

Fig. 1 Time courses of arsenic methylation by intracellular extract of strain KC42. Conditions of arsenic methylation experiments: initial As concentration, 0.10 mg As/L of As(III) or As(V); intracellular extract, 20 v/v%; SAM concentration, 2.0 mmol/L; temperature, 35°C; pH, 6.5.

The results of tests on the effects of pH and temperature on arsenic methylation are that the percentage of methylated organic arsenic compound reached a maximum of 35.9% at pH 6.5 and 35°C. This result suggests that the methylation reaction of strain KC42 was promoted by the activity of the arsenite-methyltransferase like other arsenic methylating bacterium. The percent of TMAC under these conditions was 10.8%. As a result of the analysis of TMAC produced under these conditions, TMAC was identified by TMAO and AB, and the percentage of AB was 0.5%.

To enhance the arsenic methylation reaction, glutathione and intracellular extracts from *B. megaterium* strain UM-123 (Miyatake 2009) and strain K63, which each have arsenate reducing or arsenic methylating ability, were added to the reaction solution. The results of this experiment are shown in Fig. 2. When glutathione was added to the reaction solution, the percentage of methylated organic arsenic compounds was the highest; however, almost all of it was DMAA, and the percentage of TMAC decreased to 2.0%, which was lower than when glutathione was not added. This result suggested that only methylation via glutathione complexation occurred. In contrast, when the intracellular extracts from the two aforementioned bacterial cells were added, the percentage of TMAC increased compared to when the intracellular extracts were not added. This result suggests that the oxidative methylation reaction was promoted by the activity of the arsenate reductase and the arsenite-methyltransferase contained in these two bacterial intracellular extracts. When the intracellular extracts from strain K63 were added, the percentage of AB increased compared to when the intracellular extract was not added, and was 2.3%.

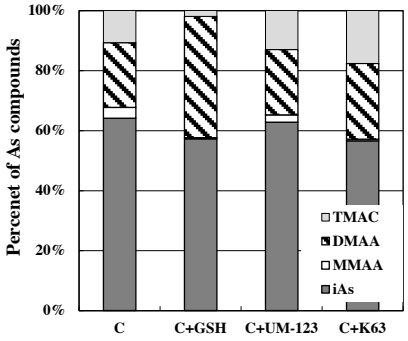

Fig. 2 Addition effect on arsenic methylation by intracellular extract of strain KC42. Conditions of arsenic methylation experiments: initial As concentration, 0.10 mg As/L of As(III); intracellular extract, 20 v/v%; SAM concentration, 2.0 mmol/L; pH, 6.5; incubate time, 2 h. The percent of arsenic compounds in reaction solution was calculated by considering the initial arsenic concentration to be 100%. C; control (no addition), C+GSH; control+7.0 mmol/L GSH, C+UM-123; control+intracellular extract of B. megaterium strain UM-123; C+K63; control+intracellular extract of Cellulomonas sp. strain K63.

These results suggest that the intracellular extracts from strain KC42 are sufficient for use in detoxification processes, and further suggest that if the conversion efficiency to AB can be improved, a technology for restoration of arsenic to nature after detoxification using intracellular extract from microorganisms can be established.

3.2 Removal of arsenic from aqueous solution using dried cells

The changes in adsorption ratios of arsenic by dried cells of strain KC42 under several different conditions, including pH, temperature, and arsenic concentration, were also examined. Although the arsenic adsorption behavior differed

according to pH, under all pH conditions the maximum adsorption ratio was observed after contact times of 2 h, respectively. The maximum adsorption was 96.9% at pH 7.0, while at pH 2.0-9.0 it was greater than 86%.

The adsorptions of arsenic under different temperature conditions were also evaluated. The adsorption was more than 83% at temperatures between 20-40ºC, but decreased significantly at temperatures above 45ºC. The maximum adsorption was observed at 30 ºC with values of 96.4%. When the adsorption of arsenic at 30ºC was evaluated by the dried cells used on adsorption experiment at temperatures above 45ºC once again, almost no arsenic was absorbed. This result may suggest that the dried cells changed by temperature.

Finally, the effects of arsenic concentration on arsenic adsorption by dried cells were investigated (Table 1). An adsorption of 96% or more was observed in solutions containing up to 1.0 mg As/L. In solutions containing a concentration of 0.5 mg As/L or lower, the final concentrations of arsenic decreased to less than 0.01 mg As/L. The maximum adsorption value was 0.179 mg As/g dw at an initial concentration of 10.0 mg As/L.

Table 1 Effect of arsenic concentrations on arsenic adsorption by dried cells of strain KC42

Initial As(III) concentration (mg As/L)	Final As concentration (mg As/L)	As adsorption	
		(%)[a]	(mg As /g dw)[b]
0.20	<0.01	99.4	0.010
0.50	<0.01	98.5	0.025
1.00	0.03	96.9	0.048
2.00	0.31	84.7	0.085
5.00	1.99	60.3	0.151
10.00	6.42	35.8	0.179

Conditions of arsenic adsorption experiments: dried cells, 20 g dw/L; pH, 7; temperature, 30°C; contact time, 2 h.

[a] Percentages of arsenic adsorption represents the ratio of the adsorbed arsenic concentration and the initial arsenic concentration.

[b] Represents the mg of arsenic per gram of dried cell weight.

The characteristics of arsenic removal by strain KC42 were identical to those previously reported for strain UM-123 and *Bacillus cereus* strain W2 (Miyatake 2011). However, as lyophilized cells of strain KC42 and strain UM-123 were unable to reduce As(V) to As(III), As(V) could not be removed from the aqueous solution. Strain KC42 was able to remove both As(III) and As(V) from the culture medium during culture, and the ratio removed was in the region of 70% in culture medium containing 0.5 mg As/L of As(III) (Miyatake 2016). When dried cells were used, strain KC42 was only able to remove As(III). But by increasing the cell density to greater than when the strain was cultured, it was possible to achieve an arsenic adsorption ratio of 97% or more in solutions of As(III) of up to 1.0 mg As/L. Strain KC42 was also able to reduce the concentrations of arsenic in solutions up to 0.5 mg As/l to 0.01 mg As/l or less, which is the environmental standard for arsenic.

Based on the present results, it appears that the dried cells of strain KC42 are effective in removing As(III), which is highly toxic and difficult to remove. Furthermore, this activity is maintained even at low concentrations of arsenic.

4. CONCLUSION

Bordetella petrii strain KC42 isolated from soil in Miyazaki prefecture was shown to be a microorganism with methylation and removal activities of inorganic arsenic. Arsenic methylation using intracellular extracts from strain KC42 at pH 6.5 and 35°C for 2 h yielded 35.9% methylated organic arsenic compound and 10.8% TMAC. When intracellular extracts from other bacterial strains with arsenic methylating ability were added to the reaction solution, the percentages of TMAC and AB increased to 17.5% and 2.3%, respectively. In experiments of arsenic adsorption by dried cells of strain KC42, the ratio of As(III) adsorption by strain KC42 ranged from 96.9 to 99.4% in solutions containing up to 1.0 mg As/L at pH 7.0 and 30°C for 2 h. Arsenic concentrations in solution decreased to 0.01 mg As/l or lower with initial concentrations of As(III) up to 0.5 mg As/L. These promising results indicate that strain KC42 can be utilized for the bioremediation.

5. ACKNOWLEDGEMENTS

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