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Characteristics to arsenic of *Bordetella petrii* strain KC42

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Abstract

The properties of *Bordetella petrii* strain KC42 isolated from the soil in Miyazaki Prefecture were investigated in terms of arsenic methylation and removal in this study. No differences were observed in the growth of strain KC42 between As(III)- and As(V)-supplemented culture medium. Strain KC42 metabolized inorganic arsenic (<5.0 mgAs/L) to nonvolatile species such as monomethylarsonic acid, dimethylarsinic acid, trimethylarsenic oxide and arsenobetaine effectively. In culture supplemented with 0.2 mg As/L As(V), 98.7% of the arsenic remaining in the supernatant was found to be methylated organic arsenic compounds. An analysis of the arsenic adsorbed to the bacterial cell walls revealed that most of the arsenic removed from culture medium was accounted by cell wall adsorption for both As(III)- and As(V)-supplementation, and that in both cases, all of the adsorbed arsenic was in the As(III) form. These results demonstrate that strain KC42 possesses arsenic-methylating and arsenic-removing capabilities, and may therefore be used in the bioremediation of arsenic.

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

1. INTRODUCTION

Water contaminated with arsenic is generally purified using physico-chemical methods, such as coagulation and coprecipitation, ion exchange, and adsorption, however, these methods are expensive and often produce secondary pollutants (Wang 2006). Furthermore, these technologies merely accumulate or concentrate inorganic arsenic; therefore, the hazardous properties of arsenic are not eliminated. In addition, these methods require separation (solidification/undergrounding) as an end process and facilities for storage or preservation.

Therefore, as there is a need for low-cost, efficient, and environmentally friendly technologies for removing arsenic from water, the use of bioremediation to counter contamination by arsenic and other metals has attracted great interest (Kapoo 1999, Sudha 2001, Loukidou 2003, Say 2003). It is anticipated that the use of microorganisms with the ability to remove environmental arsenic could either 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 detoxification 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. In this study, the properties of this bacterial strain in terms of arsenic methylation and removal were evaluated and are reported.

2. MATERIALS AND METHODS

2.1 Bacterial strain

Bordetella petrii strain KC42 was isolated from soil that

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was not polluted with arsenic in Miyazaki prefecture, and its arsenic characteristics were examined.

2.2 Culture experiments

R2A medium (Nippon Seiyaku Co., Ltd.) supplemented with 1.0 g/L glucose was used as the culture medium. Sodium arsenite (Wako Pure Chemical Industries, Ltd.) was added to the medium as trivalent arsenic (As(III)) and disodium arsenate (Wako Pure Chemical Industries, Ltd.) was added to the medium as pentavalent arsenic (As(V)), at various concentrations. Strain KC42 was pre-cultured at 30°C for 24 h with shaking at 100 strokes/min. To prepare the main culture, 10 mL of fresh medium was inoculated with 2.0% v/v of the pre-culture. Culture was incubated at 30°C with shaking at 100 strokes/min. After measuring the turbidity of the culture broth at the indicated times, the culture broth was centrifuged (10,000×g, 15 min) to separate the bacterial cells from the culture supernatant, and concentration of arsenic in the culture supernatant was measured. The pelleted cells were washed twice with purified water, lyophilized, and the dry weight of the cells was measured. To determine the amount of arsenic associated with the cell wall, lyophilized bacterial cells were suspended in purified water and then disrupted by ultrasonication. Disrupted cells were separated from the supernatant by centrifugation at 10,000 × g for 15 min, washed twice with purified water, and lyophilized. Arsenic was separated from lyophilized cells to allow its solubilization by adding 2.0 mg/ml of lysozyme (Wako Pure Chemical Industries, Ltd., Japan). After 2 h, the solution was filtered and the concentrations of arsenic were measured.

2.3 Analyses

The turbidity of the culture broth was measured at 600 nm using a spectrophotometer (Shimadzu UV-210A), and the measured values were used to evaluate the bacterial growth. 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 arsenobetaine (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 Effect of arsenic on bacterial growth

To examine the effect of arsenic on the growth of *Bordetella petrii* strain KC42, strain KC42 was aerobically cultured in a medium containing 5.0 to 50.0 mg As/L of As(III) or As(V) for 4 days. During this period, the turbidity of the culture broth was measured regularly. The results are shown in Fig. 1.

Almost no differences were observed in the bacterial growth in medium containing As (III) and that containing As (V) at the same concentration. Bacterial growth at 5.0 mg As/L was similar to that observed in media lacking arsenic and reached the stationary phase after 2 days. The effect on bacterial growth became evident at arsenic concentrations of 10.0 mg As/L, and at 50.0 mg As/L, almost no bacterial growth was observed.

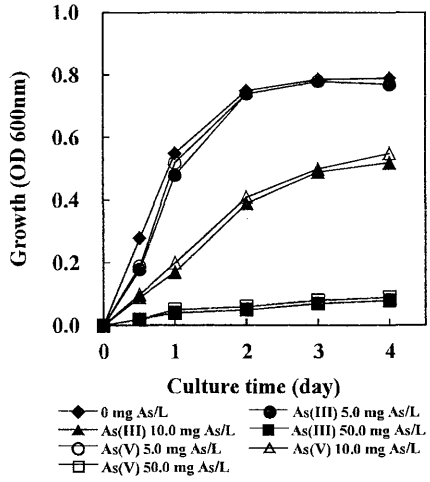

Fig. 1 Effect of arsenic concentrations on cell growth of strain KC42.

3.2 Time course of arsenic on culture

To examine changes of arsenic in the culture supernatant during culture, strain KC42 was aerobically cultured in a medium containing 5.0 mg As/L of As(III) or As(V) for 4 days. The arsenic forms in the culture supernatant were examined regularly and the results are shown in Fig. 2.

In the case of both As(III) and As(V) supplementation of the culture medium, decreases in inorganic arsenic were observed until the third day and the concentrations of inorganic arsenic were found to decrease to 1.25 mg As/L. As the inorganic arsenic levels decreased, the levels of methylated organic arsenic compounds (monomethylarsonic acid [MMAA], dimethylarsinic acid [DMAA], and trimethylarsenic compounds [TMAC]) increased from the first day. In As(III)-supplemented medium, the concentration of methylated organic arsenic compounds on the third day reached 1.41 mg As/L, while in As(V)-supplemented medium, the concentration of methylated organic arsenic compounds reached 2.81 mg As/L—twice the concentration

measured in As(III)-supplemented medium. Among the methylated organic arsenic compounds, DMAA was present at the highest concentrations: on the third day, 0.84 mg As/L and 1.69 mg As/L were measured for media supplemented with As(III) and As(V), respectively. The total arsenic concentration (i.e., the sum of the concentrations of inorganic arsenic and methylated organic arsenic compounds) in the culture supernatant was found to decrease until the third day : total arsenic decreased to 2.66 mg As/L in As(III)-supplemented medium and to 4.06 mg As/L in the case of As(V)-supplemented medium. In the case of As(III), the arsenic content decreased to 53% of the amount added to the medium.

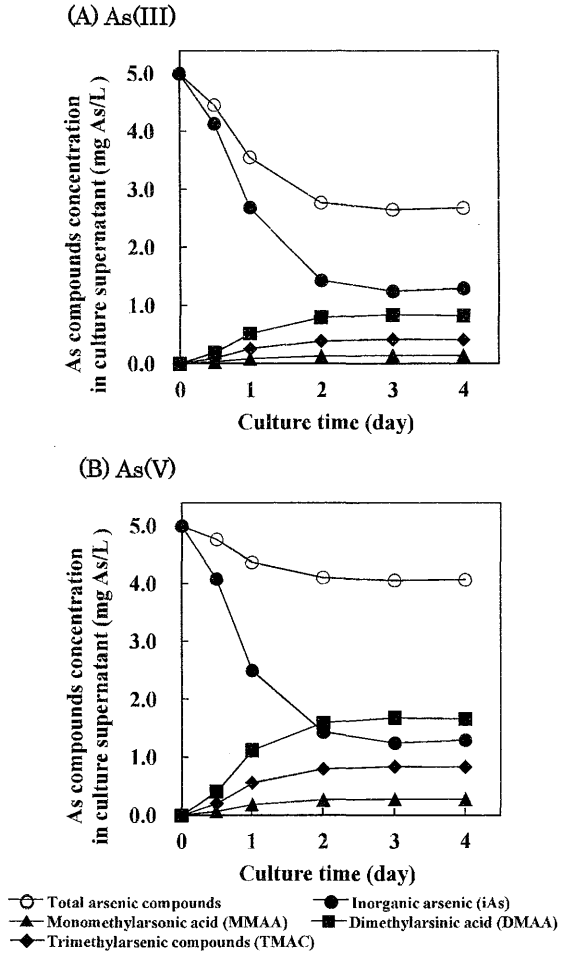

Fig. 2 Time courses of arsenic in culture supernatant by strain KC42.

3.3 Metabolism of arsenic at various arsenic concentrations

To examine the metabolism of arsenic at various arsenic concentrations in the media, strain KC42 was aerobically cultured in a medium containing 0.2 to 50.0 mg As/L of As(III) or As(V) for 4 days, after which the forms of arsenic in the cultured supernatant were examined. The results are shown in Fig. 3.

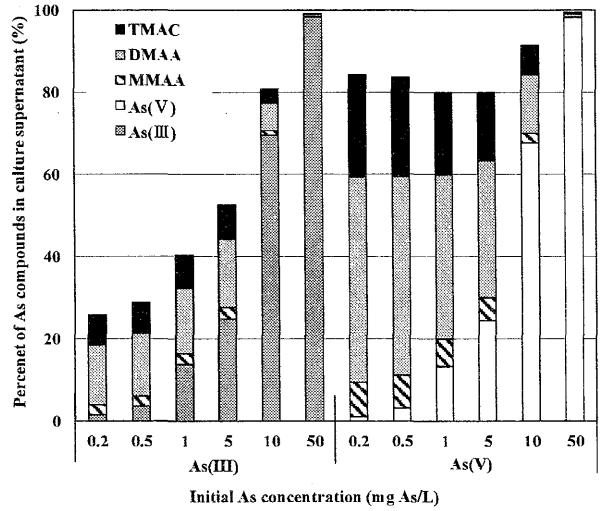

Fig. 3 Effect of arsenic concentrations on arsenic metabolism by strain KC42. The percent of As compounds in culture supernatant was calculated by considering the initial arsenic concentration to be 100%.

When As(III) was added to the culture medium, the ratio of the remaining inorganic arsenic increased with increasing concentrations of arsenic. Methylation was shown to progress efficiently as the concentration of arsenic in the medium increased to 5.0 mg As/L; however, at 50.0 mg/L arsenic, almost no methylated organic arsenic compounds were detected. When arsenic was added to the medium at 5.0 mg As/L, the yield of methylated organic arsenic reached a maximum of 1.39 mg As/L (27.8%). The ratio of arsenic remaining in the culture supernatant was lower when the amount of added arsenic was lower, and was 28.8% when the arsenic concentration in the medium was 0.5 mg As/L.

In As(V)-supplemented medium, methylation efficiently progressed as the concentration of arsenic increased to 5.0 mg As/L as was observed in the case of As(III)-supplemented medium. When arsenic was added to the medium at 0.2 mg As/L, the ratio of methylated organic arsenic compounds reached a maximum of 83.2% and in medium supplemented with 5.0 mg As/L, the yield of methylated organic arsenic compounds reached a maximum of 2.78 mg As/L (55.5%). The ratio of arsenic remaining in the culture supernatant was lowest (79.9%) when arsenic was added to the medium at 1.0 mg As/L.

The form of inorganic arsenic remaining in the culture supernatant was same as the form of arsenic supplemented culture medium for the two arsenic forms. The ratio of methylated organic arsenic compounds at each concentration of arsenic was higher in As(V)-supplemented medium than in As(III)-supplemented medium, and at an As(V) concentration of 0.2 mg As/L, 98.7% of the arsenic remaining in the culture supernatant after 4 days was found to be methylated organic arsenic compounds. The ratio of

arsenic remaining in the culture supernatant at each concentration of arsenic was lower for As(III) than for As(V); however, the ratios of the remaining inorganic arsenic were similar for the two arsenic forms at the same concentrations.

An analysis of TMAC in strain KC42 cultures showed that it is composed of TMAO and arsenobetaine (AB) as in the case of known arsenic-methylating bacteria, the *Cellulomonas* sp. strain K63 (Miyatake 2014) and the *Bacillus cereus* strain R2 (Miyatake 2009), demonstrating that strain KC42 can also produce AB from inorganic arsenic. If strain KC42 can efficiently convert inorganic arsenic to less toxic organic arsenic compounds such as AB and arsenocholine, the strain may also be able to detoxify inorganic arsenic.

3.4 Adsorption of arsenic on the bacterial cell wall

To examine the amount of arsenic adsorbed on the cell wall of strain KC42, As(III) or As(V) was aerobically cultured in a medium containing 0.2 to 50.0 mg As/L of As(III) or As(V) for 4 days, after the amount of arsenic adsorbed on the cell wall of the bacterium was measured. The results are shown in Table 1.

For both As(III)- and As(V)-supplemented culture medium, the amount of adsorbed arsenic per 1.0 g of cell wall increased with increasing concentrations of arsenic supplementation. When the culture medium was supplemented with 50.0 mg As/L, the adsorbed amounts of arsenic were 1.72 mg and 0.87 mg As/g dw in the case of As(III) and As(V), respectively. In the case of As(III), the arsenic adsorption ratio was found to increase with decreasing concentrations of arsenic supplementation. In the medium supplemented with 0.2 mg As/L of As(III), the adsorption ratio was 72.2% and strain KC42 was able to reduce the arsenic concentrations in culture medium to 0.10 mg As/L or less, which is the effluent standard for arsenic. In the case of As(V), the adsorption ratio reached a maximum of 18.5% when the concentration of arsenic in the medium was 5.0 mg As/L. All the arsenic adsorbed on the bacterial cell walls was shown to be As(III), regardless of whether the medium was supplemented with As(III) or As(V).

The sum of the arsenic adsorbed on the cell wall and that remaining in the culture supernatant was similar to the amount of arsenic added to the medium. The decreases in arsenic concentrations in the culture supernatant were therefore considered to be due to the adsorption of arsenic on the cell wall. These findings indicate arsenic compounds did not evaporate into the atmosphere from the medium, in turn indicating that volatile methylated organic arsenic compounds such as dimethylarsine (DMA) and trimethylarsine (TMA) were not produced by strain KC42. This result suggests that strain KC42 does not have a metabolic pathway for reducing DMAA or TMAO into DMA or TMA, respectively.

Table 1 Distribution of arsenic in culture broth by strain KC42

Initial As (mg As/L)	Culture supernatant (mg As/L)	(%)[a]	Cell wall (mg As/g dw)	(mg As/L broth)	(%)[a]	Total[b] (%)
As(III)						
0.20	0.05	25.8	0.06	0.14	72.2	98.0
0.50	0.14	28.8	0.14	0.35	69.7	98.6
1.00	0.40	40.4	0.23	0.58	58.3	98.7
5.00	2.63	52.6	0.94	2.31	46.3	98.9
10.00	8.08	80.8	1.13	1.88	18.8	99.6
50.00	49.55	99.1	1.72	0.44	0.9	100.0
As(V)						
0.20	0.17	84.3	0.01	0.03	13.8	98.1
0.50	0.42	83.7	0.03	0.07	14.4	98.1
1.00	0.80	79.9	0.07	0.18	18.4	98.3
5.00	4.00	80.0	0.38	0.93	18.5	98.5
10.00	9.14	91.4	0.45	0.79	7.9	99.4
50.00	49.73	99.5	0.87	0.25	0.5	100.0

[a] The percent of As compounds in culture supernatant or cell wall was calculated by considering the initial arsenic concentration to be 100%.

[b] The percent of total As compounds was added culture supernatant to cell wall.

4. CONCLUSION

The properties of Bordetella petrii strain KC42 isolated from the soil in Miyazaki Prefecture were investigated in terms of arsenic methylation and removal in this study. Almost no growth of strain KC42 was observed in medium containing arsenic at 50 mg As/L. No differences were observed in the growth of bacteria between As(III)- and As(V)-supplemented culture medium, demonstrating that the forms of arsenic did not differentially affect the growth of the bacterium. In cultures of strain KC42, the levels of inorganic arsenic in the culture supernatant decreased with increasing culture time following supplementation of the culture medium with either As(III) or As(V) at 5.0 mg As/L, while the levels of methylated organic arsenic compounds were found to increase. The total arsenic levels in the supernatant were also shown to decrease until the third day. The ratio of methylated organic arsenic compounds at each arsenic concentration was higher in As(V)-supplemented medium than in As(III)-supplemented medium, and in cultures supplemented with 0.2 mg As/L As(V), 98.7% of the arsenic remaining in the supernatant was found to be methylated organic arsenic compounds. The ratio of arsenic remaining in the culture supernatant was lower for As(III) than for As(V); however, the ratio of inorganic arsenic in the culture supernatant was similar for both As(III) and As(V) at the same concentrations. Quantification and analysis of the arsenic adsorbed to the bacterial cell walls revealed that most of the arsenic removed from the medium was accounted for by cell wall adsorption for both As(III)- and

As(V)-supplementation, and that in both cases, all of the adsorbed arsenic was in the As(III) form. These findings demonstrate that strain KC42 did not produce volatile methylated organic arsenic compounds. The production of AB observed in previously reported arsenic-methylating bacteria was confirmed in strain KC42.

These results demonstrate that strain KC42 possesses arsenic-methylating and arsenic-removing capabilities, and may therefore be used in the bioremediation of arsenic.

5. ACKNOWLEDGEMENTS

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