1. Introduction

Human intake of inorganic arsenic derived from natural sources has become a serious health and environmental problem. The number of affected victims worldwide, primarily in Asian countries, is estimated to be approximately 80 million. Some technologies have been developed for removing inorganic arsenic from the environment, such as cohesion/co-precipitation methods using polycationic chloride or ferrous sulfate, adsorption methods using cerium or lanthanum adsorbent or activated alumina, and a reverse osmosis (RO) membrane method. However, 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.

Beginning in the late 1970s, studies reported that the toxicity of arsenic compounds vary according to differences in their chemical structure or chemical form. In addition, the existence of a pathway for methylation of inorganic arsenic in marine organisms and mammals has been demonstrated, and this pathway is expected to be used to develop detoxification methods. In addition, high concentrations of arsenic compounds are present in foods such as fishery products and seaweed, which are consumed by humans. Although the arsenic intake of healthy Japanese individuals is approximately 0.15 mg, their health is not damaged. The major reason for this is that the arsenic contained in fishery products or seaweed is arsphenamine (AB) and arsosugars. The toxicity of AB (half lethal dose of 10 g/kg) is relatively low, and is 1/300 times that of inorganic arsenic (0.03 g/kg).

A safe and effective method for chemically synthesizing AB has not yet been established. However, a method for synthesizing AB from inorganic arsenic based on transmethylation by an enzyme and synthesis of the final product, AB, from inorganic arsenic via trimethylarsine oxide (TMAO) is available. One problem to be solved before this method can be of practical use is the efficiency of methylation in the pathway from inorganic arsenic to TMAO. In addition, to obtain information about the ability of sea organisms to methylate inorganic arsenic, methylation/conver-
sion of arsenic to AB has been assessed using extracts from sea organisms such as shellfish. However, this methodology is too difficult to be of practical use for low-cost synthesis, because it is complicated and the yield (activity) is low. From the viewpoint of cost, the most practical method for methylation may be the utilization of microorganisms, because large-scale preparation and purification should be easy. However, only a few microorganisms that can produce TMAO from inorganic arsenic have been found so far. Only two bacteria are known, Klebsiella oxytoca and Xanthomonas sp., and these yield only 10% TMAO or less, and arsenic detoxification processes using these microorganisms have not been developed.

To develop an effective technology for conversion of inorganic arsenic to AB by using bacteria, we have searched for microorganisms that have the ability to methylate arsenic that are adequately applicable for the detoxification process. As a result, we have successfully isolated a strain, Cellulomonas sp. strain K63 that has arsenic methylation ability from the soil in Miyazaki prefecture. In this study, the arsenic methylation characteristics of this strain were evaluated and are reported.

2. Materials and Methods

2.1 Bacterial strain

Cellulomonas sp. strain K63 was isolated from soil that was not polluted with arsenic in Miyazaki prefecture, and its arsenic methylation 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 K63 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. An aerobic culture was incubated at 30°C with shaking at 100 strokes/min. An anaerobic culture was incubated statically at 30°C after replacing the headspace with sterile nitrogen gas for about 30 seconds. 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.

2.3 Experiments using intracellular extracts

Strain K63 were aerobically cultured in R2A medium supplemented with 1.0 g/L glucose and 10.0 mg As/L of As(III) at 30°C for 2 days, and the bacterial cells were harvested by centrifugation (10,000 × g, 15 min) and freeze-dried. 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 and Bacillus cereus strain W2 were prepared as described above after aerobic culture for 1 day at 30°C in Nutrient broth (Nissui Pharmaceutical Co., Ltd.).

Arsenic methylation was carried out in 0.50 mL reactions with 0.10 mL of intracellular extract, 0.05 mL of 10.0 mg As/L As(III) solution, 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 6.0–8.0) 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 7.0) at 25°C–45°C for 2 h. To evaluate the influence of incubation time on the arsenic methylation reaction, the arsenic concentration in the reaction solution was measured after incubation in phosphate-buffered solution (pH 7.0) at 35°C for 0.5–4 h. To evaluate the influence of additives on the arsenic methylation reaction, the arsenic concentration in the reaction solution was measured after incubation in phosphate-buffered solution (pH 7.0) supplemented with 0.05 mL of 70 mM glutathione solution (GSH) or an intracellular extract of B. megaterium UM-123 or B. cereus W2 at 35°C, for 4 h.

2.4 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). 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. Result and Discussion

3.1 Effect of arsenic on bacterial growth

Cellulomonas sp. strain K63 was aerobically and anaerobically cultured in medium supplemented with 50–200 mg As/L of As(III) or As(V) for 4 days, and the turbidity of the culture broth was measured regularly to evaluate the influence of arsenic on the bacterial growth. The results are shown in Fig. 1.

There was no remarkable difference in the logarithmic phase when cultured either aerobically or anaerobically in the presence or absence of either As(III) or As(V) at 50 mg As/L. In contrast, remarkable differences were observed when either As(III) or As(V) was added to the medium at 100 mg As/L. When either As(III) or As(V) was added to the medium at 200 mg As/L, almost no bacterial growth was observed. When strain K63 was grown anaerobically, the lag phase of growth was prolonged compared to when the strain was grown aerobically, and the aerobic culture required 2 days to reach stationary phase, whereas the anaerobic culture required 3 days. When 50 mg As/L arsenic was added to the medium, in either aerobic or anaerobic culture, As(V) was found to inhibit bacterial growth compared to growth in the presence of As(III).

3.2 Time course of arsenic methylation

To evaluate the methylation of arsenic catalyzed by strain K63 during culture, strain K63 was cultured aerobically and anaerobically for 4 days in medium containing 1.0 g/L glucose and 10 mg As/L of As(III), and the arsenic speciation in the culture medium was examined at regular intervals. The results are shown in Fig. 2.

The levels of inorganic arsenic decreased in aerobic culture until the third day, and decreased to 3.01 mg As/L. The amount of dimethylarsinic acid (DMAA) increased as inorganic arsenic decreased from the first day, and the amount reached 4.12 mg As/L on the third day. The amount of trimethylarsenic compound (TMAC) rapidly increased from the third day, and it reached 2.43 mg As/L on the fourth day. Production of methylated organic arsenic compounds (monomethylarsonic acid [MMAA], DMAA, and TMAC) reached a maximum of 6.83 mg As/L on the fourth day.

In anaerobic culture, inorganic arsenic decreased until the fourth day when it reached a low of 4.87 mg As/L. DMAA increased as inorganic
arsenic decreased from the second day, and reached 2.98 mg As/L on the fourth day. TMAC increased from the third day, and reached a maximum of 1.54 mg As/L on the fourth day. Production of methylated organic arsenic compounds reached a maximum of 4.95 mg As/L on the fourth day.

The rates of bacterial growth and inorganic arsenic methylation for strain K63 were faster in aerobic culture than in anaerobic culture, and the percentage of methylated organic arsenic compounds was also higher. The decrease of inorganic arsenic was mostly in agreement with the profile of bacterial growth. This result indicates that the inorganic arsenic methylation progressed effectively at the lag phase of growth. There was no difference between the quantity of arsenic (the total of inorganic arsenic and methylated organic arsenic compounds) in the culture medium on the fourth day in aerobic and anaerobic cultures and the quantity of arsenic added to the culture medium. This result indicates that the arsenic compounds did not evaporate from the culture medium, and that strain K63 does not produce volatile organic arsenic compounds such as dimethyl arsine (DMA) and trimethylarsine (TMA). This result suggests that strain K63 does not have a metabolic pathway for reducing DMAA or TMAO into DMA or TMA, respectively.

Most methylation bacteria produce arsine gas, whereas only a few bacteria, such as strain K63, produce non-volatile arsenic compounds. Compared to *Klebsiella oxytoca*, *Xanthomonas* sp., and *Pseudomonas putida* 8,9, which produce non-volatile methylated organic arsenic compounds, strain K63 showed higher conversion efficiency from inorganic arsenic to methylated organic arsenic compounds. Furthermore, both strain K63 and *B. cereus* strain R2 10 showed nearly the same conversion efficiency and a high TMAC ratio. Microorganisms that produce arsine gas can disperse methylated organic arsenic compounds, which are less toxic than the starting material, into the atmosphere. Therefore, these organisms could be utilized in the decontamination/restoration of soil contaminated with arsenic11. However, arsine gas is unsuitable indoors because it has a garlic-like smell. In contrast, microorganisms that produce non-volatile methylated organic arsenic compounds, such as strain K63, can be used indoors, and the liquid waste generated by the processing can be discharged into the environment without causing secondary pollution. Furthermore, if the compound can be converted to an arsenic compound with low toxicity, such as AB or arsenocholine, it will be considered to be a more effective detoxification.

### 3.3 Effect of the amount of glucose added to the medium on methylation

To evaluate the effect of the initial amount of glucose added to the medium on strain K63-mediated arsenic methylation, glucose was added at 0.1–10.0 g/L to medium containing 10 mg As/L of As(III). Strain K63 was cultured aerobically in these media for 4 days, and then the arsenic in the culture medium was examined. The results are shown in Fig. 3.

The amount of inorganic arsenic decreased in up to 1.0 g/L glucose, and the ratios of DMAA and TMAC also increased. The percentage of inorganic arsenic and methylated organic arsenic compounds decreased from the second day, and reached 2.98 mg As/L on the fourth day. TMAC increased from the third day, and reached a maximum of 1.54 mg As/L on the fourth day. Production of methylated organic arsenic compounds reached a maximum of 4.95 mg As/L on the fourth day.
compounds were almost identical when the glucose concentration was greater than 1.0 g/L. The turbidity of the culture broth increased in up to 1.0 g/L glucose, and was almost identical at greater than it. These results indicate that the bacterial growth has influenced the inorganic arsenic methylation. The percent of TMAC reached a maximum of 24.8% when the glucose concentration was 1.0 g/L.

3.4 Effect of arsenic concentration on methylation

To evaluate the effect of arsenic concentration on methylation, As(III) or As(V) was added at 5.0–200 mg As/L to medium containing 1.0 g/L glucose. Strain K63 was cultured aerobically or anaerobically in these media for 4 days, and then the arsenic in the culture medium was examined. The results are shown in Fig. 4.

When As(III) was added to either aerobic or anaerobic cultures, the percentage of remaining inorganic arsenic increased as the initial arsenic concentration increased, and effective methylation was observed in up to 50 mg As/L. However, methylated organic arsenic compounds were barely detected at 200 mg As/L. The percentage of methylated organic arsenic compounds reached a maximum when 5.0 mg As/L arsenic was added, and was 90.1% in aerobic culture and 71.3% in anaerobic culture. The yield of the methylated organic arsenic compounds when 50 mg As/L arsenic was added reached a maximum of 22.41 mg As/L (44.8%) in aerobic culture and 15.22 mg As/L (30.4%) in anaerobic culture.

Similar to what was observed with As(III), when As(V) was added in either aerobic or anaerobic cultures, the percentage of remaining inorganic arsenic increased as the arsenic concentration increased, and methylated organic arsenic compound was barely detected at 200 mg As/L. The percentage of methylated organic arsenic compound reached maximum levels when 5.0 mg As/L of arsenic was added, and it was 29.9% in aerobic culture and 38.8% in anaerobic culture. The yield of the methylated organic arsenic compound reached a maximum when 50 mg As/L of arsenic was added, and was 5.15 mg As/L (10.3%) in aerobic culture and 9.05 mg As/L (18.1%) in anaerobic culture.
The percentage of methylated organic arsenic compound was higher when As(III) was added than when As(V) was added in both aerobic and anaerobic cultures. However, the percentage of the methylated organic arsenic compound was higher in aerobic culture than in anaerobic culture when As(III) was added, whereas when As(V) was added, the percentage was higher in anaerobic culture than in aerobic culture. It is generally known that As(V) is reduced to As(III) before methylation in microorganisms; therefore, the As(V) to As(III) reducing activity of strain K63 is considered to be weak. This hypothesis is supported by the fact that when As(V) was added, the percentage of methylated organic arsenic compound was higher in anaerobic conditions, which are suitable for the reduction of As(V) to As(III), and *B. cereus* strain R2 showed the same tendency.

### 3.5 Comparison to arsenic methylation by *B. cereus* strain R2

A comparison of arsenic methylation in strain K63 and *B. cereus* strain R2 is shown in Table 1. When 5.0 mg As/L of As(III) was added, 90.1% methylated organic arsenic compounds was detected in the aerobic culture of strain K63. In contrast, when 0.1 mg As/L of As(III) was added, the value was 89.0% in aerobic cultures of strain R2. These values were the maximums for each strain. The ratios of methylation from inorganic arsenic to methylated organic arsenic compound were nearly the same; however, strain K63 was able to effectively methylate arsenic at higher concentrations than strain R2.

As a result of the analysis of TMAC produced by both strains, we found that these strains produced both TMAO and AB, and the percentage of AB was higher in strain K63 than in strain R2.

### 3.6 Methylation of arsenic using intracellular extracts

An intracellular extract from strain K63 cells was prepared, and arsenic methylation reactions were performed. The results of tests on the effects of pH and temperature on arsenic methylation are shown in Fig. 5 and 6, respectively. The percentage of methylated organic arsenic compound reached a maximum of 32.9% at pH of 7.0 and at 35°C. The percent of TMAC under these conditions was 8.3%.

The results of a time course experiment for arsenic methylation is shown in Fig. 7. Inorganic arsenic decreased for the first 2 h, and methylated organic arsenic compounds, including DMAA as a major component, were produced; however, almost no change was observed after the first 2 h. Inorganic arsenic was decreased to 66.0%, and the percent of TMAC was 9.0%.

To enhance the arsenic methylation reaction, glutathione and intracellular extracts from *B. megaterium* strain UM-123 and *B. cereus* strain W2, which have arsenate reducing ability, were added to the reaction solution. The results of this experiment are shown in Fig. 8. When glutathione was added to the reaction solution, the...
The percentage of methylated organic arsenic compounds was the highest; however, almost all of it was DMAA, and the percentage of TMAC decreased to 2.7%, which was lower than when glutathione was not added. This result suggested that only methylation via glutathione complexation occurred \(^{14}\). 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 contained in these two bacterial intracellular extracts \(^{15}\).

4. Conclusion

Cellulomonas sp. strain K63 was isolated from soil in Miyazaki prefecture and was shown to be a microorganism with inorganic arsenic methylation activity. In comparison to aerobic cultures, anaerobic cultures of strain K63 showed a longer lag phase before entering into the logarithmic phase, and a longer time to reach stationary phase. Strain K63 did not grow in medium containing 200 mg As/L of arsenic in either aerobic or anaerobic culture, and As(V) inhibited bacterial growth more than As(III).

Strain K63 showed effective methylation of inorganic arsenic when up to 50 mg As/L of As(III) was added to the medium in either aerobic or anaerobic culture. When As(V) was added to the
medium, methylation was not as robust, and the percentage of methylated organic arsenic compound increased in anaerobic culture, in contrast to what was observed in As(III). This result suggests that strain K63 shows weak As(V) to As(III) reducing activity. The percentage of methylated organic arsenic compound reached a maximum 90.1% in aerobic culture when 5.0 mg As/L of As(III) was added. Production of AB (2.1%) was observed under these conditions. In addition, volatile methylated organic arsenic compounds were not produced, because there was no difference between the amount of arsenic detected in the culture medium and the initial amount of arsenic added to the medium. The methylation ability of strain K63 was similar to that of B. cereus strain R2; however, strain K63 showed more effective methylation of high concentrations of arsenic and produced a higher percentage of AB. Furthermore, arsenic methylation using intracellular extracts at pH 7.0 and 35°C for 4 h yielded 34.0% methylated organic arsenic compound and 9.0% TMAC. When intracellular extracts from other bacterial strains with arsenate reducing ability were added to the reaction solution, the percentage of TMAC increased to 14.9%.

These results suggest that Cellulomonas sp. strain K63 is a microorganism with arsenic methylation ability that is sufficient for use in detoxification processes, and further suggests that if the conversion efficiency to AB can be improved, a technology for restoration of arsenic to nature after detoxification using microorganisms can be established.

Acknowledgement

This study was supported by a Grant-in-Aid for Scientific Research (KAKENHI) (Grant Number 23510099).

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