APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. **13**, 107–111 (1999)

Accumulation of Arsenic in a Unicellular Alga *Chlamydomonas reinhardtii*

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The unicellular alga *Chlamydomonas reinhardtii* accumulated and biomethylated arsenic efficiently. A wall-less cell strain (CW-15) of *Chlamydomonas reinhardtii* proliferated in a low level arsenic-containing medium (0.01–0.1 mmol dm^{-3}) more than that in an arsenic-free medium. Although the growth of the algal cells was only slightly more inhibited in a growth medium containing arsenic at a concentration of 1.0 mmol dm^{-3} than that in an arsenic-free medium, it was completely inhibited at concentrations of 10 and 100 mmol dm^{-3}. Furthermore, transformed strains were obtained by random introduction of plasmid pJD67, carrying an Arg^+ gene, into a wall-less cell Arg^- mutant CC425 strain. Finally we selected a strain, named AS1, among the transformed CC425 of the arsenic-sensitive group. The accumulation of arsenic by the AS1 strain was about three-fold higher than that by the CW-15 strain and 80–90% of the inorganic arsenic was transformed into a dimethylarsenic compound. It is suggested that the AS1 strain is a suitable model for investigation of the accumulation and biomethylation of arsenic by microalgae in freshwater environments. Copyright © 1999 John Wiley & Sons, Ltd.

Keywords: *Chlamydomonas* species; arsenic; biomethylation; dimethylarsenic compounds

Received 2 February 1998; accepted 12 June 1998

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INTRODUCTION

Arsenic is widely distributed in the lithosphere, hydrosphere and biosphere. Marine organisms often contain high amounts of arsenic, most of which is water-soluble organic arsenic. It is postulated that inorganic arsenic in seawater is accumulated in marine algae and transformed into dimethylarsenic compounds and further transformed into trimethylarsenic compounds in animals through the food chain in marine ecosystems. Cullen *et al*. demonstrated that bioaccumulation and biomethylation of inorganic arsenic occurs in a marine unicellular alga, *Polyphysa peniculus*,[1] and also reported that methionine or *S*-adenosylmethionine was a methyl donor in the biological methylation of arsenic in marine algae.[2] Takimura *et al*. reported that the marine green alga *Dunaliella* sp. accumulated inorganic arsenic compounds and reduced them to arsenite in their cells.[3] A few reports have described the biotransformation in a freshwater environment. Kuroiwa *et al*. demonstrated the accumulation and the biomethylation of arsenic in a green alga, *Chlorella* sp.[4] Also, Kaise *et al*. have reported that the freshwater microalga *Chlorella vulgaris* accumulated arsenic in a growth medium containing disodium arsenite and experimentally converted it to dimethylarsenic compounds.[5] Maeda *et al*. reported that 95% or more of the arsenic in algal species (*Chlorella* and *Phormidium* sp.) was present as inorganic arsenic and the remaining arsenic was in the form of dimethylated arsenic (5% in *Chlorella* sp. and 0.2% in *Phormidium* sp.)[6]. It was postulated that the major arsenic species in the freshwater environment is inorganic arsenic, and inorganic arsenic is reduced to arsenite, then converted to dimethylarsenic compounds in freshwater algae. Dimethylarsenic compounds are then transformed into trimethylarsenic compounds in aquatic animals.

CCC 0268–2605/99/020107–05 $17.50

However, the mechanisms of the biomethylation of arsenic in aquatic organisms are not fully understood. From the viewpoint of environmental assessment, it is important that the circulation, the bioaccumulation and the biomethylation of arsenic in an aquatic environment are elucidated. However, the enzymic mechanism of arsenic methylation in aquatic organisms is not yet clearly known. *Chlamydomonas reinhardtii* is a unicellular eukaryotic green alga and has been used in extensive studies, such as photosynthesis, phototaxis and flagella assembly. Also, *Chlamydomonas reinhardtii* is haploid during vegetative growth, so it is easy to obtain any mutant and it is amenable to genetic analysis. In this paper, we describe the accumulation of arsenic in the wall-less strain of *Chlamydomonas reinhardtii* and the transformants for the mechanistic study of arsenic methylation in green algal cells.

MATERIALS AND METHODS

Chemicals

Sodium arsenite [$NaAsO_2$], methylarsonic acid [$(CH_3)AsO(OH)_2$], dimethylarsinic acid [$(CH_3)_2AsO(OH)$] and trimethylarsine oxide [$(CH_3)_3AsO$] were purchased from the Tri Chemical Co. (Yamanashi, Japan). Analytical-grade nitric acid (61%, w/w) and sulphuric acid (97%, w/w) were obtained from Wako Pure Chemical Co. (Osaka, Japan). Other reagents were of reagent grade. The arsenic standards of the inorganic and the methylarsenic species were prepared by serial dilutions from stock solutions.

Growth media

3/10 HSM medium

3/10 HSM medium[7] was used for the algal cell culture. Its composition was as follows: NH_4Cl 150 mg, $MgSO_4{\cdot}7H_2O$ 200 mg, $CaCl_2{\cdot}2H_2O$ 10 mg, K_2HPO_4 940 mg, KH_2PO_4 470 mg, $FeSO_4{\cdot}7H_2O$ 6.9 mg, H_3BO_4 1 mg, $ZnSO_4{\cdot}7H_2O$ 1 mg, $MnSO_4{\cdot}4H_2O$ 0.4 mg, $CoCl_2{\cdot}6H_2O$ 0.2 mg, $NaMoO_4{\cdot}2H_2O$ 0.2 mg, $CuSO_4{\cdot}5H_2O$ 0.04 mg and water 1000 cm^3 at pH 7.5.

TAP agar containing phosphoric acid

Agar (1 g) was added to 1000 cm^3 of TAP medium containing phosphoric acid at a concentration of 100 μmol dm^{-3}, and dissolved using a microwave oven. The TAP medium[8] was used for picking up transformants of the alga. Its composition was as follows: NH_4Cl 400 mg, $MgSO_4{\cdot}7H_2O$ 100 mg, $CaCl_2{\cdot}2H_2O$ 50 mg, K_2HPO_4 108 mg, KH_2PO_4 56 mg, trishydroxymethylaminomethane 2420 mg, acetic acid 1 cm^3, Hutner's trace elements solution[8] 1 cm^3 and water 1000 cm^3 at pH 7.0.

Chlamydomonas reinhardtii strains

A wall-less strain CW-15, and a wall-less cell arg^- mutant CC425 strain, were donated from the Chlamydomonas Genetic Center, Duke University, USA.

Transformed CC425 strains

Shimogawara *et al*.[9] have reported the transformation of the CC425 strain by the random introduction of plasmid pJD67, carrying the arginosuccinate-lyase Arg7 gene, using an electroporation technique. The transformants used were provided by the authors.

Culture of the wall-less strain CW-15

The algal cells were cultured in incubation bottles containing 100 cm^3 of the 3/10 HSM medium under illumination with fluorescent lumps and bubbling with filtered air enriched with CO_2 gas to 3% at 27 °C. Each bottle had sodium arsenite added at a concentration of 0, 0.01, 0.1, 1.0 10.0 or 100 mmol dm^{-3}. Growth of the cells was followed by the measurements of turbidity (optical density at 600 nm) using a JUSCO spectrophotometer (Tokyo, Japan) after 8, 15, 21 and 27 h.

Classification of the transformed CC425 strains by resistance to arsenic

The transformed CC425 was a mixture of random plasmid-introduced transformants. The transformants were classified into two groups. One was the arsenic-sensitive group which involved cells not grown on the 50 μmol dm^{-3} arsenic-containing TAP phosphoric acid agar plates under illumination with luminescent lamps at 27 °C for seven days. Another was the arsenic-resistant group which involved cells grown on the 300 μmol dm^{-3} arsenic-containing plate under the same conditions. Six strains in each group were selected for use in this experiment. These strains included AS1, AS2, AS3 and AS4 in the sensitive group and AR1, AR2, AR3, AR4, AR5 and AR6 in the resistant group.

Culture of the CW-15 strain and the transformed CC425 strains

Both strains were cultured in 500 cm^3 of the 3/10 HSM medium containing sodium arsenite at a concentration of 1.0 mmol dm^{-3} and 8.8 mmol dm^{-3} of phosphoric acid under illumination with fluorescent lamps and bubbling to aerate. After 0, 6, 12, 24 and 48 h, 100 cm^3 of the culture medium was centrifuged at 3000 rpm for 5 min at 4 °C. The alga was harvested and rinsed with an arsenic-free culture medium twice before analysis for total arsenic and arsenic species. Algal cells were weighed after the removal of water.

Total arsenic analysis[10]

An aliquot of each strain was weighed and transferred into a beaker to which an aliquot of water (1 cm^3) was added. Nitric acid (3 cm^3, 61%) and sulphuric acid (1 cm^3, 97%) were then added to the beaker. Each beaker was covered with a glass dish and left overnight. The digestion was performed on a hotplate below 100 °C until the evolution of brown fumes ceased, and then was heated until dense fumes of sulphur trioxide appeared. The degraded acid solution was transferred to a test tube, and 0.1 cm^3 of 25% (w/w) ammonium hydrogencitrate was added. The digested solution was neutralized with ammonium hydroxide. Hydrochloric acid (1 cm^3, 35% w/w), 0.5 cm^3 of 20% (w/v) ascorbic acid and 0.5 cm^3 of 20% (w/v) potassium iodide were then added to the sample solutions, followed by water to bring the solution volume to 8 cm^3. The total amount of arsenic was measured in an atomic absorption spectrophotometer (Spectra AA220, Varian) operated with a Varian Spectra AA hollow-cathode lamp at 193.7 nm equipped with a heated quartz tube, after reduction of the arsenic to arsine by an arsine generation system (VGA77, Varian) with 0.13 mol dm^{-3} sodium borohydride in 0.12 mol dm^{-3} sodium hydroxide.

Measurements of methylated arsenic species[11]

An aliquot of each strain was weighed and transferred into polymethylenepentene tubes and a 0.5 cm^3 aliquot of water was added. A 0.5 cm^3 aliquot of 2 mol dm^{-3} sodium hydroxide was added to each tube, and the top of the tube was then covered with Parafilm. After being heated in a water bath at 85 °C for 2h, the aqueous solution was neutralized with dilute hydrochloric acid and made up to a volume of 10 cm^3 with water. After centrifugation of the solution at 3000 rpm for 5 min at 4 °C, the supernatant was filtrated through a 0.45 μm membrane filter. A 3 cm^3 portion of each sample was reduced with 2% (w/w) sodium borohydride in 0.2 mol dm^{-3} sodium hydroxide and 0.6 mol dm^{-3} hydrochloric acid in a fully automated continuous arsine generation system (Hitachi HFS-2, Japan). Generated arsines were trapped on the U-shaped tube in liquid nitrogen and volatilized when the U-tube was warmed in a water bath (85 °C), then separated on a stainless capillary column (Urtla Alloy 1, 30 m, Frontier Lab. Fukushima, Japan) by gas chromatography (HP 5890). Inorganic arsenic and methylated arsenic were identified as arsine (AsH_3) methylarsine [$(CH_3)AsH_2$], dimethylarsine [$(CH_3)_2AsH$] and trimethylarsine [$(CH_3)_3As$] by single-ion monitoring at *m/z* 76, 90, 103 and 120 with MSD (HP 5972), respectively.

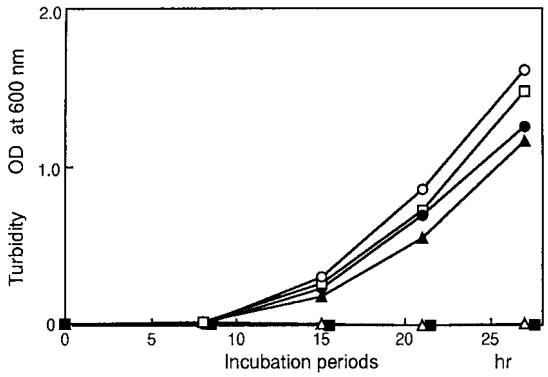

Figure 1 Growth curves of algal cells, (CW-15 strain in 3/10 HSM medium). Arsenic concentration (mmol As dm^{-3}): ●, 0; ○, 0.01; □, 0.1; ▲, 1.0; △, 10; ■, 100.

RESULTS AND DISCUSSION

Chlamydomonas reinhardtii is a unicellular autotrophic freshwater microalga. The CW-15 strain, which is a wall-less cell Arg^+ mutant of *Chlamydomonus reinharditii*, was cultured for 28 h in the 3/10 HSM medium containing sodium arsenite. The growth curves of the algal cells are shown in Fig. 1. Growth of the mutant in the medium containing 0.01 and 0.1 mmol dm^{-3} arsenic was better than that in the arsenic-free medium. Growth of the algal cells was slightly depressed in the 1.0 mmol dm^{-3}

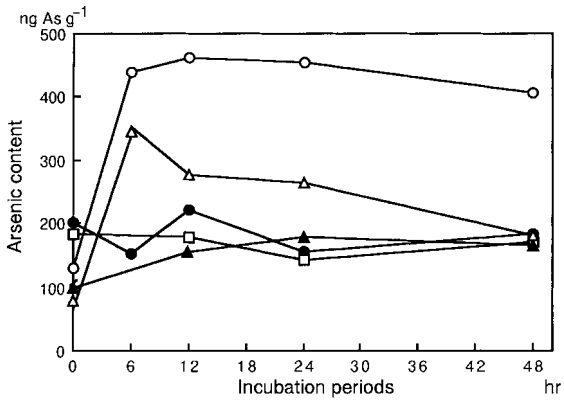

Figure 2 Total arsenic concentrations in the cells of arsenic-sensitive transformants. Arsenic content is shown as ng As g^{-1} (wet weight). Strains: ●, CW-15; ○, AS1; □, AS2; ▲, AS3; △, AS4.

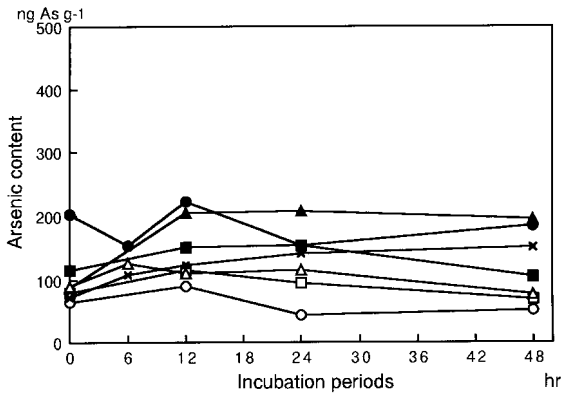

Figure 3 Total arsenic concentrations in the cells of arsenic-resistant transformants. Arsenic content is shown as ng As g^{-1} (wet weight). Strains: ●, CW-15; ○, AR1; ■, AR2; □, AR3; ▲, AR4; △, AR5; ×, AR6.

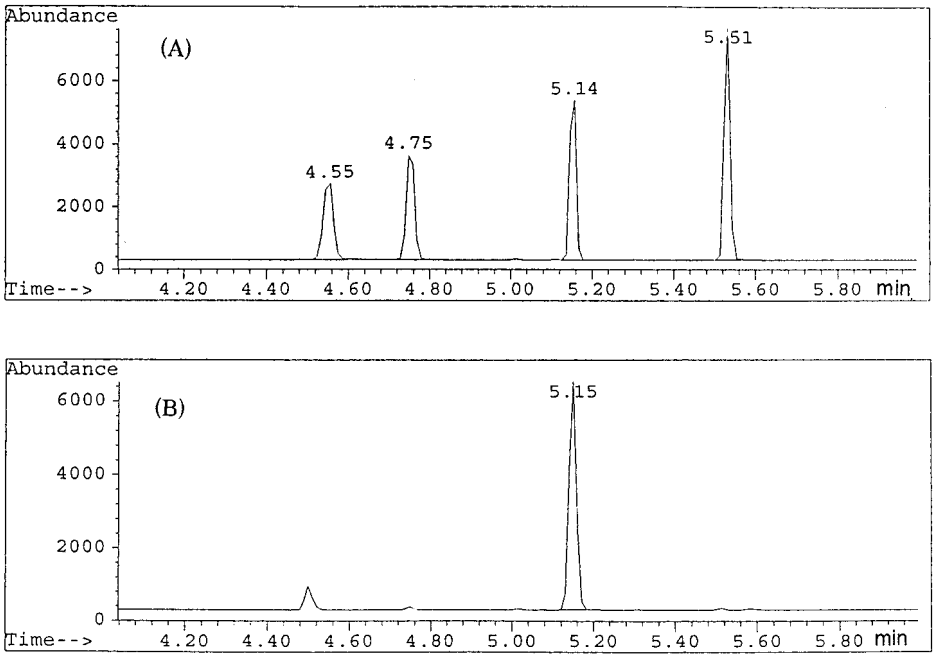

Figure 4. Total ion chromatograms of arsines in microalga *Chlamydomonas reinhardtii*. Ions of arsine were monitored at *m/z* 76, 90, 103 and 120. (A) Authentic arsines: AsH_3 (arsine) $R_t = 4.55$ min, $(CH_3)AsH_2$ (methylarsine) $R_t = 4.75$ min, $(CH_3)_2AsH$ (dimethylarsine) $R_t = 5.14$ min and $(CH_3)_3As$ (trimethylarsine) $R_t = 5.51$ min, each at 10 ng As cm^{-3}; (B) arsenic compounds in the AS1 strain.

arsenic-containing medium and was completely suppressed at a concentration of 10 and 100 mmol dm^{-3}. If the arsenic concentration was below 0.1 mmol dm^{-3}, the wall-less strain appeared to prefer the presence of arsenic.

The transformants of the CC425 strains, which were selected as described in the 'Materials and methods' section, were cultured in the 1.0 mmol dm^{-3} arsenic-containing 3/10 HSM medium. Both groups of arsenic-sensitive and -resistant transfor-

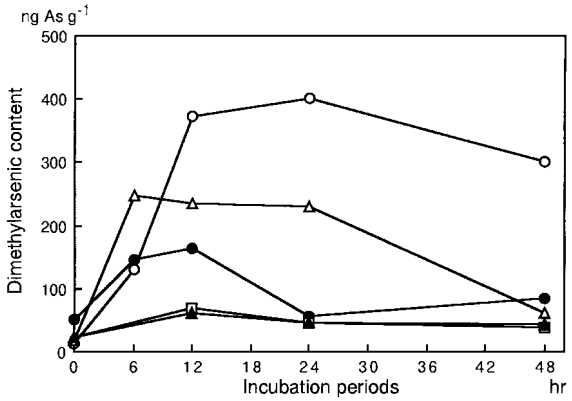

Figure 5 Arsenic concentrations of dimethylarsenic species in the cells of arsenic-sensitive transformants. Arsenic content is shown as ng As g^{-1} (wet weight) Strains: ●, CW-15; ○, AS1; □, AS2; ▲, AS3; △, AS4.

mants had proliferated more than the CW-15 strain (data not shown).

The accumulation of arsenic in these multiplied strains is shown in Figs 2 (arsenic-sensitive) and 3 (arsenic-resistant). The arsenic was reached the maximum level of accumulation in 6 h in the AS1 strain (Fig. 2).

Whereas the accumulation of arsenic in the strains of the arsenic-resistant group was lower than that of the CW-15 strain, some of the arsenic-sensitive group showed an obviously higher accumulation of arsenic than the CW-15 strain. In particular, the AS1 strain showed a rapid accumulation of arsenic, and the accumulated arsenic concentration was about three-fold higher than that of the CW-15 strain. The accumulation of arsenic reached an approximate maximum after 6 h, then the concentration was maintained for about 48 h. Figure 4 shows SIM chromatograms for the determination of the arsenic chemical species in the AS1 strain. Only one peak corresponding to dimethylarsine was obtained. It is thought that the major water-soluble arsenic compounds were the dimethylated arsenic compounds. Methylated and trimethylated arsenic compounds were not detected in the cells.

The time course of dimethylarsenic generation in the arsenic-accumulated AS1 strain is shown in Fig. 5. It is thought that approximately 80–90% of the accumulated arsenic was rapidly converted into a dimethylarsenic compound.

The microalgae accumulated the inorganic arsenic from the medium and converted it to dimethylarsenic compounds in their tissues, but the chemical structures of the dimethylarsenic species were not determined using these analytical procedures.

CONCLUSION

Chlamydomonas reinhardtii has been used recently as a model for research in plant biochemistry. We have attempted to apply it to an examination of the accumulation and biotransformation of arsenic in the freshwater environment, and we found that the strain AS1 may be suitable for this purpose. This strain, which is a variant of the plasmid pJD67 induced wall-less cell mutant *Chlamydomonas reinhardtii* CC425, efficiently accumulated arsenic and converted it nearly quantitatively into a dimethylarsenic compound.

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