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Accumulation of arsenic by *Traustochytrium* sp. CHN-1 from Seto Inland Sea†

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The accumulation of arsenic by *Traustochytrium* sp. CHN-1 (*Labyrinthulids*) was examined by using a medium [2% (w/v) glucose, 0.1% (w/v) yeast extract, 0.1% (w/v) peptone in a half salt concentration of sea water] containing arsenic as As(V), As(III). *Traustochytrium* sp. CHN-1 was grown in 1/2 sea water medium [2% (w/v) glucose, 0.1% (w/v) yeast extract, 0.1% (w/v) peptone] containing an arsenate (As(V)) at up to 1000 mg dm^{-3} and arsenite (As(III)) at up to 50 mg dm^3. The cells died even at [As(III)]-100 mg dm^{-3}. These results suggested that the order of growth inhibition of *Traustochytrium* sp. CHN-1 by arsenic was As(III) > As(V). The biomass of *Traustochytrium* sp. CHN-1 decreased with an increase of the surrounding arsenic concentration. On the other hand, the arsenic concentration in cells increased with an increase of the surrounding arsenic concentration. Arsenic compounds were extracted with methanol/water (1:1) from a freeze-dried sample of *Traustochytrium* sp. CHN-1. The extracts were analyzed by high-performance liquid chromatography, with an inductively coupled plasma mass spectrometer serving as an arsenic-specific detector. Arsenite, arsenate, monomethylarsonic acid (MMAA), dimethylarsinic acid (DMAA) and arsenosugar were identified in *Traustochytrium* sp. CHN-1. The order of arsenic species in *Traustochytrium* sp. CHN-1 was As(V) > DMAA > As(III) > MMAA > arsenosugar at [As]-10 mg dm^{-3} in the medium. Detoxification of arsenic by cells was probably achieved by methylation.

KEYWORDS: arsenic; tolerance; accumulation; methylation; *Traustochytrium*; *Labyrinthulids*

INTRODUCTION

Traustochytrium (*Labyrinthulids*) are native to a vast array of fresh water and marine environments, and can be grown in large quantities with relative ease. The very curious protists, *Labyrinthulids*, have been isolated from a wide variety of marine and fresh habitats, especially attaching to algae, vascular plants and detrital materials.[1] Porter[2] has accommodated it in a single order, the *Labyrinthulids*, with two families, the *Labyrinthulae* and *Traustochytridae*. The *Traustochytridae* contains a single genus, *Traustochytrium*.[2] Recently, we isolated the new pigment-containing strain *Traustochytrium* sp. CHN-1 from coastal sea water of Seto Inland Sea (Japan), which contained high levels of docosahexaenoic acid (C22:6, DHA).[3] In general, marine organisms accumulate arsenic at extraordinary levels compared with land organisms.[4] However, there are no reports on the characterization of *Traustochytrium* sp. including arsenic accumulation, the chemical forms of arsenic and arsenic tolerance in cells. This report describes arsenic accumulation, arsenic tolerance and the chemical forms of arsenic species in cells of *Traustochytrium* sp. CHN-1 from the Seto Inland Sea.

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MATERIALS AND METHODS

Traustochyrium

Traustochyrium sp. CHN-1 obtained from sea water of Nagahama in the Seto Inland Sea, Japan, was used throughout the experiments. *Traustochyrium* sp. CHN-1 was cultured in 250 cm^3. Ehrlenmeyer flasks containing 1 dm^3 of the following medium at 23°C, in low-intensity

fluorescent light (1000 lux) alternated diurnally with darkness, and rotary shaking at 120 rpm: 2% (w/v) glucose, 0.1% (w/v) yeast extract, 0.1% (w/v) peptone in a half salt concentration of sea water. Cells were harvested at the end of the log phase by centrifugation at 2000 rpm for 20 min, washed once with 100 cm^3 of cold reconstituted sea water, dried by freeze-drying and stored at −20 °C prior to extraction.

Accumulation experiment of arsenic by *Traustochyrium* sp. CHN-1

In the arsenic accumulation experiments, precultured *Traustochyrium* sp. CHN-1 cells (1 mg dry weight basis) were suspended in a 1 dm^3 Ehrlenmeyer flask with 1/2 sea water containing the desired amounts of arsenic ([As]-0, 1, 10, 50, 100, 500, 1000 mg dm^{-3}). Arsenic was added as Na_2HAsO_4 or $AsCl_3$. The arsenic accumulation experiments were carried out in light (1000 lux) with sterile air at 23 °C and pH 6.0. After an appropriate time, the cells were collected by centrifugation at 2000 rpm, washed three times with deionized water, and freeze-dried.

Analysis of arsenic in the *Traustochyrium* sp. CHN-1

The freeze-dried cells containing arsenic were digested with a mixed solution containing 3 cm^3 of concentrated nitric acid, 1 cm^3 of concentrated sulfuric acid and 1 cm^3 of 60% perchloric acid.[5] The amounts of arsenic were determined by means of a hydride-generation atomic absorption spectrometer system (Shimazu Model AA-6600G).

Analysis of arsenic species in the *Traustochyrium* CHN-1 cells

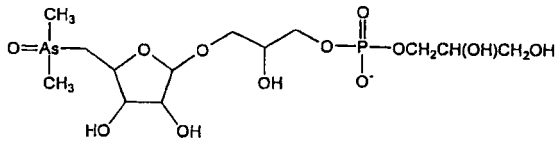
Each of the freeze-dried samples (50 to 100 mg dry weight) was weighed into a centrifuge tube. To each tube was added 5 dm^3 of methanol/water (1:1, v/v), and the tube was sonicated for 10 min. After centrifugation (2000 rpm for 10 min), the extract was removed using a Pasteur pipette. The extraction process was repeated five times for each sample; the extracts were combined, evaporated to dryness and dissolved in 2 cm^3 of water. The solution was filtered through a 0.5 μm disposable filter unit (Millipore Columnguard-LCR13), and an aliquot of the solution (25 μl) was injected into the high-performance liquid chromatography–inductively coupled plasma mass spectrometry (HPLC–ICP-MS) system (Yokogawa HP 4500 type, Japan). HPLC–ICP-MS analysis was conducted as reported previously using an Inertsil ODS column (4.6 × 250 mm^2: GL Science, Japan).[6] Arsenic compounds were eluted with 10 mM tetraethylammonium hydroxide–4.5 mM malonic acid–0.05% methanol (pH 6.8).

Quantification was performed by comparing the peak areas of each compound with those of a known concentrations of standard arsenic compounds. Interference from chemical ionization ($^{40}Ar^{35}Cl^+$ would show the same *m/z* 75 as $^{75}As^+$) and was detected by monitoring ion counts at *m/z* 77 (corresponding to $^{40}Ar^{37}Cl^+$) simultaneously. Water-soluble arsenic concentration represents the sum of all arsenic species obtained by HPLC.

(1) Arsenite AsO_3^{3-} (2) Arsenate AsO_4^{3-}

(3) Monomethyl arsonate $CH_3AsO_3^{2-}$

(4) Dimethyl arsinate $(CH_3)_2AsO_2^-$



(5) Arsenosugar

Figure 1. Arsenic standards used in the present study.

The water-soluble arsenic compounds used as standards [Fig. 1, arsenite (**1**), arsenate (**2**), monomethylarsonic acid (MMAA; **3**), dimethylarsinic acid (DMAA; **4**), and arsenosugar (**5**)] were prepared as reported previously.[6]

RESULTS AND DISCUSSION

Effects of arsenic on the growth of *Traustochytrium* sp. CHN-1

The effect of arsenic on the growth of *Traustochytrium* sp. CHN-1 was examined in various concentrations of arsenate and arsenite for 14 days, and the results are shown in Figs 2 and 3. The growth of *Traustochytrium* sp. CHN-1 decreased with an increase of arsenite (0–50 mg dm^{-3}) and arsenate (0–1000 mg dm^{-3}) concentration in the medium. The biomass of *Traustochytrium* sp. CHN-1 decreased with an increase of the surrounding arsenic concentration. Growth inhibition of *Traustochytrium* sp. CHN-1 was observed at arsenite con-

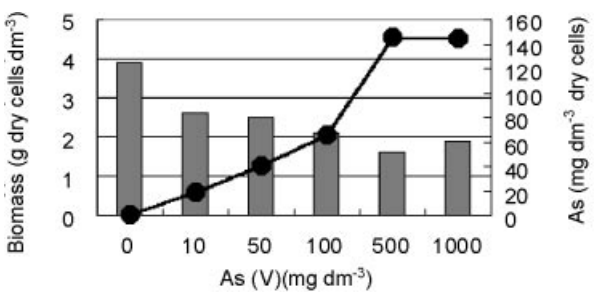


Figure 2. Effect of arsenate on the growth and accumulation of *Traustochytrium* sp. CHN-1. Conditions: 0–1000 mg dm^{-3} of arsenic(V) (as Na_2HAsO_4); glucose, 2%; yeast extracts, 0.1%; peptone, 0.1%; in 1/2 sea water; 23 °C, 1000 lux, 13 days; ●: arsenic; ■: biomass.

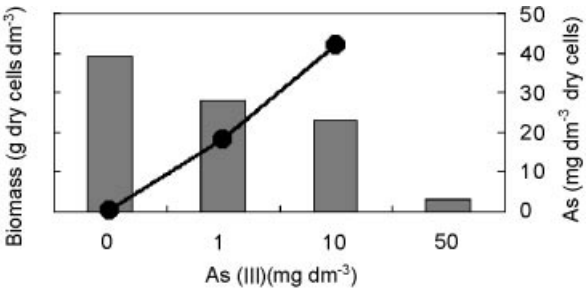


Figure 3. Effect of arsenite on the growth and accumulation of *Traustochytrium* sp. CHN-1. Conditions: 0–50 mg dm^{-3} of arsenic(III) (as $AsCl_3$); glucose, 2%; yeast extracts, 0.1%; peptone, 0.1%; in 1/2 sea water; 23°C, 1000 lux, 13 days; ●: arsenic, ■: biomass.

centrations exceeding 50 mg dm^{-3}. These results suggested that arsenic concentrations and arsenic species in the medium were largely correlated. On the other hand, the arsenic content in *Traustochytrium* sp. CHN-1 increased with an increase of arsenite and arsenate in the medium and became a maximum at [As(V)] = 500 mg dm^{-3} in the medium. Maeda *et al.*[7] and Yamaoka *et al.*[8] have recognized, for *Chlorella vulgaris*[7] and *Dunariella* sp.[8] a tendency similar to our experimental results. In conclusion, accumulation of arsenic by *Traustochytrium* sp. CHN-1 was accomplished by the common uptake pathway for arsenic. This implies that arsenic accumulation by *Traustochytrium* sp. CHN-1 depends upon biological activity and physical absorption, and the accumulation process of arsenate is different from that of arsenite.

Time course of arsenic accumulation and growth of *Traustochytrium* sp. CHN-1

Figure 4 shows the accumulation of arsenic in the growth process of *Traustochytrium* sp. CHN-1. The accumulation of arsenic by *Traustochytrium* sp. CHN-1 was approximately

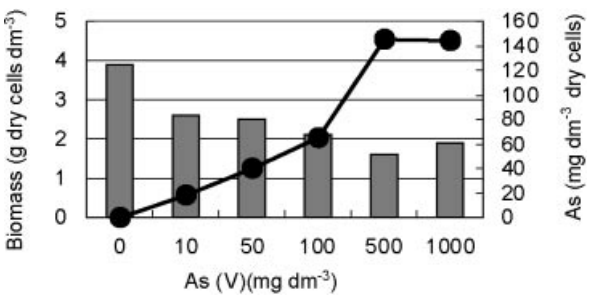


Figure 4. Time course of arsenic accumulation and the growth of *Traustochytrium* sp. CHN-1. Conditions: 10 mg dm^{-3} of arsenic(V) (as Na_2HAsO_4); glucose, 2%; yeast extracts, 0.1%; peptone, 0.1%; in 1/2 sea water; 23°C, 1000 lux, 13 days; ●: arsenic,

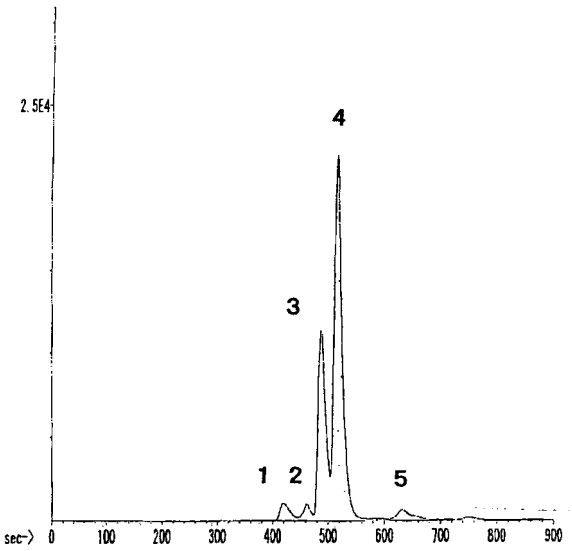


Figure 5. HPLC–ICP-MS chromatograms (m/z = 75) of the *Traustochytrium* sp. CHN-1 extracts. Column: Inertsil ODS; buffer: 10 mM tetraethylammonium hydroxide–4.5 mM malonic acid–0.05% methanol (pH 6.8); flow rate, 0.75 ml min^{-1}; 25 μl of each extract was injected. The peaks are labeled as follows: (1) arsenite; (2) arsenate; (3) MMAA; (4) DMAA; (5) arsenosugar.

43 mg dm^{-3} of dry cells within the preliminary logarithmic growth phase (2 days), but decreased to 10% or less of its logarithmic growth phase accumulation within the stationary growth phase (13 days). Arsenic concentrations in *Traustochytrium* sp. CHN-1 were relatively higher than 20 mg dm^{-3} of dry cell dry weight in *Dunaliella* sp.[8] These results suggest that arsenic was accumulated at an abnormally high concentration by *Traustochytrium* sp. CHN-1 within the logarithmic growth phase. Generally, arsenic in *Dunaliella* sp. is accumulated as As(V) within the logarithmic growth phase.[8] We presume that arsenic in *Traustochytrium* sp. CHN-1 is metabolized by similar processes to *Dunaliella* sp.

Arsenic species of *Traustochytrium* sp. CHN-1

Chromatograms of the HPLC–ICP-MS analysis of *Traustochytrium* sp. CHN-1 are shown in Fig. 5. *Traustochytrium* sp. CHN-1 clearly shows the presence of five dominant arsenic compounds in the extract. The major water-soluble arsenic species in *Traustochytrium* sp. CHN-1 were identified as arsenate (As(V)) by comparison with the standards. There are several other weak peaks in the chromatogram, and they were identified as arsenite **1**, MMAA **3**, DMAA **4**, and arseno sugar **5**. Arsenate has been widely found as a major arsenic compound in marine phytoplankton. Matutou *et al.*[9] and Maeda *et al.*[10] also documented reduction and methylated arsenic for several algae. We presume that arsenic in *Traustochytrium* sp. CHN-1 is metabolized by similar

processes. Edmonds and Francesconi[11] isolated and identified an arsenic-containing ribofuranoside (arsenosugar **5**) shown in Fig. 1 from the brown alga *Ecklonia radiata*. Also, arsenosugar **5** was identified in brown algae,[12] green algae,[12] red algae,[12] zooplankton and phytoplankton,[13] and sponges.[14] Arsenosugar **5** was found to be the major arsenic species in phytoplankton. However, these authors did not detect significant amounts of arsenosugar **5** in *Traustochytrium* sp. CHN-1. However, our findings, on the contrary, show that *Traustochytrium* sp. CHN-1 from the Seto Inland Sea, Japan, contains arsenosugar **5** as a minor water-soluble arsenic compound. These results suggest that *Traustochytrium* sp. CHN-1 cells reduced As(V) to As(III) and methylated from As(III) to MMAA and DMAA, and converted from DMAA to arsenosugar **5**:

$$\text{As(V)} \rightarrow \text{As(III)} \rightarrow \text{MMAA} \rightarrow \text{DMAA} \rightarrow \text{Arsenosugar } \mathbf{5} \quad (1)$$

In conclusion, this equation shows the detoxification process of arsenic in *Traustochytrium* sp. CHN-1.

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