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Growth characteristics and arsenic metabolism of two species of arsenic-tolerant bacteria

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Two arsenic-tolerant bacterial species were isolated from a contaminated culture of the alga *Chlorella* sp. One of the bacterial species was identified as *Pseudomonas putida*; both were aerobic heterotrophs. The bacteria grew well in a peptone medium of neutral pH at room temperature and reached the stationary phase in approximately four days. Growth was not impaired by arsenic concentration as high as 1000 mg dm^{-3} As in the form of arsenate, but decreased drastically at higher concentrations. *P. putida* grown in a peptone medium with 10 mg dm^{-3} As as arsenate, reached a maximal arsenic concentration of 67 mg kg^{-1} (dry mass) after 48 h of growth in the late log phase. Most of the arsenic in the cells was inorganic and 3% of the arsenic was in the trimethylated form. During the stationary phase the bacteria excreted arsenic largely in the inorganic, but also in the mono-, di-, and trimethylated, forms.

Keywords: Arsenic, tolerance, bacteria, accumulation, metabolism, excretion

INTRODUCTION

Several freshwater algae isolated from an arsenic-polluted environment were shown to accumulate arsenic when grown in a medium containing arsenate. The accumulated arsenic was partly methylated. The algae were able to excrete a fraction of the arsenic compounds.[1–4] Few studies on the uptake and transformation of arsenic by bacteria have been carried out. For example, the marine anaerobic bacterium, *Serratia marinorubra*, was found to produce methylarsonic acid from inorganic arsenic.[5] Methylated arsenic compounds were detected after incubation of sediments from Lake Ontario. Three anaerobic bacterial species (*Aeromona* sp., *E. Coli*, and *Flavobacterium* sp.) isolated from these sediments were able to methylate inorganic arsenic in a chemically defined medium.[6] No trimethylated arsenic compound was detected in these investigations. Mixed bacterial cultures (species not identified) obtained from estuaries and coastal marine sediments were observed to demethylate methylarsenic compounds.[7,8]

In this paper, the isolation of two species of arsenic-tolerant bacteria, their growth characteristics, and their transformation of arsenic to methylated arsenic, are reported.

EXPERIMENTAL

Bacterial cultures

Chlorella sp., a freshwater alga which had been isolated from an arsenic-polluted environment[1] and had been grown in a Modified Detmer medium[1] containing 100 mg dm^{-3} of arsenic in the form of sodium arsenate in an open system, became contaminated with bacteria. The contaminated algal suspension was centrifuged, and the resultant supernatant containing bacteria was spread on a peptone agar medium containing 100 mg dm^{-3} of arsenic in the form of arsenate and incubated overnight at 30 °C. A colony of the bacteria was isolated from the agar plate subsequently. The bacterial colony was regenerated five times on the agar medium for purification of the colony. Two bacteria were thus isolated. Both

bacteria did not grow in an algae-free or organic nutrients-free medium (Modified Detmer medium[1]). The API 20 NE test (API System SA, France) was carried out in attempts to identify the two bacterial species isolated.

Aliquots of the bacterial culture were transferred to a peptone medium of pH 7.2 prepared by dissolving 10 g of peptone and 5 g of sodium chloride in 1000 cm^3 sterilized water. The bacteria were grown under various conditions. An Erlenmeyer flask (300 cm^3) containing arsenic-inoculated peptone medium (100 cm^3) was stoppered with a microporous silicone plug, illuminated with fluorescent lamps (400–700 nm, 3000 lux), and shaken on a reciprocal shaker (100 strokes min^{-1}) at room temperature. A similar experiment was conducted in the dark. The inoculated medium (500 cm^3) in an Erlenmeyer flask (1000 cm^3) was aerated with germ-free, water-saturated air and illuminated. These experiments were carried out at 10, 20, and 40 °C. The inoculated medium (600 cm^3) kept in a 1-dm^3 commercial jar fermentor (MBF-100M, Tokyo-Rikakikai Ltd, Japan) was aerated with germ-free air (1 dm^3 min^{-1}) and stirred at 300 rpm at 30 °C. The cells were harvested by centrifuging at 11 000 ***g*** for 5 min at room temperature. The supernatants were decanted and the cells washed three times with sterilized water by centrifugation. The remaining pellets were warmed at 60 °C to constant mass.

Determination of total arsenic and methylated arsenic compounds

For the determination of total arsenic, the dry cells (10–20 mg) were mixed with 50% magnesium nitrate solution $Mg[NO_3)_2$, 2 cm^3], and the mixture was dried and mineralized by heating at 550 °C for 6 h. The mineralized samples were dissolved with 10 mol dm^{-3} hydrochloric acid (HCL, (acid 10 cm^3), 40% potassium iodide solution (KI, 1 cm^3) was added, the solution was extracted twice with chloroform ($CHCl_3$, 5 cm^3) and the $CHCl_3$ phase was then back-extracted with water (2 cm^3). Total arsenic was determined in the water phase by graphite furnace atomic absorption spectroscopy. For the determination of methylated arsenic compounds, the dry cells (*ca* 10 mg) were digested with 5 cm^3 of 2 mol dm^{-3} sodium hydroxide (NaOH) at 90–95 °C for 3 h by use of an aluminium heating block. Methylated arsenic compounds in the digest were reduced with sodium borohydride ($NaBH_4$) to the arsine compounds. The arsine gases were frozen out in a batch in a liquid-nitrogen U-trap. The arsines were successively carried out of the trap upon warming it. They passed through a quartz tube atomizer and were determined on an atomic absorption spectrometer.[2]

RESULTS AND DISCUSSION

Growth characteristics of the bacteria

When *Chlorella* sp. was grown in a Modified Detmer medium containing 100 mg dm^{-3} arsenic in containers open to air, the culture became contaminated with bacteria. The bacteria were repeatedly inoculated in a peptone agar medium containing 100 mg dm^{-3} arsenic. Two species of bacteria, both Gram-negative bacilli of approximately 0.5 μm diameter and 3 μm length, were isolated. One of the species was identified as *Pseudomonas putida* by means of the API 20 NE test (Table 1); the other species could not be identified. *Pseudomonas putida* was grown under illumination in peptone media containing up to 10 000 mg dm^{-3} arsenic for three days in an Erlenmeyer flask closed with a microporous silicone plug. The culture was shaken and illuminated. The growth of *P. putida* was unaffected by arsenic concentrations as high as 1000 mg $dm.^{-3}$ At higher arsenic concentrations the cell survived but growth was drastically impaired (Fig. 1). *P. putida* was grown under the same conditions in an arsenic-containing peptone media (100 mg dm^{-3}), the pH of which was adjusted with MES buffer (2-*N*-morpholinoethanesulphonic acid) in the pH range 4.2–7.2 and with Bicine buffer [*N*,*N*-bis(2-hydroxyethyl)glycine] in the pH range 8.0–10.1. The bacteria grew well when the pH of the medium at the time of inoculation was in the range 5–9. The optimum pH for growth was in the neutral range (Fig. 2). During the two-day growth period the pH of the media changed toward neutral as indicated in Fig. 2.

Mixtures of the two bacterial species were grown in the dark or with illumination in the peptone medium at pH 7.2 containing 100 mg dm^{-3} arsenic. The bacteria grew well in the presence and absence of light in the media that were aerated with germ-free, water-saturated air. Growth in the non-aerated cultures was drastically depressed (Fig. 3). The bacteria did not grow in a medium containing only inorganic nutrients (Modified Detmer medium). These results

Table 1 Results of the API 20 NE test for the arsenic-tolerant bacterial species

Reaction[a]	NO_3	TRP	GLU	ADH	URE	ESC	GEL	PNPG
Species 1	+	−	+	−	−	+	−	+
Species 2	−	−	−	+	−	−	−	−

Assimulation test

Reaction[b]	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC	OXI
Species 1	+	+	+	+	+	+	+	+	+	+	+	+	+
Species 2	+	+	−	−	−	−	+	+	−	+	+	+	−

[a] NO_3, nitrate reduction from NO_3 to NO_2; TRP, tryptophane–indole production ability; GLU, glucose oxidation; ADH, arginine dehydrolase; URE, urease; ESC, esculine hydrolysis (β-glucosidase); GEL, gelatin hydrolysis (protease); PNPG, *p*-nitrophenyl-β-galactopyranoside.

[b] GLU, glucose; ARA, arabinose; MNE, mannose; NAG, *N*-acetylglucosamine; MAL, maltose; GNT, gluconate; CAP, caproate; ADI, adipate; MLT, malate; CIT, citrate; PAC, phenylacetate; OXI, oxidase.

[c] Identified as *Pseudomonas putida*

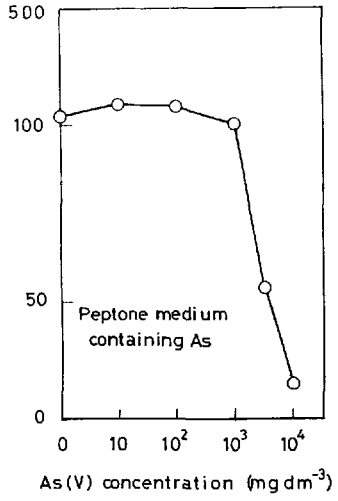

Figure 1 Growth of *Pseudomonas putida* in a peptone medium with arsenic concentrations in the range 0–10 000 mg dm^{-3} arsenic as arsenate under illumination.

indicate that *P. putida* and the unidentified species are aerobic, heterotrophic bacteria. When mixtures of the two bacterial species in the arsenic-containing peptone medium (100 mg dm^{-3} As as arsenate) were shaken under illumination for ten days at 10, 20, or 40 °C, maximal growth was observed at 20 °C (Fig. 4).

Growth curves were obtained for the two bacterial species by culturing at 30 °C in the jar fermentor in the arsenic-containing peptone medium (10 mg dm^{-3} As as arsenate) that was aerated with germ-free air. Within the first 24 h both species grew at comparable rates with an approximate doubling time of 5 h during the second 12-h period. At 48 h the unidentified species had reached the stationary phase with 55×10^8 cells per cm^3, whereas *P. putida* was still in the log phase. *P. putida* reached the stationary phase after 96 h of growth with 100×10^8 cells per cm^3 (Fig. 5). The solid line and the dotted line in Fig. 5 are calculated from the theoretical equation (logistic curve[9]). The bacterial growth data were found to be approximated well by the logistic curve.

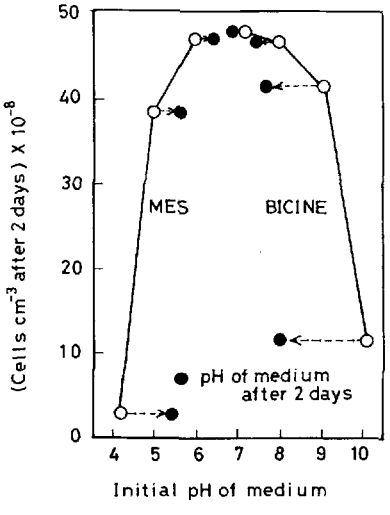

Figure 2 Growth of *P. putida* in an arsenic-containing medium (100 mg dm^{-3}) in the pH range 4.2–10.1 at room temperature shaken under illumination.

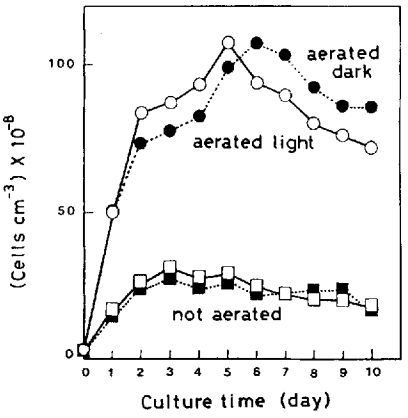

Figure 3 Growth of mixtures of the two bacterial species in a peptone medium at pH 7.2 and room temperature with 100 mg dm^{-3} As as arsenate: ○, aerated and illuminated; ●, aerated in the dark; □, non-aerated but illuminated; ■, non-aerated in the dark.

Arsenic compounds in the bacterial cells

A mixture of the two bacterial species was grown in the peptone medium in the presence of 1, 10, 100, or 1000 mg dm^{-3} As for 7, 10 and 40 days. The collected cells were dry-ashed with magnesium nitrate, and arsenic was determined in the mineralized sample. The results are summarized in Table 2. After 10 days of growth the arsenic concentration in the cells increased from 31 mg kg^{-1} (dry mass) to 137 mg kg^{-1} with a 100-fold increase (1–100 mg dm^{-3} As) of the arsenic concentration in the medium. At the 100 mg dm^{-3} As concentration in the medium, the cells

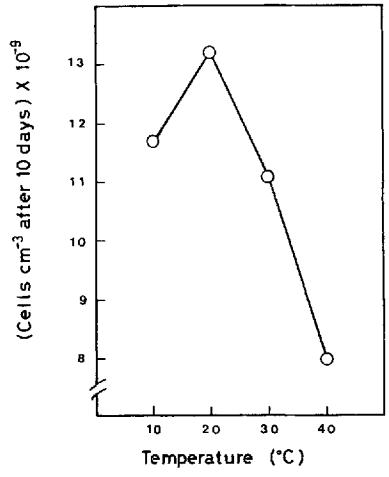

Figure 4 Effect of temperature on the growth of mixtures of the two bacterial species in a peptone medium with 100 mg dm^{-3} As at pH 7.2 shaken under illumination.

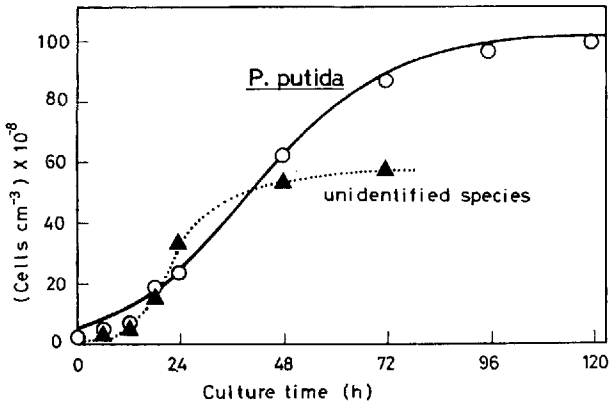

Figure 5 Growth curves for *P. putida* and the unidentified bacterial species in the aerated arsenic-containing peptone medium (10 mg dm^{-3} As as arsenate) in a jar fermentor at 30 °C.

Table 2 Effects of the arsenic concentration in the medium and of the culture time on the arsenic concentration in mixtures of cells of the two bacterial species

Arsenic as arsenate in medium (mg As dm^{-3})	Arsenic in dry cells (mg As kg^{-1})		
	7 days	10 days	40 days
1	ND[a]	31	ND
10	ND	33	ND
100	239	137	8.9
1000	ND	120	ND

[a] ND, Not determined.

appear to be saturated with arsenic, because an increase to 1000 mg dm^{-3} did not increase the arsenic concentration in the cells. Prolonged residence in the medium with 100 mg dm^{-3} As decreased the arsenic concentration in the cells from 239 mg kg^{-1} on day 7 to 8.9 mg kg^{-1} on day 40. In the stationary phase of growth the cells had excreted almost all of the arsenic. Silver and Nakahara[10] have studied the mechanism of plasmid-mediated resistance of bacteria to arsenic and concluded that resistance to arsenic in *Staphylococcus aureus* and *Escherichia coli* was caused by decreased accumulation and accelerated efflux of arsenate. The two bacterial species isolated appear to possess the efflux mechanism but not the possibility to limit accumulation of arsenic.

P. putida was cultured in the peptone medium (10 mg dm^{-3}). The cell population and the total arsenic concentration in the dry cells were determined at different times. The arsenic concentration peaked (67 mg kg^{-1}) at 48 h during the late

Table 3 Effects of the culture time on the arsenic concentration in *P. putida* when grown in a peptone medium containing 10 mg dm^{-3} As as arsenate

Culture time (h)	Arsenic content (cells cm^{-3}) × 10^{-8}	In dry cells (mg kg^{-1})
0	0.011	ND[a]
6	3.9	ND
12	6.1	11.2
18	18.2	18.8
24	23.3	22.8
48	62.0	67.0
72	86.5	46.0
96	96.0	45.0
120	99.7	ND

[a] ND, Not determined.

log phase of growth and decreased as the stationary phase was approached (Table 3). These data indicate that the bacterium accumulates arsenic during the phase of vigorous growth. The rate of arsenic excretion exceeds the rate of arsenic accumulation during the late log phase and during the stationary phase. Similar results were reported for five species of bacteria, in the cells of which most of the arsenic was associated with the protein fraction.[11] To identify the arsenic compounds formed by *P. putida* from arsenate, the bacterium was grown at 30 °C in the peptone medium containing 10 mg dm^{-3} As as arsenate for 24 h. The cells were harvested, dried and treated with 2 mol dm^{-3} sodium hydroxide solution at 90–95 °C for 3 h. The digests were treated with sodium borohydride and arsenic was determined with a hydride generation system.[2] Most of the arsenic in the cells was inorganic arsenic (22.1 mg kg^{-1}, 97%); the remainder (0.7 mg kg^{-1}, 3%) was in the trimethylated form. Mono- and dimethylated arsenic were not detected. *P. putida* was not as efficient in concentrating arsenic as the arsenic-tolerant *Chlorella vulgaris*,[3] *Nostoc* sp.[4] and *Phormidium* sp.[2] However, the ratio of methylated/inorganic arsenic is of the same order of magnitude in all of these species. In these algae, dimethylated arsenic was the predominant methylated arsenic species and trimethylated arsenic was a very minor species or could not be detected at all. The dimethylated arsenic species in the algae were further methylated higher up in the food chain.[12] Generally, dimethylated and trimethylated arsenic compounds are the predominant arsenic species in marine plants and animals. Freshwater organisms may also transform inorganic arsenic to dimethylated arsenic compounds and these could be converted to trimethylated species such as arsenobetaine by fish.

Marine and lake anaerobic bacteria could methylate inorganic arsenic to mono- and dimethylated arsenic but not trimethylated arsenic.[5,6] *P. putida*, in contrast to marine anaerobic bacteria, forms trimethylated arsenic compounds from inorganic arsenic. Mixed bacterial cultures obtained from estuaries and coastal sediments were observed to demethylate methylarsenic compounds.[7,8] The capability of demethylating arsenic compounds by *P. putida* remains to be investigated.

Excretion of arsenic compounds by *P. putida*

To identify the arsenic compounds excreted, *P. putida* was grown for two days at room temperature in an aerated peptone medium in the presence of 100 mg dm^{-3} As as arsenate. The cells were separated by centrifugation, and washed three times with sterilized water (deionized water) by centrifugation. The cells were suspended in 100 cm^3 sterilized water, the suspension was shaken for two days under illumination, and the cells were then separated by centrifugation. The supernatant was filtered through a 0.22 μm membrane filter. The filtrate was heated with 2 mol dm^{-3} sodium hydride solution at 90–95 °C for 3 h. The arsenic in the digest were determined by hydride generation.[2] The cells had excreted 144 ng of arsenic, of which 116 ng (80.8%) were in inorganic form, 5.3 ng (3.7%) monomethylated, 17.4 ng (12.1%) dimethylated, and 4.9 ng (3.4%) trimethylated. The excreted arsenic had a higher percentage (20%) of methylated compounds than the arsenic found in the cells (3%) and had an appreciable percentage of mono- and dimethylated arsenic compounds (16%), which were not detected in the cells.

The two species of arsenic-tolerant bacteria are capable of accumulating arsenic from a peptone medium with concentrations of arsenate as high as 1000 mg dm^{-3} of arsenic, transforming some of the inorganic arsenic to methylated arsenic compounds, and excreting inorganic and methylated arsenic. The growth rate of these bacteria is much higher than the growth rate of algae. Bacteria are easier to handle than algae. For these reasons, bacteria may be well suited for the biological purification of arsenic-contaminated systems.

REFERENCES

1. Meda, S, Kumamoto, T, Yonemoto, M, Nakajima, S and Takeshita, T *Sep. Sci. Technol.*, 1983, 18: 375
2. Maeda, S, Wada, H, Kumeda, K, Onoue, M, Ohki, A, Higashi, S and Takeshita, T *Appl. Organomet. Chem.*, 1987, 1: 465
3. Maeda, S, Nakashima, S, Takeshita, T and Higashi, S *Sep. Sci. Technol.*, 1985, 20: 153
4. Maeda, S, Kumeda, K, Maeda, M, Higashi, S and Takeshita, T *Appl. Organomet. Chem.*, 1987, 1: 363
5. Vidal, F V and Vidal, V M V *Mar. Biol.*, 1980, 60: 1
6. Wong, P T S, Chau, Y K, Luxon, L and Bengert, G A *Trace Subst. Environ.— Health*, 1977, 11: 100
7. Sanders, J G *Chemosphere*, 1979, 3: 135
8. Hanaoka, K, Yamamoto, H, Kawashima, K, Tagawa, S and Kaise, T *Appl. Organomet. Chem.*, 1988, 2: 371
9. Maeda, S, Fujita, S, Ohki, A, Yoshifuku, I, Higashi, S and Takeshita, T *Appl. Organomet. Chem.*, 1988, 2:353
10. Silver, S and Nakahara, H Bacterial resistance to arsenic compounds. In: *Arsenic–Industrial, Biomedical, Environmental Perspectives*, Lederer, W H and Fensterheim, R J (eds), Van Nostrand Reinhold Company, New York, 1983, pp 190–199
11. Shariatpanahi, M, Anderson, A C and Abdelghni, A A *Trace Subst. Environ. Health*, 1982, 16: 170
12. Maeda, S, Inoue, R, Kozono, T, Tokuda, T, Ohki, A and Takeshita, T *Chemosphere*, 1990, 20: 101