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Metabolism of methylated arsenic compounds by arsenic-resistant bacteria (*Klebsiella oxytoca* and *Xanthomonas* sp.)

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Two bacteria exhibiting resistance to toxic arsenic were isolated. These had been contaminated with arsenic in a *Chlorella* sp. culture medium containing arsenic. The two bacteria were identified as *Klebsiella oxytoca* and *Xanthomonas* sp., and grew well in a peptone medium at neutral pH at 30 °C, reaching the stationary phase in *ca* 100 h and 70 h, respectively. The growth of the bacteria was not affected by arsenic(V) concentrations in the medium as high as 1000 mg dm^{-3}. The bacteria bioaccumulated arsenic, a part of the arsenic being methylated. The bioaccumulation exhibited its peak around the turning point from the log phase to the stationary phase. The relative content of methylated arsenic in the excrement was greater than that in the bacterial cells. Adaptation treatment of inorganic arsenic caused an increase in the bioaccumulation of inorganic arsenic by *K. oxytoca*. Such a situation was not observed in the case of *Xanthomonas* sp. The bacteria also bioaccumulated methylated arsenic compounds, and demethylation of these species was observed. When the bacteria were killed by ethanol, arsenic was not taken up by the cells.

Keywords: Arsenic, resistance, bacteria, bioaccumulation, metabolism, methylated arsenics, metabolism, excretion

INTRODUCTION

Only a few studies have so far been carried out regarding the uptake and transformation of arsenic by bacteria. For example, uptake of arsenic by *Serratia marinorubra*, *Aeromonas* sp., *Escherichia* coli and *Flavobacterium* sp. has been reported; these bacteria transform the arsenic species accumulated in their cells to mono- and dimethylated forms.[1,2] However, to our knowledge, the transformation of arsenic to trimethylated species by bacteria has not yet been reported. This is often observed in arsenic uptake by freshwater and marine algae.[3]

In recent work we have reported the accumulation, methylation and excretion of arsenic compounds by freshwater algae which had been isolated from arsenic-polluted environments.[4–10] In the course of these studies we isolated some bacteria which exhibited a resistance to toxic arsenic. Regarding two species of these bacteria, we reported their growth characteristics and their accumulation of arsenic.[11]

In this work we take up another two arsenic-resistant bacteria, identified as *Klebsiella oxytoca* and *Xanthomonas* sp., and investigate in detail bioaccumulation of arsenic compounds, focusing especially on biomethylation of arsenic and the metabolism of methylated species. In other papers we reported a biochemical oxygen demand (BOD) sensor using an arsenic-resistant bacterium such as *K. oxytoca*.[12,13]

EXPERIMENTAL

Isolation of bacteria

An open culture of *Chlorella* sp. containing 100 mg dm^{-3} of arsenic (as elemental arsenic for Na_2HAsO_4) was contaminated with some bacteria. The contaminated algal suspension was centrifuged at 4000 rpm (3000*g*) for 20 min. The resultant supernatant containing bacteria was spread on a peptone agar medium containing 100 mg dm^{-3} of arsenic, the medium with bacteria being incubated overnight at 30 °C. Each bacterial colony was isolated from the agar medium, and regenerated five times on the agar medium of the above composition in order to purify the colony.

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Identification of bacteria

Two bacteria were isolated. They were identified in Takeda Analytical Research Laboratories Ltd (Osaka, Japan) as *Klebsiella oxytoca* and *Xanthomonas* sp.

Culture of bacteria

Aliquots of the culture of isolated bacteria were transferred to a peptone medium prepared by dissolving 10 g peptone, 5 g of sodium chloride and a certain amount of sodium arsenate in 1000 cm^3 sterilized water (pH 7.2). The inoculated medium (600 cm^3) kept in a 1-dm^3 commercial jar fermenter (MBF-100M, Tokyo Rikakikai, Tokyo) 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. After decanting the supernatant, the cells were washed three times with sterilized water by centrifugation. The resulting and remaining pellets were warmed at 60 °C to constant mass. Determinations of total arsenic and methylated arsenics in the bacterial cells were carried out in a similar manner to that described in our other work,[14,15] viz. by hydride generation–atomic absorption methods.

Adaptation to arsenic

The dilute bacterial suspension was spread on a peptone agar medium containing 100 mg dm^{-3} of arsenic in the forms of disodium arsenate (Na_2HAsO_4, DSA), monosodium methylarsonate [$CH_3As(O)(OH)(ONa)$, MSMA], dimethyl arsinic acid [$(CH_3)_2As(O)OH$, DMAA], and arsenobetaine [$(CH_3)_3AsCH_2COO$, AB], and incubated at 30 °C. A colony of the bacteria generated was isolated, resuspended in pure water, and the suspension was re-spread on the peptone agar medium containing 100 mg dm^{-3} of arsenic in the same forms. Control bacteria were incubated repeatedly on a peptone agar medium containing no arsenic.

Bioaccumulation of inorganic and methylated arsenic species and their biotransformations

K. oxytoca and *Xanthomonas* sp. were cultured for three days in four peptone liquid media containing 10 mg dm^{-3} of arsenic in the forms of DSA, MSMA, DMAA and AB, respectively. The bacterial cells were analyzed for total arsenic and methylated arsenic species accumulated by the methods described below.

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, 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 spectrophotometry.

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 spectrophotometer.[6]

RESULTS AND DISCUSSION

Bioaccumulation of arsenic

Figure 1 shows a growth curve of *K. oxytoca* and the bioaccumulation of arsenic. Figure 2 indicates similar plots for *Xanthomonas* sp. *K. oxytoca* and

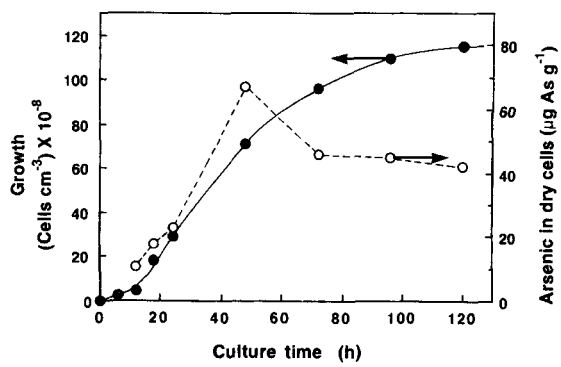

Figure 1 Growth curve of *K. oxytoca* and the bioaccumulation of arsenic from a peptone medium containing 10 mg dm^{-3} of arsenic.

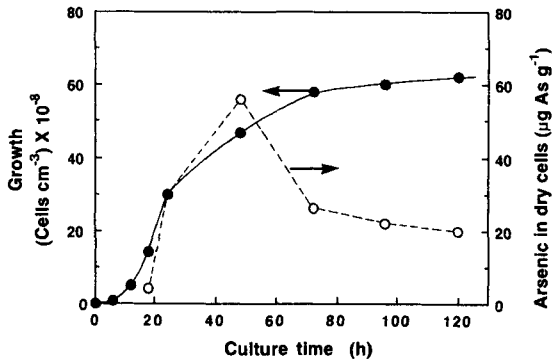

Figure 2 Growth curve of *Xanthomonas* sp. and the bioaccumulation of arsenic from a peptone medium containing 10 mg dm^{-3} of arsenic.

Xanthomonas sp. reached the stationary phases after *ca* 100 h and 70 h of growth, their growth at these phases being *ca* 120×10^8 cells cm^{-3} and *ca* 60×10^8 cells cm^{-3}, respectively.

Bioaccumulation of arsenic by *K. oxytoca* exhibited a peak around the turning point from the log phase to the stationary phase; *Xanthomonas* sp. showed similar behavior. These results suggest that the bacteria excrete the arsenic bioaccumulated in their cells, leading to a decrease in the bioaccumulation of arsenic. We have obtained similar results in bioaccumulation by *Chlorella* sp.[14]

Effect of the arsenic concentration in the medium

Table 1 summarizes the effect of the arsenic concentration in the peptone medium on the bioaccumulation of arsenic in *K. oxytoca*. The growth of the bacterium was not impaired by the arsenic at concentrations as high as 1 000 mg dm^{-3}, that of *Xanthomonas* sp. showing a similar result. In the case of three days' culture, the bioaccumulation of arsenic increased with increasing arsenic concentration in the medium. There was no distinct difference between bioaccumulation in the medium containing 100 mg dm^{-3} of arsenic and that for 1000 mg dm^{-3}. Achieving the stationary phase (after five days' culture), bioaccumulation of arsenic had then decreased to less than one-half of its initial value. This decrease in arsenic accumulation suggests excretion of arsenic by the bacteria.

Table 1 Effect of the arsenic concentration in the peptone medium on the bioaccumulation of arsenic in *K. oxytoca*

Arsenic concn in medium (mg dm^{-3})	Arsenic in dry cells (μg As g^{-1})			
	3 days	5 days	7 days	10 days
1	60	nd[a]	nd	31
10	87	42	39	33
100	510	nd	239	137
1000	530	nd	nd	120

[a] nd, Not determined.

Biomethylation and excretion of arsenic by *K. oxytoca*

Table 2 shows biomethylation of arsenic and its excetion into pure water by *K. oxytoca*. Arsenic bioaccumulated in the bacterial cells was methylated to some extent to trimethylated arsenic (TMA), and the excrement contained monomethylated arsenic (MMA), dimethylated arsenic (DMA) and TMA. The relative content of methylated arsenic in the excrement was greater than that in the bacterial cells; this situation is very similar to the bioaccumulation of arsenic by *Chlorella* sp.[15] It is proved that the methylated arsenic is excreted in greater quantity than the initial form.

Table 2 Biomethylation and excretion of arsenic by *K. oxytoca*[a]

	Arsenic species[b]				
	Total	IA	MMA	DMA	TMA
Arsenic in dry cells (μg As g^{-1})	347 [173 μg]	336 (96.9)[c]	nd[d]	nd	10.6 (3.1)
Excrement (μg)	144	116 (80.8)	5.3 (3.7)	17.4 (12.1)	4.9 (3.4)

[a] *K. oxytoca* was cultured in the peptone medium containing 90 mg dm^{-3} of arsenic for two days. The arsenic species in the bacterial cells were determined. The cells (0.498 g dry wt) were suspended in pure water (100 cm^{-3}) and shaken at 30 °C for two days. The arsenic species in the excrement were determined.

[b] IA, inorganic arsenic; MMA, monomethylated arsenic; DMA, dimethylated arsenic; TMA, trimethylated arsenic.

[c] Numbers in parentheses are percentages for arsenic compounds relative to total arsenic.

[d] nd, Not detected.

Table 3 Bioaccumulation of various arsenic compounds and their biotransformation by *K. oxytoca*

		Arsenic in dry cells (μg As g^{-1})				
		Total As	IA	MMA	DMA	TMA
IA exposure	Control	33.4	33.2 (99.4)[a]	tr[b]	0.1 (0.3)	0.1 (0.3)
	IA adaptation	80.6	80.2 (99.5)	tr	0.3 (0.4)	0.1 (0.1)
MMA exposure	Control	51.4	nd[c]	51.0 (99.2)	0.1 (0.2)	0.1 (0.2)
	IA adaptation	25.4	nd	25.0 (98.4)	tr	tr
	MMA adaptation	25.0	nd	23.0 (92.0)	tr	tr
DMA exposure	Control	3.1	nd	0.3 (10)	2.6 (84)	tr
	IA adaptation	2.4	nd	0.4 (17)	1.9 (79)	tr
	DMA adaptation	1.3	nd	0.3 (23)	0.8 (62)	tr
TMA exposure	Control	149	nd	nd	13.0 (8.7)	136 (91.3)
	IA adaptation	58.9	nd	nd	3.5 (5.9)	56.3 (94.1)
	TMA adaptation	82.6	nd	nd	9.2 (11.1)	73.4 (88.9)

[a] Numbers in parentheses are percentages for arsenic compounds relative to total arsenic. [b] tr, Detected but below detection limits. [c] nd, Not detected.

Bioaccumulation of inorganic and methylated arsenics and their biotransformation

Table 3 summarizes the bioaccumulation of inorganic and methylated arsenic compounds and their biotransformation by *K. oxytoca*. The bacterium was cultured in the peptone medium containing inorganic arsenic (IA), MMA, DMA or TMA.

When the control bacterium which had not been adapted was exposed to IA, it bioaccumulated 33.4 μg g^{-1} of arsenic. When adaptation to IA was performed, bioaccumulation of arsenic increased from 33.4 to 80.6 μg^{-1} under the same exposure conditions. Thus, adaptation to IA was found to promote its uptake into the bacterial cells. Resistance to arsenic in *K. oxytoca* is thought to arise via the detoxification of arsenic accumulated in the cells. The detoxification of arsenic is assumed to occur via biomethylation of arsenic or through a masking of arsenic by proteins, etc. However, evidence of the lesser toxicity of methylated arsenic compounds for bacteria and masking behavior has not been obtained. It is considered that the ability to detoxify arsenic is enhanced due to adaptation for arsenic, leading to an increase in the uptake of arsenic. A similar situation was also observed in the bioaccumulation of arsenic by *Chlorella* sp.[5] The biomethylation of arsenic by *K. oxytoca*, however, was not so high as that by *Chlorella* sp.[15]

On exposure to methylated arsenic species (MMA, DMA and TMA), the adaptation treatments with inorganic and the corresponding methylated arsenic species inversely reduced uptakes of arsenic to about one-half of their control values. Bioaccumulation of arsenic was the largest under TMA exposure and the least under DMA exposure. When the bacteria were exposed to DMA and TMA, demethylation of these arsenic species took place in the bacterial cells. However, no final demethylation to inorganic arsenic was observed.

Similar experiments were carried out using *Xanthomonas* sp. (Table 4). The bacterium was cultured in peptone medium containing IA, MMA, DMA or TMA. In contrast to the case of

Table 4 Bioaccumulation of various arsenic compounds and their biotransformation by *Xanthomonas* sp.

		Arsenic in dry cells (μg As g^{-1})				
		Total As	IA	MMA	DMA	TMA
IA exposure	Control	71.9	71.6 (99.6)[a]	tr[b]	tr	0.3 (0.4)
	IA adaptation	58.7	58.3 (99.5)	tr	0.2 (0.3)	0.2 (0.3)
MMA exposure	Control	1.0	nd[c]	0.8 (80)	tr	0.1 (10)
	IA adaptation	2.0	nd	1.0 (50)	tr	0.8 (40)
	MMA adaptation	1.0	nd	0.7 (70)	tr	0.1 (10)
DMA exposure	Control	2.3	nd	nd	1.3 (56)	0.8 (35)
	IA adaptatin	1.8	nd	tr	1.3 (72)	0.3 (17)
	DMA adaptation	1.7	nd	tr	1.1 (65)	0.4 (24)
TMA exposure	Control	2150	nd	0.5 (0.02)	39.5 (1.84)	2110 (98.1)
	IA adaptation	57.2	nd	0.2 (0.3)	2.6 (4.5)	54.4 (95.1)
	TMA adaptation	2010	nd	0.3 (0.01)	9.7 (0.48)	2000 (99.5)

[a] Numbers in parentheses are percentages for arsenic compounds relative to total arsenic. [b] tr, Detected but below detection limits. [c] nd, Not detected.

K. oxytoca, when IA exposure was carried out there was no significant difference between bioaccumulation of arsenic for the IA adaptation and for the control culture. This is because the mechanism of resistance in *Xanthomonas* sp. is quite different from that in *K. oxytoca*. It is noted that when *Xanthomonas* sp. was exposed to TMA, uptake of TMA was very remarkable; more than 2000 μg g^{-1} of arsenic was bioaccumulated. However, the IA adaptation in this case led to an extreme reduction in the bioaccumulation of arsenic. In both the MMA and DMA exposures, uptakes of the arsenic species by *Xanthomonas* sp. were at low levels, similar to that of DMA by *K. oxytoca* in the DMA exposure.

Uptake of arsenic by ethanol-killed bacterial cells

K. oxytoca, with or without adaptation to arsenic, was killed by 70% ethanol; it was then transferred to the peptone medium containing IA or TMA, and was shaken for three days. In these cases neither inorganic nor organic arsenic species were observed in the cells. The bacterium proved not to take up arsenic *in vitro*. With *Xanthomonas* sp. a similar result was obtained.

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