APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. 2001; **15**: 548–552
DOI: 10.1002/aoc.193

Enteric bacteria may play a role in mammalian arsenic metabolism

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The cecal content of rats administered dimethylarsinic acid for 6 months via drinking water was cultured in GAM medium with 10 mg l^{-1} of dimethylarsinic acid. Arsenic compounds in the culture were analyzed by ion chromatography with inductively coupled plasma mass spectrometry (IC–ICP-MS). Dimethylarsinic acid was metabolized. Two bacterial *Escherichia coli* strains, A3-4 and A3-6, were isolated from the culture. These strains metabolized dimethylarsinic acid and yielded two unidentified arsenic compounds, M-2 and M-3. A3-6 methylated dimethylarsinic acid to trimethylarsine oxide. Both strains metabolized trimethylarsine oxide and yielded an unidentified arsenic compound, M-1. These unknown arsenic compounds were the same compounds as detected in the urine and the feces of rats administered dimethylarsinic acid. The strains reduced arsenate to arsenite efficiently. Cysteine was required for metabolism of dimethylarsinic acid by these bacteria, but glutathione was not required. These results strongly suggested that the intestinal bacteria have a different arsenic metabolism from that in mammals and that they may play a possible role in mammalian arsenic metabolism. Copyright © 2001 John Wiley & Sons, Ltd.

Keywords: dimethylarsinic acid; trimethylarsine oxide; microorganisms; rat; cecum; metabolism; unidentified arsenic metabolite; IC–ICP-MS

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Contract/grant sponsor: Japanese Ministry of Education, Science and Culture; Contract/grant number: 11670383.

Received 1 December 2000; accepted 24 February 2001

INTRODUCTION

Epidemiological studies have indicated that arsenic is carcinogenic.[1] However, the mechanism of induction of carcinogenesis by arsenic and the chemical form of the ultimate carcinogen is still unclear. The metabolism of arsenic in mammals is well known. When inorganic arsenic is introduced into the mammalian body, it is reduced to trivalent arsenic, and is methylated to monomethylarsonic acid (MMA), dimethylarsinic acid (DMA) or trimethylarsine oxide (TMAO).[2,3] Since DMA is the principal metabolite of inorganic arsenic in rat[4] and is supposed to be a carcinogen or a promoter,[5,6] it is important to study the metabolism of DMA.

Some researchers have suggested the possibility of contribution of intestinal bacteria to the metabolism of arsenic in mammals. Intestinal bacteria of experimental animals are able to methylate inorganic and organic arsenic.[7–9] On the other hand, it appears that bacterial metabolism of arsenic differs from mammalian metabolism. Cullen *et al.*[10] reported that intestinal bacteria in ceca of rats demethylate a methylated arsenic compound. Yoshida *et al.*[11,12] detected three unknown arsenic compounds in the urine and feces of rats administered DMA, TMAO or MMA. They suggested that these unknown arsenic compounds might be produced by intestinal bacteria, since elimination of the unknown compounds is greater in rats administered arsenics orally than in those administered it intraperitoneally. It is possible that these compounds are produced by intestinal bacteria and are related to the toxic effects of DMA in mammals.

We examined the ability of culture of rat cecal

content to yield unknown arsenic compounds from DMA, and attempted to isolate the bacteria metabolizing DMA.

MATERIALS AND METHODS

Reagents and culture medium

Sodium arsenite, sodium arsenate, MMA, DMA TMAO, tetramethylarsonium iodide, arsenocholine and arsenobetaine, used for analytical standard solutions with purities of at least 99.99%, were obtained from Tri Chemical Lab. (Yamanashi, Japan). DMA for administration to rats and supplement of bacterial culture, with purity of at least 99.9%, was obtained from Wako Pure Chemical Industry (Osaka, Japan). Cysteine was also obtained from Wako Pure Chemical Industry. GAM medium and nutrient broth were purchased from Nissui Co. (Tokyo, Japan).

Instrumentation and inductively coupled plasma mass spectrometry (ICP-MS) analysis

Details are described elsewhere.[11,12] A model HP4500 ICP-MS (Hewlett-Packard, DE, USA) was used for arsenic-specific detection. The operating conditions for ICP-MS were established in accordance with those reported by Inoue *et al.*[13] ICP-MS was performed using a model IC7000 from Yokogawa Analytical Systems (Tokyo, Japan). For separation of arsenic compounds, two modes, cation- and anion-exchange, were used. In the cation-mode experiments, a Showdex NN-614 column (150 mm × 4.6 mm i.d.) was used. In the anion mode experiments, two Excelpack ICS-A35 columns (150 mm × 4.6 mm i.d.) were used.

Cultivation of rat cecal content and isolation of bacteria

The ceca were removed aseptically from four rats administered DMA via drinking water at a concentration of 100 mg l^{-1} for 9 months, and from two non-treated rats, and the cecal content was cultured in 50 ml of GAM medium supplemented with 10 mg l^{-1} of DMA under anaerobic condition at 37 °C. Arsenic compounds in the culture fluid were analyzed by ion chromatography (IC) with ICP-MS. The 6 day culture was plated on GAM–agar medium and incubated at 37 °C for 2 days under anaerobic conditions. Colonies on the plate were isolated and cultured in a tube containing 5 ml of GAM medium and 10 μg ml^{-1} of DMA for 2 days in aerobic conditions. Arsenic compounds in the culture were analyzed.

Detection of arsenic compounds in the isolated bacterial culture

The bacteria were cultured in GAM medium with 10 mg l^{-1} of an arsenic compound for 18 h at 37 °C. The culture was analyzed for arsenic compounds by IC–ICP-MS after the treatment described below.

Aliquots of the culture were centrifuged at 3000 rpm for 20 min. The supernatant was ultrafiltered by the ultrafree-MC (Millipore, MA, USA) to remove substances above 10 000 Da in molecular weight. The precipitate was resuspended in distilled water and was ultrasonicated for 5 min with Bio-ruptor (Cosmo Bio Co. Japan), then was centrifuged at 10 000 rpm for 30 min. The supernatant was also ultrafiltered.

RESULTS

Arsenic compounds in the cultures of rat cecal contents were analyzed by IC–ICP-MS using the anion columns. The cultures of both DMA-treated and non-treated rats metabolized DMA and yielded an unknown arsenic compound, M-2 (Fig. 1). M-2 was detected in the 0 day cultures that had not yet been incubated. It appeared that a portion of DMA had been metabolized, since the cultures had been left at room temperature for several hours until a portion of them was frozen for storage. The yields of M-2 in the DMA-treated rat cecal cultures were significantly higher than those for non-treated rats in the 1 day culture. In the 6 day culture, the average yield of M-2 did not differ markedly among the DMA administered rats and the non-treated rats. However, the range of M-2 concentration in non-treated rat cecal cultures was much wider than in DMA-administered cecal culture, with a maximum of 3.19 mg l^{-1} and minimum of 0.57 mg l^{-1}.

Two bacterial strains, A3-4 and A3-6, which metabolized DMA to M-2, were isolated from the 6 day cultures of the DMA-administered rat cecal content. They were Gram-staining negative rods and were identified as *Escherichia coli* at a probability of 99.99% by Minitek Cord card (BBL). Arsenic compounds in the culture of fluid

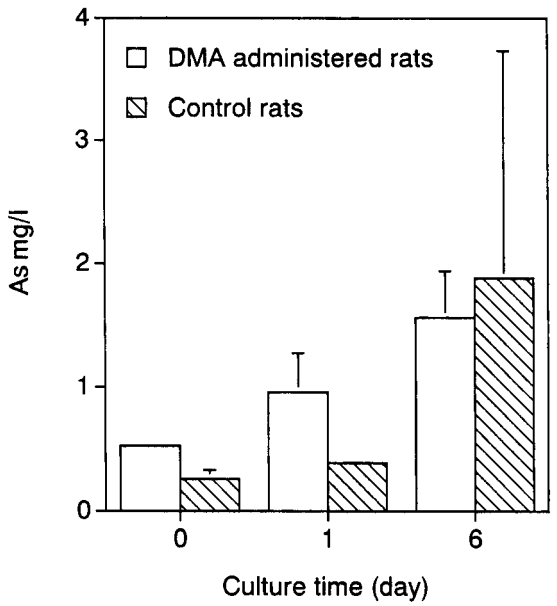

Figure 1 An unidentified arsenic compound, M-2, in rat cecal content culture with DMA. Concentration was the average for four rats administered DMA or of two control rats. Error bar is standard deviation.

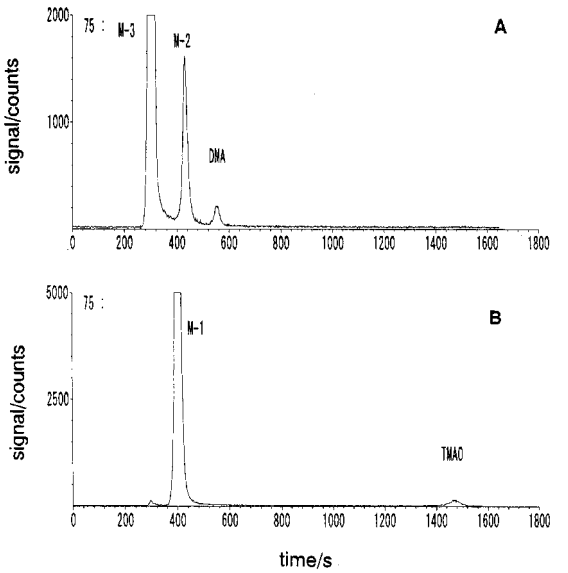

Figure 2 Cation-exchange IC–ICP-MS chromatograms of the metabolites. Column: NN-614 (150 mm × 4.6 mm i.d.); mobile phase: 5 mM HNO_3–6 mM NH_4NO_3; flow rate: 0.8 ml min^{-1}. (A) Culture supernatant of A3-4 with 10 mg l^{-1} of DMA for 18 h at 37 °C. (B) Culture supernatant of A3-4 with 10 mg l^{-1} of TMAO for 18 h at 37 °C.

of A3-4 and A3-6 were analyzed with IC–ICP-MS using a cation column. They metabolized more than 90% of DMA and yielded two unknown arsenic compounds, M-2 and M-3 (Table 1). The IC–ICP-MS chromatograms of arsenic species, obtained using a cation-exchange column, are presented in Fig. 2A. In the GAM medium without bacteria, these compounds were not detected. These compounds were also detected in the bacterial extracts, although the concentrations of the arsenic compounds in the bacterial cells were less than those in the culture fluid by about one-tenth. TMAO was detected in only A3-6 cell extracts.

Metabolites of other arsenic compounds obtained with A3-4 and A3-6 were examined. As shown in Table 2, about 50% of arsenite was oxidized to arsenate in GAM medium alone. However more than 75% of arsenite was detected in the culture fluids. Oxidization was inhibited, or the oxidized arsenic was reduced again by the bacteria. Arsenate was not changed in GAM medium. About 90% of arsenate was reduced to arsenite by the bacteria. No other arsenic compounds were detected in the culture fluid (Table 2).

When MMA was challenged to these bacteria, metabolites were not detected except for a small amount of an unknown arsenic compound (Table 3). TMAO was greatly metabolized to an unknown arsenic compound, M-1 (Table 3). The chromato-

Table 1 Arsenic compounds detected in bacterial culture with DMA

	Arsenic compound (%)				
Strain	DMA	TMAO	M-2	M-3	Total As (mg l^{-1})
GAM medium	99.7	0.0	0.0	0.0	8.03
A3-4 culture fluid	6.5	0.0	49.3	44.1	8.36
A3-4 cell extract	5.7	0.0	47.6	46.7	0.91
A3-6 culture fluid	2.8	0.0	67.3	29.9	7.61
A3-6 cell extract	10.4	37.4	37.0	13.8	0.93

Table 2 Arsenic compounds detected in bacterial culture with arsenite (As(III)) or arsenate (As(V))

Challenge compound	Culture fluid	Arsenic compound (%)		Total As (mg l^{-1})
		As(III)	As(V)	
As(III)	GAM medium	49.0	51.0	7.11
	A3-4	78.3	17.5	6.44
	A3-6	75.5	20.6	6.55
As(V)	GAM medium	0.0	100.0	4.91
	A3-4	91.4	6.3	4.29
	A3-6	87.1	11.7	4.60

gram of the IC–ICP-MS of the metabolite is presented in Fig. 2B.

Although the bacteria grew more in nutrient broth supplemented with 0.5% of yeast extract than in GAM medium under aerobic conditions, they produced very little M-2 or M-3 from DMA. GAM medium includes various components, such as amino acids, vitamin K and thioglycolate, in order to culture anaerobic bacteria. The effect of these components on microbial metabolism of DMA was investigated using nutrient broth. Addition of cysteine, which is contained in GAM medium at 300 mg l^{-1}, to nutrient broth had the same effect on metabolism of DMA as GAM medium (Fig. 3). No arsenic compounds other than DMA were detected when cysteine and DMA were incubated in nutrient broth without the bacteria. The yield of M-2 was highest at 0.25 mg ml^{-1} of cysteine, whereas that of M-3 increased in proportion to cysteine concentration up to 0.75 mg ml^{-1}. Glutathione exhibited no effect on DMA metabolism by the bacteria at concentrations less than 5 mM.

DISCUSSION

DMA and M-2 were detected in the culture of cecal content (Fig. 1). It appeared that M-3 might have been detected if a cation column had been used, since M-3 was tightly adsorbed to the anion column and had a very long retention time. The concentration of M-2 in cultures from DMA-treated rats was significantly higher than that in cultures for non-treated rats (Fig. 1). This finding suggested that DMA-metabolizing bacteria increased in number when DMA was administered long term.

In this study, three unknown arsenic compounds were produced from DMA or TMAO by the isolated bacteria (Tables 1 and 3). These compounds were different from the known arsenic compounds, arsenite, arsenate, MMA, DMA, TMAO, tetramethylarsonium ion, arsenocholine and arsenobetaine. M-1 and M-2 were the same compounds as those previously detected in the urine of rats administered 100 mg ml^{-1} of DMA or TMAO via drinking water for 7 months.[11] The concentrations of M-2 and M-3 in the urine were significantly high, being 4.1% and 10.3% of the total arsenic respectively when administered with DMA. M-3 was also detected in the feces of rats administered DMA for 20 weeks.[12] These results strongly suggest that a portion of arsenic introduced into the mammalian body via drinking water is metabolized by intestinal bacteria through a pathway different from that in mammals.

Table 3 Arsenic compounds detected in bacterial culture with MMA or TMAO

Challenge compound	Culture fluid	Arsenic compound (%)			Total As (mg l^{-1})
		MMA	TMAO	M-1	
MMA	GAM medium	98.8	0.0	0.0	5.93
	A3-4	95.1	0.0	0.0	6.22
	A3-6	92.1	0.0	0.0	6.59
TMAO	GAM medium	0.0	99.1	0.0	5.95
	A3-4	0.0	1.2	98.3	5.30
	A3-6	0.0	1.3	98.4	5.61

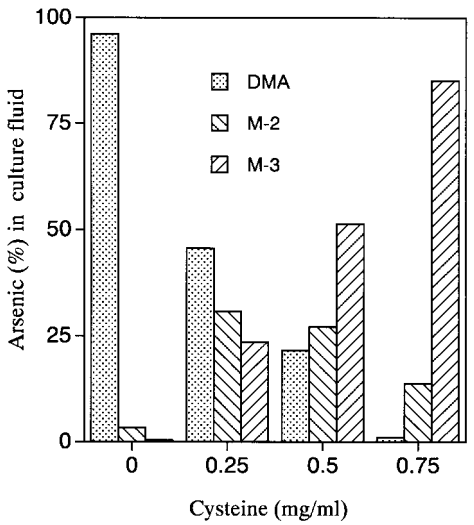

Figure 3 Effect of cysteine on DMA metabolizing ability of *E. coli* strain A3-6. The bacteria were cultured for 16 h in nutrient broth supplemented with 0.5% of yeast extract, 10 mg l^{-1} of DMA and various concentrations of cysteine.

The isolated bacteria reduced arsenate to arsenite efficiently (Table 2). The reduction might be partly due to a lowered redox-potential of the culture medium following growth of the isolated bacteria. Rowland and Davis[8] observed that arsenate was reduced to arsenite by the content of rat cecum. It is possible that bacteria similar to the isolated bacteria contributed to the results of their experiments. If the isolated bacteria also exist in the human intestine, the result suggests that individuals who ingest arsenate via drinking water for a long period will develop serious arsenicalism, since arsenite is fivefold more toxic than arsenate[14] and the isolated bacteria increase with long-term exposure. Most bacteria that are resistant to arsenic have a plasmid that reduces arsenate to arsenite in the cells to release arsenic from them.[15] The isolated bacteria may have the arsenic-resistant plasmid.

The isolated bacteria required cysteine for metabolizing DMA. However, the optimum concentrations of cysteine for M-2 and M-3 differed (Fig. 2). According to a proposed theory of arsenic metabolism in organisms,[3] a pentavalent arsenic compound must be reduced to a trivalent arsenic before it is metabolized. Cysteine is an effective reagent for reduction of DMA.[16] The above results suggest that cysteine is required for bacteria to reduce DMA. If M-2 and M-3 were produced from DMA directly, their optimum concentrations of cysteine would be the same. It appeared that these compounds participate in the same pathway and that one of them was a precursor of the other. Glutathione, a thiol compound that contributes to arsenic metabolism in mammals,[17] was not effective for metabolizing DMA by the bacteria. These results suggest that the microbial metabolism of arsenics is different from that in mammals.

Our study suggests that arsenic metabolism in mammals is partly affected by intestinal microbial flora, and that intestinal microbial metabolism of arsenic is affected by food that supplies cysteine for reduction of arsenic.

Acknowledgement This study was partly supported by a grant from the Japanese Ministry of Education, Science and Culture, no. 11670383.

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