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Demethylation of methylarsenic species by *Mycobacterium neoaurum*

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Mycobacterium neoaurum **demethylates both methylarsonic acid and methylarsonous acid to mixtures of arsenate and arsenite. After 28 days of incubation, the yields of inorganic arsenic were 27% from arsenate and 43% from arsenite. A time study of the demethylation of methylarsonic acid by *M. neoaurum* showed that demethylation occurs rapidly during the growth and stationary phases of the bacterium, and indicates that MMA(V) is reductively demethylated to arsenite. Copyright © 2003 John Wiley & Sons, Ltd.**

KEYWORDS: arsenic; demethylation; methylarsonic acid; methylarsonous acid; *Mycobacterium neoaurum*; reduction

INTRODUCTION

Although the biological methylation of arsenic has been studied extensively and is reasonably well understood,[1] few accounts of the biological cleavage of arsenic–carbon bonds have been published. Yoshida *et al*.[2] reported the presence of small amounts of dealkylated metabolites, trimethylarsine oxide (TMAO), dimethylarsinic acid (DMA(V)), methylarsonic acid (MMA(V)) and inorganic arsenic, in urine following oral administration of arsenobetaine to rats. Yoshida *et al*.[3] also found slight demethylation of DMA(V) to arsenite following oral and intraperitoneal administration to rats, and showed evidence that intestinal microflora contribute to demethylation. This is supported by the demethylation of methylarsine oxide to arsenate by mouse cecum homogenates.[4]

Several studies have examined the degradation of the organic arsenical herbicides cacodylic acid and sodium methanearsonate, and have found that these arsenicals can be demethylated to arsenate by soil microorganisms.[5,6] The demethylation of the tetramethylarsonium ion, TMAO, DMA(V) and sodium methanearsonate by sediment microorganisms has also been reported.[7,8]

In terms of isolated individual species of microorganisms, Challenger *et al*. found that *Penicillium notatum* converts $ClCH_2CH_2AsO(OH)_2$ to trimethylarsine.[1] It is probable that this conversion occurs via cleavage of the arsenic–carbon bond of the $ClCH_2CH_2-$ group and methylation. Demethylation of mono- and di-methylarsenic compounds in soils, and by isolates from soils such as *Alcaligenes* and *Pseudomonas*, has been reported.[9–12] Wine yeast demethylates dimethylarsinate to methylarsonate.[13] Cullen *et al*.[14] found that homogenates of *Candida humicola* incubated with *S*-adenosylmethionine and NADPH demethylated [^{14}C]-dimethylarsinate to [^{14}C]-methylarsonate.[14] Quinn and McMullan[15] isolated a bacterium from activated sludge which dealkylated arsonoacetate and used arsonoacetate as the sole carbon and energy source. The mechanism(s) of demethylation of methylated arsenicals by microorganisms is not known.

Here, we report the demethylation of methylated arsenicals by *Mycobacterium neoaurum* and the interaction of *M. neoaurum* with inorganic arsenicals.

MATERIALS AND METHODS

Materials

$Na_2HAsO_4 \cdot 7H_2O$ was purchased from Sigma, As_2O_3 from Fisher Scientific, $CH_3AsO(OH)_2$ from Pfalz and Bauer and $(CH_3)_2AsO(OH)$ from Aldrich. CH_3AsI_2 and $(CH_3)_3AsO$ were synthesized according to literature methods.[16,17] These arsenicals were used to prepare 1000 ppm stock solutions of arsenate (As(V)), arsenite (As(III)), MMA(V), methylarsonous acid [MMA(III)], DMA(V) and TMAO. The stock solutions were diluted to a concentration of 1 ppm as arsenic for standards for the analytical procedure involving standard additions.

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All other chemicals used were of at least reagent grade and were obtained from commercial sources. Sabouraud broth (Difco) was autoclaved for 20 min at 121 °C and 1.38 bar. Distilled deionized water was used to prepare all solutions.

M. neoaurum culture

M. neoaurum[18] (Culture Collection #364, Department of Chemistry, University of British Columbia) seed cultures were maintained in a 1 l Erlenmeyer flask with 500 ml of Sabouraud broth. *M. neoaurum* grew well in this medium, and the medium is suitable for analytical monitoring. The flasks were shaken horizontally on a rotary shaker (4.45 cm displacement, 135 rpm) and the temperature was 21 °C.

Incubation of *M. neoaurum* with inorganic and methylated arsenic species

M. neoaurum was incubated with As(III), As(V), MMA(III), MMA(V), DMA(V) and TMAO. Three 125 ml Erlenmeyer flasks were prepared for each arsenical. 45 ml of Sabouraud broth was added to each flask, the flasks were capped with foam plugs and were autoclaved. After cooling to room temperature, filter-sterilized (0.22 μm syringe filters, Pall Acrodisc) arsenical stock solution was added such that the final concentration of the arsenical in the media was 500 ppb as arsenic. The flasks were inoculated with 5.0 ml of the *M. neoaurum* seed culture. Two killed-cell controls were prepared for each arsenic species in the same way as for the samples, except that the seed cultures were added to the flasks prior to autoclaving.

All cultures were incubated on the rotary shaker for 28 days. At the end of the experiment, *M. neoaurum* was separated from the media by centrifugation for 20 min (2410*g*). The media were weighed; the biota were freeze-dried and then weighed. The inorganic arsenic species were extracted from the freeze-dried biota with 1 : 1 methanol/water, as described previously.[18] The extracts and media were frozen for later analysis by hydride generation gas chromatography–atomic absorption spectrometry (HG-GC–AAS).

Time study of MMA(V) demethylation

Six flasks were prepared, as described above, which contained 5.0 ml of the *M. neoaurum* seed culture and MMA(V) (500 ppb as arsenic). The flasks were incubated at 21 °C on the rotary shaker. At 0, 5, 10, 20, 30 and 45 days, one flask of *M. neoaurum* was removed from the shaker and stored in a freezer at −20 °C. At the end of the experiment, the flasks were thawed, and the media and biota were separated by centrifugation. The media were weighed; the biota were freeze-dried and then weighed. The media were frozen for later analysis by HG-GC–AAS.

Analysis

The media and extracts were analysed directly using HG-GC–AAS for the concentrations of inorganic arsenic (As(III) and As(V)). Aliquots of the media and extracts were also passed through a strong anion-exchange column to remove As(V) and these aliquots were then analysed using HG-GC–AAS in order to determine the concentration of As(III). The concentration of As(V) was determined by the difference between the total inorganic and As(III) concentrations.

HG-GC–AAS analysis

The samples were analysed by using semi-continuous flow HG-GC–AAS with 2% $NaBH_4$, and 1 M hydrochloric acid was used to adjust the pH.[19] The arsenicals were identified by their retention times and were quantified by the method of standard additions.

As(III)/As(V) speciation

The samples were passed through strong anion-exchange cartridges (Supelclean LC-SAX SPE tubes, 3 ml, Supelco) to retain the As(V). A new cartridge was used for each sample. The cartridges were conditioned by washing with 2 ml of methanol followed by 2 ml of H_2O. The solvents were eluted at 2 ml min^{-1} using positive pressure (syringe piston).

The sample (2.50 ml) was passed through the conditioned cartridge at 2 ml min^{-1}. The column was eluted with 2.50 ml of H_2O at 2 ml min^{-1}, which was collected with the sample. The combined eluates were weighed in order to calculate the dilution factor. The concentration of As(III) in the eluted sample was determined by HG-GC–AAS. Duplicate mixtures of 5.0 ng of As(III) and 5.0 ng of As(V) in 2.50 ml Sabouraud broth, and duplicate samples of 10.0 ng of As(III) in 2.50 ml of Sabouraud broth were washed through cartridges to verify cartridge performance.

RESULTS AND DISCUSSION

When mixtures of 5.0 ng of As(III) and 5.0 ng of As(V) in 2.50 ml Sabouraud broth were passed through strong anion-exchange cartridges and the eluate and rinsings were collected and diluted to 5.00 ml, 53% (SD = 1%) of the inorganic arsenic from the mixture was detected. When 10.0 ng of As(III) in Sabouraud broth was passed through this type of cartridge and the eluate and rinsings were collected and diluted to 10.00 ml, 108% (SD = 3%) of the inorganic arsenic was detected. These results indicate that this strong anion-exchange cartridge can be used to remove As(V) from a mixture of As(III) and As(V) in media, and that this method provides a means of determining the As(III)/As(V) speciation in the media and biota extracts from *M. neoaurum* incubations.

M. neoaurum demethylated MMA(V) and MMA(III) to mixtures of As(III) and As(V). The percentage conversions of the starting methylarsenic substrates to inorganic arsenic products in the media and in the biota, after 28 days of incubation, were determined by HG-GC–AAS of the media and of the biota extracts. These are listed in Table 1. A chromatogram produced by HG-GC–AAS analysis of the media, after 28 days of incubation of the bacterium with MMA(V), is given in Fig. 1. No demethylation or methylation of the arsenic species occurred in any of the killed-cell control

Table 1. Percentage conversion of methylarsenic species to inorganic arsenic by *M. neoaurum* after 28 days of incubation

Substrate		Media[a]	Biota extracts[a]
MMA(V)	As(III)	8(3)	0.9(0.3)
	As(V)	17(3)	0.55(0.04)
MMA(III)	As(III)	31(3)	1.2(0.4)
	As(V)	9(3)	1.3(0.4)

[a] SD (in parentheses), of three replicates.

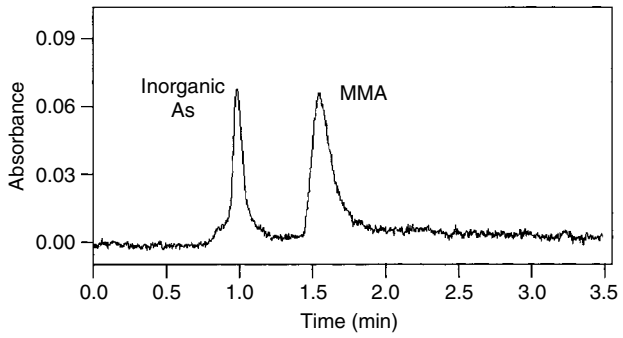

Figure 1. HG-GC–AAS analysis of 0.15 ml aliquot of media after incubation of *M. neoaurum* with MMA(V) in Sabouraud broth for 28 days.

flasks. *M. neoaurum* did not demethylate DMA(V) or TMAO; DMA(V) was not methylated. There were no differences in the growth of *M. neoaurum* with the various arsenicals.

In order to determine the relationship between the demethylation of methylarsenicals and the growth of *M. neoaurum*, the growth of liquid-media batch cultures was monitored through a demethylation experiment. The dry weights of the bacterial cells isolated throughout the experiment are plotted against the ages of the cultures in Fig. 2. The lag phase of the growth curve is very short. Although growth of this species of *Mycobacterium* is typically rapid,[20] the stationary phase is not reached until after 10 days, so other types of media may be more appropriate for the growth of this bacterium. The stationary phase lasted 10 days and was followed by the death phase.

The yield of total inorganic arsenic, As(V) and As(III), from the demethylation of MMA(V) by *M. neoaurum* was also measured against time. This was determined using HG-GC–AAS analysis of aliquots of the media collected after 0, 5, 10, 20, 30 and 45 days. These results are illustrated in Fig. 2. There is little demethylation during the initial lag phase. The yield of inorganic arsenic increases rapidly during the growth and stationary phases. Demethylation continues during the death phase, but at a much slower rate.

The results given in Fig. 2 show the yield of As(III) as a function of time. The yield increases rapidly during the growth stage, reaches a maximum just after the stationary phase and then declines rapidly. The As(V) concentration,

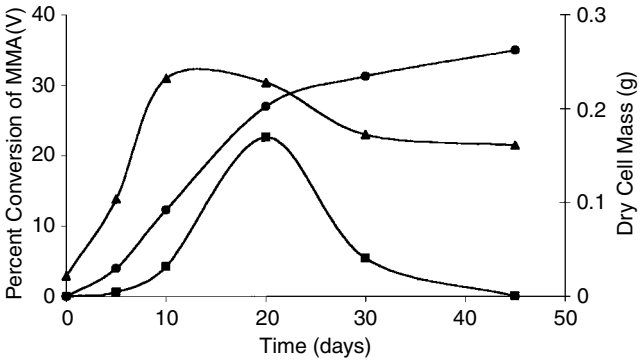

Figure 2. Incubation of *M. neoaurum* in submerged culture with 500 ppb (as arsenic) of MMA(V): ● growth curve; ▲ Demethylation of MMA(V) to inorganic arsenic (As(III) + As(V)) as a function of time; ■ Demethylation of MMA(V) to As(III) as a function of time.

calculated by the difference between the inorganic arsenic concentration and the As(III) concentration, increases rapidly after the stationary phase. This suggests that MMA(V) is reductively demethylated to As(III), the expected reverse of the Challenger mechanism.

After 28 days of incubation with 500 ppb of As(III), 23% (SD = 3%) of the inorganic arsenic in the media of the killed-cell control was oxidized to As(V). When live *M. neoaurum* was similarly incubated with As(III), 36% (SD = 7%) of the inorganic arsenic in the media was oxidized to As(V). When live *M. neoaurum* was incubated with As(V), 56% (SD = 6%) of the As(V) was reduced to As(III) after 28 days of incubation. There were no changes in the arsenic speciation of the As(V) killed-cell controls. In all of the samples, only inorganic arsenic species were present in the media. *M. neoaurum*, like many bacteria and fungi,[21,22] clearly reduces As(V) to As(III). Thus, the presence of As(V) in the medium and biota, following demethylation of MMA(V) or MMA(III), is probably the result of abiotic oxidation that occurs at the end of the growth cycle when metabolic processes that result in reduction of As(V) have slowed or ceased.

M. neoaurum converted a greater proportion of MMA(III) to inorganic arsenic than MMA(V). If demethylation follows the reverse of the Challenger mechanism,[23] then demethylation would occur only from MMA(V). This would require that either *M. neoaurum* first oxidizes MMA(III) to MMA(V), or, more likely, that the MMA(III) is oxidized abiotically, presumably by oxygen, to MMA(V). The differences in rates of demethylation, between these two methylarsenicals, would then be a consequence of the rate of oxidation. As is found for methylation, the concentrations of the metabolic products were much lower in the biota than in the media, indicating that the demethylated products are primarily excreted from the organism.

Uptake of methylated arsenicals into fungal cells is primarily via passive diffusion.[24,25] Cullen and Nelson[26]

found, from model studies of liposomes, that the permeability of DMA(V) was much greater than that of MMA(V). Thus, the lack of demethylation of DMA(V) and TMAO is likely a consequence of an MMA(V) specific demethylation process rather than an inhibited uptake of the arsenicals by the bacterium.

M. neoaurum was also incubated with 500 ppb of MMA(V) and 1.5 ppm of antimonate, antimonite, selenate, selenite, and bromide. None of these ions changed the amount of demethylation.

Mycobacterium spp. are widespread in the environment, and are found in soil, water and vegetation. Demethylation of methylarsenicals by *M. neoaurum* could certainly play a role in the environmental cycle of arsenic. More work is needed to determine the conditions under which demethylation occurs and the mechanism of demethylation.

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