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Arsenic Biomethylation by the Microorganism *Apiotrichum humicola* in the Presence of L-Methionine-*methyl-d*$_3$

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The microorganism *Apiotrichum humicola* (previously known as *Candida humicola*) grown in the presence of either arsenate, arsenite, methylarsonic acid or dimethylarsinic acid, produces arsenic-containing metabolites in the growth medium. When L-methionine-*methyl-d*$_3$ is added to the cultures, the CD_3 label is incorporated intact into the metabolites to a considerable extent to form deuterated dimethylarsenic and trimethylarsenic species, indicating that *S*-adenosylmethionine, or some related sulphonium compound, is involved in the biological methylation. Conclusive evidence of CD_3 incorporation in the arsenicals found in the growth medium was provided by using a specially developed hydride generation–gas chromatography–mass spectrometry system (HG–GC–MS).

Keywords: arsenate; arsenite; methylarsonic acid; dimethylarsinic acid; trimethylarsine oxide; methylation; extracellular; microorganism; L-methionine-methyl-*d*$_3$; *S*-adenosylmethionine; hydride generation–gas chromatography–mass spectrometry

INTRODUCTION

Since the first systematic investigation of the biotransformation of inorganic arsenic compounds conducted by Gosio in 1897,[1] many studies have shown that the biological methylation of arsenic is a ubiquitous phenomenon in nature.[2] Bacteria, fungi, plants, algae, animals, as well as humans, are all known to be able to transform inorganic arsenic compounds into methylated arsenic species; however, the biomethylation process is still not fully understood.[2]

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Challenger showed that *Scopulariopsis brevicaulis* produced trimethylarsine from arsenate, and proposed a biosynthetic pathway as outlined in Figure 1.[3,4] Evidence supporting this pathway has been provided from studies of arsenic biomethylation in microorganisms.[3–10] These investigations demonstrated that arsenate, arsenite, methylarsonic acid (MMA), dimethylarsinic acid (DMA), and trimethylarsine oxide (TMAO) are precursors to trimethylarsine.

Two basic steps are involved in Challenger's mechanism: (1) reduction of arsenic(V) species to arsenic(III) species, which is likely to be facilitated by a range of thiol-containing molecules found in living organisms;[2,11,12] and (2) subsequent oxidative methylation of arsenic(III) moieties by a methyl donor.

Challenger suggested that some methyl-containing compounds such as betaine, methionine, or a choline derivative could be the possible methyl donor for arsenic methylation.[3] To investigate this hypothesis further, Challenger *et al.*[13] added these compounds, labeled with ^{14}C, to cultures of *S. brevicaulis* growing on breadcrumbs enriched with arsenite. These experiments showed that only ^{14}C-labelled methionine, $^{14}CH_3SCH_2CH_2CH(NH_2)COOH$, was able to transfer its label to arsenite to an appreciable extent. The maximum $^{14}CH_3$ incorporation in the trapped $(CH_3)_3As$ was 28% after five days of incubation. Cullen *et al.*[7] demonstrated that the CD_3 group in L-methionine-methyl-d_3 was incorporated into the trimethylarsine that was evolved from cultures of *Apiotrichum humicola* (previously identified as *Candida humicola*) and *S. brevicaulis* grown in the presence of arsenite, arsenate, methylarsonate and dimethylarsinate. In these experiments the arsine was collected by cryofocusing in liquid oxygen, and characterized by using mass spectrometry.[7] These results strongly indicate that 'active methionine',

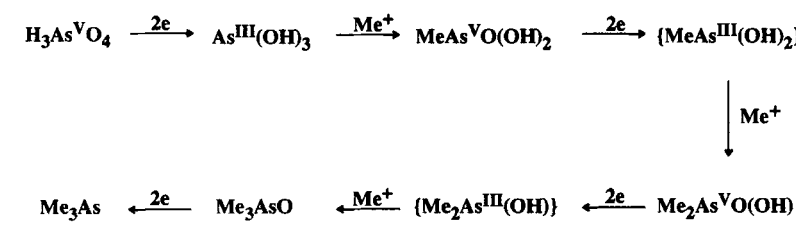

Figure 1 Challenger's mechanism for the methylation of arsenic. The intermediates in {} are unknown as monomeric species. They are formulated as $(CH_3AsO)_n$ and $(CH_3As)_2O$ respectively, when prepared by conventional methods.

S-adenosylmethionine (SAM),[14] is involved in the transfer of the methionine methyl group to arsenic during methylation.

Although arsenate, arsenite, MMA and DMA can be biotransformed by microorganisms to trimethylarsine,[3–9] intermediates in the proposed metabolic pathway had not been isolated from cultures of microorganisms until recently.[15] These arsenic metabolites were found in the pure cultures of *Apiotrichum humicola* and *Scopulariopsis brevicaulis* grown in the presence of arsenate, arsenite, MMA or DMA, and are mainly dimethylarsenic species (probably DMA) and trimethylarsenic species (probably TMAO). These results reinforce the validity of the mechanism proposed by Challenger; however, it remained to verify that SAM is the methyl donor of the extracellular arsenic metabolites produced by *A. humicola*.

In this paper we report on the effect of adding L-methionine-methyl-d_3 together with one of the four arsenic substrates arsenate, arsenite, MMA and DMA to growing cultures of *Apiotrichum humicola*. The incorporation of the deuterated methyl group, from methionine into the extracellular arsenic metabolites, was monitored by using hydride generation–gas chromatography–mass spectrometry (HG–GC–MS). The mass spectra obtained provide conclusive evidence of CD_3 incorporation in all the arsenic metabolites.

EXPERIMENTAL

Reagents

All chemicals used were of reagent grade. Distilled water was used for all dilutions. Glass and plasticware were cleaned by soaking them overnight in 2% Extran solution, followed by a water rinse, a soak in 2 mol l^{-1} hydrochloric acid, and finally a water rinse.

Arsenic standards were prepared freshly by serial dilution from stock solutions (1000 μg cm^{-3} of elemental arsenic) of the following compounds: sodium arsenate, $Na_2HAsO_4 \cdot 7H_2O$ (Baker); sodium arsenite, $NaAsO_2$ (Baker); disodium methylarsonate, $CH_3AsO_3Na_2 \cdot 6H_2O$ (Alfa); dimethylarsinic acid $(CH_3)_2AsO(OH)$ (Alfa); trimethylarsine oxide, $(CH_3)_3AsO$, was synthesized according to the literature.[16] A solution of L-methionine-methyl-d_3, $CD_3SCH_2CH_2CH(NH_2)COOH$ (Aldrich) was prepared by dissolving the compound in distilled water. Solutions of 4.0 mol l^{-1} acetic acid, and 2.0% (w/v) $NaBH_4$ in 0.1% (w/v) NaOH, were made daily.

Microorganism culture

A culture of *A. humicola* was obtained from the American Type Culture Collection (ATCC 26699). The culture was grown aerobically in a synthetic inorganic liquid medium at pH 5 as described by Cox and Alexander.[6,17]

Instrumentation

A semi-continuous mode hydride-generation apparatus connected to a gas chromatograph–mass spectrometer system was used to generate, separate and characterize arsines.[18] A peristaltic pump was used to introduce and mix the sample solution (1–3 ml) with 4.0 mol l^{-1} acetic and 2.0% (w/v) sodium borohydride solutions. In the presence of acetic acid, arsenite, MMA, DMA and TMAO can all be reduced to their corresponding arsines, but not arsenate. These experimental conditions were optimized for efficient production of the hydride $(CH_3)_2AsH$ and $(CH_3)_3As$. The arsines produced were separated on a hand-packed Porapak-PS column (80–100 mesh) by using a gas chromatograph (Varian, Vista 6000 GC) with a pre-set temperature program (Table 1), and the effluents were subsequently detected by using a quadrupole mass spectrometer (Delsi Nermag R10-10C).[18] Data acquisition and processing were performed by using a PC-based data

Table 1 HG–GC–MS experimental parameters

Hydride generation	
2.0% $NaBH_4$ solution, 4.0 mol l^{-1} acetic acid, 3 ml sample	
Gas chromatograph	
Initial temperature	50 °C
Ramp rate	40 °C min^{-1}
Final temperature	150 °C for 2 min
Helium flow rate	35 ml min^{-1}
Mass spectrometer	
Interface temperature	140 °C
Ion source temperature	160 °C
Scan range	*m/z* 74–130
Scan frequency	0.1 s per scan
Scan time	4 min

system (Teknivent, Vector 2) interfaced to the mass spectrometer. The HG–GC–MS experimental conditions are listed in Table 1.

Experimental procedures

Aqueous solutions of the appropriate arsenical and L-methionine-methyl-d_3, filter sterilized (0.2 μm membrane) separately, were added to the autoclaved growth medium. The initial concentration of the arsenical substrate and L-methionine-methyl-d_3 in the growth medium was 1.0 μg cm^{-3} and 1.3 mmol l^{-1}, respectively. A control experiment (without the addition of methionine) was also run simultaneously. Incubation conditions and the culture sampling have been previously described.[15] The incorporation of the CD_3 group from L-methionine-methyl-d_3 into the arsenic substrates arsenate, arsenite, MMA and DMA was investigated by examining the mass spectra of the arsines obtained by using the HG–GC–MS system.

RESULTS AND DISCUSSION

HG–GC–MS measurements

Hydride generation has proven to be an effective tool for detecting trace levels of arsenate, arsenite, MMA, DMA and TMAO in a variety of samples.[18–26] These arsenicals are precursors to the arsines, Me_xAsH_{3-x} ($x = 0$–3), that are ultimately detected by using either atomic absorption spectrometry (AA),[19–23] or mass spectrometry (MS).[18,24–26] With the latter method, both the selected-ion-monitoring (SIM) mode (commonly applied) and the wide-scan mode can be used for structurally characterizing arsines. In our previous work,[18] an HG–GC–MS system operating in the wide-scan mode was used to investigate the biomethylation of arsenate, in the presence of L-methionine-methyl-d_3, by the marine alga *Polyphysa peniculus*. The arsenic metabolite (presumably dimethylarsinate), which was excreted into the growth medium by the alga, was successfully characterized. Conclusive evidence of CD_3 incorporation into the dimethylarsenic compound produced was provided by this methodology.

In the present work, the mass spectrometer was set to scan from *m/z* 74 to 130 at 1 scan per 0.1 s. The upper limit of *m/z* 130 was chosen because the highest mass expected for any volatile arsine is 129, corresponding to $(CD_3)_3As$. The total ion chromatogram (TIC) resulting from a mixture of arsines generated from solution containing synthetic standards of arsenite, MMA, DMA and TMAO is shown in Fig. 2. The mass spectra of each of the arsines are presented in Fig. 3. As in our previous work[18] the wide-scan monitoring mode was used, rather than the SIM mode, because it would allow observation of all fragment ions from the deuterium-labelled arsines. This precaution was taken because the fragmentation patterns of deuterium-labelled arsines, apart from $(CD_3)_3As$, were unknown. Cullen *et al.*[7] demonstrated that the mass spectrum of $(CD_3)_3As$ has an identical fragmentation pattern to that of $(CH_3)_3As$ except that H is replaced by D. For comparison purposes, they also prepared $(CD_3)As(CH_3)_2$ and basically the same fragmentation pattern is obtained:[7] fragment ions formed by the loss of both CD_3 and CH_3 from the parent ion $[(CD_3)As(CH_3)]^+$ and $[(CH_3)_2As]^+$ were observed in high relative abundance. Although the mass spectra of standards of CD_3AsH_2 and $(CD_3)_2AsH$ are not available, it is reasonable to conclude that their fragmentation patterns would be similar to those of CH_3AsH_2 and $(CH_3)_2AsH$.

By using the HG–GC–MS methodology described above, it was possible to detect as little as 25 ng of arsenic. In the SIM mode the detection limit could be easily improved by at least an order of magnitude.

Characterization of methylated arsenic metabolites in the growth medium of *A. humicola*

In order to verify that *S*-adenosylmethionine is the probable methyl donor in the production of the extracellular arsenic metabolites of *A. humi-*

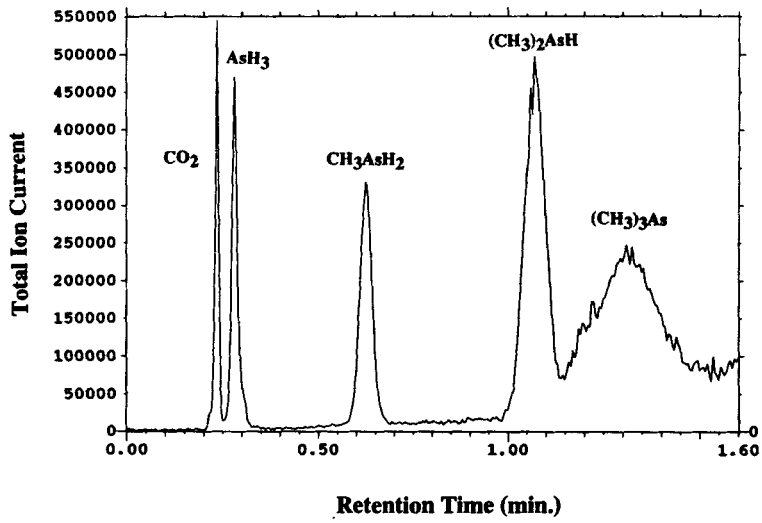

Figure 2 Total ion chromatogram (HG–GC–MS) of arsine, methylarsine, dimethylarsine and trimethylarsine. Solutions of standard arsenite, methylarsonic acid, dimethylarsinic acid and trimethylarsine oxide (100 ng of arsenic for each compound) were used.

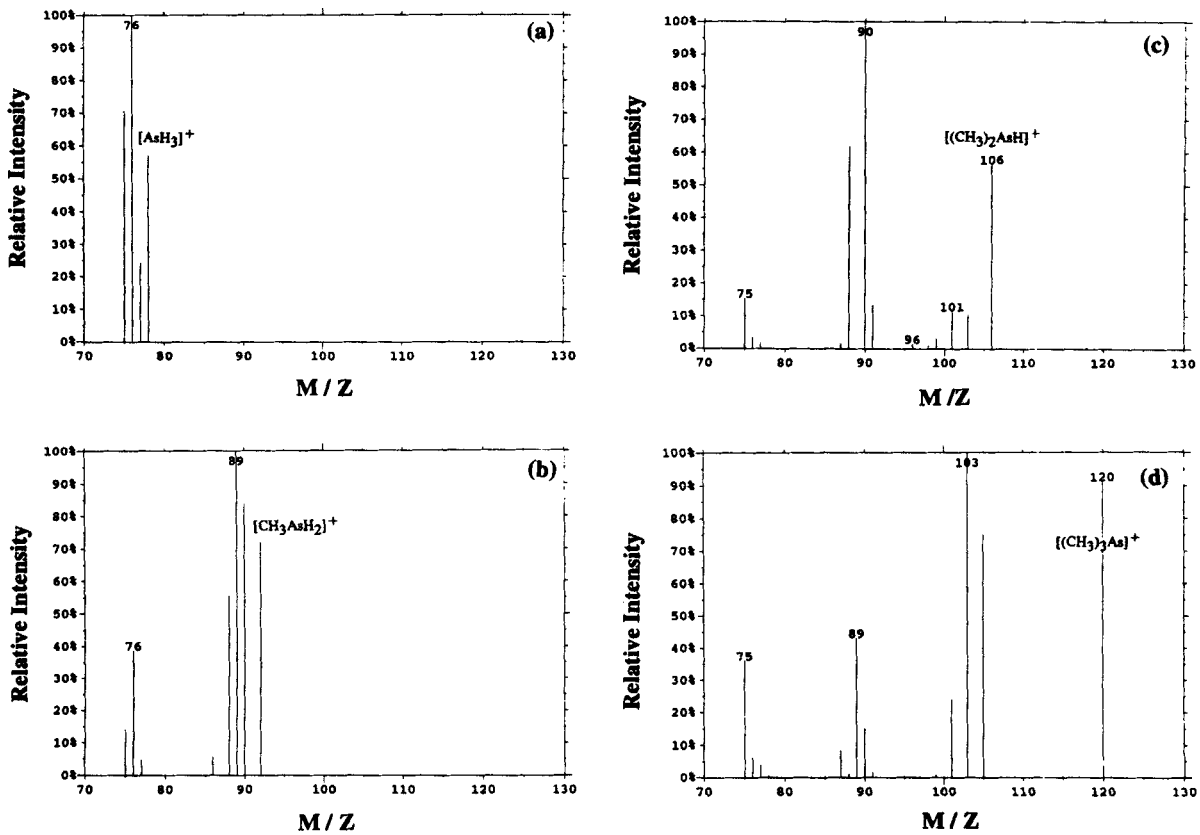

Figure 3 Mass spectra (HG–GC–MS) of arsines derived from standard solutions of arsenite, methylarsonic acid, dimethylarsinic acid and trimethylarsine oxide: (a) arsine, AsH_3; (b) methylarsine, CH_3AsH_2; (c) dimethylarsine, $(CH_3)_2AsH$; and (d) trimethylarsine, $(CH_3)_3As$.

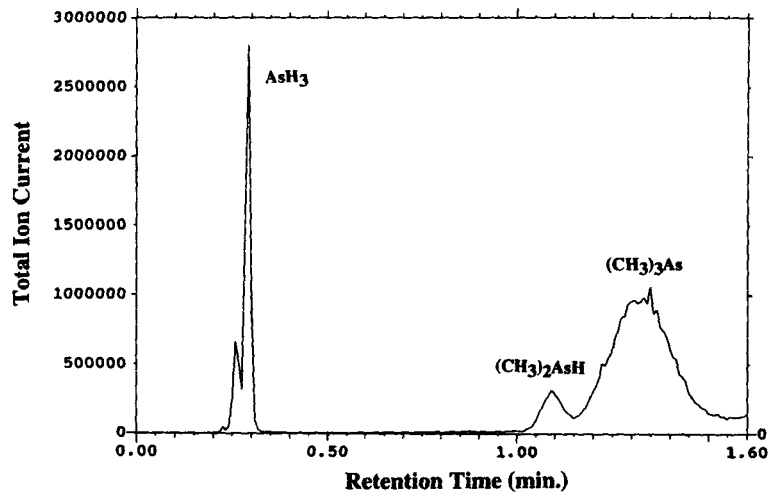

Figure 4 Total ion chromatogram (HG–GC–MS) of 2 ml of culture medium sample that was taken after 10 days of incubation. The medium was originally enriched with 1.0 μg cm^{-3} arsenate, but not with L-methionine-*methyl-d*$_3$.

cola, we added 1.0 μg cm^{-3} each of arsenate, arsenite, MMA or DMA to growing cultures of *A. humicola* both in the presence and absence of L-methionine-*methyl-d*$_3$. The aqueous arsenic metabolites in the growth medium were characterized by using HG–GC–MS in the wide-scan mode.

Transformation of arsenate

When arsenate (1.0 μg cm^{-3}) was added to the growth medium, three compounds were found to be present in the growth medium after five days of incubation as judged by using HG–GC–MS. Figure 4 shows the TIC of the sample collected on day 10. The first arsenic-containing peak (retention time 0.27–0.32 min) in the TIC was identified as arsine (AsH_3), derived from arsenite that was produced by *A. humicola* from the arsenate substrate (arsenate is not reduced to AsH_3 under current experimental conditions).[15] The second arsenic-containing peak (retention time 1.05–1.15 min) is $(CH_3)_2AsH$ derived from DMA, and the third peak (retention time 1.15–1.50 min) is $(CH_3)_3As$ derived from TMAO. The mass spectra of the latter two compounds are identical to those of the arsines derived from the standard arsenicals [Figs 3(c) and (d)].

In the presence of L-methionine-methyl-d_3, dimethylarsenic and trimethylarsenic species were also detected in the growth medium. No apparent difference in the quantity of these methylated arsenic intermediates was observed when L-methionine-methyl-d_3 was either present or absent from the medium. It has been proposed[7] that added methionine would increase the cell's internal concentration of SAM and thus could enhance the methylation process. However, later work by Cullen *et al.*[27] found that preculturing whole-cell cultures of *A. humicola* or their cell-free extracts with methionine did not affect the amount of methylated arsenic species produced from methylarsine oxide, $(CH_3AsO)_n$.

The mass spectra (HG–GC–MS) of the dimethylarsenic [Fig. 5(a)] and trimethylarsenic species [Fig. 5(b)] from cultures grown in the presence of L-methionine-methyl-d_3 exhibit additional ions, indicating that a considerable amount of CD_3 is incorporated into the arsines. The mass spectrum of the dimethylarsenic derivative contains additional ions at *m/z* 112 $[(CD_3)_2AsH]^+$, 109 $[(CH_3)(CD_3)AsH]^+$, and 93 $[CD_3As]^+$. The mass spectrum of the trimethylarsenic species shows additional ions at *m/z* 129 $[(CD_3)_3As]^+$, 126 $[(CH_3)(CD_3)_2As]^+$, 123 $[(CH_3)_2CD_3As]^+$, 111 $[(CD_3)_2As]^+$, 108 $[(CH_3)(CD_3)As]^+$ and 93 $[CD_3As]^+$.

The identification of deuterated arsenic metabolites in the growing cultures of *A. humicola* is significant, since this is the first demonstration that methionine is involved in the production of the non-volatile methylarsenic intermediates, as proposed in Challenger's pathway (Fig. 1), As described above, the labelled methyl group from methionine had been shown to be transferred to arsenate, arsenite, MMA and DMA to form labelled volatile trimethylarsine.[7,13] Our work

strongly reinforces the suggestion that *S*-adenosylmethionine or some related sulphonium compounds are the only source of the CH_3^+ shown in Challenger's pathway.

The percentage of CD_3 incorporation was determined by comparing the relative intensities of the parent ions, *m/z* 106 $[(CH_3)_2AsH]^+$, 109 $[(CH_3)(CD_3)AsH]^+$ and 112 $[(CD_3)_2AsH]^+$ for the dimethylarsenic species, and *m/z* 120 $[(CH_3)_3As]^+$, 123 $[(CH_3)_2CD_3As]^+$, 126 $[(CH_3)(CD_3)_2As]^+$ and 129 $[(CD_3)_3As]^+$ for the trimethylarsenic species. The change in the distribution of the CD_3 label in the dimethyl- and trimethyl-arsenic metabolites with incubation time is presented in Table 2. For these calculations, we assumed that the responses for the deuterated arsines are identical to those of the undeuterated arsines. However, as this assumption needs to be verified by using the appropriate deuterated methylarsenic standards, the results presented in Table 2 should be viewed for the time being as an indication of low or high incorporation.

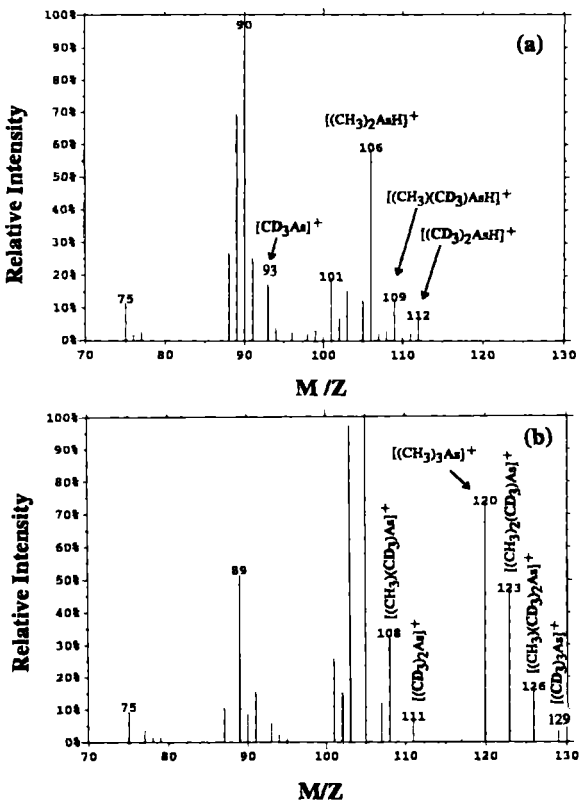

Figure 5 Mass spectra (HG–GC–MS) of the hydride derivatives of the methylated arsenic metabolites present in the growing culture, enriched with arsenate and L-methionine-*methyl*-d_3, after 10 days of incubation: (a) dimethylarsenic species; (b) trimethylarsenic species.

Table 2 Percentage distribution of dimethylarsenic and trimethylarsenic compounds detected in the growth medium

Arsenic metabolite	Incubation time: Day 5	Day 10	Day 15	Day 20
	Arsenate (1.0 μg cm^{-3}) substrate			
$(CH_3)_2AsH$	30	47	56	
$(CH_3)(CD_3)AsH$	37	28	26	
$(CD_3)_2AsH$	33	25	18	
$(CH_3)_3As$	55	61	63	
$(CH_3)_2(CD_3)As$	28	28	27	
$(CH_3)(CD_3)_2As$	11	8	7	
$(CD_3)_3AsH$	6	3	3	
	Arsenite (1.0 μg cm^{-3}) substrate			
$(CH_3)_2AsH$	51	45	51	77
$(CH_3)(CD_3)AsH$	26	30	28	14
$(CD_3)_2AsH$	23	25	21	9
$(CH_3)_3As$	45	50	52	53
$(CH_3)_2(CD_3)As$	36	34	33	33
$(CH_3)(CD_3)_2As$	19	12	12	12
$(CD_3)_3AsH$	<LOD[a]	4	3	2
	MMA (1.0 μg cm^{-3}) substrate			
$(CH_3)_2AsH$	33	66	62	64
$(CH_3)(CD_3)AsH$	67	34	38	36
$(CH_3)_3As$	42	53	58	60
$(CH_3)_2(CD_3)As$	31	29	28	28
$(CH_3)(CD_3)_2As$	27	18	14	12
	DMA (1.0 μg cm^{-3}) substrate			
$(CH_3)_3As$	59	66	69	71
$(CH_3)_2(CD_3)As$	41	34	31	29

[a] LOD, limit of detection.

Transformation of arsenite

Following the addition of the substrate arsenite to the growing culture of *A. humicola*, dimethylarsenic and trimethylarsenic species were detected in the growth medium after five days of incubation. The concentration of the arsenic metabolites was found to be independent of the presence or absence of L-methionine-*methyl*-d_3. In the absence of L-methionine-*methyl*-d_3, the HG–GC–MS traces of the dimethylarsenic and trimethylarsenic species found in the medium were similar to those of the standard $(CH_3)_2AsH$ and $(CH_3)_3As$, while in the presence of L-methionine-*methyl*-d_3, the mass spectra of the two arsenic species showed incorporation of the CD_3 group as indicated by

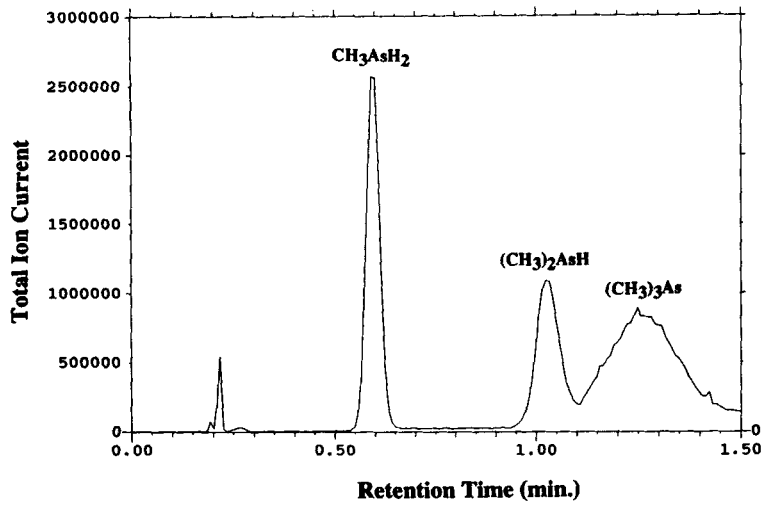

Figure 6 Total ion chromatogram (HG–GC–MS) of 2 ml of culture medium sample that was taken after 10 days of incubation. The medium was originally enriched with 1.0 μg cm^{-3} MMA, but not with L-methionine-*methyl-*d_3.

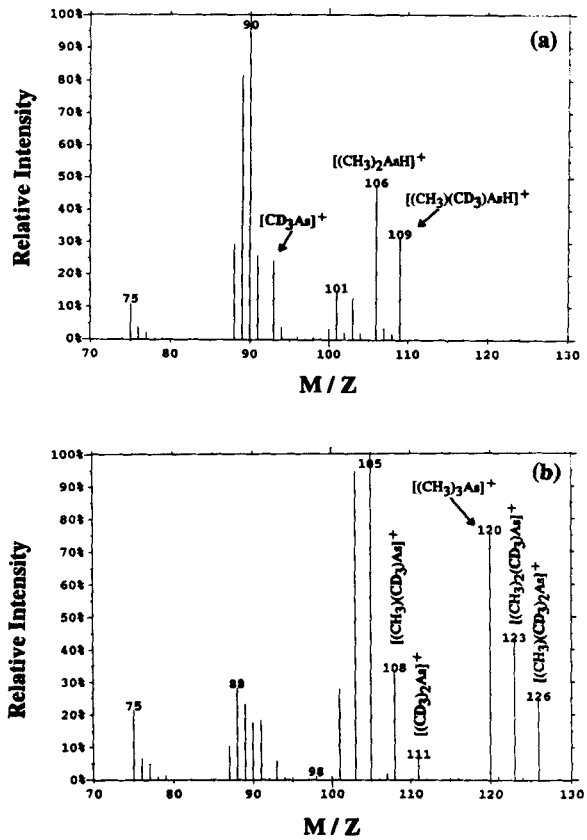

Figure 7 Mass spectra (HG–GC–MS) of the hydride derivatives of the methylated arsenic metabolites present in the growing culture, enriched with MMA and L-methionine-*methyl-*d_3, after 10 days of incubation: (a) dimethylarsenic species; (b) trimethylarsenic species.

the ions at *m/z* 112, 109 and 93 for the dimethylarsenic species, and *m/z* 129, 126, 123, 111, 108 and 93 for the trimethylarsenic species. The distribution of the CD_3 label in the dimethyl- and trimethyl-arsenic metabolites is reported in Table 2.

Transformation of methylarsonic acid

Apiotrichum humicola transformed MMA into dimethylarsenic and trimethylarsenic species in both the presence and absence of L-methionine-*methyl-*d_3 in the growth medium. Again, the presence of methionine did not significantly affect the production of arsenic metabolites. The TIC of the hydrides (Fig. 6) shows that a large amount of DMA and TMAO is present in the growing culture collected on day 10 (in the absence of L-methionine-*methyl-*d_3). The mass spectra of the hydride derivatives are similar to those shown in Figs 3(c) and 3(d).

When deuterated methionine was added to the growth medium, the CD_3 group from methionine was again incorporated into MMA to form deuterated dimethyl- and trimethyl-arsenic species. The mass spectra clearly show the presence of ions at *m/z* 109 $[(CH_3)(CD_3)AsH]^+$ and 93 $[CD_3As]^+$ for the dimethylarsenic species [Fig. 7(a)], and *m/z* 126 $[(CH_3)(CD_3)_2As]^+$, 123 $[(CH_3)_2CD_3As]^+$, 111 $[(CD_3)_2As]^+$, 108 $[(CH_3)(CD_3)As]^+$ and 93 $[CD_3As]^+$ for the trimethylarsenic species [Fig. 7(b)]. The absence of ions at *m/z* 112 $[(CD_3)_2AsH]^+$ for the dimethylarsenic species and *m/z* 129 $[(CD_3)_3As]^+$ for the

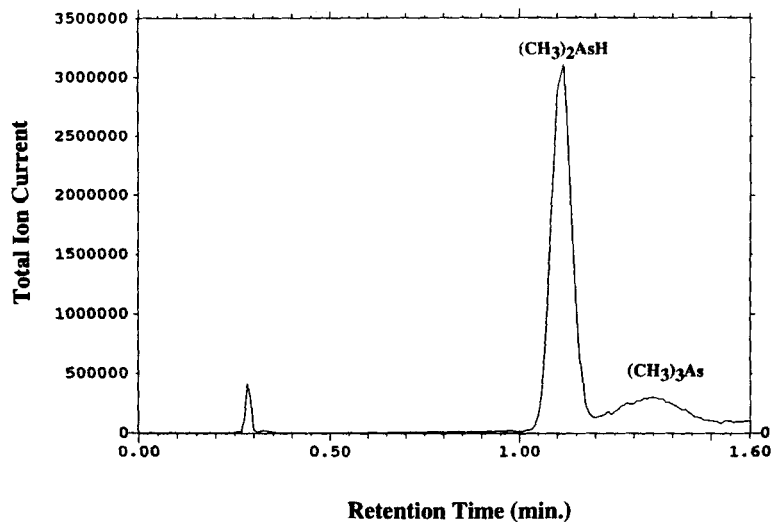

Figure 8 Total ion chromatogram (HG–GC–MS) of 2 ml of culture medium sample that was taken after 20 days of incubation. The medium was originally enriched with 1.0 μg cm^{-3} DMA, but not with L-methionine-*methyl-*d_3.

trimethylarsenic species is expected, since only one and two deuterated methyl groups can be incorported into MMA to form dimethylarsenic and trimethylarsenic species, respectively. The mass spectrum of monomethylarsenic species does not show the presence of an ion at *m/z* 95 $[CD_3AsH_2]^+$. Absence of ions at *m/z* 129, 112 and 95 indicates that biological cleavage of the H_3C–As bond is not a significant process. The distribution of the CD_3 label in the arsenic metabolites is shown in Table 2. It is important to note that the arsenic species do not undergo redistribution reactions under the experimental conditions used in these studies.

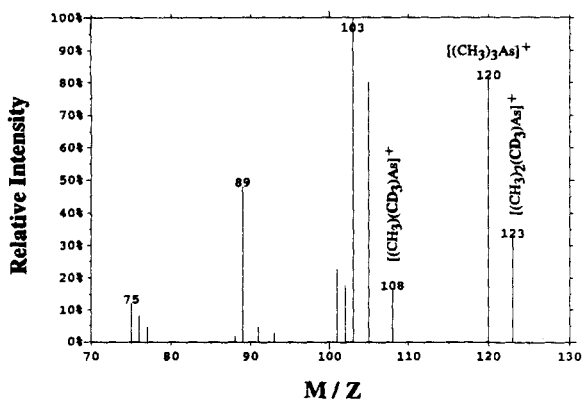

Figure 9 Mass spectrum (HG–GC–MS) of the hydride derivative of the trimethylarsenic species present in the growing culture, enriched with DMA and L-methionine-*methyl-*d_3, after 20 days of incubation.

Transformation of dimethylarsinic acid

The biotransformation of DMA to trimethylarsenic species by the microorganism *A. humicola*, in the absence of L-methionine-methyl-d_3, is a slow process compared with the formation of trimethylarsenic species from arsenate, arsenite and MMA. Most of the DMA substrate remained unchanged in the growing culture after 20 days of incubation (Fig. 8). a small amount of a trimethylarsenic species was produced, and its HG–GC–MS spectrum is similar to that of Fig. 3(d). In the presence of L-methionine-*methyl-*d_3, the production of the trimethylarsenic species was not enhanced. The corresponding mass spectrum (Fig. 9) exhibits ions at *m/z* 123 $[(CH_3)_2CD_3As]^+$, 108 $[(CH_3)(CD_3)As]^+$ and 93 $[CD_3As]^+$, indicating that the CD_3 group is incorporated into the metabolite of DMA. The distribution of the CD_3 label in the trimethylarsenic species is shown in Table 2. As expected, the mass spectrum of the trimethylarsenic species formed from DMA does not show ions present at *m/z* 129 $[(CD_3)_3As]^+$, 126 $[(CH_3)(CD_3)_2As]^+$ and 111 $[(CD_3)_2As]^+$, again suggesting that H_3C–As bond cleavage is not a significant biological process.

In conclusion, the results obtained from this study strongly suggest that methionine, or *S*-adenosylmethionine, is the source of the methyl groups in the biological methylation of arsenic by the microorganism *A. humicola*, supporting the oxidation–reduction pathway involving carbonium ions suggested by Challenger.[3,4] It should be noted that volatile trimethylarsine is not a meta-

bolite from *A. humicola* grown in the presence of low concentrations (<1 ppm) of the arsenicals. Under these conditions water-soluble trimethylarsine oxide is the ultimate metabolic product.

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