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Development and application of liquid and gas-chromatographic speciation techniques with element specific (ICP-MS) detection to the study of anaerobic arsenic metabolism

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Abstract Following the observation of volatile hydride and methylated arsenic species in the gases released from sewage treatment facilities and municipal landfills, we have developed a method for investigating the production of such gases by an anaerobic organism. Here we report the application of high performance ion chromatography (HPIC), hydride generation gas chromatography (HG-GC), and purge and trap gas chromatography (PT-GC), coupled with inductively-coupled plasma mass spectrometry (ICP-MS) to study the formation of ionic and volatile arsenic compounds produced in a batch culture of the anaerobic methanogen *Methanobacterium formicicum*. In this time course experiment we observed arsenite, mono- and dimethylated arsenic acid, arsine, mono-, di- and trimethylarsine, as well as a currently unknown volatile arsenic species.

Introduction

Metals and metalloids (e.g. As, Hg, Pb, Sb, Se, Sn) are found in wastewater as a result of household and industrial discharges, and urban storm water runoff. The metabolic activity of microorganisms in the biological treatment of wastewater can alter the mobility of these elements. The environmental mobility of arsenic in particular is affected by solubility changes resulting from methylation and redox reactions, as well as its transformation into volatile hydride and methylated arsenic species [1–3]. The biochemical pathway for the production of the volatile metal and metalloid compounds is largely unknown. Based on the study of a batch culture, we report the methylation of arsenic under anaerobic conditions by *Methanobacterium formicicum*, a species commonly found in sewage sludge [4].

The aim of this work was the development and application of chromatographic separation, coupled with ICP-MS detection, to the study of the microbiological digestion of arsenic over the course of time. High performance ion chromatography (HPIC), and hydride generation gas chromatography (HG-GC) coupled to an ICP-MS detector were employed for speciation analysis of the medium of arsenate-spiked *Mb. formicicum* cultures, and a modified purge and trap gas chromatographic (PT-GC) technique, coupled to the same detector, for speciation analysis of the headspace gas of the batch culture. The study of the solution and gas phase reveals the presence of methylated and hydride arsenic species, whose production has not previously been observed in cultures of this methanogenic microorganism.

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Experimental

Reagents

Stock solutions (1000 mg L^{-1}) of arsenite ($NaAsO_2$; Fluka), arsenate (KH_2AsO_4; Sigma), monomethylarsonic acid (from CH_3AsBr_2; Alfa), and dimethylarsenous acid (Strem), were prepared in water; a stock trimethylarsine (Strem) solution was prepared in methanol (analytical grade; Baker). Standard solutions of concentrations lower than 10 mg L^{-1} were prepared daily. Water employed in this study was purified by a Seradest SD200 purification system (Allhauser). Measurements were performed while employing a 1 μg L^{-1} Rh (III) external standard in 1% (v/v) nitric acid (Merck; Suprapur) to monitor plasma stability and sensitivity [5].

Analytical procedure

Measurements were obtained by coupling the appropriate chromatographic column effluent flow to an inductively-coupled plasma mass spectrometer detector (ICP-MS; Fisons VG, PlasmaQuad II). Liquid phase analysis was performed by HPIC (CarboPac PA-10 column; Dionex) or by HG-GC (10% SP-2100, 80/100 column packing; Supelco). The gas phase was chromatographed using PT-GC with the same column as that applied to the HG-GC work. The instrument and separation parameters are summarized in Table 1.

Solution samples were withdrawn from the culture as required with a syringe via the septum. HPIC separations were accomplished by the injection of a 50 μL culture media sample prepared by homogenization with a beadbeater (Biospec; see Table 1 for parameters). The outlet of the HPIC column (DX-500; Dionex) was connected directly to the nebulizer of the ICP-MS detector by Teflon tubing at ambient temperature. HG-GC was accomplished by injecting 100 μL of culture medium into the sample introduction flask (Fig. 1), acidification with purified HCl [6] to a pH of 2, purging for 7 min to displace arsines originating from the sample, and the addition of 0.8 mL of 6% sodium borohydride (Aldrich). Volatile arsines produced by the reduction were initially trapped on the pretrap (–196 °C) and then cryofocused on the column. Cryofocusing was accomplished by heating the pretrap to 150 °C for 5 min (1 °C s^{-1}) with He flow to the column (–196 °C; all helium gas flows were 150 mL/min). The GC was initiated by heating the column from –196 °C to 150 °C in 5 min (1 °C s^{-1}); the GC effluent was introduced into the plasma via a Teflon tube (0.8 mm i.d.; temperature of 60 °C) connected to a T-piece between the nebulizer and the torch. Six gas samples were collected by the removal of 100 mL bottles connected to the batch culture with canulas (5 mm i.d.). The gases in these bottles were transferred to the pretrap (–196 °C) in the HG-GC/PT-GC apparatus (Fig. 1) by purging with He. Following a 10 min purge at –78 °C to release matrix gases (CO_2, CH_4), the sample was cryofocused on the column and the chromatograph obtained as described above.

Table 1 Summary of the analysis parameters

HPIC	HG-GC and PT-GC
Column, precolumn: CarboPac PA-10 (Dionex)	Column: 10%SP-2100, 80/100 (Supelco)
Gradient program (flowrate: 2.0mL/min; pH = 9.8): 0-2 min: 0.85mM $NaHCO_3$/0.9mM Na_2CO_3 3-12 min: 8.5mM $NaHCO_3$/9.0mM Na_2CO_3 13-16 min: 0.85mM $NaHCO_3$/0.9mM Na_2CO_3	Temperature ramp: –196 °C to +150 °C/5min Trap: DCMS treated glass 60/80 (Alltech) Carrier gas flowrate (He): 150 mL/min
ICP-MS (Fisons PlasmaQuad 2+)	
RF-Generator power: 1500 W; nebulizer: concentric, liquid uptake 2.0 mL/min Gas flows: plasma, 13 L/min; auxiliary, 1.1 L/min; nebulizer, 1.0/-1.1 L/min	
HPIC Sample treatment	HG-GC Sample treatment
50 µL sample volume prepared by: cell destruction by shaking 1 mL sample with glass beads (100 µm) with a Beadbeater (5000 rpm, 5 min) and centrifugation (15000 g, 20 min, 4 °C) and dilution (1 : 2).	100 µm sample volume acidified with HCl (pH = 2), 7 min prepurge with He, addition of 0.8 mL 6% (m/v) aqueous $NaBH_4$ solution, 7 min trapping of produced hydrides.

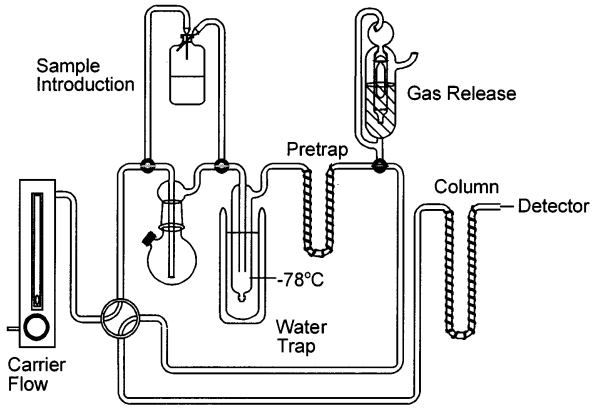

Fig. 1 HG-GC/PT-GC apparatus

Pure cultures of *Methanobacterium formicicum* (DSMZ 1535, Deutsche Sammlung für Mikroorganismen und Zellkulturen) were grown in 500 mL medium DSMZ 119, without sludge fluid [7] under an atmosphere of 80% H_2/20% CO_2, pH 6.8.The medium was reduced by the addition of a titanium citrate solution (Merck; final concentration of 1–2 mM) [8]. The culture was incubated at 37 °C in the dark and shaken at 150 rpm. After reaching the exponential growth phase, as determined by cell counts ($\sim 5 \times 10^7$ cells/mL), it was spiked with arsenate (final concentration of 200 µM). Sterilized blank control experiments, i.e. without living organism, were processed as described above.

Results and discussion

In order to test the aforementioned hypothesis that microbiological activity may account for the volatile arsenic species observed in the gases released from waste disposal sites, we have studied the arsenic metabolism of *Mb. formicicum* as a model organism. The methylation and volatilization of arsenic observed here can be attributed to this organism since blank experiments, conducted under sterile conditions, did not give rise to the arsenic species detected in living cultures of the methanogen. Based on the metabolic arsenic cycle for aerobic organisms, i.e. Challenger's mechanism and sequences derived from it [9–12], volatile arsine production occurs following consecutive reduction and oxidative methylation of arsenic in solution, ultimately resulting in the production of As(III), MMAA, DMAA, TMAO, and TMA. Although there is no evidence that anaerobic microorganisms metabolize arsenic following the same pathway, we have applied a combination of methods for detecting all of these ionic and volatile arsenic species (Table 2).

Table 2 Speciation abilities of the methods applied in this study[a]

Species	Method (ICP-MS detection)			Combined methods
	PT-GC[b]	HG-GC	HPIC[c]	
As(III)	–	Detected as AsH_3	✓	✓
As(V)	–		✓	✓
AsH_3	✓	–[d]	–	✓
MMA	✓	–[d]	–	✓
DMA	✓	–[d]	–	✓
TMA	✓	–[d]	–	✓
MMAA	–	✓	✓	✓
DMAA	–	✓	✓	✓
TMAO	–	✓	–	✓

[a] MMA: $(CH_3)AsH_2$; DMA:$(CH_3)_2AsH$; TMA: $(CH_3)_3As$; MMAA: $(CH_3)As(O)(OH)_2$; DMAA:$(CH_3)_2As(O)OH$; TMAO: $(CH_3)_3AsO$
[b] Detection limit: 20-100 pg
[c] Detection limit: 50-100 pg
[d] Volatile species are removed by solution purging prior to HG-GC

In this work, two approaches were applied to determine the different arsenic species in solution, namely HPIC and HG-GC coupled to an ICP-MS detector. The HPIC analysis of culture medium allowed the observation of arsenite in the samples, the concentration of which increased with time (Fig. 2). The HG-GC approach allowed the determination of monomethyl arse-

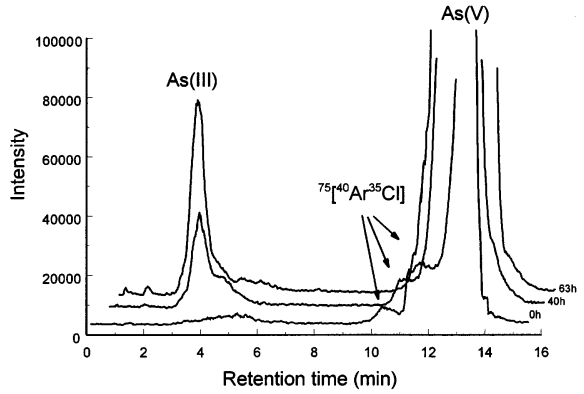

Fig. 2 Time-resolved arsenic speciation by HPIC/ICP-MS

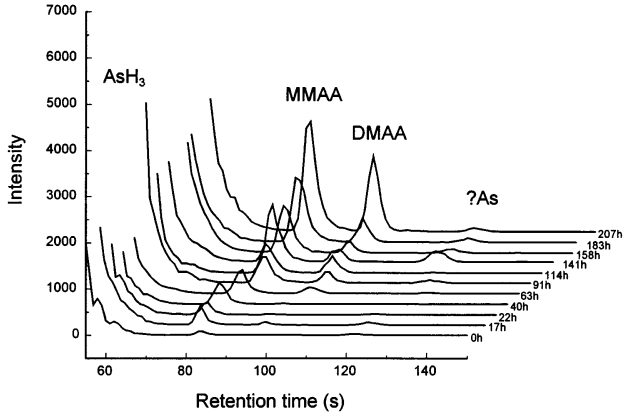

Fig. 3 Time-resolved arsenic speciation by HG-GC/ICP-MS

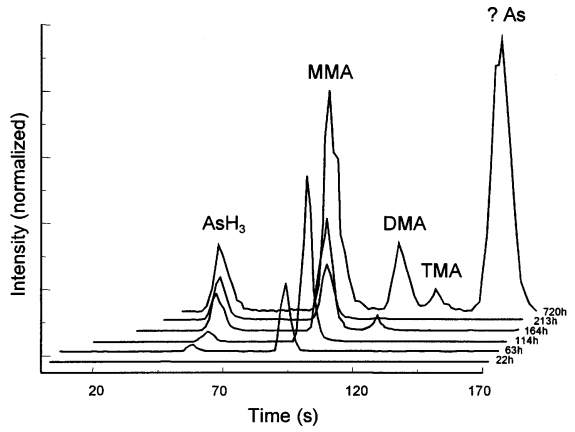

Fig. 4 Time-resolved arsenic speciation in gas samples by PT-GC/ICP-MS

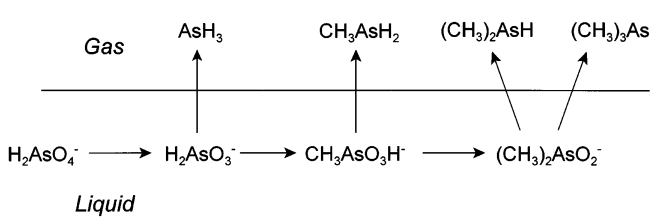

Fig. 5 Production of volatile and aqueous arsenic compounds by *Mb. Formicicum*

nous acid and dimethyl arsenic acid, detected as MMA and DMA after borohydride reduction, respectively (Fig. 3). Additionally, a peak corresponding to an unknown arsenic compound is produced by the borohydride. These different speciation analysis methods for arsenic determination in solution are complementary. The HPIC separation was capable of differentiating between As(III) and As(V), which was difficult with the HG-GC under the conditions of this experiment since some of the spiked As(V) is also reduced to arsine and is then detected together with As(III) [13]. The mono- and di-methylated ionic forms which could not be detected by HPIC, due to matrix effects and the limits of detection, were detectable using HG-GC as the corresponding monomethyl- and dimethylarsine species.

The gas phase in the headspace above the culture was analyzed by PT-GC-ICP/MS. In order to remove methane and carbon dioxide from the sample, both of which extinguish the plasma, the condensed sample was warmed to –78 °C and purged with helium prior to cryofocusing. Over 30 days, several volatile arsenic compounds were found in the gas phase; four known species, whose amounts increased with time, were identified as arsine, MMA, DMA, and TMA (Fig. 4). In addition, an unidentified arsenic compound was detected in high amounts.

The results of the solution and gas study have some mechanistic implications. We have found all of the ionic and methylated species, other than TMAO, in Challenger's mechanism, which suggests similarity between the mechanisms of arsenic metabolism. Additionally, the production of the methylated arsenic compounds appears to be consistent with the sequential reduction and methylation steps described in this mechanism. The first step in the mechanism is the reduction of As(V) to As(III) followed by oxidative methylation. In our system, both As(III) and monomethylated arsenic are produced rapidly following the As(V) spike. The rapid appearance of As(III) indicates that reduction is an early process in the batch culture; however, due to the fast production of MMAA, and the presence of As(V) during the production of the methylated species, the possible methylation prior to the reduction of As(V) cannot be ruled out. Subsequent arsenic methylation in the Challenger's mechanism proceeds via further reduction and oxidative methylation steps to produce DMAA and TMAO. Since the HG-GC method cannot distinguish between monomethylated As(III) and monomethylated As(V), it is not possible to determine whether reduction precedes methylation in this system; however, it is clear by the order of appearance (Fig. 3) that DMAA production follows MMAA production. Both MMAA and DMAA follow nearly linear production rates with respect to time. The similarity in the production rates for the two species suggests that the methylation pathway may be similar for the two species, i.e. both MMAA and DMAA may be produced from the same initial oxidation state with the same methylating agent. In addition to the ionic arsenic species, *Mb. formicicum* produces volatile arsines (arsine, MMA, DMA) which are not part of Challenger's mechanism, but have been observed above communities of microorganisms [14], and an unidentified volatile arsenic species. The structure and function of the unidentified species is currently under investigation. The production rates for the arsines are species specific and occur after the production of their solution counterparts (MMAA for MMA, DMAA for DMA and TMA). The production of these volatile species fits the arsenic metabolism sequence by the presence of hydride formation side reactions at each methylation step. We are currently obtaining data which will provide a more detailed understanding of this mechanism. An arsenic metabolism scheme for *Mb. formicicum*, including the arsine, MMA and DMA production, based on our observations, is shown in Fig. 5.

Conclusion

The combination of techniques employed in this work is suitable to study the formation of ionic and volatile arsenic metabolites in parallel. By applying these techniques we have observed that one organism can account for the production of several of the arsenic species found in nature. In this study, the anaerobic arsenic metabolism of *Mb. formicicum* resulted in the production of arsenite, and mono-, di-, and trimethylated arsenic species suggested in Challenger's mechanism, which describes the aerobic metabolism of arsenic. In addition, we have found arsine, MMA, DMA, and an unidentified volatile arsenic compound, which are not a part of Challenger's mechanism. Based on the volatile arsenic species observed, *Mb. formicicum* may be partly responsible for the arsenic compounds released from waste treatment processes. The biochemical pathway for the production of the methylated and volatile arsenic species we have observed is unknown; however, the sequential methylation of arsenic, with hydride-forming side reactions, appears to be a logical model for the process.

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