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# The Production of Methylated Organoantimony Compounds by *Scopulariopsis brevicaulis*

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**Cultures of the fungus *Scopulariopsis brevicaulis* were grown in antimony-rich media. Although volatile compounds of other elements were readily detected in the culture headspace, volatile antimony compounds were formed irreproducibly and at only ultratrace levels. In order to monitor the media for nonvolatile methylantimony compounds, a method of sample preparation was developed, based on solid-phase extraction. This enabled the separation of large quantities of soluble inorganic antimony species from trace amounts of organoantimony compounds before speciation by HG–GC–AAS. By this methodology methylated antimony compounds were detected at concentrations of 0.8– 7.1 μg $Sbl^{-1}$ in all media in which *S. brevicaulis* was grown in the presence of antimony(III) compounds. These methylantimony species were not detected in any of the nonliving or medium-only controls. Methylated compounds were not detected where *S. brevicaulis* was grown in the presence of antimony(V) compounds. This is the first study to show that antimony(III) compounds are biomethylated by *S. brevicaulis* under aerobic-only growth conditions.** 

**Keywords: *Scopulariopsis brevicaulis*; hydride generation; antimony; biomethylation; biotransformation; solid-phase extraction; sudden infant death syndrome (SIDS); volatile antimony**

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## INTRODUCTION

Years of thorough research on the Group 15 metalloid arsenic have resulted in a comprehensive understanding of its environmental and analytical chemistry. However, until recently, research on the supposedly chemically similar Group 15 metalloid antimony has been sparse. Now the experience gained by studying arsenic is being applied to antimony as researchers seek analogous species in the environment.

Some of the most interesting arsenic compounds found in the environment are organometallic, and some of these are volatile arsines. One very significant compound, historically known as Gosio gas, was identified as trimethylarsine in 1932.[1] In the mid-1970s and onwards, as analytical techniques evolved, a range of organometallic arsenic compounds were characterized, including a large number of nonvolatile species.[2]

Studies of antimony in the environment have resulted in the discovery of only a few analogues of the arsenic compounds. The earliest results, which relied on mass-transport studies, gave indications that *Penicillium notatum* was able to biomethylate the antimony(V) compounds $KSbO_3$ and phenylstibonic acid.[3] These studies have not yet been confirmed by modern analytical methods. In recent years, simple methylantimony compounds have been detected in biological and environmental systems. Volatile species have been found in landfill and sewerage gases.[4–6] Volatile species

have also been detected in headspace gas samples of anaerobic cultures of micro-organisms.[7–10] Nonvolatile species have been found in fresh-water plant extracts,[11] seawater[12] and sediments.[13] The involatile compounds are generally poorly characterized, largely because of the lack of appropriate analytical standards and because of the redistribution reactions that can occur when hydride-generation techniques are employed for speciation.[14,15]

Trimethylantimony species seem to be the most prevalent organoantimony compounds in the environment. In anaerobic systems trimethylstibine commonly occurs along with trimethylarsine.[4–6] In contrast to arsenic, only a few studies have linked specific organisms with antimony biomethylation[3,9,11] and bio-oxidation.[16,17]

The interest in volatile antimony and arsenic compounds has been heightened by a controversial hypothesis linking volatile Group 15 compounds to sudden infant death syndrome (SIDS).[18,19] The suggestion is that some micro-organisms which are found in and on infants' bedding material, in particular the cot mattress, can biotransform available compounds of Group 15 elements to toxic volatile species. The Group 15 elements are found in bedding as fire retardants, fungicides etc. Epidemiological evidence is quoted supporting the 'toxic gas hypothesis',[18,19] although it has received criticism.[20,21]

The fungus *Scopulariopsis brevicaulis* is one micro-organism commonly found in the environment; it is an opportunistic pathogen and is well known for its ability to volatilize arsenic as trimethylarsine (Gosio gas).[1,2] Indeed, this was one of the organisms that was isolated during the early studies of the toxic Gosio gas and, as part of the 'toxic gas hypothesis', it is postulated that *S. brevicaulis* is the micro-organism in bedding most likely to produce toxic gases. Consequently *S. brevicaulis* has received attention from researchers specifically interested in the 'toxic gas hypothesis', or simply interested in finding antimony analogues of organoarsenic compounds in the environment. In the SIDS context, some results have been described with *S. brevicaulis* which *imply* the formation of volatile antimony compounds,[18] but these have not been reproducible.[10,22]

The pathway for volatilization of arsenic is thought to involve intermediate methylated nonvolatile arsenic(V) and arsenic(III) compounds. It is suggested that volatile antimony compounds, notably trimethylstibine, are very susceptible to oxidation to nonvolatile compounds.[23] Consequently the presence or absence of volatile species should not be used as the sole indicator of biotransformation processes. Thus, monitoring the liquid phase for nonvolatile methylated antimony compounds is desirable.

In this investigation we studied cultures of *S. brevicaulis* growing in media containing various antimony(III) and antimony(V) species. The culture headspace, medium and cell extracts were all examined for the presence of methylated antimony compounds.

## EXPERIMENTAL

### Reagents

All reagents were of analytical grade or better. Purified water was obtained by ion exchange (Barnstead). Buffers (50 mM) were prepared by dissolving the appropriate amounts of citric acid (BDH) and ammonium carbonate (BDH) in water and adjusting the pH with potassium hydroxide (Aldrich) to 6 (citrate) and 12 (carbonate). Trimethylantimony dichloride and phenylstibonic acid were synthesized by literature methods.[24,25] A 1000 mg Sb $l^{-1}$ solution [trimethylantimony(V) stock solution] was made by dissolving the appropriate amount of solid in deionized water. In the same way solutions of potassium antimony tartrate (Fisher), and potassium hexahydroxyantimonate (Aldrich) at 1000 mg Sb $l^{-1}$ were prepared. Working solutions were made by diluting these stock solutions by the appropriate amount. Antimony trioxide (Anachemica) was added directly to the medium as a solid. Sodium borohydride reagent was prepared fresh daily by dissolving an appropriate amount of solid (Aldrich) in deionized water.

### *S. brevicaulis* culture

A submerged culture of *S. brevicaulis* (ATCC no.7903) mycelial balls was maintained in a 1-litre Erlenmeyer flask with 400 ml of Cox and Alexander medium.[26] Erlenmeyer flasks were shaken horizontally [~135 rpm,1.75-inch (4.45 cm) displacement] and maintained at 26 °C. Bioreactor inoculum (900 ml) was grown for 12 days under the same conditions. For all experiments the *S. brevicaulis* grew in the form of regular balls of mycelium which visibly increased in size throughout the experiments. All microbiological experiments were done in the Biological Service Facility

 

**Table 1** Time schedule of the bioreactor experiment (open means purged with lab air). During the times when the bioreactor was not closed it was purged with lab air (filtered).

| Time (days) | Accumulation time during closed period (h) | Comments[a] |
|---|---|---|
| −5 | — | Closed; medium only |
| −4 | 24 | Gas sample, liquid sample |
| 0 | — | Open; **addition of *S. brevicaulis* inoculum** |
| 4 | 8 | Gas sample, liquid sample |
| 7 | 6 | Gas sample, liquid sample |
| 7 | — | **Addition of antimony compounds** |
| 7.5 | 6 | Gas sample, liquid sample |
| 8 | 17 | Gas sample, liquid sample |
| 8 | — | Aeration, small flow of air |
| 11 | 19 | Gas sample, liquid sample |
| 14 | 4 | Gas sample, liquid sample |
| 18 | 22 | Open; Gas sample, liquid sample |

[a] 'Open' means purged with filtered laboratory air, during the times when the bioreactor was not 'closed'.

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## Experiments to trap volatile antimony compounds

One experiment was performed on a large scale in a 14-litre bioreactor, with batch sampling of the headspace in order to increase the yields of volatile products. A number of other experiments were performed using 1-litre Erlenmeyer flasks with either continuous or batch sampling of the headspace. All gas experiments were kept in darkness to minimize degradation by UV light.

### Smaller-scale flask experiments

A glass Erlenmeyer flask (1 litre) with a ground-glass male joint (Pyrex no.4980, stopper no.9) was filled with medium and culture (10:1) to give 400 ml total volume. The flask was capped with a female ground-glass joint (40/38) which was fitted with inlet glass tubing that reached well below the surface of the medium, and a short length of outlet tubing. Both inlet and outlet glass tubes were connected via latex tubing to glass-fibre filters (0.2 μm Acrodisc, Gelman). Various antimony compounds were added to these cultures at a range of concentrations (Table 3 below).

### Large-scale bioreactor experiment

The glass 14-litre bioreactor (Microferm, New Brunswick) containing 9 litre of medium[26] and fitted with appropriate accessories (probes, condenser, sterile air filters, inoculation flasks etc.) was autoclaved (90 min, 19 psi, 121 °C). The impeller speed was set at 150 rpm, air flow was approximately 5000 $cm^3\ min^{-1}$ and the culture temperature was maintained at 26 ± 0.5 °C. After three days of purging with sterile air, 200 ml of seed culture was added. Because of excessive foam formation, 100 μl of filter-sterilized polypropylene glycol 2025 in 5 ml distilled water was added after 2 days of incubation. Filter-sterilized antimony species $^{123}Sb(OH)_6^-$ (98.7% isotopically enriched) and potassium$^{121/123}$antimony(III) tartrate (natural abundances) were added together (100 μg of each to give 10 $\mu g l^{-1}$) to the medium after one week of incubation, and growth was allowed to continue. The gaseous phase and the liquid phase were sampled at different times, as described below and indicated in Table 1.

### Gas sampling procedure for small-and large-scale experiments

Volatile species were trapped in U-shaped traps (22 cm × 6 mm o.d.) packed with 10% Supelcoport SP-2100 on Chromosorb, and cooled in a large dry-ice/acetone-filled Dewar (−78 °C).

For batch trapping, the volatile species were allowed to accumulate over a specified time in the headspace of the Erlenmeyer flask, or the bioreactor, with the gas lines closed off. The flasks were purged for 40–60 min with air and the volatile species were trapped. The air flow rate was 100 ml $min^{-1}$ for the Erlenmeyer experiments, or 500 ml $min^{-1}$ for the bioreactor experiment. Regular filtered (0.2 μm) laboratory air was used.

For continuous trapping a peristaltic pump was used to purge the medium constantly with laboratory air (3–4 ml $min^{-1}$). The filtered (0.2 μm) air was introduced via the glass cap of the Erlenmeyer. The outlet of the glass cap had a second 0.2-μm filter attached. The volatile species were trapped in a packed U-shaped trap (−78 °C dry-ice/acetone). Generally a culture could be sampled by this means for 24–48 h before the trap became blocked with ice.

In order to check the stability of trimethylstibine in the continuous-trapping system, trimethylstibine (∼3 ng total) was slowly bubbled (1 h) through medium containing *S. brevicaulis* and then trapped. The trimethylstibine was produced by hydride generation from $Me_3SbCl_2$ in an autosampler vial, and a sample was taken using a gas syringe. The trimethylstibine was then injected into the system

 

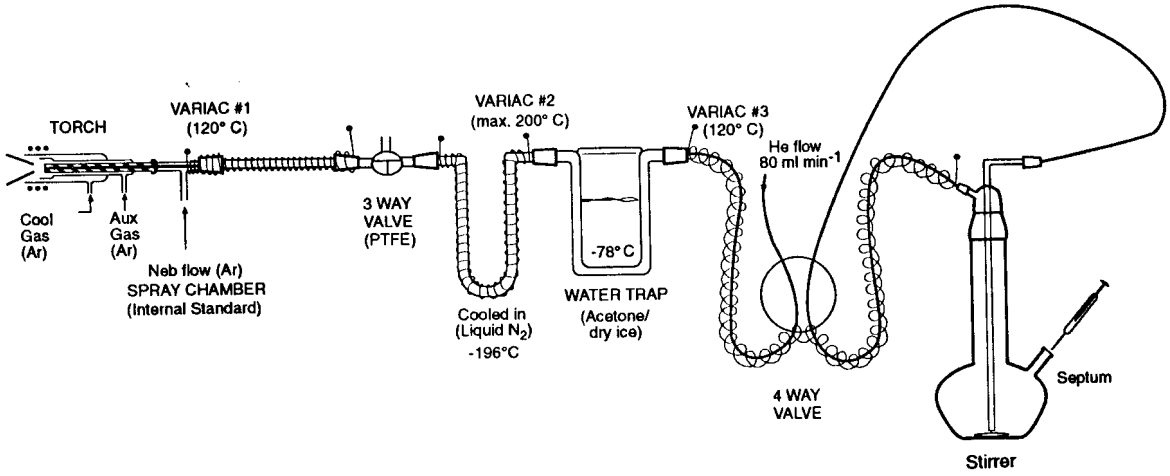


**Figure 1** HG–GC–ICP–MS apparatus.

via a rubber septum on a T-piece placed in the gas line directly after the peristaltic pump. The configuration of the trap flask and transfer line was exactly the same as in all the other experiments. In another experiment, the T-piece was placed directly before the trap and the same amount of standard was injected rapidly. The recovery of trimethylstibine was defined as the ratio of trimethylstibine measured in the first experiment to that measured in the second.

### Analytical procedure

The sampling traps were capped with rubber septa and stored at −78 °C either in a dry-ice freezer or in a dry-ice/acetone bath. For work-up, the sampling trap was connected to a helium supply and to an identical U-tube trap cooled in liquid nitrogen (−196 °C). This second trap is the one shown in Fig. 1. The sample trap was allowed to warm, the contents were volatilized by using a helium flow of 70 ml $min^{-1}$ and gentle heating (15 °C $min^{-1}$) up to 200 °C. In this way the gases were cryofocused in the second trap in order to reduce any previously broadened distribution of the volatile species on the sampling trap. After the cryofocusing step, the second trap was removed from the liquid nitrogen and heating was started (up to 200 °C) to transfer any trapped gases to the ICP–MS. Details of the interface to the ICP–MS, and the ICP–MS operation, are described in another paper.[27]

## Determination of involatile antimony compounds

Cultures of *S. brevicaulis* were presented with antimony(III) as antimony trioxide (200 mg, antimony trioxide added to 400 ml of medium to give a saturated solution, ∼4 mg Sb $l^{-1}$), antimony(III) as potassium antimony tartrate (10 and 1000 mg Sb $l^{-1}$) and antimony(V) as potassium hexahydroxyantimonate (10 mg Sb $l^{-1}$).

For each experiment two replicates of 400 ml of sterile media[26] were inoculated with 10 ml of a medium containing about 10–20 mycelial balls of *S. brevicaulis* (1–3 mm diameter). Two nonliving controls were also prepared consisting of 400 ml of medium added to a sample of autoclaved biomass. The third control contained just 400 ml of medium. An appropriate amount of antimony compound was added to the two viable cultures and all controls.

At various times during the experiments 20-ml samples of the medium were taken aseptically in a biological safety cabinet. These samples were collected in 30-ml polypropylene containers (Nalgene) and kept frozen (−76 °C) until the analysis. The sample was passed through an SPE column as described below. Analysis was performed on the eluate by HG–GC–AAS.

At the end of the experiment a 20-ml sample of the medium was taken and the remaining medium and biomass mixture was autoclaved. The biomass was removed by filtering it off from the aqueous layer and washed with deionized water. The

 

biomass was freeze-dried, then ground to a fine powder with a pestle and mortar. Extraction was performed by repeating the following steps three times.

(1) 10 ml of methanol/water (50:50, v/v) was added to the ground sample in a 20-ml polycarbonate centrifuge tube and sonicated for 10 min.
(2) The sample was centrifuged for 10 min.
(3) The supernatant solution was drawn off with a Pasteur pipette and placed in a 50-ml round-bottomed flask.

After the three extracts from this process had been combined in the 50-ml round-bottomed flask the samples were rotary-evaporated to dryness and then the residue was dissolved in 5 ml of water.

**SPE sample clean-up**

Alumina B SPE cartridges were obtained from Waters Millipore. It was found that columns prepared 'in-house' gave the same results and were more economical: these contained basic alumina (80–200 mesh Brockman activity I, Fisher Scientific), approx 5 g, in a 10-ml syringe held in place by a small glass wool plug.

The SPE column was first rinsed with 10 ml ammonium carbonate buffer (50 mM, pH 12). Then the sample (5–50 ml) was passed through the column, and the eluate was collected. No attempt was made to force the sample through the column; rather it was allowed to drip through under gravity. No attempt was made to rinse the column after the sample had been eluted.

**HG–GC–AAS procedure**

Semicontinuous-flow HG–GC–AAS methodology, as described elsewhere,[28] was used for the antimony determination. Typically, 4 ml of sample provided a signal of sufficient intensity for semi-quantitative analysis and 10–20 ml of sample was used for controls. During operation the sample was drawn into the mixing coil through the peristaltic pump: buffer solution (0.05 M citrate, pH 6) and sodium borohydride (2%) were drawn up continuously at the same rate. The mixture was combined with a flow of helium purge gas before going through the reaction loop and entering the gas–liquid separator. After passing through a dry-ice/acetone trap, the hydrides evolved were collected in a U-shaped trap in liquid nitrogen. After all the sample had passed through the system, the valve was switched from the trap to the inject position. The liquid nitrogen was then removed from the trap and replaced by hot water. This resulted in rapid injection of hydrides onto the GC column (packed with Porapak PS). Simultaneously the recorder and GC temperature program (70–150 °C at 30 °C $min^{-1}$) were started. The separated hydrides which eluted from the GC column were carried into the atomizer (a quartz tube with a hydrogen/air flame) of the AAS (Varian AA1275, 217.6 nm, or at 231.2 nm for antimony confirmation, 0.2 nm slit width). Shimadzu Ezchrom software was used for data collection and analysis, running on a PC.

**HG–GC–ICP–MS procedure**

In order to achieve a lower detection limit and also to confirm the formation of volatile antimony species, an ICP–MS was used as the detector. A batch-type hydride generation reactor was used as shown in Fig. 1. An appropriate volume of sample was placed in the reactor and made up to 10 ml in water. The reactor was installed in the system and helium was allowed to purge through the sample and out to waste for about a minute. The helium flow was then diverted to pass through the trap and sodium borohydride (0.8 ml, 6%) was injected into the reactor through the rubber septum. The hydrides evolved were purged from the reaction vessel with helium for 6 min and trapped in the U-shaped tube (22 cm, 6 mm o.d., packed with 10% SP-2100 on Chromosorb) which was immersed in liquid nitrogen (−196 °C). After the trapping was complete, the four-way valve was switched to the inject position. Simultaneously the trap was removed from the liquid nitrogen, Variac no. 2 was switched on, and the ICP–MS acquisition was started. The trap was heated to 200 °C. As the volatile species were eluted they were transported by a helium flow of about 70 ml $min^{-1}$ to the ICP–MS. The details of the interface to the ICP–MS were as described

**Table 2** Operating parameters for ICP–MS (VG Plasmaquad PQ2 Turbo)

| | |
|---|---|
| Forward radio-frequency power | 1350 W |
| Reflected power | <10 W |
| Coolant gas flow rate | 13.8 l $min^{-1}$ |
| Auxiliary gas flow rate | 0.65 l $min^{-1}$ |
| Nebulizer | DeGalan |
| Nebulizer gas flow rate | 1.00 $min^{-1}$ |
| Spray chamber | Scott, water-cooled, 4–6 °C |
| Analysis mode | Time-resolved 1 time slice |
| Expansion chamber pressure | 2 mbar |
| Intermediate chamber | $<1 \times 10^{-4}$ mbar |
| Analyser chamber | $2 \times 10^{-6}$ mbar |

elsewhere.[27] The connection units and the transfer line consisted of Teflon (PTFE and PFA). The heated transfer line was connected to a T-piece which was substituted for the elbow which normally connects the spray chamber of the ICP–MS with the torch. In this way gas samples were mixed with a nebulized solution before entering the torch of the ICP–MS. The nebulized solution (rhodium, 20 ng $ml^{-1}$) was used as a continuous internal standard for the ICP–MS. The operating parameters for the ICP–MS are given in Table 2. The ICP–MS guaranteed element-specific detection in the picogram range; further confirmation of the identity of volatile species can be made by comparison of retention times with those of standards, and by the use of GC–EI–MS.[29] The use of the chromatographic system and the sampling procedure avoided the detection of aerosols.

# RESULTS

## Production of volatile antimony compounds by *S. brevicaulis*

### Smaller-scale flask experiments

The cultures of *S. brevicaulis* appeared to be growing well in all the experiments, including the batch trapping ones, which involved closed flasks. We therefore assume that the conditions in the cultures were not oxygen-limited. The gas samples from the cultures contained a number of volatile compounds, which have undergone only limited identification and quantification to date; the results are summarized in Table 3.

In experiments 1–3 iodide was not introduced intentionally into the medium, so the volatile iodine compounds found were formed from trace iodide

**Table 3** Summary of headspace measurements for small-scale Erlenmeyer flask experiments (ND, not detected)

| Expt no. | Conditions | Results | | |
|---|---|---|---|---|
| | | Iodine | Arsenic | Antimony |
| 1 | Batch sampling, four samples, accumulation times 1, 1, 4 and 13 days: | | | |
| | Control (medium + fungus, no Sb) | MeI[a] | Various species[b] | $Me_3Sb$[b,c] |
| | 10 mg $Sbl^{-1}$ as $KSb(OH)_6$ | ND | ND | ND |
| | 10 mg $Sbl^{-1}$ as $Me_3SbCl_2$ | ND | ND | $Me_3Sb$[b,c] |
| 2 | Continuous sampling five traps per replicate over 10 days: 1 mg Sb(III) $l^{-1}$ as tartrate and 1 mg[123] Sb(V) $l^{-1}$ as $KSb(OH)_6$ | | | |
| | Replicate 1 | MeI[d] | ND | Monomethyl(?) stibine[b,c] |
| | Replicate 2 | MeI[d] | ND | Rising baseline |
| 3 | Continuous sampling, five traps over 10 days: | | | |
| | 70 mg Sb(III) $l^{-1}$ as tartrate | ND | $Me_3As$[a] | ND |
| | Control (media + antimony) | ND | ND | ND, rising baseline |
| 4 | Continuous sampling, ten traps over 10 days: 100 mg Sb(III) $l^{-1}$ as potassium antimony tartrate, 10 mg $l^{-1}$ as KI and 10 mg As(III) $l^{-1}$ | | | |
| | One flask only | MeI[d] | $Me_3As$[e] | ND, rising baseline |
| 5 | Continuous sampling, five traps over 10 days: saturated solution $Sb_2O_3$ (0.4 g) and 10 μg $l^{-1}$ as KI | | | |
| | One flask only | MeI[e] | ND | $Me_3Sb$[b,c] |
| 6 | Continuous sampling, media at pH 9, five traps over 10 days: saturated solution $Sb_2O_3$ (0.4 g), 10 μg $l^{-1}$ as KI | | | |
| | One flask only | MeI[d] | $Me_3As$[d] | $Me_3Sb$ detected in two traps, rising baseline |
| 7 | Continuous sampling, ten traps over 10 days: 10 mg Sb $l^{-1}$ as phenylstibonic acid and 10 μg I $l^{-1}$ as KI | | | |
| | Replicate 1 | MeI[e] | $Me_3As$[a] | ND |
| | Replicate 2 | MeI[e] | $Me_3As$[a] | ND |

[a] Species detected throughout experiment.
[b] Species detected only at trace levels.
[c] Species detected in one sample only.
[d] Quantities of volatile species rise to a maximum, then decrease.
[e] Quantities of volatile species increase throughout experiment.

 

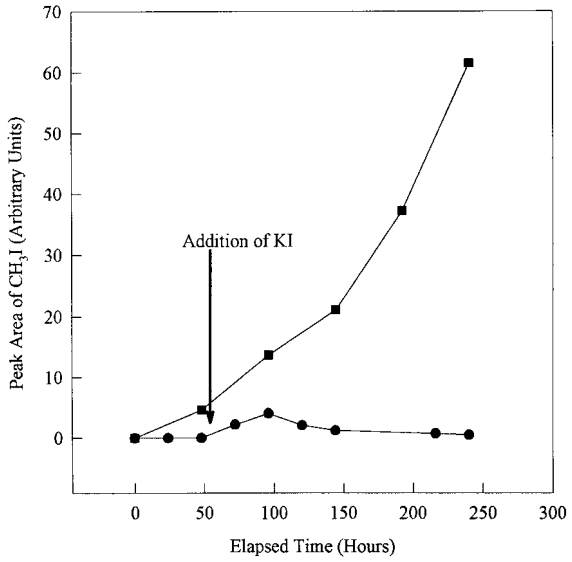


**Figure 2** Time course for the production of volatile methyl iodide. ●, Experiment 4: the medium was made up to 100 mg $Il^{-1}$ after the 48-h gas sample had been taken; peak area was determined for the signal at $m/z = 128$ ($HI^+$); $m/z = 127$ ($I^+$) was not used, because the detector response was off-scale. ■, Experiment 5: the medium was made up to 100 μg $Il^{-1}$ after the 48-h gas sample had been taken; peak area was determined for the signal at $m/z = 127$ ($I^+$). Due to the use of different channels for quantification in these two experiments the traces shown do not represent the actual relative levels of methyl iodide.

impurities in the medium. The production of methyl iodide indicated the presence of a viable strain of *S. brevicaulis* capable of methylation and also that gas trapping was efficient. Methyl iodide (boiling point 42 °C) is more volatile than trimethylstibine (boiling point 81 °C), so if methyl iodide was trapped then so should trimethylstibine have been, provided decomposition was not a problem. The stability of trimethylstibine is discussed below.

In experiments 4–7, potassium iodide was added to the medium so that methyl iodide formation could be monitored. In experiments 5–7 the antimony compounds being studied were present at a concentration 400 times greater than iodine, so it is assumed that competition between antimony and iodine was negligible. Generally methyl iodide levels are low until iodide is added to the culture, then the levels produced rise to a maximum and decline, but in some cases the production of iodine compounds continued to rise throughout the experiment and had not reached a maximum at the end of sampling (Fig. 2).

Like iodine, arsenic species were detected, formed from arsenic impurities in the medium. In experiment 4, when arsenic was added intentionally the rate of volatilization increased throughout the experiment (Fig. 3). The detection of trimethylarsine indicates that this strain of *S. brevicaulis* is capable of taking arsenic through the entire

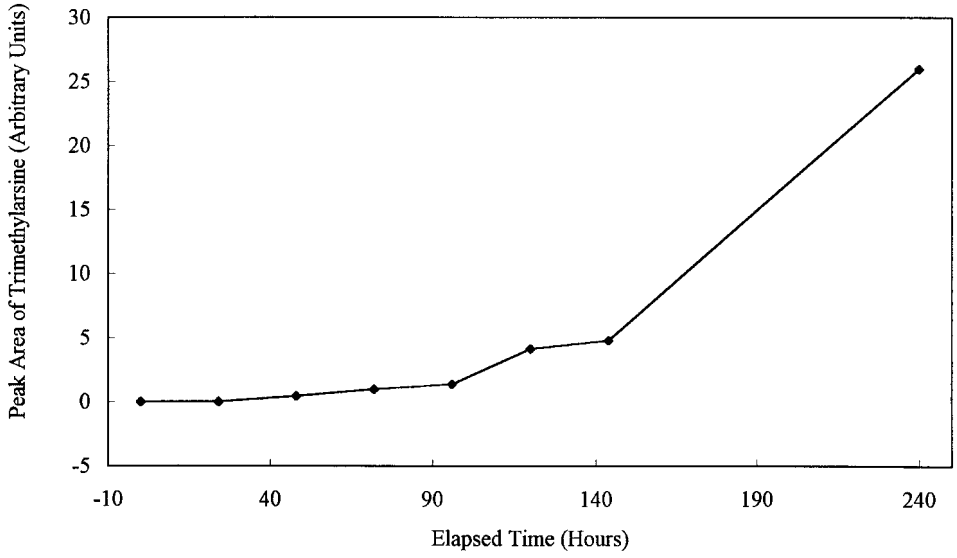


**Figure 3** Time course for the production of trimethylarsine in experiment 4. Arsenic was not added to the medium in this experiment.

 

**Table 4** Summary of results for nonvolatile antimony compounds

| Experiment | Time until methyl compounds first detected[a] | concentrations at end of expt (μg Sb $l^{-1}$) | | Time elapsed at end expt | Methylated Sb (%) |
|---|---|---|---|---|---|
| | | Dimethyl | Trimethyl | | |
| Saturated antimony trioxide | 10 days | 2 | 0.8 | 1 month | 0.001 |
| | 10 days | 1.5 | 0.8 | 1 month | 0.001 |
| Controls (3) | ND[a] | <0.2 | <0.2 | 1 month | <0.0001 |
| 10 mg Sb $l^{-1}$ as potassium antimony tartrate | 7 days | 2.1 | 3.3 | 22 days | 0.06 |
| | 7 days | 2.5 | 2.5 | 1 month | 0.05 |
| Controls (3) | ND | <0.1 | <0.1 | 1 month | <0.001 |
| 1000 mg Sb $l^{-1}$ as potassium antimony tartrate | Expt end[b] | 6.9 | 3.1 | 2 months | 0.001 |
| | Expt end | 7.1 | 5.3 | 2 months | 0.001 |
| Controls (2) | ND | <0.1 | <0.1 | 2 months | <0.0001 |
| 10 mg Sb $l^{-1}$ as potassium hexahydroxyantimonate | ND | <0.1 | <0.1 | 1 month | <0.001 |
| | ND | <0.1 | <0.1 | 1 month | <0.001 |
| Controls (3) | ND | <0.1 | <0.1 | 1 month | <0.001 |

[a] Abbrevations. ND, methyl compounds not detected; Expt end, samples were taken only at the end of the experiment.

metabolic pathway for forming trimethylarsine. Thus, if the methylation of antimony follows this same metabolic pathway [recent research (P. Andrewes, unpublished results) indicates that it does] termination at an intermediate point to form a species such as $Me_3Sb(V)$ should result from the unique chemistry of antimony, rather than being a peculiarity of *S. brevicaulis.* For arsenic, maximum volatilization rates had not been reached at the end of experiment 4. Thus there is the possibility that antimony volatilization might have been slower and occurred near the end of the experiment or after sampling had stopped.

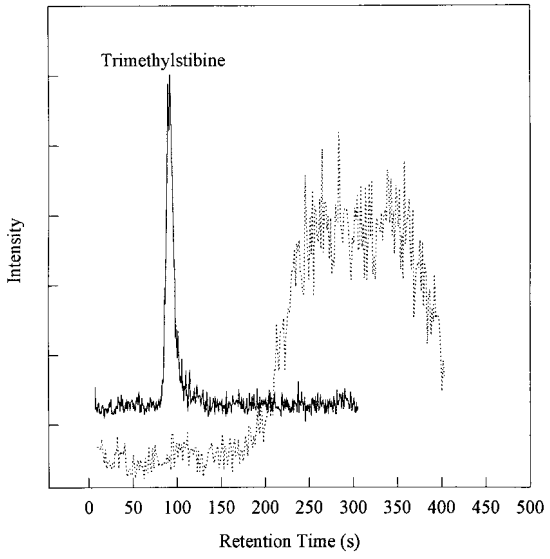


**Figure 4** GC–ICP–MS antimony (*m/z* = 121) chromatograms: ——, one of the two gas samples in experiment 6 which contained some trimethylstibine; …… sample from experiment 3 showing absence of trimethylstibine but occurrence of a baseline rise.

Volatile antimony was detected at picogram levels (Table 4) in five of the 63 gas samples collected from viable cultures of *S. brevicaulis* grown in the presence of various antimony compounds. The antimony detected corresponded to volatilization of about one millionth of the antimony added to the culture.

In addition to experiments in which the antimony compounds presented to cultures of *S. brevicaulis* were varied, experiments with batch sampling (experiment 1) and one at pH 9 (experiment 6) were performed. The experiment at pH 9 was carried out because it had been suggested to us that a high pH would be more conducive to the formation of trimethylstibine (J. Sprott, personal communication). Although in this experiment trimethylstibine was detected, the levels seen were not significantly greater than at pH 5 (experiments 1, 2 and 5).

One phenomenon observed in a few of the experiments was a rise in the baseline of the chromatograms near the end (Fig. 4), which may be associated with the presence of less-volatile antimony compounds in the trap, possibly resulting from trimethylstibine oxidation.

In the experiments to determine the efficiency of the sampling procedure, approximately 80% of the antimony added as trimethylstibine was recovered. Thus the trapping appears to be efficient, and

 

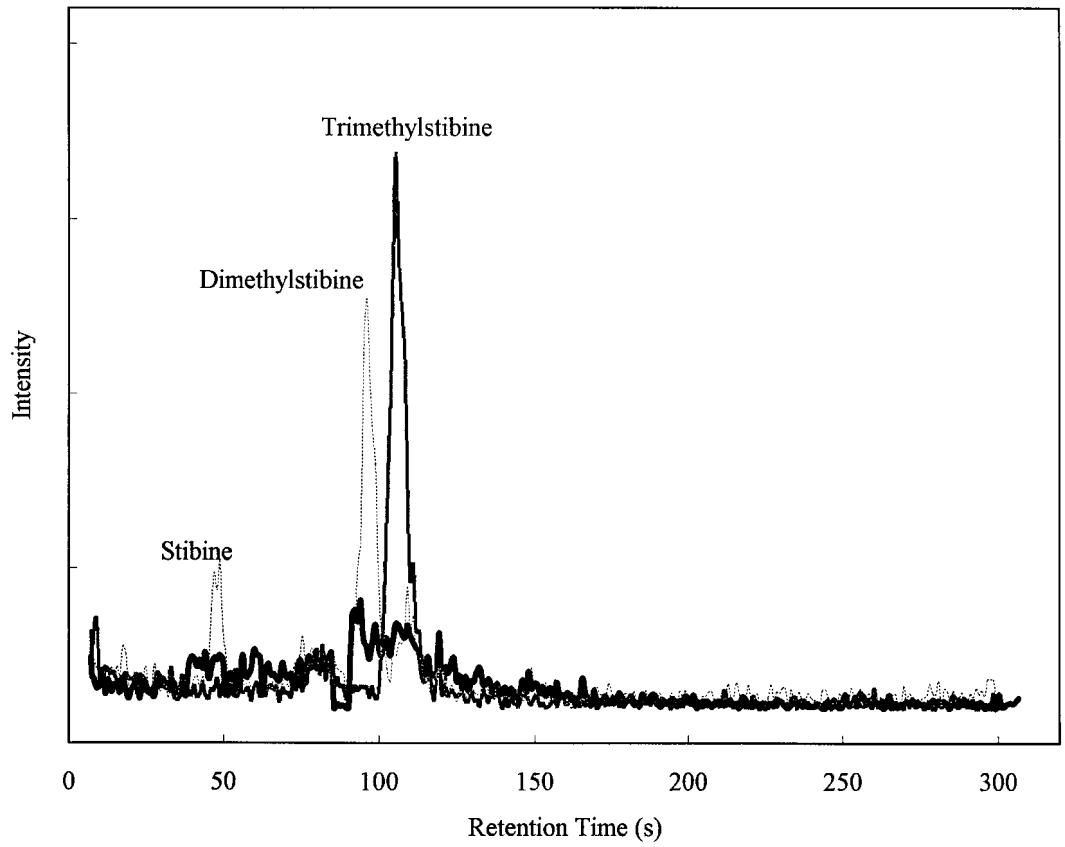


**Figure 5** GC–ICP–MS antimony ($m/z = 121$) chromatograms of bioreactor gas samples taken: ——, before inoculation with *S. brevicaulis* ——, after 8 days of fungal growth in the presence of antimony; · · · · · ·, after 18 days of fungal growth in the presence of antimony.

sorption or complexation of trimethylstibine is minimal. The concentration of trimethylstibine present in this recovery experiment was much greater than that seen in any of the sampling experiments, so the rate of oxidation should have been significantly higher in this experiment. It is presumed that once trimethylstibine is condensed in the supelcoport trap (−80 °C) it is not susceptible to oxidation. Hence in a continuous-trapping system where trimethylstibine is formed and flushed from the Erlenmeyer in less than an hour (the duration of the recovery experiment), the trimethylstibine should be readily detected. Although oxidation may occur it is doubtful that it would remove a significant amount of trimethylstibine.

### Larger-scale bioreactor experiments

The scale of the experiments was increased by about a factor of 20 (400 ml to 10 litres) in order to increase the ease of detecting and identifying trace amounts of volatile species. As was found in the smaller-scale experiments, the gas samples contained many different volatile species. In particular, antimony species were present in low concentrations. Figure 5 shows the antimony chromatograms ($m/z = 121$) of various gas samples. Figure 6 shows the time course for levels of volatile antimony in the bioreactor headspace, it can be seen that trimethylstibine was detected there before extra antimony was added, confirming the result of the smaller-scale flask experiment 1, where the control culture of *S. brevicaulis* generated volatile antimony species.

The amount of volatile antimony in the bioreactor headspace (approx 5 litres) increased after antimony(III), as potassium antimony tartrate (natural isotopic abundance) and antimony(V) as potassium hexahydroxyantimonate (isotopically enriched) had been added. However, no significant change in the isotope ratio (121:123) was found in

 

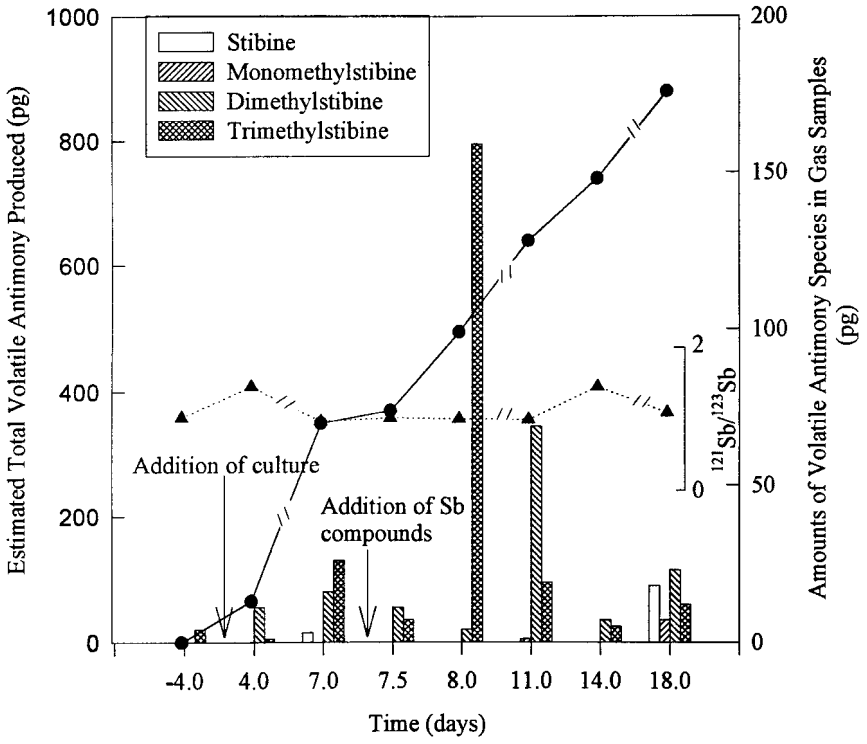


**Figure 6** Time course showing levels of volatile antimony species in bioreactor headspace. The accumulation period for gas sampling is given in Table 1. Also shown is the total estimated volatile antimony production up to and including each sample time ● and the ratio of $^{121}Sb$ to $^{123}Sb$ ▲. The natural ratio of $^{121}Sb$ to $^{123}Sb$ is 1.34.

the gas samples (Fig. 6); hence only antimony(III), from impurities or from added potassium antimony tartrate, was metabolized to volatile species.

The bioreactor was usually closed to allow gas accumulation prior to purging, so the samples represent the sum of gas production during the accumulation time and the smaller amount produced during purging. The total volatile antimony formed can be estimated by calculating the production rates (pg $h^{-1}$) at each sampling time. Total volatile antimony produced over the entire experiment (approx. 1 month) is thus estimated to be 0.9 ng (Fig. 6). The amount of antimony in the medium as an impurity, i.e. not deliberately added, is more than enough to account for this small amount of volatile antimony species. In addition to trimethylstibine, dimethylstibine, methylstibine and stibine were also detected after 18 days. However, the main species was trimethylstibine.

Volatile arsenic species, mostly as trimethylarsine but including some methylarsine and dimethylarsine, were generated by *S. brevicaulis.* No arsenic compound was added directly but the chemicals used for making up the medium contained arsenic at the nanogram/litre level. The greatest amount of trimethylarsine was detected within the first seven days, when the highest growth rate of the biomass was observed visually.

Both medium and broken cells were examined for organoantimony compounds by hydride-generation methodology involving treatment with $NaBH_4$ solution at pH 2. No significant presence of methylated species was detected in samples of either the media or the broken cells.

The total amount of antimony detected in the solution varied with time. After addition of antimony(III) as potassium antimony tartrate (natural isotopic abundance) and antimony(V) as potassium hexahydroxyantimonate (isotopically enriched) the concentration in the medium increased as would be expected, but after 24 h less than 10% of the added antimony was left in solution. This effect was shown to the same extent by both $^{121}Sb$ and $^{123}Sb$, meaning that both antimony(III) and antimony(V) were removed from solution at the same rate and that no change in

 

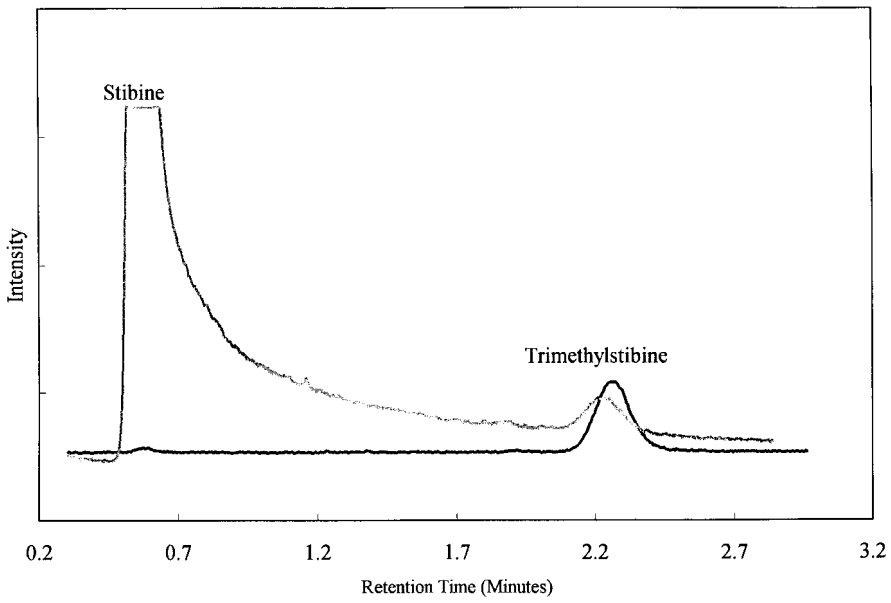


**Figure 7** HG–GC–AAS chromatograms: top trace, medium containing inorganic antimony(III) and trimethylantimony (50 ng); bottom trace, the same medium after it had passed through an SPE column.

oxidation state took place. Presumably this antimony was lost to the fungal cells.

## Production of nonvolatile compounds by *S. brevicaulis*

Samples of medium in these experiments generally contained microgram, or on occasion milligram, quantities of inorganic antimony(III). This antimony(III) was seen in the HG–GC–AAS chromatogram as a large broad stibine peak, which interferes with the much smaller peaks of the organoantimony compounds (Fig. 7). After passing the antimony(III)-containing medium through an SPE column most of the inorganic antimony was removed, whereas the organoantimony compounds remained; HG–GC–AAS on the eluent solution gave a much cleaner chromatogram (Fig. 7). Significant levels of inorganic antimony(V) could also be removed by the clean-up procedure.

In order to separate the inorganic antimony from the organoantimony compounds by solid-phase extraction, the chromatographic behaviour of all the species must be known. The choice of the SPE stationary phase was made in the knowledge that inorganic antimony binds with basic alumina,[30] the extent being dependent on pH and antimony oxidation state. During the development of this technique the only organoantimony compound available for measuring analyte recovery was $Me_3SbCl_2$. The recovery for this proved acceptable, and it was initially anticipated that the recovery for any monomethyl and dimethylantimony compounds would be similar. Subsequent investigation revealed that, as expected, the dimethylantimony species behaved very similarly to the trimethylantimony compound on the SPE column. The behaviour of monomethylantimony compounds on the SPE column is unknown. The recovery of dimethyl compounds is affected by the preparation of the SPE column; failure to make the alumina basic by using ammonium carbonate buffer results in retention of the antimony species. Unsuccessful attempts were also made to separate the organoantimony compounds from the inorganic antimony by using $C_{18}$ SPE cartridges.

Results for the production of nonvolatile methylantimony compounds are summarized in Table 4. Because no dimethylantimony analytical standards are available, the concentrations tabulated for these species are only an estimate based on the assumption that peak areas in the HG–GC chromatograms are proportional to the antimony concentration; that is, the detector response is independent of species. This assumption seemed to be justified because calibration curves for inorganic antimony and trimethylantimony are the same. A second assumption is that SPE behaviour of the dimethyl and

 

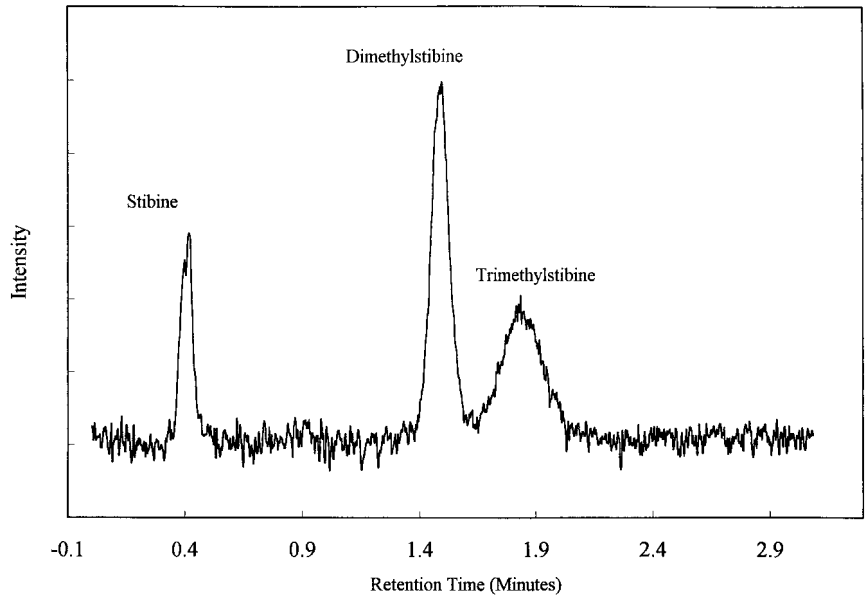


**Figure 8** HG–GC–AAS chromatogram for medium in which *S. brevicaulis* had been growing with potassium antimony tartrate (10 mg $Sbl^{-1}$).

trimethyl species found in the samples was the same as that of the trimethyl standard ($Me_3SbCl_2$) available to us. If these assumptions are not correct the extent of methylation may be greater than indicated. The final column in Table 4 represents the antimony found in the media as dimethylantimony and trimethylantimony species, as a percentage of the total inorganic antimony added to the media. These numbers are very small. The detection limit for these experiments is related to the volume of sample used; generally the detection limit was 0.1 μg $Sbl^{-1}$ as $Me_3SbCl_2$ for the typical sample volume of 10 ml that was used when the amounts of methylantimony species were low. No methylantimony compounds were detected in any of the cell extracts.

In the experiments with antimony trioxide (saturated solution ~4 mg $Sbl^{-1}$), methylantimony

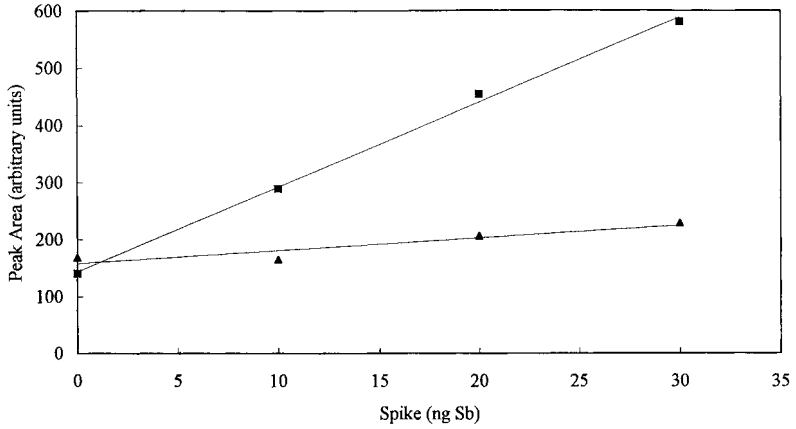


**Figure 9** Standard additions curve for the addition of trimethylantimony dichloride to samples of media (4 ml taken after 21 days of growth), from the experiment where *S. brevicaulis* was grown in the presence of 10 mg $Sbl^{-1}$ as potassium antimony tartrate. HG–GC–AAS peak areas: ■, trimethylantimony; ▲, dimethylantimony.

 

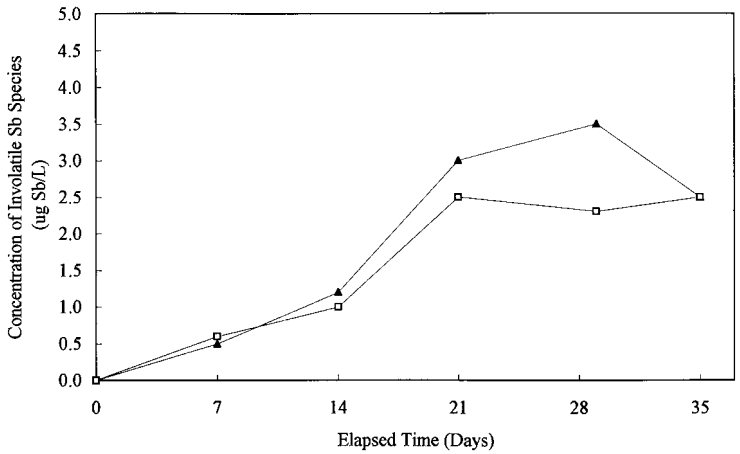


**Figure 10** Time course for the production of nonvolatile methylantimony compounds by *S. brevicaulis*. HG–GC–AAS peak areas: ▲, dimethylantimony; □ trimethylantimony.

compounds were found in the media after 10 days and higher levels were found in samples taken after a month. No methylantimony compounds were detectable in the controls at any time. The amounts detected were in the low μg $Sbl^{-1}$ range and no retardation of fungal growth was evident.

Potassium antimony tartrate is more soluble and experiments were done with media containing 10 or 1000 mg $Sbl^{-1}$ (Table 4). No organoantimony species were found in any of the controls, but they were found in samples of the media of all the viable cultures following day 7. Peaks of approximately equal area in the HG–GC–AAS chromatograms were seen for both dimethyl-and trimethyl-antimony (Fig. 8). It has been shown that demethylation of trimethylantimony species during hydride generation can result in the formation of dimethyl- and monomethyl-stibine, seen as additional peaks in the chromatogram.[14] This phenomenon is especially prevalent at low pH.[15] In the present study the dimethylstibine peak is unlikely to be an artifact because the antimony compound $Me_3SbCl_2$ under the same conditions of analysis shows negligible demethylation: only trimethylstibine is produced. Furthermore, when standard additions were made to the samples the trimethylantimony peak increased in direct proportion to the amount of $Me_3SbCl_2$ spike added, whereas the dimethylantimony peak remained effectively constant (Fig. 9).

The time course for the production of soluble methylantimony compounds (Fig. 10) seems to indicate that production is proportional to the amount of biomass observed visually. The concentrations of the trimethyl-and dimethyl-antimony species increased over time until growth had visibly ceased, after which they remained constant (Fig. 10).

The species were confirmed as antimony compounds by using AAS. The peaks seen when AAS detection was carried out at 231.2 nm had approximately half the areas of the peaks detected at the usual wavelength of 217.6 nm. This was also found for the trimethylantimony standard, the decrease in signal intensity being due to the lower extinction coefficient for antimony at 231.2 nm. In addition, no peaks were seen when the monochromator was tuned to 217.0 nm corresponding to a line in the AA lamp at which antimony atoms do not absorb, indicating that the absorption at 217.6 nm does not result from broad-band molecular absorption.

Hydride generation with ICP–MS detection also confirmed that the compounds produced were antimony compounds, and the ratio of isotopes 121 to 123 did not deviate significantly from the natural ratio. We were unable to establish the presence of monomethylantimony compounds in the sample by using ICP–MS, in spite of the greater sensitivity of the detector. This search was hindered by high levels of monomethylantimony species of unknown origin in the reagent blanks.

In performing hydride generation analysis, it is important to be sure that the stibines evolved are produced from the hydride generation process and do not just arise as a result of purging the liquid sample. Consequently all samples were run through the system without any sodium borohydride present. No peaks were observed when using AAS either or ICP–MS as detector.

 

Attempts were made to speciate the soluble methylantimony compounds by HPLC–ICP–MS, but at this stage a satisfactory technique to separate the organoantimony compounds has not been found.

For the experiment involving potassium antimony tartrate (10 mg $Sbl^{-1}$), semiquantitative determination of total antimony in the media indicates that at least 30% of the added antimony is lost from solution over a month. It is likely that the loss of antimony is due to uptake of antimony(III) into or onto the biomass.

When the antimony(V) salt was added to the media no organoantimony compounds were detected in the media or in cell extracts after 1 months growth (Table 4). In contrast to antimony(III), semiquantitative determination of total antimony in the medium indicates that the uptake of antimony(V) by *S. brevicaulis* is minimal. Others have shown that in another fungus, *Saccharomyces cerevisiae*, antimony(V) is not taken up at all, whereas antimony(III) is taken up almost completely.[31]

## DISCUSSION

Some previous studies have found methylantimony compounds in the environment,[3–13] but, only in a few cases have these compounds been linked to a specific organism.[3,9] *S. brevicaulis* was used during the present study of the biotransformation of antimony species: the same strain had previously been used for studies on arsenic methylation.[32] Gates and co-workers have recently reported that *S. brevicaulis* does not biotransform antimony to detectable levels (micrograms of antimony) of volatile compounds.[10] In the present study with a lower detection limit (picograms of antimony) we have found that *S. brevicaulis probably* produces volatile methylstibines and stibine, but in *very low yields*. However, higher (micrograms of antimony) but still very low yields are consistently obtained of nonvolatile dimethyl and trimethyl species in the aqueous phase. These methylantimony compounds were characterized by hydride generation techniques, so the actual precursor species are not known.

In the case of arsenic, the principal methyl species produced by *Apiotricum humicola* are $Me_3AsO$ and $Me_2AsO(OH)$, as determined by HG–GC–AAS, and volatile $Me_3As$ is not produced from low concentrations (ppm) of arsenic(V). Mechanistic pathways to these water-soluble compounds have been proposed, based on chemical yields and rates of diffusion of products through cell membranes.[32–34] This model accounts for the absence of MeAs(V) species in the medium. On the basis of this model the antimony derivatives produced by *S. brevicaulis* are likely to be $Me_3SbO$ and $Me_2Sb(V)$ species, although $Me_2Sb(III)$ species cannot be completely ruled out [corresponding $Me_2As(III)$ species are probably $Me_2AsOH$ or $Me_2AsSR$],[35,36] Neither $Me_2SbO(OH)$ nor $Me_2SbOH$ is a well characterized species and model compounds are not available for comparison, so the exact nature of any $Me_2Sb(V)$ or $Me_2Sb(III)$ species produced in the cultures cannot be determined.

The reduction of arsenate to arsenite is the first step in the methylation pathway of arsenic. This reduction is viewed as a detoxification of the arsenate that is actively taken up by the cell with phosphate. Many micro-organisms have this ability to reduce As(V) to As(III) but not all go on to produce methylarsenicals.[2] Antimony(V) species have very different structures in solution from phosphate and arsenate[37] so are unlikely to be taken up by the same transport processes. Indeed, *S. brevicaulis* and other fungi do not show uptake, or reduction, of antimony(V).[32] This is a major departure from the path expected if antimony methylation mirrors arsenic methylation. There is no thermodynamic barrier to the reduction process because the redox potentials of antimony species are very similar to those of arsenic. Another departure from the arsenic pathway is the finding that *S. brevicaulis* has the ability to oxidize Sb(III) to Sb(V) (P. Andrewes, unpublished results). Such behaviour is not unique; *Stibiobacter senarmontii* can oxidize antimony trioxide.[16] Furthermore *S. brevicaulis* seems to thrive in high concentrations of antimony either as antimony(III) or antimony(V). This behaviour, and the production of only low levels of methylantimony compounds, indicate that, in contrast to arsenic, methylation is a fortuitous rather than a detoxification process.

It is likely that the production of methylantimony compounds is the result of inadvertent replacement by antimony of some other element in a methylation process. This replacement becomes more probable at higher concentrations, and thus it is not surprising that no organoantimony compounds were detected in the medium for the large-scale bioreactor experiment. The concentrations of antimony in the media for the small-scale flask experiments were at least 250 times greater than for the bioreactor experiment.

 

The volatile antimony compounds $SbH_3$, $MeSbH_2$, $Me_2SbH$ and $Me_3Sb$ appear to be very minor products of *S. brevicaulis* metabolism. In the large-scale bioreactor experiment the amounts detected were so low that the source of antimony could have been impurities in the media. It has been suggested that only low levels of trimethylstibine are found because the compound is easily oxidized, more so than trimethylarsine.[23] It is possible that, in some of our attempts to detect antimony in culture headspace, some of the trimethylstibine was oxidized in the transfer lines, or in the gas trap itself, and it is possible that these oxides were collected in the gas trap. Such an accumulation may explain why on a number of occasions we observed, late in the HG–GC–ICP–MS chromatograms, an increase in the baseline antimony signal (Fig. 4) which might be attributable to these less-volatile antimony compounds. The oxidation of volatile methylantimony species could also account for both the trimethyl- and dimethyl-antimony species found in solution; however, we have shown that trimethylstibine can be manipulated without appreciable loss in our experimental set-up. Although we know little about the kinetics of the oxidation process, the rate will depend in some way on the concentration of trimethylstibine. At any time during the experiment, and especially during continuous collection, the concentration of trimethylstibine above the culture would be quite low and so the rate of oxidation would most probably be minimal. In the absence of evidence to the contrary, we prefer to believe that the methylantimony species present in the media are the final biotransformation products of antimony(III) from aerobic cultures.

It may be that under anaerobic conditions trimethylantimony(V) species can be reduced to trimethylstibine and in the bioreactor experiment, where the system was closed at times, anaerobic microenvironments may have formed where this reduction could occur. Other researchers have found that trimethylstibine was formed when a culture of *S. brevicaulis* was made anaerobic at the end of its growth in an antimony-rich media.[9]

As reports of antimony methylation are becoming more common, a number of problems surface. The nonvolatile compounds, detected within the media, need to be more fully characterized so that we may understand the process of their formation. It will be important to develop liquid-chromotographic techniques to separate these compounds, and to do this there is a need for methylantimony standards so that speciation techniques can be developed. Improved methods are also required to separate inorganic antimony from trace levels of organoantimony so that efficient recovery of all organoantimony species can be achieved. The possibility that organoantimony compounds may not be susceptible to hydride generation must be considered. Other species of fungi and different *S. brevicaulis* strains need to be studied to see if there are any differences in the rates of production and amounts of methylantimony species. Anaerobic micro-organisms also need to be thoroughly investigated. The impact of these processes on the environment need be considered. For arsenic these problems have all been solved to some extent. For antimony the solutions may be found more quickly by applying modern analytical techniques and the lessons learned from arsenic.

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