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Confirmation of the Biomethylation of Antimony Compounds

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We have evidence that an organic and an inorganic salt of antimony were reduced and methylated biologically by microorganisms in laboratory experiments. The organoantimony compound produced was trimethylstibine [$(CH_3)_3Sb$] and was detected in a culture headspace. This was confirmed by matching the compound's retention time in capillary gas chromatography, as detected by fluorine-induced chemiluminescence, with a commercial standard and by its mass spectrum determined with gas chromatography/mass spectrometry (GC–MS). $(CH_3)_3Sb$ was detected in the headspace of soil samples amended with either potassium antimonyltartrate or potassium hexahydroxyantimonate and augmented with any one of three different nitrate-containing growth media. The identity of the microorganisms in soil that accomplished this are as yet unknown. Of 48 soil samples amended with these two compounds, 24 produced trimethylstibine. Bioreduction of trimethyldibromoantimony was also detected in a liquid monoculture of *Pseudomonas fluorescens* K27 which also produced trimethylstibine. This headspace production of $(CH_3)_3Sb$ was determined to be linked to the culture's cell population as measured by optical density. This microbe, however, did not biomethylate either potassium antimonyltartrate or potassium hexahydroxyantimonate in any experiments we performed. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

The history of the biomethylation of metalloids goes back at least to the first quarter of the nineteenth century;[1–6] however, in the case of arsenic the mystery of the nature of the evolved volatile products of this process came to an end in 1933 when Challenger established that trimethylarsine was produced by fungal cultures.[7] Soon, work in his laboratory demonstrated that methylation of selenium[8] and tellurium[9] are also possible. Since then biomethylation of tin,[10–13] mercury[14–16] and possibly lead[17, 18] has been detected and confirmed.[19]

There have also been a few experiments[20–23] with negative or inconclusive findings on the biomethylation of compounds containing antimony, an element in the same group of the Periodic Table as arsenic. Challenger and Ellis[22] inoculated molds on breadcrumbs and added a solution of potassium antimonyltartrate (tartar emetic or PAT). Aspiration of the evolved products through Biginelli's solution gave neither a precipitate nor an odor after 30 days. They then inoculated the molds in six flasks with an aqueous medium that contained only the necessary inorganic salts. Into three of the flasks they added PAT and glucose, and into the other three they added only PAT. Again, there was no precipitate and no odor after 33 days. They then put the flasks away and analyzed them nine months later. When the medium was filtered it contained no antimony; whereas the washed mycelium contained opaque shiny crystalline

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particles. They proposed this compound to be antimony trioxide since it dissolved upon washing with tartaric acid. They concluded that the mold had consumed the tartrate and excreted antimony trioxide. One of Challenger's students, Barnard,[23] used the monosodium salt of phenylstibonic acid ($NaHPhSbO_3$), and potassium antimonate ($KSbO_3$) as substrates for *Scopulariopsis brevicaulis* and *Penicillium notatum.* He aspirated the volatile products of growth into nitric acid over many days and then analyzed the solution by converting any trapped antimony to SbH_3 with subsequent decomposition and detection as an elemental antimony mirror (Marsh test). Alternatively, he passed the volatile products through a filter paper impregnated with silver nitrate solution and checked for a brown stain on the paper corresponding to a silver complex, again derived from stibine and possibly methylated antimony as well (Gutzeit test). *P. notatum* produced a volatile antimony compound but the amount was small and the nonspecific nature of the test made it difficult to draw any conclusions as to its identity. Samples of *P. notatum* amended with dimethylstibinic acid salt [$(CH_3)_2SbO_2Na$] also produced positive results; however, this could have been because of the redistribution of the methyl groups or reduction to dimethylstibine [$(CH_3)_2SbH$] by the inorganic salts, rather than a biological activity. Craig states:[24] 'This latter work remains tantalizing and it is surprising that it has not been repeated with modern analytical methods for detecting and identifying the evolved products . . . This is an area which appears to offer rich dividends for a reinvestigation.'

Considering the similarities between antimony and all the elements that are known to be biologically methylated, there seems to be no compelling reason for this biological process not to occur with antimony compounds. Parris and Brinckman[25] state that 'there is no obvious thermodynamic or kinetic barrier to biomethylation and the chemical similarities between antimony, tin, lead, arsenic, selenium and tellurium which literally surround antimony in the Periodic Table, and all of which have been shown to be subject to biomethylation, would suggest biomethylation pathways for antimony.' Indeed, Brinckman and co-workers reported a bacterium able to oxidize Sb^{3+} to Sb^{5+}.[26] Thayer[27, 28] suggests that Challenger's mechanism for the reduction and biomethylation of arsenic and selenium might also apply to antimony when the similarities in the reduction potentials of these metalloids are considered: $H_2AsO_4^-/HAsO_2$, +0.662 V; $SbO_3^-/HSbO_2$, +0.678 V; $HSeO_4^-/H_2SeO_3$, +1.09 V; $HTeO_4^-/HTeO_3^-$, +0.813 V.

Andreae *et al*.[29] appear to have found methylstibonic acid, [$(CH_3)SbO(OH)_2$; MSA], and dimethylstibinic acid [$(CH_3)_2Sb(O)OH$; DMSA] in marine and estuarine environments. Among various river waters and seawaters analyzed, they reported the amounts of MSA ranged from 0.5 ppt (Sb) in Ochlockonee River, FL, up to 12.6 ppt (Sb) in Ochlockonee Bay estuary, FL. DMSA was not found in the rivers but its concentration ranged from 0.6 ppt (Sb) in Ochlockonee Bay estuary, FL, to 3.2 ppt (Sb) in the Gulf of Mexico. In later research on the Baltic Sea, Andreae and Froelich[30] found MSA and DMSA at concentrations of 6.1 and <1.2 ppt (Sb) respectively, although Thayer and others have recently called these conclusions into question.[28, 31] Finally, unidentified 'volatile antimony compounds' were anaerobically purged from soil during anaerobic incubation following potassium antimonyl tartrate amendment (75 ppm Sb^{3+}; 45-day incubation at 20 °C).[32]

Probably the most important problem in detection of trimethylstibine is its fast oxidation in the gas phase. Parris and Brinckman[25] found the gas-phase rate constants for the oxidation of trimethylstibine and trimethylarsine to be 10^3 and $10^{-6}\,\text{M}^{-1}\,\text{s}^{-1}$ respectively. With respect to the detection of trimethylstibine, this fast gas-phase oxidation points to the major downfall of Barnard's experiments in which the evolved gases were aspirated with sterile air into a solution to give a precipitate for further analysis. With such a fast oxidation rate, it is very probable that trimethylstibine was oxidized to trimethylstibine oxide [$(CH_3)_3SbO$] before it reached the solution to give a precipitate. Barnard thought that this could be his problem and therefore tried to decrease the amount of oxygen in the aspiration air to 8% by adding nitrogen. Low oxygen content, on the other hand, obstructed the growth of the molds in his experiments.[19, 23]

The possibility of biomethylation becomes more important when the current extensive use (*ca* $2\times10^7\,\text{kg y}^{-1}$ in the USA and *ca* $1\times10^7\,\text{kg y}^{-1}$ in Japan) of inorganic and organic antimony compounds in conjunction with halocarbons in fire-retardant systems is considered. Textiles, drugs, plastics, elastomers, paper, wood, paints, fire-retardant systems and coatings

are just some of the products that contain antimony and that are used daily.[33]

It is surprising that there has been so little work on the biomethylation of antimony since 1947, in spite of its importance and the potential of antimony for biomethylation. Many instruments and techniques have now been developed to study metals and organometals found at extremely low concentrations in biological and environmental samples. Flame[34, 35] and flameless[36] atomic absorption spectroscopy and high-performance liquid chromatography–inductively coupled plasma spectroscopy[37] have been used for detecting and analyzing antimony(III) and antimony(V) in various samples. The only reported detection of methylantimony compounds to date,[29, 30, 38] though, used graphite furnace atomic absorption spectrometry with hydride generation based on that successfully used for arsenic. For instance, antimony(III), antimony(V), methylstibonic acid and dimethylstibinic acid were converted into the corresponding hydrides by using sodium borohydride ($NaBH_4$) and separated by passing these hydrides through a column filled with 5% dimethyldichlorosilane in toluene. The separated stibines were then introduced into the graphite furnace by a glass connection tube and their absorbances detected at 217.6 nm. Dodd *et al.*[31] suggested that organoantimony reports based on this procedure be cautiously interpreted because of the problematic extrapolation of successful analytical methods for arsenic to similar methods for the speciation of antimony. Their criticism,[31, 39] specifically involves the problem of molecular rearrangements during the hydride generation step in batch-type generators that had not been extensively preconditioned. Quite recently these Canadian workers (Dodd *et al.*) have reported the determination of methylstibine species in extracts from a freshwater plant collected from a pond influenced by gold-mine drainage. Their method used a semi-continuous type of hydride generation–gas chromatography–mass spectrometry.[39]

Since oxidation of trimethylstibine is probably the major problem for its detection, we propose that certain cultures of bacteria that are grown under anaerobic conditions in the laboratory would produce trimethylstibine in this reducing atmosphere. Chemiluminescence detection following capillary gas chromatographic separation has been proved to be highly selective and sensitive to alkyl-sulfur, -selenium, -tellurium, -phosphorus and to a lesser degree to alkyl-tin, -lead, -mercury and -arsenic compounds.[40, 41] We also thought that because of the similarities between the above-mentioned elements and antimony, the fluorine-induced chemiluminescence detector might also be able to detect trimethylstibine with a comparable selectivity and sensitivity. In addition to the identification by retention time of a trimethylstibine standard, some of the samples examined in this research were also analyzed by a gas chromatography–mass selective detector (GC–MS).

This report, therefore, describes the search for organisms that can reduce and/or methylate antimony compounds. To this end, single cultures of a bacterium that is known to reduce and methylate selenium were examined by amending them with various antimony compounds to determine whether they exhibit this ability. Also, soil samples collected in and around Huntsville, TX, and a sample from an arsenic-contaminated site in Switzerland were examined regarding the same question. Three antimony-containing compounds were used for amendment: trimethyldibromoantimony [$(CH_3)_3SbBr_2$; TMDBA], potassium antimonyltartrate (tartar emetic; PAT), and potassium hexahydroxyantimonate(V) [$KSb(OH)_6$; PHA]—which is actually the hydrated form of the antimonate used by Barnard.[23] After chemical amendment with an antimony-containing compound, the headspaces above the amended cultures were subsequently analyzed for biologically produced organoantimony compounds by gas chromatography coupled to either fluorine-induced chemiluminescence or mass spectrometric detection.

EXPERIMENTAL

Reagents

All chemicals used in this project were of analytical reagent grade and were used without further purification unless specified differently. Ethyl ether, methyl iodide, antimony(III) chloride, bromine, carbon tetrachloride, potassium antimonyltartrate trihydrate (PAT), potassium hexahydroxyantimonate(V) (PHA), dimethyl disulfide (DMDS), dimethyl sulfide (DMS), HPLC-grade acetonitrile, potassium hydrogen

phosphate, potassium dihydrogen phosphate and sodium molybdate dihydrate were purchased from Aldrich Chemical Company, Inc. (St. Louis, MO, USA). Trimethylstibine (TMSb) was procured from Organometallics, Inc. (East Hampstead, NH, USA). Magnesium turnings, ethanol, sodium citrate (ethylenedinitrilo)tetra-acetic acid disodium salt and sodium chloride were obtained from EM Science (Gibbstown, NJ, USA). Ammonium sulfate and glycerol were obtained from J. T. Baker Inc. (Philipsburg, NJ, USA). Nitrilotriacetic acid, magnesium sulfate heptahydrate and succinic acid were acquired from Sigma Chemical Co. (St. Louis, MO, USA) and potassium hydroxide, glutamic acid and calcium chloride dihydrate were obtained from Fisher Scientific (Pittsburgh, PA, USA). Trimethyldibromoantimony (TMDBA) was synthesized in our laboratory as described below since it was not commercially available.

Biological media

All the media used in this project were derived from DM medium[42] which was prepared as follows: 7.0 g dibasic potassium phosphate, 3.0 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 10.0 g of 50% glycerol, 0.5 g sodium citrate and 0.1 g $MgSO_4 \cdot H_2O$ were dissolved in 0.9 l deionized water and the pH was adjusted to 7.4 by adding 0.1 M potassium hydroxide before deionized water was added to a final volume of 1.0 l. To provide nitrite as a biological electron acceptor, DM with nitrate medium (DM–N) was prepared by adding 1.0 g l^{-1} KNO_3 to DM medium. DMVTE medium (DM–N medium plus vitamins and trace elements) contained everything in DM–N medium plus 2.0 ml l^{-1} of vitamin solution (100 mg nicotinamide, 50 mg thiamine HCl, and 2 mg biotin in 100 ml deionized water) and 1 ml $^{-1}$ l of trace elements solution (2.8 g boric acid, 2.1 g $MnSO_4 \cdot H_2O$, 40 mg $Cu(NO_3)_2 \cdot 3H_2O$, 240 mg $ZnSO_4 \cdot 7H_2O$, 750 mg $Na_2MoO_4 \cdot 2H_2O$, and 6.0 g (ethylenedinitrilo)tetra-acetic acid disodium salt in 1000 ml deionized water). DMAATE medium was prepared by adding 20 ml l^{-1} amino acids and salt solution (10 g nitrilotriacetic acid, 2.0 g aspartic acid, 5.0 g glutamic acid, 11.0 g KOH, 29.5 g $MgSO_4 \cdot 7H_2O$, 3.3 g $CaCl_2 \cdot 2H_2O$, 100 mg $FeSO_4 \cdot 7H_2O$ in 1000 ml deionized water) and 1 ml l^{-1} trace elements solution (see DMVTE medium above) to DM–N medium.

Synthesis of trimethyldibromoantimony (TMDBA)

TMDBA was synthesized by bromination of $(CH_3)_3Sb$ (TMSb), obtained by reacting antimony(III) chloride with a Grignard reagent, methylmagnesium iodide.[43] The TMDBA crystals produced in this process were transferred to a vial for storage at room temperature. A sample of the recrystallized product was sent to Galbraith Laboratories (Knoxville, TN, USA) for elemental analysis. A small amount of the TMDBA sample was pressed in a potassium bromide pellet and the Fourier-transform infrared spectrum of TMDBA was obtained by a BOMEM DA3 FTIR spectrometer. A 60 MHz Varian EM 360 NMR spectrometer was used to take the proton NMR spectrum of this TMDBA: a few milligrams of the sample were dissolved in 10 drops of chloroform-*d* with 1% (v/v) tetramethylsilane (TMS; Aldrich Chemical Co., St. Louis, MO, USA) added. The spectrum was obtained at room temperature. A melting-point analysis could not be performed with TMDBA since at approximately 50 °C this compound decomposes into dimethylbromoantimony and methyl bromide.[43]

Culture amendment experiments

The first antimony amendment experiments used in this research project were performed with *Pseudomonas fluorescens* K27, isolated from Kesterson Reservoir, CA,[44] by Ray Fall of the University of Colorado, Boulder, CO, USA, who also supplied the strain for this project. This bacterial strain is known to reduce and methylate selenate and selenite and release organoselenium compounds into culture headspace.[41, 45] Aerobic precultures of K27 were grown on DM medium that had been previously filter-sterilized (0.2 μm membrane filter, VWR Scientific, Sugarland, TX, USA). The required concentrated stock solutions of the three antimony compounds (TMDBA, PAT and PHA) were prepared in DM–N medium and sterilized by the same method. After the bacterial inoculation was made into 50-ml sterile Erlenmeyer flasks and the mouth was closed with a sterile cotton plug, the cultures were incubated in a water-bath shaker at 30 °C overnight with shaking (105 rpm). The volumes of these precultures varied, depending on the number of tubes prepared in the next step;

however, 30 ml was a typical preculture volume. After 24 h of incubation, these aerobic precultures were diluted 1:1 with the desired stock antimonial DM–N solution in triplicate into autoclaved Hungate tubes (VWR Scientific) to give 10 ml of antimony-amended bacterial culture (5 ml preculture + 5 ml sterilized antimony-containing DM–N media) with approximately 6 ml of headspace gas. The tubes were then tightly capped with open-top screw-caps sealed with Teflon® septa (VWR Scientific). The cultures were then incubated anaerobically at 30 °C in a water bath for 24 h until the headspaces were analyzed. No effort was made to exclude oxygen from these tubes; however, *P. fluorescens* is a facultative anaerobe and quickly uses up all headspace and dissolved oxygen in these experiments, and begins anaerobic growth within a few hours. Bacterial growth was monitored (by optical density) during incubation using a Klett–Summerson photoelectric colorimeter (Klett Mfg Co., NY, USA) using a green filter ($\lambda_{max} = 526$ nm). Tubes were removed from the water bath and the optical density was measured using a matching tube containing sterile culture medium as the blank.

One set of controls for these experiments was prepared by diluting the aerobically grown preculture 1:1 with sterile filtered antimony-free DM–N medium. Another control set was prepared by mixing the sterile antimony-free DM–N medium with the desired sterile stock antimonial-containing DM–N medium 1:1. A third control contained just the sterile DM–N medium. All these controls were prepared in triplicate in Hungate tubes again to give 10 ml solutions, capped with open-top screw-caps with Teflon septa for headspace analysis, and incubated anaerobically at 30 °C in a water bath for 24 h, thereby duplicating the handling of the samples that contained antimony.

Time-course experiments

The time-course experiments that followed were carried out only for *P. fluorescens* cultures amended with TMDBA. For these experiments, a series of sample tubes was prepared as described above and the production of TMSb was measured by capillary GC–fluorine-induced chemiluminescence detection in triplicate tubes analyzed over time along with the bacterial growth which was monitored by optical density. Three tubes were analyzed at each time point and the results averaged together.

Soil enrichment experiments

Three different soil samples were used for enrichment of organisms that reduce and/or methylate the examined antimony compounds. Sample 1 (SS—Swiss soil) was collected from an abandoned leather tannery in Dübendorf, Switzerland, that was contaminated with arsenic (>1000 ppm As) and was supplied by Professor Reinhard Bachofen of the University of Zürich, Institute for Plant Biology and Microbiology, Zürich, Switzerland. Sample 2 (SPS—sewage plant soil) was collected from the sewage plant facility of Huntsville, TX, USA. Sample 3 (BSS—body shop soil) was collected from the back yard of an auto repair shop in Huntsville, TX, USA. Both of these US soil samples were collected into sterile plastic sample bags and stored in our laboratory at room temperature.

These soil samples were inoculated into three different culture media: DM–N, DMVTE, and DMAATE. Samples of 0.20 g from these soils were weighed and transferred to autoclaved Hungate tubes. A volume of 10 ml of a specific sterile antimonial stock medium with a known antimony concentration was added to each of the soil-containing tubes. The tubes were then tightly capped with open-top screw-caps with Teflon septa. The samples were incubated anaerobically at 30 °C in a water bath. After two weeks of incubation the headspace in each tube was analyzed.

Based on our results from the above preliminary experiment, we chose to continue with BSS as our soil sample and DM–N as our growth medium. Samples of 0.20 g of BSS were weighed and transferred to sterile Hungate tubes. These soil-containing tubes were divided into six groups with 24 samples in each group. To the first group was added 10 ml of sterile DM–N. To the second and the third groups were added 10 ml of sterile 0.01 mM PAT in DM–N and 0.01 mM PHA in DM–N respectively. All the tubes of groups 1, 2 and 3 were capped with open-top screw-caps with septa. The fourth, fifth and sixth groups were capped and autoclaved at 121 °C for 1 h. These soil samples were autoclaved twice more, after one and three days, to ensure sterilization of outgrown spores. Then they were opened and sterile DM–N, 0.01 mM PAT in DM–N and 0.01 mM PHA in DM–N

were added to groups 4, 5 and 6 respectively. All these tubes were then incubated at 30 °C in the water bath and their headspaces were analyzed after one month.

Gas chromatography–fluorine-induced chemiluminescence detection

A Sievers 300 sulfur chemiluminescence detector (Sievers Instruments, Boulder, CO, USA), coupled with a Hewlett Packard (Norwalk, CT, USA) 5890 Series II gas chromatograph was used for the analysis of headspaces above cultures. The chromatographic column used was a 30 m 0.32 mm i.d. capillary (Alltech Associates, Deerfield, IL, USA) with 1 μm 5% phenyl, 95% methyl polysiloxane as the stationary phase. The carrier gas used was technical-grade helium (Bob Smith Gas Products, Huntsville, TX, USA). The injector temperature was 275 °C, the inlet head pressure was 0.76 bar and the carrier flow was 1 ml min^{-1}. The fluorine-induced chemiluminescence detector has been described elsewhere.[41] The resulting signal from the detector was recorded by a Hewlett Packard 3396 Series II integrator. For all headspace analyses, the temperature program used consisted of 1 min of cryogenic trapping at −20 °C (oven cooled by using liquid nitrogen) followed by a temperature ramp of 20 °C min^{-1} to a final temperature of 250 °C.

Calibration of fluorine-induced chemiluminescence detection with trimethylstibine

The calibration of the response of the fluorine-induced chemiluminescence detector to TMSb was accomplished using standards serially diluted in a nitrogen-purged glovebox. Ten serial dilutions of TMSb were made into acetonitrile from a neat TMSb solution, used as received from the vendor. Screw-cap vials (capacity 2 ml; Alltech Associates) were used to store the standards. Every bottle or vial was kept in an ice bath during dilutions—and the subsequent analyses by GC—to prevent reagent loss and retard oxidation by atmospheric oxygen. Triplicate splitless injections of 1 μl from each standard vial using a 10-μl syringe (Alltech Associates) were made to the gas chromatograph. The chromatographic analyses were performed as described above. The linear least-squares fit for the plot of the logarithm of the TMSb peak area versus the logarithm of the mass of TMSb injected, and the relative standard deviation of each triplicate, were calculated. A signal-to-noise ratio (S/N) of 3:1 was used to calculate the detection limit. All headspace concentrations (see below) were calculated based on this solution-phase calibration.

Gas chromatography–mass spectrometry

In order to identify any biologically produced organoantimony compounds, a Hewlett Packard 5890 Series II Plus gas chromatograph, coupled to a Hewlett Packard 5972 mass spectrometer (GC–MS) was used. Ultrahigh-purity helium (Bob Smith Gas Products) was used as the carrier gas. The injector was kept at 275 °C and an electronic pressure control was used to maintain the inlet pressure at 0.76 bar and the carrier gas flow at 1 ml min^{-1}. A 30 m, 0.25 mm i.d. capillary column with a 1 μm stationary phase of 5% phenyl, 95% methyl polysiloxane was used for the chromatographic separation (J & W Scientific, Folsom, CA, USA). The oven temperature program was identical to that for the fluorine-induced chemiluminescence chromatography described above. The effluent from the column was ionized by a filament with an ionization energy of 50 eV. The resulting positively charged ions were focused into a stainless steel quadrupole. The masses from 35 to 500 amu were scanned three times every 2 s and were detected by an electron multiplier maintained at 2106 V. The signal was processed by a Hewlett Packard Vectra computer using Hewlett Packard ChemStation software which was also used to control the GC–MS. The National Institute of Standards and Technology (NIST) mass spectral library (Hewlett Packard) was used to compare the resulting mass spectrum of the compounds.

Headspace analyses

All the headspace samplings were performed using 1-ml gas-tight syringes with push-button valves (Alltech Associates). Tubes were sampled only once since pierced septa were not considered to be gas-tight. The used syringe was then cleaned in a device consisting of a simple filtration flask which was heated gently by a hotplate, while air was passed through the flask and the disassembled syringe to sweep out the

volatile compounds left in the syringe. We used three syringes and two syringe-cleaning devices at the same time, which allowed around 40 min of cleaning per syringe. Although 40 min was more than sufficient for cleaning in most cases, a syringe check run was performed by injecting 1 ml of laboratory air into the GC. Peak areas were used to calculate the gas-phase concentrations of TMSb.

RESULTS

The elemental analysis of TMDBA yielded satisfactory agreement with theoretical values (in parentheses): C, 10.88 (11.03); H, 2.64 (2.78); Sb, 36.47 (37.27); Br, 50.14% (48.92%). The IR and NMR spectra also confirmed the success of this synthetic procedure.[25, 46, 47]

The fluorine-induced chemiluminescence detector did indeed respond sensitively to TMSb as we had suspected. The calculated detection limit ($S/N = 3:1$) was 15 pg and the response was linear over three orders of magnitude. The slope of the log/log plot was 0.942 ± 0.018 at the 95% confidence level.

Results from the time-course experiments involving *P. fluorescens* amended with 0.1 mM TMDBA are displayed in Fig. 1, where headspace concentration of TMSb (in parts per million by volume; ppmv) and the cultures' optical density (OD) are plotted versus time. OD is a measure of culture cell population, below a limiting opacity, and has been confirmed as linear in this range by colony count experiments carried out in our laboratory. Time-course experiments of cultures amended with 0.01 M TMDBA were substantially similar (data shown elsewhere).[47]

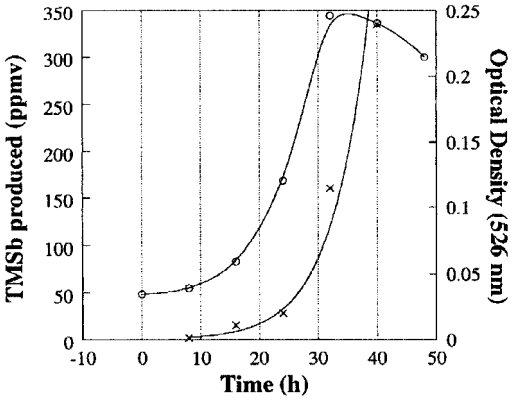

Figure 1 Time course of trimethylstibine (TMSb) production by *P. fluorescens* K27 in replicate cultures amended with 0.1 mM (12 ppm Sb) trimethyldibromoantimony (×) as measured by GC fluorine-induced chemiluminescence. The right hand y-axis (○) shows optical density and is a relative measure of the culture's cell population.

Table 1 summarizes experiments in which three soils were augmented with three different growth media and amended with PAT and PHA in order to see if biomethylation of these compounds was possible. The culture medium used to augment the soil and antimony compound with which the soil was amended is detailed along with the headspace concentrations of TMSb (in parts per billion by volume; ppbv). These are results from single-tube analyses, not averages.

Figure 2 is the chromatogram (produced using fluorine-induced chemiluminescence) of the headspace gas sampled above BSS soil amended with 0.01 M PHA (1.2 ppm Sb), augmented with DM–N medium, and incubated at 30 °C for 1 month. TMSb appears at a retention time of approximately 4.2 min and the peak in Fig. 2

Table 1 Amounts[a] of gas-phase trimethylstibine determined in the headspace of SS, SPS, and BSS soil samples in various media amended with potassium antimonyltartrate (PAT) and potassium hexahydroxyantimonate (PHA)

Soil sample	With PAT amendment			With PHA amendment		
	DM2	DMVTE	DMAATE	DM2	DMVTE	DMAATE
SS	0	0	0	0	0	0
SPS	566	2127	3348	1211	969	3003
BSS	1416	4358	896	4897	0	903

[a] TMSB gas-phase concentrations are in parts per billion by volume (ppbv).

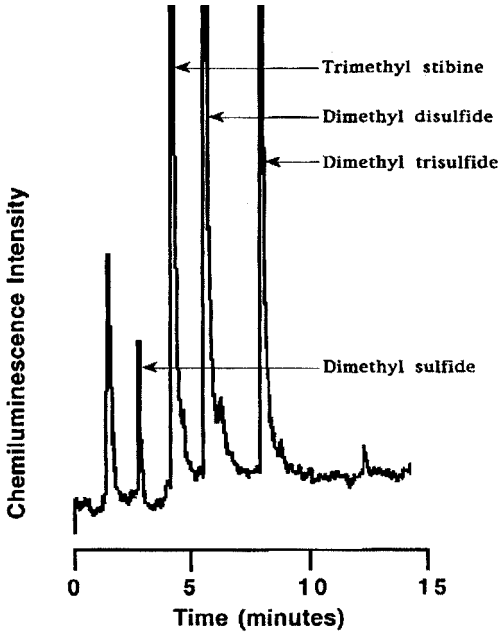

Figure 2 The GC–fluorine-induced chemiluminescence chromatogram of the headspace of BSS soil in DM–N amended with 0.01 mM (1.2 ppm Sb) potassium hexahydroxyantimonate(V). The headspace was analyzed one month after amendment.

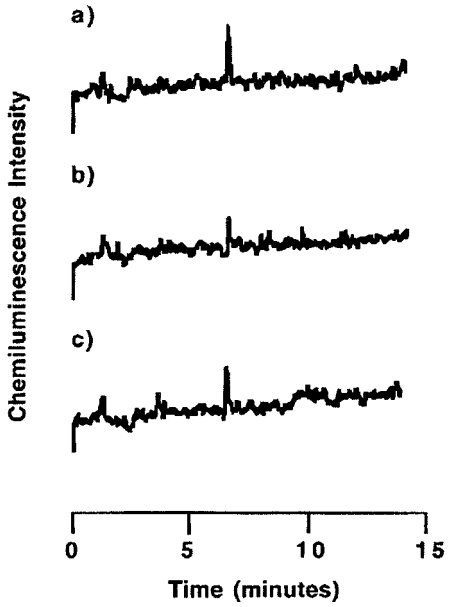

Figure 3 The GC–fluorine-induced chemiluminescence chromatogram of the headspace analyses of sterilized BSS in DM–N amended with (a) 0.01 mM (1.2 ppm Sb) potassium hexahydroxyantimonate(V); (b) 0.01 mM (2.4 ppm Sb) potassium antimonyltartrate; (c) no antimony compounds.

corresponds to a gas-phase concentration of 7.2 ppmv TMSb (5.3 ppmv Sb). Also prominent in this chromatogram are organosulfur compounds that we often see with this sulfur-selective detector when analyzing anaerobic bacterial headspaces.

Figure 3 shows three blank (chemiluminescence) chromatograms of this same soil sample, each sterilized and then amended with sterile DM–N medium and either PHA, PAT or no antimony compound at all (groups 4, 5 and 6). These chromatograms show none of the biologically produced compounds seen in the headspace above unsterilized soil samples such as those of Fig. 2. The small peak at 6.5 min was unidentified; but as Fig. 3 shows, the peak did not change substantially in size when a sample was augmented with an antimony-containing compound.

Figure 4 displays a total ion chromatogram for the GC–MS analysis of the headspace above a BSS soil sample amended with 0.01 mM PHA, augmented with DM–N and incubated for 30 days. This is not a selected ion chromatogram. Autoscaling has de-emphasized the other peaks present. Using similar chromatographic conditions to the above experiments—column carrier gas, etc.—the retention time of TMSb was 4.188 min (Fig. 4a) in this system. Figure 4b displays the mass spectrum at 4.188 min and Figure 4(c) the NIST library spectrum for trimethylstibine. Major mass fragments[50] are noted (Fig. 4b). The two major naturally occurring antimony isotopes are nominally ^{121}Sb (57.25%) and ^{123}Sb (42.75%).

DISCUSSION

The response of the fluorine-induced chemiluminescence detector to trimethylstibine expands the suite of biologically important methylated compounds that it can detect. The relatively large variability in the TMSb log/log plot determined using commercial TMSb standards actually mirrors that found with other extremely air-sensitive methylated compounds examined with this detector.[48]

When a pure culture of *P. fluorescens* K27 was inoculated in DM–N medium with 0.01 mM concentrations of either TMDBA, PAT trihydrate

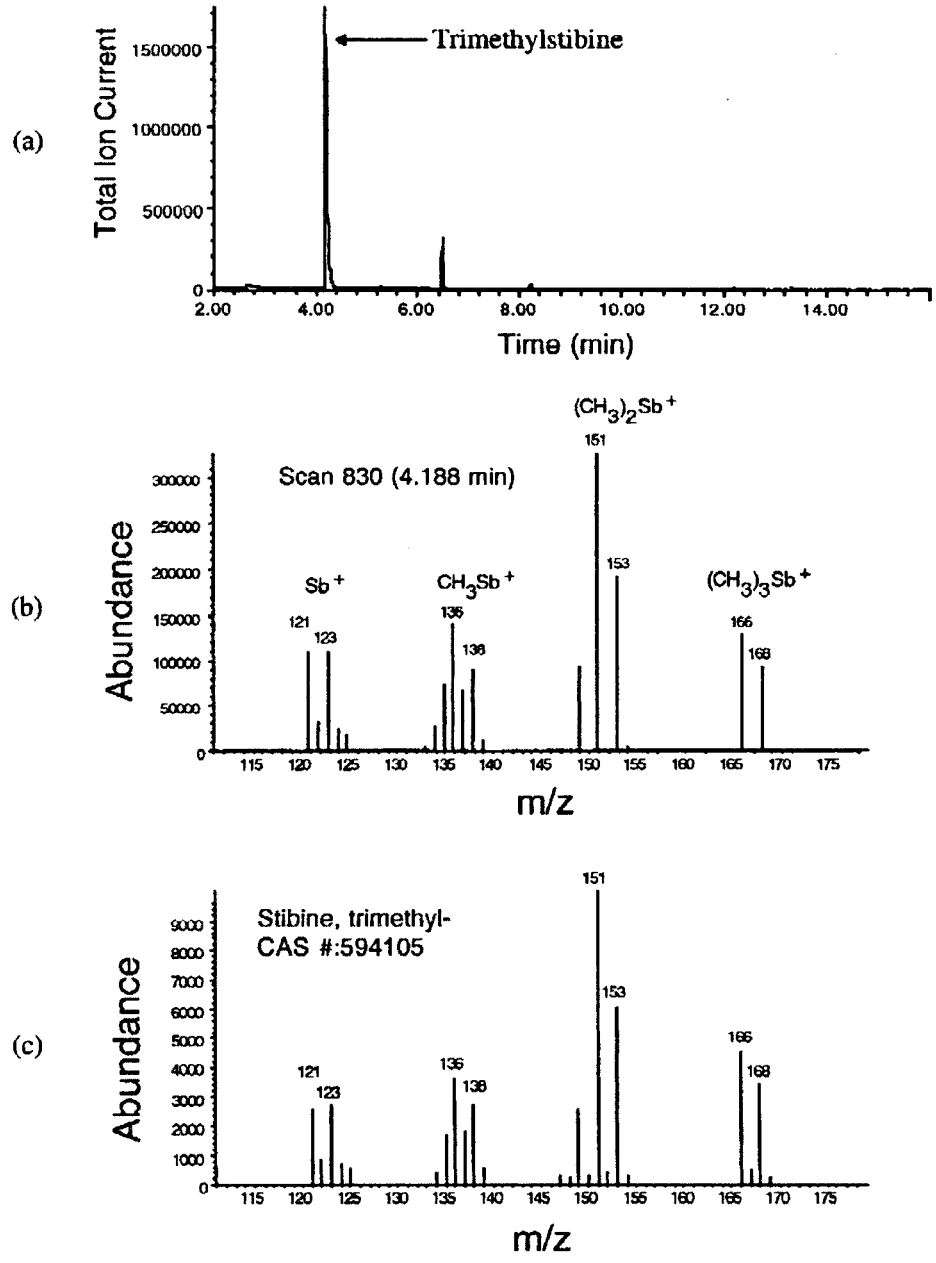

Figure 4 (a) Total ion chromatogram of the headspace above BSS soil sample amended with 0.01 mM (1.2 ppm Sb) potassium hexahydroxyantimonate(V), one month after amendment. (b) EI mass spectrum of trimethylstibine found in the cultured soil's headspace (peak at RT = 4.188 min). (c) Reference mass spectrum of trimethylstibine from NIST mass spectral library.

(2.4 ppm Sb^{3+}), or PHA (V) (1.2 ppm Sb^{5+}), TMSb was found after 24 h only in the headspace of samples that contained TMDBA. The sterile blanks for TMDBA amendment did not produce any detectable TMSb. This means that the reduction of TMDBA to TMSb in these samples is the result of biological activity. Similar bioreduction activity has been determined for this bacterium when it is amended with metalloid salts of selenite or selenate.[41, 45]

The data in Fig. 1 show that as soon as 8 h after amendment (in the early log phase of

growth) the production of TMSb becomes measurable. The production of TMSb by K27 starts to increase exponentially as the culture reaches the stationary phase of growth (as measured by optical density). In the K27 culture tubes that were amended with either PAT and PHA (see reasoning below) no bacterial growth was observed even after 1 month of incubation at 30 °C. The lack of growth in these cultures indicates that these two compounds are inhibitory to growth of *P. fluorescens* K27 (at the concentrations tested) and therefore no production of trimethylstibine from 0.1 mM PAT or PHA was observed.

Although the above experiment shows the *reduction* of an antimony compound by a bacterium, ambiguously reported in the literature up to this point, these results still lack the conclusive evidence for *biomethylation* of antimony since the compound that was successfully reduced, TMDBA, already contains three methyl groups. The next set of experiments, therefore, used two antimony compounds—that contain no methyl groups—as amendment agents to test for biological reduction *and* methylation using microbes other than K27. The first compound, potassium antimonyltartrate, was chosen since it was used by Challenger in his experiments over 60 years ago[19, 22] and in a more recent inconclusive PAT amendment experiment.[32] The second, PHA, was used (1) because it is a completely inorganic form of soluble antimony and (2) because of the historical significance of Challenger's doctoral student's (Barnard's) promising but inconclusive experiments in 1947.[23] Since *P. fluorescens* did not methylate PHA or PAT, we used soil samples to enrich for organisms that might show antimony methylation activity. Three different soil samples (SS, SPS and BSS) were collected and inoculated into different biological media (DM–N, DMVTE, and DMAATE) to increase the chances for biological growth that could result in the production of trimethylstibine. So far, no pure cultures of microorganisms responsible for this activity have been isolated. The presence of the soil in the tube made it impossible to measure the bacterial growth by optical density using the Klett meter. Therefore, we examined for growth by shaking the tubes gently without disturbing the soil and looking for a turbidity in the solution which would suggest bacterial growth. There was bacterial growth in *all* soil samples amended with either PAT or PHA; but when the headspaces in the antimony-amended tubes that contained soil from the abandoned tannery (SS) were analyzed by GC with fluorine-induced chemiluminescence detection, no TMSb was found (detection limit 9 ppbv as Sb). On the other hand, TMSb was detected in the headspaces of most of the tubes that contained the other two soil samples amended with antimony compounds (Table 1).

In order to narrow the scope of our experiments and to confirm that the process of methylation was entirely biological in nature, we chose soil collected at the automobile body shop (BSS) as our soil sample and the simplest medium (DM–N) as our augmentation medium. This combination had proved to produce significant amounts of TMSb in the experiments described above. In these subsequent experiments, out of 144 BSS samples examined (six sets of 24 samples each), 48 unsterilized soil samples were amended with either PHA or PAT (groups 2 and 3) and, of those, 15 tubes that contained PAT, nine tubes that contained PHA and one unsterilized soil in group 1 that contained no *added* antimony compounds were found to have TMSb in their headspaces when analyzed by GC with fluorine-induced chemiluminescence detection. This means that 24 of 48 antimony-amended soil samples produced TMSb. The reason for the lack of TMSb production in the remaining unsterilized yet antimony-amended soil samples is unknown, but may stem from soil sample inhomogeneity and the difficulty of successfully growing reproducible cultures inoculated from field samples.

Figure 2 is a GC–chemiluminescence chromatogram of one of the soil experiment tubes amended with PHA (0.01 mM). In addition to TMSb, DMS, dimethyl disulfide and dimethyl trisulfide were also found in the headspace. An old dimethyl disulfide standard from our lab analyzed with the chemiluminescence system yield a peak at the same retention times as the earliest-eluting peak in Figs 2. Based on this and previous work,[49] we assume that this peak is methanethiol; however, no methanethiol standard was used in this assessment. The remaining soil samples and all the sterile blanks (groups 3, 4 and 5: 72 tubes) produced no detectable TMSb. Figure 3 shows chemiluminescence chromatograms obtained from sterilized, DM–N-augmented soil samples amended with PHA (Fig. 3a), PAT (Fig. 3b) or nothing (Fig. 3c). The small peak at approximately 6.5 min did not

correspond to any known organoantimony or organosulfur compound and, while reproducible, was never identified. The headspace TMSb found in positive samples was identified by its retention time in capillary gas chromatographic runs with chemiluminescence detection as compared to a commercial standard. We also analyzed samples using a mass-selective detector. The mass spectrum of trimethylstibine was obtained from the chromatogram (TIC in Fig. 4a) acquired by the mass-selective detector and is displayed in Figure 4(b). This spectrum was then compared with the spectrum of TMSb in the NIST mass spectral library (Fig. 4c). Except for small differences in a few low-intensity fragments,[50] these spectra are in excellent agreement. This mass spectrum is also in substantial agreement with a recently published TMSb mass spectrum determined from an extract of pondweed from a lake contaminated with gold-mine effluent.[39]

We conclude that the presence of TMSb in the headspace of soil samples amended with PAT and PHA was due to biological activity. Furthermore, none of the headspace analyses of blanks that contained sterilized soil samples produced TMSb. Only one of the blanks that contained an unsterilized soil sample and no added antimony compounds produced a small amount (15 ppbv Sb) of TMSb, only 1.7 times the detection limit for this method. The presence of this TMSb could be due to the biomethylation of the native antimony content of that specific soil sample and, therefore, this may be the first report of TMSb originating from unamended soil samples; however, this production would require some native antimony content in this soil. Therefore, in order to confirm the presence of antimony in this soil, elemental analysis on BSS soil was performed to determine its antimony content. Four BSS samples were analyzed for antimony using inductively coupled plasma spectroscopy preceded by microwave and nitric acid digestion (procedure and data shown elsewhere).[47] Two out of four samples were found to contain no antimony at all (detection limit 1 ppb Sb) and two contained 18 and 409 ppb Sb; however, no efforts were taken to speciate the original form of antimony in the soils examined with ICP. Brannon and Patrick[32] reported ranges of 500 to 17 500 ppb Sb in soils from around the United States. Consequently, results from the only (nonamended) blank examined here that produced TMSb can still be considered as evidence for the biomethylation of antimony.

Finally, we have considered the possibility of the (abiotic) transmethylation of amended antimony in these soil samples by other organometals and organometalloids that are naturally present. In addition to the ICP analysis for native antimony reported above, arsenic, lead, tin and selenium were also determined in the BSS soil by ICP (data shown elsewhere).[47] Four soil samples were analyzed for each of these four elements, and concentrations ranged from 280 ppb Se to 2.9 ppm Sn, and all were within the limits expected of unpolluted crustal abundances for these four elements.[51] If one assumes that transmethylation of PAT or PHA can occur from organometals such as tetramethyl-lead or trimethylarsine, there is the possibility that the TMSb we have detected in nonsterile soil samples examined here stems from 'inorganic' processes. However, a number of conditions would have to conspire in order for this to be more than an insignificant contribution to the results reported here: (1) Most important is that a significant amount of the metal(loid)s determined would have to be present as the (organo) methylating form. No efforts were made to speciate these metal(loid)s as determined by ICP; however, the presence of significant amounts of methyl-arsenic, -selenium, -lead etc. is improbable, given that the mean summer temperatures are ~32 °C in the part of Texas where this soil was collected and that the sample was taken from topsoil clearly in the oxic zone where those reduced species would be relatively short-lived. Therefore these species were probably present as oxyanions, *not* reduced methylated species. (2) The success of transmethylation producing one molecule of trimethylstibine would require three (different) methylating molecules, given the inability (improbability) of a single chemical species to contribute all of its methyl groups to one inorganic antimony salt to produce trimethylstibine.

Moreover, the fact that none of the sterilized controls showed any TMSb also suggests that biotic processes were required. This last point can, of course, be challenged on the basis of the repeated autoclaving to which these soils were subjected (see above). This heating (in the presence of oxygen) would probably have oxidized and/or evaporated most of the organometal(loid)s present so that little would have been left in the sterilized controls. In fact, transmethylation has been described for partially

methylated lead and tin species (see, for instance, Ref. 52); however, for these species this process is slow and/or very inefficient and is dependent on temperature, concentration and pH. Finally, none of this accounts for the chemical reduction conditions concomitantly required when methylation of PAT and PHA is considered as an exchange reaction.[28]

In conclusion, we can say we have strong evidence that an organic and inorganic salt of antimony were reduced and methylated biologically by microorganisms in laboratory experiments. The organoantimony compound produced was trimethylstibine. This was found by amending soil samples with either of these two antimony-containing salts, and augmented with any one of three different nitrate-containing growth media. Bioreduction of trimethyldibromoantimony was also detected in a monoculture of *Pseudomonas fluorescens* K27 which also produced trimethylstibine. This headspace production was determined to be linked to the culture's cell population as measured by optical density.

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