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Biomethylation of bismuth by the methanogen *Methanobacterium formicicum*†

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In this study the bioconversion of bismuth to volatile derivatives was investigated in cultures of the common sewage sludge methanogen *Methanobacterium formicicum*. The production of volatile bismuth compounds was analysed during growth of *M. formicicum* with respect to the concentration and chemical formulation of the applied bismuth. The main volatile bismuth compound detected in the culture headspace was trimethylbismuth (TMBi), with a maximum conversion rate of up to 2.6 ± 1.8% from spiked 1 μM bismuth nitrate [$Bi(NO_3)_3$] in the culture media. This main compound proved to be stable under the incubation conditions in a CO_2–H_2 atmosphere. Bismuthine and the partially methylated bismuthines monomethylbismuth hydride and dimethylbismuth hydride were additionally detected in the late exponential growth phase, but only in the presence of low concentrations of spiked $Bi(NO_3)_3$ (10 nM, 100 nM). The conversion of bismuth to TMBi from the bismuth-containing pharmaceuticals Bismofalk® [containing bismuth subgallate and $Bi(NO_3)_3$] and Noemin® (containing bismuth aluminate) could also be observed, however, with a lower rate than found for $Bi(NO_3)_3$. *In vitro* experiments with crude extracts of *M. formicicum* suggest that the methylation of bismuth is mainly catalysed by enzyme-catalysed reactions with methylcobalamin as methyl donor.

KEYWORDS: bismuth; bismuthine; monomethylbismuth hydride; dimethylbismuth hydride; trimethylbismuth; stability; volatilization; sewage sludge; anaerobic

INTRODUCTION

Bismuth is regarded as the least toxic heavy element, and is also called the amazingly 'green' environmentally minded element.[1] Thus, bismuth is widely used in a variety of applications, such as in pharmaceuticals (e.g. for treatment of peptic ulcer disease), cosmetics (e.g. pigments), catalysts, industrial pigments, metallurgical alloys and ceramic additives (e.g. superconductors) and had a world production of about 5500 t in 1997.[2] The use of bismuth, especially its use in consumer products—as the pearlescent pigment bismuth oxychloride (BiOCl) applied in cosmetics—and in pharmaceutical products, including bismuth potassium tartrate, aluminate, carbonate, subgallate, nitrate and salycilate, has led to an increase in the amount of bismuth in waste-water streams, which end up in sewage sludge treatment facilities. Bismuth concentrations in sewage sludges are reported to be in the range of 1–5 mg kg^{-1} dry weight.[3,4] Although most bismuth salts are sparingly soluble in water at neutral pH, bismuth seems to exhibit a high susceptibility to biomethylation. The volatile compound trimethylbismuth (TMBi) has been found in gases released from municipal waste deposits and sewage gases[5–7] but only recently could the biogenic origin of this compound be demonstrated.[4] We could show that even low concentrations of bismuth in environmental settings, e.g. sewage sludge, are converted to volatile TMBi at a high rate.[4] Regarding the production of volatile derivatives related to the conversion of the respective element in sewage sludge, the conversion rate of bismuth to volatile trimethylbismuth is about 100-fold or even more than 4000-fold higher compared with the conversions of

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arsenic and tin respectively, to the corresponding volatile derivatives (on a weight basis).[4] The high conversion rate of bismuth might be due to an intrinsically favoured methylation of this metal by the microflora involved, or it might be caused by extrinsic factors such as complex-forming compounds facilitating a higher uptake of bismuth by microbial cells. An increased bismuth uptake of bacterial cells in the presence of lipophilic chelators could be demonstrated,[8] and, more recently, the biomethylation of bismuth by the methanogen *Methanosarcina barkeri* was shown to be dependent on the presence of lipophilic polydimethylated siloxanes[9] that are also present in sewage.[10]

Poisoning by bismuth and bismuth compounds has occurred more frequently during medical therapy than by exposure in the workplace, mainly causing renal failures or mental disorders.[11] Some 100 cases of encephalopathy — some of them fatal — were reported in France and Australia after the intake of bismuth-subgallate and -subnitrate in the 1970s.[12,13] Although the etiology of the encephalopathy caused by bismuth pharmaceuticals remains unclear, some authors speculated that the microflora of the intestine might be responsible for the conversion of such bismuth salts to compounds that are more soluble, leading to a higher absorption by the human body,[14–16] or are possibly converted to the more toxic compound TMBi, which caused encephalopathic symptoms in gassing experiments with cats and dogs.[17]

To gain more insight into the bioconversion of bismuth, we investigated the derivatization of bismuth in pure cultures of the common sewage sludge microorganism *Methanobacterium formicicum* with semi-continuous feeding of the organism with H_2 and CO_2 in the presence of different concentrations of $Bi(NO_3)_3$ and the bismuth-containing pharmaceuticals Bismofalk® and Noemin®.

MATERIALS AND METHODS

Strains and culture media

M. formicicum (DSMZ 1535^T) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) as a pure culture. Liquid cultures of this organism were grown under strictly anaerobic conditions in butyl-rubber-stoppered 120 ml serum bottles that contained 50 ml of liquid medium {1 l contains: 0.348 g K_2HPO_4, 0.227 g KH_2PO_4, 0.5 g NH_4Cl, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.25 g $CaCl_2{\cdot}2H_2O$, 2.25 g NaCl, 0.002 g $FeSO_4{\cdot}7H_2O$, 2 g yeast extract (Difco, Augsburg, Germany), 2 g casitone (Difco, Augsburg, Germany), 0.001 g resazurin, 0.85 g $NaHCO_3$, 1 g sodium acetate, 2 g sodium formate, 10 ml vitamin solution (2 mg biotin, 2 mg folic acid, 10 mg pyridoxine-HCl, 5 mg thiamine-HCl·$2H_2O$, 5 mg riboflavin, 5 mg nicotinic acid, 5 mg D-Ca-pantothenate, 0.1 mg vitamin B12, 5 mg *p*-aminobenzoic acid, 5 mg lipoic acid, 1000 ml twice-distilled water), 1 ml trace element solution [HCl (25%; 7.7 M) 10 ml, 1.5 g $FeCl_2{\cdot}4H_2O$, 70 mg $ZnCl_2$, $MnCl_2{\cdot}4H_2O$ 100 mg, 6 mg H_3BO_3, 190 mg $CoCl_2{\cdot}6H_2O$, 2 mg $CuCl_2{\cdot}2H_2O$, 24 mg $NiCl_2{\cdot}6H_2O$, 36 mg $Na_2MoO_4{\cdot}2H_2O$, 1000 ml twice-distilled water] pH 6.8}. The culture media were reduced by the addition of L-cysteine (0.3–0.5 g l^{-1}) and pressurized with CO_2–H_2 (200 kPa, 20%/80%, v/v) (Messer-Griesheim, Frankfurt, Germany). The cultures were grown in the dark in a rotary shaker (150 rpm) at 37 °C and were fed semi-continuously with CO_2–H_2 (200 kPa, 20%/80%, v/v).

All salts were purchased from Merck (Darmstadt, Germany) and all vitamins were purchased from Sigma–Aldrich (Deisenhofen, Germany). The chemicals used were analytical reagent grade or better.

Analytical methods

Determination of methane

The methane content in the culture headspace was analysed by withdrawing a gas sample with a gas-tight syringe and injecting it into a gas chromatograph (Hewlett Packard, 5890 II) equipped with a capillary column [J&W Scientific, DB 5, 30 m × 0.25 mm ID, coated with phenyl-methyl-silicon (5%, 95%)] and a flame ionization detector. The temperature of the injector port, the oven and the detector was set to 100 °C. The methane content in the gas samples was determined by comparison with a certified methane standard (Messer-Griesheim, Frankfurt, Germany; 50.3% methane in nitrogen).

Determination of volatile bismuth compounds

Volatile bismuth compounds in the headspace of liquid cultures were analysed by using a modified purge-and-trap gas chromatographic system coupled to an inductively coupled plasma (ICP) mass spectrometer (Fisons VG, PlasmaQuad II), as described previously.[4,9,18] The volatile bismuth compounds were identified at a mass/charge ratio of *m/z* 209 for the ^{209}Bi trace in ICP mass spectrometry (MS) and by comparison of the boiling point retention time correlation of bismuthine (BiH_3), monomethylbismuth hydride (MMBi), dimethylbismuth hydride (DMBi) and TMBi (b.p. = $1.2T_r - 64.8$; b.p. boiling point; T_r retention temperature; all temperatures in centigrade). According to the literature, the boiling points of BiH_3, MMBi and DMBi at 760 mmHg were estimated by extrapolation to be 16.8 °C, 72 °C and 103 °C[19] respectively, and the boiling point of TMBi is 108.8 °C.[19] The identification of the hydrides, therefore, has to be regarded as putative, whereas TMBi could be identified unequivocally by standard addition with a standard of TMBi synthesized as described elsewhere[20] and by fragment MS.[9] Quantification was performed by inter-element calibration using a ^{103}Rh solution (1 μg l^{-1}) as standard, as described elsewhere.[21]

All chemicals and solutions used for ICP-MS analysis were of certified high purity grade; water used for ICP-MS was prepared with a Seral PRO 90 CN (Seral, Ransbach-Baumbach, Germany).

Experimental setup

Determination of volatile bismuth compounds in cultures of M. formicicum

M. formicicum cultures were inoculated with 2% of a stock pure culture and the growth was followed by the production of methane. In the early exponential growth phase the cultures were spiked with $Bi(NO_3)_3$ (Sigma–Aldrich, Taufkirchen, Germany) or with preparations of the pharmaceuticals Bismofalk® (Falk Pharma, Freiburg, Germany) and Noemin® (Trommsdorf, Alsdorf, Germany). In the case of $Bi(NO_3)_3$, different concentrations of bismuth (10 nM, 100 nM, 1 μM, 5 μM, 20 μM) were added from a $Bi(NO_3)_3$ stock solution, which was prepared from a 1 mM $Bi(NO_3)_3$ solution in 1% HNO_3 with 50 mM EDTA and subsequent adjustment to pH 7.0 with NaOH. In the cases of the pharmaceuticals, one tablet of Bismofalk® [50 mg bismuth subgallate, 100 mg $Bi(NO_3)_3$] or Noemin® (200 mg bismuth aluminate), was broken up in a mortar and resuspended in 10 ml ultrapure water. After centrifugation (10 000*g*; 5 min) the bismuth concentration in the supernatant acidified with HNO_3 (1% final concentration) was determined by ICP-MS. Appropriate volumes of both solutions were applied to give a final concentration of 1 μM bismuth in the spiked cultures.

The content of volatile bismuth compounds in the headspace was determined at intervals of 12 to 48 h by purge and trap gas chromatography (PT-GC)–ICP-MS as described above in a time course of about 40 days. After each analysis of volatile bismuth compounds, the gas phase was exchanged with CO_2–H_2 (200–400 kPa, 20%/80%; v/v) and the cultures were incubated further at 37 °C in the dark. To avoid contamination with bacteria by the sampling procedure, ampicillin (100 μg ml^{-1} final concentration) was added to the cultures. All experiments were performed in triplicate.

Stability of TMBi

The stability of TMBi was determined at 37 °C in the dark in the presence of helium, CO_2–H_2 (20%/80%; v/v) or air atmosphere (100 kPa). For that purpose, TMBi was synthesized in stoppered 120 ml bottles with the respective gas atmosphere by the reaction of 4 μM $Bi(NO_3)_3$ solution in 1% HNO_3 with 10 μM methylcobalamin (CH_3-B12) for 15 min. Subsequently, the gas phase was transferred from the reaction flasks to evacuated flasks that had been previously purged with the respective gas atmosphere. The decay of TMBi was followed by determining the residual concentration of TMBi over a time course of 30 h to 40 days using the PT-GC–ICP-MS technique as described above.

Preparation of cell crude extracts

Cells of *M. formicicum* were harvested in the exponential growth phase by centrifugation (5000*g*, 5 min), resuspended in 5 ml of 100 mM *N*-2-hydroxyethylpiperazine (HEPES) (pH 7.0) (Gerbu, Gaiberg, Germany) containing 1.5 μM L-cysteine and passed three times through a French pressure cell at 200 MPa. Subsequently, the cell debris was removed by centrifugation (20 000*g*; 15 min) and the protein content of the supernatant was determined with the DC Protein Assay (Bio-Rad, München, Germany) using bovine serum albumin as a standard.

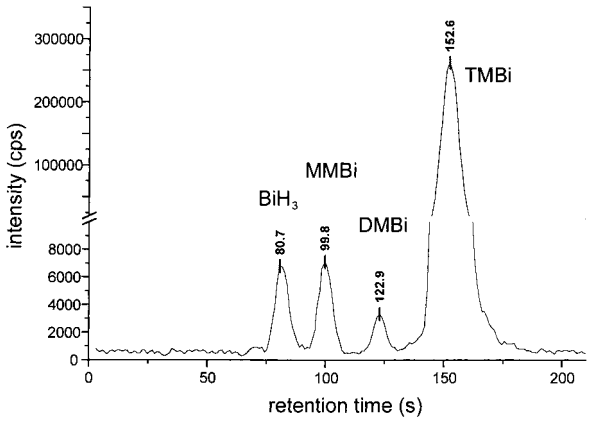

Figure 1. Chromatogram of the PT-GC–ICP-MS analysis on the *m/z* 209 trace for the detection of volatile bismuth compounds in the headspace of an *M. formicicum* culture after incubation of 35 days with 0.1 μM $Bi(NO_3)_3$.

In vitro *production of volatile bismuth compounds*

The *in vitro* production of volatile bismuth compounds was determined in butyl-rubber-stoppered 5 ml flasks under CO_2–H_2 (100 kPa; 20%/80%; v/v) in a reaction volume of 200 μl. The composition of the assay contained 100 mM HEPES (pH 7.0), 1.5 μM L-cysteine, 10 nM $Bi(NO_3)_3$ with or without 1 mg protein of the cell crude extracts of *M. formicicum*. The co-factors CH_3-B12 and *S*-adenosylmethionine (SAM) (1 μM each) were examined for their ability to transfer methyl groups to bismuth. All samples were incubated at 37 °C under moderate shaking (150 rpm) for 100 min prior to analysis by PT-GC–ICP-MS as described above. SAM and CH_3-B12 were purchased from Sigma–Aldrich (Deisenhofen, Germany).

RESULTS AND DISCUSSION

Identification of volatile bismuth compounds

Volatile bismuth compounds in the headspace of *M. formicicum* cultures spiked with different bismuth compounds were identified by matching the PT-GC–ICP-MS retention times with the boiling points of the compounds BiH_3, MMBi, DMBi and TMBi and by comparison with a TMBi standard. Figure 1 shows a chromatogram of the *m/z* 209 trace of the PT-GC–ICP-MS analysis of volatile bismuth compounds in the headspace of an *M. formicicum* culture after incubation of about 35 days with 0.1 μM $Bi(NO_3)_3$. The retention times of 80.7 s, 99.8 s, 122.9 s and 152.6 s correspond to the compounds BiH_3, MMBi, DMBi and TMBi respectively.

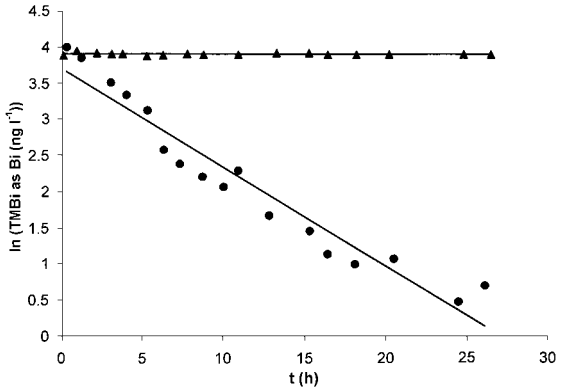

Figure 2. Stability of TMBi in CO_2–H_2 (20%/80%; v/v) (●) and air (▲) atmosphere in half logarithmic scaling.

Stability of TMBi

For stability studies, TMBi was synthesized by the reaction of CH_3-B12 with inorganic bismuth and its decay was followed in air, under CO_2–H_2 (20%/80%; v/v) or in a helium atmosphere. As documented in Fig. 2, the stability of TMBi is influenced significantly by the environmental conditions. In air, a significant decay of TMBi with a half life of <17 h could be observed, whereas under CO_2–H_2 atmosphere no decomposition occurred within 26 h. In a helium atmosphere an intermediate stability with a half life of 34 days could be determined (data not shown). Quite obviously, the CO_2–H_2 atmosphere in the headspace of the growing cultures preserves the compound, thus allowing the determination of the production rates of TMBi without being superimposed by its decay. The adsorption of TMBi on the vessel surface or the butyl rubber stoppers seems to be negligible, because there was no decrease of TMBi observed in vessels with the inert gas-phases during the experiments.

Influence of bismuth on the physiological activity of *M. formicicum*

The methane production rate of *M. formicicum* in the presence of different $Bi(NO_3)_3$ concentrations and bismuth-containing drugs was determined during growth and compared with control cultures without the addition of bismuth compounds (Table 1). As indicated by the reduced methane production rates, $Bi(NO_3)_3$ inhibits the metabolism of *M. formicicum* only at concentrations higher than 1 μM with an inhibition of up to approximately 40% at a concentration of 20 μM $Bi(NO_3)_3$.

The bismuth-containing drugs Bismofalk® and Noemin® showed different effects on methane production. The drug Bismofalk® did not affect the methanogenesis at a concentration equivalent to 1 μM bismuth, whereas the drug Noemin® caused a decrease of the methane production of about 40% at the same concentration.

The minimal inhibitory concentrations (MICs) of the bismuth compounds used in this study correspond to the range 1 to 60 μM that are applied as antimicrobial agents against microorganisms such as *Clostridium difficile, Helicobacter pylori, Pseudomonas aeruginosa* and *Escherichia coli*, depending on the compound used and the organism tested.[22,23]

Time course of the production of volatile bismuth compounds in cultures of *M. formicicum* with semi-continuous feeding

Generally, volatile bismuth derivatives were detected in the headspace of all *M. formicicum* cultures that were spiked with different bismuth salts (Table 1). In all samples, TMBi represents the main volatile bismuth species; at low, non-inhibiting $Bi(NO_3)_3$ concentrations (10 nM, 100 nM), the hydride BiH_3 and the partially methylated bismuth derivatives MMBi and DMBi were also detectable. The time course formation of volatile bismuth compounds by an *M. formicicum* culture that was spiked with 0.1 μM $Bi(NO_3)_3$ in the early exponential growth phase is depicted in Fig. 3. The volatile compound TMBi occurred already 1 day after the spike and its production continues during the incubation period. Additionally, in the middle to late exponential growth phase, the compounds MMBi and BiH_3 could be detected, followed by DMBi, which occurred only in the late stationary phase (inset of Fig. 3). The strikingly low amounts of these partially methylated bismuth hydrides could be explained, at least partially, by their low stability.[19]

As shown in Table 1, the formation of the bismuth hydride derivatives was only observed at low, non-inhibiting $Bi(NO_3)_3$ concentrations. Possibly, under these non-toxic conditions, the formation of these compounds results from hydride-generating side reactions with bismuth or its methylation intermediates mediated by electron donors that are accumulated in the stationary growth phase. At higher bismuth concentrations, where there are significant toxic effects, these side reactions are obviously suppressed — either by a general inhibition of hydride transfer reactions which also affect the methane production, or by a more specific interference with the bismuth hydride forming reactions. This shift to the more stable volatile product TMBi at higher bismuth concentrations could imply some importance for detoxification.

Yields and conversion rates of volatile bismuth compounds

The highest yield of volatilized TMBi (up to 600 ng in one culture) was detected in cultures that were initially spiked with 5 μM $Bi(NO_3)_3$ (Table 1). The overall highest rate of conversion to the respective volatile bismuth species during the incubation of 40 days, expressed as percentage of spiked inorganic bismuth, was observed at 1 μM $Bi(NO_3)_3$ (Table 1). At this concentration an average of 2.6% $Bi(NO_3)_3$ was volatilized to TMBi, with the highest production rate of TMBi being 1.5 ng h^{-1} (Fig. 4).

In a previous study, in which we analyzed the TMBi

Table 1. Methane production rate and yield of volatile bismuth compounds in the headspace of *M. formicicum* cultures in the presence of different concentrations of $Bi(NO_3)_3$ and bismuth-containing drugs

Compound	Bi (μM)	Bi in 50 ml (μg)	CH_4 production rate[a] (μmol h^{-1})	BiH_3[a] (pg)	MMBi[a] (pg)	DMBi[a] (pg)	TMBi[a] (ng)	Sum (ng)	Conversion[a] (%)
$Bi(NO_3)_3$	0	0	298 ± 96	–	–	–	–	–	–
$Bi(NO_3)_3$	0.01	0.1045	299 ± 94	0.8 ± 0.4	3.4 ± 1.9	0.2 ± 0.1	0.25 ± 0.14	0.25	0.2 ± 0.08
$Bi(NO_3)_3$	0.1	1.045	293 ± 78	3.8 ± 3.3	28 ± 25	5.1 ± 3.4	10.4 ± 6.0	10.4	1.0 ± 0.06
$Bi(NO_3)_3$	1	10.45	225 ± 75	n.d.[b]	n.d.	n.d.	272 ± 186	272	2.6 ± 1.8
$Bi(NO_3)_3$	5	52.25	192 ± 90	n.d.	n.d.	n.d.	352 ± 204	352	0.7 ± 0.6
$Bi(NO_3)_3$	20	209	180 ± 70	n.d.	n.d.	n.d.	36.5 ± 19	36.5	0.017 ± 0.009
Bismofalk®	1	10.45	309 ± 102	n.d.	n.d.	n.d.	0.25 ± 0.19	0.25	0.0024 ± 0.0018
Noemin®	1	10.45	179 ± 59	n.d.	n.d.	n.d.	0.035 ± 0.023	0.035	0.0034 ± 0.0022

[a] Mean values of three independent experiments plus/minus relative standard deviations.
[b] n.d. Not detectable.

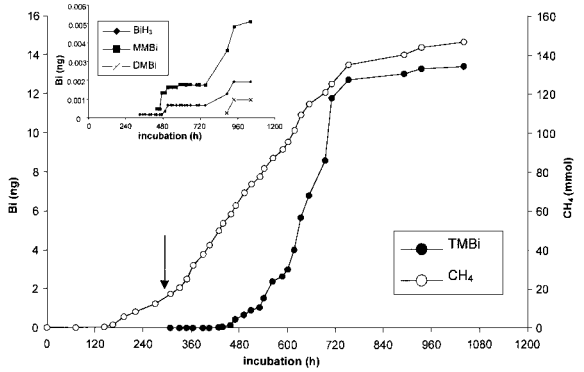

Figure 3. Time course of methane production and formation of volatile bismuth compounds of an *M. formicicum* culture spiked with 0.1 μM $Bi(NO_3)_3$ in the early exponential growth phase (arrow). The inset shows the production of BiH_3, MMBi and DMBi at 1000-fold higher sensitivity.

production in cultures of *M. formicicum* without semi-continuous feeding of the organism with H_2 and CO_2,[4] i.e. without exchanging the gas phase during the time course experiment, the conversion rate, and hence the yield, of TMBi was only one-fifth of that found in the present study. Quite obviously, the conversion and yield of volatile bismuth compounds depends not only on the concentration of the applied bismuth, but also on a sufficient supply of nutrients (i.e. H_2 and CO_2).

Biomethylation of bismuth-containing pharmaceuticals

The bismuth-containing pharmaceuticals Bismofalk® [containing bismuth subgallate and $Bi(NO_3)_3$] and Noemin® (containing bismuth aluminate) are widely used in the eradiction therapy of *H. pylori* in peptic ulcer diseases, and are applied orally in dosages of the bismuth compounds of 0.9–1.2 g day^{-1}. The biomethylation of the bismuth compounds contained in these pharmaceuticals could be shown in cultures of *M. formicicum* that were spiked with these drugs in quantities corresponding to 1 μM of bismuth. As shown in Table 1, the conversion rate of bismuth from these pharmaceuticals is significantly lower than the biomethylation rate of $Bi(NO_3)_3$ at a concentration of 1 μM. The low conversion rate from the pharmaceuticals might be influenced by (i) a lower susceptibility of bismuth in these compounds to biomethylation, (ii) a higher toxicity of the applied compounds, or (iii) a suppression of the biomethylation by additives in the drugs. In the case of Noemin®, the low conversion of bismuth accounts for a higher toxicity of this formulation to *M. formicicum* as deduced from the lowered methane production rate in this cultures; in the case of Bismofalk®, a general lower susceptibility to biomethylation or an inhibition of the conversion to TMBi seems to be more likely.

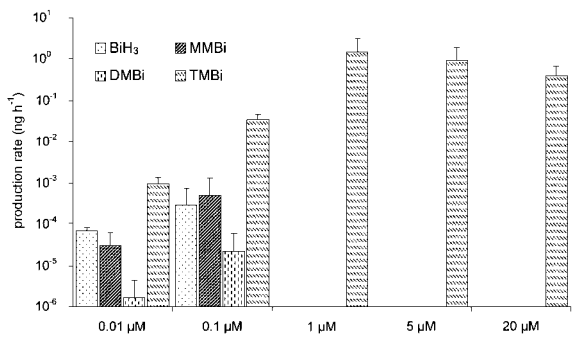

Figure 4. Maximal production rates of volatile bismuth compounds detected in the headspace of *M. formicicum* cultures in the presence of different $Bi(NO_3)_3$ concentrations. The values shown are averages of three independent experiments plus/minus the relative standard deviations.

Table 2. *In vitro* production of volatile bismuth compounds. The assay contained 100 mM HEPES (pH 7.0), 1.5 μM L-cysteine, 10 nM $Bi(NO_3)_3$ with or without (w/o) 1 mg protein of the cell crude extracts of *M. formicicum* and the co-factors CH_3-B12 or SAM (1 μM each) in 200 μl under CO_2–H_2 (100 kPa; 20%/80%; v/v)

Assay	TMBi produced[a] (ng)	Conversion (%)
CH_3-B12 w/o protein	128 ± 19.8	6.1
CH_3-B12 + protein	362.8 ± 46.3	17.4
SAM w/o protein	n.d.[b]	–
SAM + protein	n.d.	–

[a] Mean values of three independent experiments plus/minus relative standard deviations.
[b] n.d. Not detectable.

Assuming that the methanogenic flora of the intestine are also able to convert these drugs to TMBi, the application seems to be critical — despite the low conversion rate — because the high stability of TMBi under anaerobic conditions would certainly result in an accumulation of that toxic methylation product.

In vitro production of volatile bismuth compounds

As a first approach to obtaining insight into the biochemistry of bismuth methylation, we used an assay to reconstruct the biomethylation *in vitro*. For that purpose we tested the function of several compounds as methyl donors for the production of TMBi in crude extracts of *M. formicicum*.

As shown in Table 2, the production of TMBi was observed in assays containing CH_3-B12 as methyl donor, but it was not observed in the presence of SAM. Interestingly, TMBi was also synthesized in control samples in the presence of CH_3-B12 without cell crude extracts, indicating that the methyl group in this assay is transferred in a chemical reaction from the corrinoid to the metal without

enzyme catalysis. But in samples with cell crude extracts of *M. formicicum* in the presence of CH_3-B12 the yield of TMBi was threefold higher than in samples without cell proteins, indicating an involvement of enzymatic catalysed reactions in the production of TMBi (Table 2).

Challenger[24] proposed a mechanism of alternating oxidative methylation with methyl carbonium ion transfer derived from SAM, followed by reduction, for the biomethylation of arsenic. Quite obviously, this mechanism does not apply for the biomethylation of bismuth by *M. formicicum*, since (i) SAM could not be shown as a methyl donor in the *in vitro* assays and (ii) the involvement of methylated bismuth derivatives of oxidation state +5 in the biomethylation mechanism of bismuth is unlikely due to the high instability of such derivatives under these reductive conditions. The obvious involvement of CH_3-B12 in bismuth biomethylation by *M. formicicum* in the *in vitro* assays accounts for a stepwise methylation of bismuth without a change in the oxidation state of bismuth.

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REFERENCES

1. Palmieri Y. *Bull. Bismuth Inst.* 1993; special supplement.
2. Fickling M. *Bull. Bismuth Inst.* 1998; **74**: 3.
3. Merkel D, Matter Y and Appuhn H. *Korr. Abwass.* 1994; **41**: 264.
4. Michalke K, Wickenheiser EB, Mehring M, Hirner AV and Hensel R. *Appl. Environ. Microbiol.* 2000; **66**: 2791.
5. Feldmann J, Grümping R and Hirner AV. *Fresenius' J. Anal. Chem.* 1994; **350**: 228.
6. Feldmann J and Hirner AV. *Int. J. Environ. Anal. Chem.* 1995; **60**: 339.
7. Feldmann J, Krupp EM, Glindemann D, Hirner AV and Cullen WR. *Appl. Organomet. Chem.* 1999; **13**: 739.
8. Domenico P, Salo R, Novick SG, Schoch PE, van Horn K and Cunha BA. *Antimicrob. Agents Chemother.*1997; **41**: 1697.
9. Wickenheiser EB, Michalke K, Flassbeck D, Hirner AV and Hensel R. In *Metal Ions in Biology and Medicine*, vol. 6, Centeno JA, Collery P, Vernet G, Finkelman RB, Gibb H, Etienne JC (eds). John Libby Eurotext: Montrouge, France, 2000; 120–122.
10. Mueller JA, Di Toro DM and Maiello JA. *Environ. Toxicol. Chem.* 1995; **14**: 1657.
11. Slikkerveer A and de Wolff FA. *Med. Toxicol. Adverse Drug Exp.* 1989; **4**: 303.
12. Buge A, Rancurel G, Poisson M and Dechy H. *Nouv. Presse Méd.* 1974; **3**: 2315.
13. Burns R, Thomas DW and Barrow VJ. *Br. Med. J.* 1974; **1**: 220.
14. Martin-Bouyer G, Foulon G, Guerbois H and Barin C. *Thérapie* 1980; **35**: 307.
15. Martin-Bouyer G, Nevot P, Singlas E, Foulon G, Philippon A, Duret M, Fievet MH, Clavel JP and Simon P. *Thérapie* 1981; **36**: 483.
16. Chaleil D, Regnault JP, Allain P, Motta R, Raynaud G and Bouvet P. *Ann. Pharm. Fr.* 1988; **46**: 133.
17. Sollmann T and Seifert J. *J. Pharmacol. Exp. Ther.* 1939; **67**: 17.
18. Wickenheiser EB, Michalke K, Drescher C, Hirner AV and Hensel R. *Fresenius' J. Anal. Chem.* 1998; **362**: 498.
19. Amberger E. *Chem. Ber.* 1961; **94**: 1447.
20. Müller LMM. MSc Thesis, University of Essen, 1994.
21. Feldmann J. *J. Anal. At. Spectrom.* 1997; **12**: 1069.
22. Mahony DE, Lim-Morrison S, Bryden L, Faulkner G, Hoffman PS, Agocs L, Briand GG, Burford N and Maguire H. *Antimicrob. Agents Chemother.* 1999; **43**: 582.
23. Worku ML, Sidebotham RL and Karim QN. *Aliment. Pharmacol. Ther.* 1999; **13**: 753.
24. Challenger F. *Chem. Rev.* 1945; **36**: 315.

