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Connection between Multimetal(loid) Methylation in Methanoarchaea and Central Intermediates of Methanogenesis▽†

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In spite of the significant impact of biomethylation on the mobility and toxicity of metals and metalloids in the environment, little is known about the biological formation of these methylated metal(loid) compounds. While element-specific methyltransferases have been isolated for arsenic, the striking versatility of methanoarchaea to methylate numerous metal(loid)s, including rare elements like bismuth, is still not understood. Here, we demonstrate that the same metal(loid)s (arsenic, selenium, antimony, tellurium, and bismuth) that are methylated by *Methanosarcina mazei in vivo* are also methylated by *in vitro* assays with purified recombinant MtaA, a methyltransferase catalyzing the methyl transfer from methylcobalamin [CH_3Cob(III)] to 2-mercaptoethanesulfonic acid (CoM) in methylotrophic methanogenesis. Detailed studies revealed that cob(I)alamin [Cob(I)], formed by MtaA-catalyzed demethylation of CH_3Cob(III), is the causative agent for the multimetal(loid) methylation observed. Moreover, Cob(I) is also capable of metal(loid) hydride generation. Global transcriptome profiling of *M. mazei* cultures exposed to bismuth did not reveal induced methyltransferase systems but upregulated regeneration of methanogenic cofactors in the presence of bismuth. Thus, we conclude that the multimetal(loid) methylation *in vivo* is attributed to side reactions of CH_3Cob(III) with reduced cofactors formed in methanogenesis. The close connection between metal(loid) methylation and methanogenesis explains the general capability of methanoarchaea to methylate metal(loid)s.

Biomethylation and hydride generation of group 15 and 16 metals and metalloids (As, Se, Sb, Te, and Bi) by microorganisms are widespread phenomena in anaerobic habitats including landfills, sewage sludge fermentation, alluvial soils, and, as recently shown, the gut of mice and humans (5–6, 20, 22–23, 29). While these processes have a drastic effect on metal(loid) mobility and toxicity, little is known about the pathways involved in the biological formation of methyl and hydride metal(loid) species. For the methylation of arsenic, a metal(loid)-specific methyltransferase has been identified (2, 24, 28, 33). For instance, genes encoding arsenite methyltransferases such as ArsM, which catalyzes the stepwise methylation of arsenic in *S*-adenosyl methionine (SAM)-dependent reactions, are found in numerous prokaryotic and eukaryotic genomes. As ArsM confers resistance against increased arsenic concentrations and is expressed in response to elevated arsenic concentrations, arsenic methylation by ArsM is considered a deliberate detoxification mechanism (24). Furthermore, methylcobalamin [CH_3Cob(III)]-dependent methylation of As, Se, Sb, Te, Hg, and Bi has been reported for numerous anaerobic prokaryotes (4, 19, 21). In particular, autotrophic sulfate-reducing bacteria as well as methanoarchaea were suggested to be responsible for this process, as CH_3Cob(III) and CH_3Cob(III)-dependent enzymes are integral parts of physiological pathways such as carbon fixation via the reductive acetyl-coenzyme A (CoA) pathway and methanogenesis. Hence, these organisms contain high concentrations of corrinoids (17). However, it is unclear whether the different metal(loid)s are methylated by the same pathways and whether metal(loid) methylation is a deliberate enzymatic process, as in the case of ArsM, or whether it arises from side reactions of the basal physiological pathways. Interestingly, nonenzymatic methylation of some metal(loid)s, like As and Hg, by CH_3Cob(III) under reductive conditions was assumed by some authors (32, 34).

To identify metal(loid) methylation pathways which could increase understanding of the multielement biomethylation observed in anaerobic habitats, we focused on methanoarchaea. Almost all methanoarchaea studied are capable of methylating a broad spectrum of metal(loid)s (As, Se, Sb, Te, and Bi) (20). Moreover, hydride species are generated by some of the methanoarchaea investigated.

In methanogenesis, the methyl group of CH_3Cob(III) is transferred to 2-mercaptoethanesulfonate (CoM) in the penultimate step of methane formation, forming methylated CoM (CH_3CoM) and reduced cobalamin [Cob(I)] (18). In the methanol-utilizing methylotrophic pathway, this step is catalyzed by the methyltransferase MtaA (Fig. 1). Here, we studied the methyl transfer from CH_3Cob(III) to the metal(loid)s As, Se, Sb, Te, and Bi in the presence of MtaA of the methylotrophic methanogenic archaeon *Methanosarcina mazei* by purge-and-trap gas chromatography-inductively coupled plasma mass spectrometry (PT-GC-ICP-MS). Furthermore, we demonstrated the nonenzymatic methyl transfer from CH_3Cob(III) to

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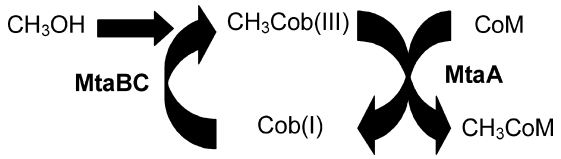

FIG. 1. Scheme of CoM methylation in the methanol-utilizing methanogenic pathway of *M. mazei*. The methyl group of methanol is cleaved off by MtaBC and transferred to the reduced cofactor Cob(I). Then, MtaA catalyzes the methyl transfer from $CH_3Cob(III)$ to CoM by deprotonating the thiol group of CoM (26).

these metal(loid)s in the presence of Cob(I), which is an intermediate product of the MtaA-catalyzed CoM methylation. Global transcriptome analyses of *M. mazei* cultures exposed to Bi were performed to underline our conclusions derived from the *in vitro* experiments.

MATERIALS AND METHODS

Standard cultivation of *M. mazei*. Cultivation of *M. mazei* strain Gö1 DSMZ 3647 was performed anaerobically (N_2/CO_2 [80:20]) at 37°C in complex medium with 250 mM methanol and 40 mM acetate as a carbon source as previously described (30). Na_2S was omitted to avoid precipitation of insoluble metal(loid)-sulfides.

***In vitro* metal(loid) volatilization assays.** For preparation of *M. mazei* crude extract, 500-ml cultures were grown under standard conditions to late exponential growth phase without addition of metal(loid)s. Cells were harvested by centrifugation (for 30 min at 6,000 × *g* and 4°C), resuspended in 50 mM HEPES buffer (pH 7), and frozen in liquid nitrogen prior to disruption using a Mikro-Dismembrator S (Sartorius, Göttingen, Germany). Insoluble cell fragments were removed by centrifugation (30 min at 13,000 × *g* and 4°C). The protein content of soluble supernatant was determined subsequently (3).

For *in vitro* assays with either crude extract or recombinant MtaA, both CoM and $CH_3Cob(III)$ were added at a final concentration of 1 mM to 1 ml of 50 mM HEPES (pH 7.0) and stored on ice prior to analysis. All preparations were performed in an anaerobic glove box (H_2/N_2, 2%/98%) under dim red light. The following metal(loid) species were added: Bi, $C_3H_5O(COO)_3Bi$; Sb, $SbCl_3$ (solubilized in ethanol); As, $AsNaO_2$ (0.1 μM each); Se, H_2SeO_3; Te, Na_2TeO_3 (each 10 μM). The concentration of trivalent Bi, Sb, and As derivatives was reduced to 0.1 μM in the reaction mixtures due to the limited dynamic range of the ICP-MS. The reaction was started by addition of either crude extract or recombinant MtaA to a final concentration of 100 μg ml^{-1} or 10 μg ml^{-1}, respectively. After a reaction time of 10 min at 30°C, volatile metal(loid) derivatives were analyzed by PT-GC-ICP-MS.

Cloning and expression of MtaA (MM_1070). The gene *mtaA* (MM_1070) of *M. mazei* was amplified by PCR using the site-specific forward primer 5′-CGC GGAATTCATATGACCGATATGAGCGAATTC-3′ and the reverse primer 5′-AGAGCGGATCCTCGAGTCAGGCGTAGAATTC-3′, which contain suitable restriction enzyme recognition sites (underlined) to facilitate the insertion of the PCR product into the NdeI and XhoI restriction sites of pET-24a (Novagen, Darmstadt, Germany). The sequence of pET24a-MM_1070 was verified by sequencing (Agowa, Berlin, Germany). The gene *mtaA* was heterologously expressed in *Escherichia coli* BL21(DE3) containing pET24a-MM_1070.

Purification of recombinant MtaA (MM_1070). Cell pellets (1 g of wet weight) of recombinant *E. coli* were resuspended in 50 mM HEPES (pH 7.0) and subsequently disintegrated by passage through a French pressure cell at 20,000 lb/in^2. Soluble cell fractions were separated from insoluble fractions by centrifugation (30 min at 13,000 × *g* and 4°C). MtaA was purified by high-performance liquid chromatography (HPLC) using a Q-Sepharose Fast Flow column (5 ml; GE Healthcare, Munich, Germany). Proteins were eluted by a NaCl gradient from 0 to 1 M in a total volume of 12 ml (MtaA fractions eluded at 300 mM NaCl) prior to a second purification step using a size exclusion column (Superdex 200, HiLoad 26/60, prep grade; Amersham, Freiburg, Germany).

Determination of cobalamin demethylation. Demethylation of 0.1 mM $CH_3Cob(III)$ and formation of Cob(I) by recombinant MtaA (10 μg ml^{-1}) in the presence of 0.1 mM CoM were measured in 1-min intervals over 20 min by UV-visible light (Vis) spectra (Specord 200; Analytik Jena, Jena, Germany) in septum-sealed quartz cuvettes (Hellma Analytics, Müllheim, Germany). The CoM methylation activity by recombinant MtaA was determined by the specific absorption at 367 nm of cyanocobalamin formed upon cyanide derivatization of demethylated $CH_3Cob(III)$ as described previously (9).

Electrochemical preparation of Cob(I). Cob(I) was formed by electrochemical reduction of aquocob(III)alamin as reported previously (15). A total of 250 mg of aquocob(III)alamin solved in 20 ml of oxygen-free 50 mM phosphate buffer at pH 7.2 was reduced in a magnetically stirred standard three-compartment electrochemical cell with a gold foil working electrode and platinum counter electrode in an anaerobic glove box (H_2/N_2, 2%/98%). Output voltage was set to 3.0 V (Model 4005, Voltcraft; Conrad Electronics, Hirschau, Germany). The final concentration of Cob(I) was determined via UV-Vis spectra.

Analysis of volatile metal(loid) derivatives. Volatile metal(loid) compounds were analyzed by a modified purge-and-trap gas chromatograph system coupled to an ELAN 6000 (PerkinElmer, Rodgau, Germany) inductively coupled plasma mass spectrometer (PT-GC-ICP-MS) as described previously (23). Species identification of volatile metal(loid) compounds based on GC-ICP-MS boiling point retention correlation was verified by using parallel molecular and elemental mass spectrometry (GC-electron impact [EI]-MS/ICP-MS) as described previously (14).

RNA isolation. To isolate total RNA from *M. mazei*, cell pellets (0.1 g of wet weight) were resuspended in 200 μl of 50 mM Tris-HCl (pH 7.0), frozen in liquid nitrogen, and disrupted by a Mikro-Dismembrator S (Sartorius, Göttingen, Germany). RNA was isolated from cell debris by using an RNeasy Midi Kit (Qiagen, Hilden, Germany) (30). DNA was digested on column by using RNase-free DNase I (Qiagen). RNA concentration and purity were determined photometrically at 260 nm and 280 nm. RNA integrity was verified after gel electrophoretic separation using 23S and 16S rRNAs as reference.

Microarray and Northern blot analysis. For both microarray and Northern blot analysis, total RNA was isolated from each of five independent cultures grown for 48 h in the presence of either 10 μM $Bi(NO_3)_3$ or 30 μM KNO_3 (see Fig. S3 in the supplemental material). For microarray analysis, RNA was labeled with Cy3 and Cy5 fluorescent dye using a CyScribe First-Strand cDNA Labeling kit (Amersham; Freiburg, Germany). The hybridization of labeled cDNA with whole-genome DNA microarrays, and the subsequent data mining was performed as previously described (13, 30). The microarray data were normalized by a ratio-based method under the assumption that the arithmetic mean of the ratios from every spot on the array should be equal to 1. Significant regulation was defined as a more than 2-fold higher or lower abundance compared to the control in at least three independent experiments (including one dye swap experiment).

Microarray chip data were validated by 16S rRNA-normalized Northern blot analyses of four selected genes (MM_1070, MM_2962, MM_2243, and MM_3188). Specific transcripts were detected and quantified after hybridization with sequence-specific ^{32}P-labeled antisense RNA probes as previously described (35). Similar expression values for these four genes for both analysis techniques were derived (see Table S1 in the supplemental material).

Microarray data accession number. Microarray data were deposited at ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) under accession number E-MTAB-787.

RESULTS AND DISCUSSION

Methylcobalamin-dependent methyltransferase MtaA of *M. mazei* is involved in multimetal(loid) methylation. *M. mazei* is capable of volatilizing several metal(loid)s (As, Se, Sb, Te, and Bi) by the formation of permethylated derivatives of these metal(loid)s *in vivo* (20). To identify possible enzymes involved in the production of methylated metal(loid) derivatives, we first examined crude extracts of *M. mazei* not previously exposed to metal(loid)s for their methylation activities. Positive results were obtained by using soluble fractions of these crude extracts amended by the methanogenic cofactors $CH_3Cob(III)$ and CoM (Table 1). For all five elements, conversion in the presence of cell crude extracts was from 2 to more than 4 orders of magnitude higher than in control experiments without crude extracts, suggesting that the required enzymes are constitutively expressed in *M. mazei*. In addition to permethylated species as found for growing cultures of *M. mazei*, volatile

TABLE 1. Relative portion of total volatilization (methylation and hydride generation) of As, Se, Sb, Te, and Bi in the presence of crude extracts of noninduced *M. mazei* and recombinant MtaA

Reactant (amt)	Mean relative recovery ± SD (%)[a]		
	Cell crude extract	Purified MtaA	Negative control[b]
As (100 pmol)	25.4 ± 11.7	34.8 ± 15.1	<0.0049[c]
Se (10,000 pmol)	12.7 ± 8.3	29.9 ± 11.7	0.14 ± 0.004
Sb (100 pmol)	35.7 ± 10.5	86.3 ± 10.3	<0.0039[c]
Te (10,000 pmol)	10.3 ± 5.2	18.2 ± 4.8	0.0047 ± 0.0039
Bi (100 pmol)	14.5 ± 10.7	23.4 ± 2.2	0.0031 ± 0.0013

[a] Assays contained 1 mM $CH_3Cob(III)$ and CoM in 1 ml of 50 mM HEPES buffer (pH 7) and inorganic metal(loid) salts at given concentrations. The reaction was started by addition of 100 μg of crude extract or 10 μg of purified MtaA. After a 10-min reaction time at 30°C, volatile metal(loid) species were analyzed by PT-GC-ICP-MS. Mean relative recoveries of the volatilized metal(loid)s and their standard deviations are given ($n = 3$).

[b] Negative control, 1 mM $CH_3Cob(III)$ and CoM without MtaA or crude extract.

[c] Below the detection limit.

hydride derivatives of As and Se were also generated by the *in vitro* assays (Fig. 2, column A).

For more detailed analyses, we focused on MtaA, a central $CH_3Cob(III)$-dependent methyltransferase in the methylotrophic methanogenesis of *M. mazei*. MtaA (MM_1070) was overexpressed in *E. coli* and purified to homogeneity by anion exchange and size exclusion chromatography. In accordance with the homologous enzyme of *Methanosarcina barkeri* (11), the recombinant MtaA of *M. mazei* exhibits a molecular mass of 37 kDa (see Fig. S1 in the supplemental material) and a specific CoM methylation activity of 7.1 U mg^{-1} at 30°C in the presence of 1 mM $CH_3Cob(III)$ and CoM.

Assays with purified MtaA resulted in methylation of all elements as methylated by *M. mazei in vivo* (Table 1 and Fig. 2, column B). In analogy to results with the crude extracts,

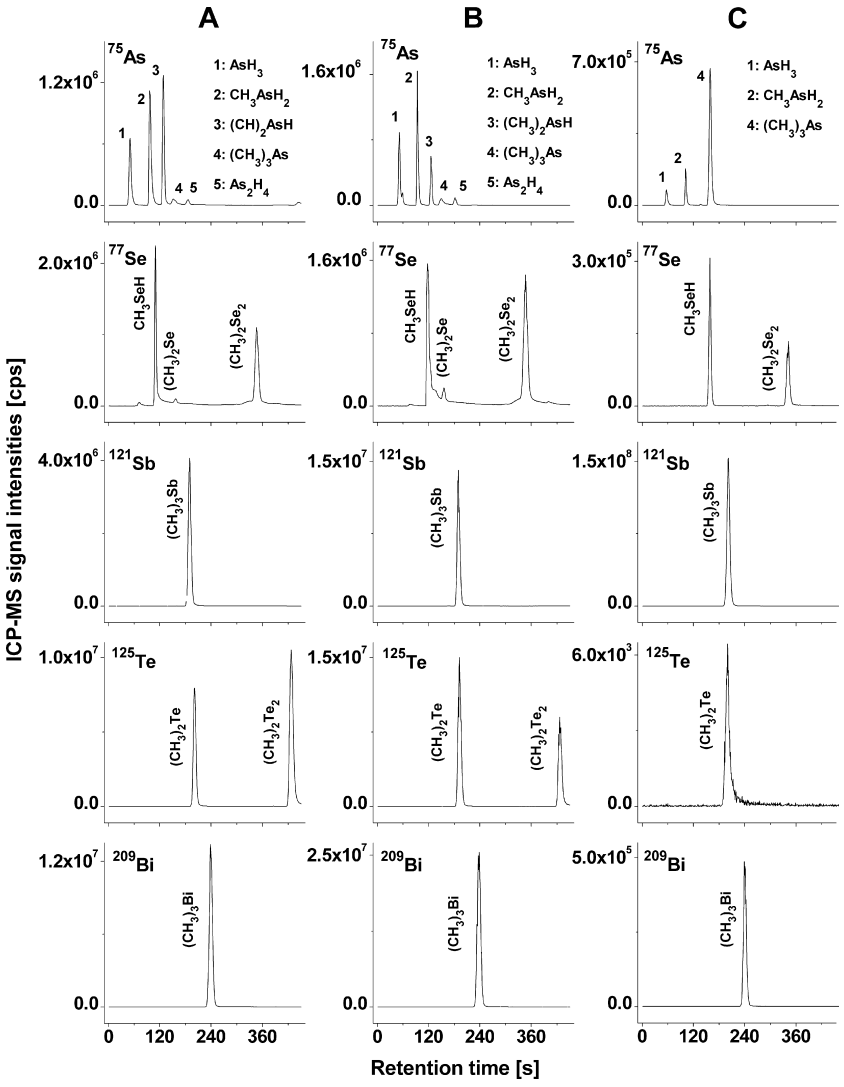

FIG. 2. Metal(loid) volatilization patterns obtained in the presence of crude extracts of noninduced *M. mazei* (column A), recombinant MtaA (column B), and electrochemically formed Cob(I) (column C). Shown are PT-GC-ICP-MS chromatograms of the volatile metal(loid) species produced under the following reaction conditions: for columns A and B, 1 mM $CH_3Cob(III)$ and CoM in 50 mM HEPES, pH 7, were incubated with 100 μg ml^{-1} *M. mazei* crude extract or 10 μg ml^{-1} purified recombinant MtaA in the presence of 0.1 μM As, 10 μM Se, 0.1 μM Sb, 10 μM Te, and 0.1 μM Bi at 30°C for 10 min prior to headspace analyses; for column C, 5 ml of 0.5 mM $CH_3Cob(III)$ in 50 mM phosphate buffer, pH 7, was incubated with 0.25 mM Cob(I) and a 0.25 mM concentration of either As, Sb, Bi, Se, or Te at 37°C for 30 min prior to headspace analyses.

these assays also produced volatile hydride derivatives of arsenic and selenium in addition to permethylated compounds. No volatile metal(loid)s were formed in the absence of CoM. The observation that a central enzyme from methanogenesis, MtaA, in the presence of its substrates, $CH_3Cob(III)$ and CoM, is capable of volatilizing all metal(loid)s, as found in *M. mazei* cultures, indicates that the pathway of methanogenesis and metal(loid) volatilization are linked in the cell.

Cob(I) is responsible for inducing the methyl transfer from $CH_3Cob(III)$ to the metal(loid)s. The mechanism of CoM methylation catalyzed by MtaA has been described as an activation of CoM through formation of a nucleophilic zinc-thiolate complex (16). This complex attacks the methyl group of $CH_3Cob(III)$, resulting in the transfer of a methyl carbocation from $CH_3Cob(III)$ to CoM and the formation of highly reduced Cob(I) (7, 18). Here, we investigated whether Cob(I) plays a role in metal(loid) methylation as previously proposed by Schrauzer et al. (27). First, we generated Cob(I) by MtaA-catalyzed demethylation of $CH_3Cob(III)$ in the presence of CoM. At CoM concentrations lower (0.05 mM) (condition A) or higher (0.14 mM) (condition B) than those of $CH_3Cob(III)$ (0.10 mM), a partial or nearly quantitative conversion of $CH_3Cob(III)$ to Cob(I), respectively, was achieved (see Fig. S2A and B in the supplemental material). The subsequent addition of arsenite led in both cases to the oxidation of Cob(I) and formation of Cob(II) as derived from the shift of the UV-Vis spectrum (Fig. 3, IA and B). In the assays with partial conversion of $CH_3Cob(III)$ to Cob(I), formation of both methyl and hydride arsine species was observed, indicating that Cob(I) is required for the methyl transfer from $CH_3Cob(III)$ to arsenite (Fig. 3, IIA). When $CH_3Cob(III)$ was nearly quantitatively converted to Cob(I) at CoM concentrations exceeding the $CH_3Cob(III)$ concentrations, arsine (AsH_3) was predominant, but almost no methylated species were formed (Fig. 3, IIB). This indicates that Cob(I) alone is capable of hydride generation and that $CH_3Cob(III)$, but not CH_3CoM, is the methyl donor for arsenic methylation.

In a second series of experiments, MtaA and CoM were replaced by Cob(I), which was formed by electrochemical reduction of aquocob(III)alamin. Cob(I) (0.05 mM) was mixed with 0.05 mM $CH_3Cob(III)$ (condition C), or 0.1 mM Cob(I) was provided without $CH_3Cob(III)$ (condition D). Again, oxidation of Cob(I) and formation of Cob(II) were observed upon arsenite addition (Fig. 3, IC and D). In accordance with the experiments with enzymatically produced Cob(I), arsenite was either converted to both methyl and hydride species in the presence of both Cob(I) and $CH_3Cob(III)$ (Fig. 3, IIC) or to nonmethylated hydride species in the presence of Cob(I) alone (Fig. 3, IID). These experiments demonstrate that both hydride generation by Cob(I) and methylation by $CH_3Cob(III)$ and Cob(I) do not necessarily require enzymatic reactions and that CoM is not needed for methylation and hydride generation of arsenite as well.

Finally, we tested the nonenzymatic methylation of Se, Sb, Te, and Bi by $CH_3Cob(III)$ in the presence of Cob(I). The pattern of volatile metal(loid) derivatives formed is very similar to the pattern formed by *in vitro* assays also containing CoM and *M. mazei* crude extract or CoM and MtaA (Fig. 2, column C). Only $(CH_3)_2AsH$ and $(CH_3)_2Te_2$ were not observed. Due to this high similarity of volatile methyl and hydride metal(loid) derivative patterns between these experiments, we suggest that the nonenzymatic reaction of Cob(I), $CH_3Cob(III)$, and the respective metal(loid) in solution represents the central pathway for the observed multimetal(loid) methylation and hydride generation by MtaA and by *M. mazei* crude extract-containing *in vitro* assays. As we observed a strong dependency of the relative formation of methyl and hydride species on the ratio of Cob(I) and $CH_3Cob(III)$ concentration in our experiments, we conclude that hydride generation is pronounced *in vitro* due to accumulation of Cob(I). In contrast, it has been assumed previously that Cob(I) is limited in the cell by rapid remethylation to $CH_3Cob(III)$ in order to prevent autoxidation of Cob(I) to Cob(II) (25). This could explain why no metal(loid) hydride generation by *M. mazei* was observed *in vivo* (20). Nevertheless, sufficient Cob(I) concentrations in the cell might be available to enable metal(loid) methylation, or another reduced cofactor from methanogenesis, like F_{420} or F_{430}, might contribute to metal(loid) methylation *in vivo* as well.

In summary, we propose that the methylation as well as hydride generation of metal(loid)s in the presence of MtaA or *M. mazei* crude extract proceeds via a two-step mechanism. In a first step, Cob(I) is produced by MtaA-catalyzed demethylation of $CH_3Cob(III)$. In a second step, Cob(I) promotes nonenzymatically the formation of nonmethylated metal(loid) hydrides in the absence of $CH_3Cob(III)$ or of both methyl and hydride metal(loid) species in the presence of $CH_3Cob(III)$.

Genes of MtaA and other $CH_3Cob(III)$-dependent methyltransferases of *M. mazei* are not induced by elevated bismuth concentrations. Our studies indicate that metal(loid) methylation in *M. mazei* is closely linked to the energy metabolism of the cell, and therefore triggering by elevated metal(loid) concentrations seems rather unlikely. However, alternative metal(loid) methylation systems might additionally exist in which methyltransferases are induced upon the exposure to metal(loid)s acting as a deliberate defense mechanism, in analogy to the ArsM system (24, 28, 33). To find indications for a specific induction of additional methyltransferases by the rare element bismuth, we studied the effect of exposure to 10 μM $Bi(NO_3)_3$ on the transcriptome of *M. mazei* by microarray analyses (see Table S1 in the supplemental material). The data were validated by Northern blot analyses.

Overall, the presence of 10 μM $Bi(NO_3)_3$ provoked only slight changes in the transcriptome compared to untreated control cultures, i.e., only up to 6.6-fold higher or lower abundance of specific mRNAs, and only 1.6% of all open reading frames (ORFs) in *M. mazei* were affected. The microarray analyses did not reveal any indication for the presence of metal(loid)-specific methyltransferases inducible by bismuth. Ten ORFs encoding proteins without annotated functions were upregulated in the presence of bismuth. Theoretically, one of these ORFs could encode metal(loid)-methylating methyltransferases, but none of these ORFs shows sequence homology to known methyltransferases. Also no ORFs encoding $CH_3Cob(III)$-dependent methyltransferases from methanogenesis, like MtaA, MtbA, MtsA, the CO dehydrogenase/acetyl-CoA synthase complex (CODH/ACS), and the N^5-methyl-tetrahydrosarcinapterin (CH_3-H_4SPT)-dependent methyltransferases (the last two of these enzyme

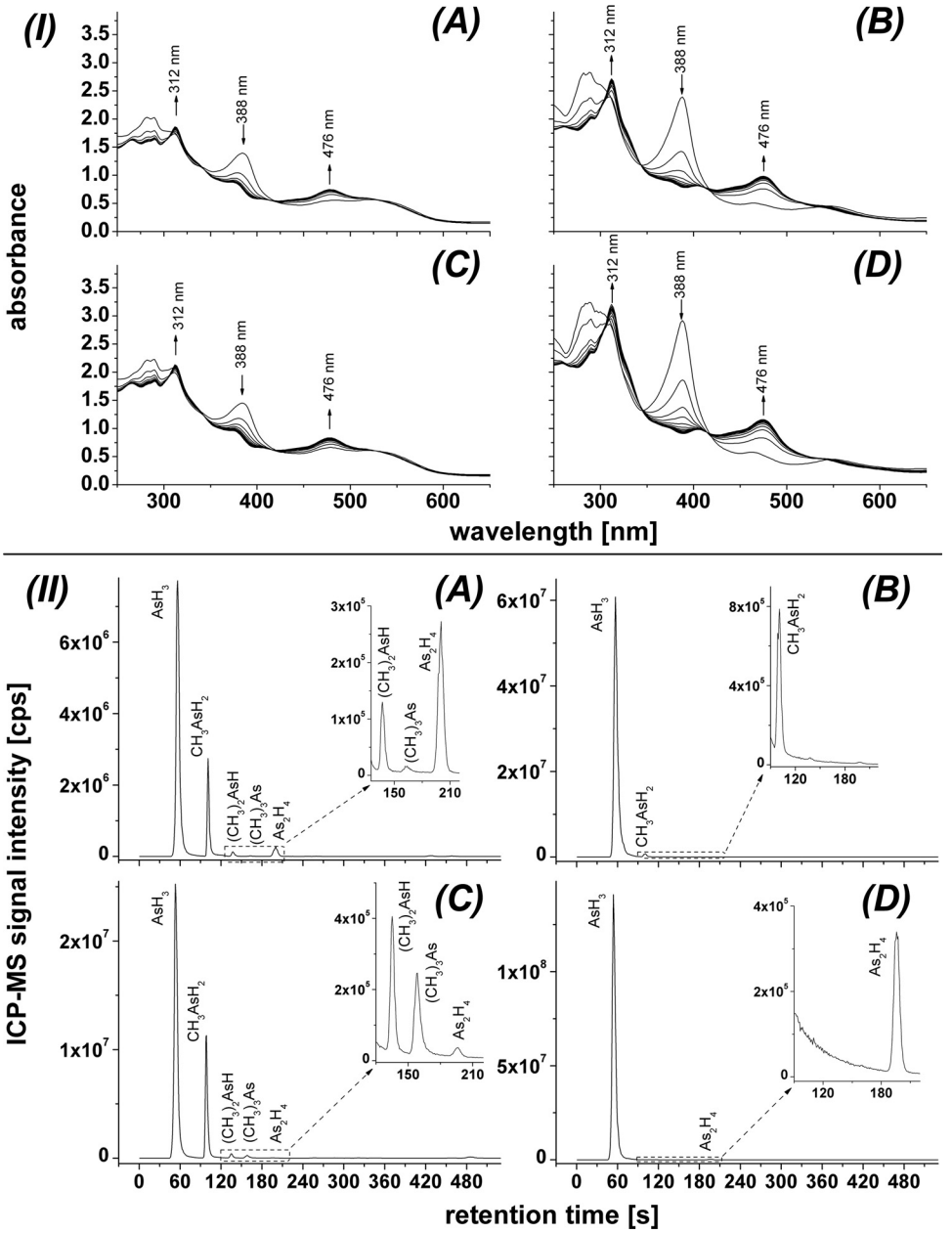

FIG. 3. Reactions of arsenite with equal amounts of $CH_3Cob(III)$ and Cob(I) (A and C in both panels; all references to the graphs apply to both panels I and II), with an excess of Cob(I) over $CH_3Cob(III)$ (B) or Cob(I) alone (D) in the presence of MtaA and CoM (A and B), or without MtaA and CoM (C and D), analyzed by UV/Vis spectra (I) and PT-GC-ICP-MS (II). MtaA at 10 μg ml^{-1} was added to assay mixtures containing 0.1 mM $CH_3Cob(III)$ (A and B) and either 0.05 mM CoM (A) or 0.14 mM CoM (B). The reaction was allowed to proceed until no further demethylation of $CH_3Cob(III)$ was observed photometrically (see Fig. S2 in the supplemental material). For the assays shown in graphs C and D, the mixture contained 0.05 mM (C) and 0.1 mM (D) Cob(I) prepared by electrochemical reduction of aquocob(III)alamin. For the assays shown in the C graphs, the mixture was additionally amended by 0.05 mM $CH_3Cob(III)$. Reaction volumes were 1 ml, and all reactions were conducted under strict anaerobic conditions in 50 mM HEPES (pH 7) at 30°C. UV/Vis spectra were measured in 1-min intervals for 10 min. Thereafter, headspace was sampled for analysis of volatile arsenic species.

complexes harbor a prosthetic cobalamin [1, 31]) showed regulation by bismuth.

Notably, some induction effects by bismuth were found for ORFs encoding enzymes which are supposed to be involved in the synthesis and regeneration of $CH_3Cob(III)$ and CoM, both common cofactors of methanogenesis and also participating in metal(loid) methylation, as shown in this study. Upregulation was observed for the following: (i) ORFs encoding putative methyltransferases involved in the methylation of Cob(I) from methanol (MM_1074, 2.9-fold) and mono-, di-, and trimethylated amines (MM_3334–5, 3.1-fold; MM_1693, 4.5-fold; MM_2051-2, 4.2-fold; MM_2961-3, 4.4-fold; MM_1689-90, 2.3-fold; MM_2047-9, 2.6-fold); (ii) ORFs MM_0133 (2.8-fold) and MM_0134 (2.8-fold), for which homologues are supposed

to be involved in CoM synthesis in *Methanosarcina acetivorans* (8, 10); and (iii) ORFs encoding the subunits of the putative heterodisulfide reductase HdrABC (MM_0387-0389, 3.7-fold) involved in the regeneration of CoM by the reductive cleavage of the CoM-S-S-CoB heterodimer (12). These findings can be interpreted as a compensation for a lower energy yield from methanogenesis caused by bismuth interfering with the methanogenic methyl transfer or by additional energy costs due to unspecific metal-induced damages of the cell.

Overall, the global transcriptome profiling data support our assumption that multimetal(loid) methylation by *M. mazei in vivo* is attributed to side reactions of central methanogenic cofactors and not to element-specific methyltransferases.

Conclusion. The present study demonstrates that the multimetal(loid) methylation capability of *M. mazei* is not attributed to metal(loid)-specific methyltransferases but is coupled to reactions of central cofactors of methanogenesis, $CH_3Cob(III)$ and its demethylated derivative Cob(I). As both $CH_3Cob(III)$ and Cob(I) are central constituents found in all methanoarchaea (1), we expect that this reaction likewise occurs in all methanogenic pathways, regardless of the enzyme catalyzing the $CH_3Cob(III)$-dependent methylation of CoM. The close link between methanogenesis and the multimetal(loid) methylation explains the outstanding capability of methanoarchaea to methylate a broad range of metal(loid)s. Though the nonenzymatic transmethylation from $CH_3Cob(III)$ to elements like Hg and As under reductive conditions was proposed earlier (27, 32), the present report is the first one that demonstrates the formation of the same volatile permethylated metal(loid) species by central methanogenic cofactors as found for growing methanoarchaea cultures. By showing that the ratio between $CH_3Cob(III)$ and Cob(I) (with the latter as a representative of a potent electron donor in methanoarchaea) is decisive whether methylation or hydride generation of metal(loid)s is preferred, our results indicate that the occurrence of volatile metal(oid) hydrides formed by hydrogenotrophic methanoarchaea, like *Methanobacterium formicicum* (21), but not in methylotrophic *M. mazei* (20) can be explained by different availabilities of reducing agents in the cell. Furthermore, it is feasible that the multimetal(loid) methylation mechanism described here can be found in other physiological groups, e.g., homoacetogenic bacteria, which possess high corrinoid concentrations similar to those of methanoarchaea (1, 17).

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