

Cobalt Limitation of Growth and Mercury Methylation in Sulfate-Reducing Bacteria

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Sulfate-reducing bacteria (SRB) have been identified as the primary organisms responsible for monomethylmercury (MeHg) production in aquatic environments, but little is known of the physiology and biochemistry of mercury (Hg) methylation. Corrinoid compounds have been implicated in enzymatic Hg methylation, although recent experiments with a vitamin B₁₂ inhibitor indicated that incomplete-oxidizing SRB likely do not use a corrinoid-enzyme for Hg methylation, whereas experiments with complete-oxidizing SRB were inconclusive due to overall growth limitation. Here we explore the role of corrinoid-containing methyltransferases, which contain a cobalt-reactive center, in Hg methylation. To this end, we performed cobalt-limitation experiments on two SRB strains: *Desulfococcus multivorans*, a complete-oxidizer that uses the acetyl-CoA pathway for major carbon metabolism, and *Desulfovibrio africanus*, an incomplete-oxidizer that does not contain the acetyl-CoA pathway. Cultures of *D. multivorans* grown with no direct addition of Co or B₁₂ became cobalt-limited and produced 3 times less MeHg per cell than control cultures. Differences in growth rate and Hg bioavailability do not account for this large decrease in MeHg production upon Co limitation. In contrast, the growth and Hg methylation rates of *D. africanus* cultures remained nearly constant regardless of the inorganic cobalt and vitamin B₁₂ concentrations in the medium. These results are consistent with mercury being methylated by different pathways in the two strains: catalyzed by a B₁₂-containing methyltransferase in *D. multivorans* and a B₁₂-independent methyltransferase in *D. africanus*. If complete-oxidizing SRB like *D. multivorans* account for the bulk of MeHg production in coastal sediments as reported, the ambient Co concentration and speciation may control the rate of Hg methylation.

Introduction

Monomethylmercury (MeHg), a potent neurotoxin which bioaccumulates to high levels in top-predator fish, is primarily formed by sulfate-reducing bacteria in freshwater, estuarine, and coastal environments (1). Although sulfate-reducing bacteria (SRB) provide the key step in transforming inorganic mercury into a more toxic organic form, the physiological

and biochemical mechanisms of mercury (Hg) methylation in SRB are poorly understood. Wood (2) first proposed that a methylcorrinoid compound is the methylating agent for inorganic mercury, and later work found that methylated vitamin B₁₂, a methylcorrinoid compound, is capable of abiotic MeHg synthesis (3). In *Desulfovibrio desulfuricans* LS, a corrinoid-containing enzyme was identified as key to Hg methylation through propyl-iodide inhibition experiments (4, 5). From ¹⁴C-labeling studies and enzyme activity experiments, Choi et al. (6) concluded that the corrinoid-containing protein responsible for Hg methylation in *D. desulfuricans* LS is involved in the acetyl-CoA pathway.

The acetyl-CoA pathway is a carbon metabolism pathway in some acetogens, methanogens, and SRB that converts acetate to CO₂. A corrinoid-containing methyltransferase has been identified in the acetyl-CoA pathway of non-Hg methylating acetogens (7) and methanogens (8). Presumably, a similar corrinoid protein is involved in the acetyl-CoA pathway in SRB. The presence of the acetyl-CoA pathway in *D. desulfuricans* LS is surprising, given that the strain was reported to be an incomplete-oxidizer. Incomplete oxidizing SRB convert carbon substrates to acetate rather than to CO₂, and do not use the acetyl-CoA pathway in major carbon metabolism. Unfortunately, the *D. desulfuricans* LS strain is no longer available and cannot be studied further.

A recent study investigated the role of the acetyl-CoA pathway in mercury methylation in SRB (9). Four incomplete-oxidizing strains were identified that methylate mercury but do not utilize the acetyl-CoA pathway for either metabolism or mercury methylation. Chloroform, an inhibitor of the acetyl-CoA pathway and corrinoid-containing enzymes, had no effect on growth or Hg methylation in these strains. All tested complete-oxidizing SRB strains that use the acetyl-CoA for metabolism also methylate mercury. Because chloroform additions to the complete-oxidizing strains completely inhibited growth, they provided no insight into the mechanism of Hg methylation in these strains.

To further investigate the mechanisms of Hg methylation in SRB, we have performed inorganic cobalt and vitamin B₁₂-limitation experiments on *Desulfococcus multivorans* and *Desulfovibrio africanus* cultures. *D. multivorans* is a complete-oxidizer that possesses a corrinoid-containing enzyme in its acetyl-CoA pathway which it may use for Hg methylation (9). *D. africanus* is an incomplete-oxidizing strain which does not use the acetyl-CoA pathway for metabolism or Hg methylation, and may not even require vitamin B₁₂ for Hg methylation (9). Corrinoids, which most SRB can synthesize (10), contain a cobalt-reactive center. Limiting the availability of cobalt, which should partially inhibit corrinoid-dependent processes, should thus lead to differential effects on growth and Hg methylation in these two strains and shed light on the methylation mechanisms.

Materials and Methods

Microorganisms and Culture Conditions. Cultures of the freshwater, incomplete-oxidizer *Desulfovibrio africanus* (DSMZ 2603) and the estuarine, complete-oxidizer *Desulfococcus multivorans* 1be1 (DSMZ 2059) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. The cultures were grown in trilaminar, 5 × 8 inch gas sampling bags consisting of an outer nylon layer, gas impermeable foil, and an inner polyethylene layer (Pollution Measurement Corporation, Oak Park, IL), which have been shown to be adequate for culturing anaerobic bacteria (11). The base media for both organisms contained 28 mM Na₂SO₄, 1.7 mM NaH₂PO₄, 4.7 mM NH₄Cl, 6.7 mM MgCl, 6.7 mM KCl,

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and 1.4 mM CaCl₂, and were passed through a Chelex 100 ion-exchange resin (Bio-Rad Laboratories) to remove trace metals, following the procedure of Price et al. (12). All media were amended with 0.5 g L⁻¹ yeast extract and 35 mM lactate from the same stock solutions, which were purified of trace metals by passing the solution through a methanol-sterilized Chelex 100 column and sterilized by boiling the solutions in a microwave for 3 min. The media were also amended with vitamins (40.9 nM biotin, 813 nM nicotinic acid, 365 nM p-aminobenzoic acid, 297 nM thiamine, 228 nM pantothenic acid, 1.22 μM pyroxidine-HCl, 36.5 nM vitamin B₁₂), 800 μM H₃BO₄, 3.6 μM FeCl₂, and 160 nM Na₂MoO₄, and buffered at pH of 7.5 with 10 mM MOPS (3-[N-morpholino]propane-sulfonic acid). The trace metal mix of the *D. multivorans* medium was the same as Aquil medium (12): 79.7 nM ZnCl₂, 121 nM MnCl₂, 50.3 nM CoCl₂, 19.6 nM CuCl₂, 19.6 nM NiCl₂, and 19.6 nM Na₂SeO₃. *D. africanus* did not grow on this metal mix and cultures were supplemented with: 500 nM ZnCl₂, 510 nM MnCl₂, 800 nM CoCl₂, 100 nM CuCl₂, 100 nM NiCl₂, and 23 nM Na₂SeO₃. Estuarine *D. multivorans* was cultured at 10‰ salinity by adding 0.17 M, chelex-purified NaCl.

Trace-metal clean methods (12) were used throughout. All media were prepared in acid-cleaned, 500 mL polycarbonate bottles (with a 150 mL headspace), microwave-sterilized (boiled for 3 min), and cooled on ice under a HEPA laminar-flow hood. The media were bubbled overnight with 0.2 μm, filter-sterilized, high purity N₂ inside a sterile, laminar flow hood until the redox indicator resazurin (1 mg/L) turned pink. After addition of 1 mg/L thioglycolate and 1.1 mM ascorbic acid, pH 7.5, the media were bubbled with N₂ until they turned clear. The solutions were microwave sterilized in the same bottles, and cooled on a dry ice/water bath for 5 min. They were quickly transferred to an all plastic, disposable anaerobic chamber filled with N₂. HPLC grade methanol was used to sterilize the outside of the cap on the medium container and the bubbling apparatus, and all connections between fillings of the culture bags.

A polyethylene tube sealed into the side of the culture bags was connected via sanitary silicone tubing to a polycarbonate three-way valve to allow for gas-tight filling and emptying. In the anaerobic chamber, the bags were first sterilized (filled with 10% HCl for 30 min and rinsed three times with microwave-sterilized Milli-Q water) and then filled with 100 mL of media each. One hundred mL of medium was pumped into each bag. In the anaerobic chamber, the media were equilibrated for 1–3 days before inoculation, and aliquots were removed to check for redox state (clear color of resazurin) and gross bacterial contamination by measuring optical density (OD) at 660 nm.

Trace-Metal Limitation and Hg Methylation Experiments. Three growth conditions were tested in triplicate for each strain: (1) metal-replete conditions with vitamin B₁₂ added (+Co/B12); (2) CoCl₂ added but not vitamin B₁₂ (No B12); and (3) neither inorganic cobalt nor vitamin B₁₂ added (No Co/No B12). To alleviate the problem of an unknown B₁₂ concentration in the yeast extract, all treatments received the same concentration of chelexed yeast extract from the same stock and were sterilized in exactly the same way. Stock cultures were maintained in standard liquid lactate-sulfate medium (9). *D. multivorans*, but not *D. africanus*, was transferred to a low trace-metal medium before inoculating the experimental bags (0.1 inoculum ratio). The effect of Co concentration was studied over three transfers in both strains. All treatments were inoculated with stationary phase cultures that were grown up between 11 and 30 days, and incubated in the anaerobic chamber at room temperature (26.7 ± 2.4 °C). Cultures of the same species contained similar initial cell concentrations after inoculation.

During the second and third transfers of *D. multivorans* cultures (5–7 h after inoculation), and the third (5 h after inoculation transfer of *D. africanus* cultures, 5 nM HgCl₂ was added to two culture bags of each treatment (ASY), with the third bag acting as a no Hg blank (BLK). Cultures were frequently sampled for cell growth (OD at 660 nm), and sampled four times for sulfide and MeHg concentrations. Sulfide samples were preserved in an antioxidant buffer, and analyzed with a silver-sulfide ion-specific probe (13). MeHg samples were frozen in acid-washed serum vials. Culture purity was monitored by epifluorescence microscopy with acridine orange (a.o.) stain. The relationship between OD and cell concentration (as measured by epifluorescence microscopy with a.o.) was determined in separate growth experiments for both strains.

MeHg was measured by distillation (14), followed by aqueous-phase derivitization and cold vapor atomic fluorescence detection (15). The average distillation recovery of MeHg in spiked autoclaved cultures was 96 ± 18% (n = 12). Abiotic Hg methylation during distillation was low (0.004 pM ± 0.013, n = 12). Check standards were included in each round of aqueous-phase derivitization.

Total Cobalt Concentration Determination. To quantify both inorganic and corrinoid-bound cobalt added directly and indirectly to the cultures, total cobalt concentrations were determined using a magnetic sector inductively coupled plasma mass spectrometer (Element 2, Thermo Finnigan, Bremen, Germany). To digest the samples, 800 μL of each culture (including cells) was combined with 1.6 mL 50% nitric acid (optima grade) in 10 mL, tightly sealed Teflon tubes, and heated below the boiling point for 4 h. The digests were diluted with 5.6 mL Milli-Q H₂O and spiked with scandium and indium as internal standards (2 μg L⁻¹ each). Analysis procedures are described in Tang and Morel (16).

Media Speciation and Cell Uptake Modeling. Visual MINTEQ version 2.21 was used to calculate the concentration of dissolved and precipitated cobalt and mercury species in the media. Equilibrium constants from the NIST (National Institute of Standards and Technology) database 46.7 were supplemented with constants for thioglycolate (17), ascorbate (Critical database, 6.0), lactate (Critical database 6.0), and sulfide-mercury complexes (13).

The maximum diffusion rate, *J* (fmole sec⁻¹ cell⁻¹), of Co from the bulk medium to the surface of *D. multivorans* was calculated as: $J = 4\pi RDC$, in which *R* is the radius of the cell (*R* = 0.75 μm), *D* is the diffusion constant (*D* = 2.0 × 10⁻⁵ cm² sec⁻¹), and *C* is the bulk cobalt concentration. *J* was divided by the average specific growth rate of each treatment to determine if diffusion could supply the Co quota to the cell. To estimate the Co quota of *D. multivorans*, we took the strain's dry weight to be 3.5 × 10⁻¹³ g, and its cobalt concentration to be equal to the corrinoid concentrations in *Desulfobacterium autotrophicum* grown with pyruvate, 200 ng g⁻¹ cell dry weight (18).

Results

Cobalt-Limited Growth Rates. *D. multivorans* maintained a steady exponential growth rate for nearly the entire duration of the experiments (5–6 days) in each transfer (Figure 1A), whereas *D. africanus* cultures only maintained a steady exponential growth rate for a day or two during each transfer (Figure 1E). In cultures of both organisms, cell densities at stationary phase were 5× higher than initial cell densities. The specific growth rates of the control cultures (+Co/B12) of both organisms decreased over the first three transfers (Table 1). However, since all treatments were inoculated from the previous transfer at the same time, differences in the specific growth rates between treatments of the same transfer can be used to deduce the effects of low Co and Vitamin B₁₂ amendments on growth.

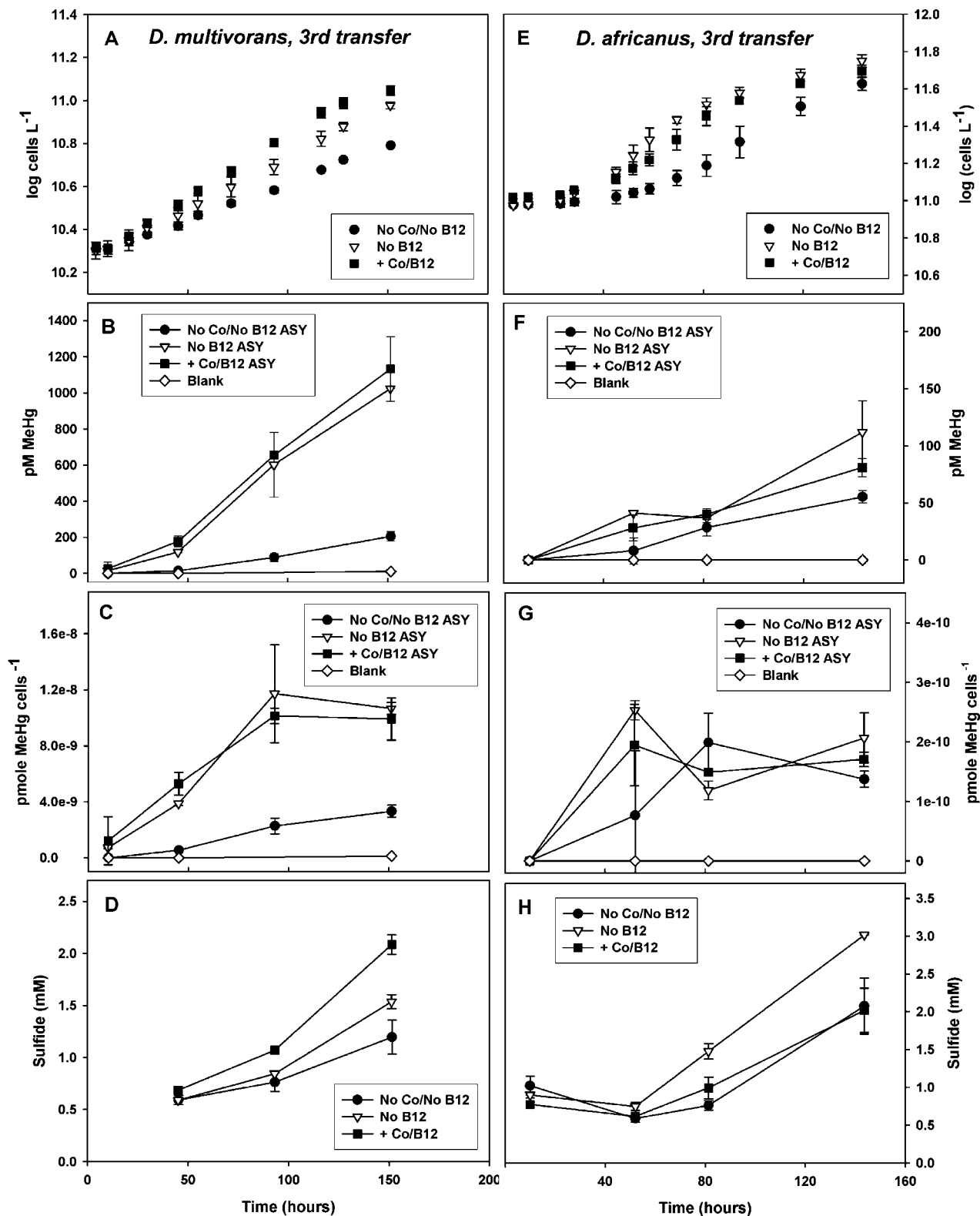


FIGURE 1. *D. multivorans*, third transfer (A–D) and *D. africanus*, third transfer (E–H) cell concentration (A, E), methylmercury (MeHg) concentration (B, F), MeHg concentration normalized by cell number (C, G), and sulfide concentration (D, H). In graph A, D, E, and H, error bars represent the standard deviation of three replicate cultures (2 assays, 1 no Hg added blank). In graph B, C, F, and G, error bars represent the range of duplicate assays and the standard deviation of triplicate blanks.

While the No B12 cultures of *D. multivorans* grew nearly as fast as the +Co/B12 cultures, the No Co/No B12 cultures grew at a slower specific growth rate in the second and third transfers (Table 1). This is illustrated for the third transfer in Figure 1A where the growth rate of No Co/No B12 cultures is only 57% of the control. Cobalt-limited *D. multivorans*

cells had a slower exponential growth rate but grew exponentially for a longer period, leading to similar cell densities in stationary phase (Table 1, Figure 1A). Low cobalt levels likely decreased carbon turnover through the acetyl-CoA pathway in this strain, but since the media contained 35 mM lactate, which can be degraded to acetate without the

TABLE 1. Specific Growth Rates of SRB Strains^a

sample name	specific growth rates (hr ⁻¹) ^a		
	transfer 1	transfer 2	transfer 3
<i>D. multivorans</i>			
No Co/No B12 1	0.0168	0.0092	0.0079
No Co/No B12 2	0.0168	0.0094	0.0076
No Co/No B12 3	0.0164	0.0091	0.0082
No B12 1		0.0159	0.0112
No B12 2	0.0177	0.0148	0.0115
No B12 3	0.0177	0.0148	0.0105
+ Co/B12 1	0.0182	0.0149	0.0136
+ Co/B12 2	0.0182	0.0144	0.0137
+ Co/B12 3	0.0185	0.0143	0.0138
<i>D. africanus</i>			
No Co/No B12 1	0.0351	0.0259	0.0169
No Co/No B12 2	0.0339	0.0273	0.0184
No Co/No B12 3	0.0343	0.0269	0.0163
No B12 1	0.0317	0.0245	0.0204
No B12 2	0.0345	0.0252	0.0202
No B12 3	0.0312	0.0239	0.0190
+ Co/B12 1	0.0362	0.0273	0.0201
+ Co/B12 2	0.0369	0.0272	0.0203
+ Co/B12 3	0.0348	0.0268	0.0205

^a Rates in bold are from Hg assays (ASY).

TABLE 2. Total Cobalt Concentrations in Culture Medium and Cells

sample	cobalt concentration (nM)		
	No Co/No B12	No B12	+ Co/B12
<i>D. multivorans</i>			
first transfer	16.7 ± 1.0	46 ± 6	73 ± 8
second transfer	10.3 ± 0.9	57 ± 10	49 ± 1
third transfer	9.4 ± 0.4	56 ± 1	74 ± 2
Milli-Q blank		0.9 ± 0.2	
<i>D. africanus</i>			
first transfer	55 ± 2	650 ± 9	630 ± 20
second transfer	7.4 ± 0.5	700 ± 27	730 ± 27
third transfer	3.6 ± 0.5	630 ± 20	720 ± 32
Milli-Q blank		0.31 ± 0.05	

acetyl-CoA pathway, metal and vitamin replete and No Co/No B12 cultures could reach stationary phase without experiencing carbon limitation. For *D. africanus*, all the treatments in each transfer grew at similar rates (Table 1). The data of Figure 1E for the third transfer show a more prolonged lag phase in the No Co/No B12 cultures compared to the +Co/B12 control but no substantial decrease in specific growth rate like that observed for *D. multivorans* third transfer No Co/No B12 cultures (Figure 1A, Table 1).

No Co/No B12 cultures of *D. africanus* contained lower total cobalt concentrations than those measured in growth-limited *D. multivorans* No Co/No B12 treatments (e.g., 3.1 vs 9.4 nM in the third transfer; see Table 2). Therefore, it does not appear that the lack of cobalt limitation in *D. africanus* cells is due to inorganic cobalt or vitamin B₁₂ carryover or contamination.

Mercury Methylation Rates. Addition of 5 nM HgCl₂ to cultures had no effect on the growth rates of either organism (Table 1). Cobalt-limited *D. multivorans* cultures (No Co/No B12) methylated mercury substantially less than the control (+Co/B12) and No B₁₂ cultures, which methylated Hg at equal rates (Figure 1B; Table 3). This lower rate of MeHg formation in the Co-limited cultures is seen even after normalization

to cell numbers (Figure 1C). In contrast, No Co/No B12 cultures of *D. africanus* produced only slightly less MeHg than No B12 and + Co/B12 cultures (Figure 1F) and this difference disappears when the data are normalized by cell number (Figure 1G).

The decrease in Hg methylation rates in Co-limited cultures of *D. multivorans* is consistent with the hypothesized role of cobalamin in the methylation reaction. To conclude that Co limitation is directly responsible for the decrease in Hg methylation in the No Co/No B12 cultures compared to the controls, however, we need to rule out possible indirect effects resulting from changes in metabolic rates or in medium composition.

If the methylation rate (d[MeHg]/dt) were simply proportional to the cell number (X) and their growth rate (dX/(dt X)), the MeHg concentration in the medium would increase linearly with the cell number: [MeHg] - [MeHg]₀ = k (X - X₀). As seen in Figure 2A, MeHg concentrations do indeed increase linearly with *D. multivorans* cell number for any given treatment but the constant of proportionality, k, is much lower in Co-limited cultures. Thus, changes in growth rate, which is an overall measure of metabolic rate, do not account for the decreased Hg methylation in these Co-limited cultures. In contrast, MeHg concentrations follow the same approximate linear trend with cell concentrations in all *D. africanus* cultures, regardless of Co concentrations (Figure 2B).

Our cultures of sulfate reducing bacteria actively produce sulfide over the course of the experiments. A number of studies have shown that sulfide concentrations affect the rate of Hg methylation by SRBs, presumably as a result of the formation of various Hg(II) sulfide complexes (19–21). Sulfide concentration during the third transfer of *D. multivorans* varied in accord with the growth of the culture: No Co/No B12 cultures grew slower and produced less sulfide, whereas + Co/B12 cultures grew faster and produced the most sulfide (Figure 1D). At 93 h, for example, the sulfide concentration in the Co-limited cultures was about 75% of the concentration in the control (Table 3). At a lower sulfide concentration in the presence of cinnabar (which is supersaturated as soon as measurable sulfide is released to the medium), the concentrations of disulfide mercury complexes such as HgS₂²⁻ and Hg(HS)₂⁰ are proportionally lower than at higher sulfide concentration. So, if the concentration of such species controlled the rate of methylation (as has been proposed for the neutral species Hg(HS)₂⁰), it would result in a decrease by approximately 25% in the methylation rate in Co-limited cultures compared to the control. This effect clearly does not account for the low Hg methylation rate in the Co-limited *D. multivorans* culture, which even after correcting for cell number and growth rate, is three-times lower than the control (Figure 2A).

Discussion

The growth rate of *D. multivorans*, a complete oxidizer which requires a cobalt-containing, vitamin B₁₂-methyltransferase in its acetyl-CoA pathway for carbon metabolism, clearly became limited at low total cobalt concentrations. To our knowledge, these are the first experiments showing metal limitation in a pure SRB culture. A previous study observed lower sulfate reduction rates under cobalt deprivation in a thermophilic anaerobic bioreactor (22). Based on our speciation calculations, this cobalt limitation of growth occurred under conditions where the bulk of the Co should precipitate as CoS(s) in the presence of excess sulfide. If equilibrium is attained under these conditions, free cobalt ([Co²⁺]), along with cobalt bound to inorganic and organic ligands, should be fixed and independent of the total Co in the medium.

Based on measurements of corrinoid concentrations in a similar complete-oxidizing SRB (18), we estimate the cobalt

TABLE 3. MeHg, Cell, and Sulfide Concentrations of SRB Assay Time Course Experiments^a

sample name	time (h)	MeHg conc. (pM)	cell no. (cells L ⁻¹)	sulfide conc. (mM)
<i>D. multivorans</i> , second transfer				
No Co/No B12 ASY	12.17	7 ± 2	2.06 ± 0.02 × 10 ¹⁰	
	31.08	44 ± 4	2.33 ± 0.03 × 10 ¹⁰	
	85.17	160 ± 27	4.59 ± 0.13 × 10 ¹⁰	
	178.5	200 ± 29	1.00 ± 0.02 × 10 ¹¹	
No B12 ASY	11.92	13 ± 2	1.97 ± 0.003 × 10 ¹⁰	
	30.83	63.3 ± 0.1	2.48 ± 0.04 × 10 ¹⁰	
	84.92	470 ± 84	5.54 ± 0.07 × 10 ¹⁰	
	178.25	760 ± 167	1.25 ± 0.02 × 10 ¹¹	
+Co/B12 ASY	12.00	13 ± 4	1.98 ± 0.09 × 10 ¹⁰	
	30.92	110 ± 13	2.50 ± 0.06 × 10 ¹⁰	
	85.00	510 ± 93	5.44 ± 0.16 × 10 ¹⁰	
	178.33	690 ± 65	1.21 ± 0.03 × 10 ¹¹	
<i>D. multivorans</i> , third transfer				
No Co/No B12 ASY	10.08	0 ± 0	1.97 ± 0.03 × 10 ¹⁰	
	45.25	14 ± 2	2.55 ± 0.05 × 10 ¹⁰	0.59 ± 0.04
	93.17	90 ± 21	3.78 ± 0.13 × 10 ¹⁰	0.76 ± 0.09
	151.42	200 ± 27	6.19 ± 0.04 × 10 ¹⁰	1.2 ± 0.2
No B12 ASY	9.92	15 ± 2	2.14 ± 0.05 × 10 ¹⁰	
	45.08	118 ± 4	3.06 ± 0.02 × 10 ¹⁰	0.59 ± 0.00
	93.00	600 ± 179	5.14 ± 0.001 × 10 ¹⁰	0.84 ± 0.00
	151.25	1023.6 ± 0.5	9.61 ± 0.39 × 10 ¹⁰	1.53 ± 0.07
+ Co/B12 ASY	10.00	30 ± 36	2.09 ± 0.05 × 10 ¹⁰	
	45.17	180 ± 30	3.34 ± 0.05 × 10 ¹⁰	0.69 ± 0.00
	93.08	650 ± 18	6.46 ± 0.17 × 10 ¹⁰	1.07 ± 0.00
	151.33	1100 ± 179	1.14 ± 0.008 × 10 ¹¹	2.08 ± 0.09
<i>D. africanus</i> , third transfer				
No Co/No B12 ASY	9.92	0 ± 0	9.36 ± 0.04 × 10 ¹⁰	1.0 ± 0.1
	52.00	10 ± 11	1.07 ± 0.02 × 10 ¹¹	0.59 ± 0.05
	81.33	28 ± 7	1.43 ± 0.02 × 10 ¹¹	0.76 ± 0.06
	143.58	55 ± 5	4.04 ± 0.008 × 10 ¹¹	2.1 ± 0.4
No B12 ASY	9.92	0 ± 0	9.66 ± 0.21 × 10 ¹⁰	0.90 ± 0.04
	51.92	41 ± 2	1.63 ± 0.03 × 10 ¹¹	0.75 ± 0.05
	81.25	37 ± 4	3.13 ± 0.05 × 10 ¹¹	1.5 ± 0.1
	143.50	110 ± 27	5.39 ± 0.24 × 10 ¹¹	3.02 ± 0.00
+Co/B12 ASY	9.92	0 ± 0	1.04 ± 0.02 × 10 ¹¹	0.77 ± 0.03
	51.83	30 ± 11	1.43 ± 0.08 × 10 ¹¹	0.62 ± 0.03
	81.25	40 ± 5	2.69 ± 0.25 × 10 ¹¹	1.0 ± 0.1
	143.42	81 ± 8	4.74 ± 0.12 × 10 ¹¹	2.0 ± 0.3

^a Error bars represent the range of duplicate assays.

quota needed to sustain growth of *D. multivorans* to be at least 7.1×10^{-5} fmol/cell. Calculations of maximum diffusive fluxes to the cell surface (see Materials and Methods) show that the diffusion of the free ion ($[Co^{2+}] = 0.3\text{--}1.0$ fM) is insufficient to supply the necessary cellular Co. The maximum diffusive flux of dissolved inorganic cobalt to the cell ($Co^+ = [CoHS^+] = 18$ fM) might be just high enough to maintain the estimated cobalt quota. However, it is likely that the actual cobalt quota of the cells is higher than the measured corrinoid concentration since significant quantities of B₁₂ may have been lost during the HPLC analytical procedures (10, 18).

Both the diffusion calculations and the fact that similar growth rates are observed in cultures in which the dissolved concentrations of Co are calculated to be identical suggest that either the medium does not reach equilibrium with CoS(s) or the cells somehow acquire Co from the solid. The mechanism(s) of metal uptake in SRB is an interesting (and unexplored) topic in view of the great insolubility of the sulfide minerals formed by most of the metals necessary for growth. In a genomic analysis of SRB, Rodionov et al. (23) found that all sequenced SRB lack homologues of any previously identified cobalt transporters, but contained a hypothetical cobalt transporter gene also found in some archaea. All sequenced SRB contain the anaerobic cobalamin synthesis pathway, *cbi*, as well as an ABC transport system for importing

vitamin B₁₂, *btuCDF* (23). Analysis of the *cbi* operon in SRB indicates that cobalt uptake, cobalt insertion into the corrin ring, and vitamin B₁₂ uptake are under tight regulatory control by cell corrinoid concentrations (23). Upon cell growth in No Co/No B12 cultures, lower in vivo corrinoid concentrations are hypothesized to cause upregulation of Vitamin B₁₂ and cobalt uptake systems.

Early data have shown that mercury methylation in SRB is likely catalyzed by vitamin B₁₂ (4, 5). Chloroform inhibition experiments have indicated, however, that this is probably not the case in incomplete-oxidizing SRB (9). Our new cobalt limitation data confirm and extend these results. Chloroform addition had no effect on the growth or mercury methylation rates in incomplete oxidizers, suggesting that neither process depends on vitamin B₁₂. Accordingly, growth and Hg methylation rates of *D. africanus* remained approximately the same at low total cobalt concentrations. Because chloroform addition completely inhibited growth, along with Hg methylation in complete oxidizers, these experiments provided no insight into the methylation mechanism in these organisms. In contrast, the cobalt-limitation experiments indicate that corrinoids play a key role in Hg methylation in complete-oxidizing SRB. The rate of Hg methylation in cobalt-limited cultures of *D. multivorans* decreased by a factor of 3–5 compared to cobalt-replete cultures. Noncorrinoid cobalt

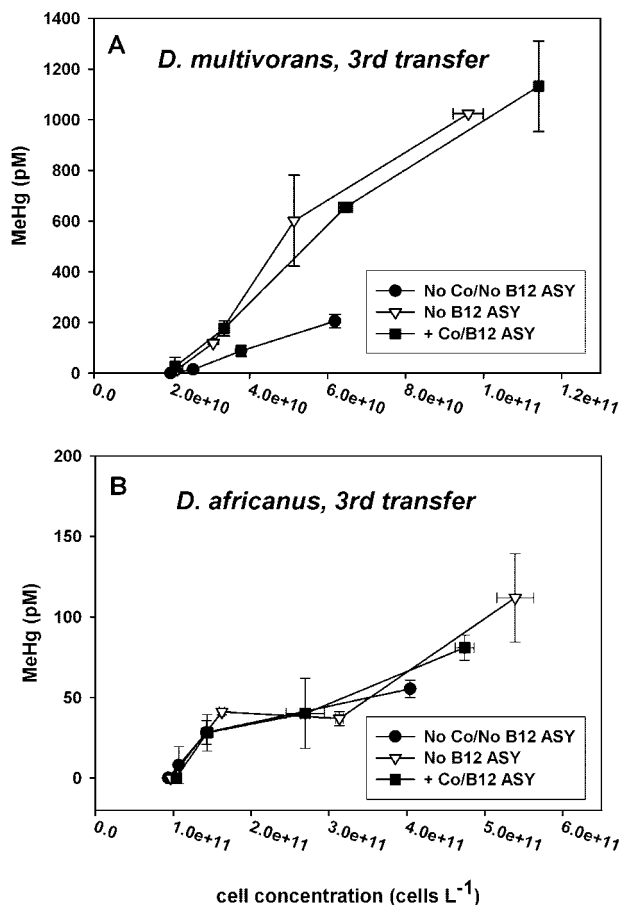


FIGURE 2. Measured methylmercury (MeHg) concentration versus cell concentration in assays of *D. multivorans* third transfer (A) and *D. africanus*, third transfer (B). Error bars represent the range of duplicate assays. The coefficient of proportionality, k , corresponds to the slope between data points.

enzymes are quite rare (24); and since a corrinoid-containing enzyme is a critical catalyst in the acetyl-CoA pathway, we infer that cobalt limitation is practically corrinoid limitation in *D. multivorans*. The decrease in cell number and in growth rate in Co-limited cultures could not account for the difference in Hg methylation; and neither could the slight decrease in sulfide concentration which might have changed the bioavailability of mercury in the medium. So it appears that Co-limitation resulted in a specific limitation of Hg methylation in *D. multivorans*, implicating cellular corrinoids in the methylation mechanism.

Together with the previous chloroform inhibition experiments (9), our Co limitation data indicate that complete and incomplete oxidizing SRB utilize different pathways for Hg methylation but they give no insight into the methylation mechanism in incomplete oxidizers. Besides B₁₂, the two coenzymes involved in methyltransferase reactions are s-adenosyl-L-methionine (SAM) and 5-methyltetrahydrofolate (CH₃-THF). Review of the KEGG database (<http://www.genome.jp/kegg/>) reveals that CH₃-THF is primarily used as a methyl-donor to vitamin B₁₂-methyltransferases. Experiments with inhibitors of SAM, which mediates most methylation reactions in the cell (KEGG database), might help elucidate the role of this compound in Hg methylation in incomplete-oxidizing SRB.

Previous research by King et al. (25) demonstrated that pure culture strains in the genera *Desulfobacterium* and *Desulfococcus*, which use the acetyl-CoA pathway for complete oxidation of carbon substrates, methylate mercury at

higher rates than other SRB genera on a per cell basis. Indeed, in our third transfer control experiments (+Co/B₁₂), *D. multivorans* made 10–50 times more MeHg per cell than *D. africanus* (Figures 1C, 1G, Table 3), even after accounting for the differences in growth rate and cell number (Figure 2). Complete-oxidizers that use the acetyl-CoA pathway are some of the most prevalent SRB in marine and estuarine sediments (refs 26 and 27), and according to calculations by King (27), could account for a major fraction of MeHg formation in coastal systems. In these environments, the rate of Hg methylation, which likely depends on the cellular B₁₂, could be influenced by in situ cobalt concentrations.

The effects of cobalt-limitation on Hg methylation could be particularly important in two unique environments: anaerobic sludge bioreactors and in permanently anoxic water columns such as the Black Sea. Elevated levels of MeHg have been measured in municipal sewage sludge (refs 28 and 29), presumably from the activity of SRB in anaerobic processes. Anaerobic bioreactors are regularly treated with trace metals including cobalt to increase bacterial activity (30). In anaerobic bioreactors digesting mercury-contaminated materials, limiting cobalt additions could help decrease Hg methylation by complete-oxidizers, albeit at the cost of decreasing the growth rate of these organisms. The anoxic water column of permanently stratified water bodies often contain low dissolved cobalt concentrations as a result of the formation and settling of sulfide precipitates. In the deep, anoxic water column of the Black Sea, measured dissolved cobalt concentrations are 20× lower than growth-limiting total cobalt concentrations measured in this study (31). The role of sulfide-induced metal limitation of anaerobes in natural environments is an interesting topic deserving further study.

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