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Bacterial growth phase influences methylmercury production by the sulfate-reducing bacterium *Desulfovibrio desulfuricans* ND132☆

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ABSTRACT

The effect of bacterial growth phase is an aspect of mercury (Hg) methylation that previous studies have not investigated in detail. Here we consider the effect of growth phase (mid-log, late-log and late stationary phase) on Hg methylation by the known methylator *Desulfovibrio desulfuricans* ND132. We tested the addition of Hg alone (chloride-complex), Hg with Suwannee River natural organic matter (SRNOM) (unequilibrated), and Hg equilibrated with SRNOM on monomethylmercury (MMHg) production by ND132 over a growth curve in pyruvate–fumarate media. This NOM did not affect MMHg production even under very low Hg: SRNOM ratios, where Hg binding is predicted to be dominated by high energy sites. Adding Hg or Hg–NOM to growing cultures 24 h before sampling (late addition) resulted in ~2× greater net fraction of Hg methylated than for comparably aged cultures exposed to Hg from the initial culture inoculation (early addition). Mid- and late-log phase cultures produced similar amounts of MMHg, but late stationary phase cultures (both under early and late Hg addition conditions) produced up to ~3× more MMHg, indicating the potential importance of growth phase in studies of MMHg production.

1. Introduction

Mercury (Hg) is a widely distributed pollutant that can be methylated by bacteria to form the neurotoxin monomethylmercury (MMHg) that can bioaccumulate up aquatic foodwebs. Interest in understanding variables controlling rates of Hg methylation by bacterial cells has been stimulated since MMHg production cannot be simply predicted by total Hg in many aquatic systems (Benoit et al., 2003; Marvin-DiPasquale et al., 2009). Notably, controls on microbial metabolic processes including activity and growth strategy (Lin and Jay, 2007), significantly affect methylation rates. Hg speciation is important in Hg methylation (Lin and Jay, 2007; Merritt and Amirbahman, 2009; Schaefer and Morel, 2009) and there are indications that different bacterial species produce MMHg under a variety of geochemical and growth conditions (Jay et al., 2002; Benoit et al., 2003; Fleming et al., 2006; Lin and Jay, 2007; Ekstrom and Morel, 2008; Merritt and Amirbahman, 2009; Schaefer and Morel, 2009). Given the incomplete understanding of connections between Hg cycling and MMHg formation in aquatic environments there is a particular need to better understand controls on bacterial production of MMHg.

Bacterial growth phase has been found to affect the rate of specific metabolic processes. In the non-Hg-methylating bacterium *Desulfovibrio vulgaris* (Hildenborough) numerous genes associated with transcription and translation were down-expressed during the transition from exponential to stationary phase, simultaneously genes related to phage and stress response, as well as iron uptake, were up-regulated. A study of the response of *Escherichia coli* to the onset of stationary phase likewise indicated the down- and up-expression of genes, but also noted changes in morphology and physiology, including development of increased resistance to physical and chemical stresses (Ishihama, 1997). MMHg production rates by *Desulfobulbus propionicus* declined in stationary phase though details of the transition from exponential into late stationary phase were not explored (Benoit et al., 2001a) In response to heavy metal stress stationary growth phase in particular appears to influence metabolic processes resulting in changes in metal speciation or concentrations in solution (Pongratz and Heumann, 1999; Sandrin et al., 2000; Kassab and Roane, 2006; Chien et al., 2007).

Recent studies of Hg methylation by a range of Hg methylating bacteria (MMB) have identified important controls on Hg uptake by bacterial cells. Numerous sulfate-reducing bacteria (SRB), and several metal-reducing bacteria produce MMHg under anaerobic conditions. The geochemistry of Hg in such laboratory experiments is an important consideration because sulfide produced as a result of sulfate reduction can influence Hg speciation via the formation of aqueous or particulate Hg sulfide species (Benoit et al., 1999; Benoit et

☆ This study investigates the effects of growth phase on methylmercury production by the known methylator *Desulfovibrio desulfuricans* ND132.

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al., 2001a; Benoit et al., 2003). Methylation potentials measured in cultures of *Geobacter sulfurreducens* vary when Hg is complexed to different low molecular weight organic ligands but these same difference are not observed with the SRB *Desulfovibrio desulfuricans* ND132 (ND132) (Schaefer et al., 2011) demonstrating that several pathways may support Hg uptake and MMHg production. Biofilm formation increased MMHg production by an order of magnitude relative to a planktonic growth strategy (Lin and Jay, 2007) indicating that microbial production of MMHg may be influenced by environment, growth strategy, or metabolic factors.

While our understanding of bacterial processes that promote Hg methylation is improving (Jay et al., 2002; Benoit et al., 2003; Fleming et al., 2006; Lin and Jay, 2007; Ekstrom and Morel, 2008; Merritt and Amirbahman, 2009; Schaefer and Morel, 2009), gaps persist in our understanding of the bioavailability of Hg in natural systems. Natural organic matter (NOM) is an important complexing ligand of Hg in natural systems influencing its mobility (Benoit et al., 2001b; Miller et al., 2007; Skyllberg, 2008; Skyllberg and Drott, 2010) and potentially affecting the availability of Hg for microbial uptake and methylation (Barkay et al., 1997; Benoit et al., 2003; Ravichandran, 2004; Hall et al., 2008; Miller et al., 2009), especially under sulfide-poor conditions (Benoit et al., 2003). Even under sulfidic conditions, there are indications that Hg could interact with NOM (Hsu-Kim and Sedlak, 2005; Miller et al., 2007; Skyllberg, 2008). Under low Hg:NOM ratios, Hg will bind to the highest energy sites and occupy progressively lower energy sites as the Hg:NOM ratio increases (Haitzer et al., 2002; Haitzer et al., 2003; Miller et al., 2009), consistent with equilibrium aqueous speciation calculations (Drott et al., 2007; Skyllberg, 2008; Dong et al., 2010; Skyllberg and Drott, 2010). Reaction kinetics of Hg binding to high energy sites in NOM are not instantaneous and may take 9 to 24 h (Benoit et al., 2001b; Lamborg et al., 2003; Black et al., 2007; Gasper et al., 2007; Miller et al., 2009). Predicting the effect of Hg complexation by NOM on its methylation is complicated both because of the range of organic molecules present in NOM and because of the complexity of interactions between Hg and NOM and their cellular uptake. Studies suggest that MMHg production in natural systems likely requires biologically active cells though a laboratory study of lysed washed *G. sulfurreducens* cells did produce MMHg (Schaefer and Morel, 2009).

Additionally, growth phase may be an important variable in understanding controls on Hg methylation. While the effect of growth phase has been investigated in a few bacterial systems, there is a need to investigate its impact on the metabolic processes controlling Hg uptake and methylation. The potential effect of NOM on Hg uptake by bacterial cells has not been directly considered even though equilibrium geochemical modeling (Dong et al., 2010) and kinetic (Miller et al., 2009) studies indicate that Hg–NOM species are expected to play an important role in natural environments. Here we address this knowledge gap through laboratory experiments investigating the effect of growth phase on methylation of Hg and Hg–NOM complexes by the known methylating SRB ND132 (Jay et al., 2002; Lin and Jay, 2007).

2. Experimental design

2.1. Microorganism and culture conditions of D. desulfuricans ND132

D. desulfuricans ND132 was isolated from an estuarine environment (Gilmour et al., 1985) and has been used by numerous investigators because it is an effective Hg methylator (Pak and Bartha, 1998a; Pak and Bartha, 1998c; Jay et al., 2002; Lin and Jay, 2007; Gilmour et al., 2011). Cultures of ND132 were provided courtesy of Dr. Cindy Gilmour (Smithsonian Environmental Research Center) and maintained in sulfate-reducing media. To minimize the effect of sulfide on Hg speciation and solubility in these experiments, ND132 was cultured in a sulfate-free anaerobic SRB media (modified from the media Yen45 (Bender et al., 2007), details in Supplementary Material) under respirative conditions using pyruvate/fumarate as electron donor/acceptor. Cultures were grown in the dark at 31–33 °C.

2.2. Hg and NOM addition to ND132 cultures and sampling

Mercury and Hg–NOM complexes were added to bacterial cultures either as early additions wherein Hg species were added contemporaneously with media inoculation or as late additions wherein Hg species were added to cultures of early log, mid log, or late stationary phase bacteria. Mercury added in early addition experiments may have initially partitioned on to bottle walls and as cell density increased partitioned on to suspended solids including cell surfaces (Benoit et al., 2001a). In late addition experiments Hg species were added to active cultures during the appropriate growth phase and given 24 h to react prior to sacrifice. Both early addition and late addition populations were sacrificed in triplicate at the early log, mid log, or late stationary phase. Control experiments in which ND132 was either omitted or autoclaved prior to bottle inoculations were conducted and showed no MMHg production.

2.2.1. Preparation of solutions

All solutions in this study were prepared using 18 MΩ (MQ) water. Glassware was baked at 500 °C and acid washed in 25% HNO_3 and 10% HCl and thoroughly rinsed with MQ prior to use. Teflon labware was acid washed following the same protocol. Anoxic solutions were prepared in an anaerobic glove box (97% N_2, 3% H_2) using degassed powders or salts and anoxic 18 MΩ water. Anoxic water was produced by flash boiling and cooling water under a flow of oxygen-free N_2 and pipetting into serum bottles sealed under N_2 headspace.

2.2.2. Preparation of Hg and NOM solutions

Mercury stock solutions (600 nM) were prepared in an anoxic 1 M K_2HPO_4–NaH_2PO_4 phosphate buffer just prior to additions to samples. Mercury stock solutions for a subset of samples were prepared in anoxic 0.5% HCl.

Unfractionated Suwannee River NOM (SRNOM; collected from Suwannee River, GA) was purchased from the International Humic Substances Society. Characterization of SRNOM from this study and the literature (Helms et al., 2008; IHSS, 2010) is provided in Supplementary material text and in Table S-1. Interactions of SRNOM with Hg have previously been characterized (Miller et al., 2007; Miller et al., 2009). Stock solutions of SRNOM were prepared in either anoxic 1 M K_2HPO_4–NaH_2PO_4 phosphate buffer or anoxic MQ water. Stock solutions of pre-equilibrated Hg–SRNOM were given 24 h to equilibrate (Lamborg et al., 2003; Miller et al., 2009) prior to being spiked into culture bottles. The stock solutions of Hg and SRNOM for these late addition spikes shared the above-mentioned 1 M K_2HPO_4–NaH_2PO_4 matrix to minimize differences in NOM during pre-equilibration periods. All stock SRNOM solutions were filter-sterilized (0.2 μm) in a glove box prior to use. Concentrations of the filtered stock NOM solutions were verified through dissolved organic carbon (DOC) measurements using a Shimadzu TOC-5000A total organic carbon analyzer.

2.2.3. Early addition of Hg and Hg–SRNOM complexes

Early addition experiments were conducted by adding Hg or Hg + SRNOM to 50 mL sulfate-free base mineral media (pH = 7.4) and allowing 24 h for equilibration prior to inoculation (Lamborg et al., 2003; Miller et al., 2009; Dong et al., 2010). Bottles were amended with pyruvate (30 mM) and fumarate (30 mM) as well as cysteine (0.3 mM) and methionine (0.3 mM) that preliminary experiments indicated were necessary to ensure growth (Supplementary material, Figure S-1). Complications that could arise from cysteine–Hg–NOM–cell interactions are addressed in section 3.3.1. Bottles were subsequently inoculated (1 mL inoculum to 50 mL media) with ND132 from an actively growing culture in the same but Hg-free media. Mercury was