ABSTRACT

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Microbial mercury methylation is an integral factor controlling methylmercury concentrations within aquatic ecosystems. This thesis explores the phylogenetic distribution and biochemistry of methylation among the dissimilatory iron-reducing bacteria (DIRB). When distribution of methylation capacity among DIRB was examined, strains of *Geobacter* spp., which are closely related to mercury-methylating, sulfate-reducing *Deltaproteobacteria,* methylated mercury while reducing iron or other substrates. In contrast, no tested strains of the *Gammaproteobacteria* genus *Shewanella* produced methylmercury above abiotic controls.

Mercury methylation by the cobalamin-dependent methionine synthase (MetH) pathway was examined. Heterologous expression of *G. sulfurreducens metH* in *E. coli* was used to evaluate involvement of MetH in methylation. Methylation by a clone expressing MetH and a non-expressing control clone was tested *in vivo* and *in vitro*. Methylation by the expressing clone was not significantly higher than either the control or abiotic assays in either experiment, suggesting that MetH is not involved in methylation in *G. sulfurreducens*.

MERCURY METHYLATION IN DISSIMILATORY IRON-REDUCING BACTERIA

By

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CHAPTER 1: INTRODUCTION

1.1 Scope of research

The production of methylmercury (MeHg) from inorganic mercury is of principal interest in the study of mercury biogeochemistry. It is this organic form of mercury which bioaccumulates most strongly through trophic levels (Hammerschmidt et al. 2004), with the potential to impart negative neurological, reproductive and developmental effects on human and wildlife populations (Clarkson and Magos 2006; Driscoll et al. 2007). Mercury methylation is a microbially mediated process thought to be dominated by dissimilatory sulfate-reducing bacteria (DSRB) (Fitzgerald et al. 2007), but was recently also recognized in dissimilatory iron-reducing bacteria (DIRB) (Fleming et al. 2006; Kerin et al. 2007, Chapter 2). The distribution of methylation capability among phylogenetic groups of bacteria is not currently known. Although methylation seems to be related to carbon metabolism, possibly through the acetyl CoA or cobalamin-dependent methionine synthase (MetH) pathways, the biochemical process of methylation within cells has not been clearly determined (Choi et al. 1994).

This study was designed to explore the phylogenetic distribution and biochemical process of mercury methylation by investigating whether: 1) methylating capability is present in multiple species of DIRB within the *Deltaproteobacteria* and 2) the MetH pathway is responsible for mercury methylation in at least one microbial species. The ability of DIRB to methylate mercury was examined in pure culture under multiple growth conditions, using enriched mercury stable isotopes, in order to better define the distribution of methylating capability among microbes. The

potential for methylation by the MetH pathway was investigated in *Geobacter sulfurreducens* using heterologous gene expression and gene deletion methods. *G. sulfurreducens* is the only methylating organism for which the complete genome sequence is currently known (Holmes et al. 2004) and for which a genetic system exists (Coppi et al. 2001). This information allows us to study methylation using genetic techniques that are not yet possible for other methylating organisms, such as *Desulfobulbus propionicus* (1pr3). Genetic sequences from *G. sulfurreducens* and *G. metallireducens* allow for the design of primers to amplify specific genes from these organisms. The existence of a genetic system for *G. sulfurreducens* makes it possible to mutate genes in this organism.

1.2. Mercury toxicity, distribution and cycling

Environmental mercury contamination is a recognized public health problem in ecosystems throughout the world and adversely affects aquatic wildlife populations (Scheuhammer et al. 2001; Clarkson and Magos 2006). Mercury exposure has been implicated as a cause of decreased reproductive success in fish and piscivorous aquatic birds (Meyer et al. 1998; Hammerschmidt et al. 2002; Heinz et al. 2006) and as an endocrine disruptor in fish (Klaper et al. 2006). The potential of mercury's deleterious effects on human populations has been recognized since the 1950s, when contamination of Minamata Bay, Japan resulted in large-scale acute poisoning and subsequent deaths of residents of that area (Harada 1995). Negative neurological, teratogenic and developmental effects on humans are also documented at lower, chronic exposure levels (Budtz-Jorgensen et al. 2006; Clarkson and Magos 2006) (Debes et al. 2006; Mergler et al. 2007); epidemiological studies suggest that these

effects may be impacting a broad human population, with as much as 8% of US women of child bearing age having blood mercury concentrations above levels considered safe by the US Environmental Protection Agency (Schober et al. 2003; Mahaffey et al. 2004). Today, human exposure occurs primarily through the consumption of fish (Mergler et al. 2007). To understand the relationship between human health and mercury contamination it is imperative to understand mercury methylation within aquatic environments.

Methylation is linked to other processes in the aquatic cycling of mercury (Figure 1). Mercury is present predominantly as complexes of inorganic divalent mercury (Hg^{2+}), as elemental mercury (Hg^0), and as complexes of organic MeHg in aquatic environments. Sources of mercury to aquatic environments include wet and dry atmospheric deposition and influx of mercury from terrestrial runoff (Fitzgerald et al., 2007). Transformations of mercury within aquatic environments play a role in the potential impact of mercury at higher trophic levels.

Mercury is highly reactive with solids, and readily interacts with sediments. It is particularly reactive with sulfides (e.g. Schwartzenbach and Widmer 1963; Dyrssen 1989; Benoit et al. 1999), and organic matter (e.g. Ravichandran et al., 1999; Haitzer et al., 2002; Skyllberg et al. 2000; Babiarz et al. 2003; Lamborg et al. 2003; Miller et al. 2007) and to a lesser extent with iron (Miller 2006) and hydroxyl groups. Divalent mercury can be methylated abiotically or microbially to form methylmercury (Benoit et al. 2003). Divalent mercury can also be reduced to Hg^0 which may remain in the water column or volatilize into the atmosphere (e.g. Fitzgerald et al. 1991; Amyot et al., 1997; Martin-Doimeadios et al. 2004; Poulain et al. 2007). This reduction occurs through abiotic and microbially-mediated pathways (Allard and Arsenie, 1991;

Mason et al., 1995; Schaefer et al., 2002). Photochemical Hg reduction is the main driver in most surface waters. (e.g. Krabbenhoft et al. 1998; O'Driscoll et al., 2004; Fitzgerald et al. 2007). Microbially mediated reduction may be mediated by the mercuric reductase gene (*merB*), which codes for an enzyme that reduces divalent mercury to elemental mercury (Schaefer et al., 2002) or by other, poorly defined pathways (Mason et al., 1995; Poulain et al., 2004). Another gene on the *mer* operon codes for the organomercurial lyase enzyme, which breaks MeHg down into methane and Hg (II) (Marvin-DiPasquale et al., 2000). Methylmercury may also be degraded microbially via the oxidative demethylation pathway (Oremland et al. 1991; Marvin DiPasquale et al., 1998). The *mer*-based pathway may operate under aerobic or anaerobic conditions while oxidative demethylation of MeHg is an anaerobic process.

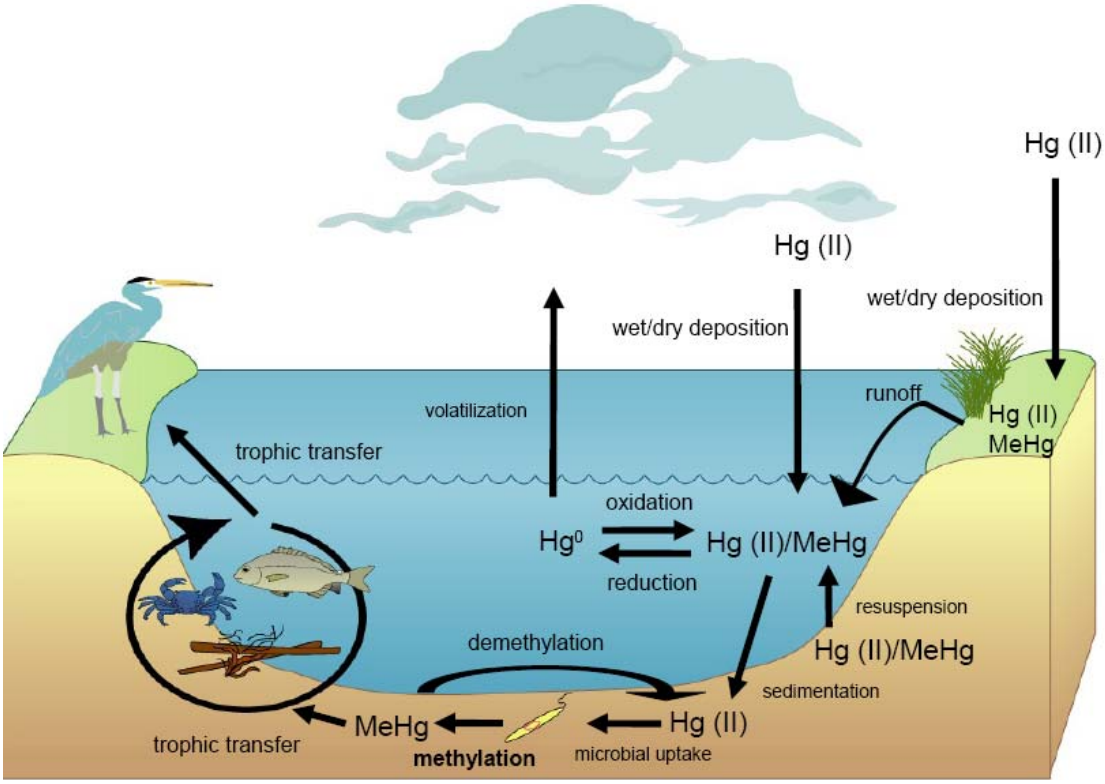


Figure 1: Conceptual diagram of mercury cycling in an aquatic environment. Mercury methylation is mediated by microbial activity in anoxic sediments, wetland soils and bottom waters.

Microbial methylation is a key component of aquatic mercury cycling. Anoxic zones in sediments, wetlands the hypolimnia of stratified waters are the most active sites of methylation in aquatic environments (Munthe et al. 2007). Although methylation can occur abiotically, microbial methylation accounts for the majority of methylation within aquatic sediments (e.g. Compeau and Bartha 1985; Callister and Winfrey 1986; Driscoll et al., 1998; Macalady et al. 2000; Heyes et al. 2006; Fleming et al. 2006). It has been shown that sediment MeHg concentrations in many environmental systems are controlled not by external supply of MeHg but rather by *in situ* MeHg production via microbial methylation (Gilmour et al. 1998; Benoit et al. 2003; Orihel et al. 2006; Munthe et al. 2007; Fitzgerald et al. 2007).

Methylation is dependent on the delivery of inorganic mercury to zones of methylation, the bioavailability of mercury for methylation, and the presence of methylating bacteria within aquatic systems (Munthe et al. 2007). The bioavailability of mercury for bacterial uptake has been linked to the complexation of the mercury. Passive uptake of neutral mercury chloride and mercuric sulfide has been observed in phytoplankton (Mason et al. 1996) and sulfate-reducing bacteria (Benoit et al. 2001). Recent studies of non-methylating bacteria suggest that charged species may also be biologically available, and that the pH of environmental conditions may affect mercury availability (Golding et al. 2002; Kelly et al. 2003; Najera et al. 2005). The mechanism of uptake has not been determined in DIRB or DRSB. Methylation rates have been correlated with the abundance of sulfate-reducing bacteria in some sediments (King et al., 2002; Macalady et al., 2000). This may be because Hg-methylating sulfate-reducing bacteria are most abundant and active in these zones. It could also be a result of geochemical conditions in these areas that render mercury

most biologically available for uptake and subsequent methylation. Correlations between methylation rates and specific strains of methylating bacteria have not been resolved. This thesis attempts to identify a biochemical marker for methylation or phylogenetic pattern of distribution that will allow this relationship to be determined. This will allow exploration of relative importance of biological and geochemical controls on methylation in the environment.

Of the inorganic and organic forms of mercury typically present in any mercury-contaminated environment, MeHg is preferentially taken up by organisms and biomagnified through the food chain (Mason et al. 1996; Lawson and Mason, 1998; Scheuhammer et al. 2007). Due to MeHg's proclivity for bioaccumulation, it is critical to understand the process of microbial mercury-methylation in order to adequately assess the potential impact of mercury in the environment.

Two potential paths to better understand microbial mercury methylation are 1) the study of the phylogenetic distribution of methylating capability among microbial communities, and 2) the study of the biochemical pathway of methylation within cells. Studies of microbial community structure that historically have been limited to culture dependent studies can now employ molecular biology techniques to examine the full diversity of communities *in situ.* Biochemical investigation of methylation pathways were difficult until the recent rise of genomic sequencing and recombinant DNA technologies that now facilitate the manipulation of functional genes for specific pathways within cells. These two applications of molecular techniques offer a new potential to comprehend a fundamental process in the cycling of mercury.

1.3. Phylogenetic distribution of mercury-methylating bacteria

1.3.1. Mercury methylation among the dissimilatory sulfate-reducing bacteria

The role of DSRB in MeHg production has been demonstrated in sediments and soils (Harmon et al., 2004; Gilmour et al. 1992; Henry et al. 1995; King et al. 1999); through amendments to lakes and wetlands (Branfireun et al. 1999, 2001; Jeremiason et al. 2006; Wiener et al. 2006), and in pure culture experiments (Compeau 1985; King et al. 2000; Benoit et al. 2001a; Benoit et al. 2001b; Ekstrom et al., 2003). In an experiment using Florida Everglades sediment, Benoit and coworkers (2003) observed a concomitant increase in sulfate reduction and mercury methylation rate in sediment cores amended with sulfate. However, inhibition of MeHg production was observed in a separate set of cores amended with sulfide. This supports the hypothesis that MeHg production is associated microbial sulfate reduction, (Compeau and Bartha 1985), but can be inhibited by sulfide, the end-product of the process (Benoit et al., 2003).

Pure culture experiments support the hypothesis that DSRB are involved in mercury methylation. Compeau and Bartha (1985) initially observed that inhibition of DSRB with sodium molybdate decreased methylation in salt marsh sediments by 95%. A *Desulfovibrio desulfuricans* strain was subsequently isolated from these sediments and demonstrated mercury methylation capability. In a later study, King and coworkers (2000) tested five DSRB strains (*Desulfovibrio desulfuricans* ATCC 13541, *Desulfobulbus propionicus* ATCC 33891, *Desulfococcus multivorans* ATCC 33890, *Desulfobacter* sp. strain BG-8 *and Desulfobacterium* sp. strain BG-33) for mercury methylation capability after incubation with 100 ng/ml inorganic mercury,

and observed MeHg production rates between 4.2 x 10^{-8} and 6.2 x 10^{-6} pg MeHg×cell^{-1}×hour^{-1}. In addition, no methylation was observed in media prepared without sulfate, indicating that the activity of the DSRB was responsible for the observed MeHg production.

Following the establishment of a relationship between pure DSRB cultures and methylation, several studies have attempted to address the relationship between DSRB community structure and *in situ* mercury methylation (King et al. 2001; Macalady et al. 2000). Using oligonucleotide probes, King and coworkers (2001) examined the correlation between MeHg production and the presence of specific DSRB groups in sediment cores from a salt marsh along the Skidaway River, GA. Results showed that the peaks in 16S rRNA hybridizing with probes designed to target *Desulfobacter* sp BG-8 and *Desulfobacterium* sp. BG-33 were found at depths corresponding to the highest MeHg production rates (King et al. 2001).

In a study of mercury methylation in Clear Lake, CA, Macalady and colleages (2000) used polar lipid fatty acid analysis (PLFA) to discern the correlation between community structure and mercury methylation potential. Based on previous studies linking PLFA composition to specific DSRB groups (Dowling 1986; Kohring et al. 1994), the authors determined that DSRB with *Desulfobacter*-like PLFA compositions were more abundant than *Desulfovibrio* and *Desulfobulbus* in sediments with high MeHg production potential. At the time there were no studies quantifying methylation rates among species of DSRB and thus the authors were unable to conclude whether these differences in abundances were controlling MeHg production rates in Clear Lake sediments.

Even though the above-mentioned studies began to address the relationship between DSRB community and *in situ* mercury methylation, there are still gaps in the understanding of *in situ* mercury methylation by DSRB. The relative contributions of geochemical and microbial controls on methylation have yet to be resolved. Although studies indicate that DSRB are important for *in situ* methylation, it is not clear that their abundance in a particular environment—or the presence or absence of a specific species of DSRB—is the factor ultimately controlling MeHg production rates. This is particularly true given the recent recognition of methylation by bacteria outside the DSRB metabolic group (Fleming 2006; Kerin et al. 2007, Chapter 2).

In a set of papers examining mercury uptake by *Desulfobulbus propionicus* (1pr3), Benoit and coworkers (Benoit et al. 2001a; Benoit et al. 2001b) examined how the complexation of mercury with sulfide affects its uptake into bacterial cells. In both studies, MeHg production was used as a proxy for mercury uptake by cells. In an experiment using pure cultures of *Desulfobulbus propionicus* (1pr3), the authors found that increasing sulfide concentrations by 10^3 fold, resulted in a fourfold decrease in methylation of filtered inorganic mercury (Benoit et al. 2001a).

Two further experiments, using natural sediment as a source of sulfide, showed correlations between the concentration of the neutral species mercury sulfide and MeHg concentration (Benoit et al. 2001b). The results of these experiments indicate that the chemical availability of mercury to uptake by cells is a predictor for methylation in pure culture. Studies of uptake and methylation by a mixed consortium of DSRB have not been conducted in a laboratory setting, but it is reasonable to assume that the same controls would play a role in mixed communities and in the environment. Thus the relative importance of these and other chemical

controls (mercury complexation with DOC, for example) (Benoit et al. 2003) in relationship to DSRB community structure remains unclear. Furthermore, some studies have also shown that microbial community structure may not always directly correlate with the aqueous chemistry of a sedimentary environment. For example, Koretsky and colleagues (2005) found that in heavily bioturbated salt marsh sediments, pore water redox chemistry varied from sulfide-dominated to suboxic, but that aerobic microbial communities coexisted with iron, manganese and sulfate-reducing bacteria. This indicates that in order to be valid, predictions of MeHg production potential must be linked to the activity of specific bacteria, rather than their mere presence.

It cannot be inferred, and in fact is unlikely, that the particular species that have demonstrated mercury methylation capability in pure culture are responsible for the methylation observed in the environment. The advent of molecular techniques in the study of microbes in the environment has revealed that the majority of organisms present in environmental samples have not been cultured (Rappe and Giovannoni 2003). Early molecular based studies of DSRB 16S rRNA in sediments revealed phylogenetic lineages of uncultured bacteria (Devereux and Mundfrom 1994) and thus it is currently difficult to evaluate the importance of these uncultured DSRB in mercury cycling without the ability to study their metabolic activities in pure culture, unless the expression of specific genes involved in methylation can be measured.

Initial attempts to relate DSRB community structure to environmental MeHg production in marine environments may have been constrained by the molecular technology available. Oligonucleotide probes used by King et al. (2001) were first developed by Devereux et al. in 1992. Using these probes, King et al. (2001)

concluded that the *Desulfosarcina-Desulfococcus* 16S rRNA make up a small percentage of total RNA present in Skidaway River salt marsh sediments, a result also found by Devereux et al. when examining estuarine sediments in Santa Rosa Sound, FL using the same probes (Devereux et al. 1996). These results are in contrast to Ravenschlag et al. (Ravenschlag et al. 2000), who used newer *Desulfosarcina-Desulfococcus* probes (designed by these authors in 2000 and by Manz et al. in 1998) and found that *Desulfosarcina-Desulfococcus* were the most abundant group *in situ* in Arctic Ocean sediments in coastal Svalbard.

Based on the conflicting results of the King et al. (2001) and Ravenschlag et al. (2000) studies, its is apparent that a clearer picture of the phylogenetic diversity of DSRB in marine sediments would help guide inferences on how this group of organisms affects MeHg production rates. In recent years, molecular-based, culture-independent research has addressed the molecular diversity of DSRB in a range of sedimentary environments (Orphan et al. 2001; Liu et al. 2003; Mussmann et al., 2005). Various approaches to community analysis in marine and estuarine sediments have been used, including construction of clone libraries using either the 16S rRNA gene (Rooney-Varga et al. 1998; Bowman and McCuaig 2003; Polymenakou et al. 2005) or dissimilatory bisulfate reductase (*dsrAB*) gene sequences, restriction fragment length polymorphism (RFLP)(Liu et al. 2003; Mussman et al., 2004; Smith et al. 2004), fluorescence *in situ* hybridization (FISH) (Orphan et al. 2001; Liu et al. 2003; Smith et al. 2004) dot blot hybridization (Ravenschlag et al. 1999), rRNA slot blot hybridization (Ravenschlag et al. 2000) and denaturant gradient gel electrophoresis (DGGE) (Llobet-Brossa et al. 2002).

Although this ample body of work does not approach the study of DSRB community structure with the aim of understanding mercury cycling, it nevertheless provides valuable insight toward that end. Overall, molecular based studies indicated 1) a high degree of diversity in sediment DSRB communities (Ravenschlag et al. 1999; Polymenakou et al. 2005) 2) prevalence of uncultured DSRB in these communities and (Smith et al. 2004; Polymenakou et al. 2005) 3) The existence of methylating strains in DRSB groups that are both environmentally rare (i.e. *Desulfovibrionales*) (Mussman et al., 2005) and dominant (i.e. *Desulfosarcinales*) phylogenetic groups (Ravenschlag et al. 1999; Ravenschlag et al. 2000; Llobet-Brossa et al. 2002).

The comparison of a *Desulfosarcina–Desulfococcus* depth profile with King et al. (2001) DSRB community composition and MMR depth profiles shows the difficulty of using the presence of specific DSRB groups as indicators of MeHg. *Desulfosarcina–Desulfococcus* probes target subgroups that may be uncultured and which have not been tested for methylation. The greatest abundance of this group (as % total DAPI cell counts) occurs at the depth range with the highest MMR and at the depth where King et al. observed the highest total abundance of DSRB. This could suggest that *Desulfosarcina–Desulfococcus*–like organisms play a role in mercury methylation. It could also mean that mercury methylation occurs where DSRB are, as a group, most prevalent, or that the geochemical conditions favorable for DSRB are coincident with those that are ideal for mercury methylation. Again, it is difficult to determine which is the case without further knowledge of the metabolic activity of DSRB present.

High DSRB diversity in MeHg producing environments suggests that a wide variety of DSRB may play a role in the methylation process. Nevertheless, without an understanding of the activity of the DSRB present or knowledge of the metabolic pathway through which methylation occurs, it is very difficult to make inferences about the relative importance of different groups of bacteria to mercury methylation *in situ*. Finally, uneven distribution of methylating and non-methyating DSRB across rare and dominant phylogenetic groups makes it tenuous to argue that the prevalence (or rarity) of specific groups of DSRB is any sort of predictor of *in situ* MeHg production.

In summary, cycling of mercury in anoxic environments is heavily influenced by microbial controls. The existing research on the effect of sulfate and sulfide on mercury methylation rates, observations of mercury methylation by DSRB in pure culture, and environmental studies linking *in situ* methylation to the presence of DSRB all indicate that DSRB are integral to the production of MeHg in these environments. A survey of molecular based DSRB community analysis research indicates that methylating DSRB genera are present at varying abundances throughout these highly diverse communities composed primarily of uncultured organisms. The key to understanding MeHg production may lie in understanding the links between the structure and activity of these DSRB communities but this challenge is further complicated by recent observations of methylating bacteria outside the DSRB.

1.3.2. Methylation outside the dissimilatory sulfate-reducing bacterial species

While much mercury methylation is thought to be driven by the activity of DSRB recent work and a theoretical understanding of microbial methylation both suggest that a broad range of anaerobic respiring bacteria may be responsible for the production of environmental MeHg. Field and culture studies increasingly suggest a role for DIRB in mercury methylation in sediments and soils (Fleming et al. 2006) (Warner et al. 2003; Mehrotra and Sedlak 2005). In a study of Mobile Alabama River basin sediments, Warner and colleagues (2003) measured methylation in areas where iron was the dominant terminal electron acceptor. Fleming and coworkers (2006) found that chemical inhibition of sulfate reduction did not result in complete inhibition of mercury methylation in sediments from Clear Lake, CA. The authors attributed this methylation under DSRB inihiting conditions to activity of dissimilatory iron-reducing bacteria. The same study also demonstrated mercury methylation by a *Geobacter* strain isolated from Clear Lake (Fleming et al. 2006). In order to study the the extent of methylating capability among DIRB, the ability of pure cultures in the genera *Geobacter, Desulfuromonas* and *Shewanella* to methylate inorganic mercury was tested (Kerin et al. 2007, Chapter 2). All strains within *Geobacteraceae* methylated mercury while *Shewanella* strains tested did not. These results, combined with those of Fleming and coworkers, suggest that mercury methylating capability may be common among the *Geobacteraceae* but is not ubiquitous within the DIRB.

There are suggestions that methylation capability may even extend beyond DIRB. Additionally, methylation of the metal bismuth, that is analogous to mercury in that it is volatile in its reduced state, has been reported in pure cultures of the

methanogen *Methanobacterium formicicum* (Michalke et al. 2002). This methylation of bismuth probably does not occur via the Challenger pathway–where S-Adenosyl methionine (SAM) donates a methyl group to a reduced metal in a coupled oxidation reduction reaction–because the reduced form of bismuth is volatile (Thayer, 2002). Indeed, Michalke and co-workers found that *M. formicicum* extracts methylated bismuth in the presence of methylcobalamin and not in the presence of SAM. Mercury is analagous to bismuth in that it is volatile in its reduced state and its methylation mediated by cobalamin. It is possible that the methylation pathways are the same for both of these elements. The discovery of mercury methylation by organisms outside DSRB and the results of Michalke and co-workers suggest a re-examination of the possibility of methylation by methanogens and other anaerobic bacteria. Although abiotic methylation by methylcobalamine could have occurred (Chapter 3)

1.3.3. Conclusions on phylogenetic distribution of methylation

The study of phylogenetic diversity of mercury methyling bacteria has advanced the understanding of mercury biogeochemistry, while leaving some compelling questions unanswered. Organisms that methylate in pure culture do not seem to be especially prevalent or rare in environmental microbial communities. The phylogenetic distribution of methylating capability is broader than was understood five years ago, and methylation seems to be distributed in a complex way among DSRB and DIRB groups. However, it is worth noting that, to the best of my knowledge, all organisms that have been shown to methylate in pure culture fall within the *Deltaproteobacteria*, and the few organisms tested for methylation outside

of the *Deltaproteobacteria* have not been capable of methylation. It is not yet possible to tell whether this putative relationship between methylation and phylogeny, is due to the small number of cultures that have been tested. Culturable bacteria represent a very small fraction of the diversity observed in environmental communities and methylation tests are relativly time consuming and labor intensive. These realities have limited the study of the distribution of methylation among phylogenetic groups as a means to determine biological controls on methylation. Understanding the structure of a microbial community is only useful in predicting methylation if the methylation capability of each species in a community is known. If a specific functional gene is shown to be involved in mercury methylation, the distribution of this gene in a microbial community could tell which species are capable of methylation. Investigation of a genetic or biochemical basis for methylation ability is therefore an alternate approach that may supplement what has been learned through studies of phylogeny.

1.4. Biochemical pathways of mercury methylation

1.4.1. Background

One approach to studying the microbial ecology of mercury methylation is to determine the phylogenetic distribution of microorganisms that are responsible for this proccess. This knowledge will be useful for studying the biological controls on the spatial and temporal production of methylmercury in a variety of geochemical environments, as linkages between methylation and certain phylogenetic groups will be better understood. Molecular advances in microbial ecology offer the potential to

provide more specific information. Identification of the gene(s) or metabolic pathway(s) responsible for mercury methylation would allow one to examine the occurrence of mercury methylation capability across phylogenetic groups and in the natural environment. Identification of the gene or metabolic pathway of mercury methylation would reveal the mechanism behind a process that affects the health of aquatic systems on a global scale.

In this study, genetic information available for *G. sulfurreducens* was used to test whether the MetH pathway is responsible for microbial mercury methylation. Methylation by the MetH pathway was suggested by Landner in 1971. In this pathway, a methyl group is transferred from methyltetrahydrofolate (MeTHF) to homocysteine via a cobalamin-containing cofactor, thus producing tetrahydrofolate and methionine. Theoretically, this cofactor could methylate mercury by transferring a methyl group to inorganic mercury rather than homocysteine. This possibility is supported by several lines of evidence, including 1) the observation of incorporation of methyl groups from MeTHF into methylmercury by *Desulfovibrio desulfuricans* LS (Bartha and others); 2) the identification of a corrinoid protein in the methylating organism *Desulfovibrio desulfuricans* LS; 3) inhibitor tests of pure DSRB cultures that suggested methylation does not occur by the acetyl CoA pathway and 4) reports of abiotic methylation by extracts of an *E. coli* strain overexpressing the *metH* gene.

1.4.2. The role of cobalamin and methyltetrahydrofolate in methylation by Desulfovibirio desulfuricans LS

Choi and Bartha (1993) hypothesized that microbial mercury methylation is not a mercury-detoxifying reaction but rather an inadvertent and relatively rare

variation in normal carbon metabolism. A series of papers by Richard Bartha's group examined methylation pathways by one mercury-methylating DSRB, *Desulfovibrio desulfuricans* strain LS. This estuarine strain was isolated from a mercury-contaminated New Jersey salt marsh (Compeau and Bartha 1985). Their work implicated methyltetrahydrofolate (MeTHF) as a methyl donor and cobalamin as a methyl carrier in this strain (Berman et al., 1990; Choi and Bartha, 1993; Choi et al., 1994a; Choi et al., 1994). Methyltetrahydrofolate and cobalamin are important elements of the acetyl coenzyme A (CoA) (Svetlitchnaia et al., 2006) and cobalamin-dependent methionine synthase (MetH) pathways (Bannerjee and Matthews, 1990) both of which transfer methyl groups within cells.

Berman et al. (1990) examined MeHg production during metabolism of serine in strain LS, using 14-C radiolabeled serine to follow the methyl group. They observed incorporation of the 14-C into MeHg and predicted that the carbon from the serine was transferred to tetrahydrofolate, which then served as the methyl donor in the mercury methylation reaction. This observation was supported by the presence of MeTHF in *D. desulfuricans* LS cell proteins and by a later study using cell extracts and showing high activity of enzymes in the pathway responsible for converting serine to MeTHF (Choi et al., 1994). These experiments implicated MeTHF as the most likely methyl donor to inorganic mercury, although they did not explain the mechanism by which the methyl group is transferred to mercury.

One possible mechanism of methylation involves transfer of a methyl group from MeTHF to mercury via cobalamin. Additional studies by Bartha's group (Berman et al., 1990; Choi and Bartha, 1993; Choi et al., 1994a; Choi et al., 1994) supported this hypothesis for *D. desulfuricans* LS. Radioimmunoassays of *D.*

desulfuricans LS cell extracts were positive for cobalamin or a related corrinoid (Berman et al., 1990; Choi and Bartha, 1993). This corrinoid was later purified, identified as cyanocobalamin by high performance liquid chromatography and shown to methylate mercury in abiotic assays (Choi and Bartha, 1993). Additional experiments with *D. desulfuricans* LS showed that methylation was decreased by the inactivation of a cobalt porphyrin and stimulated by the addition of cobaltous chloride (Berman et al., 1990; Choi and Bartha, 1993; Choi et al., 1994a). The combined results of these experiments indicate the importance of methylcobalamin in the transfer of a methyl group from donor to mercury during methylation by this organism.

The importance of MeTHF and cobalamin in mercury methylation suggests the possibility of methylation by either the acetyl CoA or the MetH pathway. In the acetyl CoA pathway, a methyl group is transferred between acetyl CoA and MeTHF by a corrinoid protein (Ragsdale et al., 1990). Corrinoid methyltransferases from the acetyl CoA pathway have been identified in the methanogenic archea *Methanosarcina thermophila* (Maupin-Furlow, 1996), the acetogenic *Moorella thermoacetica* (Hu et al., 1984), and the hydrogenogenic *Carboxydothermus hydrogenoformans* (Svetlitchnyi et al., 2004); and are likely found in other organisms as well. In the methionine synthase reaction, a methyl group from N5-MeTHF is transferred to homocysteine to produce tetrahydrofolate and methionine. In the cobalamin-dependent (MetH) form of this pathway, the methyl group is transferred via a cobalamin-containing cofactor (Banerjee and Matthews, 1990). Methylation of mercury could occur in either the acetyl CoA or MetH pathways if the methyl group

from MeTHF was inadvertently transferred to mercury by the corrinoid methyltransferase enzyme.

1.4.3. Investigations of methylation by the acetyl CoA pathway

The acetyl CoA pathway has been studied for its possible involvement in mercury methylation. The formate, pyruvate and carbonate that were shown to incorporate into MeHg in *D. desulfuricans* LS are all precursors in the formation of acetyl CoA (Berman et al., 1990; Choi and Bartha, 1993; Choi et al., 1994a). Choi and coworkers found enzymes related to the acetyl CoA pathway in *D. desulfuricans* LS, although their activity was not as high as had been measured in acetogens (Choi et al. 1994a). Acetogens are not known to be capable of mercury methylation, so these results themselves did not implicate the acetyl CoA pathway. However, Choi (Choi et al., 1994b) also found that propyl iodide simultaneously inhibited MeHg and acetyl CoA synthesis, and that the MeHg synthesis competed with the formation of acetyl CoA for methyl groups. These results suggested that MeHg and acetyl CoA form concurrently or competitively, from the same precursors, through the acetyl CoA pathway. It was suggested that these methylation would occur in this pathway by transfer of a methyl group via a cobalamin methyltransferase enzyme (Ekstrom et al., 2003) (Figure 2).

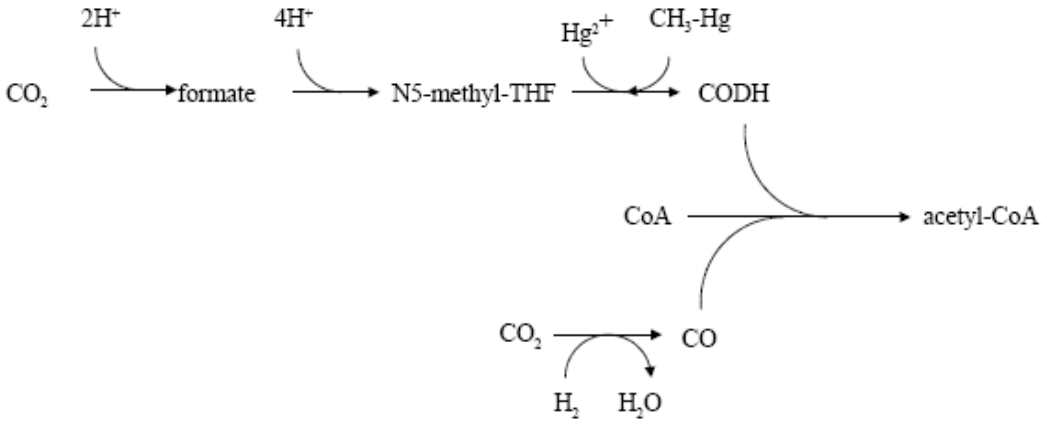


Figure 2: Potential mechanism for mercury methylation via the acetyl-CoA pathway. In this scenario, donation of a methyl group to inorganic mercury, from N5-methyl-THF would occur via a methyltransferase enzyme. In normal function of this pathway, the methyl group would be transferred to the carbon monoxide dehydrogenase enzyme (CODH).

Later work by Ekstrom et al. (2003) followed up on Bartha's suggestion that acetyl CoA pathway might be involved in methylation, but in the process identified DSRB strains both with and without the acetyl CoA pathway that are capable of MeHg production, leading the understanding of the role of the acetyl CoA pathway in mercury methylation to be somewhat uncertain. The authors selected seven strains: five strains that actively used the acetyl CoA pathway, and two that did not. Methylation capability was tested for all strains. Activity of the acetyl CoA pathway was determined by assaying the activity of carbon monoxide dehydrogenase enzyme, the enzyme splitting acetyl CoA into carbon monoxide and a methyl group. Methylation by the organisms did not correspond with the presence of the acetyl CoA pathway. Only one methylating species, *D. multivorans*, was found to have carbon monoxide dehydrogenase activity, indicating the presence of an active acetyl CoA

pathway. Next, inhibition studies were designed in which chloroform was used to stop activity of the acetyl CoA pathway. For *D. multivorans*, this inhibition decreased MeHg production. For strains without carbon monoxide dehydrogenase activity, chloroform inhibition did not affect methylation capability. This work demonstrated that microbial methylation can occur independently from the acetyl CoA pathway, but it did not rule out involvement of the acetyl CoA pathway in some strains.

1.4.4. Evidence for methylation by the cobalamin- dependent methionine synthase pathway

Since mercury methylation appears to be uncoupled from the acetyl CoA pathway in at least some *Deltaproteobacteria*, we chose to focus our first studies of methylation mechanisms in *Geobacter* on the MetH pathway. Landner suggested the possibility of Hg methylation via this pathway in 1971. The evidence suggesting that MeTHF donates a methyl group via cobalamin supports the hypothesis of methylation by MetH. An alternative interpretation of the work by Bartha and others (summarized above) strengthens this idea. Incorporation of serine, formate and pyruvate into MeHg were seen as an indication of acetyl CoA activity, but these are actually important precursors to the MetH pathway as well. Additionally, enzymes that Choi et al. (1994a) identified in *D. desulfovibrio* LS (notably the serine hydroxymethyltranserase and N5, N10-methylene-tetrahydrofolate reductase) are responsible for generating the methyl donor MeTHF used in both the acetyl CoA *and* MetH pathways (Banerjee and Matthews, 1990).

Siciliano and Lean (2002) reported MeHg production in an anaerobic *in vitro* methylation assay using *Escherichia coli* XL1-Blue/pKF5A, a strain that overexpresses the *metH* gene. Following on our previous work showing that the DIRB *G. sulfurreducens,* for which a whole genome sequence is available was a methylator, we combined genetic manipulation of the *metH* gene from *G. sulfurreducens* with methylation assays similar to those of Sicilian and Lean (2001), in order to evaluate the role of MetH in methylation by *G. sulfurreducens* (Chapter 3)**.**

CHAPTER 2: MERCURY METHYLATION BY DISSIMILATORY IRON-REDUCING BACTERIA

Kerin, E.J., C.C. Gilmour, E. Roden, M.T. Suzuki, J.D. Coates, R.P. Mason. 2006. Applied and Environmental Microbiology, 72(12): 7919-7921.

2.1. Introduction

Methylmercury concentrations in most sediments are controlled by *in situ* net microbial methylation (Benoit et al. 2003; Hammerschmidt et al. 2004). Environmental mercury methylation is an anaerobic microbial process generally driven by DSRB (Benoit et al. 2003). However, recent research suggests that DIRB may play a role in environmental methylation (Warner et al. 2003; Fleming et al. 2006). Furthermore, Fleming et al. (2006) demonstrated mercury methylation by a *Geobacter* strain isolated from Clear Lake, CA. The DSRB in the *Deltaproteobacteria*, the major group of known methylators, are phylogenetically similar to the DIRB family *Geobacteraceae*, suggesting that *Geobacteraceae* may possess the ability to methylate mercury. Consequently, an experiment was designed to screen a phylogenetically diverse group of DIRB cultures for mercury methylating capability in order to 1) develop insight into *in situ* biological methylation controls, and 2) further investigate the phylogenetic distribution of methylating bacteria.

Two studies have demonstrated net MeHg production in sediments where iron was the dominant termimal electron acceptor (Warner et al. 2003; Fleming et al. 2006), but another demonstrated inhibition of methylation by iron (Mehrotra and Sedlak 2005). Iron could potentially influence mercury methylation rates either through changes in DIRB activity, or via the impact of iron on mercury speciation

and bioavailability. In a study of estuarine wetland sediment slurries from San Francisco Bay, CA, Mehrotra and Sedlak (2005) observed decreases in mercury methylation rates with the addition of 30 mM Fe(III), and suggested that this effect was caused by decreases in dissolved mercury and sulfide due to complexation with iron. However, Warner et al. (2003) found measurable methylation in sediments where iron reduction was the dominant terminal electron acceptor, although rates of methylation were lower than those observed in sulfate-reducing or methanogenic sediments. Similarly, in sediments from Clear Lake, CA (Fleming et al. 2006) where microbial Fe(III) reduction was apparent, chemical inhibition of sulfate reduction did not result in complete inhibition of mercury methylation. This decoupling of mercury methylation from sulfate reduction suggests that another process (i.e., iron reduction) may be responsible for some amount of *in situ* mercury methylation. Mercury methylation by a *Geobacter* strain isolated from Clear Lake (Fleming et al. 2006) further supports this hypothesis.

Phylogenetic relationships between *Geobacteraceae* and the mercury-methylating DSRB also suggest a possible role for DIRB in environmental mercury methylation. The *Geobacteraceae* are found in the *Deltaproteobacteria*, branching phylogenetically between the *Desulfovibrionales* and *Desulfobaterales* orders (Holmes et al. 2004), both of which contain DSRB with methylating capability (Compeau and Bartha 1985; King et al. 2000; Benoit et al. 2001a; Benoit et al. 2001b). A wide variety of bacteria and archaea are capable of dissimilatory Fe(III) reduction (Lonergan et al. 1996; Lovley et al. 2004), including *Shewanella* in the γ-subclass of *Proteobacteria.*. This phylogenic distribution of DIRB implicates *Geobacteraceae* as possible mercury methylators and provides strains that are

phylogenetically distant from the DSRB, which may give insight into the phylogenetic distribution of mercury methylation.

2.2. Methods

To assess the role of DIRB in mercury methylation, pure cultures of *Desulfuromonas palmitatis* SDBY-1 (Coates et al. 1995), *Geobacter hydrogenophilus* (Coates et al. 2001), *Geobacter metallireducens* GS-15 (Lovley et al. 1993), *Geobacter sulfurreducens* (Caccavo et al. 1994), *Shewanella alga* BrY (Caccavo et al. 1992), *Shewanella oneidensis* MR-1 (Venkateswaran et al. 1999) and *Shewanella putrefaciens* CN-32 (Liu et al. 2003) were tested for the ability to methylate inorganic mercury while growing on a variety of electron donors and acceptors, including Fe(III), nitrate, and organic substrates (Table 1). Cultures were grown in media modified from Bond and Lovely (Bond and Lovley 2003) with electron donors and acceptors as described in Table 1, using previously described trace elements and vitamins (Laanbrock 1981).

Culture	e- Donor	e- Acceptors
D. palmitatas	20 mM acetate	55 mM Fe(III)
G. hydrogenophilus	20 mM acetate	55 mM Fe(III)
G. sulfurreducens	20 mM acetate	55 mM Fe(III); 40 mM fumarate
G. metallireducens	20 mM acetate	55 mM Fe(III); 30 mM nitrate
S. alga	30 mM lactate	55 mM Fe(III); 30 mM nitrate
S. oneidensis	30 mM lactate	55 mM Fe(III); 30 mM nitrate
S. putrifaciens	30 mM lactate	55 mM Fe(III); 30 mM nitrate

Table 1. Electron donors and acceptors and sources of DIRB cultures tested for Hg methylation.

MeHg production was assayed by measuring the amount of MeHg produced from an inorganic mercury spike during batch culture growth through stationary phase. All mercury-methylation assays were conducted in 20 ml Hungate tubes with butyl-rubber stoppers under strictly anaerobic conditions at 30^{o}C and pH 7.0. Assays using *G. metallireducens*, *G. sulfurreducens, S. putrefaciens* and S. *oneidensis* were conducted using an enriched mercury stable isotope, added as $Hg^{201}Cl_2$, at a final concentration of 10 ng/ml. Assays using *D. palmitatis, G. hydrogenophilus* and *S. alga* were conducted with natural isotopic abundance $HgCl_2$ at the same concentration. For each strain and growth condition, triplicate assays and abiotic

controls were prepared. Abiotic controls were composed of autoclaved media spiked with inorganic $HgCl_2$.

Analysis of total MeHg were performed via distillation/ethylation (Gilmour et al. 1998) /cold vapor atomic fluorescence (CVAF), using a Tekran 2500. For CVAF analysis, the method detection limit was determined by the method blank, which was generally <20 pg/sample. For analysis of a 20 ml culture sample at 10 ng Hg/L, this yields a blank equivalent to roughly 0.01% methylation. Analysis of $Me^{201}Hg$ was performed by distillation/ethylation/ICP-MS, with isotope dilution (Hintelmann and Ogrinc 2003), using a Perkin Elmer ELAN 6100 DRCII. $Me^{200}Hg$ (96.4% purity) was used as the isotope dilution standard. The concentration of $Me^{200}Hg$ was determined by reverse isotope-dilution analysis against certified standards. Methylmercury was synthesized from $Hg^{200}Cl_2$ using an aqueous methylcobalamin method (Hintelmann and Ogrinc 2003) All enriched isotopes were purchased from Oak Ridge National Labs as HgO. Method detection limits using isotope dilution-ICP/MS were generally <1 pg $Me^{201}Hg$/sample, or < 0.001% methylation.

2.3 Results and Discussion

2.3.1. Phylogenetics of dissimilatory iron-reducing bacteria methylating capability.

Methylation of inorganic $HgCl_2$ above that in uninoculated controls was observed on Fe-reducing medium in *G. metallireducens, G. sulfurreducens*, *G. hydrogenophilus*, and *D. palmitatis*, but not *S. alga,* or *S. putrifaciens* (Figure 3). The ratio of biotic to abiotic methylation was significantly higher for the *Geobacter* strains and *D. palmitatas* than for *S. alga* and *S. putrifaciens* Both *G. metallireducens* and *G. sulfurreducens* produced MeHg while growing on electron acceptors other

than Fe(III), while *S. oniedenis* and *S. putrifaciens* did not. While growing on other electron acceptors, the ratio of biotic to abiotic methylation was significantly different for each of the four cultures tested, with *G. sulfurreducens* > *G. metallireducens* > *S. putrifaciens* > *S. oniedenis*.

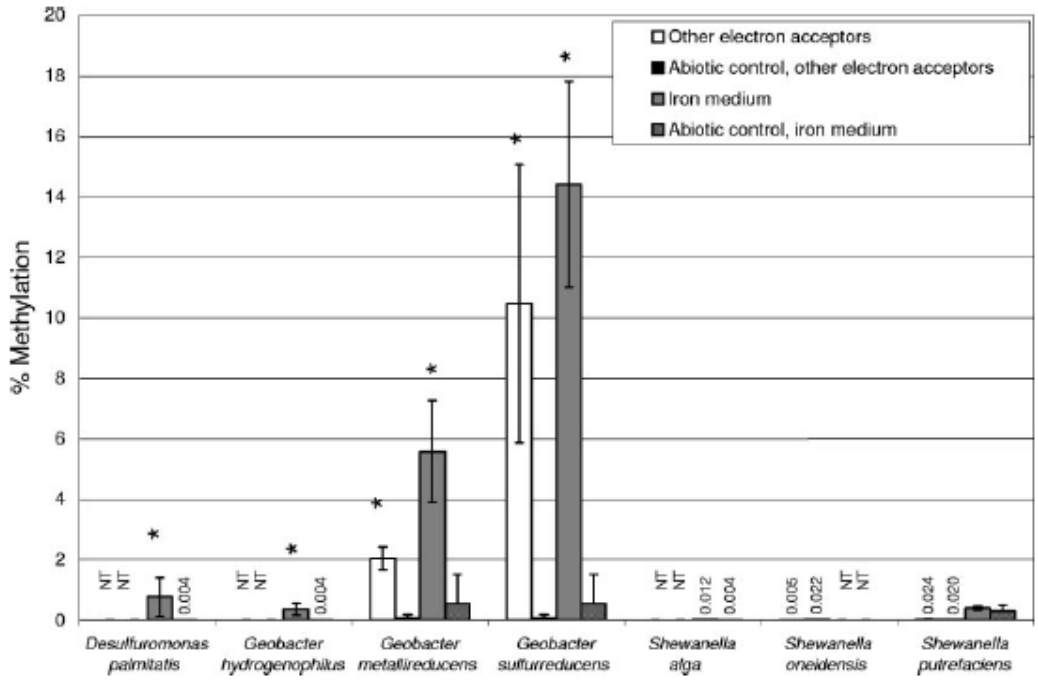


Figure.3. Observed MeHg production by pure cultures of DIRB, expressed as percent inorganic $HgCl_2$ methylated. "Other electron acceptors" refers to cultures that were grown with either nitrate or fumarate as an electron acceptor; "Iron Medium" refers to cultures grown with Fe(III) citrate as an electron acceptor (see Table 1). The small percentages of methylation observed in abiotic controls (composed of autoclaved uninoculated medium) are attributed to abiotic formation of MeHg in the experiment or during analysis (12). On both Fe(III)-reducing and Fe(III)-free media, the ratio of biotic to abiotic methylation was significantly lower for *Shewanella* strains than for *Geobacter* strains. Letters show ANOVA/SNK groupings among strains on either Fe-reducing medium (lower case) or other electron acceptors (upper case). Error bars represent the standard deviation between three separately prepared tubes for each sample. Culture and growth conditions that were not assayed for MeHg production are labeled "NT" (not tested).

These results, in combination with the observation by Fleming et al. (Fleming et al. 2006) of methylation by a *Geobacter* isolate, suggest that ability to methylate mercury may be common among the *Geobacteraceae* However, the observed lack of methylating capability among the *Shewanella* strains tested (all

Gammaproteobacteria) shows that the ability to methylate mercury is not ubiquitous among Fe(III)-reducing bacteria. To date, essentially all strains for which mercury methylation has been demonstrated fall in the *Deltaproteobacteria* (Compeau and Bartha 1985; Pak and Bartha 1998; King et al. 2000; Benoit et al. 2001b). These include DSRB from the orders *Desulfovibrionales* and *Desulfobacterales* However, it is important to note that the ability to produce MeHg is not ubiquitous among DSRB in these families. Further studies are needed to assertain whether mercury methylating capability is randomly distributed among *Proteobacteria* or related to phylogeny. Strain of DSRB and DIRB that have been tested for mercury are shown on Figure 4. Improved understanding of the phylogenetic distribution of mercury methylation capability may provide insight into the biochemical process of MeHg production within cells.

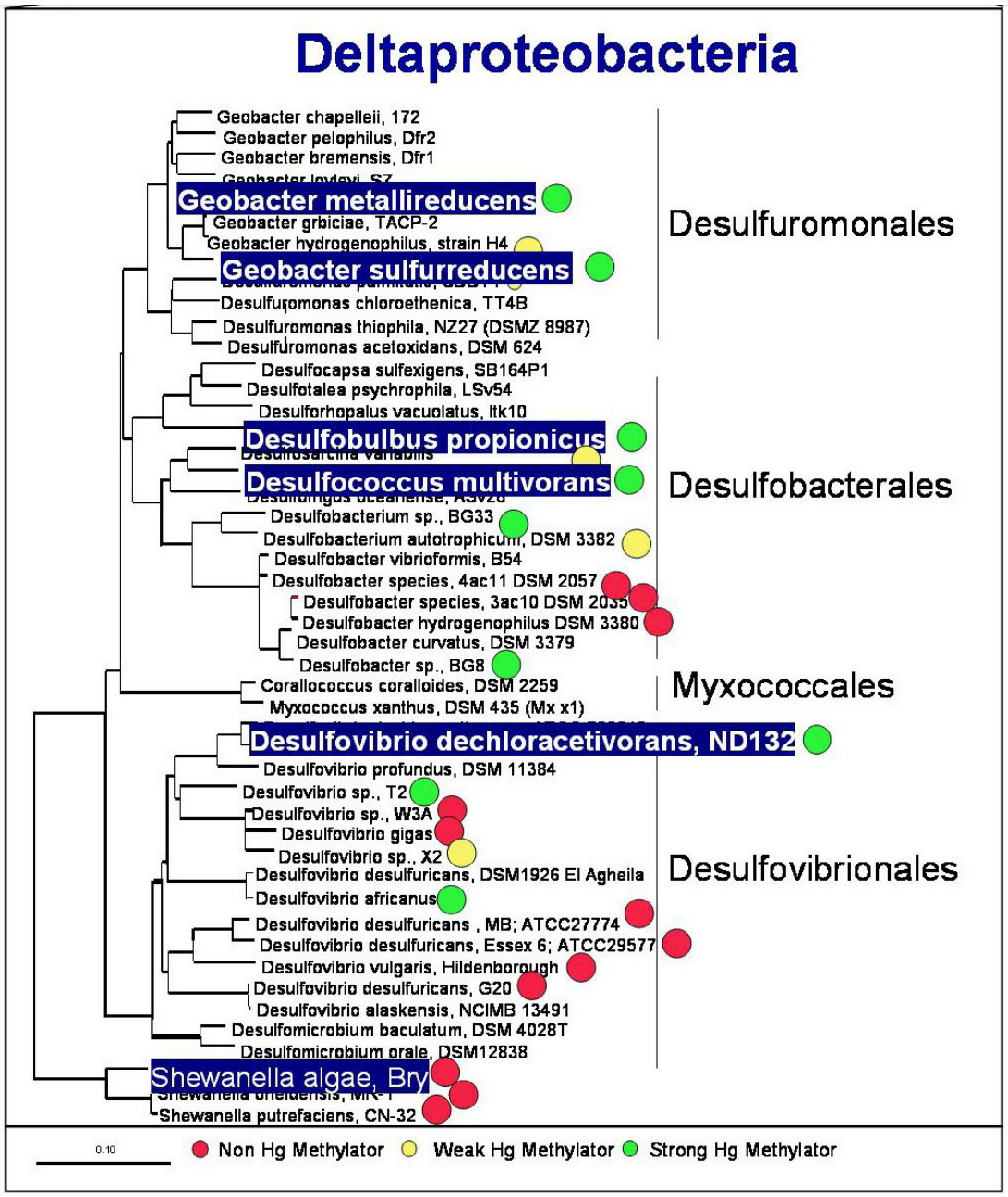


Figure 4. Phylogenetic reconstruction (Kimura Distance, Neighbor Joining, 1326 positions) of DIRB and DSRB tested for mercury methylation, and related organisms. DIRB tested in this study are highlighted in blue. Bootstrap values were based on 100 randomly resampled sets. Strong mercury methylators are indicated by green circles and methylated greater than 0.5% of added (10 to 100 ng/mL) mercury. Weak methylators (indicated by yellow circles) methylated less than 0.5% added mercury. Non-methylators methylated less than abiotic controls or produced below methylmercury below method detection limits.

It is important to note that the *Geobacter* strains tested produced MeHg during growth on either Fe(III) or other electron acceptors (nitrate or fumarate). This indicates that active Fe(III)-reducing electron-transport chains are not neccesary for mercury methylation in these strains. However, this experiment was not designed quantify the effect of electron acceptors and donors on methylation rates. Further studies would be needed to quantify these effects.

2.3.2. Environmental significance of methylmercury production by dissimilatory iron-reducing bacteria

The observation of mercury methylation by DIRB has implications for the prediction of *in situ* MeHg production. Due to the importance of DSRB as methylators, current models for methylation are based on relationships between methylation and sulfate reduction (Benoit et al. 2003). However, the finding that DIRB can produce MeHg suggests that mercury methylation may be important in sediments and soils where these organisms are dominant, e.g., iron-rich sediments with low concentrations of sulfate (Roden and Wetzel 1996). Iron can affect methylation by altering the chemistry of mercury (and hence its bioavailability) or by changing the activity of DIRB versus other groups of organisms, particularly DSRB (Roden and Wetzel 1996; Warner et al. 2003; Mehrotra and Sedlak 2005 Fleming et al. 2006). The influence of iron on both mercury complexation and microbial activity will need to be considered in order to resolve how mercury methylation by DIRB will change the paradigm for *in situ* MeHg production.

CHAPTER 3: LACK OF EVIDENCE FOR THE INVOLVEMENT OF COBALAMIN-DEPENDENT METHIONINE SYNTHASE (MetH) IN *GEOBACTER SULFURREDUCENS*

3.1. Introduction

Molecular advances have opened a new frontier of environmental mercury research. Recent work regarding microbial methylation of mercury has focused on the geochemical factors that control uptake and subsequent methylation of inorganic mercury in the environment and pure culture (Benoit et al., 2003). The biochemical mechanism of microbially-mediated mercury methylation is understudied in comparison. There are a limited number of methyltransferase mechanisms in cells, including the acetyl CoA pathway (which is cobalamin dependent) the cobalamin dependent and cobalamin independent methionine synthase pathways, and the Challenger mechanism of methylation (which methylates via *S*-adenosylmethionine rather than cobalamin) (Thayer, 2006). Of these, it has been hypothesized that cobalamin-dependant pathways would be the most likely mercury methyltransferases because a carbanion would be transferred to the mercury cation (Landner, 1971; Thayer, 2006). Other methyltransferase enzymes transfer carbocations (Thayer, 2002). Although non-methylating and methylating bacteria both have the cobalamin-dependent methionine synthase pathway, the tertiary structure of this enzyme varies between strains (Svetlitchnaia et al. 2006) and this tertiary structure may determine the affinity of the enzyme for mercury. Work to date implicates both MeTHF and MeB_{12} in methylation in at least one DSRB strain (Berman et al., 1990; Choi and Bartha, 1993; Choi et al., 1994a; Choi et al., 1994), but the biochemical pathways

have not been fully elucidated in that strain, nor have methylation mechanisms been studied significantly in other bacteria.

Observation of methylation by DIRB *Geobacter sulfurreducens* and *G. metallireducens* provides a unique opportunity to study methylation pathways, because these organisms are the first reported Hg-methylating strains for which complete genomic sequences are available (Kerin et al. 2007, Chapter 2). This chapter describes a set of experiments designed to examine the potential role of cobalamin-dependant methionine synthase (MetH) in mercury methylation in *Geobacter sulfurreducens.* The MetH pathway was chosen as the primary focus of this study instead of other cobalamin dependent methyltransferases due to previous results by others (Siciliano and Lean 2001; Ekstrom et al. 2003). In the MetH pathway, methionine is synthesized by the transfer of a methyl group from MeTHF to homocysteine by a methyltransferase containing cobalamin. It has been hypothesized that methylation of mercury could occur if this methyltransferase transferred a methyl group to mercury rather than homocysteine (Figure 5). Ekstrom et al. (2003) demonstrated that methylation by DSRB can occur independently from activity of the acetyl CoA pathway. Siciliano and Lean (2001) observed methylation by cell extracts of *E. coli* XL1-Blue/pKF5A, a strain which overexpresses the *metH* gene, suggesting the involvement of the MetH enzyme in methylation by this strain.

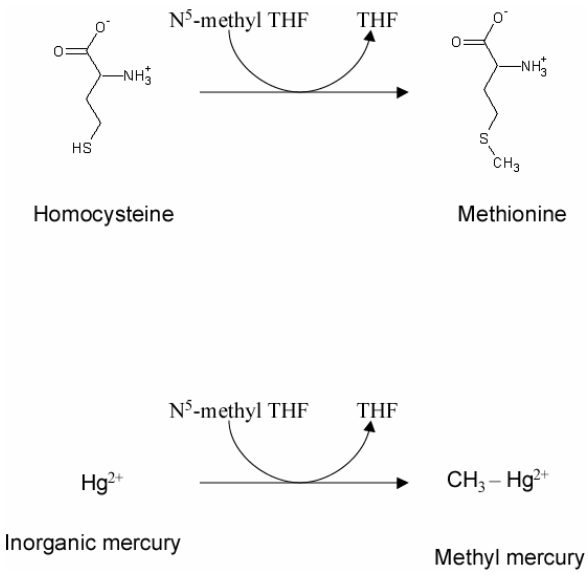


Figure 5. Potential mechanism for mercury methylation via the MetH pathway, showing methylation of mercury through substitution for homocysteine.

3.2. Approach

The *metH* gene from *G. sulfurreducens* was cloned into *E. coli* in order to investigate methylation by the MetH pathway. The clone was constructed using a standard protocol (p-Bad Topo, Invitrogen) so that expression of metH would be inducible by arabinose. Additionally a control clone was constructed with the *metH* fragment inserted in reverse. It was hypothesized that, if the MetH pathway is involved in methylation, the clone with metH inserted correctly would methylate mercury, and that the amount of methylation would be proportional to the amount of arabinose added to the methylation assays. The control clone would not be expected to methylate, as the reversed *metH* fragment would not be active and would not produce the MetH enzyme. Heterologous expression involves the insertion of DNA

from one organism into another. *E. coli* is often used in heterologous gene expression due to its culturability and high efficiency in protein expression (Greene, 2004). Heterologous gene expression can be complicated by the different intracellular environment between the organism of interest and host organism, which can affect the tertiary structure (and therefore the activity) of the proteins of interest. Finally, as there is a possibility that *E. coli* lack a mercury uptake mechanism, *in vivo* and *in vitro* experiments were used to study methylation by *E. coli* clones.

3. 3. Methods

3.3.1. Heterologous expression of G. sulfurreducens metH gene

G. sulfurreducens cells were grown until reaching stationary phase, as described in Chapter 2. DNA was extracted using the DNeasy Tissue Kit (Qiagen) from 2 µl of culture following the manufacturer's protocols for Gram Positive Bacteria. The *metH* gene from *G. sulfurreducens,* was amplified by PCR using primers GSmetH2F (5'-ATGAAACAA CCCTTTCTTCAGGCTATT-3') and GSMetH2R (5'ATCCACCCCGAGGAGC-3') designed to amplify the entire *metH* gene. In 25 µl, the PCR reaction contained 1X Platinum *Taq* High Fidelity buffer, 0.2 mM of each dNTP, 2 mM $MgSO_4$, 0.5 µM of each of the primers, 0.025 U Platinum *Taq* High Fidelity DNA polymerase and 2.5 µl of the DNA extract. Reactions were performed in a AB9700 thermal cycler programmed with a 2 min enzyme activation step at 94 °C, 30 cycles of 94 °C for 30 s, 59 °C for 30 sec and 68 °C for 3 min, with a final step of 7 min at 68 °C. 4 µl of products were ligated to the pBAD TOPO expression vector (Invitrogen), following the manufacturer's directions. The ligation

products were dialyzed and used to transform electrocompetent *E. coli* via electroporation as described in Béjà and coworkers (2000). Electroporated cells were recovered and plated on solid agar media amended with ampicillin. Individual colonies were picked from these plates and purified using the Fastplasmid Preparation kit (Eppendorf) following the manufacturer's directions and sequenced using the Big Dye V3.1 Kit, using 1:32 reaction sizes. Reactions were cleaned with the cleanseq kit (Becton-Dickson) and read in a AB 3100 Genetic Analyzer. Two clones were used for further studies: one clone containing *metH* in frame and correct orientation, and one clone with the gene inserted in reverse. Stocks of these clones were frozen for storage between methylation experiments. The clone with the gene inserted correctly was used as the positive control (+C) in this study. The clone with the gene inserted in reverse is not expected to produce the MetH enzyme and is used as a negative control (-C). To verify these insertions, the clones were grown overnight and induced with 0.2% and 2% arabinose for 2 h. A crude protein extract was produced by heating the cells at 70°C for 5 minutes and 5 µl of the extract was loaded into a precast SDS-PAGE gel and electrophoresed for 2 h at 100 V the gels were stained with Commassie blue and visualized using a FluorChem 8900 image analysis system (Alpha Innotech Co.).

3.3.2. Growth of clone cultures

For *in vivo* assays, 8 replicates of +C and –C clones were inoculated at a 1:10 dilution into Luria broth amended with 100 µg/mL ampicillin and grown at 37° C for 4 h. At this time, 2x250 mL tubes of each clone were amended with 0.02%, 0.2%, and 2% arabinose respectively (+CH, +CM, +CL and –CH, –CM, –CL) and allowed

to grow for 1 h. Two tubes of each clone were retained to serve as arabinose-free controls (+CN, -CN). All tubes were then inoculated with 10 ng/mL $Hg^{201}Cl_2$. Two abiotic controls were also prepared containing Luria broth, 100 μg/mL ampicillin and 10 ng/mL $Hg^{201}Cl_2$. These cultures were grown overnight, then frozen until mercury analysis.

For *in vitro* enzymatic assays, frozen stocks of one +C and one -C clone were thawed and inoculated separately into Luria broth amended with 100 μg/mL ampicillin and grown on a shaker overnight at about 37° C. For each clone, 10 mL aliquots of this culture was added, in duplicate, to centrifuge jars containing 110 mL of Luria broth amended with 100 μg/mL ampicillin and 5μM hydroxocobalamin. These cultures were grown for 4 hours at 37° C, at which time, for each duplicate of each clone, the media was amended with 2.0 and 0.02% arabinose and cultures grew an additional 2 hours. Crude cell extracts were prepared as follows. Cultures were centrifuged at 3400 rpm for 10 minutes and the supernatant was decanted. The pellet was rinsed in 5.0 mL phosphate buffer (1M, pH 7.2) and centrifuged again as above and the supernantant was discarded. The cells were resuspended in phosphate buffer, sonicated for three minutes to lyse cells, and centrifuged once more under the conditions above to remove cell debris. The supernatant was immediately used in the methylation assays described below (Figure 6). The use of cell extracts rather than whole cells made it possible to test methylation by the enzyme without to the assumption that *E. coli* cells could take up mercury.

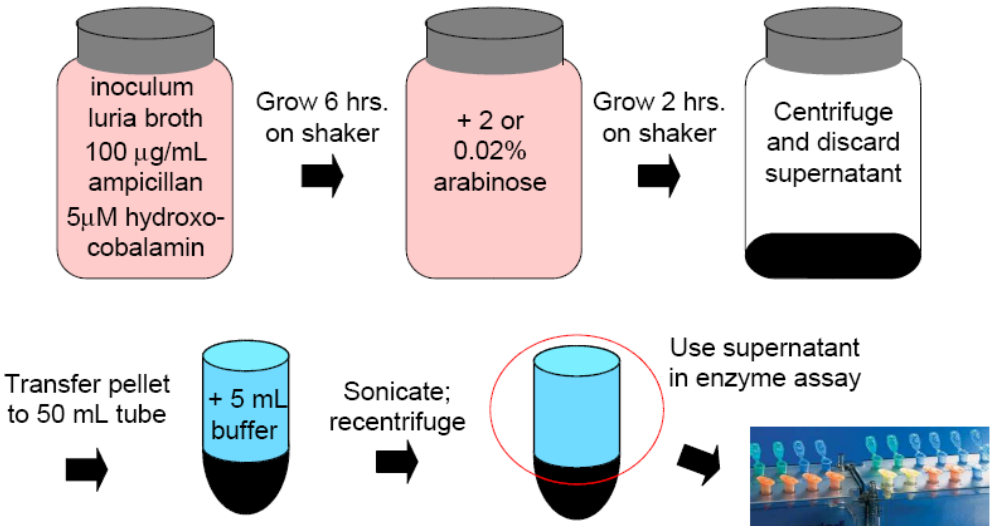


Figure 6: Method for preparation of cell extracts from *E. coli metH* clones for methylation assays.

3.3.3. Enzymatic methylation assay

The supernatant produced as described above was used to test the methylating ability of the cell extracts of the clones. Methylmercury production was assayed by measuring the amount of MeHg produced from an inorganic mercury spike. Because the MetH pathway is only active under anaerobic conditions, the methylation assay was conducted in a strictly anaerobic glove box. The following reagents were added to sterile 1.5 µL microcentrifuge tubes: 80 µL 1M potassium phosphate buffer (pH 7.2), 578.25 µL deionized water, either 40 µL 0.5 M dithiothreitol or 4 µL 25 mM Ti-nitrioltriacetate (Ti-NTA), 4.75 µL 0.0032M S-adenosylmethionine. Eighty µL of 0.5 mM hydroxocobalamin, 200 µL of cell extract and 5 µL 200 µg/L $Hg^{201}Cl_2$ were added to the appropriate tubes. After five minutes, 12 µL of 250 µM methyltetrahydrofolate was added to each tube. The mixture was incubated for 60 minutes, then were immediately frozen at -20 °C.

In order to test whether the addition of hydroxocobalamin resulted in abiotic production of MeHg within the assay, samples were prepared both with and without hydroxocobalamin. To test whether the thiol groups of dithiothreitol inhibited methylation by acting as ligands for mercury, an additional assay was completed, using Ti-NTA as an alternative reductant. For each assay, triplicate assays were performed for duplicate cultures of each clone, and abiotic controls were prepared in triplicate using potassium phosphate buffer in place of cell extracts.

3.3.4. Methylmercury analysis

We analyzed for $Me^{201}Hg$ by distillation/ethylation/ICP-MS, using a Perkin Elmer ELAN 6100 DRCII (Hintelmann and Ogrinc 2003). Method detection limits using ICP/MS were 0.3 pg $Me^{201}Hg$/sample for these analyses. This value represents three times the standard error of all analytical and method blanks run over the course of the MetH study. Percent methylation was quantified as the percent of $^{201}Hg Cl_2$ that was converted to $Me^{201}Hg$ during the course of the methylation experiment. A detection limit of 0.3 pg $Me^{201}Hg$/sample corresponds to 0.03% methylation for assays spiked with 1 ng/mL $^{201}HgCl_2$, 0.003% for assays spiked with 10 ng/mL $^{201}HgCl_2$ and 0.0003% methylation for assays spiked with 100 ng/mL $^{201}HgCl_2$. The detection limits achievable using this method can be substantially below methylation rates observed in cultures of methylating organisms. For example, *G. sulfurreducens* in batch culture can methylate over 10% of 10 ng/ml $^{201}Hg Cl_2$ provided (see Chapter 2) over the course of a few days. Determination of biologically- mediated methylation was made by comparison with abiotic controls.

3.4. Results

3.4.1 In vivo assays

Very low concentrations of $Me^{201}Hg$ were observed in methylation assays using whole *E. coli* cells containing the inserts (Figure 7). The forward and reverse clone both produced very low, similar amounts of MeHg, and methylation was not enhanced in the presence of arabinose, which stimulated production of MetH.
The highest percentages of methylation were seen in the positive and negative clones grown in the absence of arabinose (-CN and +CN). The positive clone methylated an average of 0.05% while the negative clone methylated 0.04%. Bars represent averages of two replicates for each treatment; it is difficult to determine if samples such as –CM, -CH, +CL, +CM and +CH are significantly higher than the method detection limit in this analysis.

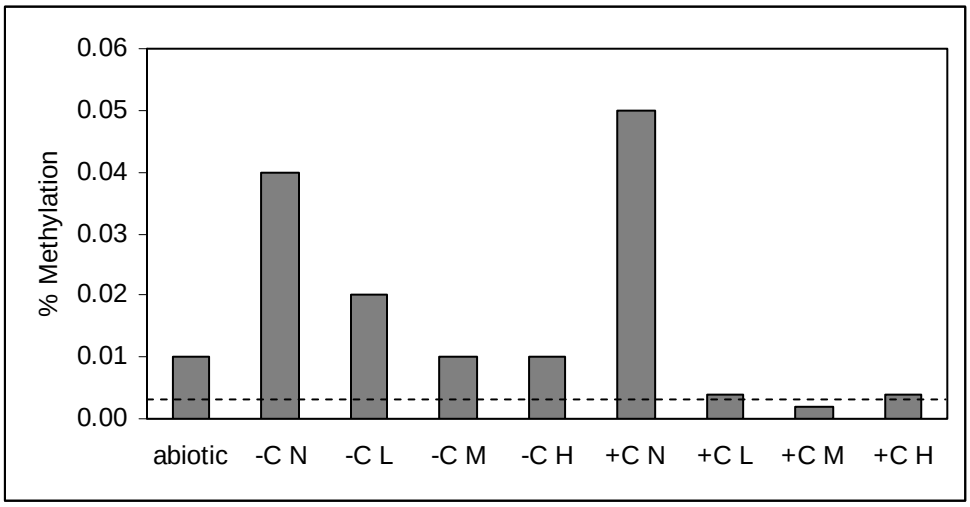


Figure 7. Percent methylation of 10 ng/mL$HgCl_2$ by whole cells during overnight incubation at 37 °C. +C refers to the clone with *metH* inserted in frame. –C refers to the clone with *metH* inserted in reverse orientation. N, L, M and H (none, 0.02%, 0.2%, 2%) refer to the concentration of arabinose in media prior to the assays. Luria broth amended with ampicillin and $HgCl_2$ was used as an abiotic control during the experiment. Each bar represents the average of duplicates. The dashed line represents the method detection limit of 0.003% methylation.

3.4.2. Cell extract assays: Assays containing hydroxocobalamin

It is not known whether *E. coli* has uptake mechanism for mercury. If whole cells of *E. coli* did not take up the mercury in the *in vivo* assay the mercury would be unavailable for methylation by the MetH enzyme. To ensure that the mercury would be available for methylation by cytosolic enzymes like MetH, assays using cell lysates were performed.

Like the in vivo assays, mercury methylation assays performed using cell extracts also showed low overall rates of mercury methylation (Figure 8). However, there was strong evidence for abiotic methylation by hydroxocobalamin in the assays. On average, assays containing hydroxocobalamin produced more MeHg than those without ($P < 0.01$). There was also some evidence for Hg methylation by the heterologously

expressed MetH enzyme. For the assays without hydroxocobalamin, the forward clone produced more MeHg on average than the reverse clone or controls ($P < 0.1$). Arabinose also stimulated MeHg production in the forward clone. The high arabinose forward clone assay produced more MeHg than the other treatments, or the abiotic control (for assays without hydroxycobalmin only; $P<0.01$). All samples were above the method detection limit for this assay of 0.005% methylation.

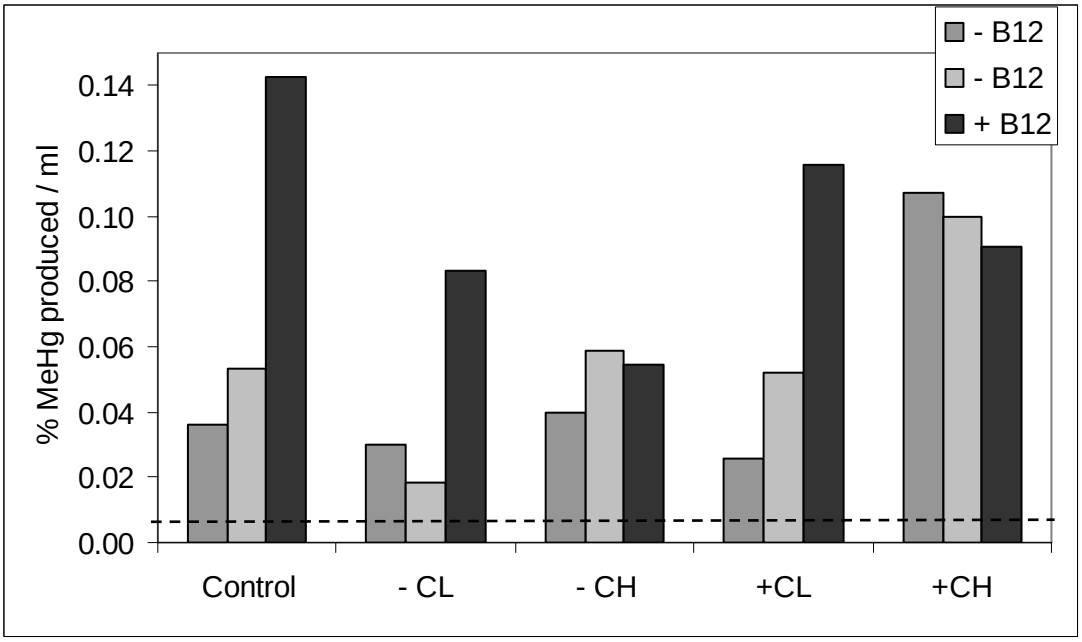


Figure 8. Percent methylation of 10 ng/mL $^{201}HgCl_2$ by *in vitro* assays over 20 min. anaerobic incubation. Grey bars on the left represent cobalamin-free assays (run in duplicate); black bars on the right represent assays containing 40 μM hydroxocobalamin (run singly). +C refers to the clone with metH inserted in frame. –C refers to the clone with metH inserted in reverse orientation. H indicates cells grown in 2% arabinose media prior to the assay experiment. L indicates cells grown on lower concentrations of arabinose (0.2%). The dashed line represents the method detection limit of ~0.005% methylation for this assay.

Since these results came from single cultures, cell extract assays were repeated. Assays were redone at 10X higher Hg concentration to potentially achieve MeHg concentrations further above the method detection limit. In assays containing

100 ng/mL $^{201}HgCl_2$ and hydroxocobalamin, methylation rates remained very low (Figure 9), and abiotic controls remained significantly higher than all other treatments ($P< 0.005$). On average, there were no significant differences between the assays with forward and reverse clones. However, for the forward clone, more MeHg was produced in assays from low arabinose cultures than in assays from high arabinose cultures ($P<0.001$). All samples were higher than the method detection limit of 0.0005% methylation.

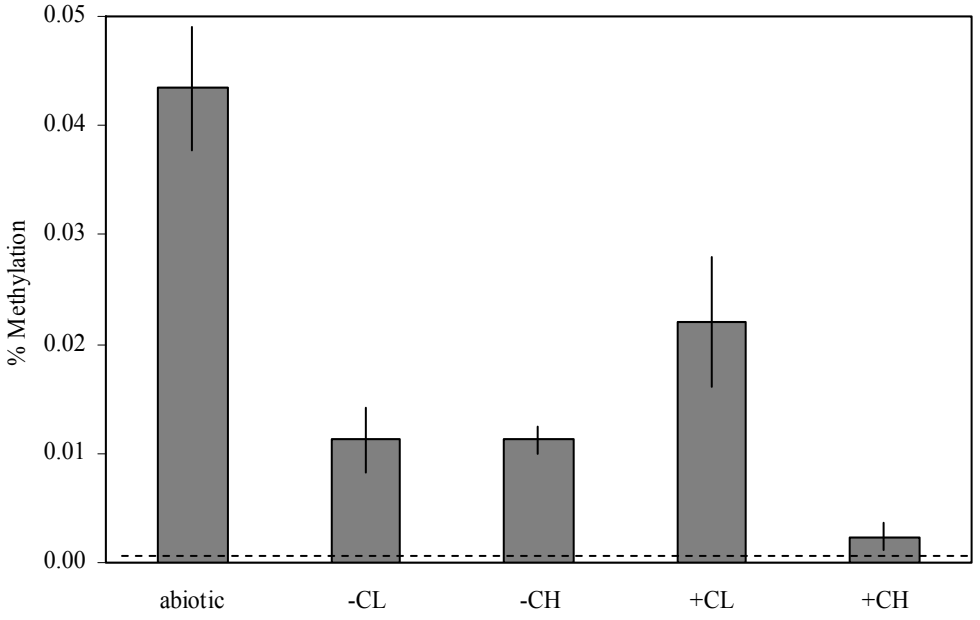


Figure 9. Percent methylation of 100 ng/mL $HgCl_2$ by cell extracts in enzyme assays containing 40 μM hydroxocobalamin over 1 hour anaerobic incubation. +C refers to the clone with metH inserted in frame. –C refers to the clone with metH inserted in reverse orientation. H indicates cells grown in 2% arabinose media prior to the assay experiment. L indicates cells grown on lower concentrations of arabinose (0.2%). Error bars represent the standard deviation of triplicate enzyme assays. The dashed line represents the analytical detection limit of 0.0005% methylation for this assay.

3.4.3. Cell extract assays: Hydroxocobalamin-free assays

Because cell-free assays conducted with the addition of hydroxocobalamin showed strong evidence of abiotic methylation, assays were repeated without the

addition of hydroxocobalamin. Again, no evidence of methylation by the MetH enzyme in cell extracts of the clones was found (Figure 10). Two separate hydroxocobalamin-free assays were conducted, using either dithiothreitol or Ti-NTA as a reductant. The Ti-NTA assays were done to avoid the potential for dithiothreitol to form complexes with the inorganic mercury in the assays, making it less available for methylation. Thiol groups are strong ligands for mercury (Ravichandran et al. 2004). All samples and controls in the hydroxocobalamin-free assays produced less MeHg (at the same ^{201}Hg spike concentration) than was produced in assays containing hydroxocobalamin. There continued to be no evidence that the forward clones produced more MeHg than the reverse clones; or that arabinose induced MeHg production. In the assays containing dithiothreitol, there were no significant differences in percent methylation among treatments or between treatments and controls ($P < 0.05$). In assays containing T-NTA, assays did not produce MeHg above the method detection limit of 0.0005%.

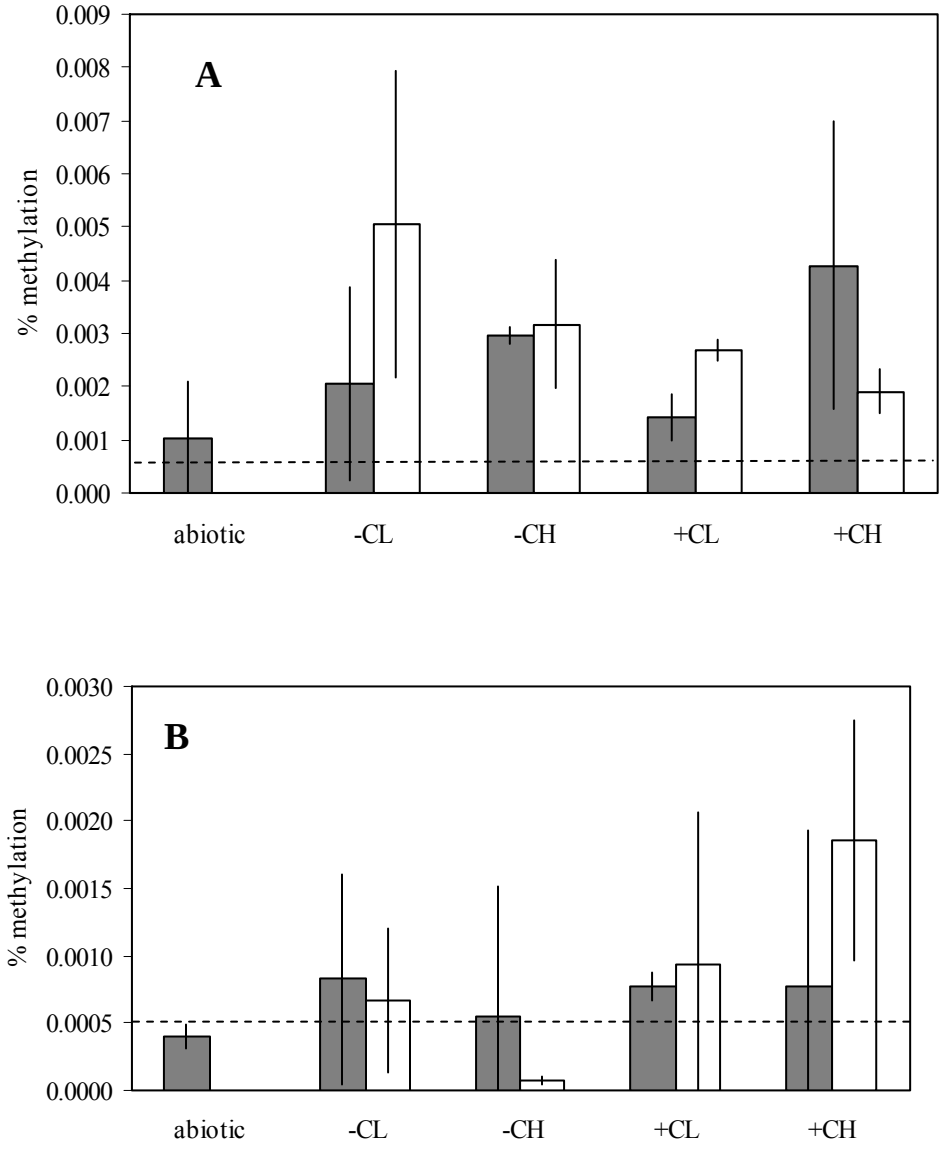


Figure 10. Percent methylation of 100 ng/L $^{201}HgCl_2$ by cell extracts in cobalamin-free enzyme assays with (A) 20 mM dithiothreitol or (B) 0.1 μM titanium nitriletriacetate as a reductant. +C refers to the clone with *metH* inserted in frame. –C refers to the clone with *metH* inserted in reverse orientation. H indicates cells grown in 2% arabinose media prior to the assay experiment. L indicates cells grown on lower concentrations of arabinose (0.2%). For +C and –C, grey and white bars represent cultures grown in duplicate. Error bars represent the standard deviation of triplicate assays for each culture or control. Note the difference in scale for percent methylation, both between (A) and (B) and between cobalamin-free and 40 μM hydroxocobalamin assays (Figure 8). The method detection limit of 0.0005% methylation for this assay is represented by the dashed lines on each graph.

3.5. Discussion

The existence of complete genomic sequences for two known methylating DIRB allows the exploration of methylation biochemistry in ways that were not

previously possible. The possibility of mercury methylation via the cobalamin-dependent methionine synthase pathway was investigated in *G. sulfurreducens*. To accomplish this, heterologous expression of the *metH* gene was used, followed by methylation tests in an assay specifically developed for biochemical investigations of the MetH enzyme. In all our assay treatments, +CH methylated less mercury than either the abiotic or –C controls and the amount of MeHg produced by +CH was never greater that 0.01 pg MeHg. These results suggest that the MetH pathway is not responsible for methylation in *G. sulfurreducens*, but further investigations are necessary to fully test this hypothesis.

Heterologous gene expression greatly alters the environment in which the gene of interest functions. An alternative explanation for these data is that despite the fact that a protein with the correct size was produced by *E. coli*, the tertiary structure (folding) was not correct or other cofactors or intracellular conditions present in *G. sulfurreducens* and not *E. coli* might be necessary for mercury methylation (Greene, 2004). The conditions of the expression of *metH* in *E. coli* could have resulted in a non-methylating organism even if MetH is responsible for methylation in *G. sulfurreducens*. *E. coli* is commonly used as a host in recombinant DNA experiments due to the ease with which it is cultured and its high efficiency in protein expression (Greene, 2004). However, the intracellular conditions of *E. coli* are very different than those of *G. sulfurreducens* and could have interfered with production of the correct MetH enzyme. In our experiments, SDS gel electrophoresis was used to verify that the *E. coli* clone was producing a ca. 85 Kd protein after induction by 2% arabinose (Figure 11). Although this indicates that MetH is being produced, the tertiary structure (folding) of the enzyme may not be correct and thus the enzyme may

not be active. Correct folding depends on both the presence of foldases and the redox state of the cell; either of these might be different enough in *E. coli* to cause a protein from *G. sulfurreducens* to misfold (Sitia and Molteni, 2004). Finally, the *G. sulfurreducens* protein must also be soluble in the experimental conditions in order to function actively (Greene, 2004).

Additionally, conditions of the enzymatic assay may have been inappropriate for mercury methylation tests, thus leading to a lack of methylation by MetH. It is recognized that the availability of mercury to methylation is strongly influenced by chemical conditions (Benoit et al. 2003). The conditions of the enzymatic assay are designed to be ideal for activity of the MetH enzyme to methylate homocysteine in *E. coli* (Drummond et al. 1995). These same conditions may not have been optimal for methylation by the *G. sulfurreducens* MetH—although assays were conducted under varying chemical conditions to limit the effects of strong ligands for inorganic mercury. Formation of a non-reactive mercury complex within the enzyme assay could be responsible for lower methylation rates in these assays. Dithiothreitol is the reductant used in the MetH assay described by Drummond et al. (1995). Since thiols have a strong affinity for mercury (Miller 2007) there was concern that mercury-thiol complexations might be forming in the assay, thus rendering the inorganic mercury unavailable for methylation. In one set of assays, Ti-NTA replaced dithiothreitol — Ti-NTA is a reductant that has been used in investigations of methylation by SRB (Benoit et al. 2003) —but we observed a lower percentage of methylation. Therefore, it does not appear that DTT was rendering mercury unavailable in the assays.

Last, it is also possible that short incubation times, used to ensure that the enzyme remained active for the entirety of the experiment, may have limited the

ability to detect MeHg production. Methylation may not have been detectable if the per hour methylation rates constant is low relative to the method detection limit, which is <0.00001 h-1. *In situ* methylation rates in sediments tend to range from 0.0001 to 0.001 per hour, while methylation rates in actively growing cultures of Hg-methylating bacteria can be as high as 0.01 h-1 (Benoit et al. 2001; 2003).

Siciliano and Lean (2002) observed mercury methylation in extracts of *E. coli* XL1-Blue/pKF5A cells overproducing MetH. Data from the *G. sulfurreducens* heterologous expression study suggest that their findings may have been confounded by the occurrence of abiotic methylation via hydroxocobalamin within the methylation assay. When Siciliano and Lean (2002) tested methylation in extracts of *E. coli* XL1-Blue/pKF5A, added 40 μM hydroxocobalamin were added to the assays, as is described by Drummond and colleagues (Drummond et al. 1995). Hydroxocobalamin is added as a catalyst to reduce oxygen to hydrogen peroxide and maintain anaerobic conditions (Drummond et al. 1995). However, as seen in our results when this assay was used to examine mercury methylation, the hydroxocobalamin may mediate abiotic methylation. Formation of MeHg has been correlated with the concentration of cobalamin present in anaerobic sediments (Regnell et al. 1996). Abiotic transfer of the methyl group from methylcobalmin is nearly stoichiometric in aqueous solution at neutral pH (Hintelmann and Ogrinc 2003). Using an anaerobic chamber and anoxic reagents allowed us to conduct anaerobic assays without the addition of cobalamin. These cobalamin-free assays produced much smaller amounts of MeHg than those containing cobalamin. This was even more pronounced for abiotic controls, where mercury may have been in a more chemically available form, since concentrations of mercury scavenging organic

compounds originating from cell debris would have been lower. It is possible that the methylation Siciliano and Lean (2002) attributed to MetH activity was in fact the result of abiotic methylation by cobalamin.

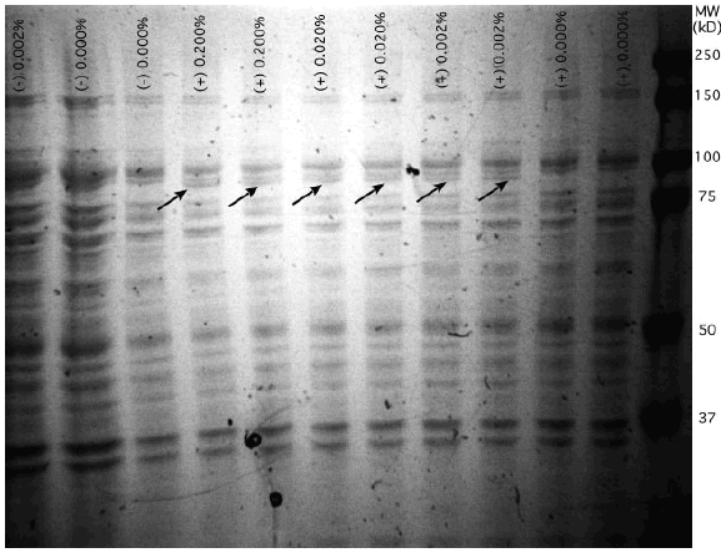


Figure 11. 12% SDS-Page gel of *E .coli* lysates from clones containing G. sulfurreducens metH gene inserted in the correct (+) and reversed (-) orientation and induced with arabinose added at different concentrations. Only three (-) lysates are shown but are indicative of all (-) lysates. Arrows indicated the putative heterologously expressed MetH, as a ca. 85 kD protein.

In summary, these initial studies suggest that the MetH pathway *in G. sulfurreducens* does not participate in mercury methylation. However, because of the limitations of the heterologous expression method, further work is necessary to completely rule out the MetH pathway as a methylating pathway mechanism in DIRB. Methyltetrahydrofolate and cobalamin are recognized as important participants in the microbial methylation of mercury. These chemicals are also key components of the MetH pathway; thus it has been suggested that the MetH pathway plays a role in mercury methylation. Further exploration of MetH—and other potential methylation pathways—is warranted. The genomic information available for methylating DIRB provides a valuable tool for this work.

CHAPTER 4: CONCLUSIONS

4.1. Phylogeny of mercury methylation

In order to study mercury-methylating ability outside of DSRB, DIRB in the genera *Geobacter, Desulfuromonas*, and *Shewanella* were examined. All of the *Geobacter* and *Desulfuromonas* strains tested methylated mercury while reducing Fe(III), nitrate, or fumarate. In contrast, none of the *Shewanella* strains produced methylmercury above abiotic controls under similar culture conditions. *Geobacter* and *Desulfuromonas* are closely related to known mercury-methylating sulfate-reducing bacteria within the *Deltaproteobacteria* (Holmes et al. 2004).

The observation of methylation by DIRB invites further exploration of both the phylogenetic distribution of methylating capability and the microbial controls on methylation in the environment. Studies of methylation by SRB have not revealed a clear relationship between phylogeny and methylation. Studies by Gilmour et al. (2006) have shown that of 44 DSRB strains, taken from environments ranging from freshwater to marine, only 10 produced MeHg. These included strains within the *Desulfovibrio, Desulfococcus, Desulfobacterium* and *Desulfosarcina*, but methylation is not ubiquitous among these genera (Gilmour et al., 2006)**.** *In vitro* studies of methylation by DIRB will likely follow a similar pattern. For the small number of iron reducers that have been screened, methylating capability is observed among all the *Geobacter* strains and is absent from all *Shewanella* However, these tests cannot be assumed to be representative of all DIRB, and consequently our ability to draw broad conclusions on the phylogenetics of methylation remains limited. Our studies of DIRB show that the distribution of methylating capability is complex, and suggests

the need to approach this problem through the study of functional genes rather than by traditional microbiological techniques

Although mercury methylation was observed by pure cultures of DIRB, the role of these organisms in environmental methylation is not known. While sulfate reducing bacteria are believed to be the primary methylators in marine and estuarine environments (Fitzgerald et al. 2007), iron reducers may play a role in mercury methylation in freshwater sediments and soils where conditions favor the process of iron reduction. Several studies have noted methylation that does not always correspond with sulfate reduction in freshwater environments (Warner et al., 2003; Fleming et al., 2006). A study on microbial mercury cycling in the San Francisco Bay estuary noted that methylation in surface sediments correlated with sulfate reduction in the winter but not in the spring or fall seasons. In this case, decoupling of methylation from sulfate reduction could be due either to inhibition of methylation by the end products of sulfate reduction during the warmer, more productive spring and fall or to activity of organisms other than sulfate reducers during these times (Marvin-DiPasquale et al. 2003). These observations suggest that iron reducers play a role in mercury methylation under certain seasonal conditions.

Isolation of an iron reducing bacteria from Clear Lake, CA (Fleming et al., 2003) suggests that iron reducers may be responsible for methylation in mining environments. Clear Lake, CA is impacted by the Sulfur Bank Mercury Mine; in this mine, cinnabar is associated with pyrite (Macalady et al., 2000). Mobilization of pyrite under oxidizing conditions can result in formation of iron oxyhydroxides, which in turn provide an electron source for iron reduction under anoxic conditions. This active iron cycling could be accompanied by methylation by DIRB. Pyrite is

frequently associated with ore deposits, and could similarly stimulate iron reduction and methylation in mining environments as well.

Methylation by DIRB may be important in a range of environments although, geochemical interactions between iron, sulfide and mercury are complex and can affect the extent to which mercury is available to microbial uptake, thus affecting methylation (Mehrotra et al. 2003; Mehrotra and Sedlak 2005). Additionally, organisms that can be isolated and shown to exhibit a certain metabolic activity in pure culture may not be the same organisms that are abundant or highly active in the environment (Rappe and Giovannoni 2003). Until relationships between the presence and activity of specific species are resolved, it is difficult to predict how microbial community structure might control mercury methylation.

4. 2. Biochemistry of mercury methylation

Demonstration of mercury methylation by *G. sulfurreducens*, the first identified mercury-methylating organism for which a full genomic sequence is a available, provided a unique opportunity to investigate the poorly understood physiological process of microbial mercury methylation The genomic sequence of *G. sulfurreducens* was initially used in heterologous gene expression studies to explore methylation by the cobalamin dependent methionine synthase pathway.

In vivo methylation tests indicated that the cloned and expressed *metH* gene did not confer mercury methylation capability by *E. coli*. However, since this could be result of lack of mercury transport into the cells, these results were not conclusive evidence that the MetH pathway is not responsible for methylation by *G. sulfurreducens*. Methylation by the expressing clone *in vitro* was not significantly

higher than that by either the control clone or controls in which phosphate buffer replaced cell extracts. This further suggests either that MetH is not involved in methylation in *G. sulfurreducens*, that the heterologously expressed protein does not mimic that in *G. sulfurreducens*, or that *in vitro* assay were not appropriate for examining mercury methylation. Methylation was higher in all treatments when assays were conducted with hydroxocobalamin and in these assays, abiotic controls methylated more than either clone. Therefore, abiotic methylation, mediated by hydroxocobalamin likely occurred in these assays. Based in these results it is clear that further research is needed to ascertain the relationship between mercury methylation and carbon metabolism within cells.

Further research is necessary to understand microbial mercury methylation both within cells and in the environment. In the study of *in situ* methylation, efforts should focus on linking the phylogenetic structure of microbial communities to the carbon metabolism and methylating capability of specific species within these communities. The development of molecular techniques to study environmentally relevant functional genes provides promise for advancement in this field of research.

An understanding of the biochemistry of mercury methylation would aid in genomic environmental studies. The observation of mercury methylation by two completely sequenced DIRB provides a means to investigate methylation biochemistry. Further work in this area could logically begin with mutation experiments in the well-characterized methylating DIRB *G. sulfurreducens*. Deletion of *metH* from *G. sulfurreducens* and subsequent methylation testing would expand on the preliminary experimentation on heterologous expression of *metH* described in Chapter 2. Technological advances in sequencing and manipulation of genetic

material will soon make these techniques feasible for the study of methylation in other organisms as well. Work should proceed to determine the genetic basis for methylation within environmentally relevant DSRB. This knowledge will provide linkages between the biochemical and environmental function of methylating organisms, and will help characterize the cycling of an element that affects aquatic ecosystems on a global scale.

Appendix: Development of methods to be used for the deletion of *metH* in *Geobacter sulfurreducens* in future research

Introduction

Measurement of mercury methylation by *G. sulfurreducens* provides a unique opportunity to investigate the poorly understood physiological process of microbial mercury methylation as the complete genetic sequence is also available for this organism. Initially, these sequences were used in heterologous gene expression in combination with an enzymatic assay to explore methylation by the MetH pathway. These experiments indicated that MetH is not involved in methylation. However, conditions of either the heterologous expression or the enzymatic assay may not have been ideal for methylation.

A genetic system has been developed for *G. sulfurreducens* (Coppi et al. 2001). This makes it possible to study how the manipulation of specific genes in *G. sulfurreducens* changes the biochemical functioning of this organism. This method avoids the potential difficulties related to expressing a gene in a different species in order to test its function. Gene deletion techniques are being pursued in order to produce a mutant lacking *metH*. Methylation tests by whole cells of this mutant will hopefully provide a more information on the possible involvement of MetH in mercury methylation. This appendix presents the methods being used to create the *metH* mutant.

Organism and culture conditions

All genetic manipulations were performed using *G. sulfurreducens* strain DL1 (ATCC 51573) (Caccavo et al. 1994). Wild type and mutant *G. sulfurreducens* strains were grown at 30°C under strict anaerobic conditions as described in Coppi

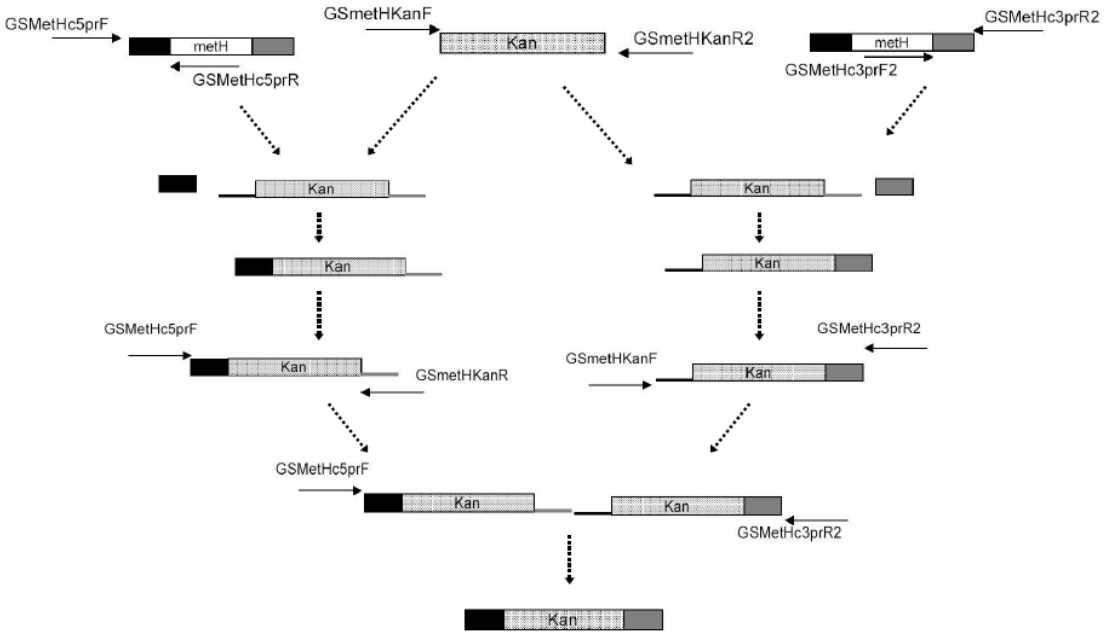
(Coppi et al. 2001). Acetate (20 mM) was supplied as the electron donor and either 40 mM fumarate or 55 mM iron (III) citrate as the electron acceptor. Incubations on solid media were performed in sealed gas-tight bags in the presence of catalysts (Anaerocult A mini, EM Science). Culture medium was prepared as in Coppi (2001) except that 5 mL/L of vitamin stock (per liter: 2 mg biotin, 20 mg niacin, 10 mg p-aminobenzoic acid, 20 mg thiamine, 10 mg pantothenic acid, 50 mg pyridoxine · 2HCl, 10 mg cobalamin), 0.5 mL/L of trace metal stock (per liter: 70 mg $ZnCl_2$, 100 mg $MnCl_2 \cdot 4H_20$, H_3BO_4, 190 mg $CoCl \cdot 6H_2O$, 17 mg $CuCl_2 \cdot 2H_20$, 24 mg $NiCl_2 \cdot 6H_20$, 36 mg $Na_2MoO_4 \cdot 2H_2O$), and 1.0 mL/L of Se/W stock (per liter: 0.5 g NaOH, 6 mg $Na_2SeO_3 \cdot 5H_2O$, 8 mg $Na_2WO_4 \cdot 2H_20$) were used in place of trace metal and vitamin stocks described in Coppi et al. (2001). Culture media were amended with 200 μg/mL kanamycin and between 1 and 10mM of methionine for growth of mutant.

DNA construct

The construct used for a knockout of *metH* gene from *G. sulfurreducens* by homologous recombination was synthesized by PCR (Figure 12). The construct synthesis method was adapted from Lloyd et al. (2003) to create a mutant in which a gene for kanamycin resistance replaced the *metH* in *G. sulfurreducens*. The kanamycin-resistance cassette and ca. 500 bp upstream and downstream regions flanking *metH* (5'- and 3'-flanks) were amplified in three separate PCR reactions using primers GSmetHKanF (CGAGGAACTCAACCTGACCCCGGATGAATGTCAGCTAC) and GSmetHKanR2 (CATGAGGGCCGAGAGCCCGAAAAGTGCCACCTG) for the kanamycin cassette; GSMetHc5prF (ACGACAACCGTGCGTC) and GSMetHc5prR

(GGTCAGGTTGAGTTCCTCG) for the 5'-flanking region; and GSMetHc3prF2 (GCTCTCGGCCCTCATG) and GSMetHc3prR2 (CGTCAAACTCGCCGTTC) for the 3' flanking region. In 10 µl, PCR reaction mixes contained 1X Platinum *Taq* High Fidelity buffer, 0.2 mM of each dNTP, 2 mM $MgSO_4$, 0.5 µM of each of the primers, 0.025 U/µl Platinum *Taq* High Fidelity DNA polymerase and either 1 µl of a genomic DNA extract (DNeasy, Qiagen) of *G. sulfurreducens* for 5'- and 3'-flanking regions or 1 µl of a 1:10 dilution of purified plasmid CB22G11 (Kan, PhD Thesis 2006 Univ. Maryland) using the Fastplasmid Prep Kit (Eppendorf). Reactions were performed in a AB9700 thermal cycler programmed with a 2 min enzyme activation step at 94 °C, 30 cycles of 94 °C for 30 s, 57 °C for 30 sec and 68 °C for 1:30 min. Products from this reactions were loaded in a 1% agarose gel modified TAE (20 mM Tris acetate, 0.1 mM EDTA pH 8.0) stained with 0.5 µg/ml ethidium bromide, electrophoresed for 1 h at 100V. Bands with the correct size were excised from the gel and DNA recovered using Ultrafree DA spin columns.

Figure 12. Method for creation of DNA construct for deletion of *metH* in *G. sulfurreducens*. Black and grey segments represent the upstream and downstream flanking regions outside *metH*, respectively. The hatchmarked segment represents the kanamycin resistant cassette. Black or grey lines adjacent to the hatchmarked segments represent overhangs homologous the flanking regions of *metH*. Horizontal black arrows represent primers, which are identified by the accompanying text. Dashed arrows represent PCR reactions. Details on primer construction and PCR conditions are described in the text.



Unlike Lloyd and colleagues (2003), two separate reactions were performed to produce a half-construct linking the 5'flank to the Kanamycin cassette and a second half-construct linking the Kanamycin cassette to the 3'flank. These reactions were consisted of two steps. In the first step either the 5'-flanking region plus the kanamycin cassette PCR products or the kanamycin plus the 3'-flanking region were linked in PCR-like reactions with no primers and taking advantage of primer overlaps designed between either GSmetHKanF and GSMetHc5prR or GSmetHKanR2 and GSMetHc3prF2 (underlined above). In 10 µl, reaction mixes contained 1X Platinum *Taq* High Fidelity buffer, 0.2 mM of each dNTP, 2 mM $MgSO_4$, 0.025 U/µl Platinum Taq High Fidelity DNA polymerase 1µl of 1:100 dilution of the gel purified Kanamycin cassette PCR product and 1 µl of either 3' or 5'-flanking regions (gel purified). Reactions were performed in an AB9700 thermal cycler programmed with a 2 min enzyme activation step at 94 °C, 15 cycles of 94 °C for 30 s, 68 °C for 30 sec and 68 °C for 2:30 min. 1 µl products of this linkage step were subject to PCR using primers combinations to amplify the entire half constructs (GSMetHc5prF/GSmetHKanR2 and GSMetHc5prF/GSMetHc3prR2. In 10 µl, reaction mixes contained 1X Platinum *Taq* High Fidelity buffer, 0.2 mM of each dNTP, 2 mM $MgSO_4$, 0.025 U/µl Platinum *Taq* High Fidelity DNA polymerase, 500 nM of primers and 1 µl of the products from the linkage reactions. Reactions were performed in an AB9700 thermal cycler programmed with a 2 min enzyme activation step at 94 °C, 30 cycles of 94 °C for 30 s, 57 °C for 30 sec and 68 °C for 2:30 min. Finally the two half constructs were gel purified and linked in two-step reactions as above. The first step consisted of 10 µl primer-less 15 cycle reactions as above. In the second step two 25 µl reactions contained 1X Platinum *Taq* High Fidelity buffer,

0.2 mM of each dNTP, 2 mM $MgSO_4$, 0.025 U/µl Platinum Taq High Fidelity DNA polymerase, 500 nM of primers GSMetHc5prF and GSMetHc3prR2 and 2.5 µl of the products from the linkage reactions. Reactions were performed in an AB9700 thermal cycler programmed with a 2 min enzyme activation step at 94 °C, 35 cycles of 94 °C for 30 s, 57 °C for 30 sec and 68 °C for 2:30 min. Reaction products were pooled and purified using the Qiaquick Spin Kit (Qiagen).

Growth and collection of cells for disruption

Cells were prepared for electroporation following methods in Coppi et al. (2001) Cells were grown for three days in 400 mL liquid media with 20 mM acetate and 40 mM fumarate as the electron donor and acceptor pair, under conditions described above, to a final concentration of 1.52×10^8 cells/mL. Cell density was calculated from measurements of optical density at 600 nm and from a relationship between optical density and cell density provided in Coppi et al. (2001). Cells were washed twice via centrifugation (4000g for eight minutes) using 50 ml falcon tubes wrapped in parafilm to maintained anaerobic conditions in the media when the tubes were removed from the anaerobic chamber and put into the centrifuge. Pellets were resuspended in 400 mL electroporation buffer (1mM HEPES [pH 7.0], 1mM $MgCl_2$, 175 mM sucrose).

Preparation of electrocompetent cells

After centrifugation, cells were resuspended in electroporation buffer, to which a 60% DMSO-40% electroporation buffer stock was added, to achieve final concentration of 10% DMSO and about 10^{11} cells/mL. All preparation procedures were carried out in an anaerobic chamber using ice cold reagents. Direct

manipulation of cells was conducted with wide bore pipette tips to minimize shearing of cells.

Electroporation and recovery

Twenty-five μL of electrocompetent cells were transformed with approximately 100 ng of the PCR construct using the Gene Pulser (BioRad). Electroporation was conducted twice using conditions described in Coppi et al. (2001) (1500 V, 25μF, 400 Ω) and in conditions typically used for transformation of *E. coli* cells (250 0 V, 25 μF, 100 Ω ohms). An aliquot of cells were preserved in glycerol immediately after electroporation and stored in anaerobic conditions at -70° C. The remaining cells were recovered for 5 hours at 30° C in 20 mM acetate/40 mM fumarate media buffered with 50 mM phosphate**.** One hundred μL of cells were then plated out on solid iron (III) citrate media described above, amended with 1mM methionine and 200 μg/mL kanamycin. These plates were incubated at 30° C in anaerobic conditions as described above in this appendix.

Check for mutant cells

The presence of correct mutant cells was tested by PCR amplification using primer constF (AGTTGATCAATGGTCCGTTG) upstream from the construct region and primer GSMetHc3prR2 in the 3' end of the construct. In 10 μl, reaction mixes contained 0.2 mM of each dNTP, 2 mM $MgSO_4$, 0.025 U/μl Platinum *Taq* High Fidelity DNA polymerase and 1 μl of a template prepared by centrifuging 200 μl of the culture used for the Hg methylation assay for 5 minutes at 13,000 g, resuspending in 1X Platinum *Taq* High Fidelity buffer, lysing for 5 min at 94°C and centrifuging for 5 min at 13,000 g to remove cell debris.

Cell growth measurements

Cell growth in liquid media was monitored by optical density (absorbance at 600 nm) and by cell counts using epifluorescence microscopy. Cells were prepared for counting by fixing 250 μL of cells in 2.2 mL of 1 mM potassium phosphate buffer (pH 4, 9g/L NaCl) and 50 μL of 49% glutaraldehyde stock. This mixture was then diluted with triple filtered deionized water if necessary, and stained with 100 μL 0.1% acridine orange per 1mL cell mixture. Cell growth on solid media was determined by observation of visible colonies or iron reduction (as evidenced by clearing of brown coloring from plates made with iron (III) citrate).

Further research

Preparation of a *metH* mutant is not complete. This method was developed as part of my thesis research, but verification of the construction of a metH mutant, and subsequent methylation testing, are beyond the scope of this project. Successful completion of this project will require that cells in glycerol stocks be checked for contamination and recovered using the methods described in the appendix. This may require more frequent additions of kanamycin, adjustment of the methionine concentration in the recovery media, or addition glycine-betaine as an alternate source of methionine for biosynthesis. The presence of mutant cells will then need to be verified by PCR amplification using primers flanking the construct region. This will hopefully yield mutant cells which can be used to test methylation in *G. sulfurreducens* lacking the MetH pathway.

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