Mercury Methylation in Whole Cells and Cell Extracts of Sulfate-Reducers and Other Bacteria

by

Xiang Wei

A thesis submitted to the
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DEDICATED TO MY FAMILY

AND

FRIENDS

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Abstract

Methylmercury is the predominant form of mercury in fish and is the only mercury species that is biomagnified. The synthesis of methylmercury occurs primarily in anoxic aquatic sediments and to a lesser extent in the water column and in soils. The methylation processes are believed to be mainly microbial activities and the sulfate reducing bacteria (SRB) have been shown to play a dominant role in mercury methylation in anaerobic sediments. However, not all sulfate reducing bacteria methylate mercury, even in different strains of the same species.

In this study, seven bacterial cultures were tested for their methylating ability in whole cells and cell extracts. Only *D. desulfuricans* B203 and *Db. propionicus* 2032 showed mercury methylation in whole cells. Other *Desulfovibrio* species, a methanogenic bacterium, *E. coli* and V*ibrio* failed. Strain B203 synthesized methylmercury when growing fermentatively or under sulfate respiration. Cells growing fermentatively produced higher levels of methylmercury than under sulfate respiration probably because HgS precipitation, produced through sulfate respiration, may cause lower Hg^{2+} availability.

In experiments with cell extracts, only *D. desulfuricans* B203 showed mercury methylation. Methyl-B_{12} added to the cell extract of B203 strain dramatically stimulated synthesis of methylmercury. However, with cell extract and methyl-B_{12}, mercury methylation was 3 times more than spontaneous synthesis of methylmercury by methyl-B_{12} when [Hg^{2+}] was at 1μg/ml, pH 7.0. Results of testing mercury methylation in

Methanobacterium thermoautotrophicum extract with or without methyl-B_{12} showed higher production of methylmercury by methyl-B_{12}'s spontaneous reaction with Hg^{2+} than the production by cell extract plus methyl-B_{12}.

3-^{14}C-serine experiment using cell extracts of *Desulfovibrio desulfuricans* B203 showed the methylmercury synthesis using serine as methyl group donor via tetrahydrofolate pathway. Both ^{14}C and ^{203}Hg experiments with B203 cell extract followed a typical kinetic curve of enzyme with an apparent K_m of milli-molar level. Methylation of mercury was completely inhibited by the presence of oxygen. The bulk protein of B203 cell extract obtained a methylmercury production at 5.7 ng/mgprotein.

INTRODUCTION

In central North America and Europe, numerous studies have shown increased concentrations of mercury in lake sediment layers deposited recently as compared with deeper, older layers (Outllet and Jones 1982; Verta *et al.* 1989; Swain *et al.* 1992; Hermanson 1993; Lockhart *et al.* 1993; Lockhart *et al.* 1995; Lucotte *et al.* 1995). In Canada, creation of hydroelectric reservoirs by enlargement of riverine lakes and flooding of adjacent forested land along rivers has led to a marked rise in rates of methylmercury production by microorganisms in sediments (Jackson 1988). A 20-year testing program that addressed methylmercury exposure in 514 native communities across Canada suggested that many people in these areas were at risk of mercury poisoning (Wheatley and Paradis 1996).

An accident involving methylmercury poisoning at Minamata Bay, Japan, in the 1960s illustrated the potential hazards associated with chronic exposure to methylmercury (Hosokawa 1993). Also in 1960s, when certain bird populations decreased drastically in Sweden, poisoning was finally correlated to the extensive use of methyl-mercury-dicyanodiamide as a fungicide in Swedish agriculture (Rosen *et al.* 1966). The fungicides containing mercury were soon widely banned for use in Europe. The ecosystem impacted by mercury contamination is very hard to be restored. In Japan, as a part of the remediation work in the mercury contaminated Bay, roughly 1,500,000 m^3 of contaminated sediment was removed from an area of 2,000,000 m^3 in the Minamata Bay 1977 to 1990. In 1990 the population of this small, beautiful city was less than 40,000 (Hosokawa 1993).

Among mercury species, methylmercury is the predominant form known to be present in fish and mammals. It was shown to accumulate at various stages of the water food chain (Nishimura and Kumagai, 1983). However, most mercury enters ecosystems in the form of inorganic mercury, through atmospheric deposition and urban discharges to waterways. The methylmercury in animals actually was converted from inorganic mercuric ions that migrated and precipitated, mostly through ambient transportation, into lakes and deposited in the sediment. Fujiki (1963) suggested that mercury could be alkylated by "plankton and other marine life".

Jensen and Jernelöv (1968) showed that mercury was methylated in sludge taken from aquaria. This observation provided the first indication that alkylation of mercury may be bacterial in origin, and that anaerobic ecosystems can effect this reaction (Wood, 1968). Evidence shows over 90% of environmental mercury methylation is associated with biological activity (Berman and Bartha, 1986) in sediments. A series of inhibition – stimulation experiments (Compeau and Bartha, 1985) confirmed the role of sulfate reducing bacteria as principal mercury methylators. The mechanism involved in microbial mercury methylation, however, is still unclear.

Although mercury methylation has been reported in a variety of sulfate-reducing bacteria species, not all sulfate-reducing bacteria methylate mercury. Even in the same genus or the same species, not all strains share this methylating ability (Gilmour and Henry 1991). King

(1999) and some other researchers found that the mercury methylation rate was coupled with the sulfate-reducing rates in several species of sulfate-reducing bacteria, *Desulfovibrio desulfuricans*, *Desulfobulbus propionicus*, *Desulfobacterium sp.*, *Desulfobacter sp. and Desulfococcus multivorans*. However, other researchers (Choi and Bartha, 1993) found high mercury methylation by sulfate-reducing bacteria growing fermenatively without sulfate supply.

Synthesis of methylmercury from 5-$^{14}CH_3$-THF and $HgCl_2$ was demonstrated previously in *Desulfovibrio desulfuricans* LS cell extract (cells were grown in Postgate's lactate-sulfate medium C, then lysed and resuspended in pH7.0 KPi buffer) and it followed an enzymatic kinetic curve with an apparent K_m of 0.872 mM $HgCl_2$ and a V_{max} of 0.728 nmol CH_3Hg^+(165ng) •minute^{-1}•mg of protein^{-1} (Choi *et al*, 1994). Under the same physiological pH but without the presence of cell extract, little methylmercury was detected. This showed that certain physiological pathways are involved in the methylating process. The remarkable high value of K_m observed in this organism may indicate that mercury methylation is an accidental reaction catalyzed by an enzyme with another primary function. Apparent high K_m and V_{max} values were also found in our studies for mercury methylation by the cell-free extracts of *D. desulfuricans* B203 growing under fermentative conditions or under sulfate respiration conditions. The detailed descriptions of our studies were described in the following chapters.

1 REVIEW OF THE LITERITURE

1.1 Mercury in the Environment

Mercury element is present in all sectors of the biosphere due to its volatility, adsorption to surfaces, and ability to form complexes. Of the several forms of mercury found in the environment, including elemental mercury, mercuric or mercurous ions, and organomercury compounds, an organic form of mercury - methylmercury - is by far the most toxic. Methylmercury, which has been found to be mutagenic under experimental condition (Friberg and Vostal 1972), has a long retention time in biological tissues, particularly brain tissue (Suzuki 1969). It is concentrated in fish and shellfish in sufficient amounts such that a continuous normal diet of the fish resulted in further concentration to neurotoxic levels in local population. The solubility of organic mercury compounds in lipids as well as their binding to sulfhydryl groups of proteins in membranes and enzymes (Berlin 1979) accounts for their cytotoxicity. Its highly toxic nature and the tendency towards biomagnification in food chains make it a potential health problem (Xun *et al.* 1987).

1.1.1 Mercury discharge in the environment

Mercury is usually present in quite low concentrations in nature. The terrestrial abundance is on the order of 50 ng/ml (Jonasson and Boyle 1971), except in mercuriferous belts and anthropogenically contaminated areas. According to a report, Mercury in Environment (1970), the average mercury content is about 100 ng/ml, with a range from 30 to 500

ng/ml; and in soil, the mercury content ranges from 10 to 20,000 ng/ml. Literature values for the natural global flux of mercury are reported around 3.0 x 10^6kg/year (Lindqvist, 1991; Nriagu and Pacyna, 1988).

Although an important fraction of the Hg introduced into the environment is due to natural processes, rarely do these processes alone produce regions of high mercury concentrations (Moore and Ramamoorthy 1984). The increasing consumption of mercury for a wide variety of commercial uses has resulted in significant pollution of aquatic environments in many parts of the world. Although the use of mercury for production of fungicides and insecticides has been banned for years in many countries, the industrial use of mercury-containing products has not stopped because of its exceptional physicochemical properties. Nriagu and Pacyna (1988) estimated that global emission of anthropogenetic mercury ranges from $910x10^3$ to $6.2x10^6$kg/yr (taking into account only the largest centers of global contamination).

Although elemental mercury is widely distributed in the atmosphere, terrestrial ecosystems, and aquatic ecosystems, it is generally most concentrated in aquatic ecosystems. Faust and Osman (1981) reported that typically 90 to 99% of the total mercury in aquatic system is associated with sediment, while less than 1% of the total mercury accumulates in the biota. Of the total mercury introduced into the aquatic environment, only a modest fraction is transformed into methylmercury (Xun. *et al.* 1987), while 90 to 99% of methylmercury accumulates in the biota (Faust and Osman, 1981). Surface waters, except where special geologic conditions prevail or are influenced by anthropogenic sources, generally contain less than 0.1ng/ml total mercury. The average content of seawater has been found to range from

0.1 to 1.2 ng/ml as total Hg (Sherbin 1979). Egawa and Tajiima (1976) reported that only a portion of 0.005-0.032 ng/ml of the total marine mercury is present as methylmercury in seawater off the Japanese Islands (Egawa and Tajima, 1976).

1.1.2 Atmospheric transport of mercury

Over the past few decades, the attention accorded mercury pollution and its effects on human health due to the consumption of contaminated food has moved from locally polluted areas to regions far removed from the human source of contamination. The migration of mercury compounds in the atmosphere and in acid rain increase methylmercury levels in freshwater fish, which is caught even in pristine lakes accepting no direct mercury pollutants (Brosset and Elsmarie 1991; Rada *et al.* 1989; Brosset 1982). It is estimated that atmospheric emissions represent 30~50% of the anthropogenetic discharges of mercury into the environment. Atmospheric transport plays a major role in their dispersion (Lindqvist 1991; Mierle 1990; Wiener *et al.* 1990; Rada 1989; Evans 1986; Lindqvist and Rodge 1985; Bjorklund *et al.* 1984). This large-scale diffuse pollution represents a serious problem for the industrialized nations. Countries like Canada, the United States and Scandinavia are particularly affected. In these countries, many natural and artificial aquatic environments contain fish with mercury contents surpassing the toxicity levels established by their governments (0.5~1.0 mg/g) (Meili 1991; Winfrey *et al.* 1990; Hakanson *et al.* 1988).

It can be shown that the creation of hydroelectric reservoirs in northern Canada was invariably followed by an increase in the

concentration of methylmercury in fish, from 5 to 6 times the natural levels (Louchouarn 1993; Verdon *et al.* 1991; Lodenius 1983). Also, flowing water brings up mercury precipitating in sediments, brings mercury from submerged forestland into water, making the mercury concentration at backwaters of river especially high.

1.1.3 Speciation of mercury in aquatic ecosystem

In natural environments, mercury is generally present in two forms: inorganic and organic. The most common mercurial organic radicals are methyl and phenyl groups; the most common inorganic complexes are with chlorine (Cl^-), hydroxyl (OH^-), nitrate (NO_3^-), and sulfur or sulphydryl (S^- or SH^-) (Lindqvist and Rodhe, 1985; Fagerstrom and Jernelov, 1971). Organo-mercuric compounds, which represent the most toxic forms of mercury, may be separated into two groups. In the first, the mercury atom is weakly bonded to the organic radical (Van der Waals or ionic bonds); in the second, the bond is strong (covalent). The first type consists of compounds commonly soluble in water, *i. e.*, it is susceptible to dissociation into a cation, $R\text{-}Hg^+$ and an anion, X^-; the most common of these organo-metallic complexes is monomethylmercury ($MeHg^+$, CH_3Hg^+). The second type includes compounds such as dimethylmercury (DMHg) and diphenylmercury (DPHg); because of the covalent bonds, these complexes are non-polar, have low solubility in water and are very volatile. The dominant mercury species in a solution will also depend on the solution's redox potential and pH, as well as the nature of the ligands present and their susceptibility to form stable complexes with mercury (Lindqvist and Rodhe 1985).

According to thermodynamic calculations, mercury dissolved in fresh water is in the form of Hg-DOC with small amount of $HgCl_2$ and $Hg(OH)_2$, with reported levels in unpolluted waters generally lower than 0.1μg/L. In seawater the dominant form should be $HgCl_4^{2-}$ (Allard and Arsenie, 1991; Benes and Havlik, 1979). The most common species of mercury in sediments is HgS due to the low redox potential and it is very insoluble (Gavis and Ferguson 1972). And 50-80% of the mercury in lacustrine systems is either absorbed on suspended particles or bound in organo-metallic complexes (Gill and Bruland 1990; Fitzgerald and Watras 1989; Benes and Havlik 1979). These complexes have an important role in the mercury cycle and in its bioavailability (Meili 1991; Gilmour and Henry 1991; Wren *et al.* 1991; Gill and Bruland 1990; Parks *et al.* 1989). Methylmercury accounts for 1-10% of the total mercury concentration in lacustrine systems, and this proportion may be higher under anoxic conditions. But DMHg is practically absent, and the low gaseous mercury concentrations are in the elemental form, Hg^0 (Fitzgerald *et al.* 1991; Lindqvist 1991; Bloom 1989).

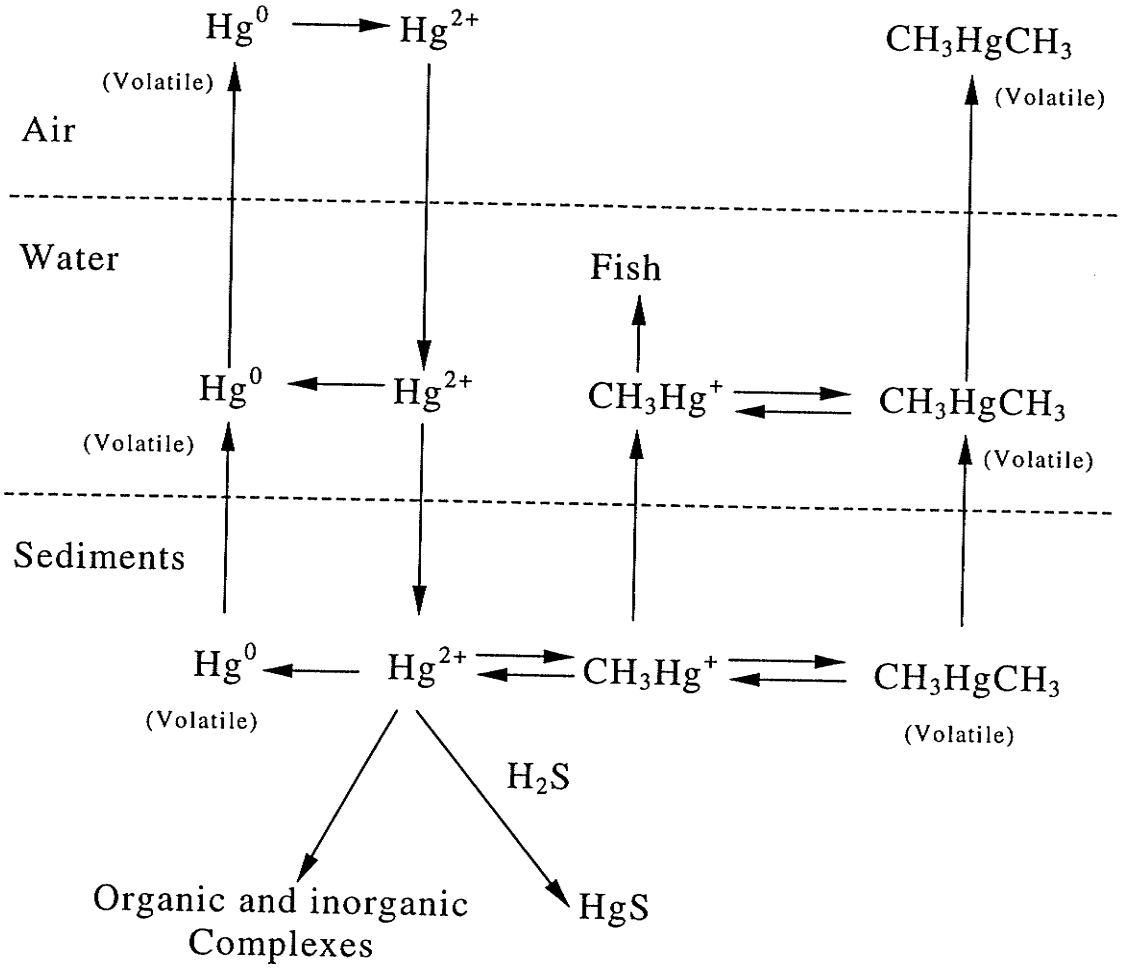

Fig. 1 Chemical forms mercury in air, water and sediment.

The major form of mercury in freshwater and seawater is inorganic mercury, whereas the predominant form found in fish is methylmercury (Westoo 1973; Gavis and Ferguson 1972; Kamps *et al.* 1972). This form of Hg is most susceptible to accumulation in organisms, i.e., the most bioavailable or with highest tendency of biomagnification (Rudd *et al.* 1980). Methylmercury in the food chain represents 2-28% of the total mercury in plants, 20-80% in invertebrates, 85-95% in fish, and 98-100% in the highest predator level, such as birds and mammals (Canaba *et al.*, 1993; Meili, 1991; Lindqvist, 1991).

Practically all the mercury in fish has been shown to be in the form of methylmercury, but modes of accumulation are not well understood. Several mechanisms have been proposed to account for the presence of methylmercury in fish. These include

(a) direct absorption of methylmercury (possibly produced by bacteria) from water and concentration due to the long retention time in tissues (Windon *et al.* 1973);

(b) direct formation of methylmercury from inorganic forms by fish tissue (Pan *et al.* 1973);

(c) indirect formation of methylmercury by intestinal bacteria or by bacteria in the surface slime of the fish, with resulting absorption into the tissues (Jernelov 1972);

(d) ingestion by fish of preformed methylmercury in their food supply (plankton for most small fish) (Saylor *et al.* 1975). In this food chain theory, the methylmercury-producing bacteria are considered to be the primary source at the bottom of the chain. Next in line in the food chain are the various larger microscopic forms, the plankton, which have been shown to accumulate mercury (Brinckman *et al.* 1975). The form of mercury in plankton, however, has not as yet been determined.

1.2 Abiotic Hg Transformation

Abiotic mechanisms, enzymatic and non-enzymatic, appear to be, at least in part, implicated in methylmercury production in nature, but little is known of these processes.

Nagase *et al.* (1982, 1984) reported methylmercury formation by abiotic transformation from humic compounds, but this process accounts for less than 1/10 of the methylmercury formed by methylation in sediment (Compeau and Bartha 1985).

Methyl-cobalamin is able to react with the mercuric ion under acidic conditions to form methylmercury spontaneously. Around more physiological pH 7.0, however, little methylmercury is formed from methyl-cobalamin and Hg^{2+}, or, the reaction is very slow (Choi 1994). This is a very important point when we are trying to look at the enzymatic mercury methylation of bacteria, as will be discussed later. Also, most sediments are pH 6.8-7.0.

Photochemical methylation of mercury was demonstrated to account for as much as a 3% conversion of mercuric acetate per day. This process is inhibited by 99.9% if $HgCl_2$ and acetic acid are used instead of mercuric acetate (Summer and Silver 1978). Research has reported that there is a quick process of photo-degradation of methylmercury formed by microorganisms in aquatic environment (Sellers *et. al.*, 1997; Samek, 1997). These results may discount the role of photochemical methylation in aquatic ecosystems and underestimate the total amount of methylmercury produced.

1.3 Biological Mercury Methylation

The biological formation of methylmercury in aquatic organisms and sediments may represent an important link in the mercury cycle. Several reports, however, have indicated poor relationships between total mercury concentrations in sediment and methylmercury in fish (Rada et al., 1986; McFarlane and Franzin, 1980). This indicates that the total mercury level is not the only factor that regulates methylmercury production because large portion of the inorganic mercuric ions are bonded with dissolved organic carbon and inorganic particulate matter, or precipitated as HgS. Only the rest dissolved form of inorganic mercuric ions are bioavailable as substrates of mercury methylation.

1.3.1 Bioavailability of mercury

Mercury in the environment can be divided into two classes: (1) bioavailable - referring to those forms which are soluble, non-sorbed, mobile, and (2) non-bioavailable - referring to those forms which are precipitated, complexed, sorbed, and non-mobile. It is the bioavailable mercury that is toxic to biological systems, since this portion of mercury is taken up by microorganisms into the cell and then converted to methylmercury *via* certain metabolic pathways. Several abiotic and biotic factors can affect mercury speciation in aquatic ecosystem, and thus affect the bioavailability of mercury to microbial populations. These factors include chemical interaction with other substances (e.g. most important, sulfide), dissolved organic carbon and salinity (Barkay

et al. 1997), pH, redox potential, and the microorganisms present. Much of the research on mercury bioavailability has been done in sediment systems in order to understand its effect on microbial uptake and methylation. Benoit *et al.* (1999) suggested the availability of mercury for methylation is controlled by the concentration of neutral dissolved mercury complexes rather than Hg^{2+} or total dissolved inorganic mercury, because uptake can occur via passive diffusion across the cell membrane. The importance of neutral chloride species has previous been demonstrated for mercury uptake by phytoplankton (Mason et. al., 1996; 1995), and mercury permeability through artificial membranes (Gutknekt, 1981).

In water-sediment environments, mercury is largely bound to organic matter. In anoxic sulfur-containing sediments, hydrogen sulfide may react with divalent mercury ions, forming Hg-S complex with extremely high formation constants (Dyrssen and Wedborg 1991). Mercuric sulfide is extremely low soluble in water (solubility product $K_{sp}=10^{-53}$). Inorganic forms of mercury are therefore less available to microorganisms thus inhibits their methylation in sediments (Winfrey and Rudd, 1990; Compeau and Bartha, 1987, 1983; Blum and Bartha, 1980).

Although methylmercury may be formed from mercuric sulfide in aerobic organic sediments, the rates are 100 to 1000 times lower than those observed for $HgCl_2$. Under aerobic conditions, sulfide is oxidized to sulfate, resulting in an increased solubility of Hg^{2+} and hence a greater availability of the Hg^{2+} for methylation. No methylmercury was formed from mercuric sulfide under anaerobic condition (Jackson and Summers, 1982), presumably because of the low redox potential. It's

hard to measure the amount of bioavailable mercury in anoxic sediments in the environment. More recent research showed (King et al., 1999), however, that despite precipitation of mercuric sulfide as well as bonding to organic compounds, there were still available inorganic mercuric ions that were observed being converted into methylmercury by bacteria in anoxic sediments. The predominant product of methylation was monomethylmercury, with dimethylmercury being formed in small amounts. The volatile dimethylmercury may also rise from the reaction of methylmercury with H_2S (Craig and Moreton, 1983).

1.3.2 Ecological location where most Hg methylation occurs

Methylation may occur in soil ecosystems (St. Louis *et al.* 1993; Rodger 1976, 1977), in water columns (Xun *et al.* 1987; Korthals and Winfrey 1987; Furutani and Rudd, 1980), in lacustrine sediments (Korthals and Winfrey 1987; Callister and Winfrey 1986), or flooded soil (Louchouarn 1993; Verdon *et al.* 1991; Jackson 1987; Lodenius 1983), in the intestines of fish and the mucus on their scales (Rudd *et al.* 1980; Jernelov 1972), and probably in many other physiologic and biogeochemical sites. However, most studies have concluded that biomethylation is primarily enhanced in anoxic sediments (Regnell, 1990; Kudo, 1976; Olson and Cooper, 1975; Andren and Harriss, 1973; Jensen and Jernnelov, 1969).

Further studies on mercury methylation in aquatic ecosystems provide us evidence that methylation of inorganic mercury in sediments is microbiological in nature and microbial communities are the principal methylator of mercury at the bottom of the food chains, contributing to

mercury biomagnification. For example, autoclaved sediments and those samples receiving no $HgCl_2$ did not produce methylmercury (Olson and Cooper 1976). Nutrient enhanced (tryptic soy broth) water and sediment floc samples increased the methylating activity of water column samples 15 times, and that of the sediment-floc samples 315 times (Furutani and Rudd 1980). It has been reported that the growth stage of microorganisms affect formation of methylmercury (King *et al.*, 1999; Ramamoorthy *et al.*, 1982).

The degradation of organic mercury and subsequent inactivation of inorganic mercury to form elemental mercury or mercuric sulfide have been considered to play an important role in the expression of bacterial resistance to organomercury (Pan-Hou *et al.* 1981a, 1981b, 1980; Schottel 1978; Summer and Silver 1978; Furukawa and Tonomura 1971). In contrast, less information is available concerning the physiological role of mercury methylation. Some research supported that the accumulation of inorganic mercury in cells and methylation is a kind of detoxifying mechanism. Although the microorganisms are producing a more toxic substance from a less toxic one, the methylmercury is more volatile, diffuses more rapidly across cell membranes than inorganic mercury ions, so that it can easily be released out of cells (Boudou *et al.*, 1991). Microorganisms surviving in high mercury environments, have been exposed to inorganic mercury for a long time, they should have evolved detoxification mechanisms. However it remains unclear to us if this is the function of mercury methylation, especially in organisms living in low mercury environments.

Experiments in many laboratories showed that microorganisms do have the potential for methylating mercury under aerobic or anaerobic

conditions. Methylmercury formation from Hg(II) ions has been reported for *Clostridium cochlearium* (Yamda and Tonomura 1972) and for laboratory cultures *of Pseudomonas fluorescens*, *Mycobacterium phlei*, *Escherichia coli*, *Enterobacter aerogenes*, and *Bacillus megaterium* under aerobic conditions (Vonk and Sijpesteijin 1973). Formation of methylmercury was also reported in *Enterobacter aerogenes* (Hamdy and Noyes 1975), and in fungi, such as, *Aspergillus niger*, *Scopulariopsis brevicaulis*, *Saccharomyces cerevisiae*, and in *Neurospora crassa* (Landner 1971). However, the methylations by these bacteria were all determined under laboratory conditions, and the mercury concentrations used were million times higher than that in the environment. Strains of some of these species were demonstrated not to methylate mercury in a previous study in our lab (Meichel, 1999).

Despite these findings, methylation in lakes was observed occurring primarily but not exclusively in anoxic aquatic sediments. Blair and Iverson (1974) detected methylmercury in the nitrogen atmosphere above two unidentified anaerobic bacterial isolates from Chesapeake Bay. A comparison of aerobic and anaerobic methylation of $HgCl_2$ in San Francisco Bay sediments indicated that methylmercury formation was faster and resulted in higher net levels under anaerobic conditions and in samples with the highest organic content (Olson and Cooper 1975). One reason for this could be because that the population densities of bacteria in lake sediments are known to be higher at the sediment-water interface than aerobic water system.

From the late 1960s to early 1980s, many studies regarding the bacterial methylation of mercury were focused on mechanism using a variety of bacteria, mostly aerobes. However, no experiments

determining the role of these bacteria with regards to methylation of mercury in the environment was performed. The bacterial strains used for study were mercury-tolerant mutants selected from media containing artificially spiked mercury whose concentrations were million times higher than that observed in the real environment. This means, the bacteria contained mercury resistance gene of the *mer* type and could reduce Hg^{2+} to Hg^{0}. The organism studied may not adequately reflect the microbial populations observed in real lake sediments or water samples methylating mercury at environmental concentration. In addition, lake sediments, where a significant portion of mercury methylation happens, are typically anaerobic environments. More recent studies have demonstrated that anaerobic microbial populations are responsible for most of the biological methylation of mercury in lake sediments. Within the microbial population, the sulfate reducing bacteria are the principal mercury methylators (Compeau and Bartha 1985).

1.3.3 Factors influence biological Hg methylation in the environment

Biological mercury methylation in the environment has received a great deal of study to determine the concentrations made by the activity of microorganisms. A strong positive correlation between the distribution of organic mercury compounds and resistant microorganisms in metal-contaminated sediment has been reported, but their exact roles are poorly understood. Numerous studies, however, have shown that methylation is influenced by a number of environmental factors, such as:

1) Binding to environmental constituents --- the availability of inorganic mercury;

2) Organic carbon (dissolved organic carbon DOC and particulate organic matter, POM) --- Numerous studies reported that methylation rate increases with the addition of easily biodegradable organic components (Mercury in Environment and Reservoirs, 1970);

3) Temperature --- Although Wright and Hamilton (1982) observed methylation in river sediments at 4 °C, methylation is normally inhibited at low temperatures and reaches its maximum at 35 °C (Choi *et al.* 1994);

4) Ion interactions, which influence the form and availability of mercury to microorganism;

5) The growth of microorganism;

6) The others: pH, redox potential, O_2, UV, and salt concentration (Compeau and Bartha, 1987);

Most of these factors contribute to microbial activity and the bioavailability of mercury, which strongly influence biomethylation of mercury.

1.3.4 Improvement of ^{203}Hg measuring method

Studies in the 1980s and 1990s used mercury radioactive tracer to measure methylation and demethylation in natural water columns or lake sediments (Gilmour *et al.*, 1992; Furutani and Rudd, 1980). Also a wide range of lab bacteria strains was tested for mercury methylation. Most of the bacteria strains used, however, are mercury-tolerant mutants induced by artificially spiked mercury whose concentrations were million times higher than observed in the real environment. So, the organisms may not typify microbial populations in the nature environment.

Net change in methylmercury is small and hard to measure, and

isotopic approaches have shortcomings when ^{203}Hg is added (need high concentrations, and the isotope might not mix with mercury in sediment or water). Researchers measured "specific rates" because the assay techniques often require the addition of mercury at levels much higher than ambient mercury concentrations (Xun *et al.*, 1987; Ramlal *et al.*, 1986).

Methods for trace amount mercury detection and measurement of methylmercury in both field and laboratory studies have improved in recent years. High specificity ^{203}Hg enables scientists to measure the small amount of methylmercury produced in environmental samples or microbial cultures. Furutani and Rudd (1990) and some other researchers have evaluated the method for its efficiency and reliability. Stordal and Gill (1995) developed two modifications so as to measure "true" *in situ* rates of mercury methylation in a water column and at sediment-water interfaces. One modification to enhance sensitivity in counting radioactive decay was to switch from liquid scintillation counting to gamma counting using a NaI detector. This change achieved between a 10- and 100-fold enhancement in sensitivity for comparable counting time. The other modification was to reduce the added radioactive mercury spike by using higher specific activity ^{203}Hg (20 to 30 mCi/mg). They detected methylation peaks at 9 and 15 hours of incubation of water column and sediment core spiked with 1~20ng/L Hg (in form of $^{203}HgCl_2$), which are at near natural levels. By using higher specific activity ^{203}Hg, only a small amount of mercury is added to samples. This provides us the opportunities to observe the methylation from the same microbial population that was active in the natural environment and minimizes the selection of a more mercury tolerant bacteria (Furutani and Rudd, 1980). A significant drawback, however, is the short half-life,

which requires most of experiments to be done within the first few months of obtaining the spike, if low levels are required for the procedure.

1.4 Methylation of Mercury by Sulfate-Reducing Bacteria

In 1980s scientists started to use sulfate-reducing bacteria to study mercury methylation in pure cultures. Compared to research on mercury speciation and the factors influencing bioavailability in aquatic ecosystem, research on methylation by SRB using pure culture seems to be quite insufficient. The results displayed much difference between one another. Most research, however, has confirmed the contribution of sulfate-reducing bacteria to mercury methylation in environment.

1.41 Principal methylators of Hg^{2+} in anoxic aquatic system

Although a wide range of microorganisms, including anaerobes as well as aerobes, have been shown capable of pure-culture methylation of Hg^{2+} at artificially high mercury concentrations, a series of inhibition-stimulation experiments gave strong evidence that bacteria are the principal mercury methylators in anoxic sediment environment. Sulfate-reducing microorganisms carried out over 95% of mercury methylations in anaerobic aquatic sediments (Compeau and Bartha 1985). Previously, it was widely believed that methanogenic bacteria contributed to mercury methylation in anoxic sediment environments (Wood *et al.*, 1968) since they were known to have the biochemical systems for

donating methyl groups, and had high concentrations of methylcobalamin, a methyl-carrying co-factor.

Wood *et al.* (1968) tested mercury methylation using cell extracts of *Methanobacterium bryanntii* MOH, but did not monitor whether or not there was methylation by whole cells. In their experiments, the addition of exogenous methylcobalamin combined with the acid deproteinization procedure at the end of the incubation period, and the lack of controls, make it difficult to support the conclusion that this methanogenic bacterium is capable of methylating mercury. Inorganic mercuric ions can be spontaneously methylated by methylcobalamin in an acidic buffer (Choi and Bartha, 1993; Desimone *et. al.*, 1973). To date no pure cultures of methanogenic bacteria have been shown to methylate mercury in whole cells.

In experiments by Compeau and Bartha (1985), Hg^{2+} spiked estuarine sediment slurries were treated with 30mM BESA (2-bromoethane sulfonic acid), which is a specific inhibitor of methanogenesis. And the contribution of sulfate reducers was assessed by treating sediment slurries with 20 mM of a specific inhibitor of sulfate reduction, sodium molybdate. Sodium molybdate inhibited sulfate reduction and completely suppressed Hg^{2+} methylation. BESA, on the other hand, inhibited methanogenesis but not mercury methylation in these estuarine sediments. Similar results were obtained using fresh water sediments (Gilmour and Henry, 1991; Gilmour *et al.*, 1992). Kerry et al. (1991) carried out similar experiments on fresh water sediment slurries, with sodium molybdate and BESA used at 10 mM and 15 mM respectively. The results were consistent with the results obtained from estuarine sediment. BESA inhibited methanogenesis thus released more carbon

sources and electron source for sulfate reducing bacteria, which contributed to mercury methylation. However, cells growing without sulfate (in the presence of pyruvate) synthesized large amounts of methylmercury. The possible reason is that the H_2S generated by sulfate respiration interferes with the methylation of mercury by precipitating it as HgS. Sulfate reducing bacteria are not obligated to use sulfate, they can also grow fermentatively. Molybdate is expected to only inhibit the sulfate reduction process of organism (Oremland and Capone, 1988). Therefore it should only inhibit those sulfate-reducing bacteria that are actively reducing sulfate, but not those that grow in the absence of sulfate, using alternate energy source (e.g. pyruvate). Previous work in our lab (Meichel, 1999) indicated methylmercury inhibition by Mo in the absence of sulfate. Thus addition of Mo to sediments should inhibit sulfate reducers growing either fermentatively or using SO_4^{2-} as an e^- acceptor in respiration.

Berman *et al.* (1986) tested the ability of *D. desulfuricans* ATCC 2774, *D. desulfuricans* Norway 4, *D. gigas* ATCC 29494, *Desulfobulbus propionicus* FP, and *Desulfotomaculum orientis* ATCC 19365 to methylate mercury in lactate-sulfate medium. Only *D. desulfuricans* Norway 4 had detectable ability to methylate mercury. King *et al.* (1999) in their pure-culture studies found the rates at which SRB methylated mercury were in the following order for the various strains tested:

Desulfobacterium >> *Desulfobacter* ≈ *Desulfococcus* >> *Desulfovibrio* ≈ *Desulfobulbus*.

The author also reported that the methylation rates were strongly correlated with the sulfate reducing rates. In contrast, a number of

reports (Choi *et al.*, 1993) indicated high mercury methylation in sulfate-reducing bacteria, mostly in *Desulfovibrio* and *Desulfobulbus*, growing in sulfate-free media.

Sulfate-reducing bacteria are recognized by their dissimilatory reduction of sulfate to hydrogen sulfide and differentiated by the ability of different species to utilize an array of electron donors including hydrogen, small organic acids such as lactate, acetate, and propionate, long chain fatty acids, aliphatic hydrocarbons, and simple aromatic compounds such as benzoate, phenol and toluene (Devereux *et al.* 1996). They are a diverse collection of Gram positive and Gram-negative strictly anaerobic eubacteria (Postgate, 1984; Pfenning *et al.*, 1981). The Gram-negative SRB were expected to be much more abundant than Gram-positive forms in marine sediment (Moriaty and Hayward, 1982). Devereux *et al.* (1996) used SRB-specific ribosomal RNA probes to determine the distribution of sulfate-reducing bacteria in anaerobic estuarine sediment. Generally Gram-negative mesophilic species similar to *Desulfovibrionaceae* were found dominant in the SRB ribosomal RNAs in the 3-4cm-depth fraction of sediment. Mercury methylation activity coincided with the peak in *Desulfovibrio* ribosomal RNA and *Desulfobulbus* species also showed mercury methylation. The test did not include *Desulfobacter* spp. *Desulfobcaterium* spp. (*D. multivorans*, *D. variabilis*, and *D. sapovorans*.). *Desulfoaruculus baarsii*, *Desulfonema* spp., *Desulfomonile teidjei*, and *Desulfotomaculum* spp. were not tested because there was no developed probe for them, but *D. baarsii* is a known mercury methylator (Gilmour, 1995).

The phylogeny of sulfate-reducing bacteria has been defined by comparative 16S rRNA sequence analysis (Devereux *et al.*, 1990, 1989;

Fowler *et al.*, 1986). Analyses have demonstrated significant phylogenetic diversity in the genus *Desulfovibrio*. The genus contains both complete and incomplete oxidizers as well as species differences in pigment content. They can oxidize lactate to acetate and use only a few other simple organic acids, hydrogen and ethanol as electron donor (Postgate 1984). Diversity also exists among strains in the species *Desulfovibrio desulfuricans* (Devereux *et al.*, 1990).

Although mercury methylation has been reported in a variety of sulfate-reducing bacteria species, not all sulfate-reducing bacteria methylate mercury. Even in the same genus or the same species, not all strains share this methylating ability (Gilmour and Henry 1991), showing that the mercury methylation seems not to be a common trait among the sulfate-reducing bacteria that have been isolated.

1.4.2 Fermentative/sulfate reduction metabolism

King and colleagues (1999b) measured the mercury methylation rate in several species of sulfate-reducing bacteria, *Desulfovibrio desulfuricans*, *Desulfobulbus propionicus*, *Desulfobacterium sp.*, *Desulfobacter sp. and Desulfococcus multivorans*, and found that the rate was strongly coupled with the sulfate reducing respiration. Bartha *et al.* (1994, 1993, 1987, 1985) found high mercury methylation rate by the sulfate-reducing bacterium *Desulfovibrio desulfuricans* LS growing fermentatively without sulfate supply, and suggested sulfate-limiting conditions favor the mercury methylation process both in pure cultures and in anoxic aquatic sediments.

In sediment, SRB may grow fermentatively or via sulfate respiration. The products of fermentative metabolism serve as substrates for sulfate reducing metabolism. Fermentation products are oxidized while the generated electrons reduce sulfate or carbonate at progressively decreasing redox potentials. The sediment slurries spiked with $HgCl_2$ and supplemented with pyruvate generated methylmercury three times more than the control and six times more than that from the slurries supplemented with lactate-sulfate. Stimulation of methylation in the presence of pyruvate agrees with the postulation that methyl-cobalamin is highly involved in the synthesis of methylmercury as a methyl donor, because pyruvate is metabolized by a cobalamin-dependant pathway (Postgate 1984) in *Desulfovibrio desulfuricans*.

Gilmour *et al* (1992) suggested there is an optimal sulfate concentration for mercury methylation by sulfate-reducing bacteria in sediments (<0.1~0.2 mM for freshwater sediments). This optimal level would vary somewhat among sediments with temperature, porosity, organic carbon and the bioavailability of mercury. Production of sulfide through sulfate reduction would inhibit methylation above this optimal sulfate concentration, while sulfate availability would limit microbial sulfate reduction and hence mercury methylation below the maximum. Kerry *et al.* (1991) found increased sulfate reduction with increased sulfate concentration (0.05~0.3mM), but no correction with mercury methylation was observed.

The *D. desulfuricans* isolated from sediment was unable to methylate pure HgS, production of methylmercury increased at low [Hg] when cells were grown in pyruvate medium. The MeHg production increased slowly in lactate-sulfate medium as the [Hg] going up, indicating the

HgS precipitation made [Hg] less bioavailable for mercury methylation. Mercury methylation can continue in the presence of high sulfate concentrations but not to the same extent as when sulfate is at lower concentrations.

1.4.3 Proposed mercury methylation process in some SRB

Choi and Bartha (1994) investigated mercury methylation by *D. desulfuricans* LS on the basis of ^{14}C incorporation from precursors and measurement of relevant enzyme activities in cell extracts. *D. desulfuricans* LS was isolated from low-salinity estuarine sediment, and was grown in Postgate sulfate-free medium D (Postgate, 1984). They found more efficient incorporation into CH_3Hg^+ of ^{14}C from [3-^{14}C]pyruvate than from [1-^{14}C]pyruvate and the C-3 serine was incorporated into methylmercury with 95% preservation of specific activity, suggesting that the C-3 serine, which is a principal methyl donor to tetrahydrofolate (THF), was most likely the source of methyl group in methylmercury (Fig. 2, left routine).

The less efficient incorporation of pyruvate C-1 into MeHg may have occurred via decarboxylation (Fig. 2, middle routine), reduction of the CO_2 to formate by a formate dehydrogenase, and formation of formyl-THF or a formyl-tetrahydroprotein. The formyl group would be reduced to the methyl level and transferred to a corrinoid or a similar compound as the ultimate donor to the mercuric ion. Label from $H^{14}COO^-$- was incorporated into CH_3Hg^+ with at least 50% retention of specific activity. The label from C-3 of pyruvate could be incorporated into

CH_3Hg^+ via serine, also could be incorporated into CH_3Hg^+ via reversal of the acetyl-CoA synthase reaction.

Propyl iodide used in the experiment blocked both the acetyl-CoA synthesis and the mercury methylation. Hg^{2+} added to cell extract inhibited synthesis of acetyl-CoA and was converted to methylmercury. methylmercury was proposed being formed by acetyl-CoA pathway. This experiment was done with cells grown on Postgate lactate-sulfate medium C (Postgate, 1984). The CH_3- group of CH_3-THF can arise from formate, from C-3 of serine, or from C-3 of pyruvate via serine and be transferred by methyltransferases to cobalt in a corrinoid protein, the last intermediate before acetyl-CoA synthase in acetyl-CoA synthesis. The CH_3 group could also arise from C-1 of pyruvate by its oxidation to acetyl-CoA, catalyzed by CO dehydrogenase, and transfer to the corrinoid protein (Berman *et al.*, 1990).

Many SRB utilize the acetyl-CoA synthesis pathway in their metabolism, such as *D. baarsii*, *D. acetoxidans*, *D. autotrophicum*, *D. multivorans*, *D. variabilis*, *D. desulfuricans*. In *D. baarsii and D. desulfurican* LS, it has been demonstrated that the pathway operates in the direction of acetyl-CoA synthesis. 16S rRNA sequence analysis showed *D. baarsii* is only distantly related to other *Desulfovibrio* species (Devereux *et al.*, 1989). *D. baarsii* and some other *Desulfovibrio* species can carry out both oxidative and reductive acetyl CoA synthesis pathway depending on the growth conditions (heterotrophic or autotrophic). Carbon monoxide dehydrogenase is the key enzyme of these pathways. Terlesky *et al.* (1986) and Lee *et al.* (1988) suggested this enzyme is likely in two different forms: One may catalyze acetyl CoA synthesis,

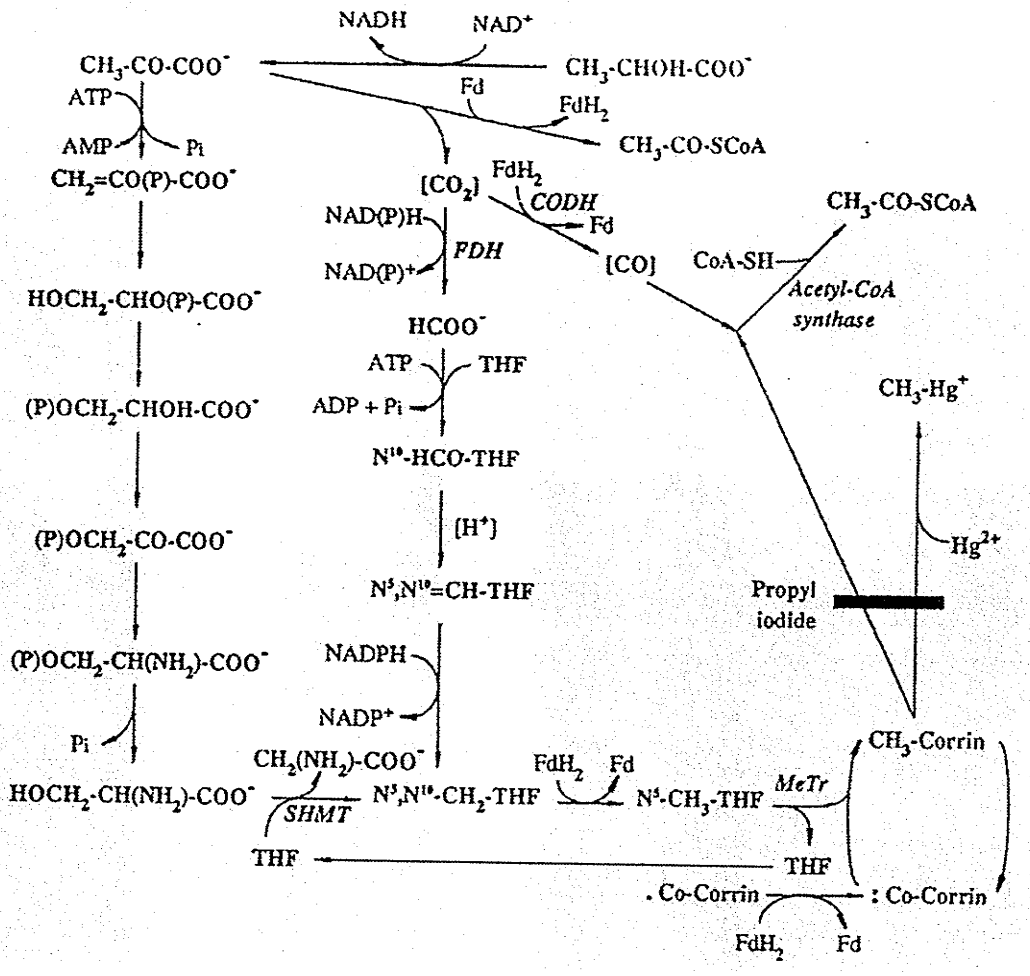

Fig 2 Acetyl CoA pathway (Choi and Bartha 1994) Proposed metabolic pathway involved in mercury methylation by D. desulfuricans LS. The propylation of corrinoid by propyl iodide blocked both acetyl-CoA synthesis and mercury methylation. Fd, ferredoxin; FDH, formate dehydrogenase; CODH, CO dehydrogenase; *MeTr*, methyltransferase; *SHMT*, serine hydroxymethyltransferase.

the other catalyzed acetyl CoA cleavage. Wood et al. (1968) suggested a CO dehydrogenase complex may catalyze the synthesis of acetyl CoA from CO_2, methyl-tetrahydropterin, coenzyme A, as well as the cleavage of acetyl CoA to these substances. One partial reaction of this enzyme complex, the oxidation of CO to CO_2 with methyl violegen as an artificial electron acceptor (Thauer et al., 1974), can be used as an indicator of the presence of the CO dehydrogenase complex.

1.4.4 Biological importance of CH_3B_{12} in Hg methylation

There are three major coenzymes that have the ability of transport methyl group in a biological system:

1) N5-methyltetrahydrofolate,
2) S-adenosylmethionine,
3) methylcobalamin.

Among them only methylcobalamin (CH_3B_{12}) has been demonstrated to interact with mercuric ions in a spontaneous chemical reaction. Regnell and Tunlid (1991) studied Vitamin B_{12} in eutrophic lake sediments. The concentrations of vitamin B_{12} and its derivatives in sediments (1~3mm) were about 0.91± 0.16 μg/g (dry weight). There was a positive and significant correlation between the concentration of B_{12} and the total concentration of phospholipid fatty acids in sediment, which included several fatty acids that are characteristic for bacteria. However, significant correlation between the concentrations of vitamin B_{12} and methylmercury was not found. Since the vitamin B_{12} derivative methyl cobalamin has been shown to function as a donor of methyl

groups to inorganic mercury (Berman et al. 1990), bacteria producing high levels of vitamin B_{12} may be involved in mercury methylation.

The addition of $CoCl_2$ and benzimidazole to a fermentative culture increased methylation activity. From *D. desulfuricans* grown in the presence of $^{57}CoCl_2$, a corrinoid was extracted and purified. Based on HPLC analysis, that cobalamin was the only corrinoid present in *D. desulfuricans* LS.

When cells of *D. desulfurican* were pre-incubated with propyl iodide, their ability to form MeHg from Hg^{2+} was blocked. Propyl iodide causes the propylation of the cobalt center of the corrinoid, blocking the Hg^{2+} methylation reaction. Exposure to light released the propyl group and restored the ability of the corrinoid to methylate Hg^{2+}.

Vitamin B_{12} (cyanocobalamin) was found to stimulate the production of methylmercury by *Clostridium cochlearium*, *Enterobacter aerogenes*, and a strain (Number 244) of *Pseudomonas* isolated from Chesapeake Bay (Blair and Iverson 1974). When the purified cyanocobalamin isolated from *D. desulfurican* was methylated by $^{14}CH_3I$, the prepared $^{14}CH_3$-B_{12} was allowed to react with mercuric ions in a pH 4.5 acetate buffer and formed ^{14}C-methyl-Hg. (No radioactivity remained in the negative control.) The specific activity of the methylmercury produced was 93.9% of specific activity of the added $^{14}CH_3I$. This indicates that the methylated *D. desulfurican* LS corrinoid spontaneously transferred its methyl group to mercuric ion under low pH condition.

The very slow spontaneous transmethylation by methyl-cobalamin at more physiological pH7.0 seems to be inconsistent with the high

methylation of Hg^{2+} in cell cultures. It indirectly supports that the mercury methylation may be an enzymatic ally catalyzed process rather than a spontaneous chemical reaction. In a sediment-water microcosm, no significant correlation between the B_{12} concentration and methylmercury production was found. In anoxic sediments, in addition to SRB, numerous methanogenic bacteria are known to contain corrinoid because of C_1 metabolism. The presence of methyl corrinoid in these microorganisms does not appear to be correlated with significant mercury methylation activity in sediments.

1.4.5 Enzymatic Process?

Since previous studies have shown that SRB are almost exclusively responsible for mercury methylation in anoxic aquatic sediment, there is no reason why homoacetogens or methanogenic bacteria should not be major methylators of mercury if the methylation is non-enzymatic or even if it is enzymatic, since they too have corrinoid and CODH. They often contain cobalamin in concentrations several orders of magnitude higher than that reported for sulfate reducing bacteria. Also, the lack of correlation between sediment cobalamin content and mercury methylation argues against the dominance of non-enzymatic mercury methylation in the environment.

Choi and Bartha (1994) found that the synthesis of methylmercury from 5-$^{14}CH_3$-THF and $HgCl_2$ by *Desulfovibrio desulfuricans* LS cell extracts followed a Michaelis-Menten enzymatic kinetic curve with an apparent K_m of 0.872 mM $HgCl_2$ and a V_{max} of 0.728nmol per minute per mg of protein. If it is true that this organism methylate mercury via acetyl CoA pathway, then there are a series of enzymes involved in the

production of methylmercury from Hg^{2+} and methyl tetrahydrofolate. The K_m represents this whole enzyme system. The cell extracts used were prepared from the cells grown in the Postgate lactate-sulfate medium C. Small amount of sulfide precipitates existed after 2 days incubation for harvest. The reaction mixture contained 10mM dithiothreitol, 190mM sodium pyruvate, 0.5~8mM $HgCl_2$, 50mM KPi buffer (pH 7.0), and cell extract (0.32mg protein/ml). The remarkable high value of K_m implicates that mercury methylation may be an abnormal behavior in the organism, that is, the organisms are going out of their way to synthesize the methylmercury under the abnormal high concentration of inorganic mercury (0~8mM), compared to unpolluted aquatic environment of nanogram per milliliter level of mercury. The enzyme is either doing this as a non-specific side reaction or most of the Hg^{2+} added is not available (*eg.* bonded to proteins or in a non available state, instead of Hg^{2+})

pH dependence was observed with methylmercury production by cell extract (Choi and Bartha, 1994). The optimal conditions for cell extract mercury methylation by *D. desulfuricans* were pH 6.5 at 35 °C. The activity was oxygen sensitive, decreasing by 44% after 5 min of exposure to air.

Bartha and colleague supposed the synthesis of methylmercury from CH_3-THF and $HgCl_2$ to be two steps and two specific enzymes are involved.

Methyltransferase I

CH_3-THF + Co-protein ----------> CH_3-Co-protein

Methyltransferase II

CH_3-Co-protein + Hg^{2+} ----------> CH_3Hg + Co-protein

They considered methyltransferase II is required, since that, if the reaction of CH_3-corrinoid protein which Hg^{2+} is a spontaneous chemical reaction, the rate of overall enzymatic methylation should be lower than or equal to the non-enzymatic rate observed with exogenously added methyl-cobalamin. But at physiological pH (7.0), the enzymatic rate was 600 folds higher than the rate of the non-enzymatic reaction. If the first reaction were rate limiting, an enzymatic kinetic curve with apparent K_m would not be observed. However whether this reaction is the physiological function of this proposed enzyme has also not been determined.

2 OBJECTIVES

Since;

1) Several sulfate reducing bacteria have demonstrated the ability to methylate mercury in pure culture, but the results seem to be highly strain specific;
2) The enzymatic capability seems to be a property of limited distribution even among strains of *Desulfovibrio* species. To date, little work has been done regarding cell extract mercury methylation, also based on the view that every single ecological behavior of an organism has its physiological dependence;
3) If the acetyl CoA pathway is involved in methylmercury synthesis pathway, CO dehydrogenase complex is very important to evaluate the effectiveness of the pathways;
4) The phylogenies of the sulfate-reducing bacteria indicate some of them possess a number of physiological abilities to grow under different conditions and display various characteristics;
5) The effect of methyl cobalamin is of such importance regarding the mercury methylation in cell extracts;
6) Mercury methylation in cell extract was investigated only in one *Desulfovibrio desulfuricans* strains (Choi and Bartha, 1994), and the synthesis was measured from CH_3-THF and Hg^{2+}. If the proposed acetyl CoA pathway for mercury is true, there are still several steps between the CH_3-THF and final methylmercury;

By using ^{203}Hg as a direct substrate I can test the formation of methylmercury from $^{203}Hg^{2+}$ and a direct methyl donor. I have proposed to;

1) investigate and compare whole cell mercury methylation in pure cultures of several SRBs, and some other bacteria;
2) investigate and compare cell extract mercury methylation in SRBs, and some other bacteria;
3) determine the CO dehydrogenase complex of these pure cultures;
4) determine the effect of different growing condition on mercury methylation in cell extracts of SRBs;
5) test the Effect of exogenous CH_3B_{12} and B_{12} on mercury methylation;
6) initiate steps for purifications of the mercury methylation activity;

3. MATERIALS AND METHODS

3.1 Organisms, Cultivation and Harvest

3.1.1 Organisms

In the study we have tested four strains of sulfate reducing bacteria for their whole-cell and cell extract ability of mercury methylation. *Desulfovibrio desulfuricans*: strain B203, strain DSM 1924, and strain DSM Essex6; *Desulfobulbus propionicus*: strain DSM 2032.

Other bacteria that were evaluated include: M*ethanobacterium thermoautotrophicum* strain Marburg, *E. coli* HMS174, which carries plasmid prb28. This strain has a mercury bio-reporter and has been confirmed to be able to uptake mercury into cells (Selifonova *et al.* 1993) and *Vibrio anguillarum*, which also carries plasmid prb28. It also has been confirmed to be able to take up mercury on the basis of the bio-reporter.

Desulfovibrio desulfuricans DSM 1924, *Desulfovibrio desulfuricans* DSM Essex6, *Desulfobulbus propionicus* DSM 2032 and *Methanobacterium thermoautotrophicum* were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH in Brunswick, Germany. The *Desulfovibrio desulfuricans* B203 was a gift from Dr. Gilmore.

3.1.2 Cultivation and harvest

Media preparation, culture transfer, incubation, and cell harvest of the sulfate reducing bacteria and *Mb. thermoautotrophicum* were

performed anaerobically. *E. coli* was handled anaerobically only when required.

Media: Three types of media were provided for the growth of sulfate reducing bacteria. Lactate-sulfate Medium C is a medium adapted from Postgate's Medium C (Postgate 1984) that facilitates growth under sulfate respiration. It has, in 1 liter of MilliQ ultra pure water, 0.5g of KH_2PO_4, 1g of NH_4Cl, 4.5/4.0g of Na_2SO_4 or Na_2SO_3, 0.06g of $CaCl_2$ $6H_2O$, 0.06g of $MgSO_4$ $6H_2O$, 6g of sodium lactate, 1g of yeast extract, 0.004g of $FeSO_4$ $7H_2O$, 0.3g of sodium citrate $2H_2O$, 1ml of resazurin and 1ml of 200mM Na_2S solution.

Postgate's Medium D (Postgate 1984) was used for fermentative growth, i.e., without SO_4^{2-} source for sulfate respiration (sulfate-free growth). It has, per 1liter of MilliQ ultra pure water, 0.5g of KH_2PO_4, 1g of NH_4Cl, 0.1g of $CaCl_2$ $2H_2O$, 1.6g of $MgCl_2$ $6H_2O$, 3.5g of sodium pyruvate, 1g of yeast extract, 0.004g of $FeSO_4$ $7H_2O$, 1ml of resazurin and 1ml of 200mM Na_2S solution. For the above two media, mineral elixer (trace elements, 1ml/L) and vitamins solution (1ml/L) were added for stimulating the growth of SRBs when recovering the cultures from storage. They may not always be necessary. Mineral elixer contains 2.02g/L of trisodium nitriloacetate, 0.21g/L of $FeCl_3$ $6H_2O$, 0.2g/L of $CoCl_2$ $6H_2O$, 0.1g/L of $MnCl_2$ $4H_2O$, 0.1g/L of $ZnCl_2$, 0.05g/L of $CaCl_2$ $2H_2O$, 0.05g/L of $CuSO_4$ $2H_2O$, 0.05g/L of Na_2MoO_4 $2H_2O$, and $NiCl_2$ $6H_2O$. Vitamin solution contains 10mg/L of pyridoxine-HCl, 5mg/L of riboflavin, 5mg/L of thiamine, 5mg/L of nicotinic acid, 5mg/L of p-aminobenzoic acid, 5mg/L of lipoic acid, 2mg/L of biotin, 2mg/L of folic acid, and 1mg/L of cyanocobalamin.

The three strains of *Desulfovibrio desulfuricans* were grown on both Medium C and Medium D. When testing for CODH, we grew them on Medium C (H_2/CO_2) using H_2/CO_2 as energy source and solo carbon source instead of sodium lactate, or grew them on Medium C (formate) using formate as sole carbon source.

Desulfobulbus propionicus was grown on medium D and medium 194 that contains sulfate. Medium 194 has, per 1 liter of MilliQ ultra pure water: 3g of Na_2SO_4, 0.2g of KH_2PO_4, 0.3g of NH_4Cl, 1g of NaCl, 0.4g of $MgCl_2$ $6H_2O$, 0.5g of KCl, 0.15g of $CaCl_2$ $2H_2O$, 1.5g of Sodium propionate, 1ml of Resazurin, 1ml of 200mM Na_2S solution, 1ml of Mineral elixer (trace elements, see description for Medium D), and 1ml of Vitamin solution (see description for Medium D).

The medium for *Mb. thermoautotrophicum* strain Marburg (DSM 2133) has, per 1 liter of MilliQ ultra pure water: 0.42g of KH_2PO_4, 0.23g of K_2HPO_4, 1g of NH_4Cl, 0.03g of $CaCl_2$ $2H_2O$, 0.04g of $MgCl_2$ $6H_2O$, 0.595g of NaCl, 0.16g of Na_2CO_3, 1ml of Resazurin, 1ml of 200mM Na_2S solution, and Mineral elixer (trace elements, 1ml/L). Growth at pH7.0 under H_2/CO_2 headspace, temperature 63°C.

For the growth of sulfate reducing bacteria and *Methanobacterium thermoautotrophicum*, every 450ml medium was put into one 1L Pyrex reagent bottle. The pH value of medium was adjusted to about 8.5 under the 20% CO_2/N_2 gas phase before autoclave. The pH is about 6.8~7.2 after autoclave. For *E. coli* and *Vibrio anguillarum*, a 10ml of LB medium (pH7.0) was used and was distributed into 20ml (anaerobic) Balch-tubes for incubation.

Incubation: *Desulfovibrio desulfuricans* strain B203 and strain DSM 1924, *E. coli* and *Vibrio anguillarum* were grown at 28 degree Celsius, after 10% inoculation. *Desulfovibrio desulfuricans* DSM ESSEX6 and *Desulfobulbus propionicus* DSM 2032 were grown at 37 degree Celsius. *Methanobacterium thermoautotrophicum* were grown in a 65 degree Celsius water bath until late log phase. Since it consumes CO_2 quickly, it needs to be fed with 20% CO_2/H_2 gas everyday. For each culture, growth curves were plotted and the optimal incubation and harvest time was determined.

Harvest: In our research, cells were collected when they reach late exponential phase by centrifugation at 8000rpm for 30min. The pellet was then suspended in the appropriate fresh medium for the test of whole-cell mercury methylation, or suspended in 50mM KPi buffer (pH7.0) for cell-extract experiments.

3.2 Preparation of cell samples

Pre-concentration of whole cell: For each sulfate reducing bacterium and *Methanobacterium thermoautotrophicum*, 1~4 bottle of cells (450ml culture) were harvested when the cell density reached an O.D. of 0.7 at 600nm. After centrifugation, the pellet was suspended into 10ml of fresh medium C or D. This 10ml cell solution was then evenly distributed into 10 tubes (10 samples).

For *E. coli* or *Vibrio anguillarum*, one tube of culture was collected after 18 hours of incubation, centrifuged and suspended in the same way as were the sulfate reducing bacteria.

Preparation of cell-free extract: For each sulfate reducing bacteria and *Mb. thermoautotrophicum*, four bottles of culture (4x450ml culture) were harvested, centrifuged and suspended into 10ml of 50mM pH7.0 KPi buffer solution. These cells were then broken with French-pressure device under 20K three times at 4 degree Celsius. The protein content was tested (~mg/ml) and the crude cells extract was put on ice ready for experiment to evaluate the effect of cell membrane bonded content on the mercury methylation. In order to obtain cell-free extract, the crude cell extract was then ultra-centrifuged at 20k RPM for 30min at 4 degree Celsius. The pellet was re-suspended into 50mM pH7.0 KPi buffer at the same volume as the crude cell extract. The cell-free extract was put on ice ready for experiments and the protein content was measured (~mg/ml). The cell-free extract may be stored in freezer at minus 60 degree Celsius, but must be used within one week. The cell-free extracts of *E. coli* and *Vibrio anguillarum* were prepared in the same way except the amount of cell being harvested was 10 to 20ml instead of 4x500ml for SRB.

Bulk protein: 10ml of cell-free extract was obtained using the method mentioned above. 2ml of it was saved for cell extract methylation as comparison to the bulk protein methylation. Protein content was measured. 8ml of it was completely precipitated with solid $(NH_4)_2SO_4$. It was carried out in ice water bath in anaerobic chamber, followed by 10^5g^min ultra-centrifugation. The pellet was re-suspended in 2.5ml 50mM pH7.0 KPi buffer. Then go through G25 1.5x7cm column to get rid of $(NH_4)_2SO_4$. It is also carried out in the anaerobic chamber. Finally 2.3ml of salt free bulk protein was collected. The protein content was measured. This bulk protein solution was used to study the methylating reaction.

3.3 Anaerobic techniques:

For this study, strict anaerobic techniques were applied because the sulfate reducing bacteria and *Mb. thermoautotrophicum* are obligate anaerobes and almost all consequent experiments were carried out under anaerobic condition. For large quantities of medium (450ml per bottle), 1L Pyrex reagent bottles that contain medium were sealed with oxygen-dispersed rubber stopper, which was kept in place by wires. 120ml Wheaton glass serum bottles and 20ml Balch-tubes of medium were used for some purposes. They were sealed with oxygen-dispersed rubber stoppers with aluminum caps. The bottles and tubes were then evacuated for 10min and gassed with 20% CO_2/N_2 for 5min, four cycles. 1ml/L of 200mM Na_2S solution was injected into bottle or tube with syringe to obtain completely reductive condition before being autoclaved.

Cell harvesting, subsequent cell re-suspending and sample distribution were carried out in the Coy-glove chamber or with syringes to ensure anaerobic condition. Before putting the bottles into the chamber, they were degassed to reduce H_2S. 400ml Nalgene bottles with rubber sealing ring in caps were used for centrifugation.

The French-pressure device was flushed with anaerobic water before use. All chemical solutions were stored in sealed bottle degassed and gassed with N_2. They were 25mM cyanocobalamin, 10mM methylcobalamin, 50mM ATP, 32μg/ml serine (no ^{14}C label), 10mM dithiothreitol, 50mM pH7.0 KPi Buffer, 100mM Sodium pyruvate, 0.5 to 8mM mercuric chloride solution (no ^{203}Hg label).

3.4 Radioactive techniques

^{203}Hg: ^{203}Hg was purchased from Amersham Inc., Arlington Heights, ILL, USA, 1ml of ~mCi/ml $^{203}HgCl_2$, and New Nuclei Company, California, USA, 1ml of ~mCi/ml $^{203}HgCl_2$. It was diluted with various concentration of anaerobic $HgCl_2$ (cold) solution to obtain different activities and concentrations of $^{203}HgCl_2$ working solution, ranging from ~1μCi/ 1μgHg^{2+}/100μl to ~10μCi/ 4mgHg^{2+} /100μl. Hamilton micro syringes were used to inject $^{203}Hg^{2+}$ into reaction tubes. Incubation of $^{203}Hg^{2+}$ spiked samples and the extraction of CH_3-$^{203}Hg^{+}$ were performed behind a lead shield. N-DEX Nitrile gloves (non-latex procedures glove) were used when handling organic solvent in CH_3-$^{203}Hg^{+}$ extraction.

3-^{14}C-serine: specific activity of 57.5mCi/mmol, purchased from Amersham Inc., Arlington Heights, ILL, USA. The working solution was 120,000 dpm/nmol. The working procedure is the same as using ^{203}Hg, but without the lead shield.

3.5 Experimental design

3.5.1 Whole-cell mercury methylation experiments

Sulfate reducing bacteria and *Mb. thermoautotrophicum* were pre-grown for 2 days and harvested at the mid-log phase. Each bottle of cell suspension (450ml medium + 50ml inoculation) was centrifuged and re-

suspended into 10ml of fresh medium. This cell sample was kept on ice ready for experiment.

The mercury methylating reaction was performed anaerobically in dark at room temperature for sulfate reducing bacteria, *E. coli* and *Vibrio anguillarum*, at 65 degree Celsius water bath for *Mb. thermoautotrophicum*, with final reaction volume of 1ml in a 20ml glass tube capped with rubber stopper. It contained 0.5ml of cell sample, 0.1ml of 50mM ATP or 100mM sodium pyruvate (as energy source), 0.1ml of 32μg/ml serine (no ^{14}C label for ^{203}Hg experiment), 1ml of 10mM dithiothreitol, spiked with 100μ of ~1μCi/1μgHg/100μl $^{203}HgCl_2$, and was supplemented with appropriate amount of 50mM KPi Buffer (pH7.0) to bring the final volume up to 1ml. It may contain 0.1ml of 25mM cynocobalamin or 10mM methylcobalamin when required. For ^{14}C label experiment, 0.1ml of 0.5 to 8mM mercuric chloride solution (no ^{203}Hg label) was used instead of the $^{203}HgCl_2$. And it was spiked with 100μl of 120,000μCi/nmol of 3-^{14}C-serine instead of adding 0.1ml of 32μg/ml serine (no ^{14}C label).

The negative controls were supplemented with 5 ml 4N HCl to kill cell immediately after it was spiked with ^{203}Hg or ^{14}C.

Samples being incubated for 2 hours to 3 days, the reaction was stopped by adding 5 ml 4N HCl. The reaction samples were ready for methylmercury extraction (See Section 3.5.3). Extraction should begin within 10-25 min after the addition of the HCl to the cell cultures. The extracted ^{14}C-methylmercury or ^{203}Hg-methyl-mercury was then detected with a liquid scintillation counter.

3.5.2 Cell-extract mercury methylation experiments

For each of the sulfate reducing bacteria and *Mb. thermoautotrophicum*, four bottles of cell suspension (4x500ml) were harvested at the middle of upper exponential phase. They were then centrifuged and re-suspended into 10ml of 50mM KPi buffer (pH7.0) ready for French Pressing (See Section 3.1.2). The lysed cells formed crude cell extract (CCE).

We obtained cell-free extract (CFE) by ultra-centrifuging and re-suspending the crude cell extract sample. Cell-free extract was kept on ice ready for experiment.

The method for preparing CCE and CFE of *E. coli* and *Vibrio anguillarum* was described in Section 3.2.

The enzymatic mercury methylating reaction was performed anaerobically in the dark at room temperature for sulfate reducing bacteria, *E. coli* and *Vibrio anguillarum*, and at 65 degree Celsius water bath for *Mb. thermoautotrophicum*, with a final reaction volume of 1ml in a 20ml Balch-tube capped with rubber stopper. It contained 0.5ml of crude cell extract or cell-free extract, 0.1 ml of 50 mM ATP or 100mM sodium pyruvate (as energy source), 0.1 ml of 32 μg/ml Serine (no ^{14}C label for ^{203}Hg experiment), 0.1 - 0.2 ml of 100 mM dithiothreitol, spiked with 100μl of ~1μCi/1μg-4mgHg/100μl $^{203}HgCl_2$, and was supplemented with appropriate amount of 50mM KPi Buffer (pH7.0) to bring the final volume up to 1ml (in some cases, it is 1.15ml). It may

contain 0.1ml of 25mM cyanocobalamin or 10mM methylcobalamin when required. For ^{14}C label experiment, 0.1ml of 0.5 to 8mM Mercuric chloride solution (no ^{203}Hg label) was used instead of $^{203}HgCl_2$. And it was spiked with 100µl of 120,000µCi/nmol of 3-^{14}C-serine instead of adding 0.1ml of 100mM serine (no ^{14}C label). The controls were put on ice to prevent enzymatic reaction from taking place.

The reaction samples were incubated for 0, 5, 10, 15, 30, 40, and 50min before being removed for methylmercury extraction (See Section 3.5.4). The extracted ^{14}C-methylmercury or ^{203}Hg-methylmercury was then detected with liquid scintillation counter.

3.5.3 Bulk protein mercury methylation experiments

For *Desulfovibrio desulfuricans* B203, 10ml of cell-free extract was obtained using the method mentioned above. 2ml of it was saved for CE methylation as comparison to the bulk protein methylation. Protein content was measured. 8ml of it was completely precipitated with solid $(NH_4)_2SO_4$. It was carried out in ice water bath in anaerobic chamber, followed by 10^5g^min ultra-centrifugation. The pellet was re-suspended in 2.5ml 50mM pH7.0 KPi buffer. Then it went through a G25 1.5x7cm column to get rid of $(NH_4)_2SO_4$. It is also carried out in the anaerobic chamber. Finally 2.3ml of salt free bulk protein was collected. The protein content was measured. This bulk protein solution was used for the methylating reaction.

3.5.4 Measurement of methylmercury:

Extraction of methylmercury formed in samples:

The technique used is based on the method of Furatani and Rudd (1980). The assay was started within 10-25 min after stopping the reaction. Each sample and control was transferred into a separatory funnel. In the funnel add 2ml of 0.5N $CuSO_4$, added, stopper and shake, add 10ml of acidic 3M NaBr, which is in 110ml concentrated H_2SO_4, stand for 30min.

Add 20ml toluene (HPLC grade), shake for five minutes and stand for thirty minutes. Drain the lower aqueous layer, add 1 - 2grams of anhydrous Na_2SO_4, gently swirl, and stand for five minutes.

Pipette out, carefully from the top layer, 10ml clear liquid and put into a scintillation tube. When it's very hard to get clear liquid, then it should be transferred into a 15ml test tube, stopper, centrifuge at 2000rpm for ten minutes.

Add 5 ml of 2.5mM $Na_2S_2O_4$ (in 20% EtOH) to the scintillation tube, shake, stand for five minutes.

Pipette out 4 ml thiosulphate layer (bottom) into 7 ml glass test tube with glass stoppers, add 1 ml 3M KI, shake, add 1 ml benzene (HPLC grade), shake five minutes, and stand for another five minutes.

Use 1ml pipetteman to transfer 500µl benzene layer (TOP) into 10 ml BCS or ACS II cocktail in a glass scintillation tube and then count with scintillation counter.

Calculation of methylmercury concentration:

$CH_3{}^{203}Hg^+$ production is expressed as the fraction of label methylated per minute.

Fraction methylated per minute = [(net CH_3Hg^+ cpm/mg)/(Hg^{2+} cpm/mg)]/(min· D· E_1· E_2· S)

Where,

min, minutes of incubation,
D, fraction of decay of the ^{203}Hg,
E1, efficiency of the scintillation counter,
E2, extraction efficiency of $CH_3{}^{203}Hg^+$,
S, the fraction of extracted sample countered

The fraction methylated was calculated as net counts in excess of the killed control.

3.5.5 Other methods

Protein content: All protein determinations were performed with the Bradford's Coomassic Blue method (Bradford 1976) on samples that had been exposed to air at 0~4 degree Celsius within one hour. Bovine serum albumin was used as a standard.

CO dehydrogenase (CODH) Assay: The standard assay is based on methyl violegen reduction under strictly anaerobic conditions at 28

degree Celsius in cuvettes containing a total 1.0ml volume solution of 40mM methyl violegen and 50mM potassium phosphate buffer (pH7.0).

The mixture was made anaerobic and degassed and then saturated with carbon monoxide. Sodium dithionate, 1.0µl of a 0.4% w/v deoxygenated solution was introduced to obtain a slight degree of methyl violegen reduction. The reaction was started by addition of cell-free extract (100µl), and absorbance at 578nm was recorded as time passed. If the CODH activity is present, it will response within 2~5 minutes. A value of $9.59 mM^{-1}cm^{-1}$ was used as the extinction coefficient at 578nm. The CO dehydrogenase activity is given as micromoles of oxidative product formed per minute per milligram of cell protein, that is, µmol MV min^{-1}mg of $protein^{-1}$.

4. RESULTS

4.1 Mercury Methylation in Whole Cells

4.1.1 Whole cell methylation between cultures

Among the selected sulfate reducing bacteria: *D. desulfuricans* B203, *D. desulfuricans* DSM 1924, *D. desulfuricans* DSM ESSex6, and *Db. propionicus* 2032, *Mb. thermoautotrophicum*, *E. coli* and *Vibrio anguillarum*, only *D. desulfuricans* B203 and *Db. propionicus* 2032 were found able to methylate mercury under fermentative growth (See Section 3.5.1 for method). After 3-day incubation starting from mid-log phase cells, *D. desulfuricans* B203 produced 619.1 ng methylmercury per gram of wet cell weight, which is about 15 times of that of *Db. Propionicus* 2032. *Mb. thermoautotrophicum*, *E. coli* and *Vibrio anguillarum* demonstrated no methyl mercury production. See Fig. 3.

4.1.2 Whole cell Hg methylation with time

During incubation of cells with 1μg/ml of mercury, we observed rapid increased methyl mercury synthesized by *D. desulfuricans* B203. Much less increase in the methyl mercury was observed in *Db. Propionicus* 2032. See Fig. 3.

Since Postgate's lactate-sulfate medium C contains a high sulfate concentration, it may not be suitable to test whole cell methylation with those sulfate-reducing bacteria in medium C because of HgS

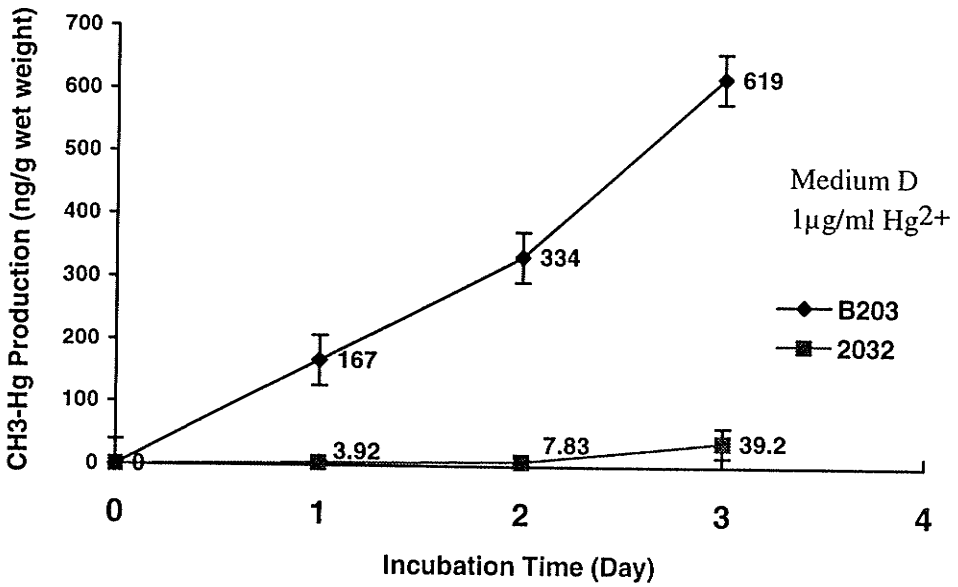

Fig. 3 ***D. desulfuricans*** **B203 and** ***Db. propionicus*** **2032 whole cell methyl mercury production. Data points are averages of triplicate samples; error bars represent standard deviations.**

precipitation. A cell extract methylation was performed for cells grown in this kind of medium.

The effect of O_2 on methylation was also determined with these bacterial cultures. Cells were harvested from anaerobic cultures, and incubated with mercury under aerobic condition. During three-days of incubation, no methyl mercury was detected in any bacterial cultures.
The mercury methylation must be related to some physiological pathway. The pathway of mercury methylation most likely would involve O_2 sensitive enzymes or cofactors.

4.2 CO Dehydrogenase (CODH)

CODH is an enzyme with two important catalytic activities, carbon monoxide dehydrogenase (CODH) (reaction 1) and acetyl-CoA synthase (ACS) (reaction 2). These reactions are key to an autotrophic pathway that has become known as the reductive acetyl-CoA or the Wood/Ljungdahl pathway. ACS also catalyzes two exchange reactions that have been valuable in elucidating the mechanism of acetyl-CoA synthesis: an exchange reaction between CO and the carbonyl group of acetyl-CoA (reaction 3) and an exchange reaction between free CoA and the CoA moiety of acetyl-CoA (reaction 4) (Ragsdale, *et. al.* 1996). The unique reactions of the reductive acetyl-CoA pathway are summarized by equation 5. The CODH must be able to bind the methyl, carbonyl, and SCoA groups of acetyl-CoA, equilibrate the carbonyl group with CO in solution, and then condense these three groups to resynthesize acetyl-CoA, i.e., the synthesis and assembly of acetyl-CoA occur on CODH, which could be named as acetyl-CoA synthase.

$$CO_2 + 2H^+ + 2e^- \leftrightarrow CO + H_2O \quad (1)$$

$$CH_3\text{-}CFeSP + CO + CoA \leftrightarrow \text{acetyl-CoA} + CFeSP \quad (2)$$

$$CH_3\text{-}{}^{14}COSCoA + {}^{12}CO \leftrightarrow CH_3\text{-}{}^{12}COSCoA + {}^{14}CO \quad (3)$$

$$CH_3CO^*SCoA + HSCoA \leftrightarrow CH_3COSCoA + {}^*HSCoA \quad (4)$$

$$CO_2 + CH_3\text{-}H_4\text{folate} + CoASH \rightarrow CH_3\text{-}CO\text{-}SCoA + H_4\text{folate} + H_2O \quad (5)$$

On the basis of a study by Choi and Bartha (1994), the methyl group used in the ACS, may also be transferred to mercury through tetrahydrofolate enzymes. Since the CODH pathway includes several tetrahydrofolate enzymes, therefore there might be a correlation between the activity of CODH pathway and mercury methylation.

D. desulfuricans B203 grew fermentatively in medium D without sulfate displayed the highest CO dehydrogenase activity at 1.59 μM MV per minute per mg of protein (See **Table 1**), which is about 4 times higher than that in medium C with formate or H_2/CO_2 as solo carbon source, respectively. CO dehydrogenase activity was not detected in cells grown in lactate-sulfate medium.

The highest CO dehydrogenase activity observed in *D. desulfuricans* DSM 1924 was 0.508 μM MV per minute per mg of protein, which is about 6-fold higher than that in cells from medium C with formate, and 3-fold higher than that in cells from medium C with H_2/CO_2.

In *D. desulfuricans* DSM Essex6, CO dehydrogenase activity was detected in cells from all media tested, including the lactate-sulfate

Table 1 CODH activity of four SRB strains in different media

Strains	CODH specific activity**, μM MV min^{-1}mg of $protein^{-1}$			
	Medium D Pyruvate	Medium C Lactate	Medium C H_2/CO_2	Medium C formate
B203	1.59	ND*	0.215	0.360
DSM 1924	0.508	ND	0.169	0.106
Essex6	0.678	0.106	0.093	0.064
DSM 2032	ND	ND	ND	ND

*: ND, not detected.

**: Data are average of four replicates, reaction at 30°C.

medium C. Cells from medium D, however, demonstrated about 6~10 fold higher CO dehydrogenase activity than that in cells from medium C.

In these three strains of *D. desulfuricans* spp., the same trend was that cells have higher CO dehydrogenase activity when growing fermentatively than under sulfate respiration pathway.

CO dehydrogenase activity was not detected in 10 minutes in cells of *Db. Propionicus* DSM 2032 from either medium D or medium C with different carbon sources.

4.3 Cell Extract (CE) Hg methylation

4.3.1 CE methylation from different bacterial cultures

For this set of experiments, cell extracts of *D. desulfuricans* B203, *D. desulfurican* DSM 1924, *D. desulfurican* DSM Essex6, *Db. propionicus* DSM 2032, *Mb. thermoautotrophicum*, *E. coli* and *Vibrio anguillarum* were tested. Only the cell extract of *D. desulfuricans* B203 demonstrated methylation of Hg with a production of 2.24ng CH_3Hg^+ per mg protein after 50-minute reaction. All the other cultures failed, no matter cell extract crude or ultra-centrifugation supernatant. In these experiments, no exogenous cyanocobalamin or methylcobalamin was added. Although *Db. propionicus* DSM 2032 showed the ability in whole cell mercury methylation, mercury methylation was not detected in its cell extract.

Since its whole cell mercury methylation was about 15 times lower than that of bacterium B203, it could be that its low methylation rate was not detected during the 50-minute assay with the amount of cell extract used.

When oxygen was present, no methyl mercury was detected with any of the cell extracts.

4.3.2 Hg-methylation experiment with cell extract of methanobacteria

Based on Wood's studies (1968) on the mercury methylation by extracts of a methanogenic bacterium, methanogenic bacteria could methylate mercury in the presence of Hg^{2+}, and thus reduced the production of methane. Since methanogenic bacteria possess high content of methylcobalamin (as methyl group donor) and CODH, it is plausible that they could be mercury methylator. Methanogenic bacteria, as with the SRBs, favor anaerobic sediment habitats. It is important to know their role in the mercury methylation in nature.

In Wood's study, however, acid precipitation was used for deproteinization before extraction of organic mercury from cell extract reaction, the dropping of pH may facilitate the abiotic formation of methylmercury. Thus we designed this experiment to determine the mercurymethylation in a methanogenic bacterium. In the experiment, crude cell extract of *Mb. thermoautotrophicum* was used with exogenous CH_3-B_{12}, reaction at 65 °C, and 35min. CH_3-B_{12} was used at 1mM final

concentration. ATP used was 5mM. Sodium pyruvate used was 10mM. Gas phase was H_2/CO_2. Data are in duplicates. See **Table 2** and **Table 3.**

In the presence of methyl-B_{12}, the CE reaction showed lower methyl-Hg production than in the absence of cell extract, suggesting that the added methylcobalamin appeared to be utilized by this methanogenic bacterium, but not for mercury methylation. See **Table 3**.

4.3.3 Time course of mercury methylation by cell extract of *D. desulfuricans* B203

Methylmercury synthesis by cell extracts of *D. desulfuricans* B203 without exogenous B_{12} or CH_3-B_{12} was recorded over time. The reaction took place at 22 °C in KPi buffer (pH 7.0), with initial 1μg/ml of Hg^{2+}added. Cells were grown on sulfate-free medium D. Data represent the averages of four replicates. It shows a slow increase of methylmercury synthesis at the first twenty minutes, followed by a nearly linear increase in methylmercury synthesis. Since the cells were harvested from medium D containing no sulfate, the low level of methylmercury synthesis was not believed to be due to the sulfide precipitation with Hg^{2+}. See **Fig. 4**. Assuming the overall methylmercury production increased linearly with time, we can calculate that there is about 50.4% of total mercury being methylated per day by cell extract (based on ~3ng/mg protein of CH_3Hg^+, 7mgprotein/ml, 1μg/ml Hg^{2+}, total 1ml reaction volume).

Table 2 Comparison of CH_3Hg^+ productions in *Mb. thermoautotrophicum* cell extract with or without exogenous methylcobalamin, with ATP

	CH_3-Hg^+ production, ng/mgprotein	
	No Cell Extract	With Cell Extract
No CH_3-B_{12} added	ND*	ND
CH_3-B_{12} added	22.1	8.31

*ND: not detected

Table 3 Comparison of CH_3Hg^+ production in *Mb. thermoautotrophicum* cell extract with or without exogenous methylcobalamin, with pyruvate

	CH_3Hg^+ production, ng/mgprotein	
	No Cell Extract	With Cell Extract
No CH_3B_{12} added	ND*	ND
CH_3B_{12} added	18.3	4.90

*ND: not detected.

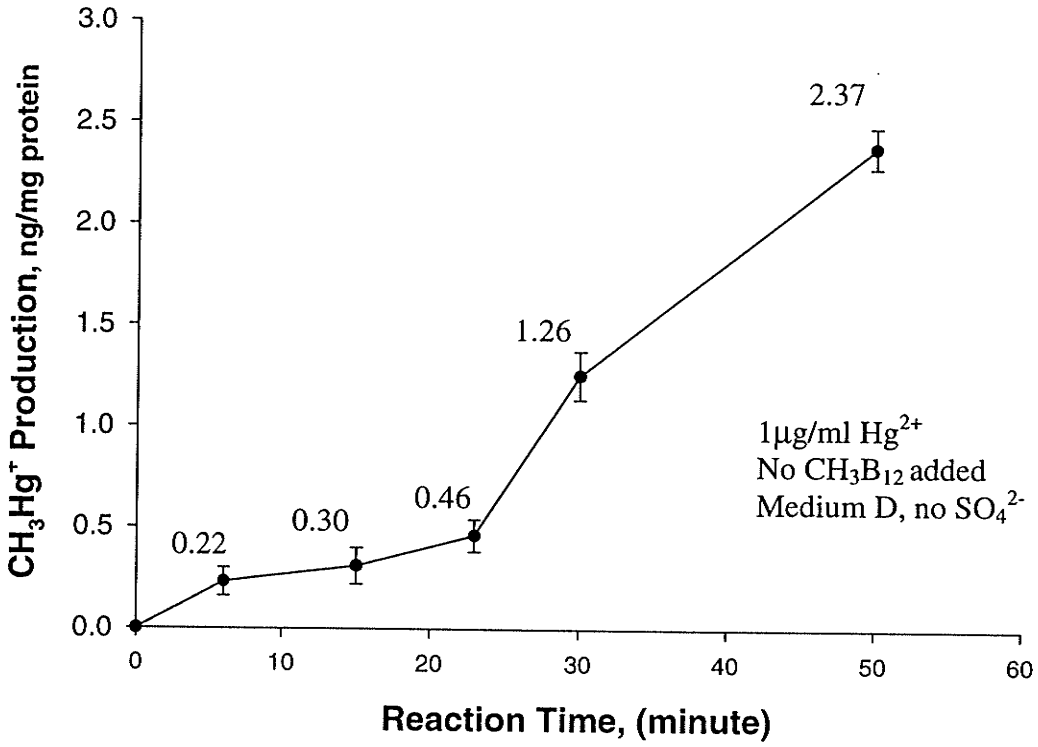

Fig. 4 Time course production of methyl mercury by cell extract of B203 from fermentative media. Data points are averages of triplicate samples; error bars represent standard deviations.

4.3.4 Methylation with cell extract of *D. desulfuricans* B203 grown on different media

With added CH_3-B_{12}, CH_3-Hg synthesis is dramatically increased. Both cell extracts from medium D and medium C had increase of CH_3-Hg^+ production along with the increase of substrate [Hg]. Medium D uses pyruvate as carbon source and is sulfate free. Medium C is lactate-sulfate medium with a gaseous headspace of H_2/CO_2 (20:80) mixture. When Hg^{2+} concentration was lower than 2μg/ml, cells from medium C has lower CH_3-Hg, probably because of lower Hg^{2+} availability due to the HgS precipitation. See **Fig. 5**.

4.3.5 B203 CE methylation with increasing initial [Hg^{2+}]

In order to eliminate the side effect of sulfide present in medium C after cell growing, cell extract of cells from medium C is ultra-centrifuged, and supernatant was used. The reaction took place in KPi buffer (pH7.0) without adding exogenous CH_3-B_{12} or B_{12}. Serine was added. Reaction time was 30 minutes. Again we observed there was only a little production of methylmercury at 1μg/ml of initial Hg^{2+}, followed by a rapid increase of methylmercury synthesis along with the increase of initial [Hg^{2+}]. But it is still much lower than that of cells from fermentative medium, or from reaction with exogenous CH_3-B_{12} or B_{12}. See **Fig. 6**.

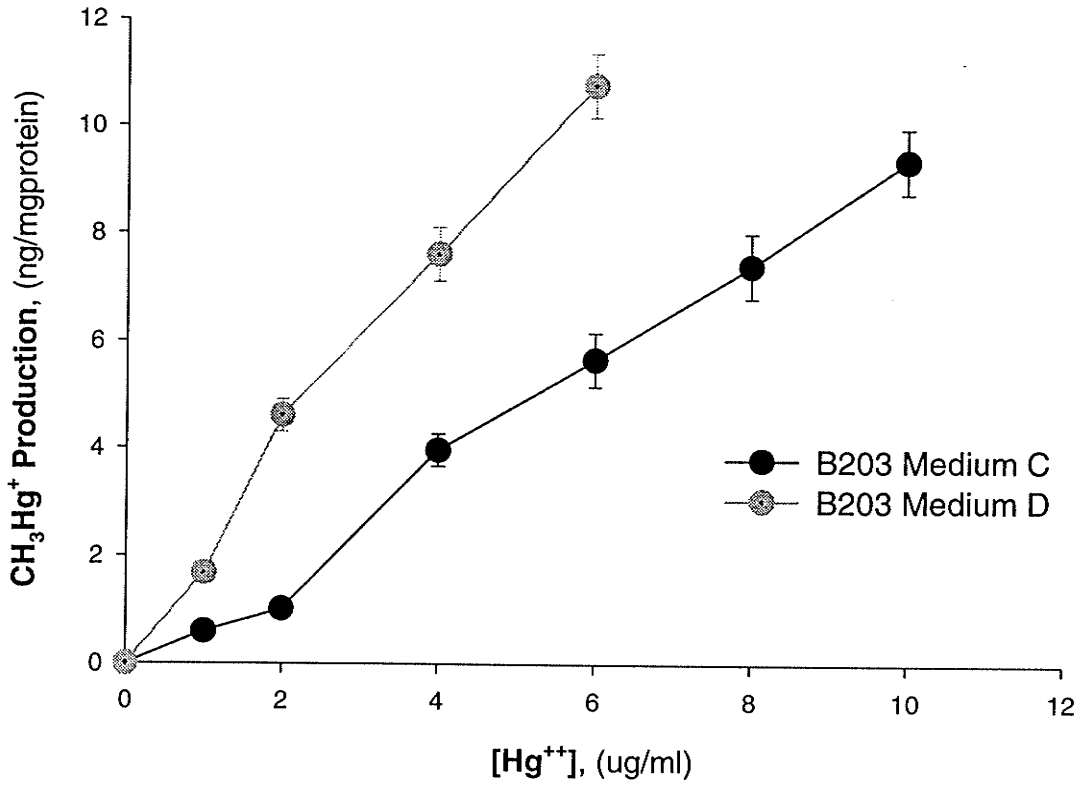

Fig. 5 Methylmercury synthesis by B203 cell extract from different media vs. Hg^{2+} concentration in the presence of methyl B_{12}. Data points are averages of triplicate samples; error bars represent standard deviations.

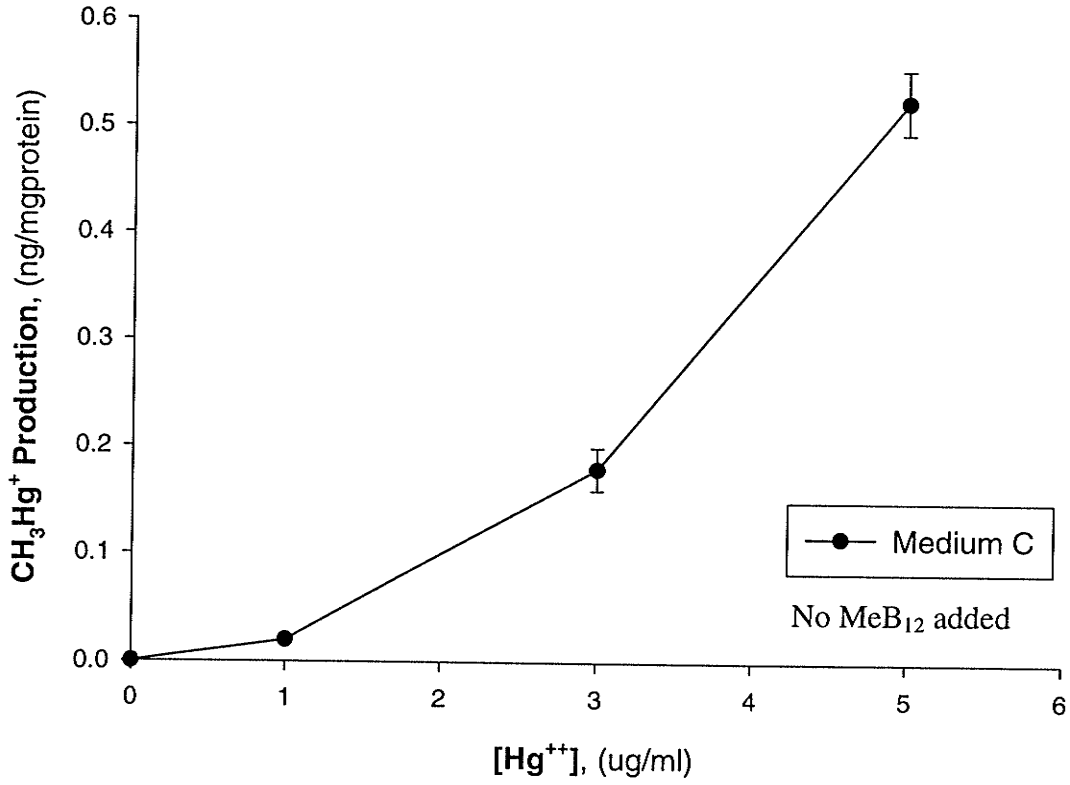

Fig. 6 Methylmercury synthesis by B203C cell extract *vs.* Hg^{2+} concentration. Data points are averages of triplicate samples; error bars represent standard deviations.

4.3.6 Effects of B_{12} or CH_3-B_{12} on mercury methylation by B203 cell extract

1) CH_3Hg^+ Synthesis by Cell Extract with added CH_3B_{12}

Without CH_3B_{12}, CH_3Hg^+ is observed at very low level when $[Hg^{2+}]$ is at 5ug/ml. On the contrary, the CH_3Hg^+ production is dramatically increased, cell extract produced 3 times more than CH_3B_{12}'s spontaneous synthesis of CH_3Hg^+ when $[Hg^{2+}]$ is at only 1ug/ml. See **Table 4**.

2) CH_3Hg^+ Synthesis by Cell Extract with added B_{12}

Hg methylation by cell extract with or without added B_{12} (2.5mM). Cell were from medium D. Triplicate samples, 1μgHg^{2+}. See **Fig. 7**.

External supplement of B_{12} seemed to have a slightly stimulating effect on CE's Hg methylation at the initial stage of reaction.

4.4. Enzyme Reaction Characteristics-V_{max} and K_m in B203

1), 3-^{14}C-serine label experiment showed the synthesis of methyl mercury from serine and Hg^{2+} followed a typical enzymatic reaction curve. See **Fig. 8**.

2), ^{203}Hg label experiment was also conduct to observe the enzyme reaction characteristics.

Table 4 Methylmercury production with Methyl-B_{12} and/or B_{12}*

	CH_3-Hg^+ production, ng/ml	
	Without Methyl B_{12}	Methyl B_{12}, 1mM
No Cell Extract	N.D.	60.9
With Cell Extract	0.67	179
With Cell extract**	3.38	N.T.***

*: reaction with 1μg/ml Hg^{2+}, no serine, in KPi buffer (pH7.0), 30min, triplicate samples.

**: $[Hg^{2+}]$ is 5μg/ml instead of 1μg/ml

***: not tested.

N.D.: Not Detected

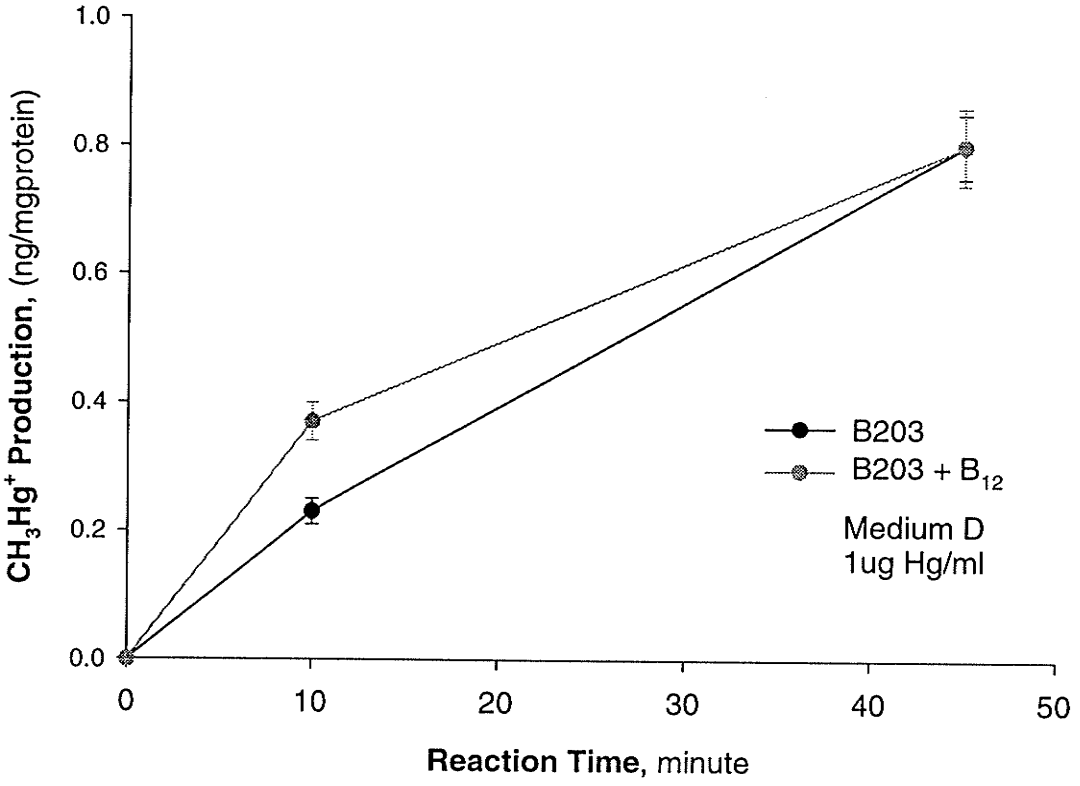

Fig. 7 Methyl Hg synthesis by B203 cell extract with or without external B_{12}. Data points are averages of triplicate samples; error bars represent standard deviations.

In order to narrow down the location of possible enzymes involved in the physiological pathway of CH_3Hg synthesis, the experiment was focused on the reaction between

$$CH_3B_{12} + Hg^{2+} \xrightarrow{\text{Cell Extract}} CH_3Hg^{+} + B_{12}$$

See **Fig. 9**.

4.5 Bulk protein mercury methylation

Reaction of mercury and methylcobalamin with bulk protein took place at room temperature, in KPi buffer (pH7.0), mercury concentration is 2μg/ml or 1.4 μg/ml, methylcobalamin concentration is 1mM, serine concentration is 3.2μg/ml, $H_2/C0_2$ (20% / 80%) headspace, reaction time 35 minutes, duplicate samples. See **Table 5**.

The detected methylation by CH_3B_{12} in control and in cell extract indicated the experiment is effective to test the methylation catalyzed by bulk protein. When the initial mercury concentration is 2μg/ml, the bulk protein and cell extract obtained a methylmercury production at 5.7 ng/mgprotein and 3.85 ng/mgprotein, respectively.

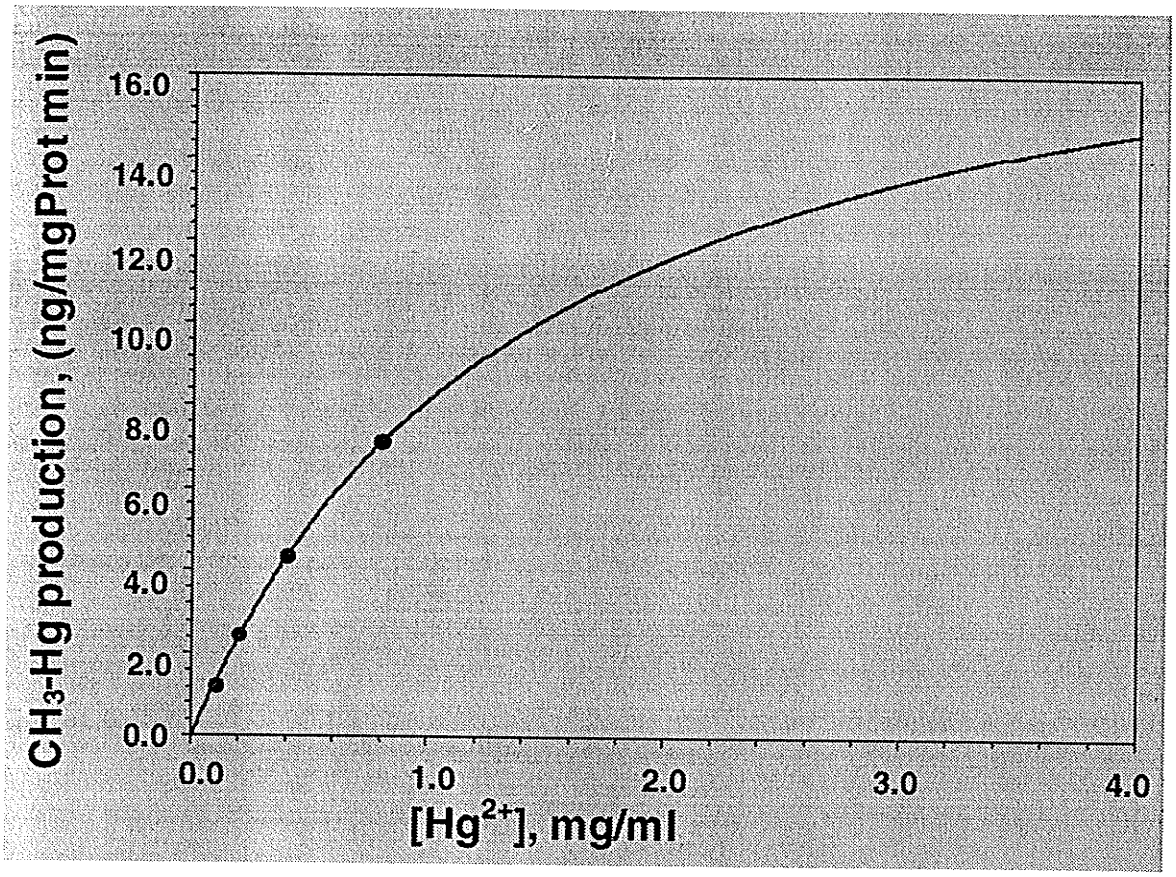

Fig. 8 Methylmercury Synthesis from 3-^{14}C-serine by Cell Extract of *D. desulfuricans* Strain B203 from medium D. Curve generated by Curve-Expert computer program simulation, giving the V_{max} and K_m as 20.0ngCH_3Hg^+ min^{-1} mgprotein^{-1} and 7.21mM of Hg^{2+} (1.45mg/ml), respectively

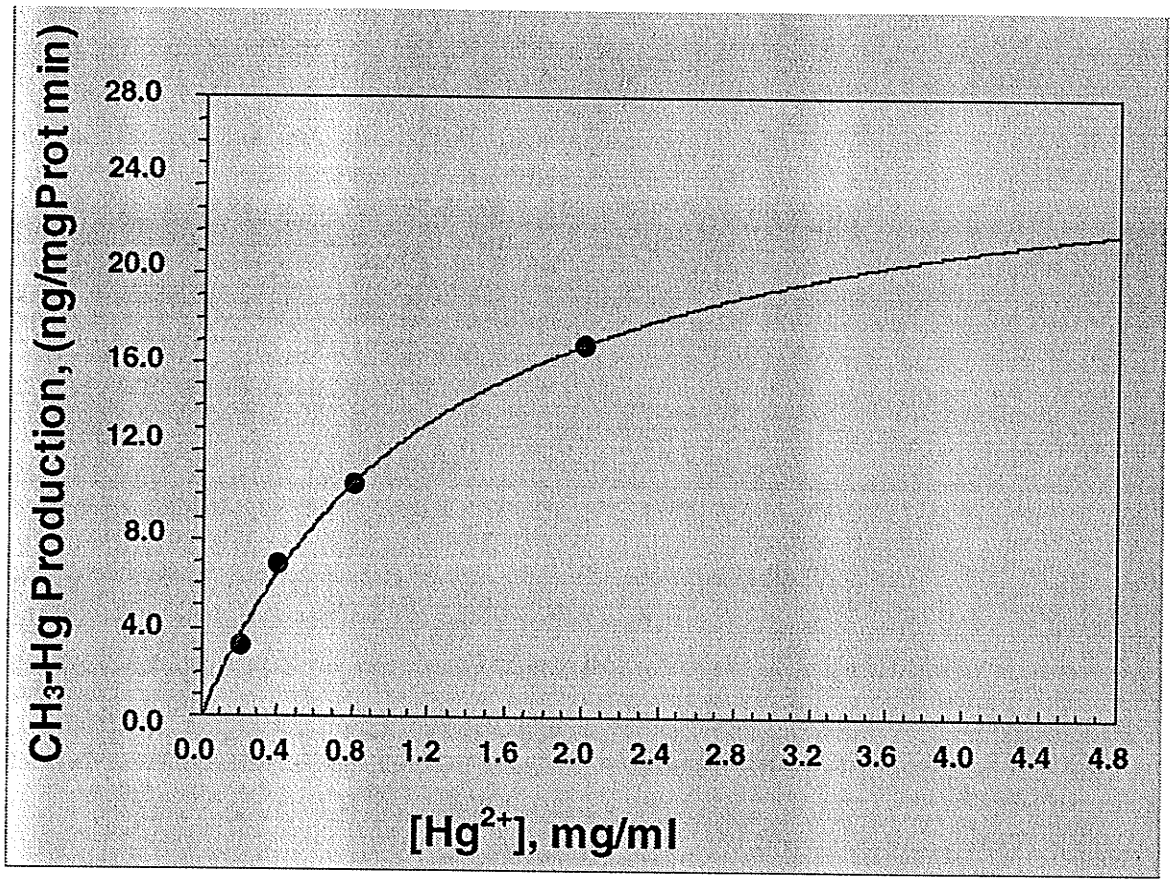

Fig. 9 Methylmercury Synthesis from methyl-B_{12} and $^{203}Hg^{2+}$ by Cell Extract of *D. desulfuricans* B203 from medium D. Curve generated Curve-Expert computer program simulation, giving the apparent V_{max} and K_m as 27.8ngCH_3Hg^+ min^{-1} $mgprotein^{-1}$ and 6.52mM of Hg^{2+} (1.31mg/ml), respectively

Table 5 Mercury methylation catalyzed by bulk protein of *D. desulfuricans* B203 (ngCH_3Hg^+/mgprotein)

	Bulk protein				Cell extract 2μg/ml Hg^{2+}	Cell extract 5μg/ml Hg^{2+}
	2μg/ml Hg^{2+}			1.4μg/mlHg^{2+}		
Serine added	3.2 μg/ml	0	0	3.2μg/ml	3.2μg/ml	3.2μg/ml
CH_3-B_{12} added	0	1mM	1mM	1mM	1mM	0
CH_3Hg^+ produced*	ND	2.00	3.85	5.70	0.95	0.483

*: Unit: ng/mgprotein;

ND: not detected.

5 DISCUSSION AND CONCLUSION

Although mercury methylation has been reported in a variety of sulfate-reducing bacteria species, not all sulfate-reducing bacteria methylate mercury. Even in the same genus or species, not all strains share this methylating ability (Gilmour and Henry, 1991). We used several sulfate reducing bacteria, namely *D. desulfuricans* B203, *D. desulfuricans* DSM 1924, *D. desulfuricans* DSM Essex6, *Db. propionicus* DSM 2032, as well as other microorganisms including *Mb. thermoautotrophicum* strain Marburg, *E. coli* and *Vibrio anguillarum* to test mercury methylation both in whole cells and in cell extracts. Only *D. desulfuricans* B203 and *Db. propionicus* DSM 2032 were found to be able to methylate mercury in intact cells. Even though *D. desulfuricans* DSM 1924 and *D. desulfuricans* DSM Essex6 are in the same species as *D. desulfuricans* B203 and have similar physiological properties, they failed to synthesize methyl mercury, suggesting that mercury methylation is not a common trait of sulfate-reducing bacteria.

Since there is a high degree of phenotypic diversity within the genus *Desulfovibrio* and some *D. desulfuricans* strains have different nutritional properties, there were researchers (Devereux, *et al.*, 1990) suggested that some strains in this genus might be misclassified. The results of partial 16S rRNA and 23S rRNA sequence determinations showed that *D. desulfuricans* Norway 4, which is a mercury methylators, and *D. baarsii*, which is also known mercury methylators, are not closely related to the type strain Essex6, which did not methylate mercury in

this study. *D. desulfuricans* Norway 4 and *D. baarsii* both methylate mercury, but they are not close related to each other.

E. coli HMS174 and *Vibrio anguillarum* used in the whole cell methylation experiments contain a mercury bio-reporter (G. Golding, personal communication). The *E. coli* strain also is known to contain B_{12} (Meichel, 1999). They are known to take up mercury into cells (Selifonova *et al.*, 1993). Neither strain showed any whole cell methylation of mercury, suggesting that the presence both of mercury and B_{12} in the same cell is not sufficient for methylation to occur.

When we designed the experiments for whole cell mercury methylation with different bacterial cultures, we did not pre-grow those cultures with inorganic mercury. We grew the cells on Postgate's sulfate free medium D until mid-log phase of growth, then the harvested cells were incubated in fresh medium D with mercury (in form of $^{203}HgCl_2$) and the methylmercury produced was measured at time intervals. We considered the mercury methylation is either a constitutive activity or the trace amount of mercury in the medium is sufficient to cause expressions of the methylation gene. During the three-day incubations of these bacteria with 1μg/ml Hg^{2+}, we observed a rapid increased methylmercury synthesized in *D. desulfuricans* B203. Methylmercury was also detected in *Db. propionicus* 2032 in much lower levels than *D. desulfuricans* B203.

As a comparison, I presented the whole-cell methylmercury production by *D. desulfuricans* B203 compared to that of incubated anaerobic sediments (Bartha *et. al.*, 1987). In a 3 day period, B203 produced 5-fold

higher amounts of methylmercury than that from incubated anaerobic sediments. See **Fig. 10**.

Methanogenic bacteria possess high content of cobalamin averaging over a million ng/g (as a methyl group donor) (Boyaniwsky, 1994). A study by Wood (1968) on the mercury methylation by extracts of a methanogenic bacterium proposed that methanogenic bacteria could methylate mercury in the presence of Hg^{2+}, and thus reduced the production of methane. It led to the conclusion that they could be mercury methylators. Methanogenic bacteria, similar to SRBs, favor the anaerobic sediment habitats in nature. It is important to know their role in the mercury methylation in nature.

In Wood's study, however, exogenous methyl B_{12} was added to cell extract, acid precipitation was used for deproteinization before extraction of organic mercury from cell extract reaction, however, no control was provided; the decrease in pH may facilitate the abiotic formation of methylmercury from Hg^{2+} and methyl B_{12}. Thus we designed an experiment to determine mercury methylation in a methanogenic bacterium. The experiment with *Mb. thermoautotrophicum* indicated that there was no methylmercury production in whole cells or in cell extracts without adding exogenous methylcobalamin. In the presence of methyl B_{12}, the cell-extract reaction showed lower methyl-Hg production than that in the absence of cell extract. This may indicate that methanogenesis used the methyl-groups on the methyl-B_{12} thus leaving less available methly-B_{12} for Hg after 40 minutes incubation. Based on these results we conclude that *Mb. thermoautotrophicum* does

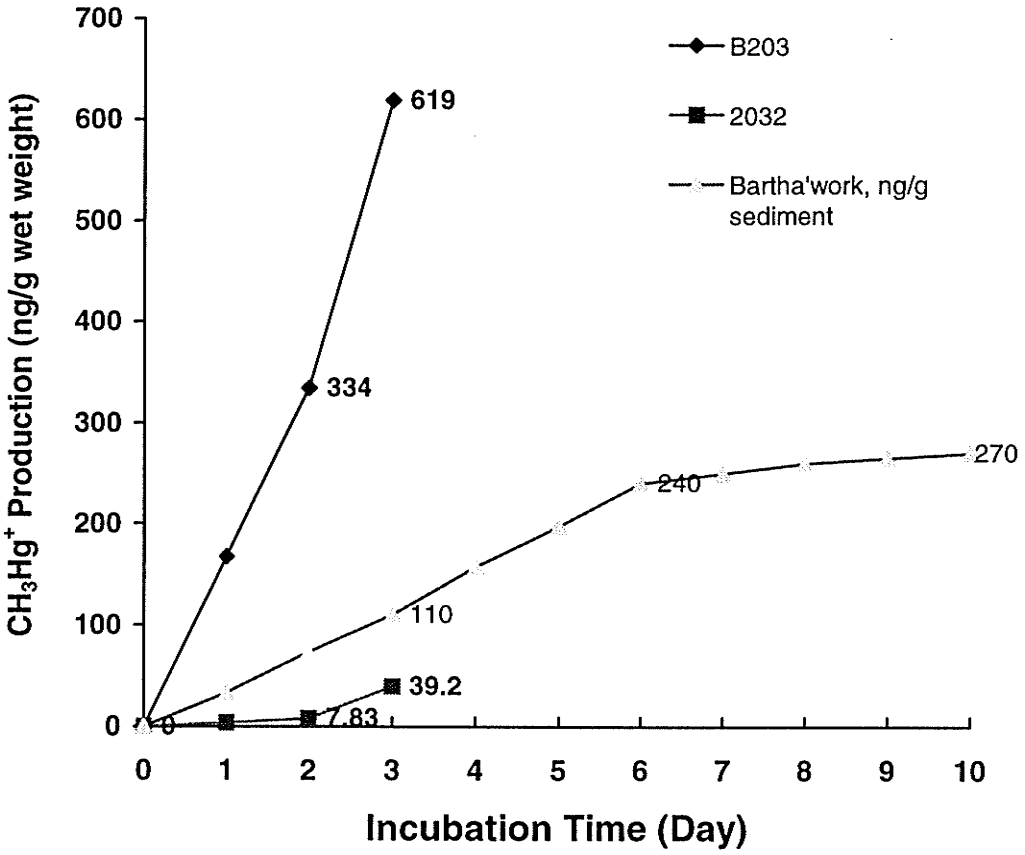

Fig. 10 ***D. desulfuricans*** **B203,** ***Db. propionicus*** **2032 whole cell methyl mercury production with comparison to that of anoxic estuarine sediment (Bartha** ***et. al.*** **1987)**

not methylate mercury either in whole cell or in cell extract. In Wood's study on the synthesis of methylmercury by extract of methanogenic bacterium, the methylmercury detected might be an artefact of the spontaneous reaction between Hg^{2+} and the methlycobalamin under acidic condition. A time zero control as provided here had not been described in Wood (1968).

Among the tested bacteria, only *D. desulfuricans* B203 demonstrated mercury synthesis in cell extract. Looking at the time course of the methylation (See Fig. 4), we found the amount of methylmercury product increased slowly for the first twenty minutes. If it is a spontaneous chemical reaction involving only mercury and methylcobalamin, the reaction should display a linear increase of methylmercury over time. What we found is that after a short period of time, there was a quick increase of methylmercury synthesized in cell extract. According to this figure (Fig. 4), we calculated there was about 50% of total mercury methylated by the cell extract per day. This value is close to what we can obtain from the environment. Kelly *et al.* (1997) reported an up to 62% of total mercury being methylated in the flooded Lake 979 system of the Experimental Lake Area (ELA) in Manitoba and Ontario, Canada. Xun *et al.* (1987) reported methylation rates of 3.8, 20-29 and up to 132% $L^{-1}h^{-1}$ in water samples from eutrophic, natural, and experimentally acidified ELA lakes, respectively.

The mercury methylation curves of cell extracts of *D. desulfuricans* B203 from different media (See **Fig. 5**) showed us significant difference between the medium D and medium C --- the much lower methylmercury production at the low mercury concentration. Cell extract from medium

D, which has little sulfate content, generated much higher methylmercury than the cell extract from sulfate medium C even at low mercury concentration. Sulfate in medium, if it is abundant, will stimulate the production of sulfide. But the H_2S generated by sulfate respiration interferes with the methylation of Hg^{2+} by precipitating it as HgS, making Hg^{2+} less available for methylation. Despite this, the two curves show much similar trend of methylmercury production. This may indicate the same mechanism of mercury methylation existing in the cells grown on the two media.

The results of CODH assays showed the CODH activities in *desulfuricans* were much higher when they were growing fermentatively than growing under sulfate respiration autotrophically. Similarly, strain B203 produced higher methylmercury when growing fermentatively than growing under sulfate respiration with H_2/CO_2. The fact that no mercury methylation was determined in cells from lactate-sulfate medium C without addition of H_2/CO_2 is consistent with the results of CO dehydrogenase activity assay. Strain Essex6 and 1924 did not show any mercury methylation either growing fermentatively or under sulfate respiration. However, CODH activities were still detected in their cell extracts, although much lower than that in strain B203. In addition to this, no CODH activity was detected in cell extract of *Db. propionicus* 2032, but this strain methylated mercury in whole cells. This indicated that the presence of CODH complex is not sufficient for the methylation. Yagi and Tamiya (1962) also reported that CO dehydrogenase of *D. desulfuricans* is not inhibited by 1 mM Hg^{2+}, which concentration is used in many laboratory experiments on mercury methylation, indicating the Hg^{2+} used in this study would not inhibit the CODH activity.

Without added Methyl-B_{12}, CH_3-Hg^+ is observed at low level when [Hg^{2+}] is at 5μg/ml (See Table 3) in cell extract of *D. desulfuricans* B203. The CH_3-Hg^+ production was significantly increased with the presence of external methyl-B_{12}. However, cell extract with methyl-B_{12} still produced 3 times more than Methyl-B_{12}'s spontaneous synthesis of CH_3-Hg^+ when [Hg^{2+}] is at only 1μg/ml. This indicated that there is certain bio-mechanism in the cell that convert inorganic mercury into methyl mercury, and this process is greatly enhanced by external methyl-B_{12}. Organisms with a functioning acetyl CoA pathway should contain catabolic amounts of corrinoid, as been verified for *D. autotrophicum* (Krautler et al. 1988). The cobalamin content in *D. desulfuricans* B203 from fermentative medium is approximate $1.0x10^5$ ng/g protein, which is about twice than that from sulfate medium (Meichel, 1999). A comparison of the methylmercury production, without adding exogenous methyl-B_{12} or B_{12}, in cells from medium D and C (Fig. 4 and Fig. 6) showed us about 60-fold higher amount of methylmercury was synthesized in fermentative cells than latter, consistent with the cobalamin content profile in this bacterium. The cobalamin contents of *D. desulfuricans* DSM1924 and *D. desulfuricans* Essex6 have the similar characteristics; both have higher levels under fermentative condition than under sulfate reducing condition. But the overall level of cobalamin, no matter in which kind of medium, is about 50-fold lower than that in *D. desulfuricans* B203. An interesting finding is that the *Db. propionicus* DSM 2032 methylated mercury in whole cells, but failed in cell extracts. Unlike *D. desulfuricans* B203, *Db. propionicus* DSM 2032 contains 9-fold higher cobalamin levels under sulfate reducing

conditions (93,900ng/g protein) than under fermentative conditions (6181ng/g protein).

Apparent high K_m and V_{max} values were found for mercury methylation by the cell extracts of *Desulfovibrio desulfuricans* B203 growing under fermentative or sulfate respiration conditions. 3-^{14}C-Serine experiment showed the methyl mercury synthesis using serine as methyl-group donor via tetrahydrofolate pathway. The apparent K_m and V_{max} (6.52mM of Hg^{2+} and 27.8ngCH_3Hg^+/ min · mg protein) we found is consistent with a series of enzymes being involved in the process of mercury methylation *in vitro*. Choi and Bartha (1994) found an apparent K_m of 0.872 mM $HgCl_2$ and V_{max} at 0.728nmol / min^{-1}· mg $protein^{-1}$ for synthesis of methyl mercury from 5-$^{14}CH_3$-THF and $HgCl_2$ by *D. desulfuricans* LS cells extract. The involvement of acetyl-CoA pathway suggests there are a series of enzymes involved in the production of methyl mercury from Hg^{2+} and methyl tetrahydrofolate. The K_m determined is based on the use of the entire enzyme system from serine. In an effort to narrow down the possible enzymes directly involved in the physiological pathway of CH_3-Hg^+ synthesis, ^{203}Hg label experiments were done focusing on the reaction of:

$$CH_3\text{-}B_{12} + Hg^{2+} \xrightarrow[\text{cell extract}]{} CH_3\text{-}Hg^+ + B_{12}$$

The result showed saturation kinetics with a K_m of 6.5mM for mercury, supporting the assumption that the mercury conversion is an enzymatically-catalyzed reaction. While the reaction clearly indicates an enzymatic reaction, the physiological purpose of such an enzyme

remains unclear. Despite the differences in K_m and V_{max} values between Bartha's work and those in this study, in either case, the remarkable high K_m value that is million times higher than the mercury concentration in natural environment. Recently we realized that the dithiothreitol used in these enzymatic Hg methylation experiments have high affinity with inorganic mercury (personal communication with George). This kind of binding made the Hg^{2+} less available for methylation. Thus high K_m values were observed in this study. This may also explain the high K_m value observed in Bartha's work.

Further we tested the methyl mercury produced by bulk (cofactor free) protein of *D. desulfuricans* B203 from fermentative grown cells. The bulk protein catalyzed the transfer of methyl group from methylcobalamin to inorganic mercury. Interestingly, the addition of serine produced nearly a 3-fold methylmercury as that of the sample without addition of serine. Providing the whole enzyme system is present in the sample, cofactors are still needed to allow the serine to act as a methyl group donor. For example, tetrahydrofolate is needed for the methyl group transforming from serine to cobal protein and then to mercury. The only explanation seems to be due to the protein concentrating technique. There might be small amount of tetrahydrofolate out with the protein when running the sample through G-25 column.

One thing we need to mention is about the ^{203}Hg we used. We assumed the mercury added is 100% bioavailable so that it's easier for us to calculate the specific activity. This did not take into account the possible precipitation of Hg with sulfide in medium or binding with organic

matter, or binding with dithiothreitol, thus we may have underestimated the percentage of methylmercury synthesized over the bioavailable inorganic mercury.

In this study with all the bacteria tested, we found no matter in whole cell or cell extract mercury methylation, they were very sensitive to O_2. Methylation could only be detected in strict anaerobic conditions.

Conclusions:

- The mercury methylation was observed only in *D. desulfuricans* and *Db. propionicus* (in whole cell only). Observed mercury methylation in whole cell, extract or bulk protein could only occur in strict anaerobic condition.

- *Desulfovibrio desulfuricans* B203 methylate mercury both in whole cell and cell extracts. B203 cell extract utilized serine or external methyl B12 to synthesize methyl mercury with a K_m at milli-molar level. This indicated that mercury methylation may not be the primary function of the enzyme causing the observed methylation, well beyond the range of mercury levels observed in environment where methylation occurs, including the environment from which the B203 strain was isolated.

- *D. desulfuricans* B203 cell extract from cells undergoing sulfate respiration produced lower level of methyl mercury. We assume it may be due to lower Hg^{2+} availability caused by HgS precipitation.

- The mercury methylation in cell extract of B203 strain followed an enzymatic reaction. The Km and Vmax were high, which was similar to that found in *D. desulfuricans* LS in Chio and Bartha's work.

- The capability of whole cell mercury methylation does not necessarily mean the bacterium can methylate mercury in cell extract (*Db. propionicus* DSM2032).

- The presence of CODH activity is not sufficient regarding mercury methylation.

- Methyl B_{12} can spontaneously react with mercury and produce methyl mercury under acidic condition (pH 4). This abiotic effect explains the methylation observed in *Methanobacterium thermoautotrophicum* extract, an organism with high B_{12} and active CODH. This artefact also may explain the observation of methylation by methanogen extracts previously reported (Wood, 1968.).

- Bulk protein experiment supports the assumption that there is certain enzyme catalyzes the one step reaction between the inorganic mercury and methylcobalamin, and provides a first step in the purification of this activity.

- *E. coli* and *Vibrio anguillarum* strains containing a mercury bio-reporter to measure the take up of mercury, however, no mercury methylation was observed in our whole cell and cell extract assays, meaning that neither the presence of B_{12} nor the presence of Hg^{2+} inside cell is sufficient for methylation of mercury to occur.

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