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Sulfate-Reducing Bacteria: Principal Methylators of Mercury in Anoxic Estuarine Sediment†

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Substrate-electron acceptor combinations and specific metabolic inhibitors were applied to anoxic saltmarsh sediment spiked with mercuric ions (Hg^{2+}) in an effort to identify, by a direct approach, the microorganisms responsible for the synthesis of hazardous monomethylmercury. 2-Bromoethane sulfonate (30 mM), a specific inhibitor of methanogens, increased monomethylmercury synthesis, whereas sodium molybdate (20 mM), a specific inhibitor of sulfate reducers, decreased Hg^{2+} methylation by more than 95%. Anaerobic enrichment and isolation procedures yielded a *Desulfovibrio desulfuricans* culture that vigorously methylated Hg^{2+} in culture solution and also in samples of presterilized sediment. The Hg^{2+} methylation activity of sulfate reducers is fully expressed only when sulfate is limiting and fermentable organic substrates are available. To date, sulfate reducers have not been suspected of Hg^{2+} methylation. Identification of these bacteria as the principal methylators of Hg^{2+} in anoxic sediments raises questions about the environmental relevance of previous pure culture-based methylation work.

The ecological and health effects of mercury pollution are greatly exacerbated by environmental transformation of the less hazardous forms of this metal to extremely toxic and biomagnification-prone methylmercury compounds (10). Synthesis of monomethylmercury (CH_3Hg^+), first reported in 1969 (11), occurs primarily but not exclusively in anoxic aquatic sediments. Although some abiotic methylation by humic compounds has been reported (14, 15), this process accounts for less than 1/10 of the methylmercury formed by methylation by sediment microorganisms (M. H. Berman and R. Bartha, Bull. Environ. Contam. Toxicol., in press). Numerous microbial isolates and their cell extracts and the microbial coenzyme methylcobalamin are capable of converting mercuric ions (Hg^{2+}) to CH_3Hg^+ (19), but the identity of the microorganisms that are actually responsible for Hg^{2+} methylation in anoxic sediments has never been determined. To assume that Hg^{2+} methylators in sediments must be identical with those that methylate Hg^{2+} in laboratory experiments performed on pure cultures would be an unwarranted and potentially misleading extrapolation.

The identity of Hg^{2+}-methylating microbial populations in anoxic sediments is of more than theoretical interest. Any selective measure to prevent or control this detrimental process requires a prior knowledge of the causative microorganisms and their metabolic pathways. We report here a series of inhibition-stimulation experiments performed directly on low-salinity anoxic estuarine sediment samples. These tests have unequivocally identified sulfate-reducing bacteria as the principal methylators of Hg^{2+} in such sediments. Previous studies on pure cultures have failed to anticipate this finding.

MATERIALS AND METHODS

Experiments on sediment. Salinity, redox potential, pH, and other sediment characteristics were measured as described previously (4). An anoxic (E_h = −220 mV), low-salinity (0.4%), neutral (pH 6.8) saltmarsh sediment (organic carbon, 17 to 20% by ignition) was collected at a depth of 10 to 30 cm by hand corer in Cheesequake State Park, N.J. The cores (5 by 25 cm) were immediately sealed in plastic liners and, after transport to the laboratory, placed in a PACE 6500 anaerobic chamber (Labline Instruments, Melrose Park, Ill.). The chamber was operated with a gas mixture consisting of 92% N_2, 3% H_2, and 5% CO_2. Methylene blue indicator remained reduced in the chamber. The H_2 and CO_2 in the chamber gas mixture increased methane production less than 5% as compared with sediment samples that had the above gas mixture replaced by 100% (O_2-free) N_2. All ensuing work with sediment samples was performed in this chamber under strictly anaerobic conditions. The sediments were slurried with deoxygenated salt solution (0.4%) prepared from a commercial sea salt mix (Seven Seas Marine Mix, Utility Chemical Co., Paterson, N.J.) which contained all of the major ionic constituents, including sulfate, in amounts proportional to those found in natural seawater. Slurries were prepared to contain approximately 16 g of dry sediment 100 ml^{-1}, a dilution of 3 parts water to 1 part wet sediment. Portions (30 ml) of this slurry were dispensed in 50-ml serum bottles sealed with butyl rubber stoppers. All additions of substrates, inhibitors, and mercury were made by syringe through the septa. Mercury was added as the bichloride ($HgCl_2$) at 75 μg g of dry sediment^{-1}. After incubation at 25°C, the slurries were extracted by the method of Longbottom (13), and monomethylmercury was determined by gas chromatography with electron capture detection (5). The lower limit of detection was 3 ng g of dry sediment^{-1} or 2 ng ml of culture solution^{-1}. Methylmercuric chloride standard was purchased from Pfalz and Bauer, Flushing, N.Y.

Methane in the headspace of sediment samples was determined by using a Hewlett-Packard 5710-A gas chromatograph equipped with a stainless steel column (0.31 by 183 cm) packed with Poropak Q (Waters Associates, Inc., Milford, Mass.). Operational conditions were as follows: N_2 carrier at 30 ml min^{-1}, flame ionization detector at 150°C, oven at 30°C. Peak areas were recorded by a Hewlett-Packard 3390A recording integrator. Methane standard (99.97%) was purchased from Matheson Gas Co., East

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TABLE 1. Effect of substrates and electron acceptors on the synthesis of CH_3Hg^+ in sediment slurries[a]

Supplement	CH_3Hg^+ produced (ng g of sediment^{-1})[b]
None	62 (10)
Glucose	ND
Acetate	40 (6)
Pyruvate	203 (12)
Lactate	40 (7)
Glucose-nitrate	13 (5)
Lactate-sulfate	30 (8)

[a] Slurries were spiked with 75 μg of Hg^{2+} g of sediment^{-1} and were anaerobically incubated at 25°C for 2 days. Substrate concentrations were 100 mM; electron acceptor concentrations were 20 mM.

[b] Average of duplicate determinations. The number in parentheses represents one-half of the range between duplicate determinations. ND, None detected.

Rutherford, N.J. 2-Bromoethane sulfonic acid (BESA) was purchased from Sigma Chemical Co., St. Louis, Mo.

Sulfide in sediment was determined iodometrically (8). Total acid-labile sulfide was 4.3 ± 0.6 mg g of dry sediment^{-1}, and free sulfide was 0.93 ± 0.18 mg g of dry sediment^{-1}. At this level, free sulfide did not completely inhibit mercury methylation. Additional Na_2MoO_4-treated and control sediment slurry samples were amended with 30 mM SO_4^{2-} and were incubated for 72 h (see Fig. 1 and 2). The sulfide formed was then determined. To ascertain that Na_2MoO_4 did not interfere with the availability of the mercury spike for methylation, during a 6-day incubation period, 1-ml amounts of the Na_2MoO_4-Hg^{2+}-spiked and control sediments were withdrawn and reacted for 1 h with 0.4 mM methylcobalamin (Sigma). The methylcobalamin was in 10-fold stoichiometric excess in relation to Hg^{2+}. The reaction was stopped by addition of acid, converting any dimethylmercury formed to the monomethyl form. CH_3Hg^+ was measured by gas chromatography.

Enrichment and isolation procedures. For enrichment of the identified principal Hg^{2+} methylators in the anoxic sediment, 50-ml quantities of various enrichment media (see Table 2) were spiked with 1 μg of $HgCl_2$ ml^{-1}, inoculated with 0.5 g (dry weight) of anoxic saltmarsh sediment, and incubated anaerobically at 25°C for 5 days. At this time, the enrichments were assayed for methylmercury. Positive enrichments were serially transferred three times in a 1:100 ratio to fresh medium of identical composition, thus eliminating residual sediment. The retention of the ability to synthesize methylmercury was monitored during these serial transfers. Pure cultures were obtained subsequently from the positive enrichments by using anaerobic techniques developed by Hungate (9) in conjunction with a diagnostic medium that contained ferrous sulfate (17). The identification of the isolate was based on microscopic observations, substrate specificity, and presence of desulfoviridin (17). Protein yield in culture solution (3) was determined by first driving off H_2S with 1 N HCl and subsequently neutralizing the solution with 1 N NaOH.

RESULTS AND DISCUSSION

Experiments on sediment. The types of microbial metabolism that are feasible in anoxic sediments are determined by the types of organic substrates and the ultimate electron acceptors that are available. Anoxic metabolism may be classified as fermentative, nitrate reducing, sulfate reducing, or methanogenic (12). The products of fermentative metabolism serve as substrates for organisms using the other three modes of metabolism: fermentation products are oxidized while the generated electrons reduce nitrate, sulfate, or carbonate at progressively decreasing redox potentials. When we supplemented $HgCl_2$-spiked sediment slurries with various substrates with and without electron acceptors (Table 1), pyruvate more than tripled CH_3Hg^+ synthesis as compared with an unsupplemented control. All other substrates actually suppressed CH_3Hg^+ synthesis. In a separate experiment, a H_2-CO_2 gas atmosphere (20 and 80%, respectively) was employed to enrich methanogenic activity. This treatment increased the amount of methane evolved from sediment samples by a factor of four over controls (data not shown), but no methylmercury was detected in these samples. Concerning the unique stimulation of Hg^{2+} methylation by pyruvate in anoxic sediment, it is important to note that this substrate can be utilized by sulfate reducers fermentatively, i.e., in the absence of sulfate (17). But faced with a universal consensus that sulfate reducers hinder rather than promote methylation by precipitating Hg^{2+} as HgS (10, 19) we initially were very reluctant to assign these organisms a positive role.

As a different approach to determine the contribution of various metabolic groups to CH_3Hg^+ synthesis, Hg^{2+}-spiked sediment slurries were treated with 30 mM BESA, a specific inhibitor of methanogenesis (7). The contribution of sulfate reducers was assessed by treating sediment slurries with 20 mM Na_2MoO_4, an inhibitor of sulfate reduction (B. F. Taylor and R. S. Oremland, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, Q41, p. 268). Na_2MoO_4 completely suppressed

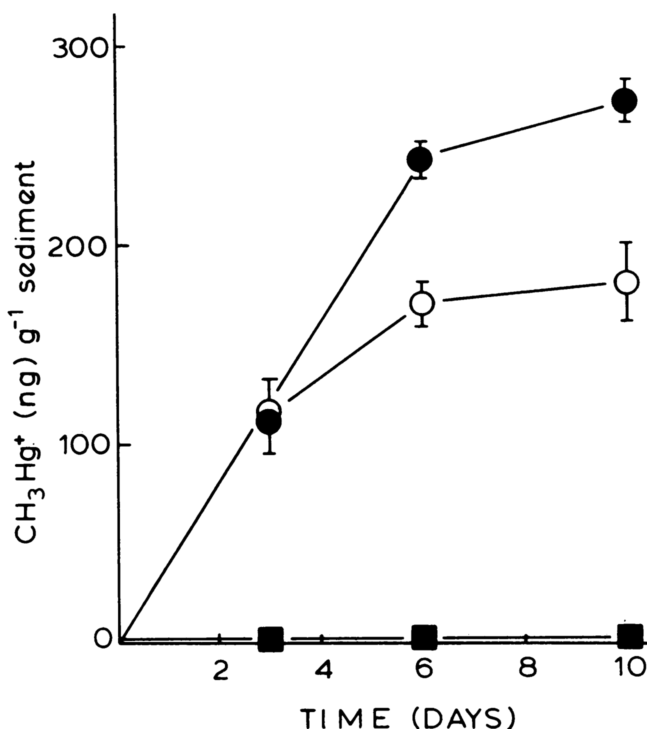

FIG. 1. Synthesis of CH_3Hg^+ in anoxic estuarine sediment slurry spiked with Hg^{2+} at 75 μg g^{-1} (dry weight) (○) and the effects on the process by 30 mM BESA (●) and 20 mM Na_2MoO_4 (■), specific inhibitors of methanogenesis and sulfate-reduction, respectively. Error bars represent the range of measurements on duplicate samples and are omitted when smaller than the symbols.

Hg^{2+} methylation whereas BESA actually increased it (Fig. 1). The effectiveness and specificity of the inhibitors were demonstrated by the patterns of methane evolution measured in the same experiment (Fig. 2). Hg^{2+} alone inhibited methanogenesis slightly, but the process was inhibited 96% by Hg^{2+} and BESA together. In contrast, Na_2MoO_4 and Hg^{2+} increased methanogenesis. Since sulfate reducers and methanogens in sediments may compete for the same carbon sources and electron donors (16), inhibition of sulfate reducers may channel the flow of electrons towards the methanogens. The same phenomenon in reverse is likely to account for the stimulation of Hg^{2+} methylation by BESA (Fig. 1). Measurements of SO_4^{2-} reduced (8) in the same experiment indicate that addition of 20 mM Na_2MoO_4 completely inhibited formation of sulfide. The baseline sulfide level of the sediment slurries was 4.3 mg g (dry weight) of sediment^{-1}. Control samples reduced 50% of the added SO_4^{2-}; no additional sulfide was formed in the Na_2MoO_4-inhibited samples.

We also considered and ruled out the possibility that the presence of Na_2MoO_4 made Hg^{2+} unavailable for methylation. There was no interference with the transfer of methyl groups from exogenous methylcobalamin added to Hg^{2+} in sediment slurries that were preincubated anaerobically for up to 6 days with Hg^{2+} and 20 mM Na_2MoO_4. The amounts of CH_3Hg^+ formed on day 3 were 680 and 750 ng ml^{-1} and on day 6 were 230 and 150 ng ml^{-1} in Na_2MoO_4-treated and control sediments, respectively. The above averages of duplicate measurements did not differ significantly from each other, indicating that Hg^{2+} remained equally available for methylation in both Na_2MoO_4-treated and control sediments.

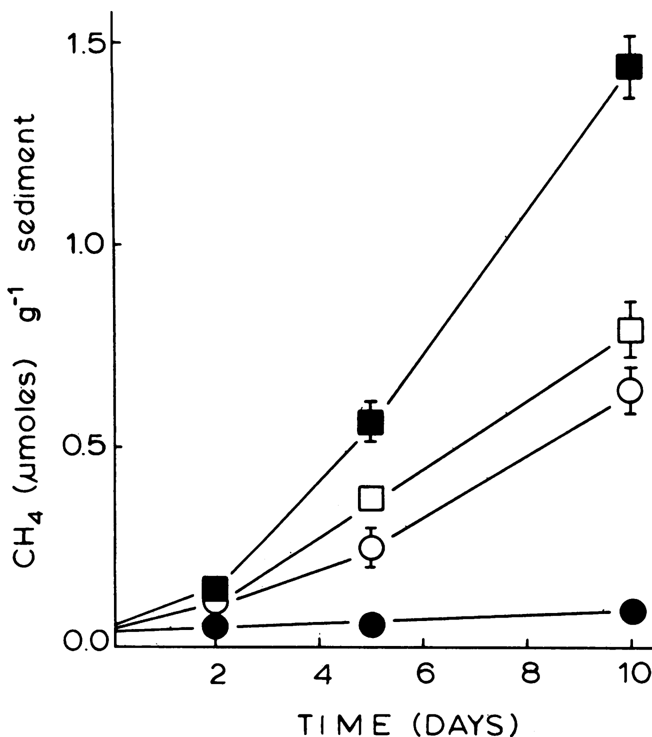

FIG. 2. Evolution of the methane from anoxic estuarine sediment slurry (□) and the effects on this process by Hg^{2+} (○), BESA plus Hg^{2+} (●), and Na_2MoO_4 plus Hg^{2+} (■) at concentrations identical to those given in the legend to Fig. 1. Error bars represent the range of measurements on duplicate samples and are omitted when smaller than the symbol.

TABLE 2. Hg^{2+} methylation in enrichment cultures incubated for 5 days

Supplement[a]	CH_3Hg^+ produced (ng g of sediment^{-1})[b]
None	60 (8)
Glucose	ND
Acetate	ND
Pyruvate	183 (22)
Lactate-sulfate	110 (13)
Peptone-nitrate	ND

[a] Only the critical components of the media are listed in the table. For complete compositions of the acetate, pyruvate, and lactate-sulfate media, see reference 17; for the compositions of the glucose and peptone-nitrate media, see reference 1.

[b] Average of duplicate determinations. The number in parentheses represents one-half of the range between the two determinations. ND, None detected.

The complete suppression of Hg^{2+} methylation by Na_2MoO_4 in anoxic sediment and the actual stimulation of the methylation process by BESA as compared with the described control experiments unequivocally point to sulfate reducers as the predominant if not exclusive methylators of Hg^{2+} in this environment. The same results strongly negate any involvement in the Hg^{2+} methylation process by methanogens. These results are surprising because pure culture experiments have never anticipated the role of sulfate reducers, and methanogens were suspected as environmental Hg^{2+} methylators ever since Wood demonstrated in vitro Hg^{2+} methylation by cell extracts of a methanogen (21).

Enrichment and isolation procedures. Concurrently with the described stimulation-inhibition experiments conducted directly in sediment, enrichments were performed to obtain a pure culture of the principal methylating population. From the enrichments described in Table 2, only pyruvate and lactate-sulfate gave positive results in terms of Hg^{2+} methylation. When serial transfers eliminated sediment, Hg^{2+} methylation activity persisted and even increased in these enrichments. On anaerobic roll tubes, five colony types were obtained, including sulfate reducers that were conspicuous by blackening of the agar medium due to FeS production. The five isolated cultures were individually tested for their ability to synthesize CH_3Hg^+. Only the sulfate-reducing isolate displayed this ability. The Hg^{2+}-methylating isolate was an obligately anaerobic, motile, polarly flagellated, gram-negative, curved rod with dimensions of approximately 3 by 0.5 μm. It formed desulfoviridin but not endospores. In the presence of sulfate, it grew on lactate and pyruvate but not on acetate. It also grew with reduced cell yields on pyruvate and choline in the absence of sulfate. These characteristics clearly identify the organism as a strain of *Desulfovibrio desulfuricans* (17).

The isolate was unable to methylate pure HgS. When grown for 48 h on lactate-sulfate medium (17) spiked with 15 μg of $HgCl_2$ ml^{-1}, the *D. desulfuricans* isolate attained a cell density of 1.4×10^8 ml^{-1} and synthesized 11.7 ng of CH_3Hg^+ ml^{-1}. Most of the CH_3Hg^+ was synthesized in the first few hours of growth when H_2S concentrations were low. On pyruvate, in the absence of sulfate, the cell yield was 3.4×10^7 ml^{-1}, and the CH_3Hg^+ yield was similar (13.5 ng ml^{-1}) to that obtained on lactate. However, based on cell protein, the CH_3Hg^+ yield was 2.5 times greater when the organic

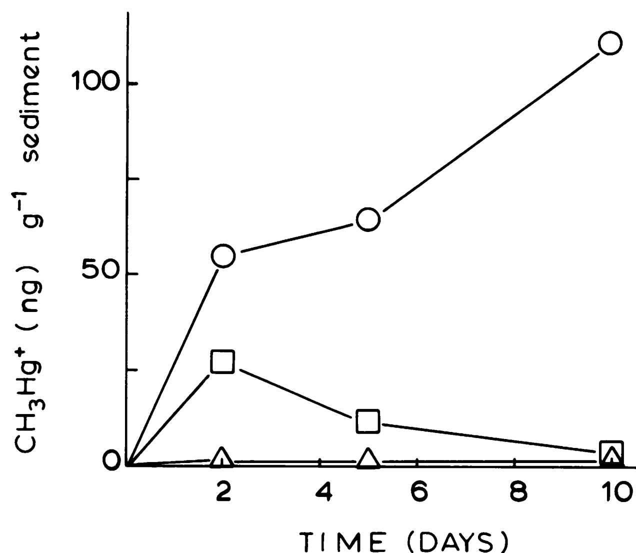

FIG. 3. Synthesis of CH_3Hg^+ from Hg^{2+} in a steam-sterilized anoxic estuarine sediment slurry amended with 10% (vol/vol) of either pyruvate or lactate-sulfate culture medium (△), or 10% (vol/vol) 48-h cultures of a *D. desulfuricans* isolate pregrown in either pyruvate (○) or lactate-sulfate (□) medium.

substrate was pyruvate than when it was lactate. These results explain why the lactate-sulfate supplement failed to stimulate CH_3Hg^+ synthesis in the experiments with the sediment slurry (Table 1). Although the results of inhibitor experiments (Fig. 1 and 2) leave no doubt that the sulfate reducers are the principal Hg^{2+} methylators in anoxic saltmarsh sediment, their methylating potential is fully expressed only when sulfate is limiting and carbon sources are available that can also be utilized in the absence of sulfate. If sulfate is abundant, organic substrates are utilized more efficiently than in its absence, but the H_2S generated by sulfate respiration interferes with the methylation of Hg^{2+} by precipitating it as HgS. In addition, product inhibition of sulfate reducers occurs at high H_2S levels (17).

To demonstrate that the *D. desulfuricans* isolate is capable of Hg^{2+} methylation in a sediment environment, 48-h cultures were reintroduced as a 10% (vol/vol) inoculum to a steam-sterilized and $HgCl_2$-spiked sediment slurry of the type used in our earlier experiments. The inoculum was pregrown either in lactate-sulfate medium or in pyruvate without sulfate. No CH_3Hg^+ was synthesized in the uninoculated slurry (Fig. 3). The lactate-sulfate-grown inoculum synthesized a limited amount of CH_3Hg^+ that subsequently declined, possibly due to disproportionation to dimethylmercury by H_2S (6). Large amounts of CH_3Hg^+ were synthesized by pyruvate-grown cells, and this product was stable for the duration of the experiment.

Our findings concerning the role of sulfate reducers in Hg^{2+} methylation do not contradict the observed inhibition of this activity in sediments by high sulfide concentrations (10). From previous work concerning mercury methylation in sediments (4) and experiments performed in pure culture reported here, it is clear that mercury methylation can continue in the presence of high sulfate concentrations but not to the same extent as when sulfate is at lower concentrations. Typically, conditions of high sulfide develop when sediments are anoxic, rich in organic matter, and high in sulfate due to the presence of sea salts. However, according to recent reports, sulfate-reducing potential is considerable even in low-sulfate sediments (20). Our experiments on *D. desulfuricans* grown on pyruvate and in the absence of sulfate show that under similar conditions, even moderate numbers of sulfate reducers may methylate considerable amounts of Hg^{2+}. Thus, the inverse relation of salinity to Hg^{2+} methylation in anoxic saltmarsh sediments (2) is satisfactorily explained.

A substantial amount of biochemical information is available on Hg^{2+} methylation by randomly isolated microorganisms (19). This work, as well as theoretical considerations (18), suggests that methylcobalamin is the most likely methyl donor for Hg^{2+}. The biochemistry of Hg^{2+} methylation by sulfate reducers has not been studied, since it was not previously suspected that these organisms perform this process. It should be noted that *D. desulfuricans* metabolizes pyruvate by a cobalamin-dependent pathway and possesses a limited capability to evolve methane (17). Nevertheless, the currently postulated Hg^{2+} methylation mechanism will require a thorough reexamination in view of our finding that a unique and previously unsuspected group of microorganisms acts as the principal methylator of polluting mercury in anoxic estuarine sediments.

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