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Enzymatic Catalysis of Mercury Methylation by *Desulfovibrio desulfuricans* LS†

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The recently defined role of methylcobalamin in Hg^{2+} methylation by *Desulfovibrio desulfuricans* LS enabled us to reexamine the question of whether the principal source of methylmercury is spontaneous transmethylation or an enzymatically catalyzed process. In cell extracts of *D. desulfuricans* LS, over 95% of the ^{57}Co label was associated with macromolecules rather than with free cobalamin. Both gel filtration and electrophoresis of cell extracts identified a single corrinoid protein of 40 kDa in size. This finding, in combination with the previously reported light-reversible propyl iodide inhibition of the Hg^{2+} methylation process, led us to propose that this 40-kDa corrinoid protein is the in vivo methyl donor in *D. desulfuricans* LS. Under reducing conditions, cell extracts containing the corrinoid protein produced $^{14}CH_3Hg^+$ from Hg^{2+} and 5-$^{14}CH_3$-tetrahydrofolate with a maximum specific activity of 0.73 nmol min^{-1} mg of cell $protein^{-1}$. The sequence of methyl transfer was from methyltetrahydrofolate to the corrinoid protein to Hg^{2+}. The rate of methylation versus the Hg^{2+} concentration followed Michaelis-Menten kinetics, with an apparent K_m of 0.87 mM $HgCl_2$. The activity was oxygen sensitive, and Hg^{2+} methylation was optimal at 35°C and pH 6.5. The observation of saturation kinetics and the 600-fold-higher rate of Hg^{2+} methylation (at pH 7.0) by cell extracts, compared with transmethylation by free methylcobalamin, proved that in vivo Hg^{2+} methylation is an enzymatically catalyzed process.

The biochemical fate of methylmercury has been studied extensively in relationship to detoxification and resistance mechanisms of microorganisms (10, 17, 21). Both aerobes and anaerobes were shown to be capable of demethylating methylmercury via organomercurial lyase-mercuric reductase, encoded in the *mer* operon. The products are CH_4 or CO_2 and volatile mercury (1, 11). However, little is known about the biochemistry of the formation of methylmercury. Methylmercury formation following an exogenous addition of methylcobalamin (23) neither proved nor excluded the existence of an enzymatic methylation process. A previous study suggested that over 90% of environmental mercury methylation is associated with biological activity (3). However, this does not necessarily imply an enzymatic catalysis, because metabolically produced methylcobalamin can spontaneously methylate mercuric ions. The identification of sulfate-reducing bacteria as principal environmental mercury methylators (7) has focused attention on these microorganisms in terms of the biochemical reaction. A light-reversible inhibition of mercury methylation by propyl iodide in *Desulfovibrio desulfuricans* LS implied methyl transfer by a corrinoid but left the question of enzyme catalysis unanswered (4).

The identification of methylcobalamin as the methylating agent of *D. desulfuricans* LS (6) raised the question of whether mercury methylation in vivo occurs as an enzymatically catalyzed process or as a spontaneous chemical reaction. To date, this question has remained unanswered because mercury methylation could not be demonstrated in cell extracts. When cells of *D. desulfuricans* LS were broken, no mercury methylation could be demonstrated either in the cell extract or in the pelleted debris or in a combination of both (2). Subsequent additions of 5-CH_3-tetrahydrofolate (THF) or *S*-adenosylmethionine to the cell extract failed to restore mercury methylation.

The aim of this study was to test for mercury methylation in cell extracts of *D. desulfuricans* LS and to document the enzymatic catalysis of this reaction under in vivo conditions.

MATERIALS AND METHODS

Cell extract. The *D. desulfuricans* LS strain used in this study was isolated from a low-salinity salt marsh sediment and has been described previously (6, 7). Cells were grown in a 20-liter carboy containing 19 liters of Postgate's lactate-sulfate medium C (12) for 2 days at 37°C in the dark. A cell yield of approximately 0.8 g (wet weight) per liter included small amounts of sulfide precipitates. After the cells were harvested anaerobically by centrifugation (15,000 × *g* for 20 min), the pellet was suspended in 15 ml of anaerobic 50 mM KP_i buffer (pH 7.0) containing 10 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and a few crystals of DNase I. Cells were broken by passing them twice through a French press at 16,000 lb/in^2 under a continuous stream of argon (Matheson Gas Products, East Rutherford, N.J.). After removal of cell debris by centrifugation at 30,000 × *g* for 1 h, the supernatant, designated as the cell extract, was transferred to a serum vial with a butyl rubber closure and stored at −80°C under nitrogen (oxygen-free grade; Matheson Gas). The cell extract was utilized within 2 weeks of its preparation.

Enzymatic mercury methylation. The enzymatic production of methylmercury from 5-CH_3-THF and $HgCl_2$ was determined by using 5-$^{14}CH_3$-THF (barium salt, specific activity of 57.5 mCi/mmol; Amersham Inc., Arlington Heights, Ill.) and analyzing for the incorporation of radiolabel into methylmercury. The reaction was performed anaerobically in the dark at 37°C, with a final reaction volume of 0.5 ml in a 1.5-ml glass vial capped with a Teflon-lined silicon stopper. The gas phase

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was nitrogen, which was passed through a hot (300°C) copper column to remove trace amounts of oxygen. The reaction mixture contained 10 mM dithiothreitol, 10 mM sodium pyruvate, 0.5 to 8 mM $HgCl_2$, 50 mM KP_i buffer (pH 7.0), and cell extract (0.32 mg of protein per ml of reaction mixture), prepared as described above. After the reaction mixture was equilibrated at 37°C for 2 min, the reaction was initiated by the addition of 16.8 μM 5-$^{14}CH_3$-THF (120,000 dpm/nmol). At various times, 100 μl of the sample was removed with a microsyringe, and the methylmercury produced was extracted with 0.5 ml of toluene per sample as described by Longbottom et al. (9). In this procedure, a single toluene extraction was found to separate $^{14}CH_3Hg^+$ from $^{14}CH_3$-THF, as confirmed by a control experiment without cell extract. The toluene phase containing all $^{14}CH_3Hg^+$ was separated by centrifugation and its radioactivity was measured by liquid scintillation (BetaTrac 6895; T. M. Analytic, Elk Grove Village, Ill.). Counting efficiency was determined by the external standard ratio method. There was no direct methylation of $HgCl_2$ by CH_3-THF alone or in combination with cyanocobalamin, unless cell extract was also added. For comparison of enzymatic and nonenzymatic Hg^{2+} methylation rates, the data were normalized on the basis of cobalamin. The determination of cobalamin in cell extracts was performed by using a vitamin B_{12}-folate dual radioimmunoassay kit, according to the instructions of the manufacturer (Amersham).

Nonenzymatic mercury methylation. The rate of nonenzymatic Hg^{2+} methylation (transmethylation) by methylcobalamin (Sigma Chemical Co., St. Louis, Mo.) was measured spectrophotometrically under the conditions described previously (6). In this experiment, the increase of the A_{350}, which is the characteristic absorption band of hydroxycobalamin generated from methylcobalamin as it donates its methyl group to mercuric ions, was recorded with time. The extinction coefficient (ε_{350}) of hydroxycobalamin is 26.5 mM^{-1} cm^{-1} (18). The measurement was carried out in a reaction mixture identical to that used in the enzymatic process, except that methylcobalamin (30 μM) was substituted for the cell extract. The reaction was initiated by the addition of a concentrated $HgCl_2$ solution to yield a final concentration of 60 μM.

Corrinoid protein. To facilitate the monitoring of corrinoid protein, cells were grown in 19 liters of lactate-sulfate medium in the presence of 0.2 mCi of $^{57}CoCl_2$ (specific activity of 5.21 mCi/μg of Co; Amersham), and the cell extract was prepared by the procedure described above. Sephadex G-150 (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) gel filtration chromatography was performed at 4°C in the dark with 2.0 ml of cell extract. The column dimensions were 1.2 by 120 cm, with V_0 and total column volume equal to 60 and 170 ml, respectively. The elution buffer contained 10 mM dithiothreitol, 10 mM PMSF, and 0.02% sodium azide in 50 mM KP_i buffer (pH 7.0). At a flow rate of 8.0 ml/h, 1.0-ml fractions were collected, and the radioactivity was measured with a gamma counter (model 1191; T. M. Analytic) with more than 90% counting efficiency. The amount of protein in the subsamples of each fraction was determined by the Bradford dye-binding method (5). The molecular weight of the ^{57}Co-corrinoid protein was estimated from the calibration curve (r^2 = 0.99), derived from known standards. The size standards (Sigma) were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa), and aprotinin (6.5 kDa).

Nondenaturing gradient gel electrophoresis (5 to 15% acrylamide) was also carried out in the dark to minimize photodegradation of the corrinoid. After electrophoresis, the gel was immediately sliced into 0.5-cm pieces and the radioactivity of each slice was measured with the gamma counter. The calibration curve (r^2 = 0.95) was derived from known standards (Sigma): hexamer (545 kDa) and trimer (272 kDa) of urease, dimer (132 kDa) and monomer (66 kDa) of bovine serum albumin, ovalbumin (45 kDa), and α-lactalbumin (14.2 kDa).

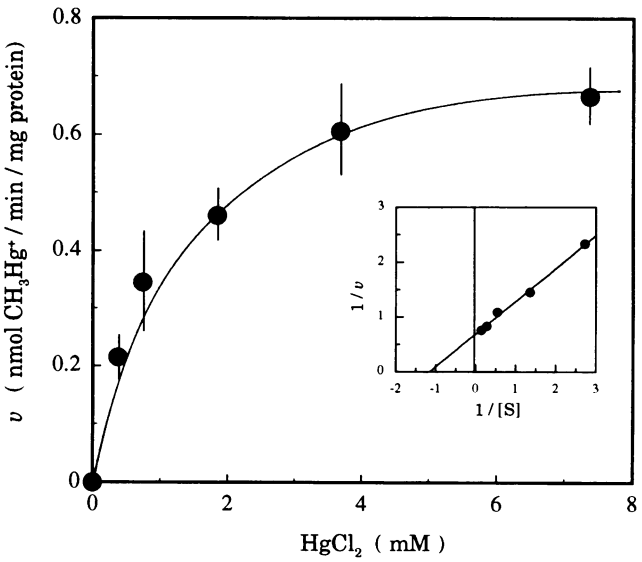

FIG. 1. Dependence of rate of methylation Hg^{2+} concentration. V_{max} and K_m were determined from the reciprocal plot (inset) as 0.728 nmol min^{-1} mg $protein^{-1}$ and 0.872 mM $HgCl_2$, respectively, by using 0.16 mg of protein of cell extract in a 0.5-ml reaction mixture volume. Data points are averages of triplicate samples; error bars represent standard deviations.

RESULTS AND DISCUSSION

Characteristics of methyltransferase reaction. Mercury methylation by cell extract of *D. desulfuricans* LS followed Michaelis-Menten kinetics, indicating methyltransferase activity (Fig. 1). The maximum specific activity was 0.728 nmol of CH_3Hg^+ min^{-1} mg of protein of cell $extract^{-1}$. In an earlier work, when intact cells were concentrated, resuspended in buffer, and spiked with 150 ppm of $HgCl_2$, methylmercury was produced at a rate of 0.011 nmol min^{-1} mg of cell $protein^{-1}$ (2). In this study, at the same concentration of $HgCl_2$ (0.7 mM) but with cell extract, a rate of 0.25 nmol of CH_3Hg^+ min^{-1} mg of $protein^{-1}$ was obtained. This suggests that we have improved the assay conditions for methyltransferase activity by the addition of pyruvate, as discussed later. The apparent K_m of $HgCl_2$ of 0.872 mM was determined from the reciprocal plot. Mercury methylation increased as the $HgCl_2$ concentration was raised to 4 mM and then reached a plateau.

Conditions were optimal for enzymatic mercury methylation at 35°C (Fig. 2) and pH 6.5 (Fig. 3). The activity was oxygen sensitive, decreasing by 44% after 5 min of exposure to air, even in the presence of 10 mM dithiothreitol (data not shown).

Without the addition of pyruvate to the reaction mixture, mercury methylation was not detected. The effect of pyruvate seems to be the generation of a reductant, such as reduced ferredoxin via pyruvate-ferredoxin oxidoreductase (22). The lack of sufficient reductant in the system may have caused the previous failure to demonstrate mercury methylation activity in cell extracts of *D. desulfuricans* LS. The acetyl coenzyme A synthesis pathway in *Clostridium thermoaceticum* includes a

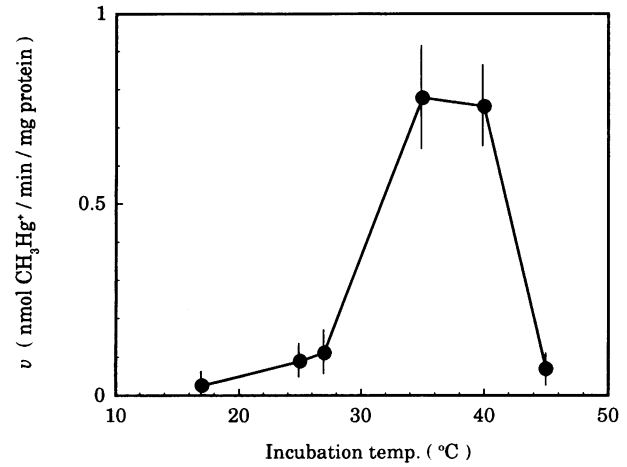

FIG. 2. Temperature dependence of methyltransferase activity. Data points are averages of triplicate samples; error bars represent standard deviations.

corrinoid-dependent methyltransferase reaction (13). The cobalt center of the corrinoid has to be in the +1 state to be competent to receive a methyl group from CH_3-THF (14). The one-electron reduction step from Co^{2+} to Co^{1+} is mediated by ferredoxin II, and its addition to the reaction mixture increased acetyl coenzyme A synthesis fourfold (16).

Corrinoid protein in *D. desulfuricans* LS. Sephadex G-150 gel filtration chromatography of the cell extract located a single major ^{57}Co-corrinoid protein with an approximate size of 40 kDa (Fig. 4). This peak represented 95% of the incorporated ^{57}Co label. The ^{57}Co-corrinoid protein fractions were pooled, concentrated, and applied to a nondenaturing gradient (5 to 15% acrylamide) gel. Electrophoresis of the pooled fractions indicated an apparent size of 41 kDa for the ^{57}Co-corrinoid protein (Fig. 5). Two-thirds of the corrinoid radioactivity

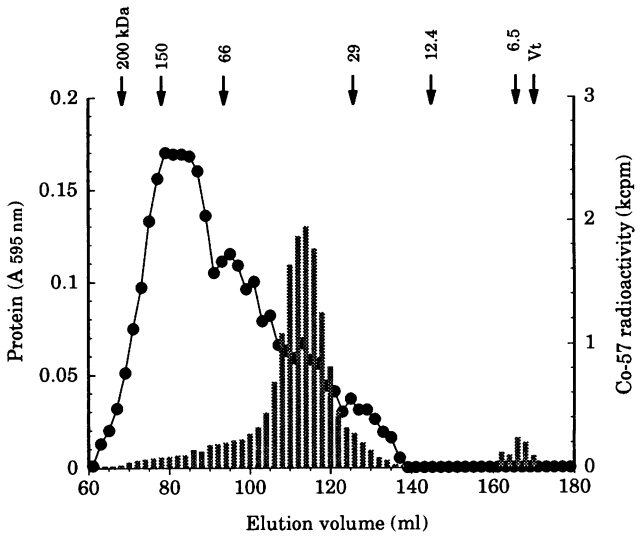

FIG. 4. Sephadex G-150 gel filtration chromatography of cell extract of *D. desulfuricans* LS. The procedure located a single major ^{57}Co-corrinoid protein with an approximate size of 40 kDa. Detached corrinoid or free Co^{2+} was eluted at the total column volume (Vt). Arrows show the positions of the size markers (in kilodaltons). Circles, protein; bars, ^{57}Co radioactivity.

became detached from the protein during electrophoresis. Neither the addition of thioglycolate to the sample buffer, the addition of dithiothreitol to the tank buffer, nor protection from light improved the stability of the corrinoid protein complex during electrophoresis. Nevertheless, the finding of a single ^{57}Co-corrinoid protein suggests that there is a specific interaction between a protein and the corrinoid.

Hatchikian (8) isolated a cobalt porphyrin-containing pro-

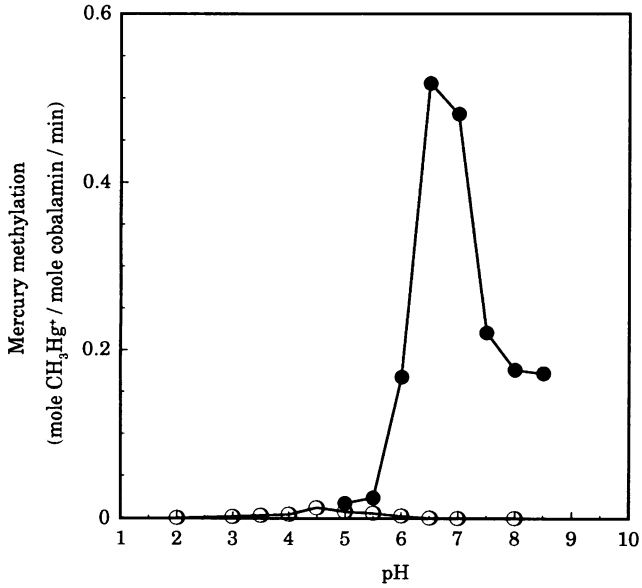

FIG. 3. pH dependence of enzymatic (●) and nonenzymatic (○) catalysis of mercury methylation on a cobalamin basis.

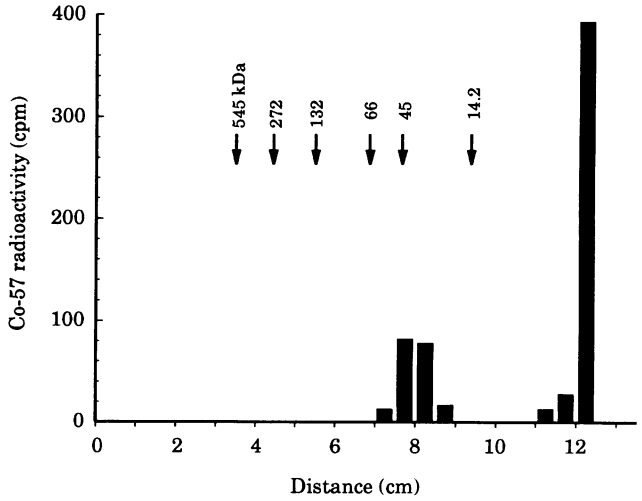

FIG. 5. Nondenaturing gradient (5 to 15%) gel electrophoresis of cell extract of *D. desulfuricans* LS. The gel was cut into 0.5-cm slices, and the radioactivity was measured. A single ^{57}Co-corrinoid protein with an approximate size of 41 kDa was located. Detached corroinoid, found at the dye front (12.3 cm), comprised two-thirds of the total radioactivity recovered. Arrows indicate the positions of the size markers (in kilodaltons).

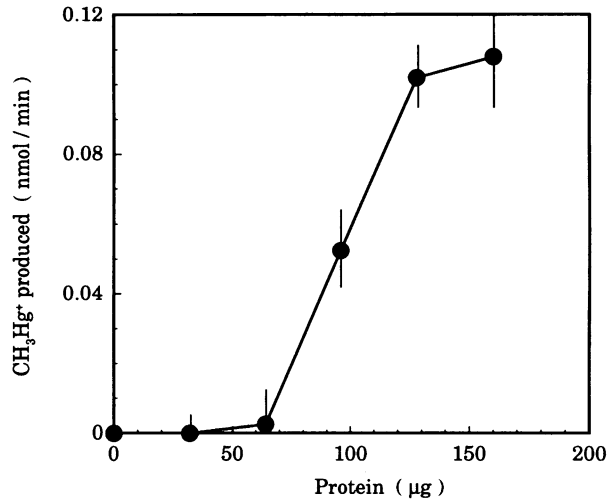

FIG. 6. Protein dependence of mercury methylation at a saturating level of substrate, $HgCl_2$ (5 mM). With increasing amounts of cell extract, methylmercury formation increased exponentially, suggesting that two or more factors may be involved in the reaction. Data points are averages of triplicate samples; error bars represent standard deviations.

tein from *D. desulfuricans* Norway. The protein was reducible by hydrogenase in the presence of cytochrome c_3, as evidenced by its spectral shifts. He speculated that the function of the cobalt protein was an electron carrier, corresponding to the redox states of the cobalt center, Co(III) and Co(II). However, it is unlikely that the only function of the corrinoid protein isolated from *D. desulfuricans* LS is to serve as an electron carrier. Instead, the 40-kDa corrinoid protein is likely also to be a methyl carrier between CH_3-THF and CH_3Hg^+, as indicated by a light-reversible propyl iodide inhibition of mercury methylation (4). In this earlier experiment, when cells of *D. desulfuricans* LS were preincubated with propyl iodide, their ability to form methylmercury from Hg^{2+} was blocked. Propyl iodide caused 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+}. In addition to the described experiment, the 40-kDa protein was the only major cobalt-containing protein detected in *D. desulfuricans* LS, as evidenced by both gel filtration chromatography and electrophoresis (Fig. 4 and 5).

Methyl transfer reaction. The actual synthesis of methylmercury from CH_3-THF and $HgCl_2$ can be divided into two steps:

$$^{14}CH_3\text{-THF} + \text{Co-protein} \xrightarrow{\text{methyltransferase I}} {}^{14}CH_3\text{-Co-protein}$$

$$^{14}CH_3\text{-Co-protein} + Hg^{2+} \xrightarrow{\text{methyltransferase II}} \text{Co-protein} + CH_3Hg^+$$

where Co-protein is corrinoid protein. If the reaction requires only Co-protein and methyltransferase I, with the second step proceeding by a spontaneous chemical reaction, the reaction rate might depend on the second power of cell extract concentration but will be linear with time. At a saturating level of substrate (5 mM $HgCl_2$), methylmercury formation increased exponentially with increasing amounts of cell extract (Fig. 6). The reaction was linear with time up to 1 h (data not shown), which suggests that only methyltransferase I is required. However, if the reaction required only methyltransferase I, with methyl transfer from $^{14}CH_3$-corrinoid protein to Hg^{2+} being a spontaneous, uncatalyzed chemical reaction, the rate of overall enzymatic methylation should be lower than or equal to the nonenzymatic rate observed with exogenously added methylcobalamin. Figure 3 shows not only that the optimal pH values of the nonenzymatic and the enzymatic reaction mixtures differed (pH 4.5 and 6.5, respectively) but also that their rates of methylation were very different. When mercury methylation rates were compared at the respective pH optima, the enzymatic rate was 40-fold higher than the rate of the nonenzymatic reaction. At physiological pH (7.0), the rate difference was even more striking (600-fold).

The fact that a K_m for Hg^{2+} can be observed (Fig. 1) suggests that the second reaction is the rate-limiting one. If the first reaction were limiting, the mercuric ion concentrations would have little effect on the rate of the overall reaction. As indicated by Fig. 1, this was not the case. This fact, together with the comparison of Hg^{2+} methylation rates in enzymatic and nonenzymatic reactions, strongly suggests that the overall Hg^{2+} methylation reaction also requires a methyltransferase II (mercury methyltransferase).

The saturable methyltransferase II activity might be a property of the corrinoid protein itself, yielding methyl transfer from $^{14}CH_3$-corrinoid protein to Hg^{2+} faster than from free $^{14}CH_3$-corrinoid without requirement for any other protein. In other words, the 40-kDa corrinoid protein itself may be methyltransferase II and not merely a methyl carrier that interacts with the transferase. Whether this is the case is not known at this time. In any case, however, the second step of Hg^{2+} methylation is enzyme catalyzed.

Inhibition studies tied mercury methylation in anoxic aquatic sediments almost exclusively to the activity of sulfate reducers (7). Should mercury methylation be nonenzymatic, there is no reason why homoacetogens or methanogens should not be major methylators of mercury, because they often contain cobalamin in concentrations several orders of magnitude higher than reported for sulfate reducers (19, 20). A lack of correlation between sediment cobalamin content and mercury methylation also argues against the dominance of nonenzymatic mercury methylation in the environment (15). The results presented here provide decisive proof that mercury methylation is catalyzed enzymatically in *D. desulfuricans* LS. This enzymatic capability seems to be a property of limited distribution even among *Desulfovibrio* strains (6). Future work will concentrate on identifying the enzyme responsible for transferring methyl groups from CH_3-corrinoid protein to Hg^{2+} and determining whether it is a unique protein or a component of the acetyl coenzyme A synthesis pathway with an unusual side reaction.

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