ENVIRONMENTAL STUDIES

Methylmercury uptake and degradation by methanotrophs

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Methylmercury (CH_3Hg^+) is a potent neurotoxin produced by certain anaerobic microorganisms in natural environments. Although numerous studies have characterized the basis of mercury (Hg) methylation, no studies have examined CH_3Hg^+ degradation by methanotrophs, despite their ubiquitous presence in the environment. We report that some methanotrophs, such as *Methylosinus trichosporium* OB3b, can take up and degrade CH_3Hg^+ rapidly, whereas others, such as *Methylococcus capsulatus* Bath, can take up but not degrade CH_3Hg^+. Demethylation by *M. trichosporium* OB3b increases with increasing CH_3Hg^+ concentrations but was abolished in mutants deficient in the synthesis of methanobactin, a metal-binding compound used by some methanotrophs, such as *M. trichosporium* OB3b. Furthermore, addition of methanol (>5 mM) as a competing one-carbon (C1) substrate inhibits demethylation, suggesting that CH_3Hg^+ degradation by methanotrophs may involve an initial bonding of CH_3Hg^+ by methanobactin followed by cleavage of the C–Hg bond in CH_3Hg^+ by the methanol dehydrogenase. This new demethylation pathway by methanotrophs indicates possible broader involvement of C1-metabolizing aerobes in the degradation and cycling of toxic CH_3Hg^+ in the environment.

INTRODUCTION

Methylmercury (CH_3Hg^+) toxin is predominantly produced by certain anaerobic microorganisms (for example, *Desulfovibrio desulfuricans* ND132 and *Geobacter sulfurreducens* PCA) having two key genes (*hgcA* and *hgcB*) necessary for converting inorganic mercury (Hg) to CH_3Hg^+ (*1*–*3*). It can accumulate and biomagnify at high levels in fish as well as in rice grains, and human consumption can cause neurological damage (*4*–*7*). Our understanding of the mechanisms responsible for Hg methylation has greatly improved recently through the identification of the genetic basis (*1*–*3*) and factors affecting Hg methylation (*8*–*12*). However, net CH_3Hg^+ levels in the environment depend on two competing biological processes—CH_3Hg^+ production and demethylation (*13*–*17*), although demethylation can also take place photochemically in surface waters (*18*).

To date, much attention has focused on Hg methylation, but fewer studies have examined microbial demethylation, except the process mediated by the *mer* operon (*17*, *19*), in which demethylation is carried out by an organomercurial lyase (MerB). MerB cleaves off the methyl group to form methane (CH_4) and Hg(II), whereas a mercuric reductase (MerA) reduces the released Hg(II) to volatile elemental Hg(0) (*7*, *13*, *20*). However, only certain aerobic prokaryotes have this CH_3Hg^+ degradation pathway. *mer*-mediated pathway is operative only at extremely high Hg concentrations (that is, micromolar) (*7*, *17*, *21*), conditions that are largely irrelevant to most natural waters and sediments, where Hg or CH_3Hg^+ concentrations are usually at picomolar to low nanomolar ranges (*7*, *17*). In addition, in vitro experiments with the isolated MerB enzyme showed that demethylation by MerB occurs generally at above neutral pH conditions, with an optimal pH of ~10 (*21*).

However, degradation of CH_3Hg^+ has been observed in anoxic sediments and in a limited number of pure cultures at relatively low Hg concentrations (for example, nanomolar) (*14*–*17*, *22*). Anaerobic sulfate reducers and methanogens are thought to be primarily responsible for this oxidative demethylation because Hg(II), CH_4, and carbon dioxide (CO_2) have been identified as major products (*14*–*16*). Despite the finding of CH_4 and CO_2 evolution, methanotrophs are not considered as important players in the oxidative demethylation of CH_3Hg^+. The possible involvement of methanotrophs has never been directly tested, and the bacteria involved and pathways leading to oxidative demethylation remain unexplored.

Methanotrophs can thrive under a wide range of redox conditions, particularly at the oxic-anoxic interface where CH_4 and CH_3Hg^+ are commonly observed (*23*–*25*). They are widespread and found in diverse locations, such as freshwater and marine sediments, bogs, forest and agricultural soils, and volcanic soils (*26*, *27*). Many methanotrophs also produce an extracellular metal-binding peptide called methanobactin that has been shown to bind CH_3Hg^+ (*28*, *29*). In addition, most methanotrophs can use methanol as a one-carbon (C1) growth substrate, and some can also grow on methylamine (*24*, *30*), and we therefore hypothesized that at least some methanotrophs can take up and possibly degrade CH_3Hg^+.

RESULTS AND DISCUSSION

CH_3Hg^+ uptake and degradation were first examined in representative strains of α-(*Methylosinus trichosporium* OB3b) and γ-proteobacterial (*Methylococcus capsulatus* Bath) methanotrophs. Both methanotrophs were found to sorb substantial amounts of CH_3Hg^+, with *M. trichosporium* OB3b showing slightly higher sorption affinity and kinetics than *M. capsulatus* Bath (Fig. 1A). Within 1 hour, ~95% of the CH_3Hg^+ was sorbed or associated with *M. trichosporium* OB3b, whereas only ~65% was associated with *M. capsulatus* Bath cells, although the sorption increased to ~85% on *M. capsulatus* Bath cells in 4 hours. Analyses of Hg species distributions indicated that a large percentage of the CH_3Hg^+ was internalized or taken up by both *M. trichosporium* OB3b and *M. capsulatus* Bath cells in 4 hours, leaving only a small percentage of the CH_3Hg^+ in solution (Fig. 1B). These results are in contrast to the rapid export and little sorption of CH_3Hg^+ observed with known mercury methylators, such as *D. desulfuricans* ND132 (*10*, *31*, *32*), suggesting that both *M. trichosporium* OB3b and *M. capsulatus* Bath have a high affinity to sorb or take up CH_3Hg^+.

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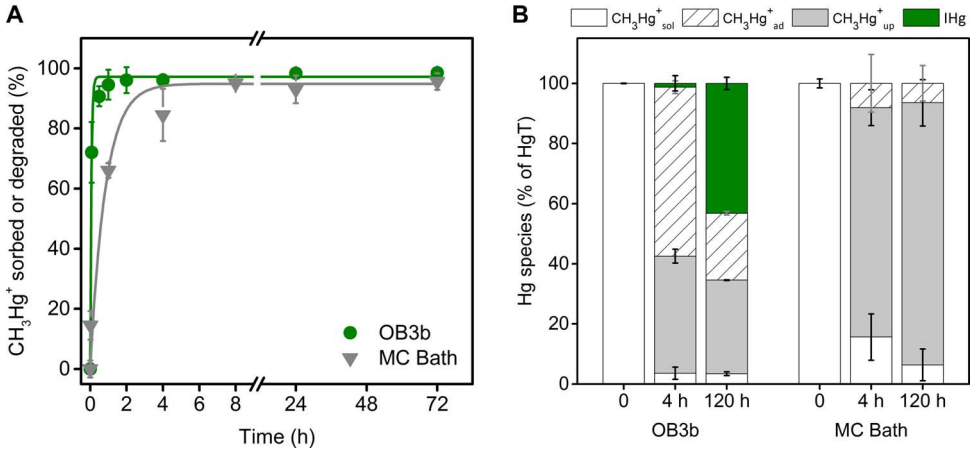

Fig. 1. Methylmercury (CH_3Hg^+) sorption, degradation, and species distribution. (**A**) CH_3Hg^+ sorption kinetics and (**B**) Hg species distributions (at 4 and 120 hours) by methanotrophs *M. trichosporium* OB3b and *M. capsulatus* (MC) Bath in 5 mM MOPS buffer. The total added CH_3Hg^+ concentration (HgT) was 5 nM at $t = 0$, and the cell concentration was 10^8 cells ml^{-1}. $CH_3Hg^+_{sol}$, soluble CH_3Hg^+; $CH_3Hg^+_{ad}$, cell surface–adsorbed CH_3Hg^+; $CH_3Hg^+_{up}$, cell uptake of or internalized CH_3Hg^+. IHg results from degradation of CH_3Hg^+. Error bars represent 1 SD from triplicate samples.

We found that, with increasing incubation time (120 hours), a substantial amount of CH_3Hg^+ (~43%) was degraded and converted to inorganic Hg (IHg) by *M. trichosporium* OB3b, but not by *M. capsulatus* Bath cell (Fig. 1B). This observation was confirmed by additional detailed time- and concentration-dependent studies of CH_3Hg^+ degradation by both *M. trichosporium* OB3b (Fig. 2, A and B) and *M. capsulatus* Bath (Fig. 2, C and D). We found no demethylation at all with *M. capsulatus* Bath cultures, regardless of the reaction time (up to 120 hours) and CH_3Hg^+ concentration (from 5 to 125 nM). However, CH_3Hg^+ was degraded progressively by *M. trichosporium* OB3b with time and CH_3Hg^+ concentrations up to 75 nM (Fig. 2, A and B). The pseudo–first-order rate constants at the initial CH_3Hg^+ concentrations of 5, 25, and 75 nM were 0.017 (±0.001), 0.032 (±0.008), and 0.037 (±0.003) $hour^{-1}$, respectively, and approximately 55, 62, and 73% of the added CH_3Hg^+ were degraded after 5 days. Again, CH_3Hg^+ was converted to IHg (fig. S1A), but no gaseous Hg(0) was observed (fig. S1B). The amount of the cell-associated CH_3Hg^+, particularly the adsorbed $CH_3Hg^+_{ad}$, decreased with time, whereas the proportion of IHg increased with time. The produced IHg mostly remained inside the cell, with less than 6% of the IHg either left in solution or sorbed on the cell surface because Hg(II) is known to strongly sorb or interact with thiol functional groups of proteins and cellular materials (*33*). Note that, at the highest added CH_3Hg^+ concentration (125 nM), the reaction rate decreased to 0.011 (±0.001) $hour^{-1}$ (Fig. 2A), and demethylation was inhibited in the first 8 to 24 hours. However, with a longer incubation time (120 hours), the cells were able to recover and degrade a substantial amount of CH_3Hg^+ (71%). This initially inhibited CH_3Hg^+ degradation may be interpreted as a result of potential toxic effects of CH_3Hg^+ on *M. trichosporium* OB3b, similar to that observed with *Geobacter bemidjiensis* Bem (*17*).

Because demethylation was observed neither in *M. trichosporium* OB3b spent medium (fig. S1B) nor in *M. capsulatus* Bath cultures (Fig. 2, C and D), the results signify that demethylation was biologically mediated and methanotroph strain specific. However, neither *M. trichosporium* OB3b nor *M. capsulatus* Bath contains a homolog of *merB* (encoding for the organomercurial lyase) in their genome, suggesting that CH_3Hg^+ degradation by *M. trichosporium* OB3b relies on an as yet unknown mechanism and that this mechanism does not exist in *M. capsulatus* Bath.

To elucidate this mechanism, we first considered the fact that both *M. trichosporium* OB3b and *M. capsulatus* Bath are sensitive to the availability of copper. That is, the copper-to-biomass ratio is a key factor in regulating the expression of the following: (i) genes encoding for the soluble and particulate methane monooxygenases (MMOs), with soluble MMO only expressed in the absence of copper (*25*, *34*); and (ii) genes encoding for the chalkophore methanobactin with expression greatest in the absence of copper (*24*, *25*). Genes encoding for the chalkophore are found in *M. trichosporium* OB3b, but not in *M. capsulatus* Bath, which contains a different class of chalkophores (*35*). Furthermore, methanobactin from *M. trichosporium* OB3b has been found to bind Hg(II) and CH_3Hg^+ (*29*) and may thus be involved in CH_3Hg^+ degradation.

We subsequently investigated CH_3Hg^+ degradation by *M. trichosporium* OB3b in the presence of a known MMO inhibitor, acetylene, but no apparent inhibitory effects were observed (fig. S2). We next considered CH_3Hg^+ degradation by cells grown either in the absence (0 μM) or in the presence (1 μM) of copper. Although CH_3Hg^+ degradation was observed under both conditions (Fig. 3 and table S1), greater degradation of CH_3Hg^+ was evident in the absence than in the presence of copper. We then examined several mutant strains of *M. trichosporium* OB3b defective in methanobactin production (*mbnA*::Gm^r and Δ*mbnAN*) (*25*) to determine whether methanobactin is directly involved in CH_3Hg^+ degradation. We also examined two additional methanotrophs—one (*Methylocystis* strain SB2) makes methanobactin and the other (*Methylocystis parvus* OBBP) does not (*36*). Results show that *Methylocystis* strain SB2 degraded CH_3Hg^+, whereas all methanobactin mutants did not regardless of the culture conditions in the presence or absence of Cu^{2+} ions (Fig. 3 and table S1). In addition, no demethylation was observed with *M. parvus* OBBP. These findings strongly suggest that methanobactin plays a critical role in degrading CH_3Hg^+.

Although methanobactin is clearly needed for CH_3Hg^+ degradation by *M. trichosporium* OB3b, subsequent studies indicate that it is not sufficient. That is, when CH_3Hg^+ was incubated with the purified methanobactin, no appreciable CH_3Hg^+ degradation was observed in the same MOPS buffer used in whole-cell studies (fig. S3). This result suggests that methanobactin likely served as a carrier or as a binding agent for CH_3Hg^+ in the cell where it is degraded by some as yet unknown

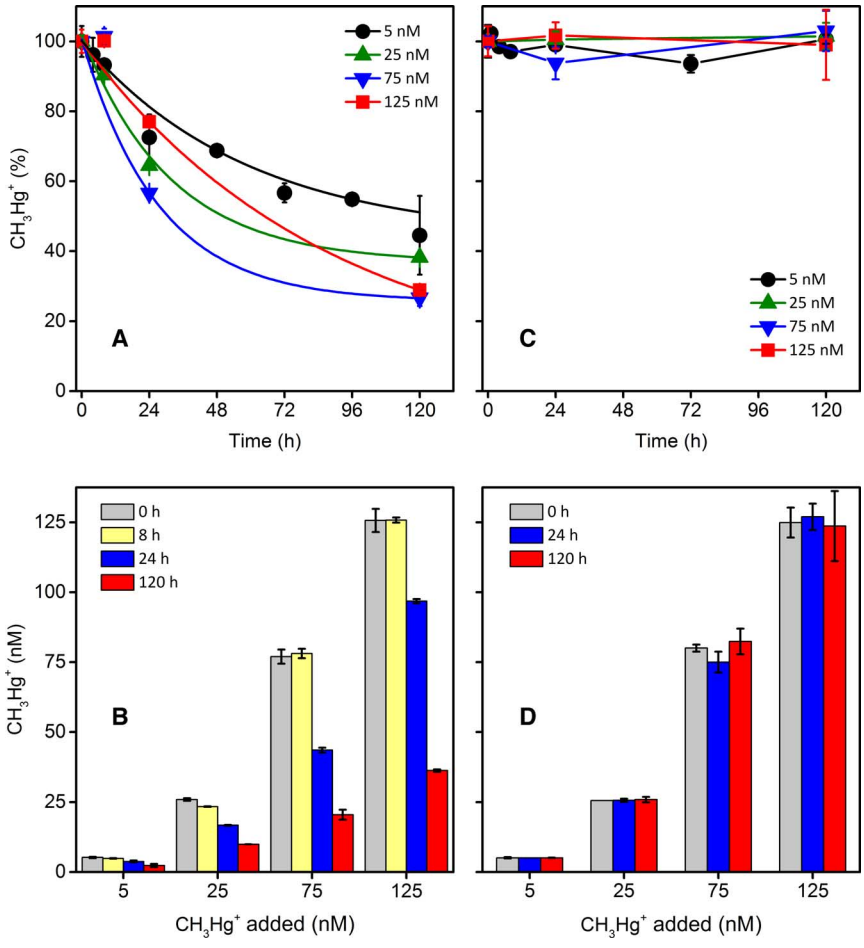

Fig. 2. Time- and concentration-dependent degradation of methylmercury (CH_3Hg^+) by methanotrophs. (**A** and **B**) *M. trichosporium* OB3b at 30°C and (**C** and **D**) *M. capsulatus* Bath at 45°C in 5 mM MOPS buffer. The added cell concentration was 10^8 cells ml^{-1} (washed), and the CH_3Hg^+ concentration was varied from 0 to 125 nM. Data points at 5 nM CH_3Hg^+ in (A) represent an average of replicate samples (10 to 15) from five independent batch experiments, and all other data points represent an average of triplicate samples. Error bars represent 1 SD from all replicate samples.

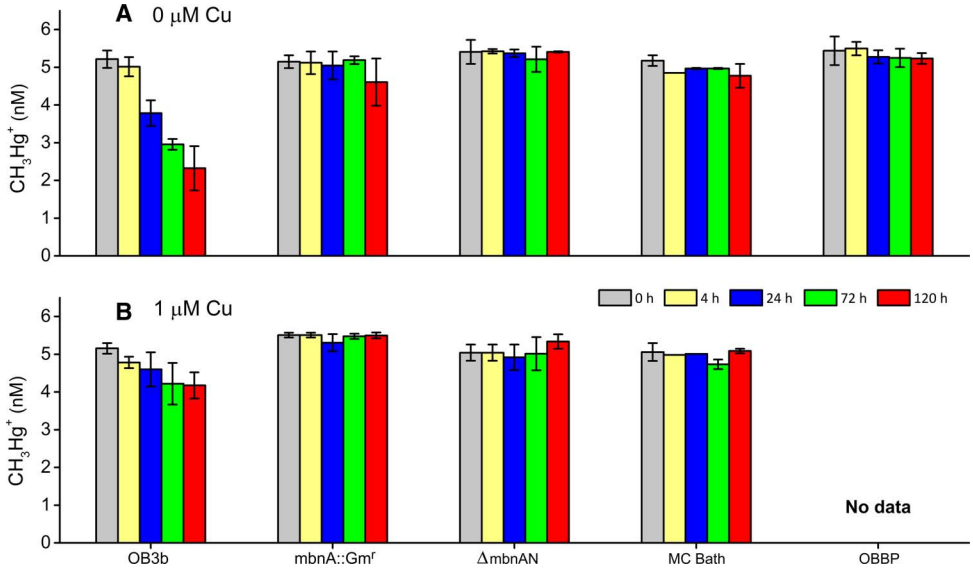

Fig. 3. Methylmercury (CH_3Hg^+) degradation by different methanotrophs and mutants. Comparisons of the time-dependent degradation of CH_3Hg^+ by washed cells of *M. trichosporium* OB3b and its mutant strains (*mbnA*::Gm^r and Δ*mbnAN*), *M. capsulatus* Bath, and *M. parvus* OBBP in 5 mM MOPS buffer. The added cell concentration was 10^8 cells ml^{-1}, and the CH_3Hg^+concentration was ~5 nM. Data points represent an average of all replicate samples (3 to 15), and error bars represent 1 SD.

enzyme(s). We subsequently considered possible involvement of methanol dehydrogenase (MeDH), which is responsible for oxidation of methanol to formaldehyde in the central pathway of CH_4 oxidation, because *M. trichosporium* OB3b may take up CH_3Hg^+ and use its methyl ($-CH_3$) group as a supplementary C1 source and energy. We found that addition of methanol (5 mM or higher) completely inhibited CH_3Hg^+ degradation (fig. S4). This inhibition cannot be attributed to decreased metabolism of *M. trichosporium* OB3b because no inhibitory effects were observed in this organism even at methanol concentrations as high as 990 mM (*37*). The result suggests that MeDH, in conjunction with methanobactin, degraded CH_3Hg^+, similar to the degradation of methanol where the methyl group is cleaved and possibly oxidized via this periplasmic enzyme. Hence, the methyl group of CH_3Hg^+ may serve as an auxiliary C1 source for methanotrophs, as speculated by others (*19*).

In conclusion, we present evidence that strongly suggests the presence of a novel biological pathway of CH_3Hg^+ demethylation by methanotrophs, which warrants further investigation. This pathway is remarkably different from the canonical organomercurial lyase found in some aerobic microorganisms (*13*, *20*). Unlike the organomercurial lyase in Hg-resistant bacteria, methanotrophs take up and degrade CH_3Hg^+ at environmentally relevant Hg concentrations (that is, picomolar to nanomolar). Methanotrophic-mediated CH_3Hg^+ degradation was also evident at circumneutral pH, unlike organomercurial lyase that has an optimal pH of ~10 (*21*). These findings suggest that methanotrophs may play an important role in controlling Hg transformation or net CH_3Hg^+ production and toxicity in situ, thereby providing new insights into as yet unknown but potentially widespread biological mechanisms of CH_3Hg^+ uptake and demethylation in the environment.

MATERIALS AND METHODS

The methanotrophs *M. trichosporium* OB3b and *M. capsulatus* Bath were grown in nitrate minimal salts medium at 30° and 45°C, respectively, either without added copper or with 1 μM copper (as $CuCl_2$) (*29*, *38*). Cells were harvested at the late exponential phase, washed once, and then resuspended in 5 mM MOPS buffer solution at pH 7.3. Methanobactin was isolated from *M. trichosporium* OB3b, as previously described (*39*).

Methylmercury (CH_3Hg^+) sorption, uptake, and demethylation assays were conducted in 4-ml amber glass vials (National Scientific) by mixing washed cells with CH_3Hg^+ in 5 mM MOPS buffer under ambient conditions. To determine whether MMOs were involved in CH_3Hg^+ demethylation, we added 100 μl of acetylene to the headspace (through a septum) and allowed it to equilibrate with the cells first for 30 min in one subset of assays because acetylene is a strong and selective inhibitor of MMO activity (*25*, *40*). CH_3Hg^+ working solution (10 nM) was prepared by diluting 5 μM stock solution (CH_3HgOH in 0.5% acetic acid and 0.2% HCl from Brooks Rand Labs) in MOPS. The reaction was initiated by mixing 0.5 ml of CH_3Hg^+ working solution with 0.5 ml of washed cells to give a final concentration of CH_3Hg^+ at 5 nM and of cells at 1×10^8 cells ml^{-1} (*17*), or otherwise specified. Samples were then placed on a rotary shaker, kept at 30°C for *M. trichosporium* OB3b and its mutants, and at 45°C for *M. capsulatus* Bath. Replicate sample vials were taken at selected time points and analyzed as follows. For CH_3Hg^+ sorption (or uptake) analysis, triplicate samples were filtered through 0.2-μm syringe filters (to remove cells) and analyzed for $CH_3Hg^+_{sol}$ (*17*, *32*). The unfiltered samples were used to determine the total Hg and total CH_3Hg^+ ($CH_3Hg^+_{Total}$) so that the cell-associated or total sorbed Hg can be calculated by their difference. For Hg species distribution analyses, six replicate samples (in separate vials) were taken, and three of them were filtered as above and analyzed for total soluble Hg (Hg_{sol}) and $CH_3Hg^+_{sol}$ (*17*, *33*). The remaining three samples were used to determine cellular uptake of CH_3Hg^+ ($CH_3Hg^+_{up}$) and cell surface–adsorbed CH_3Hg^+ ($CH_3Hg^+_{ad}$). This was accomplished by adding 2,3-dimercapto-1-propanesulfonic acid (DMPS), a Hg-chelating agent, at 150 μM to wash off the sorbed $CH_3Hg^+_{ad}$ at each time point and then analyzing CH_3Hg^+ in filtered samples (*17*, *33*), so that $CH_3Hg^+_{up}$ can be calculated by subtracting $CH_3Hg^+_{ad}$ and $CH_3Hg^+_{sol}$ from $CH_3Hg^+_{Total}$. The inorganic IHg species, resulting from degradation of CH_3Hg^+, were analyzed in the same manner, in which the adsorbed IHg_{ad} and cellular uptake of IHg_{up} were determined following DMPS washing, and soluble IHg (IHg_{sol}) was calculated by subtracting $CH_3Hg^+_{sol}$ from Hg_{sol} (*17*, *33*). Selected samples (before filtration) were determined for purgeable elemental Hg(0), but none was detected. Additional experiments were performed with cell spent medium and MOPS buffer as controls. Demethylation experiments were repeated at least once to ensure data quality, and error bars in all figures represent 1 SD of all replicate samples. Demethylation rate constants (k_{demeth}) were calculated on the basis of the pseudo–first-order rate law: $d[CH_3Hg^+]/dt = -k_{demeth}[CH_3Hg^+]$, where k_{demeth} was determined by the slope of the linear regression between natural logarithm of the CH_3Hg^+ concentration and time (*12*, *41*).

A modified EPA Method 1630 was used for CH_3Hg^+ analysis, in which isotope dilution with enriched $CH_3{}^{200}Hg^+$ was used as an internal standard, and an inductively coupled plasma mass spectrometer (ELAN DRC-e, PerkinElmer Inc.) was used to separate the various Hg isotopes to determine CH_3Hg^+ concentrations (*17*, *32*, *33*). The recovery of spiked CH_3Hg^+ standards was 100 ± 10%, and the detection limit was about 3×10^{-5} nM CH_3Hg^+. Gaseous Hg(0) was directly determined by inserting needles through the septa of the 4-ml glass vials and then purging with ultrapure N_2 for 2 min into a gaseous Hg(0) analyzer (Lumex 915+, Ohio Lumex). Total Hg and Hg_{sol} were analyzed via $SnCl_2$ reduction and detection by the Lumex analyzer after samples were oxidized in BrCl (5%, v/v) overnight at 4°C (*11*, *12*, *42*). The detection limit was ~2.5×10^{-4} nM.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/5/e1700041/DC1

table S1. Methylmercury degradation by washed cells of methanotrophs, including *M. trichosporium* OB3b and its two methanobactin (mb) defective mutants (*mbnA*::Gm^r and Δ*mbnAN*), *Methylocystis* strain SB2, *M. capsulatus* Bath, and *M. parvus* OBBP in 5 mM MOPS at pH 7.3.

fig. S1. Methylmercury (CH_3Hg^+) and inorganic mercury (IHg) species distribution during CH_3Hg^+ degradation assays with *M. trichosporium* OB3b.

fig. S2. Effects of acetylene addition (as an inhibitor of MMOs) on methylmercury (CH_3Hg^+) degradation by washed cells of *M. trichosporium* OB3b (10^8 cells ml^{-1}) in 5 mM MOPS buffer at 30°C.

fig. S3. Reactions between methylmercury (CH_3Hg^+, 5 nM) and purified methanobactin (1 μM) from *M. trichosporium* OB3b in 5 mM MOPS buffer (pH 7.3) at 30°C.

fig. S4. Effects of methanol addition on methylmercury (CH_3Hg^+) degradation by washed cells of *M. trichosporium* OB3b (10^8 cells ml^{-1}) in 5 mM MOPS buffer at 30°C.

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