nature geoscience

LETTERS

PUBLISHED ONLINE: 4 AUGUST 2013 | DOI: 10.1038/NGEO1894

Oxidation and methylation of dissolved elemental mercury by anaerobic bacteria

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Methylmercury is a neurotoxin that poses significant health risks to humans. Some anaerobic sulphate- and iron-reducing bacteria can methylate oxidized forms of mercury, generating methylmercury[1–4]. One strain of sulphate-reducing bacteria (*Desulfovibrio desulphuricans* ND132) can also methylate elemental mercury[5]. The prevalence of this trait among different bacterial strains and species remains unclear, however. Here, we compare the ability of two strains of the sulphate-reducing bacterium *Desulfovibrio* and one strain of the iron-reducing bacterium *Geobacter* to oxidize and methylate elemental mercury in a series of laboratory incubations. Experiments were carried out under dark, anaerobic conditions, in the presence of environmentally relevant concentrations of elemental mercury. We report differences in the ability of these organisms to oxidize and methylate elemental mercury. In line with recent findings[5], we show that *D. desulphuricans* ND132 can both oxidize and methylate elemental mercury. We find that the rate of methylation of elemental mercury is about one-third the rate of methylation of oxidized mercury. We also show that *Desulfovibrio alaskensis* G20 can oxidize, but not methylate, elemental mercury. *Geobacter sulphurreducens* PCA is able to oxidize and methylate elemental mercury in the presence of cysteine. We suggest that the activity of methylating and non-methylating bacteria may together enhance the formation of methylmercury in anaerobic environments.

Certain anaerobic microorganisms methylate Hg(II) to neurotoxic methylmercury, CH_3Hg^+, but the fundamental mechanisms involved in this process remain poorly understood[1–7]. So far, most bacteria known to methylate Hg(II) are sulphate- or iron-reducing *Deltaproteobacteria*[3]. Only recently have organisms outside the *Deltaproteobacteria* been predicted to generate CH_3Hg^+ (ref. 1). A recent study has shown that Hg(0) can be methylated by *D. desulphuricans* ND132 (ref. 5). However, whether other anaerobic organisms can broadly oxidize and/or directly use Hg(0) as a source for methylation in anoxic environments remains unclear. Hg(0) occurs in lake water and sediments[8–10], in surface water and groundwater[11,12], and in concentrations of up to 60 $\mu g\,l^{-1}$ in stream sediments at localized contamination sites[13]. Reduction of Hg(II) to Hg(0) has been widely suggested as a method for decreasing the bioavailability of Hg for methylation as Hg(0) is considered as relatively inert[6,14,15]. Although Hg(0) can be readily oxidized to Hg(II) in oxic environments[8,16], recent studies have also shown that abiotic Hg(0) oxidation by means of thiol-ligand-induced complexation can occur under anoxic conditions[17,18]. As bacterial surfaces and exudates contain abundant thiols[19–21], we hypothesized that dissolved Hg(0) can also be oxidized and subsequently methylated by anaerobic microorganisms.

To test this hypothesis, we selected three bacterial strains within the *Deltaproteobacteria* and compared their ability to oxidize and methylate Hg(0) under dark, anaerobic conditions. Both *G. sulphurreducens* PCA and *D. desulphuricans* ND132 are known Hg(II) methylators and *D. alaskensis* G20 is a known non-methylator[1–4,22]. *G. sulphurreducens* PCA is an iron-reducing bacterium, whereas *D. desulphuricans* ND132 and *D. alaskensis* G20 are sulphate-reducing bacteria. The experiments were carried out with washed cells in phosphate buffered saline (PBS) solutions (pH 7.4) at a cell concentration of $10^{11}\,l^{-1}$. Results (Fig. 1a–d) show that both strains ND132 and G20 (live cells) can rapidly convert purgeable Hg(0) (25 nM) to non-purgeable Hg (Hg_{NP}).

Hg_{NP} in solution is typically regarded as oxidized and this has been used to distinguish it from the reduced Hg(0) (refs 8–10,14–18). We therefore use Hg_{NP} as a proxy for oxidized Hg(II), which is also supported by X-ray absorption spectroscopic analyses[5]. We show that Hg(0) can be purged out of the cell suspension using the reductants $SnCl_2$/HCl and $NaBH_4$/NaOH (refs 23–26; Supplementary Fig. S1), but not with acid (0.5 M HCl) or base (0.2 M NaOH) in the absence of reductants. We thus exclude the possibility that Hg_{NP} contains Hg(0) that is physically associated with the cell or with the extracellular matrix.

Using this proxy, we found that >90% of the Hg(0) was oxidized within ~30 h by G20 cells (Fig. 1a,b), whereas strain ND132 required ~3–4 days for the same degree of oxidation to occur (Fig. 1c,d). In contrast, PCA cells oxidized only a small percentage (<10%) of the Hg(0) after one week of incubation (Fig. 1f). In this case, decrease in Hg(0) concentration by ~30% within a week (Fig. 1e) is primarily attributed to volatilization loss of Hg(0) from the experimental vessel. The loss of Hg(0) was verified by measurements of the total Hg concentration (Supplementary Fig. S2), which decreased proportionally to the Hg(0) concentration over the experimental period (Fig. 1e). Additionally, in all control experiments (no cells or heat-killed cells), a gradual loss of Hg(0) was observed (Fig. 1a,c,e), but Hg_{NP} remained at low or non-detectable levels (that is, no Hg(0) oxidation, Fig. 1b,d,f).

To determine whether Hg(0) oxidation might be owing to extracellular metabolites in cell suspensions, we carried out experiments to evaluate Hg(0) oxidation in the cell filtrate. Washed cells were first incubated for either 4 h or 48 h in PBS and the filtrate collected through a 0.2 μm filter to remove the cells. In the PCA filtrate, no Hg(0) oxidation was observed, similar to the results with live PCA cells (Fig. 1e,f). Nor was Hg(0) oxidation observed in

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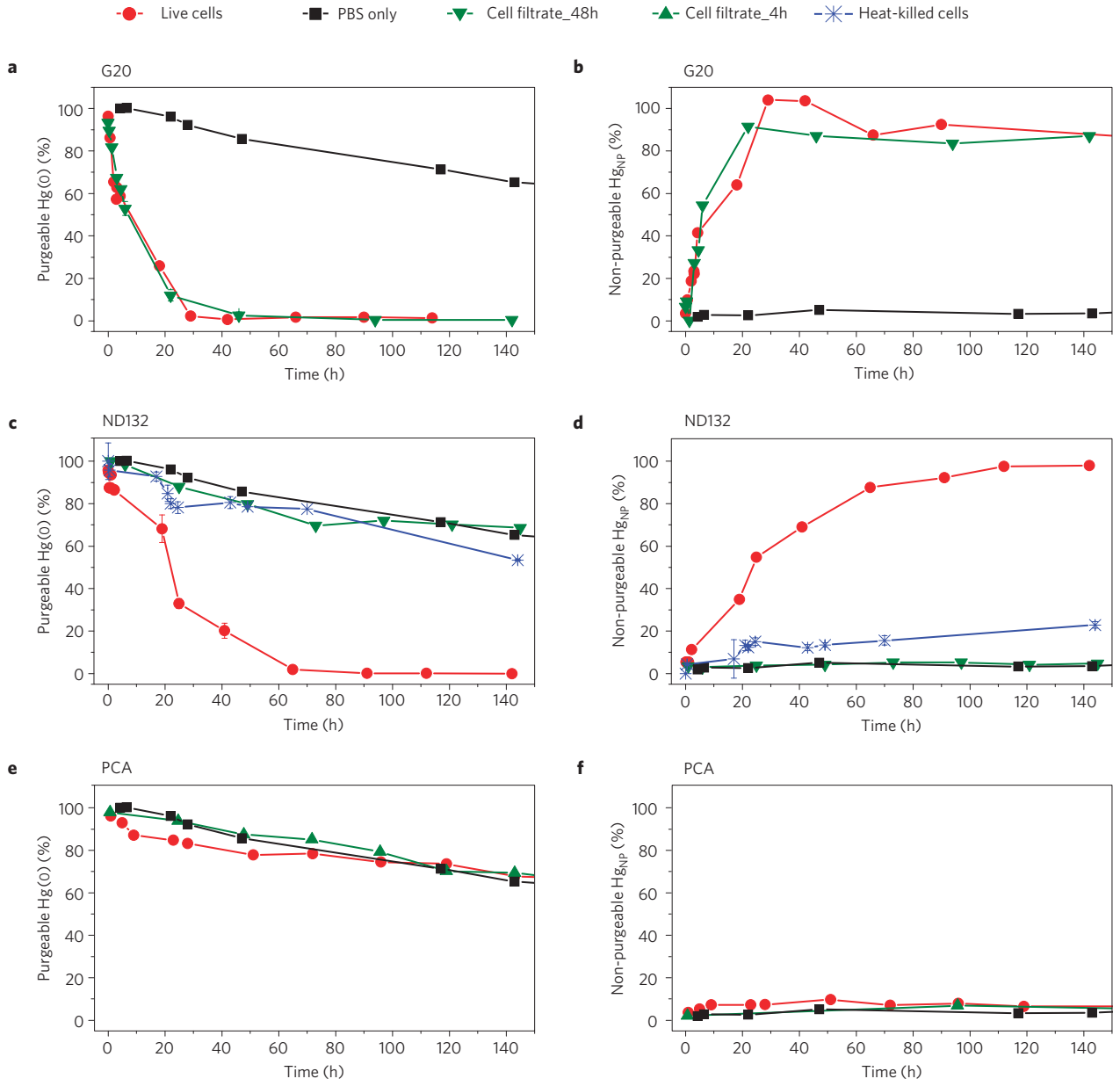

Figure 1 | Anaerobic bacterial oxidation of dissolved elemental Hg(0) in PBS. a–f, Analysis of the purgeable Hg(0) and non-purgeable Hg_{NP} over time during reactions between Hg(0) (~25 nM) and washed live cells (10^{11} l^{-1}) of *D. alaskensis* G20 (**a**,**b**), *D. desulphuricans* ND132 (**c**,**d**) and *G. sulphurreducens* PCA (**e**,**f**). Similar experiments were carried out with filter-passing cell filtrates, obtained by first incubating cells in PBS for either 4 or 48 h, then filtering through a 0.2 μm filter to remove cells. Heat-killed cells and PBS buffer solutions were used as controls. Error bars represent one standard deviation of replicate samples ($n = 2$–4).

the ND132 filtrate (Fig. 1c,d). Heat-killed cells showed lower Hg(0) oxidation than live ND132 cells, suggesting that bacterial cells or cell walls were denatured by the heat treatment. Addition of pyruvate and fumarate as the respective electron donor and acceptor in these experiments did not significantly affect the rate of Hg(0) oxidation by ND132 (Supplementary Tables S1–S3). The rate constants of Hg(0) oxidation with and without electron donor/acceptor are 31.2 (±2.1) × 10^{-3} and 33.4 (±4.4) × 10^{-3} h^{-1}, respectively, indicating that cell surface processes probably facilitated Hg(0) oxidation, rather than cellular metabolic activity that requires energy as demonstrated for Hg(II) methylation[4,27].

For strain G20, however, the cell filtrate oxidized Hg(0) to levels comparable to those in the presence of live cells; >80% of Hg(0) were oxidized in 30–40 h (Fig. 1a,b). This suggests that bacterial strains differ in the excretion of extracellular materials (with exudates of G20 probably containing thiol compounds and proteins) and/or in possessing reactive surface functional groups[19–21], which results in different affinities and reactions with Hg(0). These observations of anaerobic Hg(0) oxidation by ND132 and G20 cells (or G20 cell filtrate) are consistent with thiol-induced oxidative complexation between Hg(0) and thiols on cells or in cell exudates, as shown in abiotic studies involving Hg(0) and thiol ligands[17,18,28].

Thiol-induced Hg(0) oxidation was further validated by adding thiol cysteine (5 μM) to PCA cells, leading to a substantial increase in Hg(0) oxidation (~90%) under the same experimental conditions (Supplementary Fig. S3 and Table S1). Although cysteine is commonly known as an electron donor, its reaction with Hg(0) is driven by the formation of a strong thiolate–metal bond, leading to electron transfer or oxidation of Hg(0) (refs 18,28). The details of

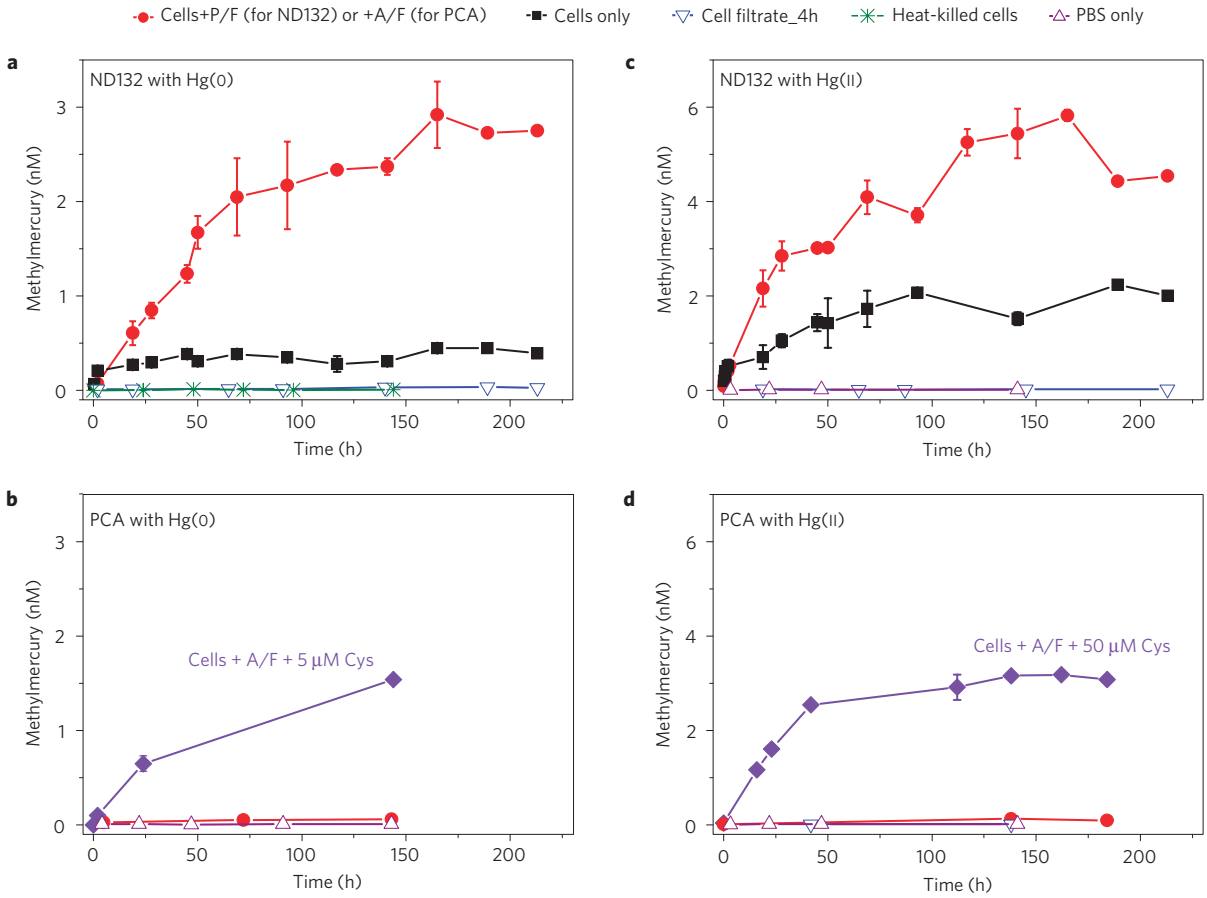

Figure 2 | Hg(0) or Hg(II) as the sole source of Hg for bacterial methylation in PBS. a–d, Bacterial methylation of Hg(0) (**a**,**b**) or Hg(II) (**c**,**d**) by washed live or heat-killed cells (10^{11} l^{-1}) and cell filtrates of *D. desulphuricans* ND132 and *G. sulphurreducens* PCA. The initial Hg(0) or Hg(II) concentration was ~25 nM. Pyruvate/fumarate (P/F; 1 mM) were provided as the electron donor and acceptor for ND132 (**a**,**c**), whereas acetate/fumarate (A/F; 1 mM) were used for PCA with (purple diamonds) or without (red circles) cysteine (5–50 μM) (**b**,**d**). Error bars represent one standard deviation of replicate samples ($n = 2$–4).

the oxidation process and mechanisms are beyond the scope of this paper, but it has been shown that, as Hg(0) is oxidized, the thiol–H^+ may be reduced[28]. In our system, nanomolar excess electrons from Hg(0) oxidation can also be consumed by other electron acceptors in the electrolytic media. Thiol-induced reactions thus offer a plausible explanation for anaerobic bacterial Hg(0) oxidation because abundant thiols are known to exist on bacterial cell surfaces[19–21]. For example, an exofacial thiol content of ~10^6 per cell has been reported for *Lactococcus lactis*[19] and up to 26.4 μmol g^{-1} of wet cells for *Bacillus subtilis*[29]. These thiols are typically associated with membrane or cell wall proteins and are thought to protect cells against oxidative stress[19].

Notably, we show that *D. desulphuricans* ND132 can produce CH_3Hg^+ with a rate constant up to $1.3(\pm0.1) \times 10^{-3}$ h^{-1} when Hg(0) (25 nM) is provided as the sole source of Hg (Fig. 2a and Supplementary Table S1). In the presence of pyruvate and fumarate (1 mM each, single addition at time zero), the maximum amount of CH_3Hg^+ produced was ~2.8 nM (>10% of the total Hg(0)) following reactions between ND132 cells and Hg(0) under anaerobic conditions. A lower but consistent amount of CH_3Hg^+ (0.3–0.4 nM) was also formed in the absence of pyruvate/fumarate under the same conditions (Fig. 2a). Conversely, in the absence of live ND132 cells (that is, PBS only, 4 h cell filtrate, or heat-killed cells), no CH_3Hg^+ was detected (detection limit is 6 pM) because Hg methylation is an active metabolic process[1,3].

As G20 is a non-methylator, production of CH_3Hg^+ is not expected in the cell culture (Supplementary Fig. S4). Although PCA is a known methylator, an insignificant amount of CH_3Hg^+ (~0.06 nM) was formed from Hg(0) in the presence of acetate and fumarate (1 mM each; Fig. 2b). The lack of Hg methylation by PCA can be explained by its inability to oxidize Hg(0) under the given experimental conditions (Fig. 1e,f). Interestingly, when PCA cells were supplemented with 5 μM cysteine, the rates and extent of Hg(0) methylation substantially increased (up to ~1.5 nM) (Fig. 2b and Supplementary Table S1). Cysteine supplementation has been shown to enhance Hg(II) methylation by PCA (ref. 30), but in our case, cysteine thiols enhanced oxidation of Hg(0) to Hg(II) (Supplementary Fig. S3), thus permitting PCA to methylate Hg(II). These results suggest that only Hg(II) may be methylated by PCA cells.

We observed higher methylation rates in both ND132 and PCA cells when Hg(II) (25 nM) rather than Hg(0) was provided as the sole source of mercury (Fig. 2c,d and Supplementary Table S1). For ND132, the methylation rate of Hg(0) was approximately three times slower than that of Hg(II) (Fig. 2c), probably owing to Hg(0) oxidation or uptake being the rate-limiting step. Even in the absence of pyruvate/fumarate, ND132 cells produced ~2 nM CH_3Hg^+ from Hg(II), compared with ~0.4 nM CH_3Hg^+ in the presence of Hg(0). Similarly, for PCA, the extent of methylation was greater using Hg(II) than Hg(0); approximately 3 nM and 1.5 nM CH_3Hg^+ were formed by PCA in the presence of Hg(II) and Hg(0), respectively. In both cases, cysteine was present in the media, although 50 μM cysteine was used in the methylation experiments of Hg(II) rather than 5 μM. Increasing cysteine from ~5 to 50 μM was previously shown to result in only a 10–15% increase in CH_3Hg^+ production from Hg(II) (ref. 30). These results again suggest that oxidation of Hg(0) to Hg(II) is necessary for methylation by PCA cells. For

ND132, it is not yet clear whether Hg(0) can be taken up directly and subsequently oxidized and methylated intracellularly. Furthermore we note that the level of CH_3Hg^+ formation is lower here than in those studies reported in the literature[4,30]; this may be owing to high chloride concentrations (143 mM) used in our buffer solution, which can compete with cysteine for Hg(II) binding[30].

Our study therefore identifies potentially important strain-specific processes involving microbially mediated oxidation and methylation of dissolved elemental Hg(0) under anoxic conditions. Although Hg(0) is methylated to a lesser extent than Hg(II), converting Hg(II) to Hg(0) may not completely prevent microbial uptake and methylation, contrary to previous assumptions[6,14,15]. We conclude that, depending on the composition of microbial communities and the geochemical conditions, dissolved Hg(0) in water and sediments may be used by microorganisms for methylation, albeit at a slower rate than Hg(II).

Although the genetic basis of Hg methylation has recently been identified, mercury cell surface interactions, specific uptake mechanisms, and biochemical pathways of methylation are still not fully understood[1,3,4]. Here we show that the ability for Hg(0) oxidation and methylation varies considerably even among closely related strains of bacteria. *D. desulphuricans* ND132 is able to oxidize Hg(0) anaerobically and uses it for methylation, whereas *D. alaskensis* G20 can only oxidize, but not methylate, Hg(0). *G. sulphurreducens* PCA shows negligible Hg(0) oxidation under given conditions, but could oxidize and methylate Hg(0) when supplemented with thiol compounds such as cysteine. These findings suggest that methylating and non-methylating bacteria may work synergistically to enhance anaerobic Hg(0) oxidation and methylation. For example, in a microbial community, oxidized Hg(II) from strain G20 or from its exudate may be available for methylation by strain PCA. However, in an environment with high thiol concentrations, the thiol-induced oxidation of Hg(0) may facilitate mercury methylation by PCA. Future studies should aim to explain how these strain-specific differences and their environmental interactions together influence microbial mercury transformation, uptake and methylation in the environment.

Received 9 November 2012; accepted 24 June 2013; published online 4 August 2013

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Acknowledgements

We thank X. Yin, Y. Qian, R. Jr Hurt and M. Drake at Oak Ridge National Laboratory (ORNL) and H. Guo at the University of Tennessee for technical assistance and support. This research was sponsored by the Office of Biological and Environmental Research, Office of Science, US Department of Energy (DOE) as part of the Mercury Science Focus Area Program at ORNL, which is managed by UT-Battelle LLC for the DOE under contract DE-AC05-00OR22725.

Author contributions

H.H. and H.L. designed and carried out the experiments; B.G. conceived the study and supervised the research; W.Z., S.J.T. and X.F. contributed experiments and analytic tools; B.G., A.J., D.A.E. and L.L. contributed data analysis and wrote the paper.

Additional information

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Competing financial interests

The authors declare no competing financial interests.