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Intracellular Hg(0) oxidation in Desulfovibrio desulfuricans ND132

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ABSTRACT

The disposal of elemental mercury [Hg(0)] wastes in mining and manufacturing areas have

caused serious soil and groundwater contamination issues. Under anoxic conditions, certain

anaerobic bacteria can oxidize dissolved elemental mercury and convert the oxidized Hg to

neurotoxic methylmercury. In this study, we conducted experiments with the Hg-methylating

bacterium Desulfovibrio desulfuricans ND132 to elucidate the role of cellular thiols in anaerobic

Hg(0) oxidation. The concentrations of cell-surface and intracellular thiols were measured, and

specific fractions of D. desulfuricans ND132 were examined for Hg(0) oxidation activity and

analyzed with extended X-ray absorption fine structure (EXAFS) spectroscopy. The

experimental data indicate that intracellular thiol concentrations are approximately six times

higher than that of the cell wall. Cells reacted with a thiol-blocking reagent were severely

impaired in Hg(0) oxidation activity. Spheroplasts lacking cell walls rapidly oxidized Hg(0) to

Hg(II), while cell wall fragments exhibited low reactivity towards Hg(0). EXAFS analysis of

spheroplast samples revealed that multiple different forms of Hg-thiols are produced by the

Hg(0) oxidation reaction and that the local coordination environment of the oxidized Hg changes

with reaction time. The results of this study indicate that Hg(0) oxidation in D. desulfuricans

ND132 is an intracellular process that occurs by reaction with thiol-containing molecules.

INTRODUCTION

The fate and transport of mercury (Hg) in contaminated groundwater is strongly affected by redox transformations.[1-3] Whereas oxidized ionic mercury [Hg(II)] binds strongly to natural organic matter via complexation with thiol functional groups,[4, 5] elemental mercury [Hg(0)] is mobile in groundwater and can migrate horizontally over long distances through the unsaturated zone as a volatile gas.[6, 7] The conversion of Hg(0) to Hg(II) also affects the bioavailability of mercury for bacterial uptake, the production of neurotoxic methylmercury [MeHg], and subsequent bioaccumulation of MeHg in aquatic food webs.[8-10] Understanding the biogeochemical processes that control Hg(0) oxidation is particularly important for predicting the formation of MeHg in industrial areas contaminated with large amounts of elemental mercury, such as historic mining sites and nuclear weapon production facilities.[11-14]

Certain anaerobic bacteria have been shown to produce MeHg from Hg(0) as the sole mercury source.[15, 16] A key step in the microbial conversion of dissolved elemental mercury to methylmercury is the cellular oxidation of Hg(0) to Hg(II).[17] The Hg-methylating bacterium Desulfovibrio desulfuricans ND132 oxidizes dissolved elemental mercury to Hg(II) and the oxidized Hg(II) covalently bonds to cellular functional groups.[15] Geobacter sulfurreducens PCA can also convert Hg(0) to Hg(II) but requires either high cell concentrations or the addition of thiol compounds in its growth medium.[16, 18] Because thiol functional groups in natural organic matter have been shown to oxidize Hg(0) via oxidative complexation,[19, 20] thiol-containing molecules associated with bacterial cells may also be important for microbial Hg(0) oxidation. Currently the role of cellular thiols, the location of Hg(0) oxidation, and the chemical forms of oxidized mercury in Hg-methylating bacteria are poorly understood.

3

47   In this study, we conducted laboratory experiments to investigate thiol-mediated Hg(0)

48   oxidation in D. desulfuricans ND132. Cells were separated into spheroplasts (cells without cell

49   walls) and cell wall fragments, and experiments were conducted with the two fractions to better

50   understand cell-surface versus intracellular Hg(0) oxidation activity. The objectives of this study

51   were: (1) to quantify the concentration and distribution of thiol functional groups in D.

52   desulfuricans ND132; (2) to determine the cellular location of Hg(0) oxidation; and (3) to

53   characterize the chemical forms of oxidized mercury with EXAFS spectroscopy. The results of

54   this study provide new insights into Hg(0) uptake and complexation of Hg(II) in anaerobic Hg-

55   methylating bacteria.

56

57   **MATERIALS AND METHODS**

58

59   **Bacterial growth conditions.** D. desulfuricans ND132 was grown anaerobically (N_2

60   headspace) in a low sulfate medium modified from Gilmour et al.[21] containing 25 mM pyruvate,

61   40 mM fumarate, 0.17 M NaCl, 6.4 mM NH_4Cl, 10 mM MOPS, 1.5 mM KH_2PO_4, 3.62 μM

62    $FeCl_2$, 6.7 mM KCl, 3.15 mM $MgCl_2\cdot6H_2O$, 1.36 mM $CaCl_2\cdot2H_2O$, 1 mL/L sulfate-free SL-7

63   trace metals,[22] 0.5g/L yeast extract, and NaOH to adjust the pH to 7.2. Growth medium and

64   buffer solutions were rendered anoxic by boiling and bubbling with O_2-free N_2 gas. Cultures

65   were incubated statically at 30℃, harvested during mid-exponential phase ($OD_{600} \sim 0.25$), and

66   washed twice with 0.5 mM MOPS buffer in an anaerobic glove box (Coy; 5:95 H_2:N_2 headspace)

67   before experimentation.

68

69   **Preparation of cell fractions**. D. desulfuricans ND132 cell walls (outmembrane and the

70   peptidoglycan layer) were removed by a procedure modified from a spheroplasts preparation

71   method for Geobacter sulfurreducens.[23] Briefly, cultures (200 mL) harvested at mid-exponential

72   phase were washed twice in an anaerobic glove box with a deoxygenated wash medium

73   containing 3.09 mM KH_2PO_4, 1.26 mM K_2HPO_4, 5.10mM KCl, 0.08M NaCl, 0.01 M MOPS,

74   350 mM sucrose at pH 6.8. The cells were then resuspended in 4 mL of 250 mM Tris buffer (pH

75   7.5) and 0.4 mL of 500 mM EDTA was added to chelate structural ions in the peptidoglycan

76   layer. After 1 min reaction with EDTA, 4 mL of 700 mM sucrose was added to the cell

77   suspension followed by the addition of lysozyme (75 mg). The cells were then incubated at 30 ℃

78   from 1 minute to 24 hours. The optimum lysozyme incubation time was determined to be 6

79   hours. To induce osmotic shock, 8 mL of ultrapure water was then added to the suspension. The

80   resulting spheroplasts were immediately harvested by centrifugation at 20,000 g for 10 min. The

81   cell wall fragments in the supernatant were collected by ultracentrifugation at 177,500 g for 2

82   hours. Both the spheroplasts and cell wall fragments were then washed twice to remove residual

83   lysozyme from the incubation. Spheroplast formation was verified by fluorescence microscopy

84   in acridine orange-stained cells and quantified by direct counting method.[24] Over 90% of the

85   cells were converted to spheroplasts. Finally, spheroplast lysate was produced by resuspending

86   the spheroplasts in a hypotonic 0.5 mM MOPS buffer solution. Heat-treated spheroplast lysate

87   was prepared by heating the lysate at 80 °C for 10 min. Protein concentrations of intact cells,

88   spheroplasts and cell walls were measured by Bio-Rad protein assay.

89

90   **Determination of thiol functional groups**. The abundance of thiol functional groups was

91   determined by a florescence-labeling method using Thiol Fluorescent Probe 4 (TFP-4) [3-(7-

92   Hydroxy-2-oxo-2H-chromen-3-ylcarbamoyl)acrylic acid methyl ester] (EMD Millipore

93   Corporation). TFP-4 is a cell-permeable fluorogenic probe that exhibits fluorescence when

94   reacted with reduced and solvent exposed R-SH compounds via an 1:1 Michael adduct

95   formation.[25, 26] A 1 mM stock solution was prepared by dissolving TFP-4 in dimethyl sulfoxide

96   and diluting with acetonitrile. Experiments were conducted with spheroplasts and cell wall

97   fragments isolated from 7.4×10^8 cells/mL cell suspensions and re-suspended in 0.5 mM MOPS

98   buffer. The protein concentration of spheroplasts and cell wall fragments were 12 µg/mL and 1.3

99   µg/mL, respectively. Whole cells experiments were conducted at a cell density of 7.7×10^8

100   cells/mL. TFP-4 titrations were performed by adding a known amount of the TFP-4 stock

101   solution to a set of sample and allowing the suspensions to react at room temperature for 2 h.

102   Fluorescence measurements were then made in 1 mL quartz cuvettes at an excitation wavelength

103   of 400 nm and peak emission intensity at 465 nm with a Molecular Devices Plate Reader. For

104   each set of samples, the TFP-4 titration resulted in a distinct inflection in the emission intensity

105   precisely at the point corresponding to the thiol concentration of the sample. The thiol content

106   per cell was determined with Avogadro's constant and normalizing the thiol concentration to cell

107   density.

108

109   **Hg(0) oxidation experiments**. The oxidation of Hg(0) by D. desulfuricans ND132 was

110   examined by exposing washed cell suspensions to a continuous source of Hg(0) gas. A drop of

111   liquid Hg(0) bead was placed in an uncapped HPLC vial inside of a 30 mL serum bottle wrapped

112   in aluminum foil and capped with a Teflon stopper. After purging the headspace of the bottle

113   with ultra-high purity N_2 for 25 min, the liquid Hg(0) bead was allowed to evaporate and

114   equilibrate with gas phase for 24 h. The liquid Hg(0) was added in excess and provided a

115 continuous supply of elemental mercury gas throughout the duration of the experiment. The

116 Hg(0) oxidation reaction was initiated by injecting 5 mL of cell suspension into the serum bottle

117 around the HPLC vial. The sample volume required for analysis was 300 µL. The reactors were

118 shaken gently at 30 ℃ and sampled periodically for formation of non-purgeable Hg(II). At each

119 time point, cell suspension (0.5 mL) was removed from the reactor to an acid-cleaned I-Chem[@]

120 vial using a long needle and syringe, and the sample was purged immediately with ultra-high

121 purity N_2 for 2 min to remove unreacted Hg(0) gas. All samples were digested with 4 M nitric

122 acid and 0.2 M BrCl. Total non-purgeable Hg contents remaining in the samples were analyzed

123 by cold vapor atomic fluorescence spectrometry using a BrooksRand[@] MERX Total Mercury

124 Analytical System (EPA Method 1631) or cold vapor atomic absorption using a Leeman Labs

125 Hydra AA Mercury Analyzer (EPA Method 245.1).

126   Hg(0) oxidation experiments were repeated for TFP-4 treated cells, spheroplasts, and cell

127 wall fragments. TFP-4 treatment was carried out by suspending washed cells in deoxygenated

128 0.5 mM MOPS buffer and reacting the cells with 233 µM TFP-4 to block cellular thiols. After 2

129 hours of reaction with TFP-4, cells were washed and resuspended with MOPS buffer, and then

130 injected into reactors for the Hg(0) oxidation experiment. Experiments with spheroplasts and cell

131 wall fragments were performed with cell suspensions at concentration of 8×10^8 cells/mL, where

132 the spheroplasts and cell wall fragments were separated by the protocol described above. The

133 protein concentration of spheroplasts and cell wall fragments were 16 µg/mL and 2.3 µg/mL,

134 respectively. The spheroplasts were suspended in a sucrose (350 mM) buffer to maintain isotonic

135 conditions throughout the experiment. Hg(0) oxidation experiments with cell wall fragments

136 were also conducted in the sucrose buffer to allow for direct comparison with the spheroplast

137 data.

138   **X-ray absorption spectroscopy**. Hg(0)-reacted D. desulfuricans ND132 spheroplasts were

139   analyzed using X-ray absorption spectroscopy. Spheroplasts reacted with Hg(0) were collected

140   from the reaction bottles and purged by ultra-high purity N_2 for 25 min and centrifuged at

141   12,000 g for 15 min. The pellets were then transferred to Teflon sample holder and sealed with

142   Kapton tape in an anaerobic chamber. The samples were placed in deoxygenated containers and

143   shipped to the Advanced Photon Source at Argonne National Laboratory for XAS analysis. Hg

144   L_{III}-edge EXAFS spectra were collected at beam line 13-ID-E, GeoSoilEnviroCARS, using Si (111)

145   monochromatic crystal with a 13 element germanium detector. Although sector 13-ID-E is capable

146   of focusing beam to 2 µm × 2 µm in vertical and horizontal directions, beam was defocused to

147   200 µm × 200 µm in vertical and horizontal directions and sample position was moved after every

148   2 scans to a fresh spot to mitigate beam induced chemistry. Spectra were collected under ambient

149   temperature, pressure, and an N_2 atmosphere. Energy calibration was performed such that the first

150   inflection points of Au foil and HgSn amalgam were assigned as 11919 and 12,284 eV respectively.

151   At least 15 spectra were collected for each sample to improve the signal-to-noise ratio. Spectral

152   features between scans were highly reproducible indicating minimal beam induced chemistry in

153   samples during data collection.

154   The data were analyzed by using the methods described in the UWXAFS package.[27] Data

155   processing and fitting were done with the programs ATHENA and ARTEMIS.[28] The data range

156   used for Fourier transformation of the k-space data was 2.5–8.5 Å^{-1}. The Hanning window function

157   was used with dk = 1.0 Å^{-1}. Fitting of each spectrum was performed in r-space, at 1.2-2.8 Å, with

158   multiple k-weighting (k^1, k^2, k^3) unless otherwise stated. Lower χ_v^2 (reduced chi square) was used

159   as the criterion for inclusion of an additional shell in the shell-by-shell EXAFS fitting procedure.

160   Hg(0) and three Hg(II) standards [Hg-cysteine, Hg-(cysteine)$_3$, and Hg-acetate] were measured to

161 fingerprint Hg species in this study. Details of the shell-by-shell simultaneous fitting approach

162 used to model the EXAFS data are described elsewhere.[29]

163

164 **RESULTS**

165

166   **Quantification of cellular thiols**. To determine the concentration of the intracellular thiols,

167 the cell wall of D. desulfuricans ND132 was removed and the spheroplast lysate was titrated

168 with the thiol-specific fluorescent probe TFP-4. The fluorescence emission increased steeply and

169 linearly until all the reduced thiols in the sample reacted with the fluorophore (Fig. 1A). After

170 stoichiometric reaction with the R-SH moieties, a decrease in slope in the titration curve was

171 observed. Best fit lines of the two linear regions of the titration curve showed an inflection point

172 at 12 µM which corresponded to the thiol concentration in the spheroplasts lysate sample. To

173 determine the concentration of thiol functional groups associated with the cell envelope, TFP-4

174 titrations were conducted with cell wall fragments. Similar to the spheroplast lysate experiment,

175 the slope of the titration curve increased steeply and linearly followed by a marked decrease in

176 fluorescence intensities at high TFP-4 concentrations (Fig. 1B). The titration curve for the cell

177 wall fragments showed an inflection point at 2 µM. Normalized to cell density, the thiol content

178 of the spheroplast (9.5×10^6 thiols/cell) was approximately 6 times higher than that of the cell

179 wall (1.7×10^6 thiols/cell). The combined thiols of the spheroplasts and cell walls was in close

180 agreement to the total thiol concentration determined for D. desulfuricans ND132 whole cells,

181 which exhibited an inflection point at 14 µM corresponding to a thiol number of 1.06×10^7

182 thiols/cell (Fig. 1C).

183

184 **Oxidation of Hg(0) to Hg(II).** We conducted experiments to determine the role of thiol-

185 containing molecules in Hg(0) oxidation by blocking the cellular sulfhydryl groups with TFP-4

186 before reacting the cells with Hg(0). TFP-4 conjugation with cellular thiols resulted in severe

187 impairment of Hg(0) oxidation activity (Fig. 2A). After 48 h, D. desulfuricans ND132 cells

188 formed 464 ppb Hg(II), while the TFP-4 treated cells produced only 60 ppb Hg(II). Over 85% in

189 Hg(0) oxidation activity was lost due to the blocking of cellular thiols with TFP-4.

190   To localize Hg(0) oxidation activity in the bacterial cells, experiments were conducted

191 with specific cell fractions of D. desulfuricans ND132. Spheroplasts lacking cell walls rapidly

192 oxidized Hg(0) with over 50 ppb of non-purgeable Hg formed in 6 h (Fig. 2B). Conversely, only

193 6 ppb was generated by cell wall fragments during this reaction time. The Hg(0) oxidized by

194 spheroplasts was approximately 8 times more than Hg(0) oxidized by cell walls. In order to

195 determine if intracellular components can account for the total Hg(0) oxidation activity observed

196 in whole cells, Hg(0) oxidation experiments were performed on lysed spheroplasts. Both whole

197 cells and spheroplast lysate exhibited similar Hg(0) oxidation capacity (Fig 2C). Heat-treated

198 spheroplast lysate retained Hg(0) oxidation activity (Fig. S1). Whole cells and spheroplasts

199 suspended in sucrose buffer showed similar Hg(0) oxidation activity, but slower reaction rates

200 were observed in sucrose buffer compared to MOPS buffer likely due Hg(0) diffusion and

201 solubility effects in the concentrated sucrose solution (Fig. S2). Interestingly, in MOPS buffer

202 the whole cells oxidized Hg(0) at a faster initial rate (8 ppb/h) compared to the spheroplast lysate

203 (6 ppb/h), suggesting that lysate conditions were sub-optimal relative to those maintained

204 natively by the cell (e.g. intact whole cells). This could be due to a number of factors including

205 the dispersal and dilution of sub-cellular components and/or the oxidation of thiol functional

206 groups upon exposure to the buffer. Despite this difference in initial oxidation rate, both

207 spheroplast lysates and whole cells oxidized a similar total amount of Hg(0) and there is no

208 statistical difference in the overall amount of non-purgeable Hg formed at the end of the

209 experiment.

210 **Chemical Speciation of Oxidized Hg**. X-ray absorption spectroscopy was performed to

211 examine the oxidation state and local binding environment of the spheroplast-associated Hg. The

212 Hg L_{III}-edge XANES spectra of the 1 h and 6 h samples were highly reproducible and showed

213 similarities with Hg-cysteine and Hg-(cysteine)$_3$ standards (Figures 3A and S1). The normalized

214 XANES spectra for spheroplast samples lack the pre-edge peak observed in the Hg-acetate

215 standard, suggesting that cell-associated Hg was not complexed to carboxyl functional groups.

216 First derivative of Hg XANES exhibited an energy separation (ΔE) between the first and second

217 energy peak for the spheroplast samples of 7.5 eV. The ΔE value was similar to that of Hg-cysteine

218 standard and significantly smaller than the ΔE value measured for the Hg-acetate standard (Fig.

219 S1b). These results indicate that the oxidized Hg is coordinated to sulfur (S) rather than oxygen

220 (O) atoms.

221 While no significant differences in the XANES spectra were observed between the 1 h and

222 6 h hours samples, the k^2 weighted $\chi(k)$ spectra showed a clear phase shift in the k^2 weighted $\chi(k)$

223 EXAFS oscillations between the 1 h and 6 h samples (Fig. 3B and S2). For the 6 h sample, the

224 phase of EXAFS oscillations shifted towards lower k values, which was manifested as longer bond

225 distance of the nearest neighbor atoms in the Fourier Transformed (FT) EXAFS data (Fig. 3C).

226 The Hg-S bond distance increased from 2.35 Å at 1 h to 2.43 Å at 6 h. The change in the Hg-S

227 bond distance was also evident from the real part of the FT EXAFS spectra (Fig. 3D). The Hg-S

228 bond distance for 1 h sample was slightly longer than the Hg-cysteine standard (2.30 Å) and the

229 Hg-S bond distance for 6 h sample was slightly shorter than the Hg-(cysteine)$_3$ standard (2.48 Å),

230   indicating there was a mixture of Hg-S species in the both samples. A shell-by-shell simultaneous

231   fitting approach was used to model the EXAFS data. Coordination numbers and bond distances

232   for the EXAFS modeling results are consistent with approximately 80% Hg-S_1 and 20% Hg-S_3 in

233   1 hour sample and 20% Hg-S_1 and 80% Hg-S_3 in 6 hour sample. Best fit values for EXAFS results

234   are shown in Table 1, and data and model fit are shown in Figure 4.

235

236   **DISCUSSION**

237

238   The experimental results presented in this study show that reduced thiols in D.

239   desulfuricans ND132 mediate Hg(0) oxidation. Inhibition of Hg(0) oxidation activity by the

240   blocking of sulfhydrl functional groups with the fluorophore TFP-4 indicates thiol functional

241   groups in biomolecules produced by D. desulfuricans ND132 chemically oxidize Hg(0) to

242   Hg(II). The oxidative process involves complexation of the thiols with Hg(0) followed by

243   electron transfer to an electron acceptor. This process is analogous to the redox reaction observed

244   with liquid Hg(0) drops reacted in thiol solutions:

245   [1] $RSH + Hg(0) \leftrightarrow RS\text{-}Hg + H^+ + e^-$
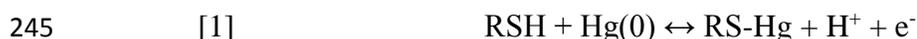
246   Open circuit potential measurements have demonstrated that the adsorption of thiol compounds

247   onto hanging mercury drop electrodes leads to spontaneous Hg(0) oxidation.[30-32] Protons have

248   been suggested as the electron acceptor for this electrochemical process,[31] with hydrogen (H_2) as

249   the reaction product. A similar mechanism has been proposed for Hg(0)$_{(aq)}$ oxidation by low-

250   molecular-weight thiol compounds.[20, 33] This oxidative mechanism would also be expected to

251   occur with biogenic thiol-containing molecules produced by D. desulfuricans ND132 in both

252   resting and metabolically-active cells.[15, 16]

253   Hg(0) oxidation in D. desulfuricans ND132 is an intracellular process rather than a cell-

254   surface mediated reaction as previously suggested.[16, 17] The experimental data implicate thiol-

255   containing molecules in either the cytoplasm or inner membrane as the reactive agents

256   responsible for Hg(0) oxidation (Fig. 2A). Because the intracellular thiol concentrations in D.

257   desulfuricans ND132 are approximately 6 times higher than the concentration of thiols on the

258   cell walls (Fig. 1), Hg(0) oxidation within the cell is significantly more favorable than in the cell

259   wall. Accordingly, our results showed that intracellular Hg(0) oxidation by spheroplasts was 8

260   times faster than cell surface Hg(0) oxidation (Fig. 2B). The fact that Hg(0) oxidation by

261   spheroplasts can account for all the Hg(0) oxidation activity observed in whole cells (Fig. 2C)

262   further supports the hypothesis that the oxidation of Hg(0) to Hg(II) is mediated by intracellular

263   reactions.

264   It is notable that the cell wall of D. desulfuricans ND132 exhibits very low reactivity

265   towards Hg(0) (Fig. 2B). Cell surface-associated thiols are reactive sites for Hg(II) adsorption in

266   gram negative bacteria,[34, 35] and surface complexation reactions form thermodynamically stable

267   Hg-S complexes in the bacterial cell wall.[36] Although surface-associated thiols have been

268   proposed as the sites for Hg(0) oxidation in G. sulfurreducens PCA,[16, 18] our data indicate the cell

269   surface thiols of D. desulfuricans ND132 do not play a central role in Hg(0) oxidation. It is

270   generally thought that Hg(0) can diffuse across bacterial membranes without undergoing

271   oxidation.[37] For example, during mercury detoxification Hg(II) by mercury-resistant bacteria,

272   Hg(II) is reduced to Hg(0) in the cytoplasm and dissolved gaseous mercury passively diffuses

273   out of the cell. The data presented in this study suggest that the reverse process can also occur,

274   whereby Hg(0) passively diffuses into the cell and undergoes oxidation to Hg(II) inside the

275   cytoplasm.

276   Hg(0) oxidation by D. desulfuricans ND132 is faster and occurs to a greater extent

277   compared to G. sulfurreducens PCA,[16] and the difference in reactivity towards Hg(0) may be the

278   result of the relative cellular thiol concentration. Thiols are five hundred times more abundant in

279   D. desulfuricans ND132 than in G. sulfurreducens, where 2.1×10^4 thiols/cell were measured

280   using the maleimide-containing probe ThioGlo-1.[38] This large difference in the number of

281   reactive cellular thiols is expected to affect the kinetics and overall extent of Hg(0) oxidation.

282   Furthermore, Hu et al.[16] showed that at low cell concentrations G. sulfurreducens PCA oxidizes

283   very little Hg(0) and requires the addition of exogenous cysteine to facilitate Hg(0) oxidation.

284   These observations suggest that biosynthesis of thiol-containing molecules and the flux between

285   oxidized and reduced thiol pools may be key controls on Hg(0) oxidation by anaerobic Hg-

286   methylating bacteria.

287   The thiol compounds in D. desulfuricans ND132 involved in Hg(0) oxidation have not

288   yet been identified. The most likely sites of reaction are the sulfhydryl functional groups

289   associated with cysteine residuals in solvent exposed proteins or small biomolecules. Oxidized

290   Hg has been shown to interact with thioredoxins[39] which are small cysteine containing proteins

291   produced by D. desulfuricans in response to oxidative stress.[40] Oxidative stress also induces the

292   expression of several thiol-specific peroxidases in Desulfovibrio including thiol-peroxidase,

293   bacterioferritin comigratory protein (BCP), and glutaredoxin.[41] Conversely glutathione, which

294   occurs at high levels in aerobic bacteria, is not produced by Desulfovibrio[42] and is unlikely to be

295   involved in Hg(0) oxidation unless assimilated from external sources.

296   The EXAFS analysis revealed that multiple forms of Hg-thiol complexes are produced

297   by intracellular Hg(0) oxidation in D. desulfuricans ND132. Linear combination fitting (LCF) of

298   the k^2 weighted $\chi(k)$ data using Hg-cysteine and Hg-(cysteine)$_3$ standards as end members

14

299   suggest a distribution of 70% Hg-S_1 and 30% Hg-S_3 in the 1 hour spheroplast sample, and 15%

300   Hg-S_1 and 85% Hg-S_3 in the 6 hour sample. These LCF estimates are in reasonably good

301   agreement with the shell-by-shell fitting results shown in Figure 4 and Table 1. A mixture of Hg-

302   S_1 and Hg-S_3 was also observed in the amplitude of the FT EXAFS data (Fig. S3). Destructive

303   interference of Hg-S_1 and Hg-S_3 signals, which are out of phase, resulted in much lower

304   amplitude for the FT EXAFS data for 1 h sample compared with the Hg-cysteine standard. The

305   chemical species Hg-S_2, which has a strong EXAFS signal, was not detected. On the other hand,

306   the amplitude for the FT EXAFS data for 6 h sample is similar to that of Hg-(cysteine)$_3$ standard.

307   Notably, the EXAFS analysis showed that the bond distance of Hg-S complexes changes

308   over time from 2.35 Å for the 1 h sample to 2.43 Å for the 6 h sample (Fig. 3B-D). This increase

309   in Hg-S bond distance is consistent with a modification of the Hg coordination environment from

310   1 S atom to 3 S atoms.[43] The Hg-S bond distance for the 1 hour sample is similar to Hg-thiol

311   bonds distances found in natural organic matter (2.34 Å) where Hg coordinates to a single sulfur

312   atom in a thiolated aromatic unit.[44] The 6 hour sample is dominated by a Hg-S_3 complex which

313   have been show to form in aqueous solutions when the cysteine ligand concentration exceeds

314   that of oxidized Hg by two fold.[45] Hg-S_4 complexes, which form in highly alkaline solutions,[46]

315   are not likely to occur at physiological pH values of strain ND132. These findings indicate in the

316   presence of thiol-containing biomolecules, there is an evolution of the local coordination

317   environment of the intracellular oxidized Hg, with the stoichiometry of Hg-S bonding changing

318   from a predominately Hg-S_1 coordination environment to a more stable Hg-S_3 configuration with

319   increasing reaction time.

320   **Environmental Implications:** The experimental set up employed in our study resembles

321   the mercury contaminant situation at many industrial areas where a significant amount of the

322 elemental mercury waste persists in soils and sediments as liquid Hg beads.[47] Constant

323 evaporation and dissolution of the elemental mercury in our experiments mimic the continuous

324 supply of Hg that is leached into groundwater and becomes available for microbial interaction as

325 dissolved Hg(0). An example of this is the mercury contamination at Oak Ridge National

326 Laboratory (ORNL), Tennessee (USA) where members of Desulfovibrionaceae have been found

327 in groundwater monitoring wells at the Field Research Center of ORNL [48, 49] adjacent to areas

328 where large amounts of elemental mercury were released into the environment.[11, 12] The

329 oxidation of Hg(0) by Desulfovibrionaceae may represent one of the pathways of mercury

330 transformation at this site.

331 Intracellular Hg(0) oxidation in D. desulfuricans ND132 has important implications for

332 the production of methylmercury from dissolved elemental mercury. Because mercury

333 methylation is a cytosolic process, intracellular oxidation of Hg(0) to Hg(II) bypasses uptake

334 limitations for importing mercury into the cell and may provide a direct pathway for methylation.

335 Conversely, oxidized mercury associated with the cell wall can undergo desorption in the

336 presence of high affinity aqueous ligands. Low-molecular-weight thiol compounds that strongly

337 bind Hg are common in anoxic aquatic systems,[50] and the competitive binding of Hg(II) by

338 fulvic acids has been shown to decrease the extent of mercury adsorption onto cell walls of

339 Bacillus subtilis, Shewanella oneidensis MR-1, and G. sulfurreducens PCA bacterial species.[35]

340 Furthermore in sulfidic waters, cell surface-associated Hg can partition into mineral forms and

341 precipitate as mercuric sulfide thus removing oxidized mercury from the cell. This is in contrast

342 to intracellular oxidized mercury which is not subject to desorption/precipitation processes. What

343 is not known is the reactivity of the intracellular oxidized mercury for methylation. An

344 interesting question is whether or not the Hg-S_1 and Hg-S_3 forms of intracellular mercury

345 detected in D. desulfuricans ND132 represent different bioavailable pools for methylation.

346 Understanding the reactive forms of intracellular mercury and the molecular reactions that

347 connect Hg(0) oxidation to MeHg production merit further investigation.

348

349 **ASSOCIATED CONTENT**

350

351 **Supporting Information.** Oxidation of Hg(0) to Hg(II) by heat-treated spheroplasts lysate in

352 MOPS buffer and by whole cells in sucrose buffer. XAS analysis of Hg(0)-reacted D.

353 desulfuricans ND132 spheroplasts including XANES spectra, k^2-weighted EXAFS spectra, and

354 the magnitude and Fourier-transformed EXAFS spectra. The supporting information is available

355 free of charge on the ACS Publication website.

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Table 1: Best fit values for EXAFS analysis of spheroplast samples.

Sample	path	CN	R (Å)	$\sigma^2*10^{-3}(\text{Å}^{-1})$	ΔE (eV)
ND132; 1 hr	Hg-S	1.36 ± 0.24	2.35 ± 0.01	2.5 ± 1.5	-1.46 ± 1.1
ND132; 6 hrs	Hg-S	2.48 ± 0.43	2.43 ± 0.01	8.1 ± 2.3	-1.46 ± 1.1

Note: CN, R and σ^2 represent coordination number, distance, and variance, respectively.

Figure 1. Fluorescence intensities of D. desulfuricans ND132 reacted with Thiol Fluorescent Probe IV (TFP-4). (A) Spheroplast lysate (open circles); (B) Cell wall fragments (open squares); (C) ND132 whole cells (closed circles). Each data point represents an individual experiment conducted in the same day.
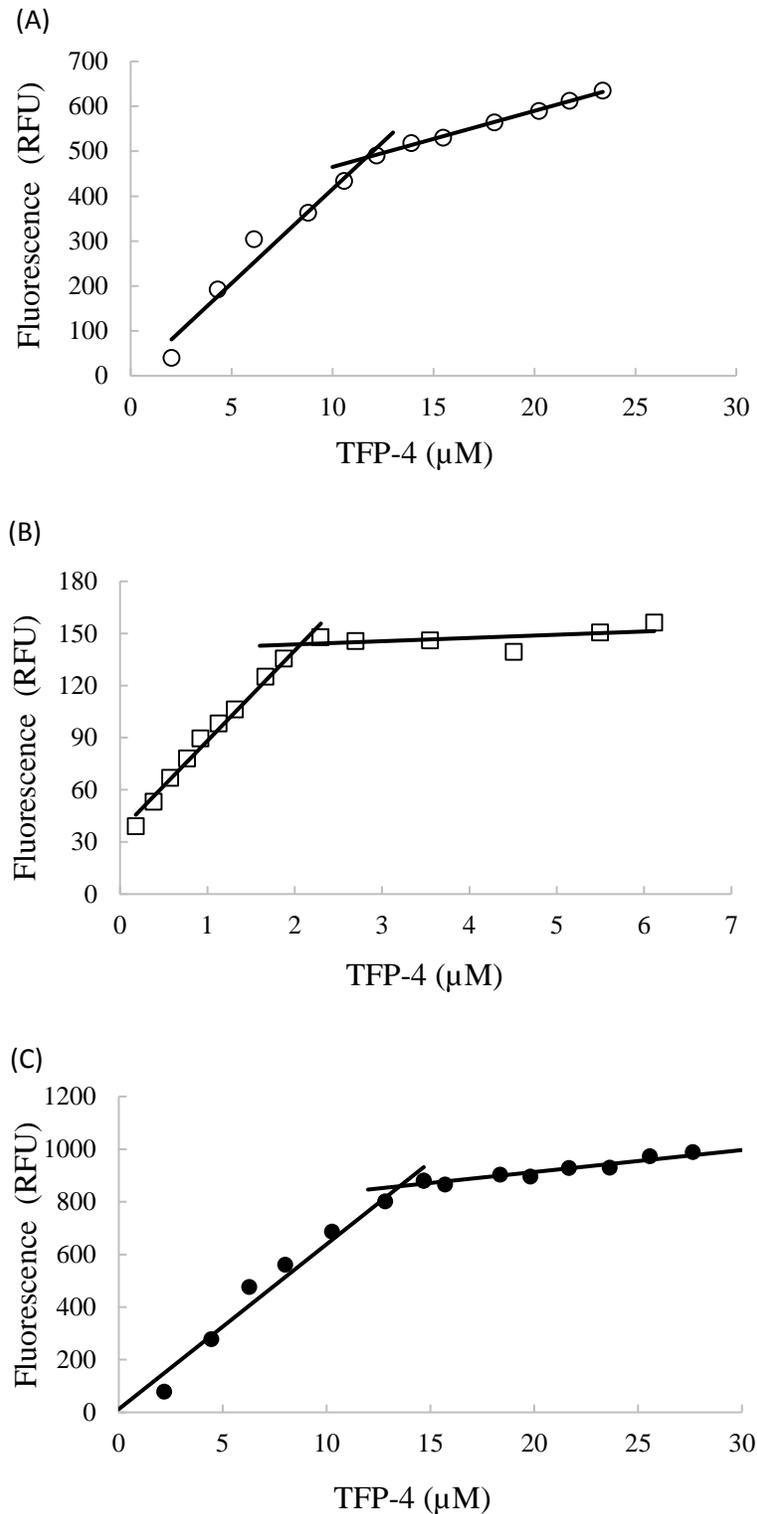
Figure 2. Oxidation of Hg(0) to Hg(II) by D. desulfuricans ND132. (A) Production of Hg(II) by D. desulfuricans ND132 whole cells (closed circles) and TFP-4 treated whole cells (closed triangle) in MOPS buffer. (B) Hg(II) formed by spheroplasts (open circles) and cell walls (open squares) in sucrose buffer. (C) Formation of Hg(II) by whole cells (closed circles) and spheroplasts lysate suspensions (open circles). All experiments were conducted with an initial cell concentration of 8×10^8 cells/mL. Points and error bars represent the means and standard deviations of triplicate experiments.
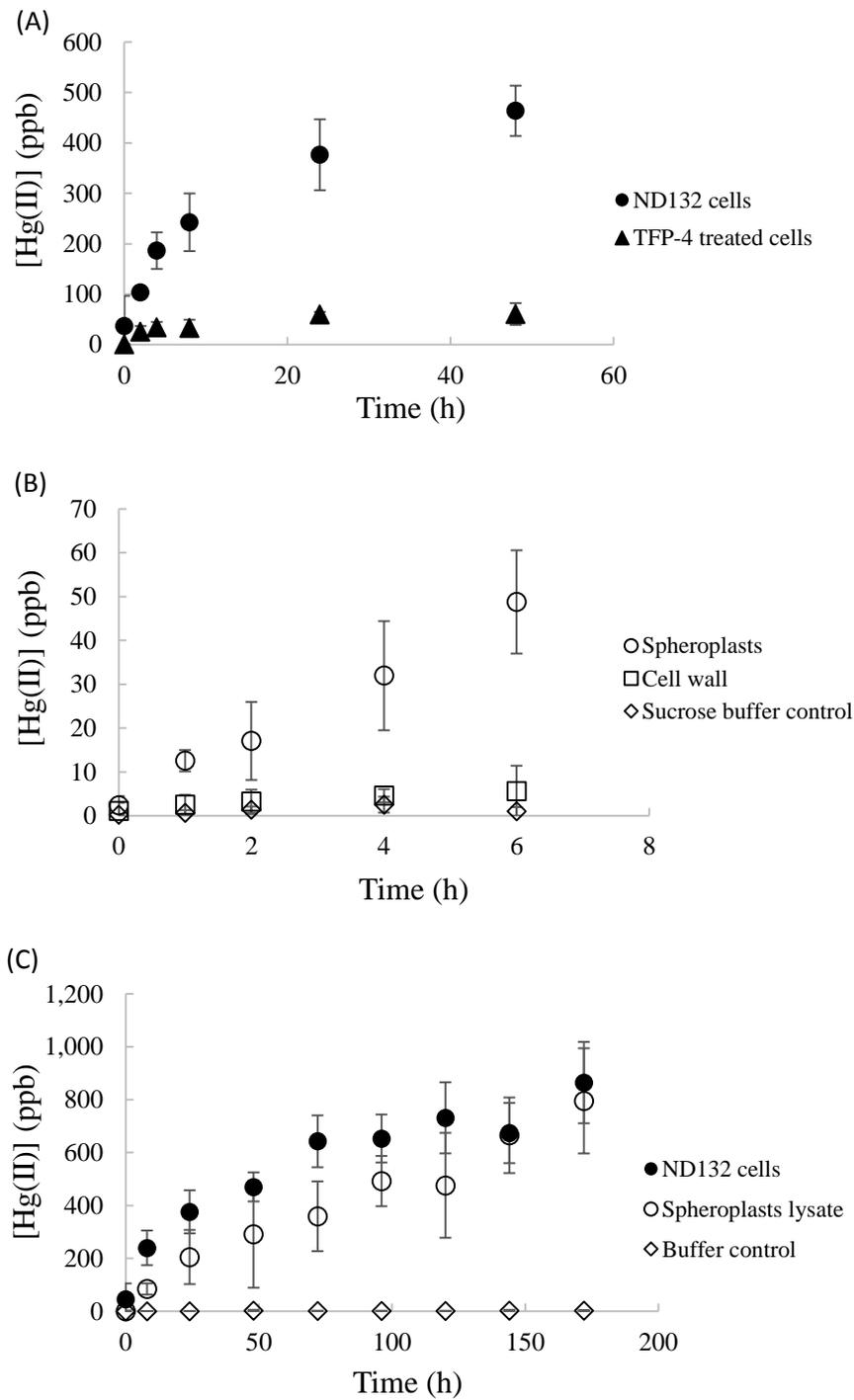
Figure 3. Structure characterization of Hg(0)-reacted D. desulfuricans ND132 spheroplasts by X-ray absorption spectroscopy (XAS) analysis at the Hg LIII-edge. (A) XANES spectra of spheroplasts samples collected at 1 h (black curve) and 6 h (red curve). The inset shows the first derivatives of the XANES spectra. (B) The k^2-weighted EXAFS spectra in k-space collected on the 1 h and 6 h spheroplasts samples. (C) Magnitude of the Fourier-transformed EXAFS spectra in R-space of 1 h and 6 h spheroplasts samples. (D) Real part of Fourier-transformed EXAFS spectra.
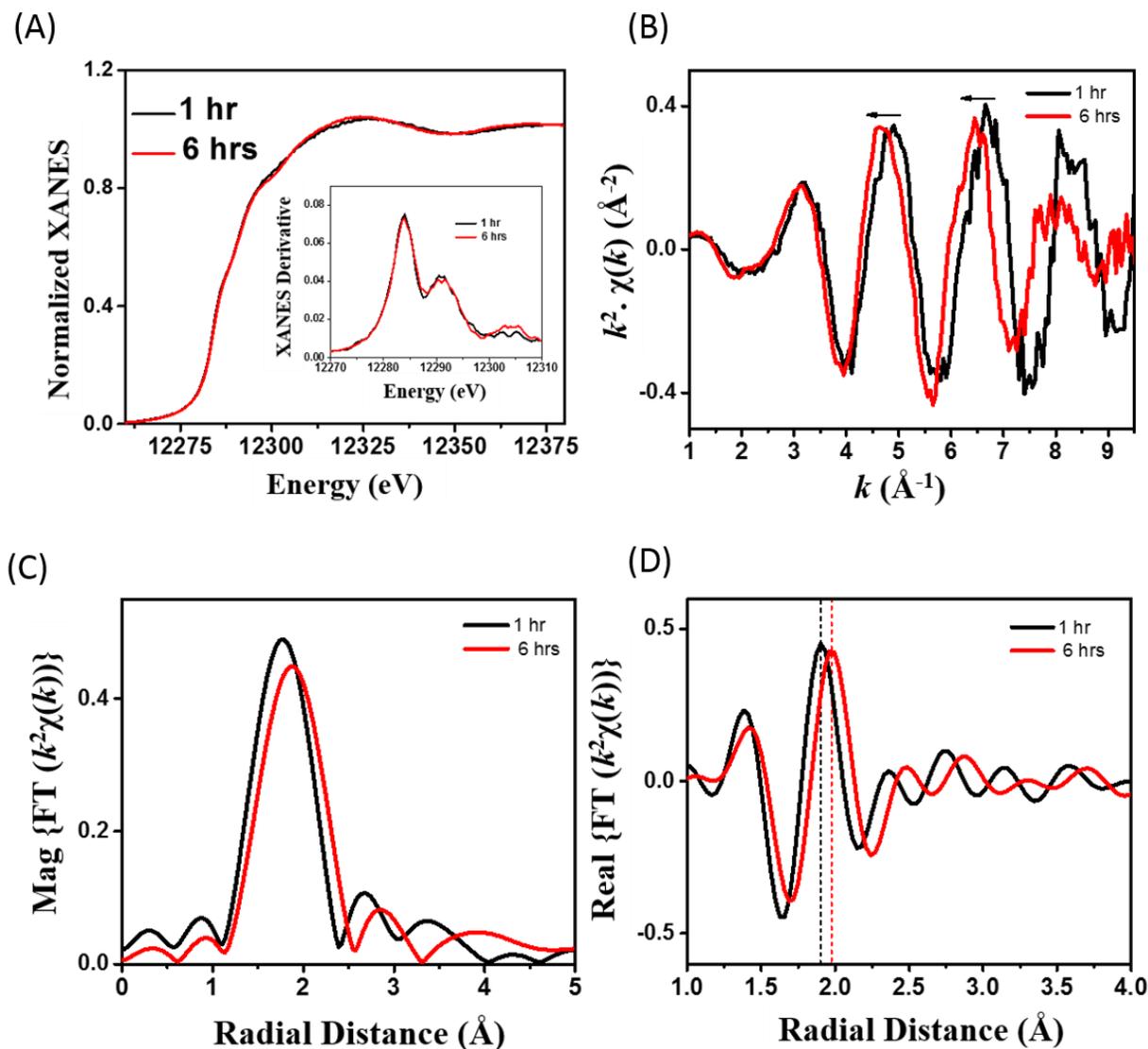
Figure 4: EXAFS data and fit for the (a) magnitude and (b) real part of the Fourier-transformed EXAFS spectra in R-space of 1 h and 6 h spheroplasts samples.
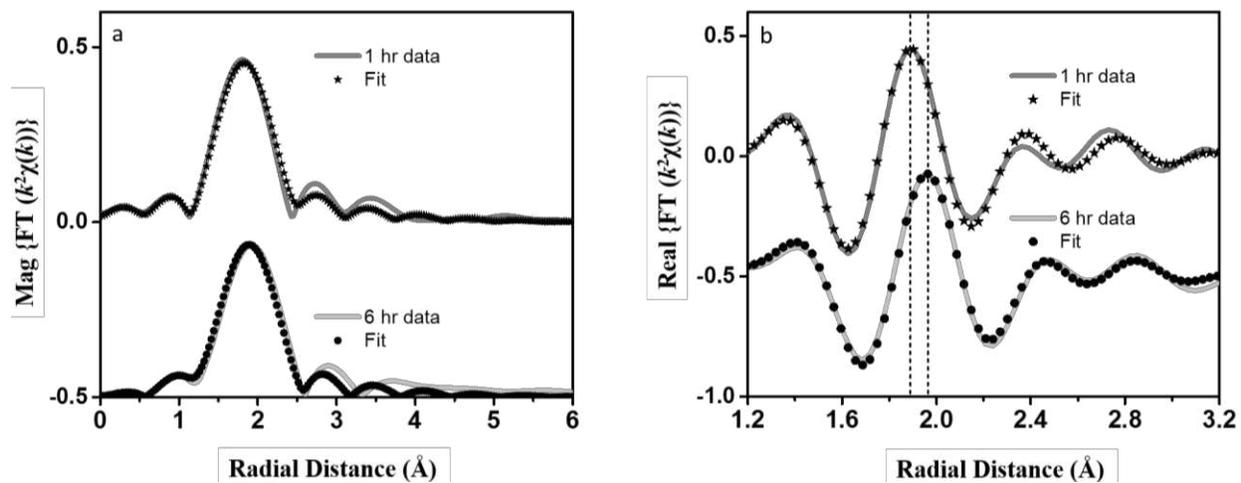
SUPPORTING INFORMATION

Intracellular Hg(0) oxidation in Desulfovibrio desulfuricans ND132

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SUPPORTING INFORMATION

Figure S1: Oxidation of Hg(0) to Hg(II) by D. desulfuricans ND132 spheroplasts lysate (closed circles) and heat-treated spheroplasts lysate (open circles). Experiments were conducted with an initial cell concentration of 8×10^8 cells/mL. Spheroplasts lysate was treated in 80 °C water bath for 10 min. Points and error bars represent the means and standard deviations of triplicate experiments.

Figure S2: Oxidation of Hg(0) to Hg(II) by D. desulfuricans ND132 whole cells (closed squares) and spheroplasts (open circles) in sucrose buffer. Experiments were conducted with an initial cell concentration of 8×10^8 cells/mL. Points and error bars represent the means and standard deviations of triplicate experiments.

Figure S3. XANES spectra of Hg(0)-reacted D. desulfuricans ND132 spheroplasts (a) Normalized XANES and (b) first derivative of XANES spectra of spheroplasts samples collected at 1 h and 6 h with Hg-acetate, Hg-cysteine, and Hg-(cysteine)$_3$ standards.

Figure S4: The k^2-weighted EXAFS spectra in k-space collected on the 1 h and 6 h spheroplasts samples with Hg-cysteine and Hg-(cysteine)$_3$ standards.

Figure S5: (a) Magnitude and (b) real part of the Fourier-transformed EXAFS spectra in R-space of 1 h and 6 h spheroplasts samples with Hg-cysteine and Hg-(cysteine)$_3$ standards.