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The role of cysteine and sulfide in the interplay between microbial Hg(II) uptake and sulfur metabolism

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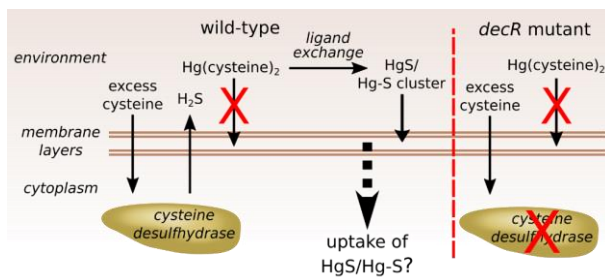
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TOC

Mercury uptake by *E. coli* in the presence of excess cysteine is facilitated by the biodegradation of cysteine to sulfide and the formation of mercury sulfide species. The deletion of a key cysteine desulfhydrase gene (*decR*) limits the uptake of mercury in the presence of excess cysteine.



Significance to Metallomics

The present study describes how the cellular sulfur metabolism can influence Hg(II) biouptake by bacteria, potentially leading to the misinterpretation of results if not considered. We demonstrate that the high Hg(II) bioavailability previously observed in the presence of excess cysteine is dependent on the biodegradation of cysteine to sulfide and the formation of cell-associated Hg(II)-sulfide species.

1 **Abstract**

2 Biogenic thiols, such as cysteine, have been used to control the speciation of Hg(II) in bacterial exposure
3 experiments. However, the extracellular biodegradation of excess cysteine leads to the formation of
4 Hg(II)-sulfide species, convoluting the interpretation of Hg(II) uptake results. Herein, we test the
5 hypothesis that Hg(II)-sulfide species formation is a critical step during bacterial Hg(II) uptake in the
6 presence of excess cysteine. An *Escherichia coli* (*E. coli*) wild-type and mutant strain lacking the *decR*
7 gene that regulates cysteine degradation to sulfide were exposed to 50 and 500 nM Hg \pm 0.5 to 2 mM
8 cysteine. The *decR* mutant released ~4 times less sulfide from cysteine degradation compared to the wild-
9 type for all tested cysteine concentrations during a 3 hour exposure period. We show with thermodynamic
10 calculations that the predicted concentration of Hg(II)-cysteine species remaining in the exposure medium
11 (as opposed to forming HgS_(s)) is a good proxy for the measured concentration of dissolved Hg(II) (i.e.,
12 not cell-bound). Likewise, the measured cell-bound Hg(II) correlates with thermodynamic calculations
13 for HgS_(s) formation in the presence of cysteine. High resolution x-ray absorption near edge structure
14 (HR-XANES) spectra confirm the existence of cell-associated HgS_(s) at 500 nM total Hg and suggest the
15 formation of Hg-S clusters at 50 nM total Hg. Our results indicate that a speciation change to Hg(II)-
16 sulfide controls Hg(II) cell-association in the presence of excess cysteine.

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22 **Introduction**

23 The bioaccumulation of alkylated mercury (Hg) species poses significant risks to ecosystem and human
24 health. However, the mechanism of bacterial uptake of inorganic Hg(II) species that leads to alkyl-
25 mercury (e.g., methylmercury – MeHg) formation remains unknown.¹ One way to gain insight into the
26 uptake mechanism, which has been extensively investigated in the literature, is to test how the Hg(II)
27 speciation in the exposure medium can affect Hg(II) bioavailability.²⁻¹² In the environment, Hg(II) is
28 expected to be bound to either thiolate groups of low molecular weight ligands/natural organic matter
29 (NOM) or sulfides due to the high affinity of Hg(II) for reduced sulfur.¹³ Among various thiols,
30 exogenous cysteine has been shown to play an important role in regulating Hg(II) uptake, greatly
31 enhancing or inhibiting it depending on the cysteine and Hg(II) concentration.^{3-5, 7, 11, 14, 15}

32 Cysteine is an amino acid that plays a central role in cellular sulfur metabolism.^{16, 17} A few of the
33 various metabolic pathways involving cysteine include the biosynthesis of methionine, the formation of
34 iron sulfur (Fe-S) clusters, and the biosynthesis of glutathione.^{16, 18-24} The thiolate group of cysteine assists
35 protein folding via the formation of disulfide bonds, exists in the catalytic sites of enzymes, and binds
36 strongly to soft acid metals (e.g., Hg(II), Cd(II), Pb(II), and Ag(I)).²⁴ However, due to its high reactivity,
37 cysteine is toxic to cells. As a result, cells have complex regulatory systems to maintain strict control of
38 intracellular cysteine concentrations.¹⁷ In response to increased intracellular cysteine concentration,
39 cysteine desulfhydrase and/or desulfidase enzymes are activated to degrade cysteine into hydrogen
40 sulfide, pyruvate, and ammonium.²⁵⁻²⁹ Although the biodegradation of biologically important thiols (e.g.,
41 cysteine and glutathione) has been well documented,³⁰⁻³² past Hg(II) uptake experiments have rarely
42 considered how the Hg(II) speciation in the exposure medium may change over time due to ligand
43 degradation, synthesis, or secretion by the bacteria. The ability of exogenous thiols to degrade into sulfide
44 and the ability of bacteria to produce sulfide endogenously (i.e., not limited to dissimilatory sulfate
45 reduction) is of particular importance for understanding Hg(II) bioavailability due to the evidence that
46 neutrally-charged dissolved and nanoparticulate Hg(II)-sulfide species may be bioavailable to bacteria via
47 passive diffusion.^{10, 33, 34}

48 Previous studies, including our own, have shown, relying on thermodynamic calculations, that the
49 sulfide released from exogenous cysteine degradation can outcompete excess cysteine and bind Hg(II) in
50 the exposure medium.^{10, 12, 35, 36} In agreement with these calculations, we have shown using x-ray
51 absorption spectroscopy (XAS) and scanning transmission electron microscopy (STEM) that cell-
52 associated HgS_(s) nanoparticles form after the exposure of *Escherichia coli*, *Geobacter sulfurreducens*,
53 and *Bacillus subtilis* to Hg(II) and excess cysteine.³⁵ Pre-equilibrated Hg(II)-cysteine complexes that are
54 added to cell suspensions will form cell-associated α -HgS_(s) or β -HgS_(s) at relatively high total added
55 Hg(II) concentrations (500 nM – 5 μ M) and short time scales (< 1 hour). At lower added Hg(II)
56 concentrations (50 nM), we also observed the formation of cell-associated Hg(II)-sulfide species from
57 Hg(II)-cysteine complexes. However, the relatively low signal to noise ratio of conventional XAS at these
58 Hg(II) concentrations limited our ability to determine the nature of the Hg(II)-sulfides (e.g.,
59 nanoparticulate β -HgS_(s) or smaller, β -HgS-like Hg-S clusters). In addition, we were unable to directly
60 relate Hg(II)-sulfide species formation to Hg(II) uptake into the cytoplasm.

61 The goal of this study is to further investigate Hg(II) bioavailability by testing the hypothesis that
62 when sulfide and cysteine coexist in the exposure medium, Hg(II)-sulfide species, and not Hg(II)-cysteine
63 species, undergo biouptake. Due to the simplicity of modifying the genome, wild-type and mutant strains
64 of *E. coli* lacking single genes involved in sulfide biosynthesis ($\Delta decR$, $\Delta sufS$, and $\Delta iscS$) were chosen for
65 these experiments. We exposed bacterial cells to 50 or 500 nM total Hg(II) that was pre-equilibrated with
66 0 to 2 mM cysteine so that Hg(II) was introduced to exposure assays as Hg(II)-cysteine complexes with
67 an excess of cysteine. Over a period of 3 hours, we quantified the total cysteine, total sulfide, and total Hg
68 concentration in the exposure medium every hour in order to predict Hg(II) speciation. To identify the
69 cell-associated Hg(II) coordination environment, we probed cell pellets that were exposed to Hg(II) \pm
70 cysteine for 3 hours with high energy resolution X-ray absorption near edge structure (HR-XANES)
71 spectroscopy.

72 **Materials and methods**

73 ***Bacterial strains***

74 Keio collection strains of *E. coli* single-gene deletion mutant JW0437 ($\Delta decR$, formerly known as $\Delta ybaO$)
75 as well as wild-type *E. coli* K-12 (BW25113) were obtained from the *E. coli* Genetic Stock Center.³⁷ The
76 cysteine desulfurase mutants ($\Delta iscS$ and $\Delta sufS$) were obtained from the BioCat team in the Chemistry and
77 Biology of Metals Laboratory (Grenoble, France) and are described in detail in Ranquet et al.³⁸ The
78 strains were regenerated from sterile filter disks or frozen glycerol stock (stored at -80 °C) onto LB agar
79 (with 50 mg/L kanamycin for mutants) at 37 °C for 24 hours. The strains were stored on LB agar plates at
80 4 °C for no longer than 4 weeks.

81 ***Growth media and cell harvesting***

82 A single colony of *E. coli* from a refrigerated LB agar plate was inoculated into ~3 mL of LB broth in a
83 sterile, 12 mL polypropylene tube and incubated aerobically at 37 °C with medium shaking for ~6 hours.
84 Subsequently, 20 – 100 μ L of the cell suspension was inoculated into 50 – 100 mL of minimal salts
85 medium (MSM; Table S1) in sterile, foil-topped 125 mL or 250 mL Erlenmeyer flasks and shaken at 37
86 °C overnight. Cells were harvested in MSM during exponential growth phase ($OD_{600} = 0.2$). Cells were
87 washed twice with minimal complexing medium (MCM) – the exposure medium for Hg(II) biouptake
88 assays – and resuspended to a density of 2×10^8 cells/mL, which is equivalent to an OD_{600} of 0.2. MCM
89 is buffered to pH = 7.1 with 20 mM MOPS and contains 1 mM Na- β -glycerophosphate, 0.41 mM MgSO₄,
90 12 mM NH₄NO₃, 0.76 mM isoleucine, 0.76 mM leucine, 3 nM thiamine, 10 mM glucose, and 9.1 mM
91 NaOH.⁸ All mutant strains of *E. coli* were grown in the presence of 50 mg/L kanamycin.

92 ***Hg(II) and cysteine exposure assays***

93 A 10 mM Hg(NO₃)₂ stock solution in 1% HNO₃ (trace metal grade; TMG) was used for all exposure
94 assays and stored at 4 °C. A 100 mM cysteine stock solution was prepared in deionized water (18 M Ω)
95 immediately before use. Hg(II) and cysteine were pre-equilibrated for 1 hour in deionized water (18 M Ω)
96 at 10 times the final desired concentrations (fixed molar ratios of 1:2,000, 1:10,000, 1:20,000, or 1:40,000

97 Hg:Cys) prior to being added to cell suspensions. Hg(II) and cysteine exposure assays were aerobic and
98 conducted in 15 mL borosilicate glass vials or 125 mL Erlenmeyer flasks with 7 mL and 50 mL total
99 volume, respectively. The assays were conducted under dark conditions at 37 °C and initiated with the
100 addition of Hg(II) solution (\pm cysteine) to the cell suspension in MCM so that the Hg(II) solution (\pm
101 cysteine) was diluted by a factor of 10. The pH was measured before and after exposure to Hg(II) \pm
102 cysteine in MCM and did not significantly vary from the initial pH of 7.1.

103 *Hg(II)-cell sorption measurements*

104 After cell suspensions mixed with Hg(II) and cysteine for 0, 1, 2, and 3 hours (7 mL total volume), ~700
105 μ L aliquots were collected at each time point for the determination of (1) total recoverable Hg (dissolved
106 + cell-bound) and (2) dissolved Hg (after filtration on a 0.2 μ m nylon filter, VWR International). As we
107 documented earlier, the nylon filters do not bind a significant amount of Hg.³⁶ The samples at 0 hours
108 were collected as soon as possible after Hg addition to cell suspensions, and thus, cells were exposed to
109 Hg(II) for up to 1 minute of mixing. The cell-bound Hg was calculated as the difference between the total
110 recoverable Hg and the dissolved Hg. Samples for determining dissolved and total recoverable Hg were
111 preserved in ~1% HCl (TMG) until the measurement of total Hg with a Direct Mercury Analyzer (DMA-
112 80, Milestone).

113 *Sulfide and cysteine detection in the exposure medium*

114 After mixing cells with cysteine for 0, 1, 2, and 3 hours (7 mL total volume), a 1 mL aliquot was
115 centrifuged (15,000 g for 5 min) for the determination of acid labile sulfide in the supernatant by a
116 method adapted from Cline^{39, 40} as well as cysteine and cystine (oxidized cysteine) by a method adapted
117 from Gaitonde.⁴¹ Both the Cline and Gaitonde methods are colorimetric and specific to sulfide and
118 cysteine, respectively. Detailed methods are reported in our previous publication.³⁶ The detection limit for
119 sulfide and cysteine was 2 μ M and 5 μ M, respectively.

120 *Bacterial samples for HR-XANES measurements*

121 After a 3 hour exposure of cells to Hg(II) ± cysteine (50 mL total volume), the cells were washed 2 times
122 by centrifugation (8,000 g for 5 min) with an equivalent volume of 0.1 M NaClO₄.⁴² During the washing
123 steps, the cell suspension was resuspended in smaller volumes in succession until cells were in a 1 mL
124 final suspension of 0.1 M NaClO₄. The 1 mL of suspension was added to a 1.5 mL centrifuge tube
125 containing a 0.2 µm cellulose acetate centrifugal filter (~8 mm diameter cut with a hole punch). The tube
126 was centrifuged briefly for 2 minutes at ~3,000 g so that the cells were collected on the filter and the
127 medium passed through the filter. The filter paper containing pelleted cells was subsequently sandwiched
128 between 2 pieces of Kapton tape and immediately plunged in liquid nitrogen. The samples were stored at
129 -80 °C for no more than 1 week and remained frozen throughout analysis.

130 *HR-XANES experimental setup and analysis.*

131 The HR-XANES experiments were performed at the European Synchrotron Radiation Facility (ESRF) at
132 beamlines BM30B FAME and BM16 FAME-UHD. All Hg standards and samples were measured in high
133 energy resolution fluorescence detection (HERFD) mode with 5 spherically bent Si(111) crystal analyzers
134 (bending radius = 1 m, crystal diameter = 0.1 m). The Hg L_{α1} fluorescence line (apparent core-hole
135 lifetime broadening of 2.12 eV)⁴³ was selected using the 555 reflection, and the diffracted fluorescence
136 was measured with a silicon drift detector (SDD, Vortex EX-90). The monochromator was calibrated with
137 a Se reference foil (K-edge = 12,658 eV), and a HgCl₂ powder was scanned at the start of each
138 experiment to maintain relative energy calibration. Hg powder standards were finely ground, diluted to
139 ~0.5 wt% with boron nitride, pressed into ~5 mm diameter pellets, and loaded onto a copper sample
140 holder. Liquid Hg reference standards were pipetted into a copper sample holder sealed on two ends with
141 Kapton tape and immediately plunged into LN₂ to minimize contact of the liquid with the copper as well
142 as to prevent the formation of ice during freezing. Frozen bacterial samples were fixed onto copper
143 sample holders with grease and quickly plunged into liquid nitrogen to prevent the sample from thawing.
144 All liquid Hg references and bacterial samples containing Hg were measured at 10 – 15 K. Powdered Hg
145 references (α-HgS_(s) and β-HgS_(s)) were measured at 10 – 15 K and room temperature for comparison.

146 Data normalization and linear combination fits of the XANES to determine the Hg speciation in bacterial
147 samples were performed with Athena.⁴⁴ Details on the preparation of Hg reference standards for HR-
148 XANES is provided in the SI (page S2).

149 *Sample preparation and imaging with TEM*

150 After mixing cells with Hg(II) ± cysteine for 3 hours, a 1 – 2 mL aliquot was collected and
151 washed 4 times with 0.1 M NaClO₄ by centrifugation (8000 g for 3 min) in a 1.5 – 2 mL microfuge tube
152 to remove Hg not associated with cells. After the final wash, the cells were resuspended in a solution of
153 200 µL filtered Milli-Q water (0.2 µm filter, VWR International). One drop (< 5 µL) was immediately
154 placed on a 200 mesh carbon-coated copper grid and allowed to air dry for ~10 minutes. TEM
155 micrographs and selected area electron diffraction (SAED) patterns were collected at room temperature
156 with a Hitachi H-8100 transmission electron microscope using an accelerating voltage of 200 kV.

157 *Thermodynamic calculations*

158 The speciation calculations for Hg(II) were performed with the program ChemEQL.⁴⁵ The equilibrium
159 constants used in the calculations are reported in Table S2.

160 **Results and Discussion**

161 *Sulfide production from cysteine degradation*

162 The transcription factor DecR (formerly known as YbaO) activates the *yhaOM* operon, where the *yhaO*
163 gene is predicted to be responsible for cysteine import and the *yhaM* gene appears to have cysteine
164 desulfhydrase (also known as desulfidase) activity.²⁸ *yhaM* is cysteine-inducible and has been shown to
165 have the most significant role in cysteine detoxification in *E. coli* among the numerous other reported
166 enzymes (e.g., TnaA, CysK, CysM, MalY, and MetC).⁴⁶ The deletion of the *decR* gene was previously
167 shown to strongly limit the desulfhydrase activity in *E. coli*.²⁸ We exposed a wild-type and *decR* deletion
168 mutant strain of *E. coli* to cysteine concentrations ranging from 0 to 2 mM and measured the total acid

169 labile sulfide concentration in the exposure medium every hour for 3 hours (Figure 1) by the Cline
170 method.³⁹

171 All added cysteine concentrations tested lead to essentially the same sulfide concentration in the
172 exposure medium at each time point for both the wild-type and *decR* mutant, indicating that cysteine
173 degradation to sulfide reaches a threshold for both strains at 0.5 mM added cysteine and above. Notably,
174 the sulfide concentration in the exposure medium reaches a maximum of ~40 μM (2 hours) for the wild-
175 type strain but only ~10 μM (1 – 2 hours) for the *decR* mutant. After just 1 hour of exposure, the
176 measured sulfide concentration in the exposure medium for all added cysteine concentrations is ~20 μM
177 and 5 -10 μM for the wild-type and *decR* mutant, respectively. Although the *decR* mutant is still able to
178 degrade cysteine and release sulfide into the exposure medium, the loss of cysteine desulfhydrase activity
179 results in a significant decrease in the concentration of total sulfide in the exposure medium.

180 The concentrations of reduced and total cysteine (i.e., reduced + oxidized) in the exposure
181 medium have also been quantified under the same conditions as the sulfide measurements (Figure 2) by
182 the Gaitonde method, which is cysteine-specific and not affected by similar thiols (e.g., glutathione and
183 homocysteine).⁴¹ A Tukey's honest significant differences test was performed on the wild-type and *decR*
184 mutant datasets for each added cysteine concentration (i.e., 0.5, 1, and 2 mM) to determine the statistical
185 significance ($p < 0.05$) between the measurements. At $t = 0$ hours, the concentration of reduced cysteine
186 is less than the total cysteine due to the known oxidation of cysteine in the exposure medium.^{35, 36} Thus,
187 all assays begin with reduced cysteine concentrations that are ~70 to 90% of the total added cysteine, with
188 cystine accounting for the remainder. With increased incubation time, the cysteine concentration in all
189 assays decreases, mainly due to cysteine biouptake/biodegradation and not oxidation because the total
190 cysteine concentration (reduced + oxidized) also decreases by nearly the same amount. The most notable
191 decrease in cysteine concentration is observed in the wild-type cells exposed to 0.5 mM cysteine, where
192 the initial reduced cysteine concentration drops from ~0.35 mM to ~0.2 mM after 2 hours of exposure.
193 The decrease in the concentration of cysteine is not as drastic for the *decR* mutant under the same

194 conditions because it has lost some ability to import and degrade cysteine. For the higher total added
195 cysteine concentrations of 1 and 2 mM, the cysteine concentration in the exposure medium decreases over
196 time, but the differences between the wild-type and *decR* mutant are not statistically significant.

197 ***Hg sorption measurements and thermodynamic calculations***

198 The concentration of dissolved and cell-bound Hg was quantified in wild-type and *decR* mutant assays as
199 a function of incubation time, added cysteine, and added Hg (Figure 3). For many measurements, the
200 sums of the dissolved and cell-bound concentrations do not add to the total added Hg, which we observed
201 in our previous studies on *E. coli*.^{35, 36} We interpreted this as a result of Hg(II) reduction to Hg(0),
202 potentially from outer membrane cytochromes,⁴⁷ and loss from volatilization. When the wild-type and
203 *decR* mutant were exposed to either 50 nM or 500 nM Hg without cysteine, the cell-bound Hg is between
204 40 to 70% of the total added Hg after 1 hour of exposure and does not change significantly after 3 hours
205 (Figure 3A,E,G, and K). The dissolved Hg for these conditions remains between 0 and 5% of the total
206 added Hg after 1 hour of exposure, demonstrating efficient Hg(II) sorption by cells in the absence of
207 added cysteine.

208 To understand whether conditions favored Hg(II)-sulfide or Hg(II)-cysteine species formation for
209 the experiments involving cysteine exposure in Figure 3, we calculated the Hg(II) speciation in the
210 exposure medium at each time point (Figure 4). Specifically, we present the sum of the concentrations of
211 the Hg(II)-cysteine species as well as HgS_(s) (dissolved Hg(II)-sulfide species were negligible) as a
212 fraction of the total added Hg. Our thermodynamic calculations incorporated the total sulfide, cysteine
213 (reduced), and total recoverable Hg concentration measured in the exposure medium (e.g., information
214 from Figures 1, 2, and 3), which we also summarize in Table S3 for reference. We did not test the
215 exposure medium for thiols secreted by the cells (e.g., glutathione). However, it is not likely that secreted
216 thiols would reach a concentration in the exposure medium that could influence Hg speciation in the
217 presence of 0.5 – 2 mM added cysteine. The measured total recoverable Hg was used in these calculations
218 so that the results reflect whether the conditions would favor HgS_(s) or Hg(II)-cysteine formation prior to

219 Hg(II) sorption to cells. Our predictions for HgS_(s) formation in the presence of excess cysteine generally
220 agree with the measured fraction of cell-bound Hg. For example, we predict a significant amount of
221 HgS_(s) formation when the wild-type strain is exposed to 50 nM Hg(II) and 0.5 or 1 mM cysteine as well
222 as 500 nM Hg(II) and 1 mM cysteine (Figure 4A,B,D), and we concurrently observe a significant amount
223 of cell-bound Hg (Figure 3B,C,F). In addition, when the *decR* mutant strain is exposed to 50 nM Hg and
224 0.5 mM cysteine as well as 500 nM Hg and 1 mM cysteine, we predict a majority of HgS_(s) formation
225 (Figure 4E,H) and observe a majority of cell-bound Hg(II) (Figure 3H,L). The one exception is the 1 hour
226 time point for the *decR* mutant exposed to 50 nM Hg and 0.5 mM cysteine where HgS_(s) is predicted to be
227 ~50% of the total added Hg (Figure 4E), but the measured fraction of cell-bound Hg(II) is <10% of the
228 total added Hg (Figure 3H). When the majority of Hg(II) is predicted to remain bound to cysteine in the
229 exposure medium (i.e., Figure 4C,F,G), we see little cell-bound Hg(II) and mostly dissolved Hg(II)
230 (Figure 3D,I,J). Thus, there appears to be a link between dissolved Hg(II) and Hg(II)-cysteine complexes
231 outside the cell due to insufficient sulfide production to shift the equilibrium to HgS_(s) formation in the
232 presence of excess cysteine.

233 ***ATP measurements***

234 In healthy cells, ATP concentrations are highly regulated and thus can be used as an indicator of
235 biological stress.⁴⁸ We assessed the potential toxic effects of cysteine exposure to the wild-type and *decR*
236 mutant strains by quantification of cellular ATP (Figure S1). The wild-type strain does not experience any
237 significant decrease in cellular ATP upon exposure to 50 nM Hg ± 1 and 2 mM cysteine or 500 nM Hg ±
238 1 mM cysteine for 3 hours (Figure S1A) compared to the cellular ATP concentration prior to exposure.
239 However, the *decR* mutant does observe a statistically significant 10 – 20% drop ($p < 0.05$) in cellular
240 ATP when exposed to 50 nM Hg + 1 and 2 mM cysteine as well as 500 nM Hg ± 1 mM cysteine (Figure
241 S1B). This decrease does not indicate a major change in cellular metabolism. Exposure of the *decR*
242 mutant to 1 and 2 mM cysteine without Hg causes the same observed decrease in ATP concentration (data
243 not shown), suggesting that the effect is due to cysteine alone. Interestingly, the greatest drop in cellular

244 ATP is observed when the *decR* mutant is exposed to 500 nM Hg without cysteine, where ATP levels are
245 $1.4 \pm 0.2 \times 10^{-18}$ mol per cell after a 3 hour exposure (a 36% drop).

246 *Cell-associated Hg(II) coordination environments*

247 While the detection limit restricts our ability to directly measure Hg(II) speciation in the exposure
248 medium, we are however able to directly probe the Hg(II) coordination environment in bacterial cells by
249 XAS for the conditions in this study. Specifically, Hg L_{III}-edge HR-XANES data were collected to
250 drastically improve the resolution of spectral features compared to conventional XANES.⁴³ The use of
251 crystal analyzers removes most, if not all, of the contribution of the background fluorescence photons, and
252 therefore, one can investigate the Hg(II) coordination environment in dilute systems (< 1 ppm).⁴⁹⁻⁵²

253 To corroborate the predictions for HgS_(s) formation and subsequent cell-association, we analyzed
254 select samples from Figures 3 and 4 by HR-XANES: wild-type and *decR* mutant exposed to 500 nM
255 Hg(II) ± 1 mM cysteine and 50 nM Hg(II) ± 1 mM cysteine for 3 hours (Figure 5). Hg L_{III}-edge HR-
256 XANES reference spectra are presented in Figure S2. The references of Hg(II) bound to two sulfur atoms
257 (i.e., α-HgS_(s) and Hg(cysteine)₂) contain a sharp, in-edge peak that represents a 2p_{3/2} → 6s/5d electronic
258 transition.⁵¹ This peak is absent in reference spectra of Hg bound to four sulfur atoms (i.e., β-HgS_(s) and
259 Hg(cysteine)₄). Because the Hg(cysteine)₂ species at pH = 11.6 also has two coordinating nitrogen atoms
260 at 2.51 Å,⁵⁰ the in-edge peak is not as sharp as those with solely 2 sulfur atoms in the coordination sphere.
261 In addition, the HR-XANES spectra of the two bulk HgS_(s) minerals – α-HgS_(s) and β-HgS_(s) – contain
262 defined peaks above the absorption edge that are lacking in the Hg(II)-thiol references. Thus, determining
263 the Hg(II)-sulfur coordination number and nature of the coordinating ligands (e.g., thiol or sulfide) can be
264 achieved with just the HR-XANES. Linear combination fit results, considering the Hg references
265 described above, are shown in red in Figures 5A,B,E,F,G. Some of the spectra are lacking best-fit curves
266 due to the absence of appropriate reference standards to explain the Hg(II) coordination environment
267 (Figure 5C,D,H). The HR-XANES of *E. coli* wild-type samples exposed to 500 nM Hg(II) ± 1 mM
268 cysteine are best fit with β-HgS_(s) and Hg(cysteine)₂ references (Figures 5A,B), confirming our previous

269 results from conventional XAS techniques for exponentially-growing *E. coli*.^{35, 36} Because we observe
270 cell-associated β -HgS_(s) when 500 nM Hg is added without cysteine, it is clear that an endogenous sulfide
271 source beyond cysteine desulfhydrase exists and binds Hg(II). The aqueous Hg(cysteine)₂ at pH = 3
272 standard was a better fit to the spectra than the Hg(cysteine)₂ at pH = 11.6, suggesting that Hg(II) exists as
273 RS-Hg-SR with insignificant Hg-N bonding. This coordination environment is expected when Hg(II) is
274 bound to 2 cysteine residues in a protein, as is the case when Hg(II) is bound to MerP,⁵³ because the
275 amine groups do not coordinate Hg(II). The spectrum of wild-type *E. coli* exposed to 500 nM Hg(II) and
276 1 mM cysteine highly resembles the β -HgS_(s) reference standard collected at room temperature. Manceau
277 et al. found that well-crystallized β -HgS_(s) HR-XANES spectra measured at room temperature resemble
278 those of nanoparticulate β -HgS_(s) measured at liquid helium temperature (~ 10 K),⁵¹ suggesting that the β -
279 HgS_(s) associated with *E. coli* is nanoparticulate. We confirmed the presence of cell-associated HgS_(s)
280 nanoparticles (~ 100 nm diameter) with transmission electron microscopy (TEM) (Figure S4). The
281 absence of diffraction spots in the selected area electron diffraction (SAED) pattern implies that the β -
282 HgS_(s) nanoparticles are amorphous (Figure S4C).³⁵

283 Surprisingly, the *decR* mutant contained more β -HgS_(s) when exposed to 500 nM Hg(II) compared
284 to the wild-type (75.6% vs. 58.7%; Figure 5A and 5E). Thus, the removal of one pathway for cysteine
285 degradation to sulfide in the cell actually increased the presence of cell-associated β -HgS_(s). This increase
286 in β -HgS_(s) formation could be a result of increased expression of other genes with cysteine desulfhydrase
287 ability to compensate for the loss of *decR*. Additionally, the Hg(II) species associated with the *decR*
288 mutant exposed to 500 nM Hg and 1 mM cysteine was primarily α -HgS_(s), unlike the wild-type where
289 cell-associated Hg(II) was primarily β -HgS_(s). We also detected the presence and phase of α -HgS_(s) with
290 TEM and SAED (Figure S5 and Table S4). We previously observed the formation of *E. coli*-associated α -
291 HgS_(s) exclusively when cysteine was present with Hg(II) and sulfide in the exposure medium, suggesting
292 that cysteine plays a role in determining the phase of HgS_(s).³⁵ We predicted that the ratio of total sulfide
293 to total Hg(II) determines the phase of HgS_(s) in the presence of cysteine. In this study, we confirmed this

294 prediction because cell-associated α -HgS_(s) forms at a total sulfide to total recoverable Hg(II) ratio of 15
295 and β -HgS_(s) forms at a ratio of 67, similar to the ratios that determined whether α -HgS_(s) (10) or β -HgS_(s)
296 (100) formed in our previous study.³⁵ Because our TEM results also reveal that the HgS_(s) particle
297 diameters are \leq 100 nm, we confirm that the cell-bound Hg(II) measurements from Figures 3F,L are
298 actually depicting cell-associated Hg(II) and not just HgS_(s) particles in solution that are trapped by the
299 200 nm pore size filter. HgS_(s) nanoparticles are lipophilic, as determined by octanol-water partitioning
300 experiments,⁵⁴ which supports our finding that HgS_(s) would prefer to attach to lipophilic cell surfaces.

301 Linear combination fits to the HR-XANES spectra of wild-type and *decR* mutant *E. coli* exposed
302 to 50 nM total Hg(II) \pm 1 mM cysteine do not fit well with the references in this study. However, a fairly
303 good fit is achieved with the *decR* mutant exposed to 50 nM Hg(II) considering 49.7% β -HgS_(s) and
304 50.3% Hg(cysteine)₂ species at pH = 11.6 (Figure 4G). The wild-type and mutant strains that were
305 exposed to 50 nM Hg(II) and 1 mM cysteine resemble the bulk β -HgS_(s) reference standard measured at
306 room temperature, with slight differences. What is clear is that a sharp in-edge peak is absent, indicating
307 that there is not a significant amount of Hg that is linearly coordinated to 2 sulfur atoms. We provide the
308 spectrum of an aqueous Hg(cysteine)₂ reference at pH = 5 (Figure S3D) made at similar concentration
309 (wt/wt) as the bacterial samples with the lowest Hg content to show that the in-edge peak characteristic of
310 2-coordinate Hg-S would be clearly visible. Because the HR-XANES also do not resemble those of
311 Hg(SR)₂, Hg(SR)₃,⁵⁵ or Hg(SR)₄ and are lacking features above the edge characteristic of HgS_(s)
312 particles,⁵¹ it is likely that Hg(II) exists as Hg-S clusters (i.e., analogous to Fe-S clusters or β -Hg_xS_y less
313 than 1 nm in diameter⁵⁶) with a coordination number of 4.

314 We also probed single gene deletion mutants of *E. coli* lacking cysteine desulfurase genes (i.e.,
315 *iscS* and *sufS*) with HR-XANES to explore the potential biogenic sulfide source that is responsible for
316 cell-associated β -HgS_(s) formation when exogenous cysteine is not added to cells. Specifically, we tested
317 the ISC and SUF systems whose role are to assemble Fe-S clusters in *E. coli*. Hg(II) displaces Fe(II) from
318 Fe-S clusters *in vivo* in *E. coli* at micromolar Hg concentrations⁵⁷ and *in vitro* from the [4Fe-4S] cluster

319 of dehydratase family enzymes.⁵⁸ Thus, Fe-S clusters could be a source of sulfide for β -HgS_(s) formation.
320 Specifically, we exposed *iscS* and *sufS* mutants to 500 nM Hg(II) for 3 hours. IscS and SufS perform the
321 same function of removing the S⁰ from L-cysteine and donating it to the scaffold protein for Fe-S cluster
322 formation, but the ISC system operates under normal conditions, while the SUF system functions under
323 stress conditions.¹⁹ From the HR-XANES spectra, the loss of the *sufS* gene had no effect on the Hg(II)
324 coordination environment for cells exposed to 500 nM Hg(II) (Figure 5A and S3A). In contrast, the linear
325 combination fit to the spectra of the *iscS* mutant exposed to 500 nM Hg(II) indicates less β -HgS_(s)
326 compared to the wild-type and *sufS* mutant. Therefore, Fe-S clusters are a likely sulfide source for β -
327 HgS_(s) formation under normal growth conditions, although more work should be done to determine the
328 effect of *iscS* loss on cell physiology. The deletion of genes involved in cysteine degradation to sulfide for
329 Fe-S cluster formation did not eliminate HgS_(s) formation in cell samples, likely due to the fact that only
330 single gene deletion mutants were tested. However, microbial cells are not viable after the elimination of
331 both ISC and SUF mechanisms for Fe-S cluster formation without significantly altering cell
332 metabolism.⁵⁹ In addition, another sulfide source in the cell that was not tested in this study could be
333 assimilatory sulfate reduction, which is the primary route of cysteine synthesis in bacteria like *E. coli* and
334 *Salmonella enterica* serovar Typhimurium.²⁷ In this pathway, which differs from dissimilatory sulfate
335 reduction by anaerobic organisms,⁶⁰ sulfate is reduced to sulfite and then sulfide via sulfite reductase.
336 Cysteine is then synthesized from sulfide and O-acetylserine with O-acetylserine (thiol)-lyase.

337 We found a striking difference in the HR-XANES spectra of the same sample (wild-type exposed
338 to 50 nM Hg + 1 mM cysteine) that was prepared by 2 different methods for HR-XANES collection. The
339 first method, which was used for all samples in this study, involved flash freezing the sample in liquid
340 nitrogen and keeping it frozen throughout the measurement. The second method involved flash-freezing
341 the sample in liquid nitrogen, freeze-drying, pressing into a pellet, and re-freezing prior to data collection
342 (compare Figure 5D and Figure S3C). We observed a similar change in Hg speciation (with XAS
343 analysis) after a flash-frozen sample thawed and then re-froze, although it was not freeze-dried.³⁶ The

344 freeze/thaw process may deteriorate the cell's membrane integrity, causing a change in Hg(II) speciation
345 in the cell. Freeze-drying bacteria without a cryo-protectant (e.g., 10% sucrose) will compromise
346 membrane integrity,⁶¹ and implies that the cell-associated Hg species, at least for that particular sample, is
347 sensitive to membrane damage. Fe-S clusters exist under reducing conditions in cells, are sensitive to
348 oxygen species, and decompose upon exposure.⁶² Hg-S clusters may have a similar sensitivity to oxygen
349 exposure.

350 *Implications for Hg(II) bioavailability*

351 Early bacterial Hg(II) uptake studies suggested that neutral Hg(II)-sulfide complexes formed the
352 bioavailable fraction of Hg(II) and were transported across the cell membrane layers by passive
353 diffusion.^{33, 63} More recently, evidence for energy-dependent Hg(II) biouptake has been reported, although
354 these experiments were all performed in the presence of thiols (mainly cysteine). Schaefer et al. proposed
355 that the entire Hg(II)-cysteine complex was taken up by an unknown metal-transport protein.⁴ Liu et al.
356 stated that cysteine may facilitate the ligand exchange of Hg(II) with a metal transport protein responsible
357 for Hg(II) biouptake.¹⁴

358 We show herein that when *E. coli* is exposed to Hg(II)-cysteine complexes in the presence of
359 excess cysteine, Hg(II) will become associated with the wild-type strain but not a cysteine desulfhydrase
360 deletion mutant under otherwise the same experimental conditions and with minimal differences in cell
361 physiology (as determined by cellular ATP concentration). The main difference between the wild-type
362 and *decR* mutant assays involving added cysteine was the concentration of total sulfide in the exposure
363 medium, and calculations for Hg(II)-sulfide species formation correlate with our measurements for cell-
364 bound Hg(II). The degradation of cysteine to sulfide is an energy dependent process, which could have
365 been mistakenly recognized as an active transport mechanism for Hg(II) biouptake in the presence of
366 added cysteine in past studies. Thermodynamic calculations for HgS_(s) formation are rather accurate in
367 predicting when Hg(II) becomes associated with the cells, indicating that the ligand exchange from
368 Hg(II)-cysteine complexes to Hg(II)-sulfides is not kinetically limited at the time scale of our experiment.

369 The Hg L_{III}-edge HR-XANES spectra never indicate that Hg(SR)₂ species are associated with either the
370 wild-type or *decR* mutant cells after exposure to Hg(II) in the presence of excess cysteine; the cell-
371 associated Hg(II) is either clearly HgS_(s) or Hg-S clusters with a Hg coordination number greater than 3.
372 This suggests that ligand exchange reactions between Hg(SR)₂ complexes outside the cell (i.e.,
373 Hg(cysteine)₂) and thiols in the cell membrane are not favorable. While our evidence suggests that the
374 Hg(II) uptake pathway in the presence of excess cysteine involves a speciation change from Hg(II)-
375 cysteine complexes to Hg(II)-sulfide species and Hg-S clusters, the uptake pathway remains unknown.

376 Understanding the formation of the cell-associated Hg-S clusters observed by HR-XANES both
377 in the presence and absence of added excess cysteine could provide some insight into the Hg(II) uptake
378 mechanism. Potentially, the Hg-S clusters are analogous to Fe-S clusters in proteins and capped by
379 cysteine, which could create the HR-XANES spectra that we observe. As suggested by Manceau et al., it
380 is also possible that Fe-S clusters serve as the scaffold for HgS nucleation, leading to the formation of a
381 HgS mineral core that is functionalized by cysteine residues.⁴⁹ Depending on the total added Hg
382 concentration, the seed HgS mineral may have only a few Hg atoms, and classify as a Hg-S cluster. There
383 might also be a biological response from the ISC and SUF systems upon Hg exposure to sequester Hg(II)
384 as HgS.⁶⁴ Although we only have evidence of Hg-S cluster formation after 3 hours of exposure and no
385 localization information, it is possible that these Hg species that dominate the cell-associated Hg at the
386 lowest tested Hg concentrations are the form that undergoes biouptake.

387 Because our results suggest that Hg(II)-sulfide species formation is a critical step for bacterial
388 Hg(II) uptake in the presence of excess added cysteine, it is interesting that previous studies have shown
389 that the presence of sulfide alone (in the absence of exogenous thiols) prevents Hg(II) uptake.^{4, 65} This is
390 likely due to the formation of HgS_(s) particles that are too large to be bioavailable.³⁵ Graham et al.
391 suggested that the coexistence of cysteine and sulfide can promote the formation of small, disordered
392 HgS_(s) nanoparticles that are able to passively diffuse through the cell's membrane layers.^{10, 12} Our
393 previous results with STEM-EDS provided direct evidence of HgS_(s) nanoparticles attached to the cell

394 surface/extracellular matrix in *E. coli* and *G. sulfurreducens* that were likely physically limited from
395 entering the cell cytoplasm.³⁵ Potentially, Hg(II)-sulfide complexes and/or small Hg-S clusters enter the
396 cytoplasm via passive diffusion, as suggested previously, and the size of the Hg(II)-sulfide species is an
397 important factor determining bioavailability.

398 **Conclusion**

399 The present study demonstrates that the bacterial sulfur metabolism (beyond dissimilatory sulfate
400 reduction) can have a large influence on cell-associated Hg(II) coordination and Hg(II) biouptake. We
401 show that the degradation of cysteine to sulfide and the formation of Hg(II)-sulfide species are critical for
402 the biouptake of Hg(II) in the presence of excess cysteine in the exposure medium. These results may
403 help elucidate Hg(II) bioavailability under sulfidic conditions where both sulfide and thiolate groups of
404 organic matter compete to bind Hg(II) in environmental systems. Although these studies have been
405 conducted with *E. coli*, which is unable to methylate Hg(II), many diverse organisms, in addition to *E.*
406 *coli*, experience enhanced Hg(II) uptake with added cysteine, including Hg-methylators (e.g., *G.*
407 *sulfurreducens*,^{4,5} *Desulfovibrio desulfuricans*,⁴ *Geobacter bemidjiensis* Bem¹⁵) and other non-
408 methylators (e.g., *Shewanella Oneidensis*³). Also, many diverse organisms are known to degrade cysteine
409 by desulphydrase enzymes.^{27, 30, 66, 67} Therefore, the findings from this study on *E. coli* likely apply to
410 organisms that are similarly affected by cysteine in their uptake of Hg(II). The role of cellular sulfur
411 metabolism, and in particular Fe-S clusters, in relation to Hg(II) biouptake should be explored further.

412 **Conflicts of Interest**

413 There are no conflicts of interest to declare.

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621 **Figures**

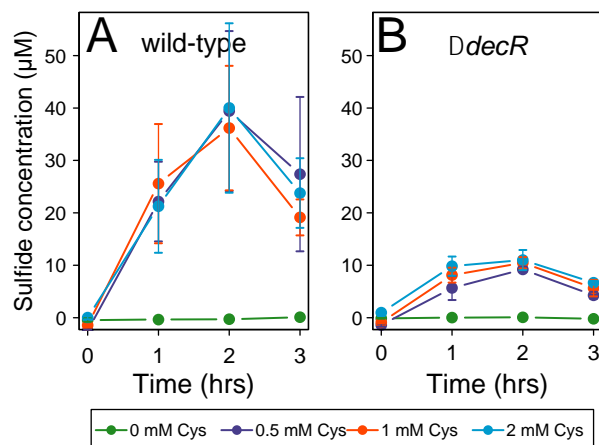
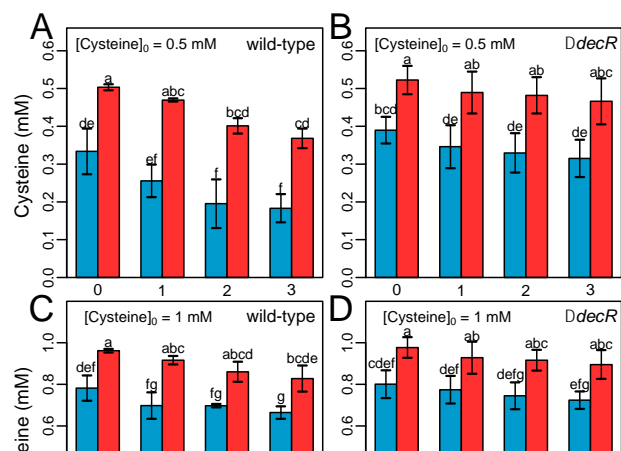


Figure 1: The concentration of sulfide in the exposure medium detected after 3-hour exposures of (A) wild-type and (B) *decR* mutant strains of *E. coli* to 0, 0.5, 1, and 2 mM cysteine (Cys). The points are averages from 3 independent experiments and the error bars are ± 1 S.D.

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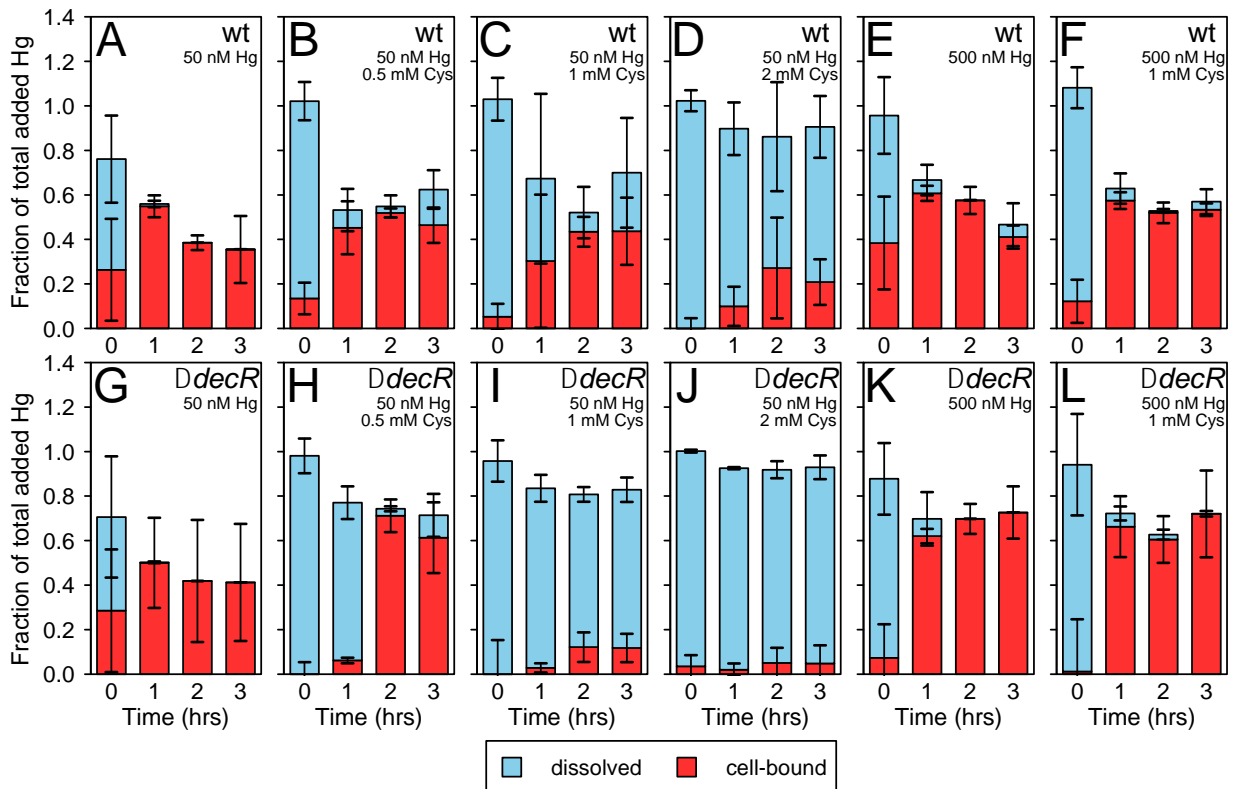
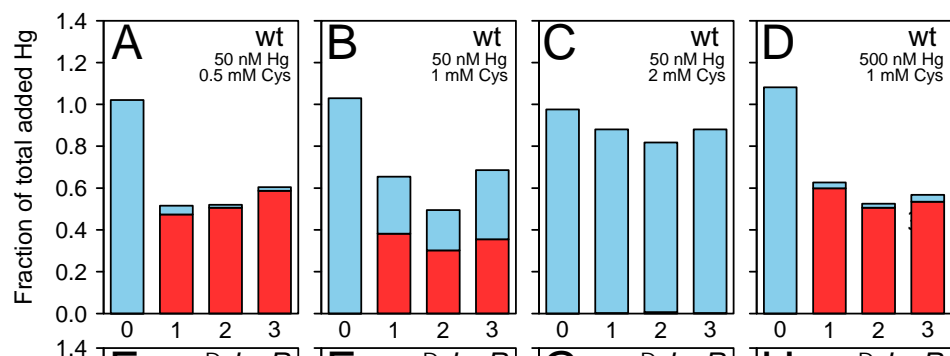


Figure 3: The dissolved and cell-bound Hg presented as a fraction of the total added Hg (i.e., 50 or 500 nM) detected after 0 to 3 hours of exposure of the (A - F) wild-type (wt) and (G - L) *decR* mutant to 50 or 500 nM total Hg. The added Hg was pre-equilibrated with either (A, E, G, K) 0 mM cysteine (Cys), (B, H) 0.5 mM Cys, (C, F, I, L) 1 mM Cys, or (D, J) 2 mM Cys. In many cases, the sum of the dissolved and cell-bound bars does not add to the total added Hg likely due to loss from Hg(II) reduction to Hg(0) and volatilization. The bars are averages from 2-3 independent experiments and the error bars are ± 1 S.D.

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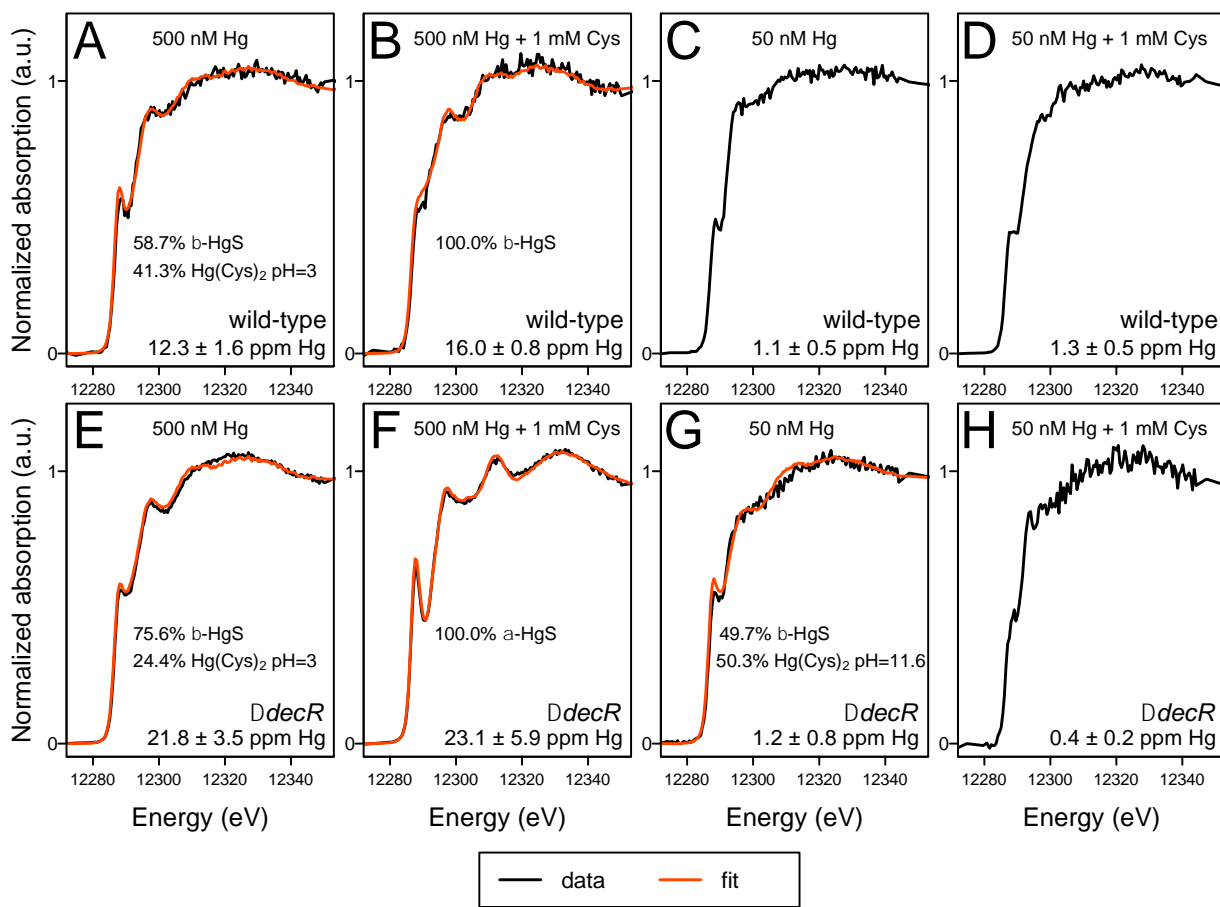


Figure 5: Hg L_{III} -edge HR-XANES of cell pellets of (A,B,C,D) wild-type and (E,F,G,H) *decR* mutant strains of *E. coli* that were initially exposed to 50 and 500 nM Hg with and without cysteine for 3 hours. The red line is the best-fit result of a linear combination fit, which included β -HgS, α -HgS, $Hg(Cys)_{2(aq)}$ at pH=3, and $Hg(Cys)_{2(aq)}$ at pH=11.6 as references. The spectra in plots C, D, and H are missing a best-fit result due to the absence of a reference spectrum that fits well; however, the spectra in plots C, D, and H do not contain prominent in-edge peaks that signify Hg coordination to 2 sulfur atoms. The concentration of cell-associated Hg (ppm) for each spectrum is included at the bottom of each plot

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