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The Acute Toxicity of Divalent Mercury to Bacteria Explained by the Formation of Dicysteinate and Tetracysteinate Complexes Bound to Proteins in *Escherichia coli* and *Bacillus subtilis*

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Abstract. Bacteria are the most abundant organisms on Earth and also the major life form affected by mercury (Hg) poisoning in aquatic and terrestrial food webs. For this reason, identifying the intracellular molecular forms and trafficking pathways of Hg in bacteria at environmentally relevant concentrations is critical to controlling Hg toxicity. We applied high energy-resolution X-ray absorption near edge structure (HR-XANES) spectroscopy to bacteria with intracellular concentrations of Hg as low as 0.7 ng/mg (ppm). Gram-positive *Bacillus subtilis* and gram-negative *Escherichia coli* were exposed to three Hg species: HgCl₂, Hg-dicysteinate (Hg(Cys)₂), and Hg-dithioglycolate (Hg(TGA)₂). In all cases, Hg was transformed into new two- and four-coordinate cysteinate complexes, interpreted to be bound, respectively, to the consensus metal binding CXXC motif and zinc finger domains of proteins, with glutathione acting as a transfer ligand. Replacement of zinc cofactors essential to gene regulatory proteins with Hg would inhibit vital functions such as DNA transcription and repair and is suggested to be a main cause of Hg genotoxicity.

INTRODUCTION

A full understanding of Hg transformation pathways in aquatic systems and Hg biomagnification in aquatic food webs rests on knowing how bacteria cope with mercury toxicity. Detoxification mechanisms are discernable if we have knowledge of the chemical forms of mercury in the microbial

cell. Bacteria having the *merA* gene from the *mer* operon eliminate Hg(II) by reduction into gaseous Hg(0).¹ The chain of chemical reactions involves proteins specialized in mercury sensing (MerR), transport (MerT, MerC, MerE, MerF, MerH, MerP), and detoxification (MerA). Hg(II) initially forms a three-coordinate cysteinyl thiol complex (Hg(CysR)₃) with the MerR protein.² This complex induces the synthesis of the transport proteins, which coordinate Hg(II) linearly as Hg(CysR)₂ complexes (MerT, MerC, MerF, and MerP³⁻⁷), or are assumed to do so (MerE and MerH^{8, 9}). The homodimeric mercuric reductase MerA¹⁰⁻¹² also docks Hg(II) linearly, but delivers it to the inner cysteines from the catalytic site via a T-shape intermediate complex (Hg(CysR)₂₊₁).¹³ Bacteria endowed with the *merA* and *merB* genes are also resistant to MeHg, which they degrade into Hg(II) before reducing it to Hg(0).¹ The Hg-C bond of MeHg is cleaved by the enzyme organomercurial lyase, MerB, which forms a Hg(CysR)₂ complex with cysteine residues from the catalytic site.¹⁴

Because almost all mercury trafficking proteins bind Hg linearly with two cysteine groups, mercury-resistant bacteria endowed with the *mer* operon should contain more Hg(CysR)₂ than mercury-sensitive microbes. Using high energy-resolution X-ray absorption near-edge structure (HR-XANES) spectroscopy, it was shown recently¹⁵ that *Pseudomonas idrijaensis*, which carries the *merA* locus, contained 56% more Hg(CysR)₂ than *Escherichia coli* and *Bacillus subtilis* exposed to 20 nM Hg as HgCl₂ (intracellular [Hg] = 4.3 ± 0.2 mg Hg/kg dry weight, dw). A minor tetrahedral species attributed to a tetrathiolate complex (Hg(CysR)₄) also was detected in the three bacteria. The same Hg coordination was observed in the muscle of fish ([Hg] = 2.20 ± 0.14 mg/kg dw) exposed to Hg complexed to dissolved organic matter (DOM).¹⁶ Mercury tetrathiolate was interpreted in the two studies as being linked to metalloprotein, perhaps metallothionein. A Hg(CysR)₂ complex along with a tetrahedral Hg-sulfide complex were identified in *E. coli*, *B. subtilis*, and *Geobacter sulfurreducens* exposed to 50, 500, and 5,000 nM Hg with or without added cysteine using XANES and extended X-ray absorption fine structure (EXAFS) spectroscopy.¹⁷⁻²⁰ The tetracoordinate species was dominant and the sulfide ligand was attributed to inorganic sulfide in nanoparticulate metacinnabar (β-HgS_{NP}). Biosynthesis of β-HgS_{NP} in the cytosol is understood to result from the reaction of Hg(II) with bisulfide (HS⁻) formed through enzymatic degradation of excess cysteine.^{17, 21-23} With a thermodynamic solubility product as low as $K_{sp} = 10^{-36.8}$ for the reaction $\beta\text{-HgS}_{(s)} + \text{H}^+ = \text{Hg}^{2+} + \text{HS}^-$ ($I = 0$, $T = 25$ °C, pH 6–10),²⁴ β-HgS forms rapidly in the presence of sulfide ions.^{25, 26} Recently, Thomas and coworkers²⁰ revisited the intracellular speciation of Hg in *G. sulfurreducens*, this time under conditions of high MeHg production enhanced by cysteine addition.^{27, 28} A tri- or tetracoordinate Hg sulfide species was identified in the cell cytosol at 50 nM Hg exposition with 100 μM cysteine, and the putative tetracoordinate species was assigned to a β-HgS-like coordination structure.

In biofilms, Hg(II) is mainly complexed to low molecular weight (LMW) thiol species other than cysteine, such as thioglycolic acid (TGA).^{29, 30} Although Hg(II)- LMW thiols are readily taken up by both mercury-resistant and mercury-sensitive bacteria,^{27, 30-32} no structural information is available on the intracellular coordination of Hg(II) when the source has a LMW:Hg molar ratio of 2:1 and the Hg-dithiolate complex is linear. Furthermore, the exact nature of the intracellular tetrathiolate complex Hg(CysR)₄ observed in previous studies is unknown. Here, these two questions were addressed by exposing Gram-positive *B. subtilis* and Gram-negative *E. coli* to HgCl₂, Hg(Cys)₂, and Hg(TGA)₂ during the exponential and stationary growth phases and determining the intracellular speciation of Hg using HR-XANES and extended X-ray absorption fine structure (EXAFS) spectroscopy. Three concentrations of Hg (20, 200, and 500 nM) were selected to delineate the conditions where mercury is bound to proteins and precipitated as β-HgS. At 20 nM, the intracellular Hg concentration was representative of contaminated systems, ranging from 0.7 to 1.8 mg/kg dw.

MATERIALS AND METHODS

Details of the experimental methods are given in section S1 of the Supporting Information (SI).¹⁵ ¹⁷Experiments were performed with the *E. coli* TOP10 strain (genotype in SI, section S2) and the *B. subtilis* ATCC 6051 strain. The exposure protocol was the same as the one described previously for *Pseudomonas idrijaensis*.¹⁵ Briefly, a bacterial biomass reaching 1 OD₆₀₀ unit (corresponding to 0.3 g/L bacteria) was prepared in LB medium at 37°C, then centrifuged and washed in minimally complexing medium (MCM buffer). The composition of the MCM buffer was 20 mM 3-(*N*-morpholino)propane sulfonic acid (MOPS buffer), 0.41 mM MgCl₂, 6 mM (NH₄)₂SO₄, and 10 mM glucose. The two bacterial species continued to grow in the MCM medium after the initial growth in LB broth (Figure S1a and S1b). After 3 h of culture in MCM medium, bacteria were fully energized, and the metabolic level was in the range of that found in LB broth (Table S1). Cells of each species were exposed at 37°C for 3 h in the exponential growth phase and under 180 rpm shaking to 20 or 200 nM HgCl₂ from a 0.1 mM HgCl₂ stock solution at pH 7.2 or to 20 or 200 nM Hg(Cys)₂ from a 0.1 mM Hg(Cys)₂ stock solution at pH 7.2. *E. coli* was also exposed to 20 or 200 nM Hg(TGA)₂ in the exponential phase for 3 h, and to 500 nM Hg(TGA)₂ in the exponential phase for 3 h, and in the stationary phase for 22 h, from a 0.1 mM Hg(TGA)₂ stock solution at pH 7.2. The MCM medium contained glucose in the exponential phase incubation and was devoid of carbon source in the stationary phase incubation.

Mercury sorbed on the cell wall and cell membrane, and in the periplasm, was removed with a two-step washing protocol.¹⁷ Cells were resuspended for 10 min in 200 mL of 50 mM EDTA and 100 mM

oxalate solution (pH = 7.5) and centrifuged at 5000 rpm in a JLA8.1 rotor for 10 min. The centrifugates were resuspended for 5 min in 50 mL of 10 mM reduced glutathione and 3 mM ascorbate solution (pH = 7), centrifuged at 4500 rpm for 10 min, and then washed with 45 mL of MCM and centrifuged again. Cell preparations were lyophilized, and about 350 to 450 mg of biomass of each bacterium was obtained. Bioaccumulation factors and the percentages of Hg(II) transferred from the medium to bacteria are given in Tables S2 and S3. For example, in *E. coli*, bioaccumulation factors were equal to 81 after 3 h exposure to 20 nM and to 75 after 3 h exposure to 200 nM Hg(Cys)₂, and the percentages of transfer of Hg(II) from the medium to bacteria were 86 % and 70 %, respectively.

Mercury L₃-edge HR-XANES and EXAFS spectra were measured at 10-15 K with high-reflectivity analyzer crystals³³ on beamline ID26 at the European Synchrotron Radiation Facility (ESRF). The XANES data were analyzed against a large database of spectra for mercury minerals and Hg(II) model complexes described in previous publications (ref.¹⁶ and references therein). The Hg(Cys)₄ complex, synthesized at a Cys:Hg molar ratio of 10 and equilibrated at pH 11.9,¹⁶ served as the reference for Hg tetrahedrally coordinated to thiolate ligands.³⁴ A “zinc finger” (znf) Hg(CysR)₄ complex bound to the RadA protein was synthesized for this study to obtain a reference HR-XANES spectrum for a protein tetrathiolate complex Hg(CysR)₄. Proportions of the Hg species were evaluated using least-squares fitting (LSF) of the experimental spectra with linear combinations of the component spectra. All reference spectra were considered, but only diagnostic spectra are discussed herein. The regression analysis was performed by minimizing the normalized sum-squared residual $NSS = \Sigma[(y_{\text{exp}} - y_{\text{fit}})^2] / \Sigma(y_{\text{exp}}^2)$. The precision of the percentage of a fit component was estimated to be equal to the variation of its optimal value when the fit was degraded by 20% (i.e., 20% increase of *NSS*).

RESULTS

L₃-edge XANES spectroscopy measured at high energy-resolution is a powerful probe of the chemical form(s) of Hg in complex samples and has already found a wide range of applications.^{15, 16, 19, 20, 35-42} A main diagnostic feature is the energy and intensity of the near-edge peak denoted A in Figure 1. This peak, which corresponds to the 2p_{3/2} → 6s/5d electronic transition, is sensitive to the Hg-ligand bonding through hybridization of the Hg 6s5d orbital with the Cl/S 3sp orbital.³⁵ It occurs at 12278.3 eV for HgCl₂, 12279.2 eV for Hg(Cys)₂ and Hg(TGA)₂, and 12279.8 eV for the methylmercury cysteinate complex (MeHgCys).^{16, 36} Its intensity depends on the probability of the electronic transition, which increases with the density of holes in the hybridized 6sd orbital, and therefore varies with the properties of the chemical bond. It is sharp and high for the Hg-Cl bond which has ionic character, and much reduced for the Hg-Cys bond which has covalent character.³⁵ Hg(Cys)₂ and

Hg(TGA)₂ have similar HR-XANES traces consistent with the high degree of similarity of the two complexes, differing essentially by the nature of the secondary O/N ligands (Figure 1).

E. coli and *B. subtilis* cells altered the initial chemical forms of Hg both extracellularly and intracellularly. When harvested immediately after exposure to 20 nM and 200 nM HgCl₂, Hg(Cys)₂, and Hg(TGA)₂, all bacteria (i.e., unwashed cells for which the associated Hg is both extracellular and intracellular) have identical XANES spectra (Figure S2a), and all of the washed cells with only intracellular Hg also have identical XANES spectra (Figure S2b). The averaged spectra for each group (Figure 2a) are statistically different from each other at the 1 σ level (Figures S2c and S2d), and noticeably distinct from the spectra for each form of mercury in the initial solutions. There are two main differences in the averaged spectra that are also visible in the unaveraged spectra. First, the near-edge peaks are reduced in intensity compared to Hg(Cys)₂ and Hg(TGA)₂, and the intracellular spectrum is reduced more (indicator region A). Second, the post-edge trace of the spectrum for the washed cells is shifted to lower energy (indicator region B in Figure 2a), which results from an increase of the interatomic distance between the photoabsorbing ion (Hg) and the neighboring atoms (S) according to the Natoli rule.⁴³ This effect is illustrated in Figure 2b with the dithiolate complex Hg(GS)₂, the trithiolate complex Hg(D-Pen)₃,⁴⁴ and the tetrathiolate complex Hg(CysR)₄. The Hg-S distance is higher on average by 0.1 Å for each added thiol ligand,⁴⁵ which is reflected in a leftward shift of the post-edge trace of the Hg(D-Pen)₃ and Hg(CysR)₄ spectra. These higher-coordinate species also lack a near-edge peak at ~12279.2 eV, which means that the near-edge peak will decrease in intensity when they occur in a mixture with a dithiolate complex (Hg(CysR)₂). Thus, the spectrum for the washed cells suggests that Hg is bonded, on average, to more than two sulfur ligands in the cytoplasm (Figure 2a). We had deduced previously that Hg occurred as a tetrathiolate complex Hg(CysR)₄ in metallothionein of *Mytilus edulis* (MT),⁴² in fish exposed to Hg(II) bound to dissolved organic matter (Fish-DOM)¹⁶, and in *P. idrijaensis* exposed to 20 nM HgCl₂ (4 μ g Hg/L)¹⁵ by similar reasoning.

The chemical form of intracellular Hg was determined by comparing the spectrum for the washed cells with those in our database. The closest resemblance was to the Fish-DOM spectrum, which is a mixture of $84 \pm 8\%$ Hg(CysR)₂ + $16 \pm 8\%$ Hg(CysR)₄ (Figure 3a).¹⁶ The best two-component fit of the intracellular spectrum was obtained with $82 \pm 5\%$ Hg(GS)₂ + $18 \pm 5\%$ Hg(CysR)₄ (Figure 3b). The proportion of Hg(CysR)₄ is also close to that of intracellular *P. idrijaensis* ($13 \pm 6\%$),¹⁵ as supported by the superposition of the washed and *P. idrijaensis* XANES traces in the B indicator region (Figure S3a). Replacing the Hg(CysR)₄ reference with the Hg(D-Pen)₃ reference degraded the fit quality by 34% ($\Delta(NSS) = 5.5 \times 10^{-5} - 4.1 \times 10^{-5} / 4.1 \times 10^{-5}$). Hg-cystine, which has a distinct HR-XANES trace from

Hg(CysR)₂, is also an incompatible model (Figure S3b). The coordination of Hg was verified by EXAFS spectroscopy. Best-fit results were obtained with 2.0 ± 0.4 S atoms at $2.36 \text{ \AA}$ and 0.9 ± 0.2 S atoms at $2.53 \text{ \AA}$, with no evidence for a trithiolate complex (Figure S4). No significant outer Hg shells are detected in EXAFS.

The chemical form of extracellular Hg is more difficult to assess because 62% to 94% of the Hg present in the unwashed cells is intracellular (82.6% on average, Table 1). There is little sensitivity to minor cell surface species, as demonstrated by the similarity of the spectra for the unwashed and washed cells (Figure 2a). The spectral difference, which represents 17.4% on average of the total Hg, provides nonetheless a clue to the binding of Hg to cell surface thiols (Figure 4a). The extracellular complex has an intense near-edge peak and the rising region C, at energies just above the near-edge minimum, is superimposed on that of Hg(GS)₂ and shifted to the left relative to the Hg(Cys)₂ pH 3 reference (Figures 4b,c). The C indicator region is sensitive to secondary coordination to N/O ligands, including amide, amine, carbonyl oxygen, and carboxyl groups.³⁸ In Hg(GS)₂, the coordination of Hg is Hg[(CysR)₂+O₂]; Hg is bonded linearly to two cysteine residues (CysR) at $2.33 \text{ \AA}$ from the two glutathione γ -Glu-Cys-Gly peptides, and perpendicularly to a carboxyl O at $2.6 \text{ \AA}$ from a Gly residue and a carbonyl O at $2.9 \text{ \AA}$ from a Gly-Cys peptide bond.¹⁵ In contrast, Hg is bonded only to two cysteinyl groups in solution with cysteine at pH 3 because the two amine groups are protonated. Thus, the C region discriminates between a Hg[(CysR)₂+(O/N)₂] coordination and a dicysteinate Hg(CysR)₂ coordination lacking secondary ligands. We conclude that Hg is bonded linearly to two cysteinyl ligands and secondarily to O/N ligands at the cell surface. A trithiolate surface complex was reported on *B. subtilis* at 50 mg Hg/kg,⁴⁶ however, this complex is not detected here at a surface Hg concentration of $0.2 \text{ mg/kg} < [\text{Hg}]_{\text{Tot}} - [\text{Hg}]_{\text{Intra}} < 5 \text{ mg/kg}$ (Table 1).

We now turn our attention to *E. coli* exposed to 500 nM Hg(TGA)₂ during the exponential and stationary growth phases. In these samples, the decrease in the peak in region A and the shift to the left in region B described previously for washed cells of microbes exposed to 20 nM and 200 nM Hg (Figure 2a) are accentuated (Figure 5a), implying that more of the intracellular Hg is tetracoordinated. The spectra of 500 nM washed cells lie in between those of the 20/200 nM washed cells (designated intracellular Hg) and nanoparticulate metacinnabar (β -HgS_{NP}). The exponential phase spectrum was fit with $64 \pm 11\%$ intracellular Hg + $36 \pm 11\%$ β -HgS_{NP} (Figure 5b). The deviation between the experimental spectrum and the reconstruction based on these two components is remarkably small ($NSS = 3.9 \times 10^{-5}$), considering the spectral disparity of the components, and engenders confidence in the overall quantitative analysis. The stationary phase spectrum was fit with the same bi-component model to yield $56 \pm 10\%$ intracellular Hg + $44 \pm 10\%$ β -HgS_{NP} ($NSS = 3.7 \times 10^{-5}$). The fit was slightly

improved with the addition of 5% $\text{Hg}(\text{CysR})_4$ ($NSS = 3.1 \times 10^{-5}$, Figure 5c) yielding $56 \pm 10\%$ intracellular Hg + $39 \pm 10\%$ $\beta\text{-HgS}_{\text{NP}}$ + $5 \pm 5\%$ $\text{Hg}(\text{CysR})_4$. Fixing % $\beta\text{-HgS}_{\text{NP}}$ to 36% in the three-component model of the exponential spectrum increased NSS four times ($NSS = 1.1 \times 10^{-4}$), demonstrating the sensitivity to the nanoparticulate metacinnabar component which has a distinct spectral shape (Figure 5d). The reason for the increase from 36% intracellular $\beta\text{-HgS}_{\text{NP}}$ after 3 hours exposure to Hg in the exponential phase to 39% after 23 hours exposure in the stationary phase is likely kinetics. Adsorption of Hg on bacterial cells is extremely rapid and approaches 100% after 3 hours,^{46, 47} which is also the time it takes for the generation of a new cell line. Cells of the following generations would adsorb less and less Hg. Most of the Hg was taken up by the first-generation cell line in the two experiments, that is on approximately the same number of cells. The main difference between the two experiments, therefore, is the ageing time: the $\beta\text{-HgS}_{\text{NP}}$ nuclei had more time to form and grow after 23 hours than after 3 hours.

The 500 nM results strengthen the interpretation of the 20 nM and 200 nM spectra regarding the attribution of the sulfur ligands for the tetrahedral Hg species to cysteine residues (CysR) instead of another form of reduced sulfur such as inorganic sulfide (S(-II)). We observed that $\text{Hg}(\text{II})$ did not form $\beta\text{-HgS}_{\text{NP}}$ at 20 nM and 200 nM exposure to HgCl_2 , $\text{Hg}(\text{Cys})_2$, and $\text{Hg}(\text{TGA})_2$ and was most likely bonded, in part, at the tetrahedral site of a protein. This raises the question of whether there was any clustering of Hg atoms as in metallothionein (MT). This can be answered by comparing the 20nM/200nM intracellular spectrum with the Hg-MT spectrum from the *Mytilus edulis* mussel.⁴² The mussel MT contains two cysteine-rich domains, an α -domain of 12 Cys residues, and a β -domain of 9 Cys residues. Hg has a higher affinity for the α -domain which comprises five CXC motifs and one CXXC motif (single-letter amino acid code, where X can be any amino acid). The chain length of the consensus CXXC motif matches the dimension of a linear $\text{Hg}(\text{CysR})_2$ complex,^{48, 49} whereas the consensus CXC motif offers the correct spacing for the formation of a $\text{Cd}_4(\text{CysR})_{12}$ ⁵⁰ and a $\text{Hg}_4(\text{CysR})_{12}$ cluster⁴² (Figure S5a). The mussel MT spectrum is distinct from the intracellular spectrum (Figure S5b), and is reconstructed with $59 \pm 9\%$ $\text{Hg}(\text{CysR})_2$ + $41 \pm 9\%$ $\beta\text{-HgS}_{\text{NP}}$ ($NSS = 5.2 \times 10^{-5}$) (Figure S5c). Nanoparticulate $\beta\text{-HgS}_{\text{NP}}$ is a good mineral proxy on the local scale for the $\text{Hg}_4(\text{CysR})_{12}$ cluster of the mussel, because the α and β metal clusters of eukaryotic cells have a metacinnabar-type mineral core.⁵¹ If the $\text{Hg}(\text{CysR})_4$ species in the interior of the bacterial cells at exposure concentrations of 20 and 200 nM were multinuclear, then such a $\text{Hg}_x[(\text{CysR})_4]_y$ cluster would have to be more disordered than the mussel cluster because the $\beta\text{-HgS}_{\text{NP}}$ reference is an incompatible component in the fit of the intracellular spectrum. Also, a cluster is unsupported because no Hg-Hg pair was detected by EXAFS (Figure S4).

DISCUSSION

This study provides new information on the dominant molecular forms of Hg(II) within bacterial cells at concentrations down to sub-ppm (sub-mg/kg) concentration. When bacteria are exposed to 20 nM and 200 nM Hg, all internalized Hg is bonded to Cys residues, of which about 82% is complexed linearly ($\text{Hg}(\text{CysR})_2$) and about 18% is complexed tetrahedrally ($\text{Hg}(\text{CysR})_4$). The proportions of the two complexes are constant up to an intracellular concentration of 18.6 mg Hg/kg obtained with an exposure to 200 nM $\text{Hg}(\text{TGA})_2$, and is independent of the chemical form of Hg in solution and at the cell surface. These observations point to a common molecular picture of Hg bonding in the cytoplasm. Intracellular $\beta\text{-HgS}_{\text{NP}}$ only occurred at an intracellular concentration > 20 mg/kg obtained with exposure to 500 nM $\text{Hg}(\text{TGA})_2$, and in association with the $\text{Hg}(\text{CysR})_2$ and $\text{Hg}(\text{CysR})_4$ species. The nature of the two thiolate species is discussed first below, after which we propose a mercury-trafficking pathway for incorporation of Hg into the bacterial cell and its cytoplasmic molecular shuttling. The reason for the acute toxicity of Hg to bacteria is discussed, and we close with a short discussion on the formation mechanism of $\beta\text{-HgS}$.

Nature of the R ligand of $\text{Hg}(\text{CysR})_2$. Mercury can bind to low molecular weight (LMW) thiol molecules and to proteins. The two most abundant LMW thiols are cysteine and glutathione. The best two-component fit to all reference spectra identified $\text{Hg}(\text{GS})_2$ at physiological pH and $\text{Hg}(\text{CysR})_4$ (RadA zinc finger, znf) as the closest model compounds to the intracellular binding environment of Hg ($NSS = 4.1 \times 10^{-5}$). Careful study of the calculated spectrum shows that it deviates from the data at 12306 eV (arrow in Figure 3b). The calculated absorption at this energy is slightly too high because the $\text{Hg}(\text{GS})_2$ complex has a flat edge maximum (Figure S6), whereas the intracellular spectrum has a rounded maximum (Figure 2a). Replacing $\text{Hg}(\text{GS})_2$ with $\text{Hg}(\text{Cys})_2$ pH 7.5 in the fit increased NSS to 6.4×10^{-5} (56%), and replacing $\text{Hg}(\text{CysR})_4$ with $\text{Hg}(\text{Cys})_4$ increased NSS to 6.0×10^{-5} (46%). The polypeptide environment of glutathione best approaches the dithiolate binding site of Hg in the cell, but does not exactly reproduce it. Thus, the dithiolate mercury complex identified by HR-XANES is likely bound to proteins in addition to dominant GSH and minor Cys.

The preference for GSH over Cys in the optimized fit to the spectrum is consistent with the equilibrium concentrations of the two complexes in *E. coli*. A GSH concentration of 86.5 mmol/kg dw was measured in *E. coli*, equivalent to 15.6×10^6 molecules per cell and a concentration of 15.5 mM.⁵² Another estimate is 17 mM.⁵³ Cysteine is toxic to the cell⁵⁴, therefore maintained at ~ 0.22 mmol/kg dw in *E. coli*, equivalent to 3.9×10^4 molecules per cell and a molar concentration of 0.0396 mM.⁵² In addition to being the most prevalent LMW thiol, GSH has a higher affinity for Hg than Cys:

$pK(\text{Hg}(\text{GS})_2) = 38.8$ and $pK(\text{Hg}(\text{Cys})_2) = 37.5$ for the reaction $\text{Hg}^{2+} + 2\text{RS}^- = \text{Hg}(\text{SR})_2$.⁵⁵ *B. subtilis* does not synthesize GSH, but instead the surrogate LMW bacillithiol (BSH),⁵⁶ which likely binds Hg in the cytosol. The best candidate proteins that could bind Hg linearly are thioredoxins Trx1 and Trx2, the thioredoxin reductase TrxR, and the glutaredoxins GrxA, GrxB and GrxC, all of which possess the CXXC sequence. Trx has a higher affinity for Hg than GSH ($K \approx 25$),⁵⁷ as expected for competition between a bidentate complex on the CXXC site of proteins and a bis-monodentate complex with GSH. Accordingly, HgCl_2 and MeHg inhibit rat Trx and TrxR activity, and human Trx1 and Grx1 lose their free thiols in the presence of HgCl_2 .^{58, 59} We conclude that the $\text{Hg}(\text{CysR})_2$ coordination detected by HR-XANES is from multiple species with GSH, thioredoxins, and glutaredoxins as dominant consortium members, among other proteins containing the consensus CXXC sequence.

Nature of the R ligand of $\text{Hg}(\text{CysR})_4$. We propose that the thiols in the tetrathiolate Hg complex are four cysteine residues within proteins. At physiological pH, GSH and Cys do not form tetrathiolate complexes with Hg(II). Tetrathiolate complexes are formed only with Cys at non-physiological alkaline pH.³⁴ GSH forms a major dithiolate complex and a minor trithiolate complex at pH 7.4 in aqueous solution.⁶⁰ We did not detect the trithiolate species in bacteria, either because it is also minor, or because it does not occur in the cytosol which has a composition far different from an ideal solution (high viscosity and salinity).

Two cysteine motifs are known to bind metals tetrahedrally in protein, the CXCXXCXC motif and the zinc finger (znf) pattern. The central XX amino acids form the turn of a loop (called a β -turn) and the two CXC motifs face each other on each strand (Figure S7a). β -turns are prone to bind Hg tetrahedrally. Zinc fingers also contain four Cys residues out of about 30 amino acid residues folded to form a compact β - β - α secondary structure.⁶¹ The $\text{Zn}(\text{Cys})_4$ complex is crucial for the structure and stability of a znf domain. Znf proteins bind only one metal (i.e., Zn), unless they also harbor the CXCXXCXC motif. A znf fold devoid of Cys₄ β -turn has been described in the GatA domain of the YdaE protein for *E. coli*.⁶² This znf is distinctive as it consists of the $\text{CX}_4\text{CX}_{29}\text{CX}_3\text{C}$ sequence, which proved to be unique in *E. coli* among all known 4-Cys-type znf proteins (Table S4). Co-occurrence of the two cysteine-rich domains, CXCXXCXC and znf, in a protein is the hallmark of a MT because together they can form a metal cluster. Several bacterial MTs have been sequenced in the last two decades,⁶²⁻⁶⁶ and the most investigated are SmtA in cyanobacteria and PflQ2 in *Pseudomonas* sp.,⁶⁷ which each harbor 9 to 10 Cys residues. The NMR structure of the Zn-bound SmtA MT for the cyanobacteria *Synechococcus* sp.,⁶³ and the Zn- and Cd-bound PFLQ2 MT for *Pseudomonas fluorescens*,⁶⁵ show that the cysteine residues form a pocket and bind four Cd atoms or three Zn atoms (Figure S7b).

We searched in the UniProt database for all proteins from the *E. coli* and *B. subtilis* genomes that contain 4-Cys-type motifs. Neither bacteria contains the CXCXXCXC motif, nor possesses a MT; however, many other 4-Cys-type motifs were identified. In *E. coli*, 46 proteins holding a 4-Cys-type motif (22 znf proteins and 24 non-znf proteins) were identified (Table S4). As some proteins contain more than one motif, the total number of 4-Cys-type motifs that could bind Hg tetrahedrally is 53 (31 znf domains and 22 non-znf motifs). Among the znf domains, the most conserved sequence type is CXXCX_nCXXC (20 motifs), followed by the sequence type CXXCX_nCX_mC (8 motifs with *m* different from 2). Unique motifs are those of the DNA repair enzyme Ada (CX₃CX₂₆CXXC),⁶⁸ the DNA helicase RecQ (CX₁₆CXXCXXC),⁶⁹ and that of the YdaE protein mentioned previously. Among the non-znf proteins that bind Zn through 4-Cys-type domains, the most represented sequence is CXXCXXCX₇C (12 motifs), followed by the sequence type CXXCX_nCXXC (7 motifs). In *B. subtilis*, 27 proteins holding a 4-Cys-type motif (12 znf proteins and 15 non-znf proteins) were identified (Table S5). The total number of 4-Cys-type motifs is 33 (17 znf domains and 16 non-znf motifs). The most represented sequence is CXXCX_nCXXC, with 13 occurrences among the znf proteins and 10 occurrences among the non-znf proteins. We conclude that the Hg(CysR)₄ species is a complex docked to 4-Cys-type motifs in multiple proteins.

Intracellular Hg(CysR)₂-trafficking and transfer to 4-Cys-type proteins. A proposed ligand exchange path for Hg-trafficking in the periplasm and cytoplasm of *E. coli* is shown in Figure 6. Hg(Cys)₂, Hg(TGA)₂, and HgCl₂ (which dissociates and converts to Hg(OH)₂ in the external medium⁷⁰), can enter the periplasm through the outer membrane porins. There, the Hg species would undergo exchange reactions with periplasmatic Cys and GSH, which are exported from the cytoplasm by the CydDC^{71, 72} (Cys+GSH) and the EamB/YfiK⁷³ and YdeD⁷⁴ (Cys) transport proteins.

Hg(GS)₂ could be carried into the cytoplasm by the glutathione (GSH)/glutathione disulfide (GSSG), and Hg(Cys)₂ by the cystine, transport systems. The periplasmic protein GsiB binds both GSH and GSSG⁷⁵ and delivers them to the glutathione-specific permease GsiCDA allowing import of the two thiol molecules to the cytoplasm.⁷⁶ Hg(GS)₂ may well be a substrate of the Gsi system. Cystine, the oxidized disulfide form of Cys (RSSR), is imported from the periplasm to the cytoplasm by two mechanisms: the low affinity TcyP/YdjN transporter and the high affinity transport system composed of the periplasmic protein TcyJ/FliY and the TcyLN/YecSC transporter.^{77, 78} Hg(Cys)₂ may be a substrate of TcyP and the TcyJLN system. A similar molecular mimicry exists for the import of Hg(Cys)₂ to eukaryotic cells, where Hg(Cys)₂ is transported as a molecular homolog of RSSR by the amino acid transporter b^{0,+}.^{79, 80}

If the Cys ligand is not replaced by GSH in the periplasm, the ligand exchange would certainly

occur in the cytoplasm as GSH is about 1,000 times more abundant and has a higher affinity for Hg than Cys. The strong binding of Hg to GSH *in vitro* raises questions of how it can be transferred *in vivo* to proteins with the CXXC sequence, such as thioredoxins (Trx) and glutaredoxins (Grx), and to 4-Cys-type proteins. The intracellular trafficking of metals fundamentally follows the local thermodynamic gradient, as described for the Cu-trafficking pathways in yeast.⁸¹ If CXXC proteins can acquire Hg directly from GSH, and likely from bacillithiol, based on their higher chemical affinity for Hg and total concentration which probably approach or surpass that of GSH, Hg transfer to 4-Cys-type proteins involves more intricate biological processes. For example, the znf site of the estrogen receptor α has an *in vitro* Hg binding pK of 7.2,⁸² about 31 orders of magnitude lower than the pK of GSH.⁵⁵ pK values can be changed *in vivo* via protein-protein docking and change of cellular GSH/GSSG redox state. This is how Zn is released from high affinity metallothioneins to low affinity enzymes.^{83, 84} Another example is provided by the GSH transfer of Co, Zn, and Cd to the 4-Cys-type znf protein XPA involved in DNA repair through the formation of a metal-XPA-GSH ternary complex.⁸⁵ The HR-XANES spectrum for the prototypic RadA znf shows a canonical tetragonal coordination of the Hg atom by the sulfur atoms from the four intervening Cys residues (Figure 2b), which means that Hg(II) can effectively replace Zn(II) within znf, and is a validation of our model.

GSH may also mediate the transport of Hg to CXXC sites. In MerA, GSH facilitates the accessibility of Hg to the N-terminus CXXC site which delivers Hg to the catalytic core domain that reduces Hg²⁺ to Hg⁰.⁵⁷ Also, because GSH is a cofactor of Grx, its binding to Grx would affect the protein conformation, increase the pK of Grx, and thereby facilitate Hg transfer to Grx. We conclude that GSH acts as a soluble cytoplasmic metallochaperone that acquires and delivers Hg to CXXC and 4-Cys-type proteins in an equilibrium percentage of 82% and 18% ($\pm 10\%$), according to the HR-XANES results. Bacillithiol (BSH) would substitute for GSH in *B. subtilis* (Figure S8).

Toxicity of Hg. The acute toxicity of Hg to bacteria may lie in its binding to 4-Cys-type proteins, because these proteins are often involved in DNA binding.⁸⁶ A survey of literature data enumerating the molecules of each 4-Cys-type protein in *E. coli* gives $67,000 \pm 25,000$ 4-Cys-type Zn binding molecules per cell (Table S6). This number is $13,000 \pm 6,000$ for *B. subtilis* (Table S7), yet it is largely underestimated because only 30% of the genes were expressed during the quantification of the cytosolic proteome. In our study, each cell contained on average 16,700 Hg atoms after exposure to 200 nM Hg(TGA)₂ (Paragraph S3, SI). The mean number of Hg atoms bonded to 4-Cys-type proteins per cell was $16,700 \times 18\% = 3,000$.

The impact of the replacement of Zn with Hg on the activity of protein involved in DNA transcription and repair can be estimated assuming that all 4-Cys centers have the same Hg binding

constant. This assumption is supported by the similarity of the equilibrium dissociation constants (K_D) of Zn bound to 4-Cys type ligands ($10^{-15.9}$ to $10^{-17.4}$).⁸⁷⁻⁹¹ Under this assumption, the distribution of Hg atoms among each type of protein only depends on the fractional amount of the 4-Cys centers in each type. The 4-Cys RNA polymerase (RNAP) subunit β' (RpoC), a protein essential to the transcriptional activity of the cell, has 7,800 4-Cys type molecules per *E. coli* cell, or $7,800/67,000 \approx 12\%$ of the total 4-Cys-type pool. Approximately 12 % of the 3,000 Hg atoms bound to 4-Cys-type proteins will be bound to RpoC, which is about 400 molecules or 5 % of the total RpoC pool. Thus, exposition of *E. coli* to 200 nM Hg may induce about 5 % inhibition of the general transcription. RNAP is also involved in the synthesis of bacterial ribosomal RNAs, a prerequisite in the formation of ribosomes, so that the velocity of action of RNAP controls in turn the overall protein synthesis in the cell,⁹² and therefore the bacterial growth rate.^{93, 94} The control by RNAP of the rRNA transcription is itself ruled by the RNAP-binding transcription factor DksA,⁹³ which possesses a 4C-type znf. The mean number of DksA molecules per *E. coli* cell is 13,000, or $13,000/67,000 \approx 20\%$ of the total 4-Cys-type pool (Table S6). Thus, RpoC and DksA represent, on average, $\sim 32\%$ of the total 4-Cys-type pool, of which approximately 5% would bind to Hg ($(32\% \times 3,000)/(7,800 + 13,000) = 5\%$).

When *E. coli* cells are exposed to micromolar doses of Hg, the proportions of RpoC and DksA that are poisoned would be too high for viable general transcription and ribosome synthesis and would lead to growth inhibition. This reasoning is supported by the experimental exposition of *Sulfolobus solfataricus* to HgCl_2 which induced 50% growth poisoning (EC_{50}) at a concentration of 0.5 μM , as a result of the inhibition of the general transcription.⁹⁵ Two znf proteins, the transcription factors TFB-1 and TFB-2, are involved in the formation of the *S. solfataricus* RNA polymerase. TFB-1 contains a Cys_2Thr_2 znf motif, and the second a Cys_4 znf motif. The former can bind Hg linearly ($\text{Hg}(\text{SecR})_2$), and the latter can bind Hg tetrahedrally ($\text{Hg}(\text{SecR})_4$). We assert that the acute toxicity of Hg resides essentially in the replacement of Zn in DNA-binding proteins involved in the transcription of tRNA genes and DNA repair (e.g., UvrA, Fpg, Table S4).⁹⁶ Nanomolar levels of Hg(II) suffice to impede the growth of *E. coli*, whereas micromolar levels are required for Cd(II) , Zn(II) , Mn(II) , and Pb(II) in minimal medium,⁹⁷⁻¹⁰⁰ a result of the replacement in Cys-rich sites of Zn cofactors essential to many cellular processes, including cell division and survival.

Formation of $\beta\text{-HgS}_{\text{NP}}$ at 500 nM Hg. A model for the formation of $\beta\text{-HgS}_{\text{NP}}$ during exposure to 500 nM Hg(TGA)_2 is depicted in Figure S9. It is based on the substitution of Hg for Fe(II) in the $[\text{Fe}_2\text{S}_2](\text{GS})_4$ complex that participates in cellular iron homeostasis and in $[\text{Fe}_2\text{S}_2]$ and $[\text{Fe}_4\text{S}_4]$ protein clusters.¹⁰⁰⁻¹⁰² At the onset of oxidative conditions inherent to Hg toxicology, the organic scaffold of the Hg-S clusters would be degraded and the clusters could aggregate to form $\beta\text{-HgS}_{\text{NP}}$.¹⁰³⁻¹⁰⁵

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Materials and methods, supplementary tables and figures (PDF).

HR-XANES spectra (Excel).

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Notes

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Table 1. Hg Complexes in Solution, Concentration of Hg in Solution and in Unwashed and Washed Bacteria, Fraction of Hg within the Cells, and Spectroscopic Method

	Hg exposure	[Hg] initial nM	[Hg] total mg/kg	[Hg] intra. mg/kg	% [Hg] intra.	Method
<i>E. coli</i>	HgCl ₂	20	0.86 ± 0.01	0.68 ± 0.01	79	HR-XANES
	Hg(Cys) ₂	20	2.04 ± 0.01	1.82 ± 0.01	89	HR-XANES
	Hg(TGA) ₂	20	2.07 ± 0.02	1.52 ± 0.07	73	HR-XANES

	HgCl ₂	200	14.2 ± 0.4	8.8 ± 0.2	62	HR-XANES
	Hg(Cys) ₂	200	18.6 ± 0.5	16.8 ± 0.1	90	HR-XANES
	Hg(TGA) ₂	200	23.1 ± 0.6	18.6 ± 0.4	80	HR-XANES
	Hg(TGA) ₂ exp.	500	na ¹	21.6 ± 3.1	-	HR-XANES + EXAFS
	Hg(TGA) ₂ stat.	500	na	27.2 ± 1.4	-	HR-XANES + EXAFS
<i>B. subtilis</i>	HgCl ₂	20	0.82 ± 0.05	0.67 ± 0.05	82	-
	Hg(Cys) ₂	20	2.01 ± 0.01	1.82 ± 0.03	90	HR-XANES
	HgCl ₂	200	8.7 ± 0.2	8.2 ± 0.2	94	HR-XANES
	Hg(Cys) ₂	200	13.7 ± 0.1	12.0 ± 0.3	87	HR-XANES

¹Not analyzed.

LEGENDS TO FIGURES

Figure 1. HR-XANES spectra of the HgCl₂, Hg(Cys)₂, and Hg(TGA)₂ complexes, to which the bacteria were exposed. Inset and top: Ball-and-stick representations of the Hg(Cys)₂ and Hg(TGA)₂ complexes at neutral pH. The Hg(Cys)₂ complex has two equilibrium structures.³⁸

Figure 2. a) Average HR-XANES spectra of total cell-associated Hg (Hg total) and intracellular Hg. The two plots on top are an enlargement of the indicator regions A (left) and B (right). b) HR-XANES spectra of two-coordinate (Hg(GS)₂), three-coordinate (Hg(D-Pen)₃),⁴⁴ and four-coordinate (Hg(CysR)₄) Hg-thiolate (RadA zinc finger in which the zinc atom is substituted by a mercury atom). The deprotonated bis-glutathione complex Hg(GS)₂ was prepared at a GSH:Hg molar ratio of 2 :1 and pH 7.5, and the Hg(D-Pen)₃ complex at D-Pen:Hg of 10 :1 and pH 11.5.

Figure 3. a) HR-XANES spectra of intracellular Hg in bacteria and fish exposed to Hg-bound dissolved organic matter (Fish-DOM¹⁶). b) Two-component fit to the intracellular spectrum with 82 ± 5% Hg(GS)₂ + 18 ± 5% Hg(CysR)₄ (NSS = 4.1×10⁻⁵).

Figure 4. a) HR-XANES spectra of extracellular Hg calculated as the normalized difference of the total cell-associated Hg and intracellular Hg. b) HR-XANES of extracellular Hg and Hg(GS)₂. c) HR-XANES of extracellular Hg and Hg(Cys)₂ at pH 3. At this pH, the two amine groups from cysteine are protonated and the complex is linear. There is no secondary Hg-N bonding, in contrast to the complex at pH 7.5 (Figure 1).

Figure 5. a) HR-XANES spectra of intracellular Hg at 20 nM and 200 nM Hg exposure in the exponential phase (blue), of intracellular Hg at 500 nM exposure in the exponential (green) and stationary (orange) phases, and of β -HgS_{NP} (purple). b) Two-component fit (black) to the 500 nM exponential phase spectrum with $36 \pm 11\%$ β -HgS_{NP} + $64 \pm 11\%$ of the 20+200 nM exponential phase spectrum (denoted Intracellular) ($NSS = 3.9 \times 10^{-5}$). c) Three-component fit (black) to the 500 nM stationary phase spectrum with $39 \pm 10\%$ β -HgS_{NP} + $56 \pm 10\%$ of the 20+200 nM exponential phase spectrum + $5 \pm 5\%$ Hg(CysR)₄ ($NSS = 3.1 \times 10^{-5}$). d) Three-component fit (black) to the 500 nM stationary phase spectrum with the fraction of β -HgS_{NP} fixed to 36% to show the high sensitivity of HR-XANES to this species.

Figure 6. A hypothetical model for the accumulation of dithiolate and tetrathiolate Hg(II) species in *E. coli*. Dithiolate- and dihydroxy-Hg can diffuse through the outer membrane passing through porins to reach the periplasm. Formation of bis-cysteinate (HgCys)₂ and bis-glutathione (HgGS)₂ complexes may occur (1) through the reaction of Hg(OH)₂ with GSH and Cys and (2) through a thiol exchange between the incoming dithiolated species and GSH and Cys. Cys is exported from the cytoplasm to the periplasm by the YdeD and EamB permeases, and the CydCD transporter which also exports GSH. Hg(Cys)₂ could be imported to the cytoplasm by two types of L-cystine transporters, TcyP and TcyJLN. Hg(GS)₂ could be bonded to the substrate binding protein GsiB which delivers it to the GsiCDA system. Hg(Cys)₂ and Hg(GS)₂ may exchange thiols in the cytoplasm, although the reaction equilibrium is shifted towards the formation of Hg(GS)₂. Hg(GS)₂ may transfer Hg(II) to the CXXC proteins Trx, TrxR, and Grx, and to the 4-Cys-type zinc binding proteins through thiol exchange. Once loaded with Hg, Trx-(HgS₂) can also transfer Hg to 4-Cys-type zinc binding proteins.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

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Supporting Information for

The Acute Toxicity of Divalent Mercury to Bacteria Explained by the Formation of Dicysteinate and Tetracysteinate Complexes Bound to Proteins in *Escherichia coli* and *Bacillus subtilis*

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S1. Materials and methods

S1.1. Growth curves and metabolic state of *E. coli* TOP10 and *B. subtilis* ATCC 6051

Cultures of the two bacteria were grown overnight. Then, four separate cultures of each bacterium were grown in LB medium starting from an OD₆₀₀ of 0.015 after appropriate dilution of the overnight cultures. Bacteria were cultured for 3.6 h upon shaking (180 rpm) at 37°C until reaching an OD₆₀₀ close

to 1 unit. Cultures were centrifuged to get rid of the LB broth and washed with one volume of MCM medium, and the cells suspended in one volume of MCM medium. Incubation was resumed for 3 more hours with shaking (180 rpm) at 37°C. The OD₆₀₀ of each culture was measured every 30 min. Results show that both bacterial species kept growing in the MCM medium following the initial growth in LB broth (Figures S1a and S1b).

The metabolic state of the bacterial cells was assessed by measuring the rate of reduction of nitroblue tetrazolium (NBT), which is linked to the activities of respiratory chain enzymes and the electron transport system.¹ The experimental protocol was adapted from Stowe et al.² Reactions were performed in two plastic cuvettes of 1 cm optical path length, containing 475 or 610 µL water, 75 or 90 µL of 10 times concentrated MCM medium, 250 or 100 µL of bacterial culture, respectively, and 100 µL Triton X-100 10 %, 50 µL of 0.2 mM phenazine methosulfate (PMS). The reaction was started with the addition of 50 µL of 4 mM NBT. Thus, the final reaction volume was 1 mL. Control cuvettes, for measuring the spontaneous reduction of NBT with time, contained the same ingredients without the bacterial cells (100 µL of 10 times concentrated MCM medium in 700 µL water, 100 µL Triton X-100 10 %, 50 µL of 0.2 mM PMS). Blank measurements were performed just before the addition of NBT for the control and bacteria-containing cuvettes, then NBT was added. The reaction volume was homogenized immediately by pipetting back and forth 2 times with a 1 mL pipette, and the OD measured at 580 nm for several min. NBT and PMS were purchased from Sigma-Aldrich (references: N6876 and P9625, respectively).

The NBT reduction rate, V_{NBT} , expressed in reduced NBT molecules.min⁻¹.bacterium⁻¹, was calculated as follows:

$$V_{\text{NBT}} = (\Delta\text{OD}_{580}/\Delta t \cdot \epsilon \cdot \ell) \times (1/1000) \times (1000/V) \times (1/\text{OD}_{600}) \times (1/D_{\text{bact}}) \times N_A$$

where ΔOD_{580} is the difference in optical densities resulting from the production of formazan molecules (reduced form of NBT) between times t_2 and t_1 (Δt in minutes, $t_2 > t_1$); ϵ is the molar extinction coefficient in M⁻¹.cm⁻¹, and ℓ is the optical path length of the cuvette (1 cm). The term 1/1000 converts liter to mL, as the final volume of the reaction medium is 1 mL; V is the volume of culture added to the cuvette (100 or 250 µL); OD_{600} is the optical density of the culture after 3 h of incubation in MCM medium; D_{bact} is the bacterial density (in cells/mL) at an OD_{600} of 1 unit; and N_A is Avogadro's number. The reduced formazan form of NBT has $\epsilon = 12,300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 580 nm.¹ D_{bact} at $\text{OD}_{600} = 1$ is 0.7×10^9 cells/mL for *E. coli* TOP10 and 0.3×10^9 cells/mL for *B. subtilis*.

The reduction rates were proportional to the amount of bacteria in the reaction volumes, so that we could calculate a mean V_{NBT} from each cuvette containing either 100 or 250 µL bacterial culture. Results show that after 3 h of culture in MCM medium, bacteria were fully energized, and the metabolic level was in the range of that found in LB broth (Table S1).

S1.2. Preparation of the Hg(Cys)₂ and Hg(TGA)₂ complexes

The complexes were prepared at $[\text{Hg}] = 0.2 \text{ mM}$, a Hg:ligand ratio of 1:2, and a pH of 7.2 from stock solutions of 20 mM Hg(ClO₄)₂ and 50 mM L-cysteine and thioglycolic acid (TGA). All solutions were constantly bubbled with nitrogen to prevent the oxidation of thiols. The complexes were stored at -20°C under nitrogen.

S1.3. Hg analysis

Mercury in the majority of samples was quantified with a DMA-80 direct mercury analyzer (Milestone Dual-cell). The accuracy was confirmed by analysis of the IAEA-436 tuna homogenate reference from the International Atomic Energy Agency. The recommended value is $4.19 \pm 0.36 \text{ mg kg}^{-1} \text{ dw}$ and the determined value was $3.98 \pm 0.13 \text{ mg Hg kg}^{-1} \text{ dw}$ ($n = 21$). Eight samples were analyzed with a DMA-80 Tri-cell analyzer (Milestone). Accuracy was confirmed by analysis of certified reference material DORM-4 (fish protein, National Research Council of Canada) which has $0.412 \pm 0.036 \text{ mg Hg kg}^{-1} \text{ dw}$. The determined value was $0.416 \pm 0.039 \text{ mg Hg kg}^{-1} \text{ dw}$ ($n = 3$).

S1.4. Preparation of a Hg-containing recombinant zinc finger

A recombinant *E. coli* RadA protein, reduced to the first 55 amino acids that contain the znf at location 11-28, was expressed in *E. coli* TOP10 exposed to HgCl_2 and purified. The recombinant truncated RadA gene construction has been inserted into the pASK-IBA13 plasmid (courtesy of Chiara Rapisarda). In this expression plasmid, the recombinant protein is fused on its N-terminal part to the Strep tag II to allow purification by affinity chromatography on a Strep-Tactin loaded adsorbant. A thrombin cleavage site was inserted between the Strep tag II and the beginning of the recombinant RadA. The recombinant gene is under control of the inducible *tet* promoter.

Four liters of LB medium containing 1 mg/L ampicillin were inoculated with the recombinant *E. coli* and incubated overnight at 37°C until a DO_{600} of 0.8 was obtained. Then the *tet* promoter was induced by adding 0.2 mg/L anhydrotetracyclin and the culture incubated for 15 h at 16°C and 180 rpm. The cells were recovered by centrifugation, washed with MCM medium, and exposed to 500 nM HgCl_2 for 3 h at 37°C in order to replace Zn with Hg in the recombinant RadA znf. The cells were washed with MCM, centrifuged, and resuspended in a lysis buffer (5 mL/g of wet cells) containing 25 mM HEPES at pH 8.0, 16 $\mu\text{g/mL}$ lysozyme, 1.25 $\mu\text{g/mL}$ DNase and one tablet of protease inhibitor cocktail (Roche). The clear lysate was loaded on a Strep-Trap HP column (5 mL, GE Healthcare), washed with the buffer (25 mM HEPES at pH 8.0, 0.1 M NaCl), and eluted with the same buffer containing 2.5 mM *d*-desthiobiotin (Merck, reference D1411). Elution fractions were concentrated with a 4 kDa Millipore concentrator and the recombinant RadA znf purified on a size exclusion chromatography column Superdex 75 (GE Healthcare). Eluted fractions were concentrated again with a 4 kDa Millipore concentrator and the final solution flash frozen in liquid nitrogen.

S1.5. HR-XANES and EXAFS spectroscopy

Bacteria were placed in a PEEK holder designed for pellets, sealed with poly (4,4'-oxydiphenylene-pyromellitimide) (Kapton) tape, and stored in a desiccator until transfer into the liquid helium cryostat of the beamline. We had verified in a previous publication that the process of freeze-drying biological cells and preparing pellets did not alter the Hg speciation.³ Effects of freeze-drying are observed when a metal is bonded to a water molecule, not when it is covalently bonded to strong ligands,⁴ as is the case with Hg. All solutions were measured in the frozen state.

The Hg L_3 -edge spectra were measured in high energy-resolution fluorescence yield detection (HERFD) mode with analyzer crystals.⁵ Rejection of higher harmonics and reduction of heat load were achieved with a white beam Pd-coated, flat mirror working under total reflection at 2.5 mrad deflecting angle. The incoming beam was monochromatized by the 111 reflection of a Si double crystal monochromator, then focused horizontally by a second Pd-coated mirror and vertically by a third Pd-

coated mirror. The flux on the sample was approximately 10^{13} photons s^{-1} in a beam footprint of ~ 700 (H) $\times$ 80 (V) μm^2 FWHM. The Hg $L_{\alpha 1}$ ($3d_{5/2} \rightarrow 2p_{3/2}$) fluorescence line was selected using the 555 reflection of five spherically bent (radius = 0.5 m)⁶ Si analyzer crystals (diameter = 100 mm) aligned at an 81.8° Bragg angle in a vertical Rowland geometry. The diffracted intensity was measured with a Si drift diode detector (SDD) in single photon counting mode. The effective energy resolution, obtained by convoluting the total instrument energy bandwidth (spreads of the incident and emitted rays), the SDD resolution (~ 160 eV), and the $3d_{5/2}$ core-hole width from the $L_{\alpha 1}$ line was about 3.0 eV, compared to an intrinsic line broadening of about 6.1 eV in conventional fluorescence yield measurement with a solid-state detector.

Spectra were collected at a temperature of 10–15 K and a scan time of 15 s (XANES) or 30 s (EXAFS) to reduce exposure, and were repeated at different pristine positions on the sample pellet to increase the signal-to-noise ratio. Scans were monitored carefully for any evidence of radiation damage. XANES spectra were measured between 12260 and 12360 eV in 0.2 eV steps and normalized to unity at $E=12360$ eV. EXAFS spectra were measured between 12200 and 13000 eV in 2 eV steps and analyzed with WinXAS.⁷

S2. Identification of the Zn(Cys)₄ protein sites in *E. coli* and *B. subtilis*

All Zn-containing 4-Cys-type proteins known in *E. coli* strains were reviewed in the UniProt protein data bank, and the identified proteins were searched afterward in the genome of *E. coli* strain TOP10. The genomes of *E. coli* TOP10 and DH10B are copied below and are identical. Both strains are derived from the MC1062 strain.⁸ The blastx comparison of the whole genome of DH10B (GenBank accession: NC_010473.1) with each 4-Cys-type protein sequence obtained from UniProt shows that several hundreds do not exist in DH10B, thus also in TOP10; therefore, these protein sequences were discarded. The missing proteins are either phage proteins or proteins involved in the conjugal transfer of plasmids or DNA between bacteria. They are in the UniProt database because of the occurrence of strains endowed with such capability as horizontal transfer of DNA. *E. coli* TOP10 does not have conjugal plasmid. The inventory yielded 44 Zn coordinated 4-Cys-type proteins, of which 21 are znf domains and 23 are not (Table S4).

The same blastx analysis was performed for *B. subtilis* strain ATCC 19217 (GenBank accession: CP009749.1). Twenty-seven 4-Cys-type Zn coordinating domains were identified, 12 of which are znf domains and 15 are not (Table S5).

***E. coli* TOP10 genotype:**

mcrA $\Delta(mrr-hsdRMS-mcrBC)$ $\phi 80lacZ\Delta M15$ $\Delta lacX74$ *recA1* *araD139* $\Delta(ara-leu)7697$ *galU* *galK* *rpsL*(Str^R) *endA1* *nupG*

***E. coli* DH10B genotype:**

F⁻ *mcrA* $\Delta(mrr-hsdRMS-mcrBC)$ $\phi 80lacZ\Delta M15$ $\Delta lacX74$ *recA1* *endA1* *araD139* $\Delta(ara-leu)7697$ *galU* *galK* λ^- *rpsL*(Str^R) *nupG*

S3. Bioaccumulation factor of Hg in bacteria and percentage of transfer of Hg from the medium to bacteria

Comparison of the amounts of Hg added to the living bacteria and measured in the biomass after 180 min exposure shows that bacteria took up about a third of the added Hg. In the 200 nM experiment (40 µg/L), 0.3 g biomass was exposed to 20 µg Hg in 500 mL giving an initial Hg concentration of 66.7 mg Hg/kg bacteria dw. The initial Hg concentration was 6.7 mg Hg/kg bacteria dw in the 20 nM experiment (4 µg/L). At the end of the Hg(TGA)₂ assay, for example, [Hg_{Tot}] = 23.1 ± 0.6 mg Hg/kg dw in the 200 nM experiment and 2.1 ± 0.02 mg Hg/kg dw in the 20 nM experiment (Table 1). Thus, bacteria retained 35% and 31% of total Hg, respectively. Furthermore, comparison of the amounts retained in the biomass and incorporated intracellularly shows that 62-94% of the Hg associated with the cells had diffused inside (Table 1).

One gram of dw bacteria contains 3.33×10^{12} cells. After exposure to 200 nM Hg(TGA)₂, 18.6 mg Hg/kg inside the cells represents $(18.6 \times 10^{-6} \times 6 \times 10^{23}) / (200.6 \times 3.33 \times 10^{12}) = 16.7 \times 10^3$ atoms of Hg/cell. In turn, knowing the volume of a bacterial cell, $1.67 \mu\text{m}^3$ during the exponential growth phase in a rich medium,⁹ we can calculate an internal Hg concentration of $(16.7 \times 10^3) / (6 \times 10^{23} \times 1.67 \times 10^{-15}) = 16.7 \mu\text{M}$, resulting in a bioaccumulation factor of $200 \text{ nM} / 16.7 \mu\text{M} = 83.5$. After exposure to 20 nM Hg(TGA)₂, 1.52 mg Hg/kg inside the cells represent 1.36×10^3 atoms of Hg/bacterial cell, making $1.36 \mu\text{M}$ Hg, therefore a bioaccumulation factor of 68.

Bioaccumulation factors calculated for all experimental conditions are reported in Table S2. *E. coli* presents a lower Hg uptake efficiency than *Pseudomonas idrijaensis*, which carries the *merA* locus, for the same density of biomass exposed to equivalent concentration of Hg. Bioaccumulation factors in *P. idrijaensis* were 196 and 189 after exposure to 200 nM and 20 nM of Hg(II) versus 39 and 30 in *E. coli*.¹⁰ These differences in bioaccumulation factors likely result from the intervening action of the three Hg transporters MerC, MerE and MerT in *P. idrijaensis* that do not exist in *E. coli* TOP10.

The transfer of Hg from the medium to bacteria has been calculated taking into account the 4-6% loss of biomass resulting from the centrifugation steps and the final wash (without chelating agent). In *E. coli*, 40-70 %, 70-85 %, and 75-85 % of the total Hg added to the medium was associated with bacteria after 3 h exposure to HgCl₂, Hg(Cys)₂, and Hg(TGA)₂, respectively (Table S3). In *B. subtilis*, the percentages were 30-35 % for exposure to HgCl₂ and 55-80 % for exposure to Hg(Cys)₂. The loss of Hg cannot be due to the production of Hg⁰, because *E. coli* TOP10 does not possess the *mer* operon and the *merA* reductase gene. Instead, part of the Hg(II) remained in the medium or was flushed out from the periplasm during the last wash (with buffer only, without chelating chemicals). An and coworkers showed that the Hg loss amounted to about 10% with *Desulfovibrio desulfuricans* after 2 h exposure.¹¹

S4. Formation of β-HgS_{NP} at 500 nM Hg(II)

A model for the formation of β-HgS_{NP} in bacteria at high Hg concentration is depicted in Figure S8. GSH and Grx bind the [Fe₂S₂] cluster, which they shuttle to the target maturing metalloproteins which contain a [Fe₂S₂] or a [Fe₄S₄] core.^{12, 13} With [Fe₂S₂], GSH forms the tetrahedral dimeric complex [Fe₂S₂](GS)₄, in which each Fe bonds two sulfide and two thiolate anions. Fe is also tetracoordinate in [Fe₄S₄] protein clusters. Exposition of *E. coli* fumarase A to Hg(II) in a 1:1 protein:metal ratio released Fe from the tetrameric [Fe₄S₄] cluster inactivating its catalytic properties.¹⁴ We suggest that Fe is totally replaced by Hg in the [Fe₂S₂](GS)₄ complex at high Hg concentration and also in [Fe₂S₂] and [Fe₄S₄] proteins. At the onset of oxidative conditions inherent to Hg toxicology, the organic scaffold of the Hg-S clusters would be degraded and the clusters could aggregate to form β-HgS_{NP}. The biomineralization of Hg may also occur by self-assembly of the [Fe₂S₂](GS)₄ and [Fe₄S₄]-protein scaffolds, as commonly

observed for the biosynthesis of metallocusters, such as the biomineralization of Fe in ferritin.¹⁵⁻¹⁷ Bacillithiol (BSH) would substitute for GSH in *B. subtilis*.

S5. Supplementary figures

Figure S1. Growth curves *E. coli* (a) and *B. subtilis* (b). Four separate cultures of each bacterium were grown in LB medium for 3.6 h and afterward in MCM medium for 3 h. Arrows indicate the time of replacement of LB with MCM medium.

Figure S2. HR-XANES spectra of total cell-associated (a) and intracellular (b) Hg at 20 nM and 200 nM Hg exposure to HgCl_2 , $\text{Hg}(\text{Cys})_2$ and $\text{Hg}(\text{TGA})_2$ in the exponential phase. c) Average spectrum of total cell-associated Hg with standard deviation among the nine intracellular Hg spectra. d) Average spectrum of intracellular Hg with standard deviation among the nine total Hg spectra. Plots c) and d) show that the difference between the two average spectra is statistically significant at the 1σ level. The total Hg spectrum does not differ much from the intracellular spectrum because 62% to 94% of the Hg is intracellular in unwashed cells.

Figure S3. Average HR-XANES spectrum of intracellular Hg in *E. coli* and *B. subtilis* exposed to 20 nM and 200 nM Hg and HR-XANES spectrum of *P. idrijaensis* exposed to 20 nM Hg (Pi-4-Intra).¹⁰ *P. idrijaensis* has a more intense near-edge peak (region A), because it contains a higher proportion of dithiolate Hg(CysR)₂ complex ($87 \pm 6\%$) than *E. coli* and *B. subtilis* ($82 \pm 5\%$) as a result of the presence of the *mer* operon in *P. idrijaensis*. Region B is sensitive to tetrathiolate complex, and the superposition of the two spectra in this region is consistent with the presence of similar fractional amounts of Hg(CysR)₄ in *P. idrijaensis* and in *E. coli* and *B. subtilis* (i.e., $13 \pm 6\%$ and $18 \pm 5\%$, respectively).

Figure S4. Hg L₃-edge EXAFS spectrum (left) and radial structure function (right) of intracellular Hg measured on a mixture of *B. subtilis* exposed to HgCl₂ (8.2 mg Hg/kg) and *E. coli* exposed to Hg(TGA)₂ (18.6 mg/kg) with model-fits. a) Peak at $R + \Delta R = 4 \text{ \AA}$ modeled with Hg-Hg pairs. b) Peak at $R + \Delta R = 4 \text{ \AA}$ modeled with a four scattering leg S-Hg-S path.

Figure S5. a) Schematic representation of the distribution of the Cys residues in the amino acid sequence of the α -domain of the metallothionein (MT) for *Mytilus edulis*, and polyhedral representation of the metal cluster with a β -HgS-like linkage.³ b) HR-XANES spectra of intracellular Hg and the mussel MT. c) Two-component fit to the mussel MT with 59 $\pm$ 9 % Hg(Cys)₂ pH 7.5 + 41 $\pm$ 9 % β -HgS_{NP} (NSS = 5.2 $\times$ 10⁻⁵).

Figure S6. HR-XANES spectrum of Hg(GS)₂ at pH 7.5.

Figure S7. a) Predicted tertiary structure from I-Tasser^{18, 19} of the CXCXXCXC motif of the PflQ2 metallothionein for *Pseudomonas fluorescens* Q2-87. The amino acid sequence is NELRCGCPDCHCKVDPE (single-letter amino acid code). b) NMR structure of the whole PflQ2 MT fold showing the cysteine-rich site, which binds four Cd or three Zn atoms.²⁰

Figure S8. A model for the accumulation of dithiolate and tetrathiolate Hg(II) species in *B. subtilis*. This bacterium has a periplasm, although it belongs to the Gram-positive class.²¹ Dithiolate- and dihydroxy-Hg can diffuse through the cell wall to reach the periplasm, while Cys is exported from the cytoplasm to the periplasm by the YdeD permease. There, ligand exchange may occur yielding Hg(Cys)₂, which can be imported to the cytoplasm by the L-cystine transporters TcyP, TcyABC, TcyJKLMN.²² In the cytoplasm, Hg(Cys)₂ can exchange thiol with bacillithiol (BSH). *B. subtilis* does not synthesize GSH but BSH, a tripartite molecule consisting of a cysteine linked to a glucose-2-amine itself linked to a malic acid.^{23, 24} The cellular concentration of BSH in *B. subtilis* varies between 1 to 5 mM along the growth phase.²⁵ The concentration of Cys is low in the cytoplasm, like in *E. coli*, whereas BSH reaches $\sim 20 \pm 8$

μM .²⁶ BSH acts as the major buffer of labile Zn^{2+} , binding Zn^{2+} as a $\text{Zn}(\text{BS})_2$ complex with a binding constant of $1.9 \times 10^{12} \text{ M}^{-2}$.²⁷ It is estimated that $\text{Zn}(\text{BS})_2$ can account for $\sim 1/3$ of total Zn^{2+} in cell, which ranges from 0.1 to 0.8 mM.^{27, 28} Bacterial strains lacking bacillithiol and/or thioredoxins are more sensitive to Hg than the wild type. Exposure to $3 \mu\text{M}$ $\text{Hg}(\text{II})$ consumed all intracellular free reduced BSH and Cys, leading to $\text{Hg}(\text{II})$ sequestration.²⁹ $\text{Hg}(\text{BS})_2$ can transfer $\text{Hg}(\text{II})$ to Trx, TrxR, Grx, and 4-Cys-type zinc binding proteins through thiol exchange, similarly to the $\text{Hg}(\text{GS})_2/\text{protein}$ exchange in *E. coli*. Once loaded with $\text{Hg}(\text{II})$, Trx-(HgS_2) is also able to transfer $\text{Hg}(\text{II})$ to 4-Cys-type zinc binding proteins. The metal chaperone activity of BSH has been established by showing that it facilitates $\text{Zn}(\text{II})$ dissociation from the metal-sensing protein CzrA.²⁷

Figure S9. A model for the formation of β -HgS_{NP} in bacteria at high Hg concentration. Hg is tetrahedrally coordinated to four sulfide anions in β -HgS_{NP}, and this coordination is suggested to derive from the tetrahedral coordination of Fe to sulfide and thiolate anions in [Fe₂S₂] clusters bound to GSH ([Fe₂S₂](GS)₄) and to the [Fe₂S₂] and [Fe₄S₄] clusters bound to Cys residues in proteins.^{30, 31} GSH plays the central role in this model. Monothiol glutaredoxins (Grx) are able to bind iron, sulfur and GSH, forming [Fe₂S₂] clusters that are shared between two subunits of a Grx homodimer and two GSH molecules.^{32, 33}

Step 1. GSH reacts *in vitro* with Fe³⁺ and cysteine to form [Fe₂S₂](GS)₄. The reaction is catalyzed *in vivo* by the sulfur transferase SufS enzyme which transfers two sulfur atoms from Cys to the cluster.^{34, 35}

Step 2. a) In *E. coli*, GSH is a molecular chaperone for the cytosolic Fe²⁺ pool and forms a GS-Fe^{II} complex which constitutes about 90 % of the total cellular iron pool.¹² GS-Fe^{II} can bind to monothiol glutaredoxins.³⁶ b) By analogy with step 1, the [Fe₂S₂](GS)₄ dimeric complex can be formed *in vivo* by the reaction of GS-Fe^{II} with Cys and GSH.

Step 3. The [Fe₂S₂](GS)₄ complex can deliver *in vitro* the [Fe₂S₂] cluster to an apo-protein.^{13, 37, 38} *In vivo*, this process is catalyzed by the cluster assembly complex SufEBCD in *E. coli* and SufUBCD in *B. subtilis*.³⁹

Step 4. At an 1:1 stoichiometric enzyme:metal ratio, Hg(II) inactivates *in vitro* *E. coli* fumarase A by attacking the [Fe₄S₄] cluster of the enzyme and releasing Fe.¹⁴

Step 5. The [Fe₂S₂](GS)₄ complex is degraded under oxidative conditions.¹³

Step 6. We propose that Hg replaces totally Fe in [Fe₂S₂](GS)₄ and the protein clusters upon Hg excess. The nascent dimeric and tetrameric Hg-S clusters resulting from the Hg for Fe substitution may seed the germination of β -HgS_{NP}.

S6. Supplementary tables

Table S1. Metabolic state of bacteria after 3h of culture in MCM medium^a

Culture	<i>Escherichia coli</i>				<i>Bacillus subtilis</i>			
	1	2	3	4	1	2	3	4
100 μ l culture	9.5	10.1	8.7	10.1	78	36	nd ^b	54
250 μ l culture	7.1	7.1	7.4	8.3	39	53	50	56
Mean $\pm$ SD	8.6 $\pm$ 1.3 (n = 8) ^c				52 $\pm$ 14 (n = 7)			

^aMeasured with the reduction rate of NBT, V_{NBT}, expressed in megamolecules.min⁻¹.bacterium⁻¹.

^bnd: not determined.

^cFor comparison, after 3.6h of culture in LB broth, V_{NBT} was 5.9 $\pm$ 0.7 for *E. coli* (n = 8) and 52 $\pm$ 13 for *B. subtilis* (n = 8).

Table S2. Bioaccumulation factors of Hg(II) in *E. coli* and *B. subtilis*

	Hg exposure	[Hg] _i medium nM	[Hg] intra. ^a mg/kg	[Hg] atoms/cell x 10 ³	[Hg] intra. μM	Bioacc. factor
<i>E. coli</i>	HgCl ₂	20	0.68	0.61	0.61	30
	Hg(Cys) ₂	20	1.82	1.63	1.63	81
	Hg(TGA) ₂	20	1.52	1.36	1.36	68
	HgCl ₂	200	8.8	7.9	7.9	39
	Hg(Cys) ₂	200	16.8	15.1	15.1	75
	Hg(TGA) ₂	200	18.6	16.7	16.7	83
	Hg(TGA) ₂ exp.	500	21.6	19.4	19.4	39
	Hg(TGA) ₂ stat.	500	27.2	24.4	24.4	49
<i>B. subtilis</i>	HgCl ₂	20	0.67	0.60	0.60	30
	Hg(Cys) ₂	20	1.82	1.63	1.63	81
	HgCl ₂	200	8.2	7.4	7.4	37
	Hg(Cys) ₂	200	12.0	10.8	10.8	54

^aIntracellular**Table S3. Percentage of transfer of Hg(II) from the medium to bacteria**

	Hg exposure	[Hg] _i medium nM	[Hg] total mg/kg	% Hg bound to bacteria ^a
<i>E. coli</i>	HgCl ₂	20	0.86 ± 0.01	41 ± 2
	Hg(Cys) ₂	20	2.04 ± 0.01	86 ± 4
	Hg(TGA) ₂	20	2.07 ± 0.02	87 ± 4
	HgCl ₂	200	14.2 ± 0.4	67 ± 2
	Hg(Cys) ₂	200	18.6 ± 0.5	70 ± 3
	Hg(TGA) ₂	200	23.1 ± 0.6	74 ± 3
<i>B. subtilis</i>	HgCl ₂	20	0.82 ± 0.05	32 ± 2
	Hg(Cys) ₂	20	2.01 ± 0.01	78 ± 4
	HgCl ₂	200	8.7 ± 0.2	34 ± 5
	Hg(Cys) ₂	200	13.7 ± 0.1	54 ± 3

^aThe calculation takes into account the loss of biomass during the different centrifugation steps and the final washing step (with buffer only, without chelating chemicals), that amounts to 4-6 %.

Table S4. Tetracoordinated zinc in the proteome of *Escherichia coli*

Gene names and accession numbers ^a	C4-type zinc finger locations	Cysteine arrangement within zinc fingers
4-Cys-type zinc finger proteins		
UvrA (J7QB19), 940 aa	253-280; 740-766	CXXCX ₂₀ CXXC and CXXCX ₁₉ CXXC
DNA topoisomerase TopA (P06612), 865 aa	599-630; 662-689; 711-736	CXXCX ₁₆ CX ₁₀ C, CXXCX ₁₇ CX ₅ C and CXXCX ₁₆ CX ₄ C
Carbamoyltransferase HypF, (P30131), 750 aa	109-134; 159-184	Both CXXCX ₁₈ CXXC
Primosomal protein N' PriA (A0A4S5AYQ0), 732 aa	436-448; 463-479	CXXCX ₅ CXXC and CXXCX ₉ CXXC
Ribonucleoside-triphosphate reductase, NrdD (P28903), 712 aa	644-665	CXXCX ₁₄ CXXC
ATP-dependent DNA helicase RecQ (P15043), 609 aa	380-403	CX ₁₆ CXXCXXC
DNA repair helicase RadD (P33919), gene yejH, 586 aa	384-425	CXXCX ₂₃ CX ₁₃ C
DNA repair protein RadA (P24554), 460 aa	11-28	CXXCX ₁₀ CXXC
ATP-dependent Clp protease ATP-binding subunit ClpX (P0A6H1), 424 aa	15-40	CXXCX ₁₈ CXXC
Chaperone protein DnaJ (C3TRJ7), 376 aa,	144-164; 183-200	CXXCX ₁₃ CXXC and CXXCX ₁₀ CXXC
DNA repair enzyme Ada (P06134), 354 aa	38-72	CX ₃ CX ₂₆ CXXC
Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta (C3T1Y2), AccD, 304 aa	27-49	CXXCX ₁₅ CXXC
Formamidopyrimidine-DNA glycosylase (P05523), Fpg or MutM, 269 aa	238-269	CXXCX ₁₆ CXXC
Endonuclease 8 (P50465), Nei, 263 aa	232-263	CXXCX ₁₆ CXXC
Recombination protein (P0A7H6), RecR, 201 aa	57-72	CXXCX ₈ CXXC
DNA topoisomerase, YrdD (W8STP9), 180 aa	13-49; 62-98; 104-142; 145-167	CXXCX ₁₆ CX ₅ C, CXXCX ₁₆ CX ₅ C, CXXCX ₁₈ CX ₅ C, and CXXCX ₁₇ CX ₄ C
RNA polymerase-binding transcription factor (C3TPZ2), DksA, 151 aa	114-138	CXXCX ₁₇ CXXC

Thioredoxin 2 (P0AGG4), TrxC, 139 aa	5-18	CXXCX ₁₆ CXXC
Antitoxin MqsA (Q46864), 131 aa	3-40	CXXCX ₃₀ CXXC
C4-type zinc finger protein (P41039), YbiI, 88 aa	39-63	CXXCX ₁₇ CXXC
DNA gyrase inhibitor YacG (P0A8H8), 65 aa	9-32	CXXCX ₁₅ CXXC
Protein YdaE (P38394), 56 aa	7-46	CX ₄ CX ₂₉ CX ₃ C
4-Cys-type non-zinc-finger proteins^a	C4-type zinc binding site locations	Arrangement of coordinating cysteines
RNA polymerase subunit β', RpoC (P0A8T7), 1407 aa	70-88; 814-898	CXCX ₁₂ CXXC, CX ₇₃ CX ₆ CXXC
Ribonuclease E, Rne (P21513), 1061 aa	404-411	2 protomers share a single zinc ion: CXXC
Isoleucine-tRNA synthase, IleS (P00956), 938 aa	901-924	CXXCX ₁₆ CXXC
Methionine-tRNA synthase, MetG (P00959), 677 aa	146-162	CXXCX ₉ CXXC
Ribonuclease G, Rng (P0A9J0), 489 aa	405-412	2 protomers share a single zinc ion: CXXC
Alcohol dehydrogenase YbdR (C3TJW2), 412 aa	90-104	CXXCXXCX ₇ C
S-(hydroxymethyl)glutathione dehydrogenase, FrmA (P25437), 369 aa	92-106	CXXCXXCX ₇ C
Alcohol dehydrogenase YphC (A0A449CWR4), 364 aa	111-125	CXXCXXCX ₇ C
Alcohol dehydrogenase, YdjL (P77539), 358 aa	93-107	CXXCXXCX ₇ C
Alcohol dehydrogenase, YdjJ (P77280), 358 aa	95-109	CXXCXXCX ₇ C
Aldehyde reductase YahK (P75691), 349 aa	93-107	CXXCXXCX ₇ C
Galactitol 1-phosphate 5- dehydrogenase, GatD, 346 aa	89-103	CXXCXXCX ₇ C
L-threonine 3-dehydrogenase, Tdh (P07913), 341 aa	93-107	CXXCXXCX ₇ C
Aldehyde reductase Ahr (P27250), 339 aa	96-110	CXXCXXCX ₇ C
Starvation-sensing protein RspB (P38105), 339 aa	89-103	CXXCXXCX ₇ C

Alcohol dehydrogenase AdhP (P39451), 336 aa	89-103	CXXCXXCX ₇ C
Hsp33 family HslO chaperone (P0A6Y5), 292 aa	230-266	CXCX ₃₀ CXXC
NADH pyrophosphatase, NudC (P32664), 257 aa	98-119	CXXCX ₁₄ CXXC
Sulfur carrier protein ThiS adenylyltransferase (P30138), ThiF, 251 aa	169-243	CXXCX ₆₇ CXXC
Molybdopterine-synthase adenylyltransferase MoeB (P12282), 249 aa	172-247	CXXCX ₆₈ CXXC
Aspartate carbamoyltransferase regulatory chain, PyrI (P0A7F3), 153 aa	109-141	CX ₄ CX ₂₃ CXXC
Hydrogenase maturation nickel metallochaperone HypA (P0A700), 116 aa	73-93	CXXCX ₁₃ CXXC
Hydrogenase maturation factor HybF (A0A4V1GQU3), 113 aa	73-93	CXXCX ₁₂ CXXC

^aUniprot accession number are indicated between parenthesis.

Table S5. Tetracoordinated zinc in the proteome of *Bacillus subtilis*

Gene names and accession numbers ^a	4-Cys-type zinc finger locations	Cysteine arrangement within zinc fingers
4-Cys-type zinc finger proteins		
UvrA (O34863), 957 aa	252-279; 738-764	CXXCX ₂₀ CXXC and CXXCX ₁₉ CXXC
Primosomal protein N' (P94461), ATP-dependent helicase PriA, 805 aa	510-522; 537-553	CXXCX ₅ CXXC and CXXCX ₉ CXXC
ATP-dependent Clp protease, ATP-binding subunit ClpE (O31673), 699 aa	3-32	CXXCX ₂₂ CXXC
DNA topoisomerase 1 (P39814), TopA, 691 aa	579-605 ; 619-647 ; 660-683	CXXCX ₁₆ CX ₅ C, CXXCX ₁₈ CX ₅ C and CXXCX ₁₆ CXXC
ATP-dependent DNA helicase RecQ (A0A2I7TCG1), 592 aa	375-398	CX ₁₆ CXXCXXC
DNA repair protein RadA (P37572), 458 aa	10-27	CXXCX ₁₀ CXXC
ATP-dependent Clp protease ATP-binding subunit ClpX (P50866), 420 aa	15-40	CXXCX ₁₈ CXXC

Chaperone protein DnaJ (P17631), 375 aa	145-165; 188-205	CXXCX ₁₃ CXXC and CXXCX ₁₀ CXXC
Acetyl-coenzyme A carboxylase, carboxyl transferase subunit beta (C0SP93), AccD, 290 aa	32-54	CXXCX ₁₅ CXXC
Formamidopyrimidine-DNA glycosylase (O34403), Fpg or MutM, 276 aa	249-272	CXXCX ₁₆ CXXC
DNA repair enzyme Ada, (P19219), 211 aa	54-88	CX ₃ CX ₂₆ CXXC
Recombination protein RecR (P24277), 198 aa	57-72	CXXCX ₈ CXXC

4-Cys-type non-zinc-finger proteins^a

C4-type zinc binding site locations

Arrangement of coordinating cysteines

RNA polymerase subunit β', RpoC (P37871), 1199 aa	60-78; 818-902	CXCX ₁₂ CXXC, CX ₇₃ CX ₆ CXXC
Alcohol dehydrogenase (Q70KK7), 378 aa	89-103	CXXCXXCX ₇ C
Alcohol dehydrogenase AdhB (A0A5Q3E9A3), 378 aa	90-104	CXXCXXCX ₇ C
Sorbitol dehydrogenase GutB (Q06004), 353 aa	100-114	CXXCXXCX ₇ C
Alcohol dehydrogenase AdhA (S6FCG4), 349 aa	97-111	CXXCXXCX ₇ C
Alcohol dehydrogenase (A0A5Q3E4B8), 348 aa	89-103	CXXCXXCX ₇ C
L-threonine dehydrogenase, Tdh (A0A5Q3KSJ1), 348 aa	98-112	CXXCXXCX ₇ C
Alcohol dehydrogenase YjmD (A0A5Q2UXZ7), 339 aa	90-104	CXXCXXCX ₇ C
Molybdopterin-synthase adenylyltransferase MoeB (A0A235BIC9), 339 aa	166-243	CXXCX ₇₀ CXXC
Ethanol dehydrogenase (A0A5Q2UX45), 338 aa	90-104	CXXCXXCX ₇ C
Molybdopterin-synthase adenylyltransferase ThiF (A0A5Q3DXQ2), 336 aa	166-241	CXXCX ₆₈ CXXC
Heat shock protein Hsp33 (A7Z0J4), 291 aa	235-271	CXCX ₃₀ CXXC
50S ribosomal protein L31 (Q03223), 66 aa	16-39	CXCX ₁₇ CXXC

30S ribosomal protein S14 (P12878), 61 aa	24-43	CXXCX ₁₂ CXXC
50S ribosomal protein L32 (O34687), 59 aa	30-46	CXXCX ₉ CXXC

^aUniprot accession number are indicated between parenthesis, along with the length of the protein in amino-acids (aa).

Table S6. Number of 4-Cys-type zinc binding protein molecules per *E. coli* cell^a

	Schmidt <i>et al.</i> ⁴⁰		Li <i>et al.</i> ⁴¹		
	Glucose	LB	MOPS minimal	MOPS complete	Mean
4-Cys-type zinc finger proteins					
UvrA (J7QB19)	127	228	206	621	295.5
TopA (P06612)	355	890	677	2792	1178.5
HypF (P30131)	nd ^b	nd	14	38	26
PriA (A0A4S5AYQ0)	3	4	59	105	43.75
NrdD (P28903)	30	52	92	256	107.5
RecQ (P15043)	nd	nd	63	155	109
RadD (P33919)	nd	nd	nd	nd	
RadA (P24554)	39	48	148	428	165.75
ClpX (P0A6H1)	3251	5494	2475	5857	4269.25
DnaJ (C3TRJ7)	688	2011	938	5688	2331.25
Ada (P06134)	nd	nd	15	21	18
GatD (P0A9S3)	128	1229	2360	643	1090
AccD (C3T1Y2)	1660	3440	2475	8774	4087.25
MutM (P05523)	3	21	86	684	198.5
Nei (P50465)	0	0	93	248	85.25
RecR (P0A7H6)	76	257	226	1342	475.25
YrdD (W8STP9)	3	3	169	347	130.5
DksA (C3TPZ2)	5844	19594	5438	21225	13025.25
TrxC (P0AGG4)	257	393	569	1704	730.75
MqsA (Q46864)	34	181	319	448	245.5
YbiI (P41039)	3	4	81	4	23
YacG (P0A8H8)	95	255	292	724	341.5
YdaE (P38394)	nd	nd	0	0	0
4-Cys-type Zn- chelating proteins, non- zinc-fingers					

RpoC (P0A8T7)	2779	7164	3715	17553	7802.75
Rne (P21513)	848	1624	1061	2729	1565.5
IleS (P00956)	2166	2886	2270	6143	3366.25
MetG (P00959)	2082	2572	1864	6555	3268.25
Rng (P0A9J0)	85	98	370	440	248.25
YbdR (C3TJW2)	19	23	53	4	24.75
FrmA (P25437)	551	1528	736	4204	1754.75
YphC (A0A449CWR4)	2	1	39	62	26
YdjL (P77539)	nd	nd	10	17	13.5
YahK (P75691)	1474	877	2250	154	1188.75
YdjJ (P77280)	nd	nd	10	7	8.5
IdnD (P39346)	274	382	9	8	168.25
Tdh (P07913)	181	2388	263	4448	1820
RspB (P38105)	1	2	24	20	11.75
Ahr (P27250)	1	0	nd	nd	0.5
AdhP (P39451)	26	48	773	20	216.75
HslO (P0A6Y5)	2363	5556	953	6405	3819.25
NudC (P32664)	147	133	318	593	297.75
ThiF (P30138)	nd	nd	713	5	359
MoeB (P12282)	94	106	107	325	158
PyrI (P0A7F3)	19076	3040	26096	1082	12323.5
HypA (P0A700)	nd	nd	3	1	2
HybF (A0A4V1GQU3)	nd	nd	9	47	28
Total	44731	62351	58122	102478	66920 ± 24866

^aSince in the present study the bacterial biomass was grown in LB and the exposure to Hg(II) was performed in a minimal medium containing glucose, and since the number of protein molecules vary according to the medium (for most proteins it is higher in a complete rich medium), we assumed that the real number would be in between those given in these two conditions. Therefore, we have calculated the mean number and used the two available sets of data.^{40, 41}

^bnd: not described. In the work published by Schmidt *et al.*⁴⁰, there are 2359 genes for which the expression has been quantified out of 4494 genes that contains the *Escherichia coli* species.

Table S7. Number of 4-Cys-type zinc binding protein molecules per *B. subtilis* cell

	S ^a	CH ^a	Mean
<u>Zinc finger proteins</u>			

UvrA (O34863)	212	nd	
PriA (P94461)	nd ^b	nd	
ClpE, (O31673)	165	420	292.5
TopA (P39814)	324	1123	723.5
RecQ (A0A2I7TCG1)	nd	nd	
RadA (P37572)	nd	nd	
ClpX (P50866)	1690	6358	4024
DnaJ (P17631)	353	461	407
AccD (C0SP93)	1119	1271	1195
MutM (O34403)	nd	nd	
AdaA (P19219)	nd	nd	
RecR (P24277)	nd	nd	

4-Cys-type Zn-chelating proteins, non-zinc-fingers

RpoC (P37871)	2487	8264	5375.5
Alcohol dehydrogenase (Q70KK7)	nd	nd	
AdhB (A0A5Q3E9A3)	nd	nd	
GutB (Q06004)	nd	nd	
AdhA (S6FCG4)	nd	nd	
Alcohol dehydrogenase (A0A5Q3E4B8)	nd	nd	
Tdh (A0A5Q3KSJ1)	nd	nd	
Alcohol dehydrogenase (A0A5Q2UXZ7)	nd	nd	
MoeB (A0A235BIC9)	nd	nd	
Alcohol dehydrogenase (A0A5Q2UX45)			
ThiF (A0A5Q3DXQ2)	323	790	556.5
Hsp33 (A7Z0J4)	263	728	495.5
Ribosomal protein L31 (Q03223)	nd	nd	
Ribosomal protein S14 (P12878)	nd	nd	
Ribosomal protein L32 (O34687)	nd	nd	
Total	6936	19415	13175 ± 6239

^aTwo growth media were made⁴²: a minimal one containing glucose as the carbon source and ammonium salt as a nitrogen source (condition S), and a rich medium containing 10 g/L of casein hydrolysate and supplemented in Glu, Ala and Asn (condition CH). ^bnd: not described. In the work published by Muntel *et al.*⁴², there are 1280 genes for which the expression has been quantified out of 4422 genes that contains the *Bacillus subtilis* species.

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