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**A Synthetic Circuit for Mercury Bioremediation Using Self-Assembling Functional
Amyloids**

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Abstract

Synthetic biology approaches to bioremediation are a key sustainable strategy to leverage the self-replicating and programmable aspects of biology for environmental stewardship. The increasing spread of anthropogenic mercury pollution into our habitats and food chains is a pressing concern. Here, we explore the use of programmed bacterial biofilms to aid in the sequestration of mercury. We demonstrate that by integrating a mercury-responsive promoter and an operon encoding a mercury-absorbing self-assembling extracellular protein nanofiber, we can engineer bacteria that can detect and sequester toxic Hg^{2+} ions from the environment. This work paves the way for the development of on-demand biofilm living materials that can operate autonomously as heavy-metal absorbents.

Keywords

biofilm engineering, curli, mercury sequestration, biosorption, protein engineering, amyloid

Mercury is widely circulated throughout the environment as a by-product of industrial processes such as mining, materials processing, coal combustion for power, and as components of chemicals and electronics. These anthropogenic sources of environmental mercury result in the unnatural dissemination and accumulation of mercury compounds in land, freshwater, and marine habitats.¹ Mercury is a particularly insidious pollutant, as its accumulation in ecological niches increases up through the food chain, a process known as biomagnification.² Humans are typically exposed to toxic mercury compounds through the ingestion of contaminated food sources such as fish or shellfish, leading to damage to tissues of the brain, kidney, and lung.³ *In utero* exposure results in severe developmental abnormalities, resulting in EPA-FDA advisories against eating fish during pregnancy.⁴ Recent studies have found that mercury contamination in the environment is much more prevalent than previously thought,⁵ hence the need for innovative

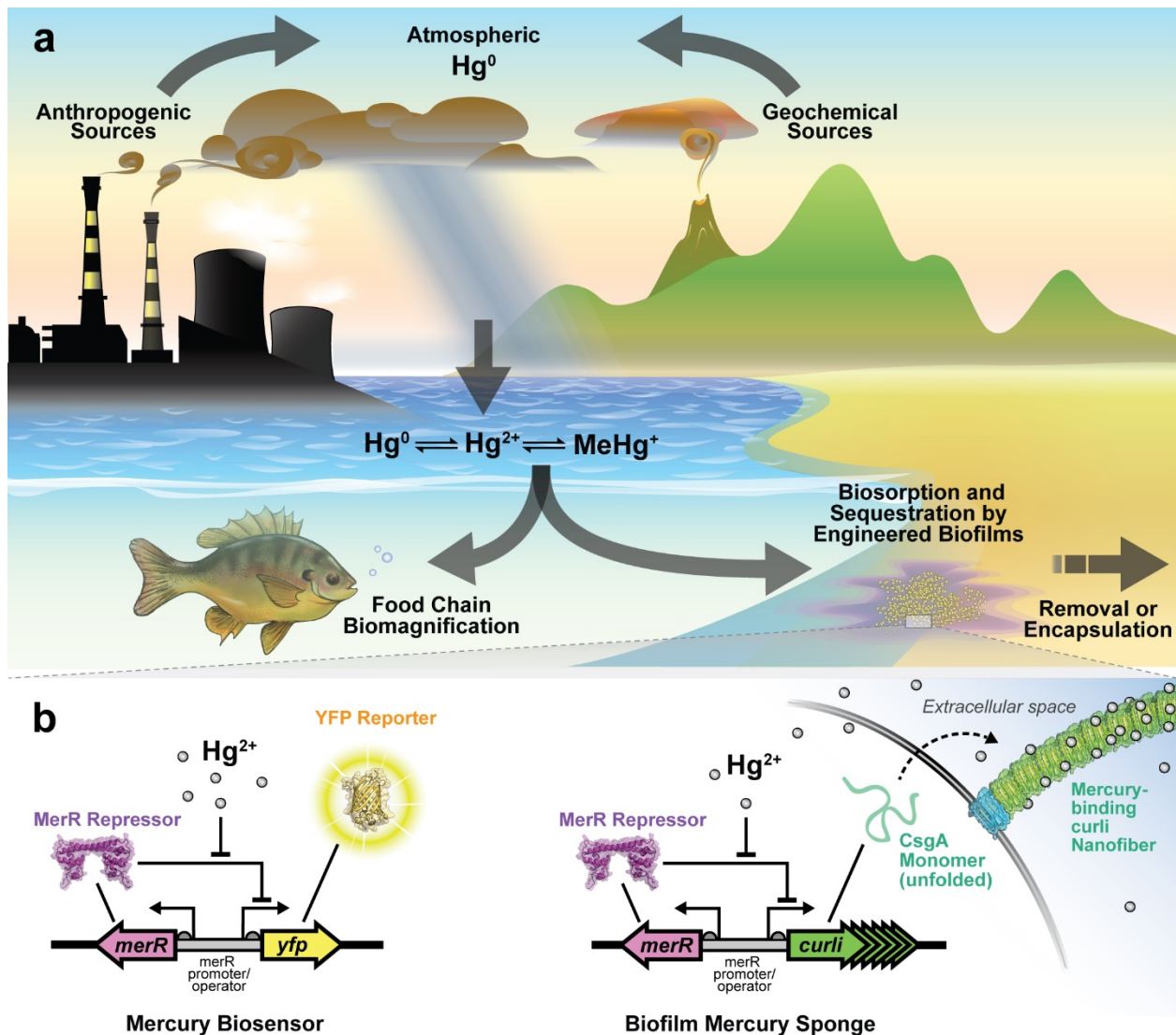


Figure 1. Engineering a synthetic mercury bioremediation circuit. (a) Man-made and natural geochemical processes result in mercury accumulation in the environment and food chains, where it is biomagnified. Engineered synthetic biofilms acting as a mercury sink could be deployed to bind and sequester environmental mercury for bioremediation. (b) Schematic of the MerR-regulated Hg^{2+} -binding curli biofilm circuit. The reporter gene in the MerR-based mercury biosensing circuit is replaced with a curli operon encoding the synthesis and export of self-assembling functional amyloids that are able to bind mercury ions.

approaches to remediating contaminated sites. Of particular interest are scalable, low-cost, and sustainable biological approaches for the detection and sequestration of mercury ions.

Bioengineered mercury sensor circuits employ naturally occurring mercury-responsive transcriptional regulators, such as the MerR regulator.⁶ MerR regulates the expression of the *mer* operon—a widespread and ancient bacterial operon family found in plasmids and transposons—which encodes enzymes for mercury detoxification.⁷ Upon binding to Hg²⁺, the MerR repressor undergoes a conformational change resulting in de-repression of the *mer* operon. By replacing the *mer* operon genes with a reporter such as luciferase⁸ or GFP,⁹ mercury-inducible biosensors have been developed that allow for bacterial reporting of environmental mercury. Besides mercury reporters, a number of attempts have been made to use bacteria to bind and sequester mercury. For example, intracellular accumulation of mercury has been engineered into bacteria by the overexpression of heavy metal-binding metallothioneins, with the goal of remediating mercury contaminated water.¹⁰ However, it was found that the addition of a Hg²⁺ transport system, encoded by the *merT* and *merP* genes, were essential for mercury sequestration. An alternative strategy uses cell-surface displayed mercury-binding proteins, such as a metallothioneins,¹¹ phytochelatins,¹² or the MerR metal binding domain,¹³ to create engineered cellular biosorbents. These examples of engineered bacterial circuits for sensing and absorption of mercury demonstrate the exciting potential of green biological strategies for mercury remediation of contaminated environments. However, the sequestration strategies described above employed externally added chemical inducers (e.g., IPTG) rather than having the cell react dynamically to environmental mercury. Furthermore, these strategies use the cell biomass itself as the mercury sink, which requires continuous energetic investment in biomass synthesis, and would end up poisoning the cell. One promising approach is the use of the extracellular material (ECM) of bacterial biofilms to act as a biosorbent for the extraction of environmental mercury. The high surface area of the ECM could potentially provide much larger absorption capacities than cell surface-based strategies and prevent mercury-induced toxicity to the cell, allowing sustained production of the mercury-binding material. A number of studies have investigated naturally occurring biofilms for their heavy metal absorption characteristics,^{14, 15} but to our knowledge, there has not been a rationally designed dynamic gene circuit for the production of a mercury-absorbing bacterial ECM. A robust and autonomously operating biofilm that is able to sequester mercury could act as a sink to remove anthropogenic and geological sources of mercury contamination, for field deployment to reduce the mercury burden in ecological food chains and remediate heavily polluted sites (Figure 1a). Our strategy builds upon efforts from others that use renewable biomaterials, often purified and assembled into a filter matrix *in vitro*, as materials for heavy metal adsorption. These materials include animal¹⁶ and plant-derived¹⁷ biomaterials such as keratin or cellulose fibers as binding agents for the sequestration of various heavy metal contaminants. Of particular interest is the recent exploration of synthetic composites containing self-assembling amyloid fibers as a powerful technology for the removal of heavy metal pollutants.¹⁸ Amyloids have been known to interact specifically with heavy metals¹⁹⁻²¹ and these complexes can be redox active.^{18, 22, 23} Such properties have led to a number of amyloid-based emerging technologies.²⁴⁻²⁶ Although using purified amyloids to create purification membranes has the advantages of precise control over the final composition of the material and its properties, it still requires purification and engineered assembly steps which may add to cost and complexity of the system. Here, we explore the potential of integrating such amyloid materials technology with synthetic biological principles to create an engineered living

material^{27, 28} capable of fabricating a functional sequestration material *in situ* only when the pollutant is detected.

Escherichia coli and *Salmonella* spp. biofilms contain functional amyloids called curli, which are self-assembling extracellular protein nanofibers.²⁹ We and others have recently re-engineered curli fibers for the functional display of peptides and proteins to create dynamic engineered living materials.³⁰⁻³³ Curli and similar functional amyloids have evolved as a key biofilm component enabling substrate adhesion,³⁴ structural reinforcement of the biofilm,³⁵ and host cell invasion.³⁶ A recent study by Hidalgo and colleagues suggested that curli might also serve a protective function, specifically by shielding bacteria in biofilms from extracellular mercury through adsorption of the heavy metal.³⁷

Based on these findings, we designed and engineered a synthetic circuit that is able to detect mercury in the environment (via MerR) and direct the synthesis of curli nanofibers to sequester mercury ions in an extracellular matrix. The circuit utilizes the divergently regulated MerR promoter (P_{merR}) derived from a *Shigella flexneri* plasmid, engineered such that MerR is constitutively expressed and represses transcription of either a reporter (YFP) or the curli operon. When present, mercury ions bind to MerR to trigger an allosteric change and allow transcription and expression of the desired output (Figure 1b).

To demonstrate that our circuit responds to mercury, the pET30a- P_{merR} -curli plasmid was transformed into a previously engineered *E. coli* strain, PQN4, in which the entire curli operon has been deleted.³⁸ The parental *E. coli* strain is the MC4100 strain, which advantageously does not produce any other extracellular materials, such as polysaccharides, other fimbriae, or flagella, that may complicate analysis. The MC4100 strain has thus been used extensively in mechanistic and genetic studies of the curli operon.³⁹⁻⁴¹ In the wild-type MC4100 background, as in most other wild *E. coli* strains, induction of the genomically-encoded curli operon occurs only under conditions of low osmolarity and/or starvation.^{42, 43} By placing this operon instead under the control of a mercury-inducible promoter, we have decoupled curli production from these narrow conditions and have now coupled them instead to the presence of environmental mercury, creating an engineered living material that fabricates a heavy-metal sequestering nanomaterial in response to the detection of that specific pollutant.

The pET30a- P_{merR} -YFP and a pET30a control vector were used as controls. Colonies of overnight cultures were spotted onto minimal media agar containing the amyloid-specific dye Congo Red, with or without ionic mercury (Hg^{2+}), and left to grow at 30°C overnight. Minimal media was chosen to reduce the effect of media components on metal binding. As shown in Figure 2a, curli production was tightly regulated by P_{merR} and high expression required the presence of Hg^{2+} . The P_{merR} -YFP biosensor transformants exhibited mercury-induced fluorescence, indicating proper functioning of the MerR-regulated promoter (Figure S1). There was a graded response, with more curli produced at higher Hg^{2+} concentrations. This concentration-dependent expression was further demonstrated by culturing cells in suspension overnight with exposure to a range of Hg^{2+} concentrations. The curli content of the cultures was measured using a Congo Red quantification assay and normalized to the OD_{600} of the cultures. We confirmed that the presence of mercury did not affect Congo Red binding to curli (Figure S2). In our MerR-regulated curli circuit, induction in liquid media occurred at 600 ppb and above. Induction of curli plateaus at 1000 ppb and was sustained at the maximum concentration

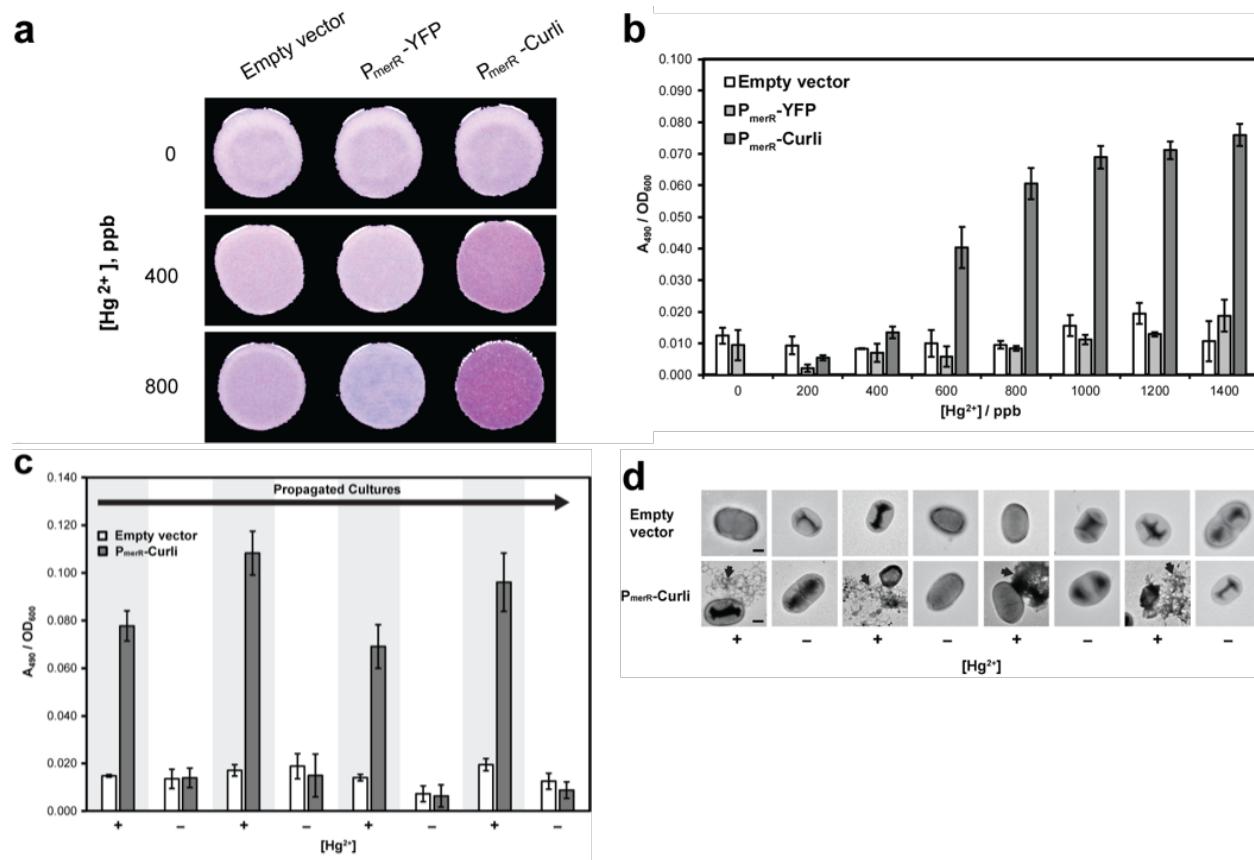


Figure 2. Curli nanofiber production is regulated by environmental mercury (Hg²⁺) concentration. (a) The MerR circuit activates the output genes in the presence of Hg²⁺, as seen with P_{merR}-curli cells spotted on plates containing the amyloid-specific dye Congo Red. (b) Cells grown in suspension were exposed to a range of Hg²⁺ concentrations overnight. Quantitation of curli production showed a distinct concentration dependence. (c) Curli production in individually propagated cultures exposed to alternating conditions of 1000 ppb of mercury and no mercury, demonstrating dynamic control of nanofiber production only in the presence of environmental mercury. For each transformation type, individual clones were propagated in quadruplicate. (d) Representative transmission electron micrographs of cells from (c), showing the presence of curli (arrows) only for the P_{merR}-curli cells exposed to mercury. Scale bars, 500 nm.

tested, 1400 ppb (Figure 2b), but significant amounts beyond background was not detected in the absence of Hg²⁺ as measured by our CR-binding assay. To examine potential toxic effects of high mercury concentrations on the viability of the cell, we measured the density of the cultures after 24 hours growth for each of the concentrations. At mercury concentrations above 1000 ppb, cell densities only decreased for the P_{merR}-curli expressing cells. In contrast, transformants harboring the P_{merR}-YFP or negative control plasmid (Figure S3a) showed no reduced cell density up to 1400 ppb. Previous reports have established an HgCl₂ MIC of 2 ppm for *E. coli*.⁴⁴ The results demonstrate that at these higher mercury concentrations, any negative impact on growth for the P_{merR}-curli cells is likely due to the metabolic burden of induced protein overproduction rather than mercury toxicity effects on the cell. We also observe similar negative impacts on cell health when the curli operon under control of the strong P_{T7/lacO} promoter is highly overexpressed by IPTG induction (Figure S3b). These findings lend support to the utility of our sensing feedback-regulated circuit, in which a metabolically costly nanofiber matrix is fabricated by the cell for sequestering mercury only when mercury is detected in the environment.

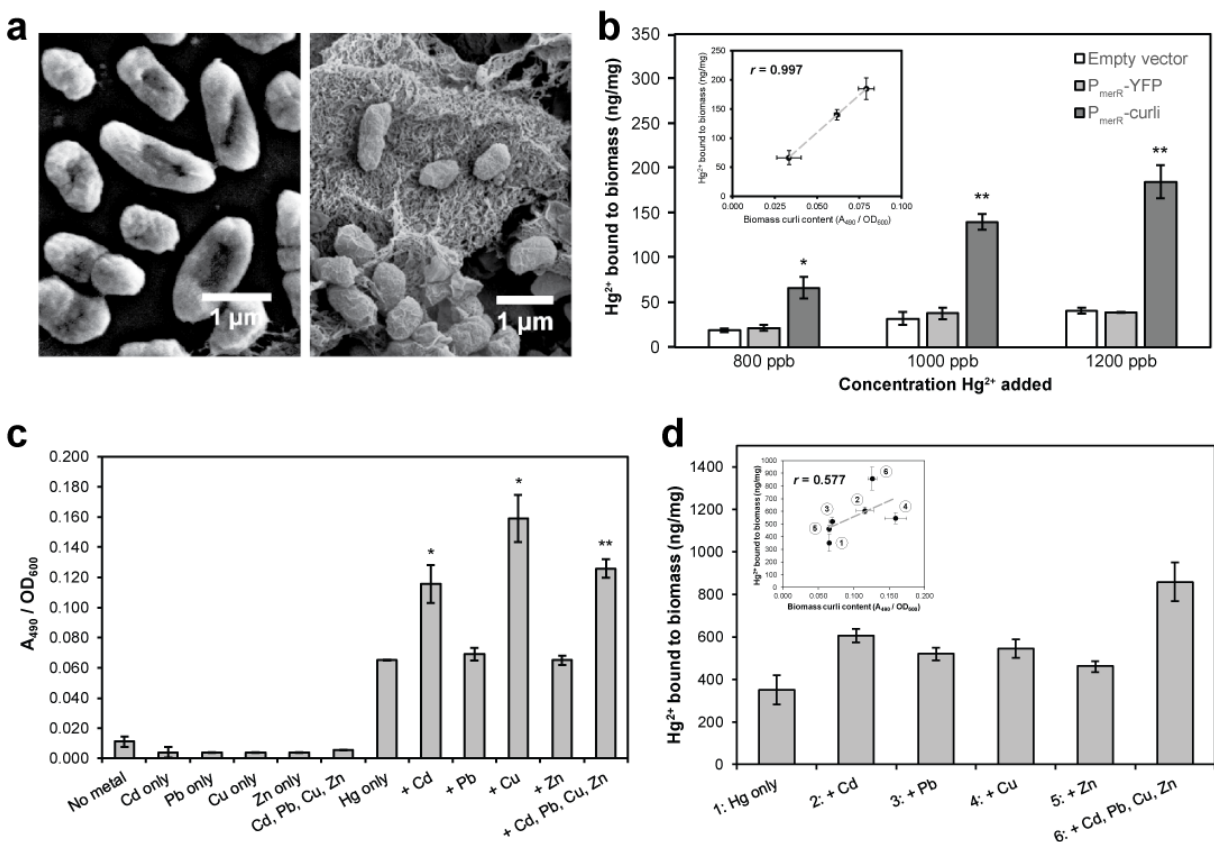


Figure 3. Mercury-induced curli nanofibers sequester mercury from the environment. (a) Scanning electron micrographs of cells containing empty plasmid (left) and P_{merR}-curli plasmid (right), exposed to Hg²⁺. Only the latter showed abundant production of extracellular curli nanofibers. (b) Cultures expressing curli showed higher Hg²⁺ sequestration after overnight exposure compared to non-curliated cultures, as measured by ICP-MS quantitation of bound mercury. The amount of Hg²⁺ bound correlated positively with the curli content of the culture (inset, $r = 0.997$ = calculated Pearson correlation coefficient). (c) The MerR circuit is selective for Hg²⁺, although some divalent metals (Cd²⁺, Cu²⁺) could act synergistically with Hg²⁺ to further enhance curli production. All metals were added at 5 μ M. (d) Hg²⁺ sequestration by curli fibers was not compromised in the presence of a metal mixture as determined by ICP-MS. Inset shows the Pearson coefficient between the amount of Hg²⁺ bound and the curli content. * represents $p < 0.05$; ** represents $p < 0.01$.

As a proxy for solid media such as contaminated soil, mercury-laden agar was used to perform induction experiments on macrocolonies (Figure S4). On solid media, the curli induction response as detected by a quantitative congo red assay appears to be more sensitive to mercury concentrations, occurring at 200 ppb and above. This lower induction threshold in comparison to liquid media is likely due to altered gene expression between the different modes of growth that may influence P_{mer} mercury induction and/or the increased likelihood for curli polymerization on solid media due to retarded diffusion. These results demonstrate that such a circuit for generating a mercury-absorbing extracellular matrix can be applied to different forms of contaminated media.

We visually confirmed the presence of dense nanofibers by scanning electron microscopy (Figure 3a). Our circuit was active at mercury concentrations defined for mercury-contaminated sites,⁴⁵ thus making it potentially useful for environmental Hg²⁺ remediation. Further, the dynamic response of the circuit, as shown for single clonal populations propagated through

multiple generations, persisted through repeated changes in environmental mercury concentration (Figure 2c).

We next investigated the extent of mercury sequestration by curli-producing cultures. Cultures exposed overnight to Hg^{2+} were pelleted, dried and analyzed for their mercury content by inductively coupled plasma mass spectrometry (ICP-MS). Curli-expressing bacteria bound 4.5x more mercury on a dry weight basis than cells containing the empty vector when exposed to 1200 ppb Hg^{2+} (Figure 3b). Cells expressing the P_{merR} -YFP circuit showed the same low level of mercury binding as cells with the empty vector (Figure 3b), demonstrating that activation of the circuit alone was not responsible for enhanced mercury sequestration, and the latter was a consequence of curli production. Furthermore, IPTG-induced curli fibers generated by a $P_{T7/lacO}$ promoter instead of P_{merR} also bound to mercury at equivalent levels (Figure S5a,b), indicating that mercury adsorption is due only to the curli fibers and that these nanofibers are functionally identical regardless of the promoter system. TEM analysis also indicates the same ultrastructure for curli fibers produced regardless of the regulating promoter (Figure S5c). The dependence of mercury binding on curli synthesis was more apparent when we looked at P_{merR} -curli cells exposed to different concentrations of mercury. The quantity of mercury bound in the biomass correlated significantly to the curli content of the cultures (Figure 3b inset), thus bacteria exposed to higher levels of mercury also sequestered more mercury via the production of more extracellular curli fibers, creating a self-governing mercury-binding system. Curliated cultures were able to retain mercury for over ten days, even after several washes (Figure S6). The mechanism of mercury binding to curli is unclear; there are no cysteine residues in CsgA, though the presence of multiple glutamic and aspartic acid residues along the backbone of assembled fibers suggests an electrostatic interaction. CsgA could also have an inherent ability to reduce Hg^{2+} , as has been demonstrated for the amyloidogenic $\text{A}\beta$ peptide and its reduction of Cu^{2+} .⁴⁶ Because mercury-contaminated sites could also contain other metal pollutants,⁴⁷ we exposed P_{merR} -curli cells to different metal cocktails to determine their impact on circuit activation and mercury binding to curli. MerR is known to be cross-selective for several other metals (Au^{3+} , Zn^{2+} , Ag^+ , Cd^{2+}), though higher concentrations of those metals (2-3 orders of magnitude relative to mercury) are required for transcriptional activation.⁴⁸ We tested four divalent metals (Cd^{2+} , Pb^{2+} , Cu^{2+} , Zn^{2+}), none of which induced curli production when used individually or as a mixture at concentrations equivalent to 1000 ppb Hg^{2+} (Figure 3c). Interestingly however, Cd^{2+} and Cu^{2+} gave a further increase in curli production when used in equimolar combination with Hg^{2+} , even though Cu^{2+} is not known to interact with MerR.^{48, 49} This hitherto undescribed hetero-bimetallic activation of MerR expands the range of environments in which our circuit could be useful (for instance, in mercury-contaminated sites near copper mines) and its mechanism warrants further investigation. Importantly, the amount of mercury bound by curli-expressing cultures as measured by ICP-MS was not compromised in mixed-metal environments and was actually substantially higher in all cases, although it no longer scaled with curli content, possibly due to interference from the other metals (Figure 3d). One possibility is that the metals could be forming multi-metallic complexes on the curli fibers, which would facilitate Hg^{2+} deposition and explain improved mercury binding from metal cocktails.

Flocculation of cellular biomass driven by mercury-induced biofilms would particularly aid in the sequestration of mercury by generating a precipitated mass that would consolidate and extract the heavy metal when the contaminant media is liquid, such as leachate or mine tailings

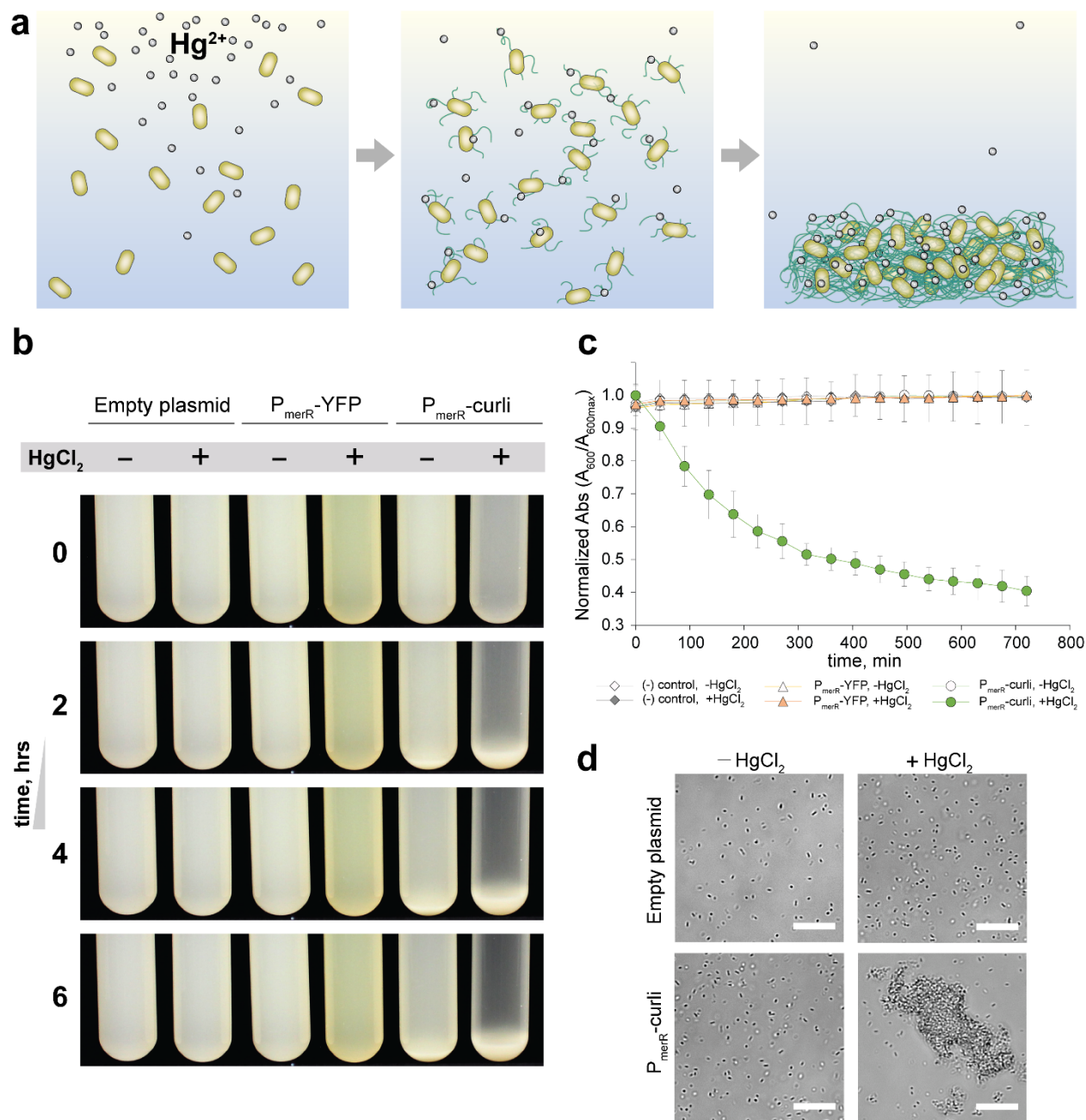


Figure 4. Mercury-induced biofilm flocculation removes mercury-bound biomass from suspension. (a) Schematic showing induction of curli production leading to mercury binding and flocculation of the cells and curli, resulting in sedimentation of the biomass and bulk removal of mercury from the suspension. (b) Images of transformants with no mercury or exposed to 800 ppb of mercury that have been allowed to flocculate and settle over 6 hours. (c) Quantitative sedimentation of the cultures by absorbance measurements. Shown as $n=3$, S.D. (d) Representative microscopy images of cell cultures showing flocculation for P_{merR} -curli transformants either exposed to no mercury or 800 ppb of mercury. Scale bars, 15 microns.

(Figure 4a). Rapid flocculation was observed for P_{merR} -curli transformants cultured in the presence of Hg^{2+} , whereas this was not observed for P_{merR} -YFP or empty vector transformants (Figure 4b-c). Microscopy examination of the cultures showed the presence of large cellular aggregates only when cells containing the P_{merR} -curli circuit were exposed to Hg^{2+} (Figure 4d).

Further studies are warranted to establish any potential influence of mercury cations on curli aggregation, as heavy metals have been found to participate in the aggregation of other amyloids.⁵⁰

Current heavy metal bioremediation strategies face several limitations: where natural biofilms are used, large quantities of biomass are often necessary to compensate for unpredictable and non-specific metal sorption, and even with bacteria genetically engineered to improve their metal binding capacity, the need for costly chemical inducers to sustain the expression of metal binding groups has limited large-scale deployment of these organisms. These engineered systems could theoretically be designed for constitutive or bistable (toggle) expression, but this would result in metabolically wasteful protein production when no heavy metal is present, and compromise the viability of the bacteria in field applications. Such metabolic burden manifests as impaired cellular growth which we observe when the curli operon is overexpressed. These barriers to scalability and robustness have led to synthetic biology efforts exploring the design and engineering of self-regulating dynamic circuits capable of efficient feed-back controlled gene expression.⁵¹⁻⁵³ For metabolically costly products such as a large-scale extracellular protein matrix, one of the most efficient regulatory strategies is a feedback-controlled graded response. A number of studies have shown that implementation of in-cell dynamic feedback systems increases the robustness and predictability of engineered synthetic biology circuits and leads to productivity increases.^{54, 55} By combining a metal sensing promoter and a metal binding effector protein nanofiber in a single genetic circuit, we have created bacteria that dynamically generate mercury-binding curli amyloid fibers in the presence of environmental mercury. The circuit described herein is responsive to mercury in a sensitive range (above 400 ppb) that is below that of the trigger threshold for contaminated sites (which ranges from 1 – 10 ppm mercury, depending on country) and the mercury MIC (2 ppm) of *E. coli*,⁴⁴ yet above the experimentally established tolerable limit (0.13 ppm) for soil health in terms of plants and micro-organisms.⁵⁶ This adds a level of precise autonomous selectivity to bioremediation efforts, where metal removal efficiently occurs *in situ* only in response to a detected contaminant. Our circuit was also selective for mercury even in mixed metal environments which commonly occurs for contaminated sites of previous mining or metal processing facilities,^{57, 58} and the curli fibers produced induced flocculation of the cells, further facilitating mercury sequestration and biomass retrieval. The bound mercury was not easily washed off the biomass, thus acting as a strong bio-generated sink for mercury. Curli nanofibers are highly stable, being resistant to proteases,⁵⁹ harsh chemicals,³⁰ and detergents and high temperatures.⁶⁰ This makes them ideal for sequestering mercury in a matrix that will not easily break down. Furthermore, the curli fibers form dense nanofibrous networks with an extremely high surface area that would be ideal as an engineered ‘sponge’ for adsorbing mercury. Although the mechanism of mercury binding and the specific adsorption capacity per mass of curli have yet to be determined, we anticipate future biophysical studies to elucidate these intriguing questions. Additional engineering of the CsgA protein to incorporate metal binding groups could further improve the efficiency of metal removal, although attempts to engineer a small set of mercury-binding motifs into our BIND curli display system resulted in poor secretion and mercury binding inferior to wild-type curli (data not shown).

Here we have presented a synthetic biology circuit in which a mercury-absorbing extracellular self-assembling nanomaterial is fabricated under the control of a mercury-sensing regulatory system. Our initial characterization of this circuit was performed in *E. coli*, as the

genetics⁶¹ and biophysics⁶² of the curli functional amyloid system in *E. coli* is the most well characterized to date. However, one potential drawback is the relatively low mercury toxicity threshold of *E. coli*, at 2 ppm. Given that the curli functional amyloid is phylogenetically widespread,⁶³ exploration of other bacterial chassis that may confer specific phenotypic advantages could greatly expand the operational range, induction sensitivity, and robustness of this circuit for practical deployment. In particular, the circuit could potentially be introduced into a microbe that is uniquely adapted for widespread colonization of the target environment, for instance, soil bacteria such as *Bacillus* spp. or *Shewanella* spp., or one that is highly tolerant to mercury¹⁴, allowing for improved organismal fitness in contaminated environments. One key area for future optimization of our circuit would be improving the induction response of the merR promoter to further increase the sensitivity or alter the response dynamics of induction. This could be undertaken by introducing a transporter for mercuric ions to increase the intracellular mercury levels¹⁰ or engineering of the MerR regulatory protein.^{64, 65} Further engineering to induce toxin precipitation or mineralization within the biofilm^{66, 67} would facilitate toxin removal upon disposal of the biofilm or sequester the mercury to prevent it from mobilizing through the biosphere. Given further engineering efforts such as that described above, a mercury-sensing and absorbing engineered living material could be practically implemented in a variety of ways.

One implementation is known as ex situ bioremediation (ESB), which employs fixed- or moving-bed bioreactors and have been implemented in the field for heavy metal decontamination of various media.^{68, 69} However, ESB requires excavation of the contaminated media for feedstock as well as downstream separation of the contaminants from the soil or water, which often increases costs. In contrast, in situ bioremediation (ISB) efforts have been investigated as cost-effective green solutions for environmental remediation, and numerous pilot studies have been performed in which bacteria have been injected (in a process known as ‘bioaugmentation’) into contaminated soil⁷⁰⁻⁷² or even deep into the bedrock.^{73, 74} While most ISB efforts attempt to utilize unengineered bacterial isolates that can be surprisingly competitive with the indigenous microbial population⁷⁵, there is immense potential for the development of remediation-focused synthetic organisms that can be readily programmable for specific growth conditions or contaminants.⁷⁶ Such genetically-modified bacterium specifically engineered for enhanced bioremediation have already undergone field testing at contaminated sites.^{77, 78}

We can envision sentinel bacteria populations capable of responding to a variety of environmental toxins by the *in situ* production of biofilm sponges to sequester toxins at their source, thus preventing significant leaching into surrounding soil or water bodies. Such solidification/stabilization (S/S) strategies focus on binding or sequestering the toxins at their source in a stabilized mass, trapping the toxins in an insoluble format and reducing mobilization throughout the ecosphere, preventing leaching into highly mobile media (e.g., groundwater) that would facilitate poisoning of food chains. S/S approaches are the most frequently used strategy to treat soil, sludge, and liquid that is contaminated with mercury.⁶⁹ A synthetic biology approach for the implementation of a genetically engineered living material for bioremediation that is able to sequester mercury in a highly stable amyloid matrix at contaminated sites for different forms of media could be considered to be a hybrid approach of ISB and S/S strategies. As contaminated sites are often highly heterogeneous with spatially localized hot spots, the engineered biofilms would selectively populate the regions around the hot spots that are below

their toxicity threshold, biosynthesizing mercury-adsorbing curli nanofibers *in situ*. As the biofabricated highly stable nanofibers sequester the local mercury, the cells would be able to colonize further and produce more mercury-sequestering curli. Given the diversity of metal-responsive promoters,⁷⁹ the range of biofilm-specific functional amyloid proteins available for genetic manipulation,⁸⁰ and the recent advances towards displaying functional heterologous peptide and proteins domains on these amyloid scaffolds, this strategy of environmentally-triggered production of engineered biosorptive extracellular matrices could be adapted for the remediation of other toxic metals and environmental pollutants.

METHODS

Cloning. The P_{merR}:YFP mercury reporter plasmid was constructed by Gibson assembly of synthesized DNA fragments (Thermo Fisher Scientific). The *merR* gene and promoter region was taken from the *mer* operon of Shigella flexneri 2b plasmid R100. The *csgBAC* and *csgDEF* divergent curli operons derived from *E. coli* LSR10 were subcloned into a pET30a vector to obtain a single synthetic *csgBACEFG* operon as previously described.³⁸ To create the P_{merR}:curli operon, the *csgBACEFG* operon was subcloned in place of *yfp* gene. The negative control plasmid was obtained by completely excising the T7-LacO promoter region from pET30a using Gibson cloning. All plasmids were transformed into PQN4 cells, an engineered *E. coli* MC4100 strain in which the curli operon was removed by lambda Red recombineering and the T7 RNA polymerase gene integrated into the genome using a DE3 lysogenization kit (Merck Millipore). All cells were plated on LB agar or grown in bacterial medium supplemented with 50 µg/mL kanamycin.

Cell culture and metal exposure. Metals were diluted from 1000 ppm stocks in 2% HNO₃ (High Purity Standards) to the desired final concentrations. Overnight cultures were expanded to OD₆₀₀ ~0.7 at 37°C in LB. All subsequent experiments involving mercury exposure were performed in supplemented minimal media (GCMM) comprising: 6.78 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 0.5 g/L NaCl, 1 g/L NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂, 0.4% w/v glucose, and 1% (w/v) casamino acids. Congo Red plates were prepared with GCMM and agarose (Lonza) supplemented with 25 µg/mL Congo Red, 10 µg/mL Coomassie Brilliant Blue and the appropriate concentration of Hg²⁺. Agarose was used to reduce non-specific binding of mercury to normal bacteriological agar. For protein expression on plates, expanded cultures were pelleted and resuspended in GCMM, and 5 µl spotted on Congo Red plates, which were incubated at 30°C overnight. For protein expression in suspension, metal was added to GCMM-resuspended cultures to the appropriate final concentration and cultured for at least 18 hrs in 1 mL deep-well plates (30°C, 900 rpm). Three replicate cultures were used for each condition tested. Statistical analysis was performed using the Student's t-test with a 95% confidence interval. For experiments involving cycling of cells between different mercury concentrations, four single clonal colonies were expanded in LB to an OD₆₀₀ ~0.7. The cultures were pelleted and resuspended in GCMM with or without 1 ppm Hg²⁺, and allowed to grow for at least 18 hr. Samples were collected for TEM imaging, OD₆₀₀ measurement and quantification of curli expression using the Congo Red assay as described below. The cultures were then normalized to OD₆₀₀=1, diluted 250x into fresh LB, and the cycling repeated four times.

Quantitation of protein expression. 100 µL of metal-exposed cultures were passed through a 96-well filter plate (MultiScreen Isopore, Millipore). Wells were washed once with PBS and

shaken with 100 μL of 15 $\mu\text{g}/\text{mL}$ Congo Red solution for 5 min. The suspension was filtered and the absorbance at 490 nm of the unbound Congo Red in the filtrate was read on a BioTek H1 plate reader, and used to determine the amount of Congo Red bound to the cultures. Wells were subsequently shaken with 100 μL deionized water, and YFP fluorescence was determined on the plate reader (Ex: 485 nm / Em: 550 nm).

Transmission electron microscopy. Cell culture samples (5 μL) were applied to plasma-cleaned formvar/carbon film nickel TEM grids for 1 minute, then washed with 5 μL of ultrapure water for 1 minute, and subsequently negative-stained with fresh 2% uranyl formate for 15 seconds. The samples were allowed to dry for 10 minutes, and then imaged on a JEM-1400 Transmission Electron Microscope at 80 kV accelerating voltage.

Scanning electron microscopy. Nuclepore filter membrane discs containing immobilized metal-exposed cultures were fixed overnight in 2% glutaraldehyde / 2% *para*-formaldehyde at 4°C. The discs were immersed in a series of dehydrating ethanol solutions (25%, 50%, 75%, 100% v/v ethanol), then dried on a Tousimis Autosamdri-931 CO₂ critical point dryer. SEM images were obtained on a Zeiss Ultra Plus FE-SEM.

Quantitation of metal binding. 350-450 μL of metal-exposed cultures were pelleted, frozen and lyophilized and the dry weights measured. Pellets were taken up in 250 μL concentrated HNO₃ (69% v/v, trace-metal grade, Fisher), then briefly heated to 95°C and sonicated for complete resuspension. The mixture was left to digest at 25°C for 1 hr with shaking. Acid-digested samples were diluted in 2% HNO₃ and their metal content analyzed on an Agilent 7700x ICP-MS. Bismuth was used as an internal standard. Statistical analysis was performed using the Student's t-test with a 95% confidence interval.

Cell flocculation studies. Liquid cultures of PQN4 transformants with the negative control plasmid, P_{merR}-YFP, or P_{merR}-curli were grown for 24 hours in LB with and without 800 ppb Hg²⁺. The resulting cultures, after thorough resuspension, were allowed to settle at ambient conditions and were photographed every 2 hours. The flocculation was also quantitatively measured in triplicate by monitoring the absorbance at 600 nm of 1 ml cultures in cuvettes in an Agilent Cary 300 UV-Vis spectrophotometer over the course of 720 minutes. Representative microscopy images of the cultures showing cell suspensions or flocs were imaged on an EVOS FL Cell Imaging System.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Figures that provide additional data for YFP expression under the MerR promoter, the effect of mercury on the Congo Red assay, mercury sequestration by curli fibers expressed using different promoters, mercury retention by the biomass, and plasmid maps of the constructs used in this work, and listing of sequence information.

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Author Contributions

P.Q.N. and N.S.J. conceived of the concept, P.R.T. and P.Q.N. performed the experiments, and all authors contributed to the manuscript.

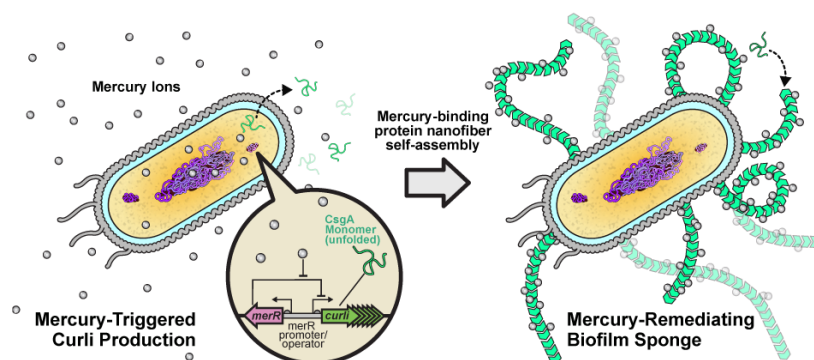
Notes

The authors declare no competing financial interest.

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