Caulobacter crescentus as a Whole-Cell Uranium Biosensor

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We engineered a strain of the bacterium Caulobacter crescentus to fluoresce in the presence of micromolar levels of uranium at ambient temperatures when it is exposed to a hand-held UV lamp. Previous microarray experiments revealed that several Caulobacter genes are significantly upregulated in response to uranium but not in response to other heavy metals. We designated one of these genes urcA (for uranium response in caulobacter). We constructed a reporter that utilizes the urcA promoter to produce a UV-excitable green fluorescent protein in the presence of the uranyl cation, a soluble form of uranium. This reporter is specific for uranium and has little cross specificity for nitrate (<400 μM), lead (<150 μM), cadmium (<48 μM), or chromium (<41.6 μM). The uranium reporter construct was effective for discriminating contaminated groundwater samples (4.2 μM uranium) from uncontaminated groundwater samples (<0.1 μM uranium) collected at the Oak Ridge Field Research Center. In contrast to other uranium detection methodologies, the Caulobacter reporter strain can provide on-demand usability in the field; it requires minimal sample processing and no equipment other than a hand-held UV lamp, and it may be sprayed directly on soil, groundwater, or industrial surfaces.

The role of nuclear technology in our energy resource portfolio is likely to become increasingly important as the global demand for energy continues to rise. Uranium processing, both for energy and for nuclear weapons, has resulted in a multitude of contaminated sites worldwide. In the United States specifically, there are more than 120 uranium-contaminated sites containing approximately 6.4 trillion liters of waste (22). As a heavy metal as well as a radionuclide, enriched uranium is toxic to cellular functions. Remediation strategies have utilized plants to extract uranium from contaminated sites (15) and recently have focused on containment to minimize migration of uranium in groundwater and prevent infiltration into drinking water supplies (6). The uranyl ion (α2−) is the most water-soluble and bioavailable form of uranium (38) and poses the greatest threat to human health. Due to the ease of uranyl ion spread through groundwater systems, most bioremediation strategies attempt to prevent contaminating uranium spread by utilizing microorganisms to reduce the oxidation state of uranium from U(VI), found in uranyl, to less soluble forms of uranium, including U(IV) (17, 36, 37).

Detecting the presence of low concentrations of uranium is necessary to identify contaminated areas and to assess the progress of remediation efforts. Methods employed to detect and quantify uranium concentrations often exploit the physical properties of uranium, including phosphorescence kinetics (5), atomic emission (16), and mass spectrometry characteristics (4). These methods are extremely sensitive and selective for uranium, but they allow low sample throughput and are not very portable. Additionally, these technologies measure the total amount of uranium present in a given sample, as opposed to the quantity of bioavailable uranium. Recently, several uranyl biosensors have been reported; these biosensors include a uranium immunosensor in which a fluorescently labeled monoclonal antibody selectively binds to chelated uranyl (3) and a catalytic beacon sensor consisting of a DNA enzyme that in the presence of uranyl catalyzes DNA cleavage, leading to an increase in fluorescence (19). The catalytic beacon biosensor rivals the most sensitive analytical instruments for uranium detection, with a detection limit of 45 pM, a linear detection range of up to 400 nM, and extremely good specificity for the uranyl ion. However, catalytic beacon sensor measurement must be performed with individually isolated and prepared samples.

In addition to biosensors based upon in vitro methods, such as the immunosensor and catalytic beacon sensor described above, there are precedents for whole-cell heavy metal biosensors (1, 7, 18, 28, 30, 33) utilizing either luciferase or fluorescent protein reporters. In contrast to other methodologies, whole-cell biosensors may be dispensed directly on the site of interest, detecting the presence of a bioavailable heavy metal in situ. This is possible because whole-cell biosensors that utilize a UV-excitable green fluorescent protein, GFPuv (8, 33), require little or no sample preparation. The heavy metals detectable thus far by whole-cell biosensors include mercury, chrome, arsenic, and copper, but to the best of our knowledge, no whole-cell biosensor for uranium has been reported previously.

In the work presented here, we bioengineered a strain of the bacterium Caulobacter crescentus to become fluorescently green in the presence of toxic levels of uranium. Caulobacter is a widely distributed bacterial genus that is able to survive in low-nutrient environments, including freshwater, seawater, soil (29), contaminated groundwater (21), wastewater (20), and habitats where contamination with uranium may be present.

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(27). This great diversity of viable habitats, including habitats contaminated with uranium, suggests that a Caulobacter whole-cell in situ uranium biosensor could robustly function across a wide spectrum of environmental conditions and ambient temperatures. Caulobacter is particularly resistant to the lethal effects of uranium up to a uranyl nitrate concentration of 1 mM (13), and previous microarray experiments have demonstrated that several Caulobacter genes are significantly upregulated in response to uranium but not in response to other heavy metals (13). Building upon these results, we constructed a uranium reporter that places GFPuv under the control of the promoter that is most strongly upregulated under uranium stress conditions.

MATERIALS AND METHODS

Materials. T4 DNA ligase, shrimp alkaline phosphatase (SAP), and endonucleases were purchased from Fermentas (Hanover, MD) and New England Biolabs (Ipswich, MA). DNA oligonucleotides were purchased from the Stanford Protein and Nucleic Acid Biotechnology Facility (Stanford, CA). OneShot Top10 chemically competent Escherichia coli and 0.1-cm electroporation cuvettes were purchased from Invitrogen (Carlsbad, CA). DNA sequencing was performed by Sequetech (Mountain View, CA). KOD Hot Start DNA polymerase kits were purchased from Qiagen (Valencia, CA). An ND-3300 fluorospectrophotometer was purchased from NanoDrop (Wilmington, DE). Cadmium sulfate (CdSO₄), potassium chromate (K₂CrO₄), lead nitrate [Pb(NO₃)₂], and depleted uranyl nitrate [UO₂(NO₃)₂·9H₂O] were purchased from Sigma-Aldrich, and stock solutions were prepared as described previously (13).

Pₘₐₙₜ lacZ reporter strain. The genomic region containing the αucA promoter was amplified from C. crescentus CB15N genomic DNA with KOD Hot Start DNA polymerase and oligonucleotides NJH144 and NJH121 (see Table S1 in the supplemental material). The 50-μl PCR mixture, containing 5% dimethyl sulfoxide, was made using the manufacturer’s protocol. The PCR was initiated by 1.75 min of melting at 94°C, followed by 32 cycles of 15 s of melting at 94°C, 30 s of annealing at 58°C, and 70 s of extension at 68°C. The PCR product was then purified by electrophoresis through 1.2% agarose gel and gel extraction, and then reamplified with oligonucleotides NJH120 (see Table S1 in the supplemental material) and NJH121 as described above. The second PCR product was then digested with BglII and KpnI (using the protocol recommended by the manufacturer), ligated overnight at 16°C with T4 DNA ligase (using the protocol recommended by the manufacturer) with the similarly digested pPR9TT vector (31) backbone, and then transformed into OneShot Top10 chemically competent E. coli cells (using the protocol recommended by manufacturer). The sequence of the resulting plasmid, pNJH123, was confirmed by primer extension sequencing using oligonucleotides NJH155 and NJH156 (see Table S1 in the supplemental material). C. crescentus CB15N ΔC₆₃₄ (strain LS4358) was transformed with plasmid pNJH123 by electroporation as previously described (10) to obtain the αucA promoter LacZ reporter strain NJH199 (Table 1). The in-frame ΔC₆₃₄ deletion reduces the background β-galactosidase activity of Caulobacter (J. C. Chen, unpublished results).

Pₘₐₙₜ lacZ reporter activity assays. Cultures of strain NJH199 were grown at 28°C in M2G medium (10). Overnight cultures were diluted to an optical density at 660 nm (OD₆₆₀) of 0.1 with fresh M2O medium and then grown for an additional 2 h at 28°C to obtain exponential growth again before the cells were stressed with either a mock treatment or indicated concentrations of uranyl nitrate, sodium nitrate, lead nitrate, cadmium sulfate, or potassium chromate. The stressed cultures were then grown on an orbital shaker for 2 h (see Fig. 3B) or the amount of time indicated below (see Fig. 3A) before liquid culture β-galactosidase assays were conducted, as previously described (24).

Pₘₐₙₜ gfpuv reporter strain. An Ncol/Nhel DNA fragment containing gfpuv was amplified from plasmid pBAD-GFP (8). An Ncol restriction site internal to gfpuv was silently mutated using splicing by overlap extension (SOE) (12). The pBAD-GFP template was amplified with KOD Hot Start DNA polymerase using oligonucleotides NJH122 and NJH237 (5′ SOE PCR) (see Table S1 in the supplemental material) or NJH236 and NJH123 (3′ SOE PCR) (see Table S1 in the supplemental material), as described above. The first-round SOE PCR products were mixed 1:1 and used as the template for the second-round SOE PCR

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TABLE 1. Plasmids and strains used in this study
and then amplified using oligonucleotides NH122 and NH123, as described above. The second-round SOE PCR product was then digested with NcoI and NheI.

An AesCl/NcoI DNA fragment containing the urcA promoter was amplified from plasmid pNJH293 with KOD Hot Start DNA polymerase using oligonucleotides NH144 and NH258 (see Table S1 in the supplemental material). The PCR product was then digested with AesCl and NcoI, ligated with the NcoI/NheI-digested gfpuv fragment (described above) and the AesCl/NheI-digested low-copy-number prVVYPF-2 vector (M. Thanbichler, unpublished) backbone (triple ligation), and then transformed into OneShot Top10 chemically competent E. coli cells. The sequence of the resulting plasmid, pNJH193, was confirmed by primer extension sequencing using oligonucleotides NH237 and NH241 (see Table S1 in the supplemental material).

In an effort to enhance folding and stability, we added a His6 tag to the N terminus of GFPuv. To do this, oligonucleotides NH246 and NH247 (see Table S1 in the supplemental material) (100 pmol/µl each) were mixed 1:1 to obtain a 50-µl (total volume) mixture, heated at 94°C for 2 min, and then annealed at room temperature. The annealed mixture of NH246 and NH247 was diluted 1:400 and then mixed 1:1 with the NcoI-digested, SAP-treated pNJH193 vector backbone for ligation. This ligation mixture was then transformed into OneShot Top10 chemically competent E. coli cells. The sequence of the resulting plasmid, pNJH198, was confirmed by primer extension sequencing using oligonucleotide NH241 (see Table S1 in the supplemental material).

In an attempt to increase the strength of the ribosome-binding site within the GFPuv reporter gene, we designed a new ribosome-binding site by making a triple ligation (see Table S1 in the supplemental material), as described above except that the extension time was 2.5 min. The PCR product was then reamplified using oligonucleotides NH200 and NH232 (see Table S1 in the supplemental material), as described above except that the extension time was 30 s. The second PCR product containing urcA promoter GFPuv reporter constructs. The results of the microarray experiments localized the urcA + 1 transcriptional start site to a 10-bp window (13) (Fig. 1A). CC3302Hyp_r_at probe 3 is the most upstream probe in the Affymetrix array that matches the urcA transcript, placing its +1 site approximately 5 to 15 bp upstream from the end of the immediately adjacent probe 2 (chromosomal position 3552896). A uranium-inducible promoter sequence motif, present in the promoter regions of 11 Caulobacter genes, has been identified (23). The urcA promoter contains two matches to this uranium-specific m_5 motif, located 107 and 55 bp upstream of the putative +1 site (Fig. 1A and C). The urcA transcript overlaps the opposing strand of the CC_3302 gene in the original annotation of the Caulobacter genome (26), but the revised Glimmer (9) and GeneMark (2) annotations of the Caulobacter genome have identified urcA as the true open reading frame (spanning from chromosomal position 3552927 to position 3555280 on the positive strand), dispensing with the originally annotated CC_3302 gene. The
The urcA gene encodes a predicted 12.7-kDa protein. The signal sequence prediction tool SignalP (25) predicted with nearly 100% certainty that UrcA contains an N-terminal signal sequence whose most likely cleavage site is located between residues 30 and 31, and the prokaryotic subcellular protein localization tool SubLoc (14) predicted with 96% expected accuracy that UrcA is a periplasmic protein. To experimentally test the bioinformatic prediction of UrcA’s periplasmic localization, we constructed a xyleose-inducible UrcA-mCherry fusion (32). After xylose induction, the P
\_ylo urcA-mcherry strain was imaged by deconvolution microscopy (Fig. 2). The fluorescence images are consistent with the hypothesis that UrcA localizes to the cell periphery and to the cell stalk.

To determine if the urcA promoter could be a candidate uranium biosensor, we constructed a plasmid-borne LacZ fusion reporter containing 1 kb of the promoter region upstream of the urcA start ATG codon through the first 8 amino acids of UrcA fused to LacZ to create strain NJH199 (Fig. 1B). The resulting P
\_urcA lacZ reporter strain was exposed to 0, 2.5, or 20 μM uranyl, and the resulting kinetics of β-galactosidase activity was assayed in liquid culture (Fig. 3A). The P
\_urcA lacZ reporter was able to detect the presence of 2.5 μM uranyl, and maximal β-galactosidase activity occurred by 2 h after exposure to either 2.5 or 20 μM uranyl nitrate. To test the sensitivity and specificity of the reporter, the P
\_urcA lacZ strain was exposed to a panel of heavy metals for 2 h and then assayed for β-galactosidase activity (Fig. 3B). The P
\_urcA lacZ reporter’s detection limit for uranyl after 2 h of exposure was about 1.0 μM. The maximum increase in the signal of the P
\_urcA lacZ reporter was 65-fold over the background with 20 μM uranyl. The P
\_urcA lacZ reporter was not stimulated by the presence of lead (150 μM) or chromium (41.6 μM), but it showed cross sensitivity to cadmium at a concentration of 48 μM. In addition to the analysis of the panel of heavy metals, the P
\_urcA lacZ reporter response to nitrate was assayed as a negative control to ensure that the nitrate component of uranyl nitrate salt does not contribute to P
\_urcA lacZ reporter activity.

After our success with the P
\_urcA lacZ reporter, we constructed a reporter strain (NJH371) in which plasmid-borne P
\_urcA drives the expression of UV-excitible green fluorescent protein fluorescence. The P
\_urcA gfpuv reporter construct is shown in Fig. 1B. The time course of GFPuv signal kinetics for P
\_urcA gfpuv after induction with uranyl nitrate is shown in Fig. 3C. The fluorescence activity of the P
\_urcA gfpuv reporter strain reached a maximum between 3 and 4 h after exposure to uranyl, but one-half the maximum activity was observed after about 2 h. It should be noted that as the P
\_urcA gfpuv reporter strain reached a high cell density at 6.5 h (OD
\_660 > 0.9), entering stationary phase in the absence of uranium, the basal activity level of P
\_urcA gfpuv began to increase. This result indicates that high-density cultures of the P
\_urcA gfpuv reporter strain should not be used, because they could lead to false
The P_{urcA} gfpuv reporter strain was exposed to a panel of heavy metals for 4 h and then assayed for fluorescence activity (Fig. 3D). The P_{urcA} gfpuv reporter exhibited specificity for uranium, and there was little cross specificity for nitrate (<400 μM), lead (<150 μM), cadmium (<48 μM), or chromium (<41.6 μM). The P_{urcA} gfpuv reporter’s detection limit for uranyl after 4 h of exposure was around 0.5 μM. The mean signal increase for the P_{urcA} gfpuv reporter was 4.2-fold over the background with 100 μM uranyl. Despite sizeable standard deviations in reporter fluorescence activity, it should be pointed out that the minimum measured activities of the reporter for uranyl concentrations above 0.5 μM (n = 7) were all greater than the maximum activities measured for nitrate, lead, cadmium, or chromium (n = 3). Interestingly, we did not observe low-level stimulation of the GFPuv reporter by cadmium, in contrast to the LacZ reporter results (Fig. 3B). An inhibitory effect of 41.6 μM chromium on GFPuv activity was observed for the P_{urcA} gfpuv reporter (Fig. 3E), but cadmium levels less than 48 μM did not appear to significantly affect P_{urcA} gfpuv reporter activity in the presence of 10 μM uranyl.

Figure 4 demonstrates that the P_{urcA} gfpuv strain distinguished uranium-contaminated groundwater samples (4.2 μM uranium) from uncontaminated groundwater samples (<0.1 μM uranium) collected at the Oak Ridge Field Research Center. Adding 50 μM uranyl nitrate to the uncontaminated water sample yielded comparable photoemission. Using a hand-held UV lamp as the light source, the naked eye alone was sufficient to distinguish P_{urcA} gfpuv reporter strain cultures exposed to the contaminated water (4.2 μM uranium) from cultures exposed to the uncontaminated water (Fig. 4B), although filtering out the blue region of the spectrum (as shown by isolating the green channel of the RGB image) facilitated discrimination. This key result provides proof of principle that the P_{urcA} gfpuv reporter strain may be used to detect the presence of uranium contamination in real-world water samples, that the reporter’s output may be successfully monitored with the naked eye without resorting to a fluorimeter, and that the background chemical composition of the water samples tested does not appear to induce false-positive or -negative results.

**DISCUSSION**

In the work presented here, we constructed a whole-cell uranium biosensor that can report the presence of micromolar amounts of the uranyl cation in situ with nothing other than a hand-held UV lamp. To accomplish this, we utilized a reporter construct that placed GFPuv under the control of the promoter of the Caulobacter gene urcA, which is strongly upregulated upon exposure to uranium. A promoter motif was identified in 11 Caulobacter genes that were induced in response to uranyl nitrate. The urcA promoter contains a tandem repeat of this uranium response motif, m_5, which may explain why urcA is upregulated under uranium stress so much more strongly than any other Caulobacter gene. The m_5 uranium response motif is similar to the cell cycle regulation promoter motif cc_1, which appears to be stress induced (23). As the OD_{660} of a P_{urcA} gfpuv reporter strain culture begins to exceed about 0.92 at 6.5 h, the fluorescence activity becomes quantitatively comparable to the reporter’s activity output after 4 h of exposure to 2.5 μM uranyl (Fig. 3C). The increase in the basal activity level...
High levels of contaminating chromium (41.6 mg/L from the Oak Ridge Field Research Center, demonstrating uranium from uncontaminated groundwater samples acquired from groundwater samples contaminated with micromolar levels of uranium. The maximum rescence activity of the P_{urcA} gfpuv reporter was observed after 3 to 4 h of exposure to uranium, but the assay time could confidently be reduced to 2 h at the expense of increasing the detection limit of the P_{urcA} gfpuv reporter strain from a lyoph- sized powder could greatly enhance its on-demand usability in the field. In conjunction with bioremediation efforts, the P_{urcA} gfpuv reporter could complement analytical uranium detection methodologies by rapid screening of many locations in parallel for toxic levels of bioavailable uranium contamination.

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