Biomining of Uranium by PhoY Phosphatase Activity Aids Cell Survival in *Caulobacter crescentus*

Mimi C. Yung, Yongqin Jiao

Biosciences and Biotechnology Division, Physical and Life Sciences Directorate, Lawrence Livermore National Laboratory, Livermore, California, USA

Caulobacter crescentus is known to tolerate high levels of uranium [U(VI)], but its detoxification mechanism is poorly understood. Here we show that *C. crescentus* is able to facilitate U(VI) biomineralization through the formation of U-P_i precipitates via its native alkaline phosphatase activity. The U-P_i precipitates, deposited on the cell surface in the form of meta-autunite structures, have a lower U/P_i ratio than do chemically produced precipitates. The enzyme that is responsible for the phosphatase activity and thus the biomineralization process is identified as PhoY, a periplasmic alkaline phosphatase with broad substrate specificity. Furthermore, PhoY is shown to confer a survival advantage on *C. crescentus* toward U(VI) under both growth and nongrowth conditions. Results obtained in this study thus highlight U(VI) biomineralization as a resistance mechanism in microbes, which not only improves our understanding of bacterium-mineral interactions but also aids in defining potential ecological niches for metal-resistant bacteria.

Uranium (U) is a widespread environmental contaminant, with major sources coming from energy and nuclear weapon production (1). With its oxidizing nature and high water solubility, hexavalent U(VI) is extremely toxic and carcinogenic (2). Chemical and physical techniques for waste treatment or removal of U are challenging and expensive. An alternative method for U remediation is microbially mediated, *in situ* U immobilization, which has added benefits of reduced cost and environmental friendliness relative to other approaches (3, 4).

The most-studied form of microbial U(VI) immobilization is through microbial reduction by dissimilatory metal-reducing bacteria (DMRB) (5, 6). These organisms can directly or indirectly (e.g., by coupling with iron or nitrate redox chemistry) reduce soluble U(VI) to less soluble U(IV) under anaerobic conditions, resulting in immobilization. The biogenic U(IV) minerals (in the form of uraninite) generated by phylogenetically and metabolically diverse bacteria are chemically and structurally similar, suggesting a common mechanism for U(VI) reduction (7). Recent studies suggest that DMRB rely on high-molecular-weight, *c*-type cytochromes associated with the outer membrane for U(VI) reduction, similar to the reduction of other metals, such as chromate and ferric iron (8). Many researchers have confirmed that uraninite is present both associated with the cell wall and in the periplasm (5, 9), with the exception of some rare reports of cytoplasmic uraninite, suggesting that U complexes do not generally have access to intracellular enzymes (10–12). The stability of thus-formed uraninite was later evaluated under environmental conditions. When exposed to oxygen or other electron acceptors, U(IV) complexes are readily reoxidized to the more mobile U(VI) form (13, 14), defeating the purpose of immobilization. Therefore, reduction of U(VI) is unlikely to be a successful long-term strategy for the immobilization of U.

Besides reductive precipitation under anaerobic conditions, other mechanisms of microbe-mediated U immobilization that are redox insensitive include reactions with enzymes or polysaccharides excreted on the cell surface by many microorganisms found in natural waters (15, 16). In particular, phosphatase activity, both acid and alkaline, from various bacterial species such as *Serratia* sp. strain N14 (formerly *Citrobacter* sp. strain N14), *Sph-*

ingomonas sp. BSAR-1, *Arthrobacter*, *Rahnella*, and *Bacillus* has been found to facilitate U(VI) precipitation through the formation of uranium phosphate complexes (17–19). Phosphatases from these organisms have been cloned and expressed in *Escherichia coli* and other organisms, and the resulting engineered strains have been reported to efficiently precipitate uranium (20–22). Among all these systems, however, little attention has been paid to the cellular benefits of the native or heterologously expressed phosphatases toward U(VI) resistance.

In this study, we examined the phosphatase-facilitated uranium tolerance mechanism in *Caulobacter crescentus* NA1000, providing a link between phosphatase activity, U biomineralization, and cell survival. *C. crescentus* is an aquatic, aerobic bacterium that is able to survive under low-nutrient conditions (23). *Caulobacter* species are able to tolerate high concentrations of U(VI) (24) and have been found in U-contaminated sites (25). Exposure of *C. crescentus* to U(VI) has been shown to elicit U-specific cellular responses on both the transcriptional and proteomic levels (24, 26, 27). We show here that *C. crescentus* is able to tolerate uranium through its native phosphatase activity enabled by a periplasmic enzyme PhoY, demonstrating the potential for *C. crescentus* to be used for U(VI) bioremediation under aerobic conditions.

MATERIALS AND METHODS

Materials, bacterial strains, and growth conditions. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Peptone, yeast extract, and agar were purchased from Amresco (Solon,

Received 30 March 2014 Accepted 25 May 2014

Published ahead of print 30 May 2014

Editor: J. E. Kostka

Address correspondence to Yongqin Jiao, jiao1@llnl.gov.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.01050-14>.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AEM.01050-14

TABLE 1 Plasmids and bacterial strains used in this study

Plasmid or strain	Description	Reference or source
Plasmids		
pNPTS138	Nonreplicating vector for integration and allelic replacement; <i>oriT</i> , <i>kan</i> (Km ^r), <i>sacB</i>	M. K. Alley, unpublished
pBXMCS-2	High copy no. xylose-inducible expression vector; <i>kan</i> (Km ^r)	32
pRVCHYC-2	Low-copy-no. vanillate-inducible expression vector containing coding region for a C-terminal mCherry construct; <i>kan</i> (Km ^r)	32
pMCY10	pNPTS138-derived vector for Δ <i>phoY</i> allelic replacement	This study
pMCY21	pBXMCS-2-derived vector for expression of <i>phoY</i>	This study
pMCY23	pBXMCS-2-derived vector for expression of <i>phoY-mcherry</i>	This study
<i>C. crescentus</i> strains		
NA1000	Wild-type <i>C. crescentus</i> , a synchronizable derivative of CB15	48
YJ0010	NA1000 Δ <i>phoY</i>	This study
YJ0021	NA1000 Δ <i>phoY</i> harboring pMCY21	This study
YJ0023	NA1000 Δ <i>phoY</i> harboring pMCY23	This study

OH). Uranyl nitrate hexahydrate [(UO₂)(NO₃)₂ · 6H₂O] was obtained from SPI Supplies (West Chester, PA). A stock solution of uranyl nitrate (100 mM) was prepared in 0.1 N nitric acid. All PCRs were amplified using iProof polymerase from Bio-Rad (Hercules, CA) supplemented with 5% dimethyl sulfoxide (DMSO) according to the manufacturer's instructions. *Caulobacter crescentus* NA1000 (Table 1) was maintained on solid PYE medium (0.2% peptone, 0.1% yeast extract, 0.5 mM MgSO₄, and 1 mM CaCl₂) supplemented with 1.5% agar (28). Liquid cultures were grown in either (i) PYE medium or (ii) modified M5G minimal medium lacking inorganic phosphate (P_i) and containing 5 mM glycerol-2-phosphate as the sole phosphate source (M5G-GP; pH 7.0) (29). Where applicable, kanamycin was supplemented at 25 μg/ml in solid medium and 5 μg/ml in liquid medium for *C. crescentus* and 50 μg/ml in solid and liquid media for *E. coli*.

For growth in PYE medium, *C. crescentus* cells were first precultured in 2 ml of PYE medium from a single colony at 30°C overnight. Overnight cultures were diluted to an initial optical density at 600 nm (OD₆₀₀) of 0.1 and cultured for an additional 2 h at 30°C to an OD₆₀₀ of 0.2, at which point uranyl nitrate was added to the medium to a final concentration of 200 μM. Cells were grown for an additional 30 min, after which the cultures were analyzed. Growth of cells was performed in biological triplicate and monitored using OD₆₀₀.

For growth in M5G-GP medium, *C. crescentus* cells were precultured in 2 ml of PYE or PYE supplemented with kanamycin when appropriate from single colonies at 30°C overnight. Overnight cultures were diluted in the morning in PYE medium to an initial OD₆₀₀ of 0.04 and incubated for another 7 h until the OD₆₀₀ was about 0.5 (late exponential phase). Cells were harvested, washed once with 10 mM NaCl, and inoculated into M5G-GP supplemented or not with 50 μM uranyl nitrate to an initial OD₆₀₀ of 0.02. M5G-GP was not supplemented with kanamycin due to kanamycin inactivation by uranium (data not shown). Growth of cells was performed in biological triplicate and monitored using OD₆₀₀.

Construction of Δ *phoY* mutant. The ~400-bp regions upstream and downstream of the *phoY* open reading frame (CCNA_02545) were PCR amplified and cloned into pNPTS138 (M. K. Alley, unpublished data).

TABLE 2 Primers used in this study

Primer	Sequence (5' to 3')
phoY_URfor	GGCTGGCGCCAAGCTTCGGTCACGATATCGGCGA CGAG
phoY_URrev	ACGTTGCATATGGCGCAAGAACGGAAGCGT
phoY_DRfor	CGCCATATGCAACGTTGCTAACGGCGGTTAG
phoY_DRrev	CGAAGCTAGCGAATTCATTGAAACGCCCGGCTAGC
BXphoY_for	GGGGAGACGACCATATGTTGCGCGAAGGGCGGC TTC
BXphoY_rev	CGGGCTGCAGGAATTCCTTAGCAACGTTGGCCGTAG ACCAGC
RVphoY_for	GCGAGGAAACGCATATGTTGCGCGAAGGGCGGC TTC
RVphoY_rev	CGTAACGTTCGAATTCCTCGAACGTTGGCCGTAGA CCAGC
BXphoYch_rev	TGGCGGCCGCTCTAGATTACTTGTACAGCTCGTCC ATGCCGC

The upstream fragment was amplified using primers phoY_URfor and phoY_URrev (Integrated DNA Technologies, Coralville, IA), and the downstream fragment was amplified using primers phoY_DRfor and phoY_DRrev (Table 2). A three-way Gibson assembly (Clontech In-Fusion HD Cloning Plus kit, Mountain View, CA) of the upstream and downstream fragments into the HindIII and EcoRI sites of pNPTS138 generated pMCY10 (Table 1). The sequence of the cloned regions was confirmed by DNA sequencing (Elim Biopharmaceuticals, Hayward, CA). An in-frame deletion of *phoY* (YJ0010, Table 1) was generated using pMCY10 through standard homologous recombination methods as previously described (30, 31), leaving behind only the first two and last three codons of the *phoY* gene. Deletion of *phoY* was confirmed by DNA sequencing.

Construction of *phoY* and *phoY-mcherry* complement strains. To generate the *phoY* complement strain, the *phoY* open reading frame was first PCR amplified using primers BXphoY_for and BXphoY_rev (Table 2). The *phoY* fragment was then inserted in frame using Gibson assembly into the NdeI and EcoRI sites downstream of the xylose-inducible promoter (P_{xyI}) in pBXMCS-2 (32) to generate pMCY21 (Table 1). The sequence of *phoY* was confirmed by DNA sequencing. The Δ *phoY* mutant was transformed with pMCY21 to obtain the P_{xyI}*phoY* complement strain YJ0021. Transformants were selected on PYE agar supplemented with kanamycin.

To generate the *phoY-mcherry* complement strain, the *phoY* open reading frame was PCR amplified using primers RVphoY_for and RVphoY_rev (Table 2). This *phoY* fragment was inserted using Gibson assembly into the NdeI and EcoRI sites upstream of the *mcherry* coding region in pRVCHYC-2 (32). The resulting *phoY-mcherry* construct was then PCR amplified using primers BXphoY_for and BXphoYch_rev (Table 2) and was subsequently inserted into the NdeI and XbaI sites downstream of the P_{xyI} promoter in pBXMCS-2 (32) to generate pMCY23 (Table 1). The sequence of *phoY-mcherry* was confirmed by DNA sequencing. The Δ *phoY* mutant was transformed with pMCY23 to obtain the P_{xyI}*phoY-mcherry* strain YJ0023. Transformants were selected on PYE agar supplemented with kanamycin. Experiments with YJ0021 and YJ0023 were conducted without xylose supplementation since leaky expression from the xylose promoter provided sufficient PhoY activity.

Cell-bound phosphatase assay. *C. crescentus* NA1000 was grown in PYE medium with and without U addition as described above. Cells were harvested, washed once with 10 mM NaCl, and resuspended in 100 mM Tris-HCl (pH 7.0) to a final OD₆₀₀ of 0.5 in a final volume of 700 μl. Phosphatase assays were started by addition of glucose-6-phosphate, fructose-1,6-bisphosphate, or glycerol-2-phosphate to a final concentration of 5 mM. At each time point, aliquots of assays were removed to quantitate the total P_i content as described below.

TABLE 3 Uranium and inorganic phosphate distribution during growth of *C. crescentus* in PYE medium with U^a

Type	Uranium (nmol)		Inorganic phosphate (nmol)		Mol U/P _i ratio	
	WT cells	Abiotic	WT cells	Abiotic	WT cells	Abiotic
Insoluble	308 ± 3 (81% ± 2%)	220 ± 20 (63% ± 8%)	340 ± 20 (22% ± 1%)	170 ± 30 (13% ± 3%)	0.90 ± 0.04	1.32 ± 0.15
Soluble	72 ± 7 (19% ± 2%)	130 ± 20 (37% ± 6%)	1,210 ± 10 (78% ± 1%)	1,090 ± 10 (87% ± 2%)	0.06 ± 0.01	0.12 ± 0.02
Total	380 ± 8 (100%)	350 ± 30 (100%)	1,550 ± 20 (100%)	1,260 ± 30 (100%)	0.245 ± 0.003	0.275 ± 0.002

^a Uranyl nitrate (200 μM) was added in early-exponential phase to 2-ml cultures, and measurements of soluble and insoluble uranium and inorganic phosphate amounts were taken 30 min after U addition. An abiotic, no-cell control was included for comparison. Results are expressed as means ± standard deviations of results from three biological replicates.

U biomineralization assay. *C. crescentus* NA1000, YJ0010 (Δ *phoY*), and YJ0021 (Δ *phoY/pMCY21*) cells were individually precultured in 500 μl of PYE or PYE supplemented with kanamycin from single colonies at 30°C for 8 h. Cells were then diluted to an initial OD₆₀₀ of 0.001 and cultured for 16 h until the OD₆₀₀ was about 0.6 (late exponential phase). Cells were harvested, washed once with 10 mM NaCl, and resuspended in 50 mM PIPES (pH 7.0) to a final OD₆₀₀ of 0.5 in a final volume of 700 μl. Biomineralization assays were started by the addition of glycerol-2-phosphate and uranyl nitrate to final concentrations of 5 mM and 500 μM, respectively, and incubated at 30°C. We note that U(VI) is fully soluble under these assay conditions, presumably by complexation with glycerol-2-phosphate. Controls without glycerol-2-phosphate or uranyl nitrate were also conducted. At each assay time point, aliquots were removed to quantitate the total, soluble, and insoluble U and P_i contents, as described below. Aliquots were also removed for cell survival analysis. Serial dilutions of 10¹ to 10⁶ of the aliquots were prepared in PYE, and a 10-μl aliquot of each dilution was spotted on PYE agar. Images of the spots were taken after 2 days of incubation at 30°C.

Measurement of U and P_i. To measure total U and P_i contents, a 45-μl aliquot of sample was directly quenched with 45 μl of 12.5% trichloroacetic acid (TCA). Samples were centrifuged at 20,000 × g to remove cell debris, and the supernatant was analyzed for total U and P_i via Arsenazo III and molybdate colorimetric assays, respectively (33, 34). Briefly, to measure U, supernatant (40 μl) was added to 60 μl of filtered 0.1% Arsenazo III in 6.25% TCA. Absorbance at 652 nm was measured and compared to standards to determine the U content. To measure P_i, supernatant (40 μl) was added to 60 μl of 1% ammonium molybdate and 7.2% FeSO₄ in 3.2% H₂SO₄. Absorbance at 700 nm was measured and compared to standards to determine the P_i content.

To measure soluble U and P_i contents, a 45-μl aliquot of sample was immediately centrifuged at 20,000 × g for 5 min. The supernatant was quenched with an equal volume of 12.5% TCA and then analyzed for soluble U and P_i contents as described above. To measure the insoluble U and P_i contents, the pellet after centrifugation was resuspended in 45 μl of degassed 300 mM NaHCO₃ in water and mixed at room temperature for 5 min. The sample was then centrifuged again at 20,000 × g for 5 min. The supernatant was quenched with an equal volume of 12.5% TCA and analyzed for U and P_i contents as described above.

XRD sample preparation and analysis. For X-ray powder diffraction (XRD) analysis, cell culture (40 ml) grown with 200 μM U in PYE medium for 30 min was harvested by centrifugation at 20,000 × g for 10 min. The pellet was washed with water three times. Clear separation of cells and precipitates was observed. The top layer of cells was resuspended in residual liquid by gentle pipetting and carefully removed. The bottom layer was resuspended in water, spread on an XRD disk, and dried overnight in a desiccated chamber. To control for chemical- and cell surface-induced precipitation, abiotic and heat-killed cell controls were included. The heat-killed cell sample was prepared by heating cells at 70°C for 10 min. XRD analysis was performed on a Bruker D8 X-ray diffractometer (Billerica, MA), and spectra were compared to references from the International Centre for Diffraction Data (ICDD).

Transmission electron microscopy (TEM) sample preparation and imaging. For cells grown with 200 μM U in PYE medium for 30 min, 3 ml of culture was collected. For cells originating from U biomineralization assays, 1.6 ml of cell suspension with 5 mM glycerol-2-phosphate and 250 μM uranyl nitrate was collected after 5 h.

Samples were harvested at 20,000 × g for 1 min and fixed in 1 ml of 4% paraformaldehyde in 100 mM sodium cacodylate (pH 7.2) for 1 h at room temperature, with rocking. The pellet was washed once with 1 ml of water and then dehydrated sequentially in 1 ml of each of the following for 10 min: 50% ethanol, 70% ethanol, 90% ethanol, and 100% ethanol. The 100% ethanol dehydration was repeated twice. The cell pellet was then dislodged in 1 ml of 50% LR White resin (Electron Microscopy Sciences, Hatfield, PA) in ethanol and incubated at room temperature overnight with rocking, protected from light. The cell pellet was then infiltrated with 1 ml of fresh 100% LR White for 1 h at room temperature two times. Finally, cells were embedded in LR White anaerobically at 65°C for 2 days.

Thin sections, 90 nm in thickness, were cut from embedded samples using a Leica Ultracut UC6 ultramicrotome (Buffalo Grove, IL) with a diamond knife. Sections were collected using 200-mesh, Formvar/carbon-coated copper grids (Ted Pella, Redding, CA). TEM was conducted using a FEI/Philips CM300 transmission electron microscope equipped with energy-dispersive X-ray spectroscopy (EDS). Images were collected at an accelerating voltage of 300 kV.

Fluorescence microscopy. Strain YJ0023 (Δ *phoY/pMCY23*) was cultured from a single colony at 30°C in PYE supplemented with kanamycin until the cells reached an OD₆₀₀ of 0.25 (mid exponential phase). Cells were harvested and resuspended in 1/10 of the culture volume. Concentrated cells (5 μl) were spotted on a PYE-1% agarose pad and imaged using an Axiovert 200M microscope (Zeiss, Minneapolis, MN) equipped with a Photometric CoolSNAP HQ charge-coupled-device (CCD) camera. Images were acquired with a 100× objective and a Texas Red filter set (Chroma, Bellows Falls, VT; filter 41004). Images were processed using ImageJ (35).

RESULTS

***C. crescentus* produces extracellular, crystalline U-P_i precipitates.** To determine if *C. crescentus* is able to facilitate uranium phosphate (U-P_i) precipitation, wild-type strain NA1000 was grown in PYE medium to early exponential phase, at which time uranyl nitrate was added to a final concentration of 200 μM. After an additional 30 min, the amounts of U and P_i present in the soluble and insoluble fractions were determined (Table 3). As a control for chemical precipitation, abiotic samples with no cells were also prepared.

The biotic, cell-containing samples contained larger amounts of both U and P_i in the insoluble fraction (308 ± 3 nmol of U and 340 ± 20 nmol of P_i) than did the abiotic control (220 ± 20 nmol of U and 170 ± 30 nmol of P_i), suggesting that cell metabolism induced U-P_i precipitation (Table 3). The larger amount of U present in the biotic insoluble fraction was consistent with the

FIG 1 Analysis of *C. crescentus* collected during growth in PYE medium with U. (A) An XRD spectrum of U precipitates produced in the presence of wild-type cells. Reference spectra are shown below for comparison. (B and C) Representative TEM images showing U precipitates present on the cell surface and in the bulk medium. Scale bars, 500 nm. (D) EDS analysis of areas labeled by arrows in the micrograph above. Scale bar, 500 nm.

lower U content in the soluble fraction. The amounts of P_i in all fractions (soluble, insoluble, and total) were higher for the biotic than for the abiotic samples, indicating that P_i was produced and released from cells during growth. P_i production by *C. crescentus* during growth was confirmed by monitoring P_i concentration in the medium throughout growth in the absence of U (data not shown). The fact that the insoluble P_i made up a greater proportion of the total P_i in the biotic sample compared to the abiotic sample ($22\% \pm 1\%$ versus $13\% \pm 3\%$) indicates that some of the P_i produced by the cells was precipitated in the insoluble fraction. Further calculations indicate that the biotic sample had a lower molar ratio of U to P_i in the insoluble fraction than did the abiotic sample (Table 3), suggesting that the biotic precipitates may be chemically different from the abiotic precipitates and that the cellular production of P_i is responsible for the biomineralization process.

XRD analysis was used to determine the crystallinity and identity of the U precipitates. Only precipitates produced in the pres-

ence of cells generated a diffraction pattern, indicative of crystalline material (Fig. 1A); samples from abiotic or heat-killed cell controls did not produce any detectable diffraction pattern. The XRD spectrum of the biotic precipitates confirmed the presence of meta-autunite [uranyl phosphate species in the U(VI) oxidation state], likely in the form of uramphite $[(NH_4)(UO_2)(PO_4) \cdot 3H_2O]$, ICDD 00-042-0384, potassium sodium uranyl phosphate hydrate $[Na_{0.43}K_{0.57}(UO_2)(PO_4) + xH_2O]$, ICDD 00-059-0399, and/or potassium uranyl phosphate hydrate $[K(UO_2)(PO_4) \cdot 3H_2O]$, ICDD 00-049-0433].

In order to determine where U precipitates reside relative to cells, TEM analysis of the samples collected after 30 min of U exposure was performed. The majority of the precipitates were found to be located extracellularly in the bulk medium (Fig. 1B). Occasionally, we observed precipitates surrounding the surface of the cells (Fig. 1C). EDS analysis revealed that all precipitates contained primarily U and P (Fig. 1D). Based on peak intensities of the EDS spectra, the cell surface-associated precipitates appeared

to have a lower U/P ratio than did those present in the bulk medium. While the lower U/P ratio may be indicative of the P_i released and precipitated with U on the cell surface, the presence of phosphate-containing macromolecules on the cell surface, such as lipopolysaccharides and phospholipids, may also contribute to the lower ratio. The interior of the cells appeared to have negligible amounts of U but significant amounts of P, as expected given the prevalence of P in nucleotides and proteins.

Uranium biomineralization is catalyzed by PhoY. Given that U precipitates formed in the presence of cells contained larger amounts of P_i than did the abiotic control (Table 3), we hypothesized that P_i production by phosphatases facilitates U biomineralization, which has been observed in other systems (17–19). Whole-cell phosphatase activity of *C. crescentus* grown in PYE medium with and without U was tested with three different organic phosphate substrates: glucose-6-phosphate, fructose-1,6-bisphosphate, and glycerol-2-phosphate (see Fig. S1 in the supplemental material). The results revealed phosphatase activity toward all three substrates, indicating broad substrate specificity. Surprisingly, cells grown with and without U exhibited the same phosphatase activity toward each substrate tested (see Fig. S1), indicating that phosphatase activity is neither induced nor inhibited by U. In addition, we found no evidence for extracellular phosphatase activity in the spent medium with and without U (data not shown), confirming that the phosphatase activity is cell associated.

To identify the gene(s) encoding the enzyme(s) responsible for the whole-cell phosphatase activity and thus the U biomineralization observed, we searched the annotated genome of *C. crescentus* for alkaline phosphatases (36). We focused on alkaline instead of acid phosphatases because the activities that we observed had an optimal pH of 7.5 (see Fig. S2 in the supplemental material). A genome search revealed 4 annotated alkaline phosphatases in *C. crescentus* NA1000. The presence of cell surface-associated U- P_i precipitates (Fig. 1) suggested that phosphatase activity and U biomineralization occur at the cell surface. Thus, we hypothesized that the responsible phosphatase would be located at the cell periphery. Each of the 4 annotated proteins was therefore analyzed for an N-terminal export signal sequence using SignalP 4.1 (37). Only CCNA_02545 (herein named PhoY) had a predicted signal sequence for export across the cytoplasmic membrane. In addition, the prokaryotic subcellular protein localization tool PSORTb (38) predicted with 98% expected accuracy that PhoY is a periplasmic protein. To experimentally test the bioinformatics prediction, we constructed a xylose-inducible *phoY-mcherry* fusion strain, which expresses a fluorescent mCherry protein fused to the C terminus of PhoY. Fluorescence microscopy revealed that PhoY-mCherry was indeed localized to the cell periphery (Fig. 2). Thus, based on its annotated activity and its cellular localization, PhoY is a likely candidate for catalyzing U biomineralization in *C. crescentus*. We should note that there is a slight discrepancy in the translational start site of *phoY* in the currently available versions of genome annotation for *C. crescentus* in NCBI (36, 39). However, this discrepancy does not affect the mature protein sequence after export and thus the results of this study. Phylogenetic analysis of the mature protein sequence of PhoY (see Fig. S3 in the supplemental material) revealed that it is most similar to PhoK in *Sphingomonas* sp. BSAR-1 (39% identity and 51% similarity), an alkaline phosphatase previously shown to catalyze U biomineralization (18). However, notably, the pH optimum for phosphatase

FIG 2 Subcellular localization of PhoY-mCherry. The $\Delta phoY$ strain harboring *phoY-mcherry* on a plasmid was grown to early exponential phase in PYE medium supplemented with kanamycin without xylose induction. Bright-field (left) and epifluorescence (right) images are shown. Inset, zoom-in of a single cell. Scale bars, 1 μ m.

activity in *C. crescentus* (pH 7.5) differs significantly from that of *Sphingomonas* sp. BSAR-1 (pH 9.0) (see Fig. S2 in the supplemental material) (18). Nevertheless, PhoY and PhoK belong to a group that is clearly distinct from acid phosphatases (e.g., PhoN in *Salmonella enterica*) that have been implicated in U biomineralization as well as other annotated phosphatases in *C. crescentus* (17, 19, 20).

In order to test if PhoY is responsible for U biomineralization in *C. crescentus*, we compared the abilities of wild-type NA1000 and a PhoY deletion mutant ($\Delta phoY$) to biomineralize U (Fig. 3). The biomineralization assay was conducted with glycerol-2-phosphate as the organic phosphate source at pH 7.0 in order to prevent chemical precipitation of U. Wild-type cells clearly exhibited biomineralization activity, as evidenced by the decrease in soluble U and increase of insoluble U over time (Fig. 3). Although both soluble and total P_i increased over time, the amount of soluble P_i was consistently lower than that of the total P_i , consistent with the increase in insoluble P_i . In controls without glycerol-2-phosphate, no P_i production or U precipitation was observed, as expected (see Fig. S4 in the supplemental material). In contrast to the wild type, the $\Delta phoY$ strain did not produce any P_i or precipitate any U, indicating that PhoY is responsible for whole-cell phosphatase activity and U biomineralization in *C. crescentus*. Furthermore, a complement strain (YJ0021), in which $\Delta phoY$ harbors a xylose-inducible *phoY* plasmid, rescued P_i production and U precipitation activities. Notably, the *phoY* complement strain has a higher phosphatase activity (480 ± 10 nmol/h) than does the wild type (260 ± 10 nmol/h). As a result, the rate of U precipitation was higher for the complement strain (136 ± 3 nmol/h) than for the wild type (80 ± 10 nmol/h). Comparison of P_i production with and without U confirmed that the presence of U did not affect phosphatase activity (see Fig. S5 in the supplemental material), demonstrating that endogenous phosphatase activity is responsible for U biomineralization.

To further examine the U biomineralization process, we performed TEM analyses of samples collected at the 5-h time point of the biomineralization assay (Fig. 3E). TEM images of both the wild type and the *phoY* complement strains revealed the presence of extracellular U- P_i precipitates. In contrast, the $\Delta phoY$ strain was bare of extracellular precipitates. Intracellular dark deposits appeared to be present in the $\Delta phoY$ strain, and EDS analysis revealed that these deposits do contain U (data not shown). However, due to the weak contrast of the image and potential

FIG 3 Comparison of U biomineralization among wild-type, $\Delta phoY$, and *phoY* complement strains. (A) Total and soluble uranium. (B) Insoluble uranium. (C) Total and soluble P_i . (D) Insoluble P_i . Circles, wild type; squares, $\Delta phoY$ strain; triangles, *phoY* complement strain. In panels A and C, empty symbols represent total U or P_i and solid symbols represent soluble U or P_i . Error bars denote standard deviations from three biological replicates. (E) TEM analysis of samples collected at 5 h during the biomineralization assay. Extracellular U precipitates were observed in wild type and *phoY* complement strains but were absent in the $\Delta phoY$ strain. wt, wild type; $\Delta phoY$, *phoY* deletion mutant; $\Delta phoY + phoY$, *phoY* complement strain. Scale bars, 500 nm.

nonspecific staining inherent to TEM sample preparation, we cannot exclude the possibility that these dark deposits are artifacts.

PhoY aids cell survival under U exposure. Several studies have established that phosphatase activity facilitates U biomineralization (17–19); however, few studies have directly examined whether the biomineralization process affects cell survival and function (40). To address this question, we compared both growth and cell survival of the wild-type, $\Delta phoY$, and *phoY* complement strains under U biomineralization conditions. Growth tests in M5G-GP medium with glycerol-2-phosphate as the sole phosphate source indicated that while all three strains grew in the ab-

sence of U, only the $\Delta phoY$ strain showed a lack of growth in the presence of U (Fig. 4A and B). The initial growth lag observed with the $\Delta phoY$ strain in the absence of U was likely caused by P_i limitation due to a lack of extracellular phosphatase activity. The fact that the $\Delta phoY$ strain eventually grew suggests the presence of alternative phosphatases. We suspect that *C. crescentus* is able to transport glycerol-2-phosphate into the cell and generate P_i through intracellular phosphatases as an alternative pathway to obtain P_i for growth. Consistently, we did not observe P_i accumulation in the medium with the $\Delta phoY$ strain with or without U (data not shown). The *phoY* complement strain also exhibited an

FIG 4 Comparison of growth and cell survival among wild-type, $\Delta phoY$, and $phoY$ complement strains under U biomineralization conditions. (A and B) Growth of wild-type, $\Delta phoY$, and $phoY$ complement strains in M5G-GP medium in the absence (A) or presence (B) of 50 μ M uranyl nitrate. Circles, wild type; squares, $\Delta phoY$ strain; triangles, $phoY$ complement strain. Error bars indicate standard deviations from three biological replicates. (C) Cell spotting for survival with samples collected at different time points (indicated on the left) during the biomineralization assay. Controls with glycerol-2-phosphate alone (GP) and uranium alone (U) were included. Serial dilutions of 10^1 to 10^6 (left to right) were spotted on PYE agar. Cell spotting was performed for three biological replicates, and one representative replicate is shown for each condition. wt, wild type; $\Delta phoY$, $phoY$ deletion mutant; $\Delta phoY + phoY$, $phoY$ complement strain.

initial growth lag in the absence of U, which is likely due to the metabolic burden and/or toxicity associated with PhoY overexpression. This general growth defect likely explains why growth of the $phoY$ complement strain in the presence of U was not restored to the wild-type level. Notably, PhoY-stimulated growth under U occurs only when P_i is initially absent in the growth medium; we observed no difference in growth between the wild type and the $\Delta phoY$ strain in PYE medium with and without U (see Fig. S6 in the supplemental material).

To test PhoY-induced cell survival during U exposure under nongrowth conditions, aliquots of the wild-type, $\Delta phoY$, and $phoY$ complement strains during the biomineralization assay were serially diluted and spotted on PYE agar to test for survival (Fig. 4C). Controls with no glycerol-2-phosphate or U were included for comparison. Compared to that of the wild type, the $\Delta phoY$ strain exhibited an ~ 100 -fold increase in cell death at 7.5 h, demonstrating that PhoY confers a survival advantage during U exposure under nongrowth conditions. The $phoY$ complement strain restored survival in the biomineralization assay; no significant cell death was observed compared to the no-U control. We note that the $phoY$ complement strain did exhibit a general survival disadvantage in the absence of U, which again is likely attributable to metabolic burden and/or toxicity from PhoY overexpression, consistent with the strain's slower growth in M5G-GP medium. Finally, the most severe cell death was observed for all strains in controls with U alone without glycerol-2-phosphate, with almost complete cell death after 1.5 h. This observation suggests that free uranyl nitrate species in solution is more toxic than U complexed with glycerol-2-phosphate, which, in turn, is more toxic than U- P_i precipitates. These results thus highlight the importance of U speciation when examining U toxicity to microbes.

DISCUSSION

In this study, we demonstrated that the aquatic bacterium *Caulobacter crescentus* is able to catalyze the formation of uranium phosphate precipitates at the cell surface when exposed to U(VI). The biogenic U precipitates are crystalline, with a higher P/U ratio than chemically produced precipitates, characteristics that may allow analytical differentiation of the biogenic minerals from their abiotic counterparts found in the environment. XRD analysis showed that the precipitates are in the form of meta-autunite, with no alteration of the U(VI) redox state during the biomineralization process, in contrast to reductive precipitation by DMRB under anaerobic conditions (5, 13). Immobilization of U as U(VI) phosphate minerals under aerobic conditions offers the possibility of long-term U stability, since meta-autunite minerals have been shown to be stable for long periods of time, over a wide range of pHs (41). These results thus highlight the potential utility of *C. crescentus* for U immobilization in the oxic zones of contaminated sites.

Based on the results described in this study, we propose a model for U biomineralization by *C. crescentus* (Fig. 5) in which the nonspecific, alkaline phosphatase PhoY is responsible for the production of P_i in the periplasm, which, in turn, precipitates with U(VI) to form meta-autunite minerals on the cell surface and in the bulk medium. While the cell surface-deposited uranium phosphate precipitates observed by TEM (Fig. 1) likely result from *in situ* precipitation facilitated by PhoY, we cannot exclude other possibilities. U is known to interact with bacterial cell surfaces through phosphate-containing macromolecules such as lipopolysaccharide and S-layer protein (42, 43). S-layer protein has been shown to be important for U resistance in *Bacillus sphaericus* JG-7B (43); however, it does not appear to contribute significantly

FIG 5 Model for U biomineralization by *C. crescentus*. PhoY, an alkaline phosphatase located in the periplasmic space, catalyzes U biomineralization by cleaving organic phosphate to produce inorganic phosphate, which, in turn, precipitates with uranyl ion to produce uranium-phosphate precipitates on the cell surface in the form of meta-autunite. S, S-layer; OM, outer membrane; PS, periplasmic space; IM, inner membrane.

to U immobilization or cell survival in *C. crescentus* (see Fig. S7 in the supplemental material). This difference is probably due to lack of phosphorylated S-layer protein in *C. crescentus*, in contrast to *B. sphaericus* (43).

Remarkably, PhoY-induced U biomineralization aids survival of *C. crescentus* toward U under both growth and nongrowth conditions. Given the periplasmic localization of PhoY, we hypothesize that the production of P_i by PhoY impedes U transport into the cytoplasm by local precipitation in the periplasm as a first line of defense, preventing U toxicity to the organism. TEM images showing a lack of intracellular uranium deposits in the wild type and the *phoY* complement strain support this hypothesis (Fig. 3). It is becoming apparent that biominerals produced by certain organisms, ranging from prokaryotes to eukaryotes, play an important protective role by acting as critical detoxification sinks to efficiently remove potentially toxic species from the immediate environment (44, 45). Surprisingly, however, the phosphatase activity in *C. crescentus* is not induced by the presence of U, suggesting that U biomineralization is likely a fortuitous consequence of native phosphatase activity.

Another example that reflects the intricacy between U resistance and phosphate metabolism in *C. crescentus* is the role of a phytase enzyme (CCNA_01353), previously found to be upregulated in response to U and able to facilitate growth in U when phytate served as the sole phosphate source (24, 27). The protection mechanism of the phytase, however, is likely different from that of the PhoY phosphatase found in this study. In the case of phytase, we found no detectable amount of P_i released into the medium and no U precipitation from wild-type cells grown in the presence of U with phytate as the sole phosphate source (data not shown). Furthermore, phytase activity was found to be 10 times lower than the PhoY activity in whole-cell assays (data not shown), which may explain why almost no U biomineralization was detected. Thus, the protective role of phytase is likely through supply of P_i for growth under these conditions rather than U biomineralization.

In the PhoY system, in contrast, cells do not experience significant P_i limitation, due to the presence of highly active PhoY and/or other intracellular phosphatases, as evidenced by the relatively normal growth rate of the $\Delta phoY$ strain in M5G-GP medium in the absence of U. Thus, supply of P_i for growth is unlikely

the primary mechanism for resistance. We hypothesize that U biomineralization by PhoY is likely the primary mechanism for resistance through a precipitation-induced shift of U solubility. Consistently, the increase in cell survival in the presence of PhoY was not apparent until sufficient P_i was produced and U was biomineralized (Fig. 3 and 4C). While the relationship between U speciation and bioavailability is complex (1), evidence indicates that free UO_2^{2+} and UO_2OH^+ are the major forms of U(VI) available to organisms, rather than U in chelation complexes or adsorbed to colloidal and/or particulate matter (4). Consistently, our results showed that U(VI) complexes with both organic and inorganic forms of phosphate greatly reduce U toxicity, with the inorganic phosphate form being the least toxic (Fig. 4C). By converting organic phosphates to inorganic phosphates, PhoY shifts the pool of U from chelating with organic phosphate to precipitation with inorganic phosphate, resulting in a decrease in U toxicity.

Finally, we should note that while the concentrations of U used in this study are within the range of U concentrations found in contaminated sites (up to ~ 0.2 mM) (46), the concentrations of organic phosphates are far above those found at contaminated sites (41). Since U biomineralization and toxicity are dependent on the ratio of U to organic phosphate (Fig. 3 and 4C), we would expect higher toxicity from the unchelated form of U and slower conversion to U- P_i precipitates at contaminated sites, as previously observed with *Serratia* sp. N14 under phosphate-limiting conditions (47). Recent studies by Beazley et al., however, suggest that U- P_i biomineralization can be efficiently stimulated in contaminated soil samples from the Oak Ridge Field Research Center (ORFRC) by addition of organic phosphates (41), demonstrating that stimulated biomineralization can be a viable remediation strategy for environments with low organic phosphate concentrations.

The findings presented in this report help define and characterize biogenic U minerals as well as U trafficking during biomineralization in *C. crescentus*, establishing a model for this process (Fig. 5) and demonstrating the potential utility of this organism in U bioremediation. We further identified PhoY as an alkaline phosphatase that plays a central role in U biomineralization and resistance. Knowledge gained in this study thus not only improves our understanding of bacterium-mineral interactions on the surfaces of metal-resistant bacteria but also helps us in defining ecological niches for metal-resistant bacteria.

ACKNOWLEDGMENTS

We thank Mavrik Zavarin for assistance with XRD analysis. We thank Mark Wall for help with TEM-EDS analysis. We thank Catherine Lacayo for help with fluorescence microscopy. We thank Dan Park for critical review of the manuscript.

This work was performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract DE-AC52-07NA27344 (LLNL-JRNL-652320). This study was supported by a Department of Energy Early Career Research Program award from the Office of Biological and Environmental Sciences (to Y.J.).

REFERENCES

- Markich SJ. 2002. Uranium speciation and bioavailability in aquatic systems: an overview. *ScientificWorldJournal*. 2:707–729. <http://dx.doi.org/10.1100/tsw.2002.130>.
- National Research Council. 2008. Review of toxicologic and radiologic risks to military personnel from exposure to depleted uranium during and after combat. The National Academies Press, Washington, DC.

3. Phillips EJP, Landa ER, Lovley DR. 1995. Remediation of uranium contaminated soils with bicarbonate extraction and microbial U(VI) reduction. *J. Ind. Microbiol.* 14:203–207. <http://dx.doi.org/10.1007/BF01569928>.
4. Turick CE, Knox AS, Leverette CL, Kritzas YG. 2008. In situ uranium stabilization by microbial metabolites. *J. Environ. Radioact.* 99:890–899. <http://dx.doi.org/10.1016/j.jenvrad.2007.11.020>.
5. Wall JD, Krumholz LR. 2006. Uranium reduction. *Annu. Rev. Microbiol.* 60:149–166. <http://dx.doi.org/10.1146/annurev.micro.59.030804.121357>.
6. Williams KH, Bargar JR, Lloyd JR, Lovley DR. 2013. Bioremediation of uranium-contaminated groundwater: a systems approach to subsurface biogeochemistry. *Curr. Opin. Biotechnol.* 24:489–497. <http://dx.doi.org/10.1016/j.copbio.2012.10.008>.
7. Sharp JO, Schofield EJ, Veeramani H, Suvorova EI, Kennedy DW, Marshall MJ, Mehta A, Bargar JR, Bernier-Latmani R. 2009. Structural similarities between biogenic uraninites produced by phylogenetically and metabolically diverse bacteria. *Environ. Sci. Technol.* 43:8295–8301. <http://dx.doi.org/10.1021/es901281e>.
8. Marshall MJ, Beliaev AS, Dohnalkova AC, Kennedy DW, Shi L, Wang Z, Boyanov MI, Lai B, Kemner KM, McLean JS, Reed SB, Culley DE, Bailey VL, Simonson CJ, Saffarini DA, Romine MF, Zachara JM, Fredrickson JK. 2006. c-Type cytochrome-dependent formation of U(IV) nanoparticles by *Shewanella oneidensis*. *PLoS Biol.* 4:e268. <http://dx.doi.org/10.1371/journal.pbio.0040268>.
9. Xu H, Barton L, Zhang P, Wang Y. 2000. TEM investigation of U⁶⁺ and Re⁷⁺ reduction by *Desulfovibrio desulfuricans*, a sulfate-reducing bacterium. *Mat. Res. Soc. Symp. Proc.* 608:299–304. <http://dx.doi.org/10.1557/PROC-608-299>.
10. Suzuki Y, Kelly SD, Kemner KM, Banfield JF. 2004. Enzymatic U(VI) reduction by *Desulfosporosinus* species. *Radiochim. Acta* 92:11–16. <http://dx.doi.org/10.1524/ract.92.1.11.25404>.
11. Liu C, Gorby YA, Zachara JM, Fredrickson JK, Brown CF. 2002. Reduction kinetics of Fe(III), Co(III), U(VI), Cr(VI), and Tc(VII) in cultures of dissimilatory metal-reducing bacteria. *Biotechnol. Bioeng.* 80:637–649. <http://dx.doi.org/10.1002/bit.10430>.
12. Lovley DR, Phillips EJ. 1992. Reduction of uranium by *Desulfovibrio desulfuricans*. *Appl. Environ. Microbiol.* 58:850–856.
13. Senko JM, Istok JD, Suflita JM, Krumholz LR. 2002. In-situ evidence for uranium immobilization and remobilization. *Environ. Sci. Technol.* 36:1491–1496. <http://dx.doi.org/10.1021/es011240x>.
14. Finch R, Murakami T. 1999. Systematics and paragenesis of uranium minerals, p 221–253. *In* Burns PC, Finch R (ed), *Uranium: mineralogy, geochemistry and the environment*, vol 38. Mineralogical Society of America, Washington, DC.
15. Kalin M, Wheeler WN, Meinrath G. 2005. The removal of uranium from mining waste water using algal/microbial biomass. *J. Environ. Radioact.* 78:151–177. <http://dx.doi.org/10.1016/j.jenvrad.2004.05.002>.
16. Haferburg G, Kothe E. 2007. Microbes and metals: interactions in the environment. *J. Basic Microbiol.* 47:453–467. <http://dx.doi.org/10.1002/jobm.200700275>.
17. Macaskie LE, Bonthron KM, Rouch DA. 1994. Phosphatase-mediated heavy metal accumulation by a *Citrobacter* sp. and related enterobacteria. *FEMS Microbiol. Lett.* 121:141–146. <http://dx.doi.org/10.1111/j.1574-6968.1994.tb07090.x>.
18. Nilgiriwala KS, Alahari A, Rao AS, Apte SK. 2008. Cloning and overexpression of alkaline phosphatase PhoK from *Sphingomonas* sp. strain BSAR-1 for bioprecipitation of uranium from alkaline solutions. *Appl. Environ. Microbiol.* 74:5516–5523. <http://dx.doi.org/10.1128/AEM.00107-08>.
19. Martinez RJ, Beazley MJ, TAILLEFERT M, Arakaki AK, Skolnick J, Sobecky PA. 2007. Aerobic uranium (VI) bioprecipitation by metal-resistant bacteria isolated from radionuclide- and metal-contaminated subsurface soils. *Environ. Microbiol.* 9:3122–3133. <http://dx.doi.org/10.1111/j.1462-2920.2007.01422.x>.
20. Basnakova G, Stephens ER, Thaller MC, Rossolini GM, Macaskie LE. 1998. The use of *Escherichia coli* bearing a *phoN* gene for the removal of uranium and nickel from aqueous flows. *Appl. Microbiol. Biotechnol.* 50:266–272. <http://dx.doi.org/10.1007/s002530051288>.
21. Powers LG, Mills HJ, Palumbo AV, Zhang C, Delaney K, Sobecky PA. 2002. Introduction of a plasmid-encoded *phoA* gene for constitutive overproduction of alkaline phosphatase in three subsurface *Pseudomonas* isolates. *FEMS Microbiol. Ecol.* 41:115–123. <http://dx.doi.org/10.1111/j.1574-6941.2002.tb00972.x>.
22. Kulkarni S, Ballal A, Apte SK. 2013. Bioprecipitation of uranium from alkaline waste solutions using recombinant *Deinococcus radiodurans*. *J. Hazard. Mater.* 262:853–861. <http://dx.doi.org/10.1016/j.jhazmat.2013.09.057>.
23. Laub MT, Shapiro L, McAdams HH. 2007. Systems biology of *Caulobacter*. *Annu. Rev. Genet.* 41:429–441. <http://dx.doi.org/10.1146/annurev.genet.41.110306.130346>.
24. Hu P, Brodie EL, Suzuki Y, McAdams HH, Andersen GL. 2005. Whole-genome transcriptional analysis of heavy metal stresses in *Caulobacter crescentus*. *J. Bacteriol.* 187:8437–8449. <http://dx.doi.org/10.1128/JB.187.24.8437-8449.2005>.
25. Utturkar SM, Bollmann A, Brzoska RM, Klingeman DM, Epstein SE, Palumbo AV, Brown SD. 2013. Draft genome sequence for *Caulobacter* sp. strain OR37, a bacterium tolerant to heavy metals. *Genome Announc.* 1(3):e00322–13. <http://dx.doi.org/10.1128/genomeA.00322-13>.
26. Hillson NJ, Hu P, Andersen GL, Shapiro L. 2007. *Caulobacter crescentus* as a whole-cell uranium biosensor. *Appl. Environ. Microbiol.* 73:7615–7621. <http://dx.doi.org/10.1128/AEM.01566-07>.
27. Yung MC, Ma J, Salemi MR, Phinney BS, Bowman GR, Jiao Y. 2014. Shotgun proteomic analysis unveils survival and detoxification strategies by *Caulobacter crescentus* during exposure to uranium, chromium, and cadmium. *J. Proteome Res.* 13:1833–1847. <http://dx.doi.org/10.1021/pr400880s>.
28. Ely B. 1991. Genetics of *Caulobacter crescentus*. *Methods Enzymol.* 204:372–384. [http://dx.doi.org/10.1016/0076-6879\(91\)04019-K](http://dx.doi.org/10.1016/0076-6879(91)04019-K).
29. Domian IJ, Quon KC, Shapiro L. 1997. Cell type-specific phosphorylation and proteolysis of a transcriptional regulator controls the G1-to-S transition in a bacterial cell cycle. *Cell* 90:415–424. [http://dx.doi.org/10.1016/S0092-8674\(00\)80502-4](http://dx.doi.org/10.1016/S0092-8674(00)80502-4).
30. Ried JL, Collmer A. 1987. An *nptI-sacB-sacR* cartridge for constructing directed, unmarked mutations in gram-negative bacteria by marker exchange-eviction mutagenesis. *Gene* 57:239–246. [http://dx.doi.org/10.1016/0378-1119\(87\)90127-2](http://dx.doi.org/10.1016/0378-1119(87)90127-2).
31. Stephens C, Reisenauer A, Wright R, Shapiro L. 1996. A cell cycle-regulated bacterial DNA methyltransferase is essential for viability. *Proc. Natl. Acad. Sci. U. S. A.* 93:1210–1214. <http://dx.doi.org/10.1073/pnas.93.3.1210>.
32. Thanbichler M, Iñiesta AA, Shapiro L. 2007. A comprehensive set of plasmids for vanillate- and xylose-inducible gene expression in *Caulobacter crescentus*. *Nucleic Acids Res.* 35:e137. <http://dx.doi.org/10.1093/nar/gkm18>.
33. Holman WI. 1943. A new technique for the determination of phosphorus by the molybdenum blue method. *Biochem. J.* 37:256–259.
34. Fritz JS, Bradford EC. 1958. Detection of thorium and uranium. *Anal. Chem.* 30:1021–1022. <http://dx.doi.org/10.1021/ac60138a002>.
35. Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH image to ImageJ: 25 years of image analysis. *Nat. Methods* 9:671–675. <http://dx.doi.org/10.1038/nmeth.2089>.
36. Nierman WC, Feldblyum TV, Laub MT, Paulsen IT, Nelson KE, Eisen JA, Heidelberg JF, Alley MR, Ohta N, Maddock JR, Potocka I, Nelson WC, Newton A, Stephens C, Phadke ND, Ely B, DeBoy RT, Dodson RJ, Durkin AS, Gwinn ML, Haft DH, Kolonay JF, Smit J, Craven MB, Khouri H, Shetty J, Berry K, Utterback T, Tran K, Wolf A, Vamathevan J, Ermolaeva M, White O, Salzberg SL, Venter JC, Shapiro L, Fraser CM. 2001. Complete genome sequence of *Caulobacter crescentus*. *Proc. Natl. Acad. Sci. U. S. A.* 98:4136–4141. <http://dx.doi.org/10.1073/pnas.061029298>.
37. Petersen TN, Brunak S, von Heijne G, Nielsen H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* 8:785–786. <http://dx.doi.org/10.1038/nmeth.1701>.
38. Yu NY, Wagner JR, Laird MR, Melli G, Rey S, Lo R, Dao P, Sahinalp SC, Ester M, Foster LJ, Brinkman FSL. 2010. PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* 26:1608–1615. <http://dx.doi.org/10.1093/bioinformatics/btq249>.
39. Ely B, Scott LE. 2014. Correction of the *Caulobacter crescentus* NA1000 genome annotation. *PLoS One* 9:e91668. <http://dx.doi.org/10.1371/journal.pone.0091668>.
40. Plummer EJ, Macaskie LE. 1990. Actinide and lanthanum toxicity towards a *Citrobacter* sp.: uptake of lanthanum and a strategy for the biological treatment of liquid wastes containing plutonium. *Bull. Environ. Contam. Toxicol.* 44:173–180. <http://dx.doi.org/10.1007/BF01700133>.
41. Beazley MJ, Martinez RJ, Webb SM, Sobecky PA, TAILLEFERT M. 2011. The effect of pH and natural microbial phosphatase activity on the speciation of uranium in subsurface soils. *Geochim. Cosmochim. Acta* 75:5648–5663. <http://dx.doi.org/10.1016/j.gca.2011.07.006>.

42. Barkleit A, Foerstendorf H, Li B, Rossberg A, Moll H, Bernhard G. 2011. Coordination of uranium(VI) with functional groups of bacterial lipopolysaccharide studied by EXAFS and FT-IR spectroscopy. *Dalton Trans.* 40:9868–9876. <http://dx.doi.org/10.1039/c1dt10546a>.
43. Merroun ML, Raff J, Rossberg A, Hennig C, Reich T, Selenska-Pobell S. 2005. Complexation of uranium by cells and S-layer sheets of *Bacillus sphaericus* JG-A12. *Appl. Environ. Microbiol.* 71:5532–5543. <http://dx.doi.org/10.1128/AEM.71.9.5532-5543.2005>.
44. Newman DK, Beveridge TJ, Morel F. 1997. Precipitation of arsenic trisulfide by *Desulfotomaculum auripigmentum*. *Appl. Environ. Microbiol.* 63:2022–2028.
45. Beveridge TJ. 1989. Role of cellular design in bacterial metal accumulation and mineralization. *Annu. Rev. Microbiol.* 43:147–171. <http://dx.doi.org/10.1146/annurev.mi.43.100189.001051>.
46. Brooks SC. 2001. Waste characteristics of the former S-3 ponds and outline of uranium chemistry relevant to NABIR field research center studies. Rep. ORNL/TM-2001/27. Oak Ridge National Laboratory, Oak Ridge, TN.
47. Yong P, Macaskie LE. 1995. Role of citrate as a complexing ligand which permits enzymatically-mediated uranyl-ion bioaccumulation. *Bull. Environ. Contam. Toxicol.* 54:892–899.
48. Evinger M, Agabian N. 1977. Envelope-associated nucleoid from *Caulobacter crescentus* stalked and swarmer cells. *J. Bacteriol.* 132:294–301.