Biomineralization of uranium by PhoY phosphatase activity aids cell survival in

*Caulobacter crescentus*.

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ABSTRACT:

*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-Pi precipitates via its native alkaline phosphatase activity. The U-Pi precipitates, deposited on the cell surface in the form of meta-autinite structures, have a lower U/Pi ratio compared to 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 to *C. crescentus* towards U(VI) under both growth and non-growth conditions. Results in this study thus highlight U(VI) biomineralization as a resistance mechanism in microbes, which not only improves our understanding of bacterial-mineral interactions, but also aids in defining potential ecological niches for metal-resistant bacteria.
INTRODUCTION:

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, they are readily re-oxidized 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. N14 (formerly Citrobacter sp. N14), Sphingomonas sp. BSAR-1, Arthrobacter sp., Rahnella sp., and Bacillus sp. 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 was paid to the cellular benefits of the native or heterologously expressed phosphatases towards 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, OH). Uranyl nitrate hexahydrate [(UO$_2$)(NO$_3$)$_2$•6H$_2$O] 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 PCR reactions were amplified using iProof polymerase from Bio-Rad (Hercules, CA) supplemented with 5% dimethyl sulfoxide (DMSO) according to manufacturer’s instructions. Caulobacter crescentus NA1000 was maintained on solid PYE medium (0.2% peptone, 0.1% yeast extract, 0.5 mM MgSO$_4$, and 1 mM CaCl$_2$) supplemented with 1.5% agar (28). Liquid cultures were grown in either 1) PYE or 2) modified M5G minimal medium lacking inorganic phosphate (P) 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 pre-cultured in 2 mL of PYE from a single colony at 30 °C overnight. Overnight cultures were diluted to an initial optical density at 600 nm (OD$_{600}$) of 0.1 and cultured for an additional 2 h at 30 °C to an OD$_{600}$ 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 OD$_{600}$.

For growth in M5G-GP medium, C. crescentus cells were pre-cultured 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 to an initial OD$_{600}$ of 0.04 and incubated
for another 7 h until the OD$_{600}$ was about 0.5 (late exponential phase). Cells were harvested, washed once with 10 mM NaCl, and inoculated into M5G-GP supplemented with 0 or 50 µM uranyl nitrate to an initial OD$_{600}$ of 0.02. M5G-GP media were not supplemented with kanamycin due to kanamycin inactivation by uranium (data not shown). Growth of cells was performed in biological triplicate and monitored using OD$_{600}$.

Construction of ΔphoY.

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). 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\textsubscript{xyl}) in pBXMCS-2 (32) to generate pMCY21 (Table 1). The sequence of \textit{phoY} was confirmed by DNA sequencing. \textit{ΔphoY} mutant was transformed with pMCY21 to obtain the P\textsubscript{xyl} \textit{phoY} complement strain YJ0021. Transformants were selected on PYE-agar supplemented with kanamycin.

To generate the \textit{phoY}-mcherry complement strain, the \textit{phoY} open reading frame was PCR amplified using primers RV\textit{phoY\_for} and RV\textit{phoY\_rev} (Table 2). This \textit{phoY} fragment was inserted using Gibson assembly into the NdeI and EcoRI sites upstream of the \textit{mcherry} coding region in pRVCHYC-2 (32). The resulting \textit{phoY\textendash mcherry} construct was then PCR amplified using primers BX\textit{phoY\_for} and BX\textit{phoYch\_rev} (Table 2) and was subsequently inserted into the NdeI and XbaI sites downstream of the P\textsubscript{xyl} promoter in pBXMCS-2 (32) to generate pMCY23 (Table 1). The sequence of \textit{phoY\textendash mcherry} was confirmed by DNA sequencing. \textit{ΔphoY} was transformed with pMCY23 to obtain the P\textsubscript{xyl} \textit{phoY\textendash 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.

\textit{Cell-bound phosphatase assay.}  

\textit{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 re-suspended in 100 mM Tris-HCl pH 7.0 to a final OD\textsubscript{600} 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<sub>i</sub> content as described below.

**U biomineralization assay.**

*C. crescentus* NA1000, YJ0010 (∆phoY), and YJ0021 (∆phoY/pMCY21) cells were individually pre-cultured 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 OD<sub>600</sub> of 0.001 and cultured for 16 h until the OD<sub>600</sub> was about 0.6 (late exponential phase). Cells were harvested, washed once with 10 mM NaCl, and re-suspended in 50 mM PIPES pH 7.0 to a final OD<sub>600</sub> 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<sub>i</sub> content as described below. Aliquots were also removed for cell survival analysis. Serial dilutions of 10<sup>1</sup> to 10<sup>6</sup> of the aliquots were prepared in PYE and 10 µL of each dilution were spotted on PYE-agar. Images of the spots were taken after 2 days of incubation at 30 °C.

**Measurement of U and P<sub>i</sub>**

To measure total U and P<sub>i</sub> content, 45 µL of sample was directly quenched with 45 µL of 12.5% trichloroacetic acid (TCA). Samples were centrifuged at 20,000 x g to remove cell debris and supernatant was analyzed for total U and P<sub>i</sub> via Arsenazo III and molybdate colorimetric
assays, respectively (33, 34). Briefly, to measure U, 40 µL of supernatant 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 concentration. To measure P, 40 µL of supernatant 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 concentration.

To measure soluble U and P content, 45 µL of sample was immediately centrifuged at 20,000 x g for 5 min. Supernatant was quenched with an equal volume of 12.5% TCA and then analyzed for soluble U and P concentrations as described above. To measure the insoluble U and P content, the pellet after centrifugation was re-suspended in 45 µL of degassed 300 mM NaHCO₃ in water and mixed at room temperature for 5 min. Sample was then centrifuged again at 20,000 x g for 5 min. Supernatant was quenched with an equal volume of 12.5% TCA and analyzed for U and P concentrations as described above.

XRD sample preparation and analysis.

For X-ray powder diffraction (XRD) analysis, 40 mL of cell culture grown with 200 µM U in PYE medium for 30 min was harvested by centrifugation at 20,000 x 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 re-suspended in residual liquid by gentle pipetting and carefully removed. The bottom layer was re-suspended 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 defractometer (Billerica, MA), and spectra were compared to references from the International Centre for Diffraction Data (ICDD).

**TEM sample preparation and imaging.**

For cells grown with 200 µM U in PYE medium for 30 min, 3 mL of culture was used. For cells originating from U biomineralization assays, 1.6 mL of cell suspension at 5 h assay time with 5 mM glycerol-2-phosphate and 250 µM uranyl nitrate was collected.

Samples were harvested at 20,000 x 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. 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 of 90 nm 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). Transmission electron microscopy (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 (ΔphoY/pMCY23) was cultured from a single colony at 30 °C in PYE supplemented with kanamycin until the cells reached an OD<sub>600</sub> of 0.25 (mid exponential phase). Cells were harvested and re-suspended in 1/10 of the culture volume. Five microliters of the concentrated cells were spotted on a PYE-1% agarose pad and imaged using an Axiovert 200M microscope (Zeiss, Minneapolis, MN) equipped with a Photometric CoolSNAP HQ CCD camera. Images were acquired with a 100x objective and a Texas Red filter set (Chroma, Bellows Falls, VT, filter #41004). Images were processed using ImageJ.

RESULTS:

C. crescentus produces extracellular, crystalline U-P<sub>i</sub> precipitates.

To determine if C. crescentus is able to facilitate uranium phosphate (U-P<sub>i</sub>) 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<sub>i</sub> 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 higher amounts of both U and P<sub>i</sub> in the insoluble fraction (308 ± 3 nmol U and 340 ± 20 nmol P<sub>i</sub>) compared to that of the abiotic control (220 ± 20 nmol U and 170 ± 30 nmol P<sub>i</sub>), suggesting that cell metabolism induced U-P<sub>i</sub> precipitation (Table 3). The higher amount of U present in the biotic insoluble fraction was consistent with the lower U content in the soluble fraction. The amounts of P<sub>i</sub> in all fractions (soluble, insoluble, and total) were higher for the biotic versus abiotic samples, indicating that P<sub>i</sub> was produced and released from cells during growth. P<sub>i</sub> 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 ($22 \pm 2\%$ 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 compared to that of 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 presence 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 (uranil phosphate species in the U(VI) oxidation state), likely in the form of uramphite ($(\text{NH}_4)(\text{UO}_2)(\text{PO}_4)\cdot 3\text{H}_2\text{O}$, ICDD 00-042-0384), potassium sodium uranyl phosphate hydrate ($(\text{Na}_{0.43}\text{K}_{0.57})(\text{UO}_2)(\text{PO}_4)\cdot 3+x\text{H}_2\text{O}$, ICDD 00-059-0399), and/or potassium uranyl phosphate hydrate ($K(\text{UO}_2)(\text{PO}_4)\cdot 3\text{H}_2\text{O}$, ICDD 00-049-0433).

In order to determine where U precipitates reside relative to cells, TEM analysis of the samples collected after 30 min 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 intensity of the EDS spectra, the cell surface-associated precipitates appeared to have a lower U to P ratio compared to 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 appears 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 presence of cells contained higher amounts of P, compared to the abiotic control (Table 3), we hypothesized that P 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 (Fig. S1). The results revealed phosphatase activity towards all three substrates, indicating broad substrate specificity. Surprisingly, cells grown with and without U exhibited the same phosphatase activity towards each substrate tested (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 (Fig. S2). A genome search revealed 4 annotated alkaline phosphatases in *C. crescentus* NA1000. The presence of cell-surface associated U-P, 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 (Fig. S3) revealed that it is most similar to PhoK in Sphingomonas sp. BSAR-1 (39% identity, 51% similarity), an alkaline phosphatase previously shown to catalyze U biomineralization (18). However, notably, the pH optimum for phosphatase activity in C. crescentus (pH 7.5) differs significantly from that of Sphingomonas sp. BSAR-1 (pH 9.0) (Fig. S2) (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 (ΔphoY) 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 the soluble and total P$_i$ concentrations increased over time, the soluble P$_i$ was consistently lower than 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 (Fig. S4). In contrast to wild type, ΔphoY did not produce any P$_i$ nor precipitate any U, indicating PhoY is responsible for whole-cell phosphatase activity and U biomineralization in *C. crescentus*. Furthermore, a complement strain (YJ0023), in which ΔphoY 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 wild type (260 ± 10 nmol/h). As a result, the rate of U precipitation was faster in the complement strain (136 ± 3 nmol/h) than in 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 (Fig. S5), 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 wild type and the phoY complement strains revealed the presence of extracellular U-P$_i$ precipitates. In contrast, ΔphoY was bare of extracellular precipitates. Intracellular dark deposits appear to be present in ΔphoY and EDS analysis revealed that these deposits do contain U (data...
not shown). However, due to the weak contrast of the image and potential non-specific 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 wild type, ΔphoY, and the phoY complement strain 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 absence of U, only ΔphoY showed lack of growth in the presence of U (Fig. 4A and B). The initial growth lag observed with ΔphoY in the absence of U is likely caused by Pi limitation due to lack of extracellular phosphatase activity. The fact that the ΔphoY strain eventually grew suggests the presence of alternative phosphatases.

We suspect *C. crescentus* is able to transport glycerol-2-phosphate into the cell and generate Pi through intracellular phosphatases as an alternative pathway to obtain Pi for growth. Consistently, we did not observe Pi accumulation in the medium with ΔphoY with or without U (data not shown). The phoY complement strain also exhibited an 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 only occurs when Pi is initially absent in the growth medium; we observed no
difference in growth between wild type and ΔphoY in PYE medium with and without U (Fig. S6).

To test PhoY-induced cell survival during U exposure under non-growth conditions, aliquots of wild type, ΔphoY, and the phoY complement strain 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 wild type, ΔphoY exhibited a ~100 fold increase in cell death at 7.5 h, demonstrating that PhoY confers a survival advantage during U exposure under non-growth 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 attributed 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-Pi precipitates. These results thus highlight the importance of 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 pH (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 non-specific, 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 interaction with phosphate-containing macromolecules such as LPS and S-layer (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 to U immobilization or cell survival in *C. crescentus* (Fig. S7). 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 non-growth 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 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 up-regulated in response to U and able to facilitate growth in U when phytate served as the sole phosphate source (24, 27). The protection mechanism by the phytase, however, is likely different from that of the PhoY phosphatase presented in this study. In the case of phytase, we found no detectable amount of P, 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, for growth under these conditions rather than U biomineralization.

In the PhoY system, in contrast, cells do not experience significant P, limitation, due to the presence of highly-active PhoY and/or other intracellular phosphatases, as evidenced by the relatively normal growth rate of ∆phoY in M5G-GP medium in the absence of U. Thus, supply of P, 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 speciation. Consistently, the increase in cell survival by 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 is dependent on the ratio of U to the initial organic phosphate concentration (Fig. 3 and 4C), we would expect higher toxicity from the un-chelated 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 study 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 bacteria-mineral interactions on the surfaces of metal-resistant bacteria, but also helps us in defining ecological niches for metal-resistant bacteria.

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pH and natural microbial phosphatase activity on the speciation of uranium in subsurface

Coordination of uranium(VI) with functional groups of bacterial lipopolysaccharide

Complexation of uranium by cells and S-layer sheets of Bacillus sphaericus JG-A12.


## Table 1. Plasmids and bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Plasmid/Strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pNPTS138</td>
<td>Non-replicating vector for integration and allelic replacement; oriT, kan (Km'), sacB</td>
<td>M.R.K. Alley, unpublished</td>
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<tr>
<td>pBXMCS-2</td>
<td>High copy number xylose-inducible expression vector; kan (Km')</td>
<td>(32)</td>
</tr>
<tr>
<td>pRVCHYC-2</td>
<td>Low copy number vanillate-inducible expression vector containing coding region for a C-terminal mCherry construct; kan (Km')</td>
<td>(32)</td>
</tr>
<tr>
<td>pMCY10</td>
<td>pNPTS138-derived vector for ΔphoY allelic replacement</td>
<td>This study</td>
</tr>
<tr>
<td>pMCY21</td>
<td>pBXMCS-2-derived vector for expression of phoY</td>
<td>This study</td>
</tr>
<tr>
<td>pMCY23</td>
<td>pBXMCS-2-derived vector for expression of phoY-mcherry</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. crescentus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA1000</td>
<td>Wild type C. crescentus, a synchronizable derivative of CB15</td>
<td>(48)</td>
</tr>
<tr>
<td>Y30010</td>
<td>NA1000 ΔphoY</td>
<td>This study</td>
</tr>
<tr>
<td>Y30021</td>
<td>NA1000 ΔphoY harboring pMCY21</td>
<td>This study</td>
</tr>
<tr>
<td>Y30023</td>
<td>NA1000 ΔphoY harboring pMCY23</td>
<td>This study</td>
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## Table 2. Primers used in this study.

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<tr>
<td>phoY_URrev</td>
<td>GACGAG</td>
</tr>
<tr>
<td>phoY_DRfor</td>
<td>ACGTTGCATATGGCGCAAGAACGGAAGCGTCAGATATCGGC</td>
</tr>
<tr>
<td>phoY_DRrev</td>
<td>GACGAG</td>
</tr>
<tr>
<td>BXphoY_for</td>
<td>CGCCATATGCAACGTTGCTAACGGCGGTTAG</td>
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<tr>
<td>BXphoY_rev</td>
<td>CGAAGCTAGCGAATTCATTGAAACGCCCGGCT</td>
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<tr>
<td>RVphoY_for</td>
<td>GGGAGAAGCATGATGGTGCCGAAGGGCG</td>
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<tr>
<td>RVphoY_rev</td>
<td>GCTTC</td>
</tr>
<tr>
<td>BXphoYch_rev</td>
<td>CCGAGTCAGGAATTCTTAGCAACGTGGCCG</td>
</tr>
<tr>
<td>AGACCAGC</td>
<td></td>
</tr>
<tr>
<td>BXphoYch_rev</td>
<td>TGGCGGCCGCTCTAGATTACTTGTACAGCTCGT</td>
</tr>
<tr>
<td>CCAATGCCGC</td>
<td></td>
</tr>
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Table 3. Uranium and inorganic phosphate distribution during growth of *C. crescentus* in PYE medium with U. 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. Error bars denote standard deviations from three biological replicates.

<table>
<thead>
<tr>
<th></th>
<th>Uranium (nmol)</th>
<th>Inorganic phosphate (nmol)</th>
<th>Mol ratio U/P</th>
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<tbody>
<tr>
<td></td>
<td>Wt cells</td>
<td>Abiotic</td>
<td>Wt cells</td>
</tr>
<tr>
<td>Insoluble</td>
<td>308 ± 3 (81 ± 2%)</td>
<td>220 ± 20 (63 ± 8%)</td>
<td>340 ± 20 (22 ± 1%)</td>
</tr>
<tr>
<td>Soluble</td>
<td>72 ± 7 (19 ± 2%)</td>
<td>130 ± 20 (37 ± 6%)</td>
<td>1210 ± 10 (78 ± 1%)</td>
</tr>
<tr>
<td>Total</td>
<td>380 ± 8 (100%)</td>
<td>350 ± 30 (100%)</td>
<td>1550 ± 20 (100%)</td>
</tr>
</tbody>
</table>
Fig 1. XRD and TEM 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.
Fig 2. Subcellular localization of PhoY-mCherry. ΔphoY 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 µm.
Fig 3. Comparison of U biomineralization among wild type, ΔphoY, and phoY complement strains. (A) Total and soluble uranium. (B) Insoluble uranium. (C) Total and soluble Pi. (D) Insoluble Pi.  Wild type, circles; ΔphoY, squares; phoY complement strain, triangles. In (A) and (C), empty symbols represent total U or Pi and solid symbols represent soluble U or Pi.  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 the phoY complement strain, but were absent in ∆phoY. wt, Wild type; ∆phoY, phoY deletion mutant; ∆phoY+phoY, phoY complement strain. Scale bars, 500 nm.
Fig 4. Comparison of growth and cell survival among wild type, ΔphoY, and phoY complement strains under U biomineralization conditions.  (A and B) Growth of wild type, ΔphoY, and phoY complement strains in M5G-GP medium in the absence (A) or presence (B) of 50 μM uranyl nitrate. Wild type, circles; ΔphoY, squares; phoY complement strain, triangles. Error bars denote standard deviations from three biological replicates.  (C) Cell spotting for survival with samples collected at different time points (denoted 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; ΔphoY, phoY deletion mutant; ΔphoY+phoY, phoY complement strain.
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.