Title
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Metabolic engineering of microorganisms for actinide and heavy metal precipitation

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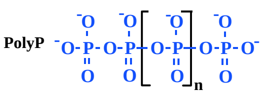
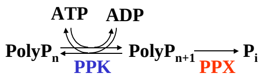
Actinide precipitation by phosphate secretion

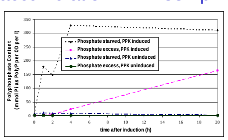
Engineering polyP metabolism



Shown above is the structure of polyphosphate (polyP). It is a molecule of phosphates linked by high energy phosphoanhydride bonds. PolyP is reversibly lengthened by polyphosphate kinase (PPK), using ATP as the phosphate donor. It can be degraded by exopolyphosphatases (PPX), shortening the molecule by one phosphate group, releasing that phosphate as inorganic phosphate. *E. coli* possessing multiple copies of *ppx* and *ppk* gain higher metal tolerance.

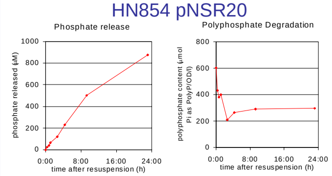


PolyP accumulation in HN854 pNSR20



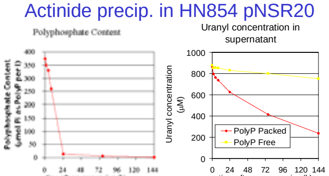
A pMMB206 based plasmid containing the native PPK under control of a $P_{tac/lac}$ promoter (pNSR20) was transformed into *Pseudomonas aeruginosa* HN854. Cells were grown to an OD of approximately 0.2 in MOPS minimal media under varying phosphate conditions. Phosphate starved cells were initially fed 0.132 mM rather than 1.32 mM Pi. 13.2 mM Pi was added to all cells upon induction w/ 1 mM IPTG. The resulting polyP accumulation was 100 fold higher in induced cells, and approximately twofold higher in phosphate starved cells. Replacement of inorganic phosphate with an organic phosphate source, such as glycerol-2-phosphate resulted in a further doubling in polyP levels (data not shown).

Polyphosphate degradation in HN854 pNSR20



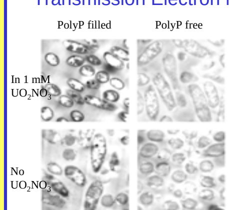
HN854 pNSR20 was filled with polyP using phosphate starvation conditions and glycerol-2-phosphate as a phosphate source. Cells were resuspended in MOPS media lacking phosphate and carbon sources. Phosphate release was unchanged when PPX expression was induced, suggesting the native overexpression of PPX or the presence of an alternate mode of polyP degradation.

Actinide precip. in HN854 pNSR20



Cells were filled with polyP as previously stated, washed, and resuspended in a 1 mM uranyl nitrate solution. Nearly 80% of the uranyl was bound to the cells, resulting in a loading of 40%w/w – favorable to the ion exchange resins currently used.

Transmission Electron Microscopy



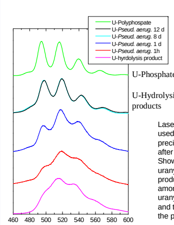
Shown are polyphosphate filled and free cells (in columns) 8 hours after being challenged with a 1 mM uranyl nitrate solution or after being resuspended in sterile distilled water. Small circular dark and white spots (examples of which are marked by blue arrows) are polyphosphate granules that have remained in the section or have fallen out, respectively. Notice the darkness of the membrane in the PolyP filled, metal-challenged cells, suggesting the presence of an electron dense precipitate on the membrane.

Energy-dispersive X-ray spectroscopy

Stoichiometric Composition:
48% P
52% U

Energy-dispersive x-ray spectroscopy was used to identify the elemental composition of the electron dense precipitate on the membrane. Shown is a line scan along the red line highlighted by the green arrow. Shown in the line scan are counts of chlorine (to show the location of the membrane), phosphorous, and uranium. The red dot in the micrograph corresponds to the location of the line in the scan – the cell membrane. The precipitate analyzed contained stoichiometric amounts of uranium and phosphorus, suggesting UO_2HPO_4.

Fluorescence spectroscopy of metal challenged cells

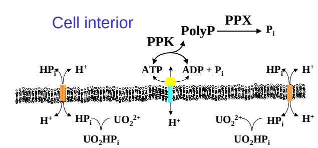


Laser-induced fluorescence spectroscopy was used to further confirm the identity of the precipitate and its genesis. Times given are time after resuspension in 1 mM uranyl nitrate. Shown at the top and bottom are spectra of uranyl phosphate and the uranyl hydrolysis products (a mixture of UO_2OH^+ and $UO_2(OH)_2$, among others) at pH 4.5. It appears that the uranyl binds the cell initially as uranyl hydroxide and then is converted to uranyl phosphate as the phosphate is released by the cell.

Project Goals

- Biologically precipitate aqueous actinides on *Deinococcus radiodurans* as phosphate complexes.
  - Engineer accumulation and degradation of intracellular stores of polyphosphate under highly radioactive and stressful conditions
  - Precipitate uranyl phosphate from uranium waste on the cell surface in a bioreactor
  - Combine bioprecipitation with organic bioremediation functionality

Precipitation of uranyl phosphate on the cell wall



Heavy metals and actinides are significant contaminants at many DOE sites and industrial locations in the U.S. Most of these sites contain metals in combination with organics. Due to the costs associated with excavating, transporting, and remediating contaminated sediments at remote locations, an economically viable alternative is to mineralize the organic contaminants *in situ* and immobilize the metals and actinides to prevent migration. There are few reports in the literature of organisms capable of all of these functions. Besides their potential use *in situ*, these organisms should find use in bioreactors treating wastes tanks at such sites as Hanford that contain mixed organics, metals, and actinides.

The goal of our work is to engineer heavy metal and actinide precipitation in *D. radiodurans* for treatment of DOE sites contaminated with heavy metals, actinides, and/or organics. Specifically, we propose (1) to engineer polyphosphate synthesis and degradation into *Deinococcus radiodurans* for removal of uranium(VI) and plutonium(VI, V); (2) to test removal of actinides; actinides and heavy metals; and actinides, heavy metals, and organics using the engineered organisms; and (3) to confirm the functionality of the system under high radiation fields.

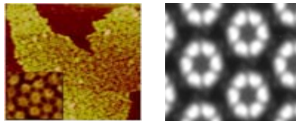
During the previous grant period, we overexpressed polyphosphate kinase in *E. coli, D. radiodurans,* and *P. aeruginosa* to enable these organisms to accumulate high levels of polyphosphate during phosphate excess and exopolyphosphatase for polyphosphate degradation and concomitant secretion of phosphate from the cell. *P. aeruginosa* was shown to be capable of removing relatively high levels of metals from solution; *D. radiodurans* was shown to secrete phosphate under a 40 Gy h^{-1} radiation field and has potential for actinide removal from contaminated waste streams or immobilizing these elements *in situ.*

Since the desirability of *D. radiodurans* as a bioremediation tool depends on its striking robustness to ionizing radiation, we characterized the effects of growth phase, growth conditions, and irradiation conditions on its survivability. We found a large variability in survivability depending on radiation type, rate, and media; in addition, exponentially growing cells were much more sensitive to radiation than fully mature cultures, as may be expected from known radiation effects on living cells. These results are crucial to effectively tailoring the design of a potential bioreactor for maximal results.

The cellular structure of *D. radiodurans* is complicated and fascinating; its surface is covered with a hexagonally packed surface layer (S-layer) of protein. We have shown that the chemistry and stability of uranium interaction with the *D. radiodurans* cell surface is best explained by a carboxylate moiety displayed on the cell surface, consistent with reports that the C-terminus of the protein envelope is oriented outward. New spectroscopic techniques are being explored to confirm this hypothesis.

Actinide chemistry and P secretion of *Deinococcus radiodurans*

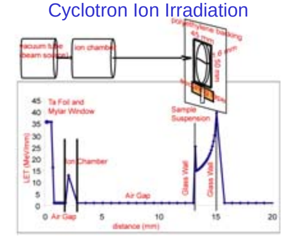
HPI protein layer displays C-term



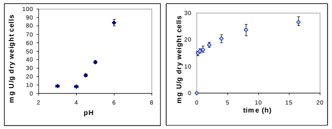
1. Müller et al. **PNAS** vol. 96, no 23, **1999** 2. Moller et al. **Biophys J.**, vol 77, no. 2, **1999**.

The self-assembling, hexagonally packed protein S-layer of *D. radiodurans* is known to display its hydrophilic C-terminus on its outer surface (1). Microbes often have negatively charged surfaces as a general defense against disruptive metal cations, immobilizing them on the surface to minimize damage. The stability constants of uranyl-carboxylate complexes are typically much less than those of uranyl-phosphates, however, making the engineering of a uranyl-phosphate precipitation system, nucleated on the surface uranyl complexes, desirable for bioimmobilization.

Cyclotron Ion Irradiation

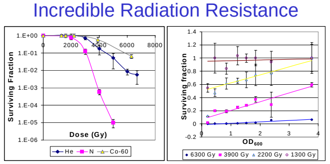


UO_2^{2+}-Cell Chemistry



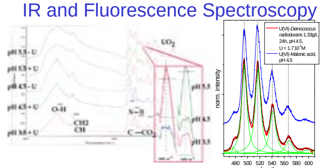
The pH sorption curve of *Deinococcus*-uranyl at 10^{-6} M $U^{233}O_2^{2+}$ is consistent with the pK_a value of a carboxylate, which is typically 4 – 5. Sorption kinetics at pH 4.5 and 10^{-6} M uranyl indicate a quick initial binding, followed by gradually increasing concentration. The pattern and loading capacity are independent of whether the cell is live, heat killed, or formaldehyde fixed, growth phase, and relative biomass concentration, indicating a passive sorption mechanism in equilibrium with the dissolved uranyl. The increasingly complicated chemistry of uranium with increasing pH, due to the formation of hydroxy, oxy-hydroxy, and carbanato species, as well as quick cell death and loss of structural cohesion at pH lower than 4, limits the experimental pH range.

Incredible Radiation Resistance



D. radiodurans has a well-documented ability to survive and even thrive under high radiation fields. In an *in situ* application or waste-treatment bioreactor, however, the cells will have several types of ionizing radiation and multiple stressors to contend with simultaneously. We determined its relative radioresistance to heavy ion radiation (alpha particles and N nuclei) as well as the dependence of its gamma raidation survivability on factors such as growth phase and irradiation rate.

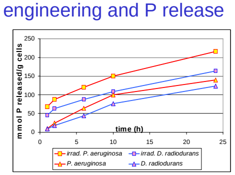
IR and Fluorescence Spectroscopy



Synchrotron IR of bulk cells and cells challenged with uranyl at varying pH values show little difference in the spectra other than pH-dependent changes in a weak peak at 1400 cm^{-1}. This peak has several possible assignments, but since the transition is between pH 3.5 and 4.5 the most fitting is the C-C bond between aliphatic and carboxylate carbons. This peak is also changed by the addition of uranyl at any pH, indicating that this functional group is involved with uranyl binding and is likely on the cell surface. Laser fluorescence analysis of the uranium-cell complexes also indicate similarity to uranyl-carboxylate model compounds.

Metabolic engineering and P release

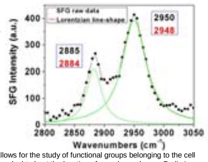
The Cmp^R pRADz1 *D. radiodurans – E. coli* shuttle vector (Meima and Lidstrom, **Appl. Environ. Microbiol.**, vol 66, No. 9, **2000**) was transformed by collaborators with the putative ppk gene under the control of a native constitutive promoter.



A *D. radiodurans* metabolic construct has been shown to secrete phosphate after being grown under selective conditions. The rates of P release for both engineered *D. radiodurans* and engineered *P. aeruginosa* increase upon exposure to a 40 Gy h^{-1} ^{60}Co gamma radiation field, indicating a radiolytic increase the number of available sites for exophosphatases. The *D. radiodurans* survived at rates similar to non-engineered strains under identical conditions; the *P. aeruginosa* demonstrated no colony forming units after 1 hour of irradiation.

Sum Frequency Generation IR

Sum Frequency Generation Infrared (SFG-IR) spectroscopy is an emerging tool for studying IR-active functional groups at areas of broken inversion symmetry, such as at surfaces or phase interfaces.



SFG-IR spectroscopy allows for the study of functional groups belonging to the cell surface alone, eliminating doubt about the location of uranyl complexes. Preliminary studies of *D. radiodurans* indicate that we can obtain usable spectra from hydrated cells; however, the intensity of the SFG-IR signal is proportional to the cube of the bond energy, making study of N-H and C=O bonds more difficult. We are currently investigating methods to increase the range of obtainable signal.