FINAL REPORT

Genes for Uranium Bioremediation in the Anaerobic Sulfate-Reducing Bacteria

Natural and Accelerated Bioremediation Research Program

US Department of Energy

By

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Statement of Problem:
Bioremediation of heavy metal and radionuclide contaminated environmental sites involves the removal of these metals from the biological food chain. Microorganisms play a role in the removal by bioadsorption, bioaccumulation, metabolic alteration of complexing agents, or changing the metal solubility through reduction/oxidation reactions. Among these bacteria are the sulfate-reducing bacteria (SRB), members of the genus *Desulfovibrio*, that can convert the soluble uranyl ion to the insoluble mineral uraninite by reduction with two electrons. Thus the source of electrons, the path of electrons to the radionuclide, the rate of electron flow, and the competing electron acceptors are all important factors in the efficiency of the process. In bacteria, electron flow is coupled to ion gradients that are used to do work, *e.g.* transport of nutrients, flagellar rotation, and ATP synthesis. Thus to decipher the path of electron flow to various terminal electron acceptors, including toxic metals, the broader energy transduction mechanisms must be known.

Results:
In *Dv. desulfuricans* strain G20, we confirmed the importance of the tetraheme cytochrome c_3 by disruption of the gene encoding that cytochrome, *cycA*, and demonstration of a decrease in the ability of the mutant (I2) to reduce U(VI). The lack of cytochrome c_3 almost completely blocked U(VI) reduction with hydrogen but only partially blocked electrons from lactate or pyruvate. Curiously, mutants lacking c_3 were also differentially altered in use of these latter electron donors, lactate versus pyruvate, that are connected by a single redox reaction. Further studies with I2 have shown that all electrons generated from pyruvate oxidation (pyruvate oxidoreductase or pyruvate formate lyase) apparently find their way to the periplasm and need a functional type 1 tetraheme cytochrome c_3 to be returned to the cytoplasm for sulfate or fumarate reduction. I2, lacking this cytochrome, can only ferment. When given pyruvate and sulfate, I2 does not reduce sulfate. It is also not capable of generating the reduced organic products that are made by the wild type strain under fermenting conditions. Succinate is a significant fermentation product of the wild type strain but not of I2.

To obtain spectroscopic information for or against a functional interaction of the type I tetraheme cytochrome c3 and uranium in whole bacterial cells, we attempted to obtain structural information about the protein and its interaction with redox active metals. That is, was this cytochrome c3 the uranium reductase? We purified the protein, obtained crystal structure of the oxidized cytochrome refined to the 1.5 Angstrom level, obtained the structure of a crystal reduced with sodium dithionite, and attempted to determine the site of interaction of U(VI) or Mo(VI) (a proxy for chromate) with the protein in the crystals.

During these efforts a report appeared (Assfalg et al., PNAS USA 99:9750-9754, 2002) that established an apparent biofunctional site of interaction of metal complexes to the triheme cytochrome c7 (cyt c7) from *Desulfuromonas acetoxidans*. Cyt c7 shares a conserved arrangement of hemes with type I tetraheme cytochromes with the exception of a deletion of Heme II. Molybdate, isostructural and isoelectronic to chromate, was redox inert with cyt c7 and, therefore, was used to identify the site of metal binding to cyt

c7. When we added sodium molybdate to reduced cyt c3 and obtained a UV-Vis spectrum, we were surprised to find that cyt c3 was oxidized. Crystals of oxidized cyt c3 soaked with this anion had a unique occupancy site that was not shared between monomers of the crystal. Because molybdate was effective in electron exchange with cyt c3, we suggested that the site of binding might be the site on the protein at which electrons were delivered to substrates, near the exposed edge of Heme IV.

Additional insight into the interaction of uranyl and molybdate ions with reduced cyt c3 was sought through an analysis of the fast kinetics of oxidation of the protein. Stopped flow analysis for oxidation of cyt c3 by uranyl acetate revealed a dissociation constant of 58.8 µM for uranyl ion versus 112.2 µM for sodium molybdate. Thus there was a higher affinity for the uranyl ion and a faster reaction. The molybdate ion is negatively charged while uranyl is positive, even when hydroxylated. Thus both the charge and the relative redox properties of the ions will effect their molecular interactions with cyt c3. Future experiments were to pursue competition experiments, pH profiles, and ionic strength effects to explore facets of the reaction.

Site directed mutations of the cycA gene were constructed. They include F19A, Y27A, C45A, K66A, K72A, and M80K. The Y27A mutation rendered the protein sufficiently unstable such that we were unable to obtain mutant protein from the *Escherichia coli* recombinant strain. All others were purified by immunoaffinity chromatography and the ability of each to reduce U(VI) established. While we did not generate kinetic data for this process, all the mutant proteins were still able to be oxidized by U(VI) and by Mo(VI).

Finally a mutation of one of three lysines present at the exposed edge of Heme IV, K14A, was created. Interestingly this mutant protein was unable to be oxidized by molybdate, confirming the crystallographic location of the molybdate ion as the functionally interacting site. Curiously, the uranyl ion still oxidized the protein. It remains unclear whether the kinetics of oxidation of the mutant protein by uranyl were the same as those of the wild type protein. Analysis of the interaction of carbonate complexes of the uranyl ion with the mutant proteins will be needed to determine whether those potentially more environmentally relevant complexes can oxidize each.

Whole cells of a mutant strain of *Desulfovibrio desulfuricans* G20 that lacks type I cytochrome c3 are still capable of reducing U(VI) to U(IV) with organic acids as electron donors. With hydrogen as electron donor, this cytochrome is the primary donor to this toxic metal in the periplasm. The search for alternative uranium reductases and the pathways of electron flow remains incomplete.

Published papers and a few abstracts derived from this project:

Rapp-Giles, B. J., L. Casalot, R. S. English, J.A. Ringbauer, Jr., A. Dolla, and J.D. Wall. 2000. Cytochrome c_3 Mutants of *Desulfovibrio desulfuricans.* Appl. Environ. Microbiol. 66:671-677.

Payne, R. B., L. Casalot, J. A. Ringbauer, Jr., B. Rapp-Giles, and J.D. Wall. 2002. Uranium reduction by cytochrome mutants of Desulfovibrio. Appl. Environ. Microbiol. 68:3129-3132.

Hemme, C.L., and J.D. Wall. 2004. Genomic insights into the gene regulation of *Desulfovibrio vulgaris* Hildenborough. Omics 8:1-13.

Payne, R.B., L.Casalot, T.Rivere, J.H. Terry, and J.D. Wall. 2004. Interaction between uranium and the cytochrome c3 of Desulfovibrio desulfuricans strain G20. Arch. Microbiol. 181:398-406.

Pattarkine, M.V., J.J. Tanner, C.A. Bottoms, Y.-H. Lee, and J.D. Wall. 2006. *Desulfovibrio desulfuricans* G20 tetraheme cytochrome structure at 1.5 Å and cytochrome interaction with metal complexes. J. Mol. Biol. 358(5):1314-1327 .

Payne, R.B., C.L. Hemme, and J.D. Wall. 2004. A new frontier in genomic research. World Pipelines 4:53-55.

Hemme, C.L., 2004. Examination of metabolic and regulatory networks of *Desulfovibrio* species. PhD Dissertation, University of Missouri-Columbia.

Payne, R. B., 2004. Energy metabolism and uranium (VI) reduction by *Desulfovibrio.* PhD Dissertation, University of Missouri-Columbia.

Miller, S. M., 2005. Examination of specific amino acid residues of *Desulfovibrio desulfuricans* cytochrome c_3 in electron transfer. MS Thesis, University of Missouri-Columbia.

Drury, E.C., R.B. Payne, & J.D. Wall. 2006. Ion transport systems in *Desulfovibrio vulgaris* Hildenborough. Abstr. 106th Gen. Meet. Amer. Soc. Microbiol. Orlando, FL, Q-358.

Ringbauer, Jr., J. A., R. B. Payne, H. Zhili, Q. He, W. Liyou, J. Zhou, M. W. Fields, E. Alm, K. Huang, T. C. Hazen, A. Arkin, & J. D. Wall. 2005. Transposon mutagenesis of *Desulfovibrio vulgaris* yields insight into sodium and pH stresses. Abstr. 105th Gen. Meet. Amer. Soc. Microbiol. Atlanta, GA, K-068

Giles, B. J., K. E. Hart, H. C. Yen, & J. D. Wall. 2005. Electron flow in cytochrome c_3 mutant of *Desulfovibrio desulfuricans* G20. Abstr. 105th Gen. Meet. Amer. Soc. Microbiol. Atlanta, GA, K-049.

Equipment purchases on this grant:

6/29/00 Gas chromatograph: Continues to be used for products of electron flow that may be pertinent to metal reduction. 100% cost covered.

10/08/04 Scintillation counter: 6.65% of cost covered as a multiuse instrument. Still functioning well. Generally used by Wall group for contamination monitoring from DNA hybridizations.

09/17/04 Sorvall RC-5C Plus low speed centrifuge. 22.09% of cost covered as a multiuse instrument as backup for Wall lab centrifuge.

1/27/05 Sorvall RC-5C Plus low speed centrifuge. 100% of cost covered. Laboratory workhorse, still being used for genetic studies of toxic metal stresses in sulfate-reducing bacteria. Fiberlite rotor was found to be less useful and was traded for an alternative. That rotor was replaced last year after a centrifuge accident destroyed it.