Project #1026860

Title: Molecular Mechanisms of Uranium Reduction by Clostridia and its Manipulation

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Results To Date: The objective of this grant is to examine and manipulate the molecular mechanisms in Clostridia to make them better agents for uranyl [U(VI)] bioremediation. We have made progress on two fronts in this project period towards this end.

1. Cloning and expression of an oxidoreductase gene from C. acetobutylicum. Our previous work with other bacteria (1, 2, 3, 4) has established that obligatory two-electron reductases with certain characteristics possess a general capacity to reduce chromate and we had hypothesized that they can also reduce uranyl. As discussed below, this turned out to be the case and we therefore decided to clone a selected NAPDH-quinone oxidoreductase gene from C. acetobutylicum, produce the protein it encodes (protein ID: NP_346665) in pure form and in large quantities to carry out further studies. Genomic DNA was extracted from a 2-ml aliquot of a culture of Clostridium acetobutylicum ATCC824. Cells were precipitated by centrifugation (16,000g) for 10 minutes and genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen). DNA concentration and integrity were checked by electrophoresis. Two primers were designed to amplify the above gene from C. acetobutylicum. The forward primer incorporated a NheI restriction site. The reverse primer contained two stop codons in tandem to ensure the termination of translation; it also contained a restriction site for HindIII. In addition, the reverse primer incorporated a hexahistidine coding sequence (6His-tag) to permit protein purification by affinity chromatography. The resulting protein thus had a C-terminal his tag. The amplified gene of approximately 600 bp was directionally cloned into pET28a(+) between the sites of NheI and HindIII in the multiple cloning site. The recombinant plasmid (pMMB67EH containing the cloned gene) was used to transform E. coli BL21(DE3) competent cells. Clones were selected on LB-agar plates containing ampicillin. The presence of the insert was verified by colony PCR, using the primers employed in cloning the gene, and subsequent excision and sequencing of the gene. A single positive colony was inoculated in a 250-ml culture flask containing 50 ml of LB medium amended with ampicillin. Cells were grown overnight (for approximately 14 hours) under agitation (250 rpm) at 37C until an OD660nm of 10.0 when IPTG was added for induction of protein expression. Cells were allowed to grow for further 3 hours under the same conditions described above. An aliquot (1ml) of this culture was removed for analysis of protein expression. An aliquot of a culture of E. coli transformed with the empty plasmid was also removed for comparison of protein profiles. Following centrifugation, cell pellets were frozen overnight. Cell lysis was performed using the Bugbuster reagent (Novagen). The cell lysate was centrifuged (13,000 rpm) for 20 minutes at 4C and the supernatant (soluble fraction) utilized for protein purification. Affinity chromatography was performed using the His-binding resin (Novagen) for small-scale purification (batch method). SDS page confirmed purification of a protein of 22.2 kDa. Since a hallmark characteristic of enzymes in which we are interested is obligatory two-electron reduction of chromate, we have examined the reduction of

this metal using our standard procedure (1, 2). The specific activity of this enzyme for chromate is 0.742 nmol/min/mg protein (5). Uranyl reduction as well as generation of reactive oxygen species during these reductions is now being studied. This is a diagnostic test of the obligatory two-electron reduction of metals and radionuclides which is the desired manner of reduction to enhance bacterial bioremediation capacity because it eliminates or greatly minimizes oxidative stress during such reductions.

2. Development of capacity for uranyl reduction in a previously purified and studied enzyme. Since our effort includes development of generic capacity for improving metal and radionuclide reduction in bacteria, that is to say, to develop improved enzymes for this purpose that can function in different bacteria, we focused on the enzyme under study in my lab for the past several years. This enzyme (YieF) is versatile. In its wild type state, it has not only active chromate reductase activity but also uranyl reductase activity. We conducted site-directed mutagenesis combined with rational approaches to carry out directed evolution of this gene in order to enhance these activities. These procedures generate libraries of genes with infinite sequence space and we thus devised a colorimetric screening procedure for high throughput screening to find genes with superior capacity to reduce uranyl and chromate. This has led to the isolation of several genes which encode proteins with several-fold higher activity than the wild type enzyme for the reduction of both of the above pollutants. These genes are now being examined in C. acetobutylicum for effective expression and ability to enhance its remediation activity for these metals and radionuclides. A paper based on this work has just been submitted (6).

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