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ENHANCED BIOREMEDIATION OF SUBSURFACE CONTAMINATION: ENZYME RECRUITMENT AND REDESIGN

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Enhanced Bioremediation of Subsurface Contamination: Enzyme Recruitment and Redesign

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INTRODUCTION

Over the last 50 years large quantities of wastes have been disposed at Department of Energy (DOE) sites. Drainage from cribs, trenches, and ponds and direct injection of contaminants to the subsurface or to aquifers has resulted in groundwater contamination and subsurface transport of contaminants. Over 4000 locations representing a broad range of climatic and geohydrologic conditions have been identified for remediation. Both surface and subsurface systems have been contaminated with complex mixtures of radionuclides, metals, nitrates, chlorinated hydrocarbons and other organic solvents with varying properties and solubilities in water, and other miscible and immiscible materials. Many of these contaminants and waste mixtures are unique to DOE sites and are not found in the private sector. Furthermore, contamination in the deep subsurface is commonly dilute, highly dispersed, inaccessible, and exists in large volume. Thus, the nature of deep contamination substantially limits or precludes the application of many remediation strategies that are currently being applied to remediate surface and near-surface contamination. Remediation of deep subsurface environments is of importance not only to DOE, but also to the private sector since deep subsurface, or shallow inaccessible, environments beneath industrial sites in the private sector have probably also been contaminated.

SHORTCOMINGS OF CONVENTIONAL REMEDIATION TECHNOLOGIES FOR THE DEEP SUBSURFACE

Remediation technologies based on abiotic (e.g., soil washing, air or steam stripping,

vacuum extraction, incineration, grouting, vitrification) and biotic processes (e.g., pump and bioreactor treatment at the surface, soil slurry, air venting, and landfarming) have been successfully applied to the remediation of surface and near-surface contamination. However, these technologies were developed for surface or near-surface applications where the areal contamination is relatively small and engineering systems can be relatively easily applied to manipulate the soil environment. Technologies based on biological processes are often more advantageous because they have the potential to destroy organic contaminants (rather than merely transfer the contaminants from one phase or location to another), because they decrease contaminant concentrations to lower levels than can solely abiotic processes, and because they often are more cost-efficient and timely than abiotic methods. Clearly, cost and time considerations also necessitate the use of *in situ* remediation methods for deep subsurface contamination. However, there is a paucity of demonstrated or available methods and tools for remediation of widespread contamination at depth. The stimulation of indigenous subsurface microorganisms and the high-density application of microorganisms possessing useful traits are two potentially useful *in situ* strategies for cost-effective and timely remediation of contaminated deep subsurface environments.

STRATEGIES FOR REMEDIATING DEEP SUBSURFACE CONTAMINATION

Metabolically active and diverse microbial populations have been found within deep (up to 500 m) saturated zones of the southeastern Atlantic coastal plain. (1-5) Microbial density did not decrease with depth, and populations of 10^6-10^7 colony forming units (CFU) g^{-1} sediment were typical in the more conductive zones. Metabolically active microorganisms are also present in deep vadose zones (50 to100 m) of the arid western United States. (6-8) These studies suggest that the indigenous microflora may be exploited to assist in the degradation or immobilization of subsurface contaminants. Because metabolic rates *in situ* are exceedingly

slow with large portions of the population in a dormant state due to nutrient limitation, engineered systems to deliver electron donors, electron acceptors, and other nutrients will be necessary in order to allow high metabolic rates and promote controlled microbial growth. (9)

Engineered systems could also deliver microorganisms possessing useful traits to contaminated subsurface zones after mass-culturing at the surface. Useful traits could include the ability to degrade specific contaminants; ability to bioaccumulate heavy metals and radionuclides (thus attenuating their transport); or resistance to heavy metals, radioactive fields, or pH conditions. Microorganisms introduced in this manner could be initially isolated from subsurface sediments or obtained from other sources such as contaminated surface soils. In addition, indigenous microorganisms could be engineered by introduction of traits that may not have evolved or been selected for in the subsurface because of nutrient limitation and very slow generation times over geological time periods. Introduction of microorganisms with engineered traits may be particularly useful in deep subsurface environments because indigenous microorganisms may require very long periods of time to evolve the necessary trait (or co-evolve multiple necessary traits in the presence of complex mixtures of contaminants) to carry out a particular function or survive. Given the high cost of remediation activities and the likely slow evolution of new traits in deep subsurface environments, a reliance on evolution and/or adaptation of deep subsurface microbial communities may not be a wise approach. An approach which also uses introduced microorganisms known to possess the trait(s) of interest would likely have a greater assurance of success.

The use of engineered microorganisms to assist in the removal of anthropogenic environmental contaminants is becoming a viable alternative for economic, regulatory, and social reasons. The burgeoning costs of remediation technologies and site monitoring and the increasingly stringent regulatory requirements on contaminant concentrations for site closure demand relatively rapid remediation technologies that are able to effectively remove trace

levels of contaminants. With time, in the face of increasing concern over the effects and fate of environmental contaminants, both society as a whole and the Environmental Protection Agency appear to be displaying a more lenient attitude towards the selected release of microorganisms engineered to degrade contaminants. However, a critical question is to what extent engineered microorganisms will be able to maintain their intended activity and survive in the environment.

We are using combined experimental and theoretical approachs to improve the biodegradative potential of subsurface microorganisms. Two general approaches are being utilized. The first involves the introduction (i.e., recruitment) of genes-encoding enzymes with specificity towards DOE priority contaminants into indigenous subsurface microorganisms. The second approach involves the use of molecular modeling and molecular dynamics simulations to design a biodegradative enzyme for specificity against a recalcitrant DOE contaminant that the native enzyme does not act upon.

Enzyme Recruitment

Toluene dioxygenase, which co-metabolically degrades trichloroethylene (TCE), was selected as our initial model system for enzyme recruitment. The *todC1C2AB* genes have been recruited into a variety of indigenous subsurface microorganisms and expressed using broad host-range, regulatable expression vectors. (10) Because expression of TCE degradation by this enzyme requires induction by toluene, the genes were placed under the control of a promoter that can be induced by a compound that can be used in the environment. Thus, genetic engineering allowed the uncoupling of the requirement for a toxic inducer from the expression of the enzyme, enabling the co-metabolic reaction to be actively expressed under a wide variety of conditions. Of principal interest is how well the recruited enzymes will be expressed in microcosms that simulate the subsurface environment. Contaminant degradation will be investigated in flow-through columns and intermediate-scale flow cells inoculated with

engineered subsurface bacteria and containing subsurface materials and the attendant indigenous microflora. Hydrodynamic flow, temperature, and nutrient concentrations will be maintained at values considered to be representative of deep subsurface aquifer systems undergoing remediation. Experiments will also examine the effect of gene copy number by using microorganisms containing the regulatable expression vector and the same microorganisms containing a stable chromosomal insertions of the gene(s).

Enzyme Redesign

Since enzyme "lock-and-key" or "induced-fit" mechanisms have evolved to be very specific for their particular substrate(s) and product(s), microbial degradation of mankind's synthetic substances will probably only occasionally occur at significant levels due to coincidental co-metabolism or fortuitous evolution.

It is generally assumed that the genetic traits necessary for biodegradation of synthetic compounds can be readily selected for under either laboratory or field conditions, in relatively short time intervals. While it is less than straightforward to obtain a quantitative time estimate, genealogical analysis of different evolutionarily related proteins suggests that proteins evolve at more or less characteristic rates. The unit evolutionary period (UEP) is the time in millions of years needed to establish a 1% difference between the amino acid sequence between divergent lines; the UEP range for several enzymes is 2 to 20. (11) Thus, the evolution of an enzyme with enhanced specificity and efficiency for a non-native substrate may proceed very slowly if multiple and specific genetic changes are simultaneously required for adaptation. (12-13) On the one hand, microorganisms with rapid generation times and in the presence of appropriate selection pressure could perhaps evolve new enzyme functions in a much shorter period of time than suggested above. On the other hand, subsurface microorganisms existing at considerable depth and in a nutrient-limited environment may

require long periods of time to similarly evolve significantly different enzyme functionality due to very slow generation times. Therefore, a basic premise for undertaking an enzyme redesign research program is that "designing" multiple changes into an enzyme under laboratory-controlled conditions may be the only systematic and timely alternative to waiting for essentially geological timescales for such rare changes to occur *in vivo* under natural conditions.

We have recently discussed the role of rational protein engineering in relation to developing biodegradative enzymes with enhanced remediation potential. (12-14) In order to have a reasonable chance to successfully carry out rational redesign, to alter a biodegradative enzyme's specificity, and/or enhance its efficiency, it is necessary to have adequate structural data about the native enzyme and native enzyme-substrate interactions, an understanding of the underlying catalytic mechanism(s), a cloned gene for site-directed mutagenesis experiments, and reliable simulation methods for studying protein dynamic fluctuations. Studying the dynamic motions of relevant enzyme-ligand complexes for adequate time periods is necessary for rational redesign since an experimental [x-ray crystal or solution nuclear magnetic resources (NMR)] structure is a static, time-and-space average structure. Considerable progress has been made in the area of validating the effectiveness of computationally efficient protein molecular dynamics simulation methods that permit useful enzyme-ligand simulations. (12,15-16)

The objective of the theoretical work is to rationally redesign an appropriate biodegradative enzyme for optimal activity for degradation of one or more targeted contaminants. For reasons given elsewhere, cytochrome P450cam has been the focus of our initial redesign efforts. (12-14) Initial molecular dynamics simulations have focused on obtaining a fundamental molecular understanding of the complementarity and dynamic motions involved in P450cam interactions with its native substrate camphor (17-18), a substrate

analogue (19), and a substrate analogue with P450cam mutants. (20) In addition, the stereochemistry and efficiency of ethylbenzene hydroxylation, a non-native substrate, was predicted from molecular dynamics simulations and found to be in good agreement with the experimentally observed product distribution. (21) These results confirm and expand the validity of computational methods in characterizing and predicting the metabolism of small substrates by cytochrome P450cam.

This understanding serves as a guide for the rational "fitting" of P450cam to targeted contaminants. Following construction and *in vitro* testing for activity of the redesigned P450 enzymes (in the laboratory of Professor Steven G. Sligar at the University of Illinois at Urbana), experimental activities will be used to plan further iterations of design if necessary. If adequate *in vitro* activity is obtained, the next step will involve sub-cloning and expression of redesigned enzymes in subsurface bacteria. Plasmid vectors containing genes encoding the degradative enzymes will be introduced into a wide variety of subsurface bacteria and the degradation of target contaminants quantified under cultural conditions. The last step will address the ability of subsurface bacteria containing redesigned (or recruited) enzymes to degrade target contaminants under conditions that more closely approximate the subsurface environment.

SUMMARY

Subsurface systems containing radionuclide, heavy metal, and organic wastes must be carefully attended to avoid further impacts to the environment or exposures to human populations. It is appropriate, therefore, to invest in basic research to develop the requisite tools and methods for addressing complex cleanup problems. The rational modification of subsurface microorganisms by enzyme recruitment and enzyme design, in concert with engineered systems for delivery of microorganisms and nutrients to the contaminated zone, are

potentially useful tools in the spectrum of approachs that will be required for successful remediation of deep subsurface contamination.

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