The Development and Application of Engineered Proteins for Bioremediation

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Executive Summary

Jill Trewhella

Clean up of the toxic legacy of the Cold War is projected to be the most expensive domestic project the nation has yet undertaken. Remediation of the Department of Energy and Department of Defense toxic waste sites alone are projected to cost ~$1 trillion over a 20-30 year period. New, cost effective technologies are needed to attack this enormous problem. Los Alamos has put together a cross-divisional team of scientists to develop science based bioremediation technology to work toward this goal. In the team we have expertise in:

- molecular, ecosystem and transport modeling;
- genetic and protein engineering;
- microbiology and microbial ecology;
- structural biology; and
- bioinorganic chemistry.

We have targeted our study on chlorinated organic compounds – the largest class of soil and groundwater chemical pollutants with a total annual release of 200 million pounds per year. One reason for the persistence of chlorinated hydrocarbons, like trichloroethylene (TCE), in the environment is that microorganisms are not naturally adapted to cleave carbon-halogen bonds. There are several genera of bacteria that contain enzymes with limited ability to either cleave such bonds, or to oxidize the carbon atom causing fortuitous release of the halogen substituent. These enzymes are hydrolytic dehalogenases and oxygenases, respectively. The effectiveness of these enzymes in bioremediation schemes is limited by their catalytic and binding properties. For example the affinity of these enzymes for halogenated hydrocarbons is poor and they simply will not bind the target pollutants at concentrations that are orders of magnitude greater than is required for regulatory compliance. Because these enzymes act fortuitously on the man-made (xenobiotic) halocarbons, the catalytic function of interest here has not been optimized via natural selection. Therefore, significant improvements could be anticipated through the application of protein engineering. The Los Alamos approach is to:

- Understand and subsequently engineer enzymes that naturally transform some halogenated hydrocarbons in order to design enhanced enzymes that are more effective at degrading the pollutants (at low pollutant concentrations);
- Understand and engineer the regulatory systems used by native microorganisms for gene expression in order to develop effective means for production of the enhanced enzymes;
- Characterize the microorganisms present at a contaminated site, understand their ecology and gene transfer processes in order to design effective mechanisms to introduce and use the enhanced catalytic efficiency of the engineered enzymes;
• Model the microbial ecology and transport of chemicals and microorganisms at the site in order to implement effective bioremediation technologies that will degrade halogenated hydrocarbons in soil and water to below regulatory limits.

The purpose of this Workshop was to bring together the Los Alamos team with outside experts working in different aspects of bioremediation technology development to learn of the latest advances made in their respective areas, to present the Los Alamos proposed project, and to discuss strengths, weaknesses, and approaches to overcoming problems. This Report presents summaries of the talks given by the invited outside experts. David Goodin and Stephen Sligar both presented their ideas on engineering heme enzymes to modify their functions for applications in bioremediation. David Goodin focused on cytochrome c peroxidase, a heme enzyme that oxidizes another heme protein, cytochrome c, as part of the respiratory pathway in yeast. He and his colleagues have successfully modified the activity of this enzyme by site directed mutagenesis in ways that modify the chemistry and accessibility of the active site, as well as the size of the active site cavity that can be occupied by substrate. Steve Sligar talked about cytochrome P-450, a heme enzyme that catalyzes the hydroxylation of camphor and is a member of a larger superfamily of mono-oxygenases. P-450 also catalyzes the reductive dehalogenation of chloroalkanes, albeit at very low rates of turnover. The conclusions of both Sligar and Goodin’s talks were that heme enzymes are excellent targets for protein engineering for bioremediation because there is a wealth of structural data available, their dynamics and function are well suited for study using spectroscopy, they are demonstrably robust to engineering, and they carry out a wide range of activities including reductive dehalogenation.

Robert Steffan from Envirogen gave an industrial perspective of bioremediation technology development. His company is focusing on hard-to-treat compounds, and he indicated this was a very good area for Los Alamos to focus on in partnership with industry since the national laboratories are able to undertake the long-term, high-risk basic research such as genetic engineering approaches to bioremediation. Envirogen has a big effort aimed at the bio-degradation of TCE in situ in which high performance, “designer” organisms are added to the site. They are focusing on developing bacteria that constitutively express degrading activity, have reduced adhesion properties (hence are readily transported in the aquifer), and carry with them their own “sack lunch,” (i.e., an internal energy source that facilitates survival in the carbon depleted aquifer).

Malcolm Shields discussed the use of a co-metabolic enzyme system to remediate TCE. The target system was toluene ortho-monoxygenase (TOM) which is known to degrade a number of substrates including toluene and TCE. The strategy is to transfer the constitutive TOM producing plasmid to an alternative host that has greater potential for survival in situ. The approach involves selecting hosts from the field that naturally dominate after supplementation with the carbon source required for co-metabolic degradation. While this approach appears to be very promising, the questions that remain are: what should one select for when identifying the “dominant organism,” and under what conditions? Answering these questions will require a huge amount of site characterization in terms of microbial population.
James Tiedje talked about general questions of practical importance in designing bioremediation schemes concerning evidence for the degradability of the target, whether the environment is hospitable to the microbes, and what might be rate limiting parameters. He reviewed technologies that have been successfully implemented at field (and in one case meso-) scale sites to degrade TCE, perchloroethylene, polychloro-biphenyls (PCBs), benzene/toluene/ethyl benzene/xylene (BTEX) and carbon tetrachloride. The practical difficulties of adapting experiences at one site to another were discussed, and the problems of our incomplete understanding of how ecology and microbial diversity at specific sites relates to the success or failure of specific bioremediation strategies. Successful bioremediation in the field requires that the contamination transformations occur in an open, heterogeneous environment populated by multiple organisms. In addition to understanding diversity and ecology, a basic understanding of how microbial communities respond and function under field conditions is rudimentary. The bottom line is that significant basic research into microbial ecology is needed to bring the practice of bioremediation to full fruition.

The Discussion Summaries that follow the Invited Talks in this proceedings briefly describe the subcomponents of the Los Alamos Science Based Bioremediation project, and then summarize the comments and suggestions of the participants. Overall there was strong consensus among the external participants of the Workshop that Los Alamos can play a very important role in the research and development of bioremediation technologies, in partnership with industry and academia. In general the national laboratory environment is well suited to undertake very large, multidisciplinary projects that require long term commitment to a difficult and important problem. In the area of bioremediation, Los Alamos has a broad set of relevant skills and technologies to contribute.

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Los Alamos remains an institution founded on and committed to creating and applying the best possible science and technology to our national mission. That mission today—post-Cold War and post-nuclear testing—is centered on what we have titled Reducing the Nuclear Danger. This means that Los Alamos is not only applying its science to ensuring the nuclear stockpile is reliable, secure and safe, but is also applying it consciously to the broader aspects of the nuclear world—nuclear materials management, non- and counter-proliferation and environmental stewardship.

The environmental programs at Los Alamos constitute a large fraction of our budget. This reflects the national and DOE recognition that there is a real and significant challenge to first restoring, and then maintaining, our environment. Los Alamos has broad expertise to address this environmental challenge—including modeling and simulation, analysis and assessment, nuclear and advanced materials, earth and environmental sciences, chemical science and technology and, of course, bioscience and biotechnology. This year, Los Alamos has deliberately chosen to invest a significant portion of its internal discretionary research and development funds into what we call a scientific thrust in the area of bioremediation. The intent is to explore those aspects of bioremediation that are somewhat higher risk, beyond what we can immediately field, but that are of great potential benefit. This is exactly the type of role that Congress envisaged as appropriate for discretionary Laboratory investment.

To begin that exploration, we have assembled a very strong Laboratory team incorporating diverse strengths from several technical divisions. That team comprises the most capable individuals already active and widely recognized in their fields. At this workshop, we have further gathered national experts from industry and academia to help set the course for the project and to start out with the broadest possible knowledge and support. Continuing external communication and participation in our deliberations will be very important to the success of the project. This workshop is a good beginning.

It, therefore, gives me great pleasure, on behalf of the Laboratory, to voice strong support for this bioremediation project. It exemplifies well, the Los Alamos National Laboratory tradition of commitment to applying our brightest individuals, and our best science and technology to important national problems.
Invited Talks
Introducing Novel Function into a Heme Enzyme: Engineering by Excavation

David B. Goodin

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Talk summary by Tom Terwilliger and Jill Trewhella

One approach to designing proteins for novel function is to begin with a natural enzyme and then use systematic mutagenesis experiments to modify the reactivity of the active site. It is an interesting question to see how far one can push an enzyme to modify specificity and introduce new functions. Heme enzymes are important targets for applications in bioremediation. They carry out a variety of functions in nature, including oxidation, hydroxylation, epoxidation, and electron transfer. In doing so, they bind a variety of substrates; from ions, to amino acids and small organic molecules, to proteins. Heme enzymes are good candidates for protein engineering in part because the stereochemical requirements are more relaxed than for engineering general catalysis. As long as the substrate is in the right neighborhood near the heme, the correct chemistry can occur. Further, the direction in which substrates can approach a heme determines, in large part, the type of chemistry that occurs.

Peroxidases are heme enzymes in which the reactive intermediate is an oxyferryl (Fe$^{4+}$=O) that is produced upon addition of peroxide to the ferric (Fe$^{3+}$) form of the enzyme. Peroxidases oxidize substrate by a mechanism involving transfer of electrons to an aromatic amino acid.

Three distinctive examples of heme enzymes in which the reactive center, the heme, is accessed differently by substrate are:

**Cytochrome c Peroxidase:**
- Substrate is another protein (cytochrome c).
- Electron transfer occurs at the protein/protein interface, from the cytochrome c to a Trp radical near the heme (Trp$^{51}$).

**Horse radish peroxidase:**
- Substrate is peroxide.
- Electron transfer occurs as substrate approaches the heme at its edge to be oxidized.

**Cytochrome P-450:**
- Substrate is a hydrocarbon, which accesses the heme on its distal side in order to accept the radical.
Three distinctive examples of heme enzymes in which the reactive center, the heme, is accessed differently by substrate are:

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Substrate is another protein (cytochrome c). Electron transfer occurs at the protein/protein interface, from the cytochrome c to a Trp radical near the heme (Trp51).

**Cytochrome P-450:**
Substrate is a hydrocarbon, which accesses the heme on its distal side in order to accept the radical.

Dr. Goodin presented the results of his group's research on engineering the heme protein cytochrome c peroxidase (CcP), an enzyme that oxidizes cytochrome c as part of the respiratory electron transfer pathway in yeast. Specifically, he has asked if it is possible to introduce aspects of the functioning of horse radish peroxidase and/or cytochrome P-450 into CcP. The overall objectives are to:

- understand and control the heme redox potential,
- control reactivity with substrates,
- introduce new, artificial substrate binding sites,
- eventually construct chimeric peroxidases, and
- use a protein scaffold to design artificial metal binding sites.

In initial mutagenesis experiments designed to test the feasibility of manipulating CcP activity, Asp235 was changed to Ala. Asp235 normally forms a hydrogen bond to the proximal His. By abolishing this hydrogen bond, the redox potential is shifted by 100 mV, and the pH dependence of the redox properties of the mutant enzyme is shifted two pH units to more alkaline values. The results demonstrate that mutations can be made near the heme moiety without destroying the enzymatic function.

Another set of experiments were aimed at modifying the access of substrates to the heme. CcP will oxidize small molecule substrates (poorly and non-specifically) that can get to the heme via a channel through the protein that is evident in the crystal structure. Mutations at the mouth of the channel would be expected to modify substrate access. To test this, Ala147 at the mouth of the protein channel was mutated to Met and to Tyr. Tyr mutations do, indeed, dramatically reduce both the rate of oxidation of ferrocyanide...
ent molecules occupying the cavity show that they bind in specific orientations in the interior of the protein. Surprisingly, the related compounds imidazole and dimethyl imidazole do not bind with the imidazole ring in exactly the same location. Instead, the methyl groups on dimethyl imidazole are in the position of the ring atoms in imidazole. Interestingly, the reconstituted proteins containing imidazole or dimethyl imidazole are capable of carrying out the function of the natural protein, though more slowly.

Consistent with the proposed role of a positively charged tryptophanyl radical in the mechanism of CcP, the cavity forms when the Trp side chain is removed, binds a positively charged potassium ion and the imidazole molecule binds most tightly at low pH where it is positively charged. The most important properties for binding of a molecule in the cavity are a complementary shape fit and a cation to pair with Asp235.

Another surprising finding is that the imidazole and dimethyl imidazole compounds can be reconstituted into the cavity mutant even when the protein is in a crystal. There is no solvent-accessible channel to the outside of the protein evident. These observations, along with kinetic measurements of the rate of incorporation of these compounds, indicate the protein must be quite dynamic, rearranging on a relatively fast time scale to allow access of the large compounds to the interior of the protein. The crystal structure of the protein with benzimidazole gives a clue as to how this might occur. In this crystal structure, a loop on the surface containing a Pro is "flipped out" via a cis/trans isomerization.


Enzymes can be used to do work either as constituents of intact micro-organisms, or as purified entities, sometimes immobilized on a substrate. Enzymes have already found significant application in both research and industry. About 20% of industrial catalysts are enzyme-based, and we have probably only tapped a small fraction of the potential in this arena. Today there are approximately 3000 enzymes that have been isolated, and 300 to 400 of these are available in mg quantities with 20 to 30 available in large scale. Engineering enzymes for increased stability and modified activities is likely to significantly expand their potential role in many application areas. Dr. Sligar talked about rationally engineering enzymes that catalyze oxidations, specifically cytochrome P-450, for applications in bioremediation.

The cytochrome P-450 superfamily of mono-oxygenases includes a number of heme enzymes, which fall into two broad classes. The first class, found as integral or membrane-associated proteins at the mitochondrial wall, are important for steroid biosynthesis. These enzymes perform hydroxylations with strict regio- and stereo-specificity as part of the pathway that produces appropriate subtly differing male or female hormones. For example, aromatase which is critical in estrogen synthesis, and as such is a target for breast cancer drugs, falls into this class. The second class, found in abundance in liver microsomes, is involved in metabolism of a wide range of xenobiotics (such as drugs or carcinogens). In many cases, these P-450's activate the xenobiotic with the end result being either anaesthesia or cancer.

The second class includes the soluble P-450’s which have much less specificity in their substrate activity. The most studied soluble cytochrome P-450 is P-450cam (Figure X) which catalyzes the hydroxylation of camphor in the first step of a catabolic process in the bacterium Pseudomonas putida, an organism that can live on camphor as its sole carbon source. The soluble cytochrome P-450 enzyme class is a suitable target for engineering of enzymes to both alter substrate binding specificity (K_m) and the overall rate of catalysis (k_cat). Crystal structures for four distinct members of the superfamily are available, and there is considerable depth of knowledge of the system, including a body of mutant and mechanistic studies. With regard to their potential in bioremediation, this class of enzymes carries out a wide range of reactions (including reductive dechlorination of halocarbons under anaerobic conditions). Further, they are good candidates for engineering and introduction into bacterial hosts, as proposed in the bioremediation effort at Los Alamos. The strongest disadvantage of P-450’s for bioremediation applications is they require expensive reductants like NADPH, and hence may not be suitable for use as extracellular degradatory enzymes. However, the advantages
Crystal structure of P-450cam showing the heme, and residues Thr^{251} and Asp^{252} lying above the heme, and the heme coordinating Cys^{357}.

at this point indicate P-450’s are good targets for investigation of the potential of engineered enzymes for bioremediation.

A number of critical issues can be identified that need to be addressed in a rational engineering effort designed to get a P-450 to degrade a novel substrate:

**Fit of the substrate in the active site.** In general, if the fit is too loose the reaction becomes uncoupled, or undesired side reactions occur. If the fit is too tight, formation of reactive oxygen intermediates is hampered. In the reaction of P-450_{cam} with norcamphor (a molecule somewhat smaller than the normal substrate) substrate disorder at the active site leads to many different products. Presumably this might lead, in a bioremediation application, to the formation of unwanted intermediates such as epoxides that would proceed to either poison the P-450 or do damage elsewhere. In other cases, the flexibility of a loose-fitting substrate at the active site can turn P-450_{cam} from a mono-oxygenase into a rather short-lived oxidase. Indeed, lifetime of P-450’s in processing unusual substrates seems to be a significant issue.

**Water accessibility to the heme active site must be taken into account in any modification.** Water bound to the heme iron in the ferric state prevents electron transfer from occurring prematurely. Substrate binding acts as a switch to initiate the catalytic cycle. The substrate displaces the heme-bound water and raises the heme redox potential sufficiently to permit electron transfer steps to occur, at the same time water is excluded from the area immediately around the heme iron in order to avoid destructive oxidase activity. 

**The mechanism of oxygen activation is still poorly understood.** P-450 mechanism is at a much lower level than for other mono- and di-oxygenases, because of the difficulty of obtaining appreciable populations of intermediates, even by fast kinetic techniques. Model systems are probably required for better understanding of the reactive intermediates for rational design of new enzyme functions.

The Development and Application of Engineered Proteins for Bioremediation
Although, a great many studies of P-450 mechanism have been made to date, there are several key intermediates that cannot be observed, because their rates of disappearance are significantly more rapid than their rates of formation. Attempts to block the reaction pathway expose three separate shunt pathways back to the enzyme resting state that further limit our ability to observe the reactive intermediates:

- superoxide formation in low-turnover reactions is analogous to auto-oxidation in hemoglobin;
- the “peroxide shunt” that occurs when water is free to roam around the active site; and
- the “uncoupling” reaction that occurs with loosely-coordinate substrates (i.e., when substrate hydroxylation by the oxyferryl intermediate is blocked).

In the uncoupling reaction the yield of the reaction is identical to the reduction of oxygen to water by oxidases such as cytochrome c oxidase, however, no useful chemistry occurs in P-450 under these conditions. From studies of kinetic solvent isotope effects, it has been established that at least two protons are involved in the mechanism, which has lead the Sligar group to propose specific H-bonding roles for two residues in the binding pocket (Thr$^{252}$ and Asp$^{251}$, in P-450$^{\text{cam}}$).

The mechanistic studies that have elucidated the shunt pathways have been greatly facilitated by site-directed mutants of residues surrounding the camphor binding site in P-450$^{\text{cam}}$. The Sligar group has followed the strategy of modifying the binding site in three tiers.
occur is hindered. Thus, reactive oxygen intermediates are more difficult to form, but their reactivity once formed is high.

Reductive dehalogenation of chloroalkanes is catalyzed by P-450s. For example, 1,1,1,2-tetrachloroethane can be dehalogenated by a P-450, but the turnover rate is quite low. The product of this and several other reductive dehalogenation reactions is trichloroethylene (TCE), which binds with a $K_d$ of 0.7 mM. The reductive degradation pathways of P-450 complement the strategies for bioremediation of TCE discussed elsewhere in this workshop.

Dr. Sligar concluded that despite the difficulty of balancing the requirement for expulsion of water from the active site to initiate the reactive cycle and the need to ensure that some water can access the active site for subsequent protonation steps, cytochrome P-450's are ideal systems to engineer for bioremediation applications. They carry out a wide range of reactions including reductive dechlorination of halocarbons under anaerobic conditions. The availability of crystallographic, and the depth of knowledge of the system provide fertile ground for development of new substrate binding sites with specific reactivity for new and more complex environmental contaminants. He emphasized the need for an understanding of protein dynamics as critical to rational design of P-450's. Several specific types of studies are needed to obtain information pertinent to design of new enzyme function, including spectroscopic studies of intermediates in model systems, structural intermediates by X-ray crystallography, and theoretical studies of reactivity and hydration. These are all areas where Los Alamos has significant, if not unique, capabilities and expertise.

For Further Reading


Gerber, N. C., & Sligar, S. G. (1992) “Catalytic mechanism of cytochrome P-

Selection and Development of Bacterial Strains for in situ Remediation of Chlorinated Solvents

Robert J. Steffan
Bioremediation Technologies Department
Envirogen Inc.
Newtown, PA 18940

Talk summary by Laura Vanderberg-Twary

Envirogen is a hazardous waste treatment company that was started six years ago by an entrepreneur. The company is working toward field applications for toxic waste removal, including bioreactor treatment of waste effluent, and cleanup of contaminated aquifers using bioaugmentation.

Chlorinated hydrocarbons are primary contaminants at all of the “Superfund” sites targeted by the Environmental Protection Agency (EPA) for cleanup. Our ability to clean up these sites using currently available technology ranges from relatively easy for solvents to somewhat more difficult for BTEX (benzene/toluene/ethyl benzene/xylene), to moderately difficult for TCE (trichloroethylene) and the nitroaromatic compounds, to very difficult for the PCBs (polychloro-biphenyls) and dioxin (depicted in chart below).

Envirogen is focusing its bioremediation research on the hard-to-treat compounds. Dr. Steffan suggested this was a good area for the Los Alamos effort to focus on also, in partnership with the private sector. Long term high risk, high pay off basic research on, e.g., genetic engineering approaches to bioremediation is exactly the type of work where Los Alamos contributions could be of great importance. Dr. Steffan also emphasized the industrial viewpoint that in all approaches to the development of bioremediation technologies, it is of paramount importance to consider cost—the lowest priced effective technology will always be the technology of choice.

Current research efforts at Envirogen focus on TCE as a model compound for the bioremediation of chlorinated alkanes. Because of its chemical nature, TCE migrates through the vadose zone (zone of unsaturated soil located between the surface soil and the water table, having decreased levels of oxygen, organic matter and bacteria) into the ground water. TCE also adheres tightly to clays and forms dense non-aqueous phase liquid zones (DNAPLs). TCE also sorbs to aquifer materials and leaches from clays into waters as aqueous levels decline.

Two approaches to in situ bioremediation are biostimulation and bioaugmentation. Biostimulation is the process of adding nutrients to soils to enhance the activity of microbes already present at a given site to degrade contaminants. This approach gene

BIOREMEDIATION SPECTRAS

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<td>Petroleum (BTEX)</td>
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<td>TCE</td>
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<td>Dioxin</td>
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Bioaugmentation involves the addition of high performance organisms to contaminated sites, to treat source contamination in solids (soils). In this treatment method, there is no attempt to establish a new microbial population at the site over a long period, rather the idea is to add high performance organisms, added as “a cheap bag of enzymes” to quickly degrade the contaminant. The bioaugmentation approach has the advantage of allowing for improved process control, more efficient use of additives, and shorter treatment times. However, many factors can negatively affect bioaugmentation schemes:

- introduction of “sticky” bugs leads to inadequate penetration of soils;
- inducing substrates may be required for the desired enzymatic activities;
- DNAPLs are difficult to access; and
- competition and predation, as well as, oxygen depletion can be destructive to microbial activities.

Further, because contaminated sites are heterogeneous, cleanup approaches need to be considered on a site-by-site basis. In order to overcome these difficulties, Envirogen has been developing a designer microbe strategy for cleanup of TCE-contaminated aquifers using bioaugmentation.

Envirogen’s bioaugmentation research program is focusing on three important properties of the candidate bacteria required for success: organisms that constitutively express degrading activity, are adhesion deficient (hence are readily transported in the aquifer), and able to carry an internal source of energy for survival in the carbon depleted aquifer environment. Specifically their strategies are as follows:

**Development of bacteria that constitutively express degrading activity.**

*Pseudomonas mendocina* strain KR-1 utilizes toluene by the *para*-monooxygenase pathway. It has been suggested that part of the toluene molecule and the TCE molecule have similar chemical structures. Whether this is the reason that toluene oxidizing enzymes have the ability to oxidize TCE is not known. Regardless of the reason, the toluene monooxygenases require a significant amount of inducer to be active. Constitutively expressed toluene monooxygenase has been produced by transposon mutagenesis as well as chemical mutagenesis. Many microbes harboring constitutively expressed enzymes have been developed and are ready to go, however, there is some question whether release of these altered microbes will be approved by the EPA.
In an interesting side light, it was found that levels of TCE as low as 0.1 μM induced the genes for toluene monooxygenase in an environmental isolate. Phenol or toluene (normally inducers for this enzyme) were not needed. In spite of this, TCE did not support microbial growth, so a carbon source such as glutamate was needed. Transposons with TCE inducible promoters have been identified, but little is known about the regulatory elements involved. Alkanes also induce TCE degradation in isolates suggesting that hydrophobicity has a role in induction of these promoters. The TCE inducible promoters may also be part of a global stress response.

Reducing Bacterial Adhesion to Aquifer solids to promote transport Burkholderia cepacia strain G4 also degrades TCE and utilizes toluene but, in contrast to Pseudomonas mendocina strain KR-1, by the activity of an ortho-monooxygenase. This microbe adheres to sand in column experiments, so an adhesion deficient strain was developed for better dispersion in bioaugmentation schemes. Adhesion deficient strains were selected for by passing a culture over a sand column several times. While 90% of the B. cepacia G4 cells adhered to the column, an adhesion-deficient strain was identified where only 3% of the population bound to the column. This strain moved through sand columns at the same rate as a chloride tracer and it degraded TCE and toluene as well as the wild type G4. Another approach to overcoming adhesion of microbes is to pretreat clays with pyrophosphate.

Providing introduced bacteria with an energy source for survival in the carbon depleted aquifer environment.

Envirogen's approach to this problem is to load bacteria with polyhydroxybutyrate (PHB) storage granules that can serve as a "sack lunch" for bacteria released into the nutrient-starved environment. These PHBs are stored in the bacteria and allow growth to extremely high densities (on the order of 65 optical density units). Energy enriched bacteria degraded more TCE than energy depleted bacteria. A bacterial strain has been developed by Envirogen that is capable of producing PHBs at 65% of its dry cell weight. This strain maintains its TCE-degrading capacity much longer at this level of PHB production.

In another investigation, soluble methane monoxygenase (sMMO) from Methylosinus trichosporium was compared with the toluene para-monooxygenase at the molecular level. sMMO has a broader substrate range than para-monooxygenase, and so PCR mutation of the para-monooxygenase was undertaken to broaden the substrate specificity of this enzyme. A threonine to serine alteration in the enzyme resulted in 280% rate of toluene oxidation in whole cells when compared with wild type bacterium. An in vitro assay has just been developed to better understand the effect of this alteration.

Dr. Steffan also described a phage chelating system for heavy metals. Envirogen developed an M13 phage library and mutated regions on the surface of the phage particle. Phage mutations were generated by random PCR, transfected into E. coli cells, and tested on metal affinity columns for their ability to bind mercury, chromium, cadmium, and europium. Preliminary screening identified phage that could bind chromium and europi-
um. Phage can be employed in solution or bound on columns to remove metals.

Dr. Steffan’s talk demonstrates an industrial viewpoint of bioremediation technology development. Envirogen’s research is aimed at practical solutions to treat recalcitrant wastes such as TCE. Clearly novel approaches are needed to solve such problems as are indicated in Envirogen’s development of microbes equipped with a “sack lunch,” microbes containing altered or constitutive enzymes and microbes lacking adhesion properties.

For Further Reading


Steffan, R. J. “Methods for decreasing the concentration of toxic materials in biological wastewater treatment.” US Patent Number 5439590 A 950808


Genetic Analysis and Preparation of the Toluene Ortho-Monooxygenase for Field Application in the Remediation of Trichloroethylene

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Talk summary by Paul Jackson and Laura Vanderberg-Twary

The goal of the project described by Dr. Shields was to investigate use of a co-metabolic enzyme system to remediate a site contaminated with trichloroethylene (TCE). The target system is toluene ortho-monooxygenase (TOM) which is known to degrade a number of substrates including toluene and TCE. The specific objectives were to:

- isolate bacteria with a greater potential for environmental survival and selection than the inducible TOM expressing Burkholderia cepacia (strain G4) or its mutant PR131 that constitutively expresses TOM. These organisms are well known for their ability to degrade TCE, but not to the low concentrations required for environmental cleanup;
- transfer the constitutive toluene ortho-monooxygenase (TOM) producing plasmid (TOM31c) to these alternate hosts; and
- screen the TOM31c recipients for phenotypic expression of the TOM gene, and evaluate their ability to co-metabolize, and, hence, degrade TCE.

The implications of field use and release of these alternate hosts were also investigated, and possible strategies outlined for such a release. The table indicates the potential contaminated matrices where this approach might be usefully applied.

<table>
<thead>
<tr>
<th>Field Treatment Methods</th>
<th>Contaminated Matrices</th>
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<tbody>
<tr>
<td></td>
<td>Surface Soils</td>
</tr>
<tr>
<td>Groundwater</td>
<td></td>
</tr>
<tr>
<td>Recycle</td>
<td>+</td>
</tr>
<tr>
<td>Bioventing</td>
<td>+</td>
</tr>
<tr>
<td>Biosparging</td>
<td>+</td>
</tr>
<tr>
<td>Nutrient Perfusion</td>
<td>+</td>
</tr>
<tr>
<td>Bioreactors</td>
<td>+</td>
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<tr>
<td>Land farming</td>
<td>+</td>
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</tbody>
</table>

One critical problem to be addressed in this approach is that co-metabolic degradation of TCE by native TOM containing bacteria requires an additional carbon source for energy (e.g., toluene or phenol). Current TOM constitutive B. cepacia strains that co-metab-
olize TCE have repeatedly demonstrated a reluctance to compete and/or undergo positive selection in native environments. A TCE degrading organism is needed, therefore, that is more competitive. The approach used by the Shields group involved isolation of putative field application vectors (FAV) (environmentally stable hosts) from aquifers, and selection of those that were dominant following supplementation with a carbon source. In this way, it was hoped to isolate strains that would predictably achieve dominance when introduced into a contaminated soil or aquifer environment along with the selective carbon source (e.g., glucose, lactate, Igepal or ethanol). The TOM31c plasmid was transformed into these strains and following chemical or transposon (Tn5) mutagenesis screened for constitutive TOM expression. The effectiveness of strains containing constitutive TOM expression was assessed following release into native soil and groundwater from West Florida.

The genes on the plasmid were mapped using the Tn5 sequences as a probe. The genes encoding for the enzymes required for toluene degradation in the TOM31c plasmid are clustered in one region that is flanked upstream by a short sequence with homology to Tn5, and downstream by a longer sequence with similar homology. The upstream Tn5 sequence appears to contain a weak constitutive promoter that may be responsible for expression of the TOM genes. This suggests that such transposable elements may play a role in constitutive synthesis in other catabolic pathways.

TOM can degrade at least 14 different substrates ranging from toluene to vinyl chloride. The Shields group has cloned the gene encoding TOM, sequenced it, and compared it to other gene sequences for toluene oxidizing enzymes, such as the dmp region of Pseudomonas putida CF600. A comparison of the TOM and dmp gene sequences facilitated identification of the TOM open reading frames and encoded functions. There is 60 to 70% homology (at the amino acid level) between TOM and dmp.

A common first step in toluene degradation pathways involves attachment of a hydroxyl group to the toluene ring by an Fe-S reductase/protein bridge system (see Figure). Oxygen and toluene are used as substrates in this pathway. The oxygen binding affinities of the dmp and TOM encoded enzymes are very different, separating them into two distinct classes. This difference may be important when oxygen is limiting—as it often is in the environment.

To M

\[ \text{NADH}^{2-} \rightarrow \text{NAD}^{+} \]

\[ \text{2Fe - 2S} \rightarrow \text{O}_{2} \rightarrow \text{Fe - O - Fe} \rightarrow \text{H}_{2} \text{O} \]

\[ \text{dmp} \rightarrow \text{K L M N O P} \]
Organisms cannot use oxygen when it drops below a certain concentration in the environment. This severely restricts the use of oxygenase enzymes for bioremediation. To address this problem, the gene for a bacterial hemoglobin found in the microaerophilic bacterium *Vitriocella* was cloned into the *B. cepacia* strain PR131, and expressed. The hope was it would confer an advantage to the organism in low oxygen environments. It was found that the transformed PR131 strain did utilize oxygen and degrade toluene more rapidly. However, the final concentration of TCE remaining after degradation was unchanged. This approach could, therefore, be valuable under conditions for which the rate, rather than the extent, of TCE biodegradation is critical.

**Field testing**

Trial releases of *B. cepacia* (G4) were attempted at eight sites. G4 was added to a column of soil along with toluene as a co-metabolite and TCE was observed to be degraded. Toxic amounts of toluene were added to sterilize the column, after which no toluene degradation occurred, although the TCE continued to be degraded implying the presence of either a toluene resistant organism or residual active enzymes. Attempts to attach G4 to surfaces (in order to localize their activity in a well defined environment) were unsuccessful. Strains were, therefore, selected that would grow as biofilms (that might then be used to coat surfaces) and the selected strains were transformed with the TOM31C plasmid. These transformants did constitutively degrade TCE in bioreactors, but when released in column microcosms containing soil from a test site, the control and experimental columns degraded TCE. These results indicate that there were already naturally occurring TCE degraders at the site.

Many phenol degraders were also found to be naturally occurring at the site. Addition of toluene to the test column was sufficient to “turn on” the naturally occurring TCE degraders. Through a series of experiments a strategy was developed to select for the dominant microbe at a site, transform them with TOM31C, and screen for those that constitutively degrade TCE in order to re-introduce them back into soils. A large number of plasmid recipients were obtained, and 50% of them expressed genes on TOM31C constitutively. Examples included:

- NEG-3 maintained itself in the environment for at least 10 days and represented 0.1 - 1% of the total microbial population, i.e. it was very dominant;
- PR131 (TOM31C) also maintained itself in the environment, but was only marginally better at TCE degradation than G4;
- MFG-2 did not degrade TCE;
- MFI-1 (TOM31C) survived and degraded TCE to non-detectable concentrations. However, it did not degrade TCE that was subsequently added to the site; and
- NFG-2 (TOM31C) survived and degraded the TCE present, and continued to degrade additional TCE as long as glucose was also added.

All the organisms did well at 15°C, and required glucose as a carbon source for energy.

The results presented by Dr. Shields suggest that the alternative host approach described here appears to be more promising than other methods investigated. The problem that remains to be addressed is: what should one select for (when trying to identify a “dominant organism”) and under what conditions?
Answering this question will require a huge amount of site characterization in terms of microbial population, which is an important aspect of the Los Alamos Bioremediation project.

**For Further Reading**


Microbial Ecology and Diversity Important to Bioremediation

James Tiedje

Center for Microbial Ecology
Michigan State University
East Lansing, MI 48970

Talk summary by James R. Brainard

Dr. Tiedje suggested that there are three questions of practical importance in designing and implementing bioremediation schemes.

Is there evidence that the chemical is degradable? Is the chemical a growth substrate, or is it co-metabolized? Are the biodegrading organisms numerous?

Is the environment hospitable? Is there toxicity? Are there sufficient nutrients?

What is the rate-limiting parameter and can it be modified? Are there enough organisms, environmental resources, substrate?

Microbial diversity and ecology are intimately involved in each of these questions; for example, if the chemical is readily biodegradable, the implication is that organisms with biodegradative capability are numerous and widespread in nature, and efforts to enhance effective bioremediation should be focused on other parameters. If the rate limiting parameter is the number of organisms at the site with biodegradative potential, strategies to enhance the numbers of organisms are likely to be beneficial. Another example is the difference between degradation of contaminant that can serve as growth substrates and degradation of contaminants by co-metabolism.

The contrast between biodegradation of a growth substrate and a co-metabolic substrate is profound. When a contaminant is a growth substrate, the catalyst for destruction (the microbe) grows logarithmically on the contaminant, natural selection results in maximum growth in all environmental niches and the growth substrate is almost always completely mineralized. In contrast, co-metabolism requires addition and delivery of a co-metabolic substrate, and the contaminant is almost never completely mineralized. Further, the selection of proper co-metabolizing organisms is frequently challenging as the co-substrate can competitively inhibit degradation of the contaminant.

The relationships between microbial ecology and diversity and the application of bioremediation can be visualized in Figure 1. The time to application and the research focus of bioremediation are strongly correlated to the prevalence of biodegraders in microbial communities. For example, the occurrence of toluene and phenol degraders in microbial communities is fairly widespread, representing generally greater than 1% of the culturable bacteria from most sites. Because toluene biodegradation is not generally limited by the availability or presence of organisms with degrading capacity, the research focus is primarily on the environmental factors that may be rate limiting (i.e., the presence of proper nutrients, electron acceptors, etc.). In contrast, degraders of fluorene and 2,3,6,7-tetrachlorodibenzodioxin (TCDD) are very rare and the research focus for these contaminants
BIOREMEDIATION FROM AN ECOLOGICAL PERSPECTIVE

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Widespread</th>
<th>Common</th>
<th>Rare</th>
<th>Unknown</th>
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<tbody>
<tr>
<td>&gt;1/10²</td>
<td>1/10⁵</td>
<td>1/10⁸</td>
<td>1/10¹¹</td>
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</tbody>
</table>

Example phenol naphthalene TCE naphthalene 2,4-D bibenzofuran fluorene Aroclor 1260 TCDD

Application

<table>
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<th>Time to Application</th>
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<tbody>
<tr>
<td>Short Range</td>
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<td>Long Range</td>
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<tr>
<td>Exploratory</td>
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Research Focus

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<th>Focus of Research or Evaluation Important to Application</th>
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<tr>
<td>Environmental</td>
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<tr>
<td>Organismal</td>
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<tr>
<td>Molecular</td>
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Figure 1. Relationship of frequency of biodegraders in the community to application of bioremediation.

is at the molecular level (i.e. understanding mechanisms for dehalogenation and ring cleavage, engineering enzymes with these activities).

There are a number of very promising technologies for bioremediation that are now being tested. Some of these include:

Co-metabolic removal of trichloroethylene (TCE)- This process involves the fortuitous transformation of TCE under aerobic conditions by broad specificity oxygenases; commonly those that oxidize methane, ammonia, phenol or toluene. At Moffet Field Naval Air Station, investigators demonstrated that phenol was better than toluene which was better than methane in enhancing the degradation of TCE. With phenol injection, initial TCE concentrations in the aquifer were reduced by 65%. However, reactive intermediates generated by the transformation of TCE are toxic, which results in declining remediation activity in the community unless the degrading populations are regrown.

Anaerobic degradation of perchloroethylene (PCE) and other chlorinated solvents
Highly chlorinated hydrocarbons, such as PCE are only degraded under anaerobic conditions. Although anaerobic degradation rates are usually slower than aerobic rates, PCE degradation in which dechlorination is coupled to electron transport and microbial growth (chlororespiration), is faster than aerobic co-metabolism. Growth of active organisms can be stimulated by addition of organic carbon substrates serving as electron donors, but the results are not always predictable in that seemingly similar sites and substrates can give very different rates and extents of
degradation. This technology was successfully demonstrated by Dupont in Victoria, Texas.

**Sequential anaerobic-aerobic degradation of polychloro-biphenyls (PCBs)** Degradation of PCBs has been tested using an anaerobic phase in which the more highly chlorinated PCB conjugers are degraded, followed by an aerobic phase, where the lower chlorinated PCB conjugers and intermediates produced during the anaerobic phases are degraded. Intrinsic bioremediation in the form of reductive dehalogenation may already be underway at many anoxic sites. For example, in the Hudson river, General Electric achieved a 50% reduction of PCBs by the application of oxygen in a single phase aerobic treatment, suggesting that the highly chlorinated conjugers present at the site had already been degraded in an intrinsic anaerobic process.

**Anaerobic degradation of benzene/toluene/ethyl benzene/xylene (BTEX)** This technology takes advantage of the capability of microbial communities to use alternate electron acceptors such as sulfate, nitrate and iron (III) in the degradation of organics. Nitrate amendment, sulfate in sea water or high concentrations of Fe(III) at the site can stimulate toluene, xylene and ethyl benzene removal. This approach using nitrate as the alternate electron acceptor, was successfully demonstrated at the Air National Guard Station in Traverse City, Michigan.

**Niche adjustment for carbon tetrachloride (CCl₄) removal by Pseudomonas KC.** Pseudomonas KC is an organism that degrades CCl₄ without formation of chloroform as a byproduct. CCl₄ degrading activity is stimulated by the presence of acetate, denitrifying conditions and an alkaline pH (8.2). Craig Criddle and co-workers have demonstrated the selection, survival and biodegradative activity of this organism at laboratory mesoscale by introducing it into aquifer material and adjusting the environmental conditions to favor Pseudomonas KC. It is scheduled to be tested at field scale this year (1995).

All of the technologies discussed here have been successfully implemented at field (or in the case of Pseudomonas KC niche adjustment at meso-) scale, but it is difficult to apply the experiences at these selected sites to others. In part this difficulty lies in an incomplete understanding of how ecology and microbial diversity at specific sites relates to the success or failure of specific strategies for implementing bioremediation. For example, we have little knowledge of how microbial communities respond during bioremediation treatments.

In a study of the effects of co-metabolic treatment on the microbial ecology and diversity at a field site Teidje, with Perry McCarty and Malcolm Shields, monitored how the microbial community responded to treatment of TCE at a test site at Moffet Field. The test plot was a shallow sandy gravel aquifer, with injection and extraction wells 7 m apart and 4 monitoring wells in between located at distances from 1-4 m from the injection well. This test site has been described previously (see Hopkins, et al 1993). The investigators placed bags of glass beads at the bottom of the third monitoring well, to which colonies of microorganisms became attached during treatment. Subsequently, the investigators removed the bags to characterize the microbial communities attached to the beads under various treatment regimes.

When relatively high levels (600 ppm) of toluene were injected as the inducer of the degradation, 10 to 50% of the total organisms
isolated from the beads could degrade toluene at low levels, but the majority of these isolates were unable to tolerate very high levels of the inducer; total heterotrophs from the site were cultured at \(10^7\) to \(10^8\) gm, whereas isolates that grew at 50 ppm toluene could be cultured at only \(10^4\) gm.

These investigators also used DNA probes for the enzymes catalyzing the first step in the five known pathways for aerobic toluene degradation to probe the prevalence of different pathways among the organisms selected for by the bioremediation strategy. These probes were based on the DNA sequences for the oxygenases and hydroxylases from the organisms listed in the table below.

Based on southern and SLOt blot analysis of DNA from strains isolated from the Moffet Field test site, ~35 to 40% of the isolates showed the presence of the gene for an ortho-hydroxylase similar to Pseudomonas (Ps.) strain JS-150. Organisms containing the dioxygenase pathway were the next most prevalent at ~10%. In agreement with the finding that most of the organisms used the toluene orthomonooxygenase pathway, o-cresol was detected as an intermediate of toluene degradation at the site.

Notably, the most competitive toluene and phenol degraders at Moffet field were denitrifiers; almost half of the isolates produced nitrogen gas, approximately one quarter produce nitrite and 5% produce NO. Only 20% of the isolates were non-denitrifiers, suggesting that the conditions at this test site (low oxygen and high carbon) select strongly for denitrifying degraders.

The investigators also evaluated the number of bacteria and species richness using REP PCR (Repetitive Extragenic Palindromic sequences and the Polymerase Chain Reaction) after several different substrate additions at the Moffet Field test site. During this test, the site was initially injected with TCE and phenol. When 1,1 dichloroethylene (DCE) was added at toxic levels to the injection mix, the bacterial numbers and species richness decreased dramatically. When the 1,1 DCE was removed the bacterial numbers returned to original levels, but the species richness did not. Only when phenol in the injection mixture was replaced by toluene did the species richness recover to the original mixture of groups. Representatives of six major groups were found on the beads: Camamonas, Burkholderia, Azotarcus, Nocardia, a gram positive unknown and a unspecified group. Notably, this suite of organisms did not contain Psuedomonas, or other g-proteobacteria.

These investigators also compared the restriction fragments from PCR amplification of the 16S rDNA gene from DNA isolated directly from the glass beads with the restriction patterns from the 6 dominant groups of organisms. Only one or two bands of the restriction fragment patterns from the DNA extracted from the beads could not be accounted for from the six groups of dominant organisms

<table>
<thead>
<tr>
<th>Organism</th>
<th>Enzyme</th>
<th>First Intermediate in Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ps. putida PaW1</td>
<td>Toluene methylmonooxygenase</td>
<td>Benzyl Alcohol</td>
</tr>
<tr>
<td>Ps. putida F1</td>
<td>Toluene dioxygenase</td>
<td>cis-Toluene Dihydrodiol</td>
</tr>
<tr>
<td>Ps. mendocina KR</td>
<td>Toluene parahydroxylase</td>
<td>p-cresol</td>
</tr>
<tr>
<td>Burkholderia picketti PKO1</td>
<td>Toluene metahydroxylase</td>
<td>m-cresol</td>
</tr>
<tr>
<td>Ps. strain JS-150</td>
<td>Toluene orthohydroxylase</td>
<td>o-cresol</td>
</tr>
</tbody>
</table>

The Development and Application of Engineered Proteins for Bioremediation
that could be cultured. This observation sug-
gests that the groups of dominant organisms
cultured from the beads represent fairly well
the groups of organisms actually present on
the beads.

A potential tool for predicting the success of
strategies for bioremediation is the use of
specific gene probes for enzymes involved in
degradative pathways to demonstrate poten-
tial for the desired activity. At the Moffet
Field test site, TCE degradation was stimulat-
ed by toluene and the most common pathway
detected for toluene degradation was the
orthomonooxygenase pathway. Consequently,
the investigators wanted to know if the probe
for the orthomonooxygenase could be used to
detect organisms with enhanced TCE degrada-
ing capabilities. Shown in Figure 2 is a plot
of the distribution of TCE degrading activity
(100% of TCE degraded) versus the cumu-
lative frequency (the rank order normalized to
one) for orthomonooxygenase (TOM) posi-
tive and TOM negative isolates from the test
site. Generally, the TOM positive strains
showed more TCE degradation than the TOM
negative strains, although many TOM nega-
tive strains showed equivalent TCE degrada-
tion as the best TOM positive strains under
these culture conditions. In addition, some
TOM positive strains were very poor TCE
degraders and isolates that were equivalent
toluene degraders showed very different TCE
degradation. These findings suggest that the
presence of the TOM gene was loosely corre-
lated with the ability to degrade TCE, but
there are other factors which confound a
direct relationship.

One of the most significant concerns about
bioremediation is whether it can achieve
destruction at the very low concentrations of
contaminants required by the regulatory lim-
its. In order to begin to address this question,
Dr. Tiedje’s lab selected for isolates that
could grow on very low (10^-1 ppm) concen-
trations of benzene in a chemostat by gradu-
ally decreasing the influent concentration.
They were successful in obtaining isolates
from the chemostat that were able to colonize
at these relatively low concentrations of ben-
zene. When they characterized these isolates
with respect to their constants for Michaelis-
Menton kinetics, the majority of isolates fell

![Figure 2. TCE degrading activity (expressed as a percentage of total TCE degraded) vs cumulative frequency for TOM positive and TOM negative isolates from the Moffat test site.](image)
into 2 classes: those with relatively high $V_{\text{max}}$ and $K_m$ (a high rate of degradation and a low affinity for benzene), and those isolates that had relatively low $V_{\text{max}}$ and $K_m$ (a low rate of degradation and a high affinity for benzene). This observation supports the general assumption that isolates that have very low $K_m$'s also have relatively slow rates of degradation and isolates that have high rates of degradation have poor substrate affinities. Consequently, a suite of organisms, some with high capacities (that can degrade the majority of contaminant at relatively high concentrations) and some with high affinities (that can degrade the small fraction of contaminant left at very low concentrations) is necessary to achieve regulatory limits at most sites. Notably, there were a few isolates (1 to 2) that had both a high affinity ($K_m < 1 \text{ mg benzene/L}$) and a high rate of degradation ($high V_{\text{max}}$). A comparison was made between that degradation of 1 ppm benzene achieved by two of the isolates from the chemostat with that achieved by *Pseudomonas putida* F1 (as an “ordinary” benzene degrader). The two isolates from the chemostat were able to degrade benzene in glass culture tubes to less than the detection limit -1 to 2 ppb. In contrast, *Pseudomonas putida* F1 was much slower in degrading benzene, and was capable of reducing concentrations to only 40 ppb.

A very interesting group of toluene degraders is the *Azourcus* group. This group of isolates degraded toluene under anaerobic conditions using a novel pathway. Based on metabolites detected during growth on toluene and putative intermediates and precursors in the pathway, Tjide’s lab was able to suggest that toluene in this organism is degraded through condensation with acetyl CoA to form Hydrocinnamoyl-CoA, and subsequent reduction, acetylation and reduction to E-Phenylitaconyl-CoA. Attack on the benzoyl carbon by CoA-SH gives succinyl CoA and benzoyl CoA which then enter pathways for intermediary metabolism. This pathway is characterized by two acetyl CoA additions and two reductions. All members of this family fix N$_2$. Notably these isolates grow very poorly on rich media such as tryptic soy agar and grow best anaerobically on toluene. This same family was isolated at Moffet Field as aerobic toluene degraders.

To investigate the prevalence of biodegradative ability in pristine ecosystems worldwide, Tjide’s lab has screened for isolates with the ability to degrade metachlorobenzoate from six sites; California, South Africa, Chile, Australia, Russia and Saskatchewan. These sites were selected to have similar ecosystems, but to be geographically distinct. The compound benzoate is not widespread in nature and it is unlikely that any of the pristine sites were exposed to this compound. A continuum of activities in 600 isolates was found from these sites, implying that the capability to degrade metabolized benzene is relatively widespread in nature. However, degradation of metabolized benzene in many of the isolates gave colored products, suggesting that degradation was incomplete. The existence of incomplete pathways in many of the isolates suggests that consortium activity may be important at these sites in order to completely mineralize xenobiotics.

The data reviewed by Dr. Tjeide in his talk underscore the importance of microbial ecology and diversity to bioremediation. Successful bioremediation in the field requires that the contamination transformations occur in an open, heterogeneous environment populated by multiple organisms. This talk also underscores the fact that despite the importance of diversity and ecology, basic understanding of how microbial communities respond and function under...
field conditions is rudimentary, and significant basic research into microbial ecology is needed to bring the practice of bioremediation to full fruition.

For Further Reading


Ka, J.O., Holben, W. E. & Tiedje, J. M. (1994) “Genetic and phenotypic diversity of 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacteria isolated from 2,4-D-
treated field soils,” Appl. Environ. Micorbiol. 60(4), 1106-1115.


The Development and Application of Engineered Proteins for Bioremediation
Discussion Summaries
Engineering Haloalkane Dehalogenase for Bioremediation

Discussion Leader: Clifford J. Unkefer

Chemical Science and Technology Division
Los Alamos National Laboratory

Synopsis of Subproject
Industrial, agricultural, and military use of chlorinated hydrocarbons has led to their wide spread dispersal in soils and ground water. Cleanup of halogenated hydrocarbons is particularly challenging because these compounds constitute a health threat even at concentrations of a few parts per billion. 1,2-dichloroethane, in particular, is produced at a rate of 12 billion pounds per year, and was found in ground water at 14% of 358 sites tested in one EPA study. While bioremediation strategies are potentially the most cost effective, the persistence of chlorinated hydrocarbons in the environment is evidence that microorganisms are not naturally adapted to degradation of these compounds. A lack of appropriate catabolic enzymes rather than a fundamental thermodynamic limitation is responsible for the recalcitrant nature of many halogenated compounds. In systems that have been studied, the rate of dehalogenation limits the rate of microbial growth on halogenated compounds. This component of the Los Alamos Science Based Bioremediation program will use experimental and theoretical tools to design, express, and test enzymes based on the naturally occurring protein haloalkane dehalogenase. These modified proteins will have the enhanced capabilities required for in situ bioremediation strategies including: increased substrate affinity, enhanced catalytic rate, and a broader range of substrate specificity. Specifically, we will engineer novel dehalogenases that have increased catalytic rate at the low concentrations of dichloroethane required for bioremediation. In addition, we will design a series of enzymes that together have broad specificity and will have the capability of degrading a broad range of halogenated alkanes. Haloalkane dehalogenase provides an experimentally accessible system that will be used to develop protein engineering strategies.

The bacterium Xanthobacter autotrophicus is capable of growth using 1,2-dichloroethane as its sole source of carbon and energy. This unique ability derives from the organism’s expression of haloalkane dehalogenase which hydrolyzes one of the carbon-haloge bonds in 1,2-dichloroethane to yield 2-chloroethanol. Although this bacterium is capable of degrading dichloroethane, the rate of catalysis, about 6 turnovers per second, is very slow. Furthermore, the enzyme is ineffective when the dichloroethane concentration drops below millimolar concentrations (100 ppm). The low catalytic rate and requirement for high concentrations of dichloroethane for efficient catalysis limit the usefulness of this enzyme in bioremediation, particularly considering the maximum contaminant level allowed in the U.S. for 1,2-dichloroethane is just 5 ppb.

Haloalkane dehalogenase catalyzes the overall hydrolysis by a water molecule of primary haloalkanes to yield a primary alcohol, a proton, and halide. This enzyme is attractive for bioremediation schemes because no cofactors or co-substrates are required. The enzyme acts on primary chlorinated alkanes...
with chain lengths up to four carbons. The related compounds, dichloromethane, 1,1-dichloroethane, 2-chloropropane, and 1,2-dichloropropane do not serve as substrates.

Our goal is to develop efficient enzyme catalysts that degrade aliphatic halogenated compounds. This goal will be achieved via a two-pronged approach utilizing both combinatorial and rational design approaches. We will produce a combinatorial library of dehalogenase mutants, which we can enrich for mutants enhanced enzymatic capability using a chemostat, and then screen for mutants with tighter substrate affinity, broader substrate specificity and/or faster turnover. In a rational approach we will use theoretical modeling to determine the structures and relative energies of intermediates along the reaction pathway. From the calculated energies for stable complexes and the barriers for transition states along the reaction pathway, one can determine the rate-determining steps. A detailed analysis of various components contributing to the energetics (e.g., solvation, polarization, hydrogen bonding and electrostatics) along the reaction pathway will suggest mutations in the enzyme that should increase the efficiency of the catalytic conversion. Kinetic isotope effects will be used to experimentally confirm which steps in the mechanism are rate determining. We will use this information to predict how changes in residues in the vicinity of the active site can alter the course of the reaction. This information will be used to direct the rational design of improved haloalkane dehalogenases. In addition, we will evaluate rational design approaches will provide the shortest path to our goal.

Specifically our plan is to:

- We will use three methods involving the polymerase chain reaction have been developed for generating large libraries (10^6 to 10^8) of mutations in a single gene. We will introduce random mutations into the haloalkane dehalogenase in Xanthobacter autotrophicus then screen for lower alkylhalide K_M and improved k_cat by enriching for growth on 1,2-dichloroethane. In addition, we will screen the combinatorial library for altered substrate specificity by enriching for growth on 1,2-dichloropropane.

- Dehalogenation by haloalkane dehalogenase involves two distinct steps: The halide is displaced by the carboxylate of an aspartate residue to yield an intermediate ester. The ester is hydrolyzed by the attack of hydroxide on the ester. The hydroxide is generated by abstraction of a proton from water using a charge-relay mechanism similar to that found in serine proteases. We will use solvent and substrate kinetic isotope effects to determine which of these steps is rate limiting.

- We will develop a theoretical model for the haloalkane dehalogenase reaction which will contain a realistic, atomic level, description of its reactivity, dynamics, and thermodynamics. This model will be used
What strategies can be used to both decrease $K_M$ and increase $k_{cat}$?

Dr. Tiedje thought that the Los Alamos effort to engineer better catalysis for bioremediation should focus on degrading compounds at low concentration (low $K_M$). The regulatory limits on particularly halogenated solvent low (< 5 ppb) Using continuous culture techniques, Dr. Tiedje enriched for organisms that can degrade benzene at low concentration. From the natural variation of organisms that grow on benzene a number of strains were isolated that grew well on benzene at low concentration. Dr. Tiedje found that the organisms contained a dioxygenase, responsible for the first step in benzene catabolism, with altered kinetic properties. Some of these isolates contained a dioxygenase that bind benzene at lower concentration (lower $K_M$) and others that had a higher rate (increased $k_{cat}$). A few of the isolates contained a dioxygenase with both decreased $K_M$ and increased $k_{cat}$. These organisms effectively remove benzene from solution to concentrations below 1 ppb.

The Los Alamos group will use in vitro evolution techniques to generate large groups of randomly altered haloalkane dehalogenases. From this group of mutant enzymes, those with greater affinity and/or altered substrate specificity will be identified. What is the best selection/enrichment/screening strategy to identify useful mutants?

Selections for altered substrate specificity can be straight forward. For example, by altering the specificity of haloalkane dehalogenase so that it converts 1,2-dichloropropane to 2-chloro-1-propanol a potential growth substrate, you change a recalcitrant compound (1,2-dichloropropane) into a growth substrate. After mutating the haloalkane dehalogenase, one can screen for the ability to grow on 1,2-dichloropropane. Selections or screens for lower $K_M$ or increased $k_{cat}$ for a normal substrate can be more difficult. Dr. Tiedje concurred that an enrichment in a chemostat, would make this approach successful. As discussed above he used this strategy to enrich for benzene degrading organisms grow well at low concentrations of benzene. These organisms contained a benzene dioxygenase with both decreased $K_M$ and increased $k_{cat}$.

Summary Comments

There was general agreement among the panel members that engineering enzymes and organisms for bioremediation was potentially very important. The application of engineered enzymes requires the use of genetically engineered microorganisms (GEMs) in the field which presents the following problem. At present the federal regulatory agencies forbid the application of GEMs in the field. These regulatory barriers make it impossible for industry to pursue GEMs. On the other hand, this is just the kind of high risk and very high payoff project that is appropriate for the national laboratory. This was viewed as an advantage for the Los Alamos Science Based Bioremediation Program. After significant discussion there was consensus among the workshop participants that given a GEM that had an advantage in the field, regulatory approval could be obtained. It was pointed out that this scenario lead to the regulatory approval of genetically engineered proteins and organisms in agricultural applications.

In addition, the panel felt it was important that efforts to re-engineer an enzyme or organism for bioremediation should involve targets that cannot be remediated by current technologies. In general, it was felt that halocarbons are a good choice. Dr. Sligar pointed out that if the Los Alamos effort to engineer enzymes for bioremediation contributed to our overall understanding enzyme design, it will have
been a success. In addition to bioremediation, rationally designed enzymes will have important application in the synthesis fine chemicals and in the pharmaceutical industry.
Enzymes for Oxidative Biodegradation

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Synopsis of Subproject
The focus of this subproject is to study the catalytic mechanism and the structures of oxygen intermediates in protein models of oxygenase activity. The insights gained will be used to engineer a more flexible oxygenase to carry out oxidative degradation of a difficult halo-hydrocarboin substrate (such as TCE) with high catalytic efficiency.
Specifically we propose to:

- Determine the fundamental mechanism of oxygen activation in model systems with monooxygenase activity and in heme monooxygenases themselves. We wish to understand the nature of the activated oxygen intermediate, which is critical to knowing where to place a substrate with respect to the active sites to obtain maximum reactivity;

- Apply quantum chemistry techniques to study the electronic properties of the reactive oxygen intermediate to provide guidance for interpreting and extending the experimental results, and to determine the critical factors involved in the redox chemistry of the activated species;

- Determine the influence of the proximal ligand and heme environment on the generation of reactive oxygen species in the enzyme active site through studies of model heme and protein systems. Determine the reactivity with model substrates. Determine the changes in protein structure for intermediates along the reaction pathway for cytochrome P-450 as a model oxygenase;

- Engineer a metalloenzyme such as cytochrome P-450 ery-F to tailor reactivity and substrate specificity for a targeted halo-hydrocarbon such as TCE or a PCB, while keeping the enzyme lifetime high.

Cytochrome P-450 has been chosen as our model system for a number of reasons, many of which were delineated by Dr. Sligar in his talk at this workshop. We know from several studies that there are cytochrome P-450 enzymes capable of hydroxylating xenobiotics (man-made halocarbons) such as TCE. Further, the energetics of hydroxylation of a wide range of chlorinated hydrocarbons is favorable so that cytochrome P-450s with modified substrate binding sites can be used to attack a variety of molecules. However, there are several problems that need to be overcome; the specificity of cytochrome P-450s that have been structurally characterized is not high for the xenobiotics we wish to oxidize, the turnover rates are typically slow ($k_{cat} = 1 \text{ s}^{-1}$), and the products of side reactions such as epoxidation of TCE are toxic to the cell and will destroy the enzyme itself. Despite these problems, the fact that cytochrome P-450 is a heme enzyme is a tremendous advantage. The heme prosthetic group acts as a platform aiding in the visualization of strategies for systematic modification of the enzyme. Several cytochrome P-
450s have been extensively characterized including X-ray crystal structures and a wealth of information on substrate binding. This information can be used to study less well understood cytochrome P-450s, model the active site using molecular models, and modify these cytochrome P-450s to obtain the desired enzyme function: hydroxylation of specific xenobiotic. For example, it is known that epoxidation of TCE is not obligatory. By studying TCE binding and enzyme reactivity we hope to understand the factors that determine whether epoxidation or hydroxylation dominates reactivity.

We have already made progress towards a cytochrome P-450 model system using a so-called cavity mutant of myoglobin, in which the ligand to the heme iron has been genetically altered from histidine to glycine allowing exogenous ligands to bind to iron. When ethanethol is used as a model for cysteine in native cytochrome P-450, the iron active site properties have been shown to be essentially identical to those of cytochrome P-450 with bound substrate. We plan to further develop the myoglobin cavity mutant to study reactive intermediates in this model system where the active site is not strongly coupled a substrate and where protonation rates of oxygen intermediates are reduced by the protein structure in the heme environment. Myoglobin is an ideal candidate for these model system studies exactly because it is designed to reversibly bind oxygen without allowing destructive reactions to occur at the binding site. This means that there is a greater chance of protecting reactive intermediates in genetically modified myoglobins than in many other systems. There are several other candidates for model systems, including cytochrome c peroxidase (CcP) and microperoxidase which consists of the functional portion of CcP obtained from a protease digest. The model systems will be studied using several techniques in collaboration with Dr. Harry Gray’s group at California Institute of Technology. The technique of photoinjection of electrons into the heme active site will be exploited to create unstable intermediates, that can then be studied by time-resolved Raman and infra-red spectroscopy. The characterization of the activated oxygen species using these vibrational spectroscopies will test hypotheses of oxygen activation in cytochrome P-450.

Los Alamos capabilities for continuous wave (cw) and time-resolved vibrational spectroscopy are excellent. We have a number of independent laser systems capable of studying reaction dynamics on a time scale from picoseconds to milliseconds using both infrared probe (and excitation) and Raman probes of molecular vibrational states. We, also, have Fourier-transform infrared spectrometers and Raman spectrometers with tunable Raman probe sources from 400 to 1000 nm. We have experience with heme proteins and have extensively characterized cytochrome c oxidase and myoglobin dynamics. More recently a number of studies of the myoglobin cavity mutant have yielded a wealth of information.

**General Discussion**

*How do the studies of myoglobin dynamics relate to the goal of understanding oxygen activation in cytochrome P-450?*

Dr. Goodin noted that the stability of the oxyferryl intermediate in various heme enzymes is not well understood. In order to understand the energetics of oxidation by P-450 it is necessary to create intermediates transiently or in model systems that allows one to study their reactivity towards substrate. Dr. Franzen added that vibrational spectroscopy allows us to probe not only active site structure but also protein backbone structural changes in
response to specific changes of state. In the proposed research we hope to develop a spectroscopic approach to more reactive intermediates using the same techniques that have been shown to work in myoglobin.

Dr. Berendzen stated that there are many modifications of an enzyme that can be imagined. Since we do not have the time to determine the structure of all of them, spectroscopy can be used to tell us what is working and what is not, so that we reduce the search space of the protein design process. Dr. Goodin concurred that there are many factors that one could choose to look at. For example, if one wants to know how a charge is transferred through a protein one could ask, should we remove the oxidizable residues or rather change their environment? Dr. Sligar commented that cytochrome P-450 intermediates are not well understood. It would be exciting to be able to access reactive intermediates using spectroscopy. One might imagine using a spectroscopic screen of a random mutagenesis library. They have created a number of random libraries and usually the problem is finding a creative screen for the function you are interested in. Effective screens for myoglobin mutants are known and hopefully it will be possible to learn from these how to approach P-450 mutant screens.

Oxygen is in short supply in underground aquifers and soil where much of the bioremediation effort will be directed. Is the choice of oxidative enzymes for bioremediation biased by the environment we know with its oxidizing atmosphere?

Dr. Sligar answered that while it may be a concern, P-450 is worth studying anyway because it has excellent potential to degrade substrates anaerobically, as well as aerobically. This is known in therapeutic applications as well as in studies of the degradation of chloroalkanes. The redox potential of the oxidized form is in a good range act as a reductant for many substrates.

What you can do about damage from epoxides or reactive intermediates in P-450s?

Dr. Sligar noted that the heme in P-450 is not oxidized by highly reactive oxygen intermediates even when the substrate is loosely bound and the reaction is uncoupled. This is a concern in engineering a novel enzyme, because we know hemes in other enzymes such as myoglobin are attacked by reactive oxygen intermediates.

Is the use of denitrifying bacteria a good strategy to overcome the problem of low oxygen concentration?

Dr. Steffan responded that the answer is complicated because not enough is known about the enzymes responsible for denitrification.

Would it be possible to send down a gas bottle with enzymes? For example, if the organisms were engineered to express high levels of vitreoscilla hemoglobin they could be sent into the ground with a quantity of stored oxygen analogous to the “sack lunch” idea Dr. Steffan has engineered?

Dr. Tiedje opined that a better strategy would be to lower $K_m$ of P-450 for oxygen so that more of the oxygen goes to the degradation system. Provided that cell is getting energy from the degradative process, this could enhance the ability of an organism to compete and reduce the level of contaminant at the same time. This is one of the goals of the proposed research at Los Alamos.

Summary Comments
The proposed activities involving the use of oxygenase enzymes as candidates for oxida-
tion of halocarbons were outlined. While several oxygenase/hydroxylase enzymes have been observed to degrade halocarbons including TCE, limiting factors for these enzymes include substrate inhibition of alkyl halide degradation, low $K_M$ values and slow $k_{cat}$ rates, and durability limited to few turnovers toxic products. The Los Alamos approach is to employ spectroscopic probes on model systems (myoglobin and cytochrome c mutants) to glean information about several key intermediates that lie in the “terra incognita” region of the standard cytochrome P-450 oxidation cycle. Knowledge about the identity and lifetimes about these species would provide information about how one might alter the process in desired ways through mutagenesis and other approaches.

In the discussion, it was pointed out that the stability and lifetime of the proposed ferriy intermediates appear to vary greatly between the cytochrome P-450 and peroxidase systems. The talk by Dr. Sligar illustrated the possibilities of cytochrome P-450s for carrying out both oxidation and reduction of hydrocarbon species. The spectroscopic probes were viewed as offering useful information to guide mutagenesis experiments, as well as clues about competing pathways that could occur.

As opposed to the “rational” approach of trying to understand the as yet unknown details about the catalytic oxidative cycle, the complementary random mutagenesis approach was also put forth by the discussion participants as an equally viable option. If altered enzyme structures were obtained using random changes that showed greater activity, such structures would provide a starting point for further investigation, even if the reasons for the improved performance would not be forthcoming. Knowledge of the relative performance of a variety of forms could aid modeling activities in terms of which factors are most important.

Other questions arose concerning whether fluoro- as well as chloro-carbons could be candidates, but these species were viewed as even tougher cases for bioremediation. Another major limitation is providing oxygen in sufficient concentrations, and the idea of an equivalent anaerobic enzyme system was proposed. Cytochrome P-450 is known to be an anaerobic reducer. A question about whether denitrifiers would be good candidates for halocarbon remediation was answered negatively based on experiences at Moffett Field.
The overall goal of this section of the Los Alamos Bioremediation project is to develop sufficient understanding of the microbial ecology, genetics, and physiology of a selected 1,2-dichloroethane-contaminated field site to rationally design and test strategies to establish and express engineered genes within the indigenous microbial community. In order to meet this goal, specific project objectives have been identified:

(i) identification of a field site or sites in which to manipulate and model the microbial ecology of haloalkane degradation,
(ii) investigation of genetic and physiological characteristics of specific indigenous bacterial populations in order to identify promising candidates as hosts for engineered proteins,
(iii) development of strategies for the introduction and expression of these proteins,
(iv) development of laboratory microcosms for use in testing host survival, growth, expression, biodegradative activity, etc. in site-derived soils and sediments, and
(v) development of numerical models for the chemical, physical and biological processes occurring in the field.

In order to focus discussion on the above goals, specific questions were presented to the group for consideration. The first broad topic concerned (government) regulatory considerations: what factors must be considered, how important is it that these issues be addressed early in the project, and can the selection of field sites and the initial design of gene constructs address or ameliorate any of these issues? The second broad topic was the use of indigenous bacteria as hosts: what bacterial characteristics are most desirable for this process, what factors should be considered when selecting potential hosts from a site, what are the relative merits of using a mixture or consortium of hosts versus a single species, and what factors must be considered when developing native plasmids as vectors? The last set of questions focused on strategies of gene maintenance, expression, and regulation: which strategies have the highest potential for success in a soil or subsurface environment, predicting the metabolic costs of harboring new constructs, how to ensure that desired traits will be maintained in situ, and miscellaneous issues such as catabolite repression, preference for alternate carbon sources, promising gene promoters, and limiting factors at the genetic level.

Government regulatory issues.

A number of recombinant microorganisms have been licensed, but these have primarily gone through the USDA, which licenses agricultural and pesticidal uses of recombinants. Licensing for field application for non-agricultural uses is less-developed, and is proceeding on a case-by-case basis with initial temporary guidelines still being followed, rather than a set of specific regulations. Little has been done in the area of field release of recombinant microorganisms to enhance bioremediation, primarily because strains...
“worth releasing” have not as yet been developed. The primary disincentive to the commercial development and field testing of recombinants for bioremediation is cost, particularly to a small company. Thus, a national laboratory is a better venue for such development and testing, and a group such as this, working on the current proposal “may be the best group to do this sort of thing.” There was a general consensus among those who have dealt and are dealing with this issue that this is not one which we should be greatly concerned with at this time. It was felt that this group should focus on the science of developing a useful strain or strains for bioaugmentation and allow the successful development of strain(s) be the driving force for overcoming future regulatory hurdles. However, the planned use of “natural” components—species, vectors, etc.—indigenous to the site will help ameliorate regulatory problems later on, as will the use of well-studied bacterial groups and plasmids coupled with a thorough understanding of their genetic behavior in situ.

Selection and use of bacteria indigenous to the field site.

A primary focus of the proposed research will be the use of bacteria native to the contaminated field site under study. This approach is intended to maximize the likelihood of establishment of the engineered genetic construct in the field. It was pointed out that there is often misunderstanding prevalent with regard to the survival of introduced microorganisms. Huge numbers of microorganisms are added, then it is expected that these will stay at these (artificially) high levels. Instead, these nearly always either die back to stable levels, presumably to the normal carrying capacity of the environment, or numbers are reduced below detection levels. Thus, efforts directed first at survival and establishment of the organism(s), then possibly at increasing the carrying capacity for that organism, would be expected to be most successful.

Selection of one or more specific field bacterial populations, perhaps belonging to a known and well-studied group (such as rRNA Group I Pseudomonas) or groups will be necessary. It is important that as much as possible be known about the biology of the microorganisms selected as target hosts for the genetic construct. Also, from a regulatory standpoint, using well-studied species would be far superior to targeting so-called “odd bugs” about which little is known. The approach of Monsanto was discussed, in which much thought was put into the critical criteria in host organisms for delivery. An initial criterion for selecting populations for study would be numerical dominance at the study site. For methodological considerations, the initial focus should be on dominant culturable bacteria. However, non-classical dilute media, such as R2A (Difco), should be used. Media and culturing conditions can also be used which reflects important characteristics of the site (e.g., low oxygen tensions, varied levels of specific nutrients or metals, etc.). Also, the dominant populations already carrying out the biodegradative activity of interest, albeit in less-than-ideal fashion, can be targeted as potential hosts. It was pointed out that there will be a range of pollutant concentrations at the field site and that, as the concentration drops, environmental conditions will shift. Thus, an organism adapted or optimized to survive and degrade the contaminant at one concentration may not be optimal at another. One has to, therefore, settle for optimization at some common concentration at a site or sites.

It may be useful to manipulate the environment to give host organisms a selective advantage. An example is the Criddle group’s work
on “niche adjustment” to facilitate the survival and competitive ability of an introduced Pseudomonas strain (Dybas, J. J., G. M. Tatar, W. H. Knoll, T. J. Mayotte, and C. S. Criddle. 1995. Niche adjustment for bioaugmentation with Pseudomonas sp. Strain KC. In R. E. Hinchee, J. Fredrichson, and B. C. Alleman (Eds.), Bioaugmentation for Site Remediation. Battelle Press, Columbus, OH. pp. 77-84). Malcom Shields pointed out that most of their current research in this area employs some manipulation of the host environment (e.g., addition of surfactants, changes in pH, etc.).

Vectors.

Using known plasmids as vectors will save time, as it can be difficult and time-consuming to characterize native plasmids for this purpose. Also, cloning into a native plasmid may turn out to be impossible. Getting the construct into site populations “quick and dirty” will be important. Thus, the use of a mini-transposons “cassette” system, such as that developed in the Timmis laboratory (e.g., Herrero M., V. De Lorenzo, and K. N. Timmis. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. 172: 6557-6567) to insert the construct into a suite of strains was suggested. These will insert at single copy into many gram-negative bacteria and a number of markers, including non-antibiotic ones, are available. With regard to mobilization of the vector, mating generally is most effective with large DNA elements, while electroporation is useful for smaller elements such as cloning vectors.

Study site selection.

Although groundwater-contaminated sites get the most attention, many sites exist in which soil and vadose zones are contaminated with chlorinated hydrocarbons. However, methods other than bioremediation exist which are probably better for remediating vadose zone contamination (e.g., bio-sparging). Categorizing sites according to important common abiotic and biotic factors may prove useful.

Other issues.

Use of multiple vs. single species - In agricultural bioaugmentation studies, a single species was never used. DuPont and others all used mixed cultures.

Regulation of expression - The main disadvantage of regulated expression is that high levels of inducer are normally needed. For this reason, constitutive expression is generally preferable. Instances of catabolite repression in biodegradative bacteria have been observed, including catabolite repression of toluene catabolism by lactate in B. cepacia G4 (M. Shields). However, catabolite repression is generally not a problem unless it is engendered by a co-substrate.

Excreted enzymes - The question was raised whether an excreted enzyme (either naturally excreted or engineered) might be useful in field situations. Free enzymes are relatively rare in soil. Generally, extracellular enzymes are quickly either consumed or bound up in clays and organic matter.

Endemism - Can one expect to find the same target strain in soil or subsurface samples collected globally or even locally, such as throughout a specific contaminated field site? J. Tiedje’s work on the worldwide biogeography of bacteria indicates that the degree of endemism is dependent on the level of taxonomic differentiation.
examined. For example, when repetitive sequence PCR fingerprinting (REP-PCR, J. Versalovic, T. Koeuth, and J. R. Lupski. 1990. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucl. Acids Res. 19:6823-6831) was used to distinguish isolates at fine (i.e., subspecies) levels. No overlap was seen in bacterial strains cultured from similar, but geographically dispersed sites from around the globe. However, strains similar at the level of species, genus, and higher taxonomic levels could be found. There is little data available, however, on the distribution of specific bacterial populations in localized soil or subsurface habitats.
Performance of Indigenous Bacteria, Hosting Engineered Proteins in Microbial Communities

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Synopsis of Subproject
The Los Alamos Science Based Bioremediation Project is very broad in scope. Two parallel efforts will be initiated to change the kinetic parameters of two potentially useful enzymes, haloalkane dehalogenase and cytochrome P-450 oxidase. The gene sequences encoding altered enzymes will be cloned into plasmid vectors and expressed in E. coli to evaluate the new enzyme’s performance. Promising candidates will also be expressed from a variety of promoters. Plasmid constructs, each with a promoter and altered gene, will be used to confer the new degrading activity to a series of bacterial hosts indigenous to a contaminated area. In this manner, we can compare the effects of altering degrading ability in a single genetic background (a single bacterial host), as well as the effects of different genetic backgrounds (several different bacterial species), on the ability of these bacteria to survive, compete, and degrade target compounds in the environment.

This discussion addressed questions at the population and community level, to identify factors essential for successful bioremediation in the field. Our proposal is unique in two ways that affect our research strategy in bioremediation ecology. The first is that we will be working with genetically altered microorganisms that allow us to examine the effects of subtle, specific changes in genetic makeup on species function at the population level (instead of comparing isolated strains that could vary in many genetic characters). Second, our project is not tied directly to cleaning up a specific contaminated site. This allows us to investigate the consequences of adding genetically altering bacterial species into a complex community, and to critically address questions at the community level that will help us devise successful cleanup strategies. We can explore the basic mechanisms of microbial community processes that affect bacterial survival. This information will allow us to model and make predictions about the success of different strategies.

We have four general objectives:
(1) identify candidate indigenous bacterial species, adapted to contaminated areas, and introduce novel/enhanced metabolic abilities into them;
(2) investigate their ability to survive, function, multiply, and compete in contaminated environments, to determine critical factors affecting their in situ bioremediation use;
(3) determine whether introduced traits can be further spread by in situ plasmid transfer to other indigenous bacterial populations; and
(4) develop models incorporating microbial community parameters and microbial transport processes in the field environment.

An Earlier discussion (p. 40) focused on objective 1. The discussion outlined below...
centered on four topic areas related to achieving goals 2, 3, and 4—to identify key players in the microbial community that would affect bioremediation success, to explore community dynamics for optimized activity of an introduced bacterial species, and to explore possible mechanisms of genetic flow within the bacterial community.

Selecting and characterizing an experimental field site.

To predict the success of introducing a bacterial species for bioremediation, one needs to have some knowledge of the physical and nutritional characteristics of the site. Bioremediation has been considered on a site-by-site basis because the physical, chemical, and microbial components differ tremendously between sites. Characterization of the microorganisms indigenous to contaminated sites has been difficult because we have not had very good methods to enumerate, identify, and map them. Bioremediation success or failure has been difficult to predict, in part, because we have not had a good idea of the microbial community already present at the site.

The discussion focused on the difficulties of characterizing the microbial community in soils and water. The traditional plating methods used only (a sample) a small sub-population of the total community. Only ~1% of the bacterial cells that one can see in a soil sample under a microscope are cultureable on nutrient media. This suggests that either most of the bacteria in the soil are not cultureable or are dead. DNA yields from soils are often at least twice what would be expected from microbial culture assays. Several investigators have identified new groups of bacteria by isolating and sequencing the 16S ribosomal RNA genes directly from soil DNA, and most of the genetically identified species do not match the cultured species from a given site.

Why do we need to characterize the rest of the microbial community? There was some disagreement on whether it was necessary to characterize microbial community processes to achieve successful bioremediation schemes. Those in the discussion group closer to the actual application of bioremediation technology suggested that a “shot-gun” approach of simply adding large amounts of contaminant-degrading bacteria worked well in certain situations. Others felt that if we could understand the microbial community dynamics in general (through our microcosm studies), we might be able to more quickly characterize a site, and better predict the outcome of bioaugmentation in different environments.

Grazing by protozoa has been a major problem when high populations of bacteria have been introduced into field sites. Bacteria evade protozoan predators by attachment to soil colloids and other substrates, and we may be able to avoid this problem by the use of surfactants, known to inhibit protozoa, or by attaching the introduced bacteria to a solid carrier such as vermiculite or alginate beads. It was pointed out, however, that a balance needs to be achieved between protection from predation and the negative effect on transport of microorganisms.

Factors important in the design of, and assessment using soil/water microcosms.

We need to establish small-scale soil and aquifer environments to examine bioremediation processes. Microcosms reported in the literature range from paper cups of soil to complex bioreactors. The general consensus was that it is very difficult to predict...
soil/aquifer processes in the laboratory, and that the most efficient microcosms are simple batch designs that are small, repeatable and easy to use. When studying matrices with structure, such as soils, a preferred approach is the use of intact cores. Sandy aquifers, on the other hand, generally have little structure and, therefore, are not as affected by physical disturbance.

We also discussed ways to measure performance, survival, competition, and fitness. Again the conversation was directed toward a comparison of traditional plating techniques and gene probe techniques. Both have drawbacks; plating assays can bias for specific bacterial groups that may not be predominant or functionally important. Gene probes can identify the presence of a specific microbe or group of microbes, but these assays are more difficult to quantify. A combination of both approaches seems warranted to follow microbial populations in microcosm experiments. These should be coupled with biochemical analysis to measure biodegradation.

*Horizontal plasmid transfer from an introduced host into the indigenous bacterial community.*

Genetic transfer between different bacterial species has been demonstrated in the laboratory, and has been suggested by field isolation of a single gene in different bacterial species. Conjugal plasmid transfer requires cell-to-cell contact, and occurs at very low frequency, because of natural barriers to transfer between organisms of different species. Tiedje's group has done some work in this area (unsuccessful graduate student thesis projects). His appraisal was that it did not occur at significant rates in nature, and would be difficult to successfully employ for bioremediation. Shields suggested that in a biofilm situation, such as a stream where cells are in direct contact, the approach might be more feasible. Use of bacterial phage as a genetic delivery system was also discussed. The primary impediments to this approach were discussed. Most phage are lytic (destroy the cell) and are extremely host specific. If we were able to find and engineer a broad host range phage, the regulatory problems of releasing such a phage would be a problem.

*Modeling bacterial performance in microcosms and in the field.*

It was agreed that there is a real need to incorporate microbial processes into the current models of contaminant transport and soil nutritional status. We should be able to generate some of the needed data through our microcosm studies.
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