# 2004 Project Report

## Directed Evolution of Hydrocarbon Utilizing Bacteria for High Molecular Weight Substrates

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The purpose of this project is to improve the activity of the enzyme Alkane Monooxygenase from *Pseudomonas aeruginosa* towards large alkanes by directed evolution. The recipient strain is Pseudomonas putida, which has a highly homologous AlkB gene and high alkane degrading activity. Two oligonucleotide primers were designed by using NCBI nucleotide data for Pseudomonas aeruginosa PAO1 and BLAST, for PCR amplification of the Alkane Monooxygenase gene. This enzyme was selected because it is the slowest as well as the most specific enzyme in the alkane-degrading pathway. The upstream primer used was 5'ggcacccg**aagctt**ccgtttcc3' and the downstream primer used was 5'gtctga**gaattc**tcctccc3' With HindIII and EcoRI restriction sites, respectively. The PCR products were checked by gel electrophoresis and a second ~300 nucleotide band was discovered, a BLAST search did not find any other regions on the PAO1 genome with the selected primers. A plate based hydrocarbon degradation assay was developed. A thin layer of hydrocarbon substrate was deposited onto M9 media by dissolved in ether and spraying onto the plate. Within 48 hours, zones of clearing form. This assay only works with M9 media as on LB the bacteria will preferentially utilize other carbon sources. The optimal concentration was found to be 0.05g substrate/ml ether and a total of 0.25g/plate. The substrates screened were C10-15, Hexane, Pyrocatechol, Eicosane and Napthalene. 28 known hydrocarbon utilizers were screened by stamping onto pre-prepared M9+hydrocarbon plates. 12 strains were found to be capable of degrading the largest tested alkane, eicosane ($C_{20}H_{42}$). The activity, or rate of clearing was lowest on eicosane with over 4 days necessary to see >1mm ZoC's. The mutant library was created by a combination of PCR and gene shuffling. A range of PCR products was produced by varying the Mg concentration. The PCR products were digested with DNAaseI and then recombined by thermal cycling, a second PCR reaction produced the final point and shuffled mutants. The resulting DNA was then ligated into pUC 28T pseudomonas vector. Pseudomonas putida was then transformed by electroporation. After 24 incubation the mutants were stamped onto assay plates.

### 2 PURPOSE

The ubiquitous use of plastics such as polyethylene and other hydrocarbon like compounds has lead to a growing problem due to their persistence in the environment. Microbial degradation of most polyaromatic and other high molecular weight compounds is slow due to limitations such as toxicity and bioavailability **[8]**, and others are extremely resistant to degradation do to their novelty in the biosphere and are xenobiotic. Although microbial bioremediation methods have over two decades of research they are not widely applied. The direct application of enzymes capable of oxidizing and cracking alkanes (straight saturated chains) and aromatics (benzene like rings) might have many advantages over the microbial methods. Bacteria are limited by the toxicity of some hydrocarbons and by the fact that in general, most bacteria which attack hydrocarbons, must first absorb them through their cell membrane, thus large hydrocarbons like many synthetic polymers are off limits to bacteria.

One of the primary reasons for the resistance of plastics to degradation is the scarcity of available chain ends. All of the known enzymes isolated from hydrocarbon utilizing bacteria attack the hydrocarbons terminally or just sub-terminally (at the ends of the hydrocarbon chains). If a set of enzymes was engineered to attack in the middle of alkanes it could have enormous potential in the bioremediation of biodegradation resistant pollutants. Multiple enzymes are necessary to degrade hydrocarbons, from initial oxygen insertion by oxygenases to dehydrogenation with dehydrogenases and cleavage by an enzyme such as thiolase (from beta-oxidation). If all of the enzymes required for hydrocarbon pollutant degradation could be engineered into a single multi-enzyme complex that would make direct enzyme application a far more attractive alternative. The multi-enzyme complex would be simpler to purify (purifying a single protein is preferable to purifying several separate ones). It is very interesting to see if there is any increase in

stability between free enzymes and enzyme bonded to one another. At this point there is insufficient literature to know how the activity of enzymes bound together with flexible poly-glycine or poly-proline linkers would be affected. Ideally, most of the activity would be retained and the overall rate would be increased by substrate channeling due to favored diffusion of substrate into the next enzyme in the pathway in the multi enzyme complex instead of into the bulk phase. Often, multiple strains termed a 'consortia' are used in concert to degrade pollution with a wide

range of structures, the large set of enzymes are together capable of degrading many compounds. The obvious limitation of a small set of enzymes is their substrate specificity. Most enzymes are specific to a single substrate, a very important feature in cells which cannot afford to have random reactions occurring, however for bioremediation, specificity is a limiting factor. Most enzymes are size and structure specific, reducing this specificity is necessary to make the enzymes more practical. Directed evolution have been successfully used to reduce the specificities of numerous enzymes **(H.Yang et all)**. The goal of directed evolution is to modify any binding pockets or residues, which recognize the molecule, while maintaining the enzymes catalytic properties. Other methods of reducing enzyme specificity include substituting in less bulky residues, this allows for larger substrates, also, the active site can be made shallower. The engineering of a multi-enzyme complex with wide substrate specificity could have enormous applications in bioremediation and commercial applications such as upgrading.

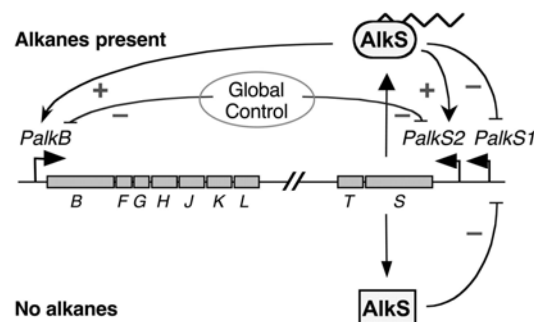
## 3 OBJECTIVES

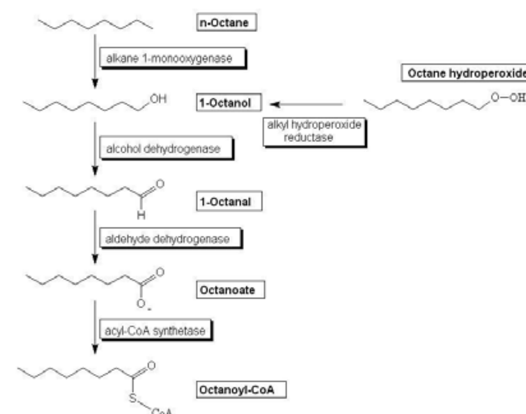
To reduce the substrate specificity and terminality of enzymes involved in degradation of hydrocarbons by directed evolution. Enzymes with preferential activity towards larger substrates will se selected in an attempt to push the pathways towards degradation of high molecular weight substrates

To attempt to engineer a multienzyme complex. This is a large modular protein with several catalytic domains (the individual enzymes) with polyglycine or polyproline linkers, the optimal length for these linkers would be determined.

The limitation of currently available enzymes is that they add onto the terminal or second carbons of alkanes. If these terminal ends are unavailable, bioremediation is limited, thus enzymes capable of breaking carbon-carbon bonds in the middle of a alkane would make a huge impact on bioremediation of polymers and compounds such as asphaltenes.

## 4 BACKGROUND




Directed Evolution of Hydrocarbon Utilizing Bacteria for Degradation of High Molecular Weight Substrates. The purpose of this project is two fold, to improve activity of Alkane-1-monooxygenase towards large n-alkanes by random mutagenesis and screening and to attempt to engineer a multienzyme complex capable of catalyzing all steps in the degradation of n-alkanes independently of bacteria other then for cofactor regeneration. The known hydrocarbon utilizers will be screened for sub-terminal activity by assaying their activity of octane with blocked chain ends. The majority of alkane degradation pathways are terminal, the use of a sub-terminal pathway greatly improves the chances of the pathway being active on extremely large hydrocarbons because often the number of chain ends is limited thus limiting the rate of bioremediation.

Many of the most recalcitrant and thus environmentally harmful and persistent compounds are of high molecular weight, polymers such as PVC's and hydrocarbon fractions such as asphaltenes **[4]**. Bacteria are severely limited in their degradation because of the low rate of diffusion across the cell wall. Fungi overcome this by excreting their enzymes extracellularly. There are many bacteria, which can degrade hydrocarbons with chain length less than 20 but above that the list becomes much shorter. Bacteria are limited to medium chain length compounds because light fractions are toxic and heavy fractions have very low water solubility.

Directed evolution involves introducing mutations into a gene or genes and then looking for improvements over the original or wild type enzyme. Methods of introducing and screening vary widely but some of the most common include mutator strains, radiation and error prone PCR, screening methods are usually colorimetric or spectrophotometric. Because of the complexity of enzyme structure and function, most mutations, even single conservative mutations distant from the active site can have very significant effects of all parts of catalysis, from substrate recognition to catalysis itself. Thus it is necessary to screen very large numbers of mutants. A typical enzyme of 300 amino acids has a total of 20^300 possible combinations, a truly staggering number which is far beyond any screening method. In order to succeed the screening method must be simple and fast so that large numbers of mutants can be screened, in excess of 10 000 at minimum.

The plate-based assay developed for this project involves spraying hydrocarbon substrates dissolved in ether onto M9 media plates. A great number of methods where unsuccessful, many different concentrations, conditions and agar mixtures where attempted before a successful system was discovered. Even on 1/8 LB agar plates, no hydrocarbon degrading activity was visible, the idea was to limit the food of the bacteria, encouraging them to express alternative pathways. M9 media was successful because it limits the carbon source to only the hydrocarbon substrate supplied. Of the 28 known hydrocarbon utilizers and two negative controls E. Coli and Salmonela Typherium 12 showed significant degradation of naphthalene, C10-C15 and Eicosane C20. No degradation or growth was detected with procatechol when sprayed, possibly due to toxicity at high concentrations. The Eicosane assay was selected for further screening due to good visibility of zones of clearing over the other substrates. Both naphthalene and the C10-C15 fractions were unreliable in the assay.

Two primers were used to amplify the AlkB or Alkane Monooxygenase gene, they were designed by using the BLAST program with NCBI sequence data. A Taq Polymerase was used to introduce mutations. The PCR program included 36 cycles. All PCR product analyses was conducted on 1% agarose gels at 70 V. A second band was discovered on the gel, the source of which is unknown. Again using BLAST the genome of Pseudomonas aeruginosa PAO 1 was searched for the primers used, no matches were found other then Alkane-1-monooxygenase and the band is not the chromosomal DNA so the source of the second band is currently unknown.

For further experimentation the most active mutants were pooled and then selected by screening using the M9 plate assay except instead of using the 96 well plate replicator a pin was used. Thus the rate of degradation was more visible in the rate of expansion of the zone of clearing. By comparison of the diameter of the zones of clearing after 24h at 37 degrees Celsius the three most active strains are Pseudomonas Putida LQ16, an unknown strain called Environmental Isolate 46 and Bacillus subtilis. The Pseudomonas putida and Bacillus subtilis strains were selected because their genomes are completely sequenced, allowing great flexibility in manipulation of their genes.

Pseudomonas putida and Bacillus subtilis Alkane Monooxygenase homologs were found in their genomes and the adjacent proteins were observed. Their seems to be operon organization on both. An operon is a set of genes which is controlled by single promoter, by looking between the genes for proter sequences it is possible to determine if the genes are operon organized. This allows for two things, first of all it allows for ease of cloning, instead of cloning the mutated genes into the source bacteria the operon can be cloned into competent E. Coli with the TOPO Cloning Kit. Secondly, and more importantly it allows creation of the multi-enzyme complex, because there is certainty that all the necessary enzymes are present and the whole thing can be cloned with just one set of primers.

The results so far are encouraging, while the primers tested so far did not work for most of the Alkane Monooxygenase homologs the ones that did show differences in activity. Because the homologs are not identical the primers designed for PAO1 did not anneal to most of the strains

and the PCR reaction did not work. From one PCR reaction approximately one hundred active clones resulted of which two showed improvements over the wild type PAO strain. This strain is from one of the university of Calgary's strain banks, Ruth Biofilm Box 7 C4. To confirm the improvement the wild type and pcr mutant were grown on M9 media with Eicosane for 24 hours at 37 degrees celcius. The mutant zone of clearing rate was 0.9 cm/day and the wild type was 0.6cm/day.

The project is currently in its most rapidly progressing phase, once primers are designed and received for the Pseudomonas putida and Bacillus subtilis strain alknae-1-monooxygenase homologs believed to be crucial for their degrading activity then directed evolution can be applied once the gene is confirmed as necessary by looking for inactive clones, inactive clones mean that an inhibitory mutation is present in the homolog enzyme. Also because the gene's are operon organized engineering of a multi enzyme complex will be attempted in the near future. The C and N terminal regions of all the enzymes have been confirmed as being on the surface, this allows connection of the enzymes without loss of activity. The hypothesis is that the multienzyme complex will have higher activity then the individual enzymes in solution due to substrate channeling.
The primary obstacle is that many enzymes involved in the initial oxidation are membrane-bound such as the P450 enzymes. One thing which can be done with a multienzyme complex gene is introducing mutations into all of the genes with a single PCR reaction. While this would drastically increase the sequence space that needs to be searched, it could result in very interesting enzymes.

Directed evolution of a single enzyme has been demonstrated in this project however, directed evolution of an entire pathway is different. In terms of improving the activity of an enzyme it is not difficult, however if one enzyme gains the ability to react with a new substrate then all the other enzymes in the pathway must also gain this ability in order for the pathway to be functional. As you can imagine the sequence space or probability of this happening are truly fantastically small. However since evolution of pathways occurs in nature and that 10^11 mutants can be made in just a few hours by PCR this should be possible to do.

The applications of this project are many, degradation of high molecular weight compounds and polymers can be very useful in many industrial applications, environmental applications are equally numerous, for degradation of toxic or recalcitrant to bacteria compounds **[12]**. The advantages of this method over the application of bacteria are numerous. There are no transport limitations, much fewer limits on application to mixed and or toxic environment, most compounds inhibitory to microbial growth would not affect an enzyme, unless it was an enzyme inhibitor. Also it is better suited for industrial applications such as continuous flow reactors etc. The limiting factor is the need for cofactors are proton donors/acceptors reducing and or oxidizing agents.

For the future, hybrid enzymes and reverse micelles should be considered for modifying the solubility of the multienzyme complex. If the enzyme was soluble in oil, it would probably have a much higher activity then enzymes which can only operate at the oil/water interface. Also there is currently ongoing research into degradation pathways which require few or even no cofactors, if this could be achieved then the direct application of enzymes to both industrial and environmental applications would become a reality very rapidly as cofactor expense is the truly the limiting factor.

# 6 MATERIALS AND METHODS

**Error Prone PCR**
PCR buffer (5µL), MgCl2 (2µL), dNTP's (1µL 2.5mM), alkB2-up (0.5µL), alkB2-down (0.5µL), Taq Polymerase (0.5µL), H2O (40µL), Template (0.5 µL). A thermal cycler program called KP was used, 95C for 10 minutes, 55C for 10 minutes, 60C for 10 minutes, 36 cycles, total run time = 3hours.

**M9 Media**
[Na2HPO4 1.2g, KH2PO4 0.6g, NaCl 0.1g, NH4Cl 0.2g, H2O 100ml ]+[Agar 3g, H2O 100ml]+ MgSO4 (1M) 0.4ml, CaCl (1M) 0.02ml. Per 10 plates.

**Surface Deposition of Hydrocarbon Substrates**
Done in fume hood. 0.5g C10-C15, Napthalene, Eicosane (C20) / 10ml ether. 500µL x 2 spray at a height of 30cm above the plate from a narrow bore syringe, rapidly expel to aerosolize. Move syringe in a circular motion to ensure even layer or use plate spinner.

This project has been exciting and successful thanks to the participation of many people. I especially want to thank Dr. Surrette and Dr. Kanti for their patience and the huge amount of time they spent teaching me the basics which allowed me to pursue this project. I want to thank my good friend Andrew Stagg and in fact his entire family for their support in the creation of my application video, without Andrews experience and hardware, the video would not have been very interesting! I would also like all of the graduate students, professors and other random acquaintances who contributed to my understanding of the project.

**APPLICATIONS**

- In-situ and surface remediation of persistent hydrocarbon compounds from plastics to pesticides.
- Upgrading of high molecular weight hydrocarbons.
- Conversion of biological matter into useful short chain hydrocarbons
- Industrial polymer processing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