1. COVER SHEET:

FINAL REPORT
U.S. Department of Energy

Genetic Analysis of Stress Responses in Soil Bacteria for Enhanced Bioremediation of Mixed Contaminants

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3. EXECUTIVE SUMMARY:

Bioremediation is the technological process whereby biological systems are harnessed to effect the clean-up of environmental pollutants. Bioremediation has been demonstrated to be an appropriate alternative to conventional clean-up strategies in many cases. For example, aerobic bioremediation of trichloroethylene (TCE) at the Savannah River Integrated Demonstration site (Hazen, 1992) was shown to save time and be cost-effective when compared to baseline technologies, such as pump and treat (Saaty et al., 1995). The use of indigenous and/or genetically engineered bacteria to accelerate bioremediation provides two very attractive approaches. The wide metabolic and physiological versatility of microorganisms can be used to degrade many pollutants such as TCE and carbon tetrachloride. However, in a natural environment or contaminated sites, the number of such bacteria is usually too scanty to have an impact on the bioremediation process. When indigenous bacteria that are capable of degrading the pollutant exist, nutrient or electron acceptor can be delivered *in situ* to stimulate the growth of this bacterium to enhance bioremediation. However, the knowledge on the genetic responses of starved indigenous bacteria to nutrient or electron acceptor stimulation is still poorly understood. Thus, whether supplement will enhance the bioremediation activities still needed to be empirically determined. Moreover, in the case of using engineered microbes, special gene expression system needs to be developed.

In our study, we have chosen two soil bacteria, *Deinococcus radiodurans* and *Sphingomonas aromaticivorans*, as models for experimentation. These organisms represent two distinct groups of soil bacteria, each of which has specific features of interest for bioremediation. *D. radiodurans* exhibits high resistance to external stress including radiation and solvent; *S. aromaticivorans* is a deep subsurface (Savannah River Site) organism with unique degradative capabilities.

To understand the growth response of starved *S. aromaticivorans* to nutrient supply, competitive growth experiments were performed. We have observed that different starved subsurface bacteria (both pollutant degraders and non-degraders) respond differently to the dose of nutrient provided. As a result, biostimulation may not necessarily stimulate the preferential growth of those indigenous bacteria that are capable of cleaning up the pollutants. Our result indicates the importance of understanding the physiology of the microbial consortium before biostimulation is deployed. Thus, more effective strategies can be devised for enhanced bioremediation by biostimulation.

Using a molecular biology approach, we have identified several starvation-inducible genes and solvent-inducible genes. We have also determined the complete DNA sequence of a 180 kb conjugative plasmid pNL1, from which we have identified clusters of genes responsible for the degradation of a variety of pollutants. The starvation and solvent-inducible genes will be useful for expressing bioremediating genes into these organisms, and can be further developed as molecular probes for monitoring bioremediation strategies. The bioremediating genes on the plasmid pNL1 are also potentially useful for engineering *D. radiodurans*.

We have established a gene transfer system in *D. radiodurans*, and attempted to express a toluene monooxygenase gene complex into *D. radiodurans*. Although the gene complex was introduced into *D. radiodurans*, we were not able to detect enzyme activity or TCE degradation activity in the engineered bacteria. On the other hand, the study of promoter structure in *D. radiodurans* may help to develop a highly effective gene expression system. We have also developed a gene transfer system in *S. aromaticivorans*, which will be useful for engineering *Sphingomonas* strains to be more resistant to higher level of solvent so that it will be more effective in degrading aromatic compounds in the subsurface environment.

In summary, our studies have provided relevant scientific knowledge and tools for addressing

OEM's applications. Knowledge derived from the studying of the genetic responses of starved subsurface bacteria to biostimulation will guide us to devise better strategies, specifically for promoting the growth of indigenous bacteria that are capable of clean-up at the contaminated subsurface environment. Genetic tools for developing GEMS will be applicable to bioremediation of DOE's mixed waste sites where bacteria with enhanced capabilities are needed.

4. RESEARCH OBJECTIVES:

In order to realize the full potential of bioremediation, an understanding of microbial community and individual bacterial responses to the stresses encountered at contaminated sites is needed. Knowledge about genetic responses of soil and subsurface bacteria to environmental stresses, which include low nutrients, low oxygen, and mixed pollutants, will allow extrapolation of basic principles to field applications, either using indigenous bacteria or genetically-engineered microorganisms. Defining bacterial responses to those stresses presents an opportunity for improving bioremediation strategies, both with indigenous populations and genetically-engineered microbes, and should contribute to environmental management and restoration actions that would reduce the cost and time required to achieve OEM's clean up goals.

Stress-inducible genes identified in this project can be used as molecular probes for monitoring performance of indigenous bacteria as well as the effectiveness of bioremediation strategies being employed. Knowledge of survival and catabolic plasmid stability of indigenous bacteria will be needed for devising the most effective bioremediation strategy. In addition, stress-inducible regulatory elements identified in this project will be useful for creating genetically-engineered microorganisms which are able to degrade hazardous wastes under stress conditions at contaminated sites.

One of the model organisms, *Deinococcus radiodurans*, is a stress-resistant bacterium. Thus, in addition to serving as a model for gene regulation in Gram-positive organisms, it may have specific application at aerobic DOE sites where combinations of contaminants produce a particularly stressful environment. Similarly, the use of *Sphingomonas* F199, isolated from a depth of 407 m at the Savannah River site (Fredrickson et al., 1991), may have relevance to deep subsurface bioremediation applications, where indigenous or engineered microorganisms adapted to the that environment are needed. In addition, F199 contains aromatic oxygenases that are relevant to degradation of contaminants at that site and is representative of a large class of similar organisms from Savannah River

Identification of the genes responsive to different stresses encountered at contaminated sites will provide a basic understanding of stress responses in soil bacteria and can lead to improved strategies for bioremediation. Enhanced *in situ* removal of hazardous wastes by stimulating growth of indigenous bacteria with nutrients or electron acceptors such as oxygen has been demonstrated. However, how much and how often to apply these supplements has largely been determined empirically. As a result, a controlled, reproducible, and properly managed degradation of pollutants in the environment is difficult to achieve. Genes inducible by

low nutrient and low oxygen conditions can serve as markers for determining the minimal amount of supplements needed. The disappearance and reappearance of such stress responses will determine how much and when nutrients and oxygen are needed to be applied or reapplied. Similar applications of stress-inducible markers are already being applied in bacterial cultures in solution (Selifonova and Eaton, 1996). Stress responses induced by pollutants also have potential use as a biological index for the performance of indigenous bacteria during bioremediation as well as a microbiological risk assessment index for environmental pollutants. For instance, measurement of the stress responses of contaminant-degrading microorganisms would provide information complementary to measurement of enzymatic activity. This more complete picture of the physiological state of the desired organisms can be used to predict their performance. Finally, prior knowledge of the stress responses of competing bacteria could be used to predict their environmental competitiveness.

Promoters from stress inducible genes will facilitate the construction of genetically-engineered microorganisms in which the expression of the catabolic genes is uncoupled from both microbial growth and the utilization of the pollutant as the carbon source. The application of genetically engineered organisms in bioremediation requires the design of gene expression systems that function under environmental conditions and are cost effective. The promoter, the genetic regulatory element that directs the use of the gene, plays the central role in gene expression systems. The ideal promoter for environmental applications should possess two qualities: (1) it does not require the addition of exogenous compounds for activation, and (2) it is active under nutrient-limited conditions and not dependent on cell growth for activity.

Promoters that are expressed constitutively meet the first quality. However, such promoters usually require active cell growth for expression and thus incur the increased cost of constant nutrient addition. Furthermore, constitutive expression of some enzymes at the high desired levels is itself inhibitory to cell growth.

To overcome these limitations, inducible promoters can be used. Inducible promoters widely used in the laboratory are activated by the addition of isopropyl-ß-D-thiogalactoside (IPTG). However, the prohibitive cost of such inducers limits the use of these promoters for environmental remediation. Promoters that use low cost inducers have been identified and inserted into expression vectors. These include promoters such as the P_m promoter of the meta operon pathway from the TOL plasmid which is activated by the addition of the low cost inducer benzoate (Mermod et al. 1986; Ramos et al, 1988). Promoters which are induced by the pollutant itself have also been used, including the XylR/P_u promoter, which is induced by toluene and xylene, components commonly found in fuel contaminants (DeLorenzo et al., 1993a). However, these inducers can be competitive substrates for the induced enzyme. Another limitation with these promoters, however, resides in the fact that they couple the induction of the promoter to cell growth. This coupling can create problems for *in situ* remediation. For example, it necessitates the addition of large amounts of nutrients to contaminated environments, potentially resulting in over-expansion of the biomass and consequent biofouling of the matrix.

Promoters which are responsive to specific environmental signals have been pursued by many investigators for various bacteria (DeLorenzo et al., 1993b; Little et al., 1991; Matin, 1992). Starvation-inducible promoters from *E. coli* have been used to express toluene 4-monooxygenase to effect TCE and phenol degradation under limited growth conditions (Matin et al., 1995); however, *E. coli* is not appropriate for *in situ* remediation and its promoters will not necessarily function in soil bacteria. Starvation-induced promoters have been cloned from the soil bacterium *Pseudomonas* (Kim et al., 1995), but these promoters have not yet been shown to regulate other genes. Furthermore, as for *E. coli*, these promoters will not necessarily function in other bacteria that are relevant to bioremediation. Thus, it is important to obtain a broader view

of stress responses of soil bacteria including deep subsurface microbes.

Knowledge of stress responses can be applied to innovative design of gene expression systems for bioremediation. Where GEMs are an appropriate approach for bioremediation, it is necessary to regulate gene expression in a selective manner. The proposed work will identify genetic regulatory elements that can be used to express desired degradative enzymes under nutrient-limited or other environmental conditions. The utilization of stress-induced promoters to control expression of degradative genes allows induction under the environmental conditions encountered and effectively decouples the activity of the promoter from the requirement for cell growth, thus eliminating the problems with biofouling and creating a more cost-effective remediation strategy.

Our study has provided initial relevant scientific knowledge and tools for addressing OEM's applications. Knowledge derived from studying the genetic response of starved subsurface bacteria to biostimulation will help us devise better strategies for specifically promoting the growth of indigenous bacteria that are capable of clean-up at the contaminated subsurface environment. Genetic tools resulted from this proposed works will be applicable to develop GEMS for bioremediation of DOE's mixed waste sites where bacteria with enhanced capabilities is needed. Promoters from starvation inducible or solvent inducible genes may be useful for further development of engineered *D. radiodurans* currently constructed by other institutes (Lange et al., 1998; Brim et al., 2000).

5. METHODS AND RESULTS:

In our ESMP studies, we have chosen two different bacteria as models for the understanding of their genetic responses to starvation and solvent. One of the model organisms, *D. radiodurans*, is a stress-resistant bacterium. We have identified several genes that respond to organic solvent and others that are required for oxidative or radiation resistance (Markillie et al., 1999). The other model organism is *S. aromaticivorans*, which is a subsurface bacterium with a 184 kb catabolic plasmid, is able to degrade many organopollutants. We have completely sequenced the 184 kb catabolic plasmid (Wong et al., 1996; Romine et al., 1999), and were able to identify many genes involved in the degradation of organopollutants as well as genes related to the conjugative transfer of this plasmid to other bacteria.

1. Identification of solvent inducible genes in *D. radiodurans*.

Soil bacteria with enhanced metal or solvent resistance will be desirable at mixed-contaminant sites with higher contaminant levels. *D. radiodurans* can tolerate a relatively high level of organic solvent; however, the molecular mechanism of its tolerance is still unknown. By studying the solvent tolerance of *D. radiodurans*, we might be able to use those identified genes to enhance the solvent tolerance of other bacteria useful for bioremediation. Moreover, the promoters of such genes will be useful for expressing biodegradative genes from other bacteria into *D. radiodurans*.

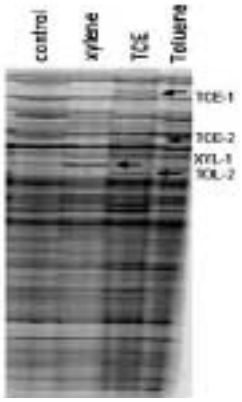

Figure 1. Identification of solvent inducible genes by RNA fingerprinting.

To identify solvent-inducible genes, total RNA was extracted from *D. radiodurans* that have been treated with xylene, trichloroethylene, or toluene. An RNA fingerprinting technique (Wong and McClelland, 1994) (Figure 1) was used to identify the RNA transcripts that were induced by solvent. Four putative solvent-inducible genes were identified and were cloned. Two of these genes were found to be the rhamnosyl transferase and methyl transferase by sequencing analysis; the other two genes were unknown. Since the sensitivity of bacteria to solvent is related to their cell surface hydrophobicities, it is possible that these induced genes may be involved in changing the cell surface hydrophobicities. However, further studies are needed to prove that hypothesis.

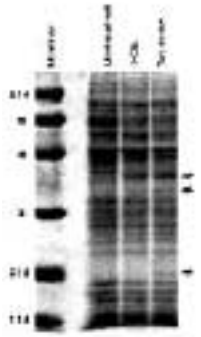

Figure 2. SDS-PAGE analysis of total proteins from solvent treated cells. Arrows indicates solvent inducible proteins.

By studying the protein expression of TCE and toluene treated *D. radiodurans*, we were able to identify proteins that are induced by solvent stress (Figure 2.) One of the proteins was identified to be homologous to phage stress protein (PspA) *in E. coli*, but two other proteins were unknown. Using targeted mutagenesis (discussed below), we further confirmed the role of *pspA* in solvent resistance.

By overexpressing the *pspA* gene in other bacteria, such as solvent degrader *S.*

aromaticivorans, one may enhance their solvent resistance, and thus their degradation capability.

2. Identification of starvation inducible gene in *D.* radiodurans.

To identify starvation inducible genes from *D. radiodurans*, bacterial cultures of *D. radiodurans* were grown to saturation in a minimal media (Raj, et al. 1960), harvested, washed, and suspended to a density of 1 X 10^{10}cells per liter in the minimal media minus glucose. The starvation cultures were incubated at room temperature without shaking for 0,4, 14, or 21 days. Direct cell viability counts showed that *D. radiodurans* was maintained in this media without growth for at least 21 days. The RNA was isolated from cells at each time point and RAP-PCR performed. Five different arbitrary primers were screened and six tentative differentially expressed genes identified. An example of the RNA fingerprint is shown in Figure 3. Putative differentially identified bands were cut out of the gels, cloned into the pCR-Blunt vector (InVitrogen), and sequenced using an Applied Biosystems 377 automated sequencer. Three of the putative clones were identified as either 16S or 23S rRNA. Two clones share sequence homology with *glgX*, glycogen debranching enzyme and *hbd*, 3-hydroxybutyryl coenzyme A dehydrogenase. The enzymes are involved in the utilization of glycogen and fatty acid stored within the cell. The results indicated that *D. radiodurans* may utilize reserved glycogen and fatty acid during starvation condition. This may also explain the difference in fatty acid profile between starved bacteria and normal bacteria.

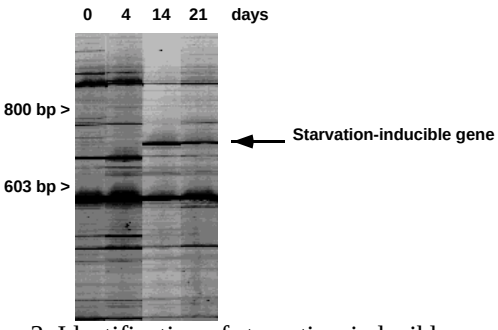

Figure 3. Identification of starvation-inducible genes by RNA fingerprinting analysis.

3. Functional analysis of stress-inducible genes in *D. radiodurans* by targeted mutagenesis.

We have developed a targeted mutagenesis method in *D. radiodurans* (Markillie et al., 1999) to define the function of identified genes. Mutants with mutations in *pspA* (phage stress protein), *katA* (catalase) or *sodA* (superoxide dismutase) were generated. The result indicated that the wild-type R1 strain is very resistant to organic solvent but the *pspA* mutant is very sensitive (Figure 4.). We speculate that by over-expressing *pspA* in other bacteria may enhance their solvent resistance.

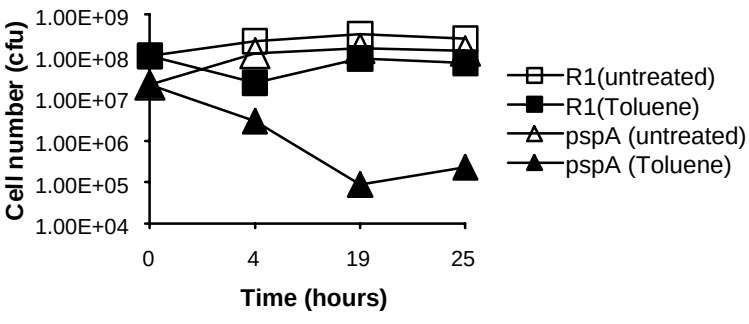

Figure 4. Solvent Sensitivities of *pspA* mutant and the wild-type strain R1

As shown in Figure 5 and 6, wild-type R1 strain is very resistant to hydrogen peroxide and ionizing radiation. However, the *katA* mutant becomes more sensitive to hydrogen peroxide. Since hydrogen peroxide has been used to stimulate growth of indigenous bacteria during bioremediation, it is an advantage to deliver engineered *D. radiodurans* when it is ready to be

deployed for bioremediation in the field.

Beside reduced hydrogen peroxide resistance, *katA* mutant is also less radioresistant to ionizing radiation (Figure 6). *SodA* mutant is even more sensitive to ionizing radiation (Figure 6.) due to the lack of superoxide dismutase. The results indicated that catalase and superoxide dismutase are required for *D. radiodurans'* extreme hydrogen peroxide and radiation resistance.

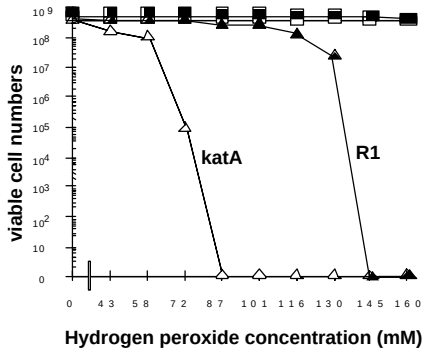

Figure 5. Hydrogen peroxide sensitivity of wildtype R1 and katA strains. Δ, treated cell; □, untreated control.

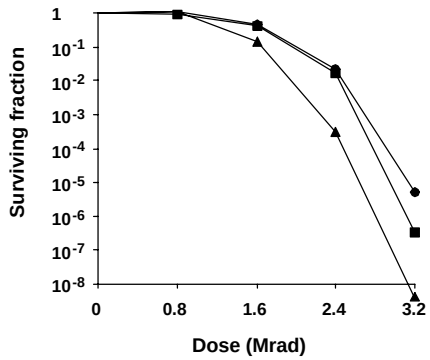

Figure 6. Radiation sensitivities of *katA* and *sodA* mutants. □, wild-type; ■, katA; ▲, sodA. (Markillie *et al*., 1999)

4. Analysis of biochemical changes during biostimulation of *S. aromaticivorans F199*.

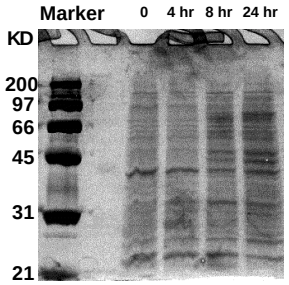

Figure 7. Analysis of total proteins of resuscitated starved *Sphingomonas* cultures.

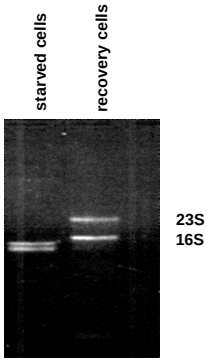

Figure 8. Analysis of rRNA from starved culture.

The starvation responses of *S. aromaticivorans* F199 were also analyzed. Using *S. aromaticivorans* as a model, the effects of adding nutrient to starved cells were investigated in terms of protein and gene expression. Cells were starved in reconstituted well water without carbon and nitrogen sources for nine months. Nutrient (TGY medium) was added to the starved cell, and proteins were extracted from the cell culture after 4, 8, and 24 hours (Figure 7). Only a few major proteins were present and few changes were detectable four hours after the nutrient was added, but several prominent proteins appeared after eight hours, suggesting that there is a lag time before the starved cells respond to the nutrient. These identified proteins may be useful for monitoring the bioremediation process during biostimulation with nutrient supplements.

Furthermore, we also investigated the ribosomal RNA. Twenty-four hours after nutrient was added, the size of rRNA molecules of recovery cells had increased (Figure 8). The rRNA may have undergone fragmentation under starvation conditions. Future efforts will focus on determining the sequences of the rRNA from starved and recovery cells. Comparing these sequences may enable scientists to develop

oligonucleotide probes to monitor the number of recovery cells that are metabolically active during bioremediation.

5. Sequencing analysis of catabolic plasmid pNL1 from *S. aromaticivorans* F199.

The complete 184,457-bp sequence of the aromatic catabolic plasmid, pNL1, from *S. aromaticivorans* F199 has been determined (Romine et al., 1999). A total of 186 open reading frames (ORFs) are predicted to encode proteins, of which 79 are likely to be directly associated with catabolism or transport of aromatic compounds. Genes that encode enzymes associated with the degradation of biphenyl, naphthalene, m-xylene, and p-cresol are predicted to be distributed among 15 gene clusters. The unusual coclustering of genes associated with different pathways appears to have evolved in response to similarities in biochemical mechanisms required for the degradation of intermediates in different pathways. A putative efflux pump and several hypothetical membrane-associated proteins were identified and predicted to be involved in the transport of aromatic compounds and/or intermediates in catabolism across the cell wall. Several genes associated with integration and recombination, including two group II intron-associated maturases, were identified in the replication region, suggesting that pNL1 is able to undergo integration and excision events with the chromosome and/or other portions of the plasmid. Conjugative transfer of pNL1 to another *Sphingomonas* sp. was demonstrated, and genes associated with this function were found in two large clusters. Approximately one-third of the ORFs (59 of them) has no obvious homology to known genes (Romine et al., 1999).

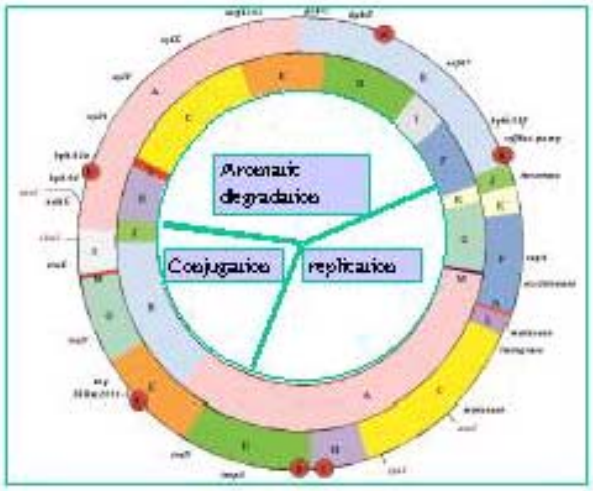

Figure 9. Physical map of pNL1. The 184 kb plasmid contains clusters of genes for aromatic compound degradation, conjugation and plasmid replication (Romine et al., 1999)

6. Competitive growth analysis of starved subsurface bacteria during biostimulation with nutrient.

Insufficient nutrient in the environment is one of the reasons limiting the degradative ability of microbial communities. Four subsurface bacteria, *Terrabacter* sp. B511, *Arthrobacter* sp. B518, *S. aromaticivorans* B522, and *Acinetobacter* sp. B525, were chosen for the study. All the subsurface bacteria were isolated from the same sample, but strain B522 is an organic degrader while the other strains are not. The rationale of this study is to test whether we can preferentially stimulate the expansion of organic degrader by supplying different amounts of nutrient. Can we predict the relative expansion of each bacterium by nutrient stimulation if we know the growth rate of each bacterium in different media?

Figures 10 and 11 show the growth curves of subsurface bacteria in the presence of 1% PTYG (low nutrient supply) and 10% PTYG (high nutrient supply) media. Under low nutrient

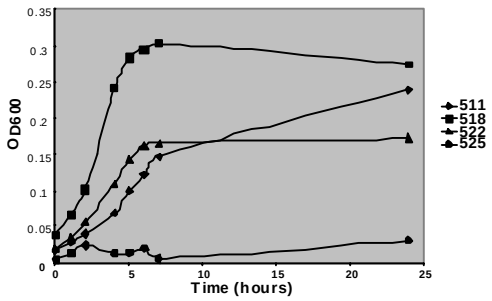

Figure 10. Growth Curves of subsurface bacteria in 1% PTYG (low nutrient).

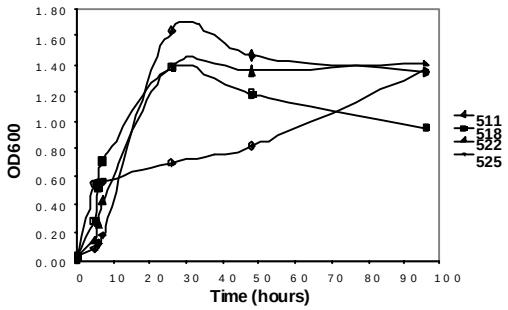

Figure 11. Growth curves of subsurface bacteria in 10% PTYG (high nutrient).

supply (Figure 10), strain B518 had the highest growth rate, but strain B525 was not able to grow. Strain B511 and B522 (organic degrader) have intermediate growth rates. On the other hand, under high nutrient supply (Figure 11), all the strains have similar growth rates except B525. These results indicate the heterogeneity in growth rates of subsurface bacteria. We hypothesize that, by stimulating the growth of starved cocultures (two or more bacteria strains mixed together) with different levels of nutrient supplies, we can manipulate the microbial community structure to enhance bioremediation.

To test this hypothesis, we carried out two experiments to study the effect of starvation and nutrient supply on the relative expansion of bacteria existing as cocultures. Strain B518 (bacterium with the highest growth rate in low nutrient supply) and strain B522 (organic waste degrader) were both grown in 10% PTYG medium, and then equal numbers of each bacterium were mixed together. Nutrient was stripped from the cocultures by washing the bacterial cells with minimal salt water (10 mM $CaCl_2$ and 10 mM $MgCl_2$). After 24 hours of starvation, viable cell numbers of each bacterium were obtained by serial dilutions of the cocultures. B518 appeared as white colonies, and B522 appeared as yellow colonies. We observed that the viable cell numbers of B522 have decreased from 9.3 x 10^5 to 6.8 x10^5, while B518 has increased from 9.75 x 10^5 to 2.14 x 10^6 (Figure 12).

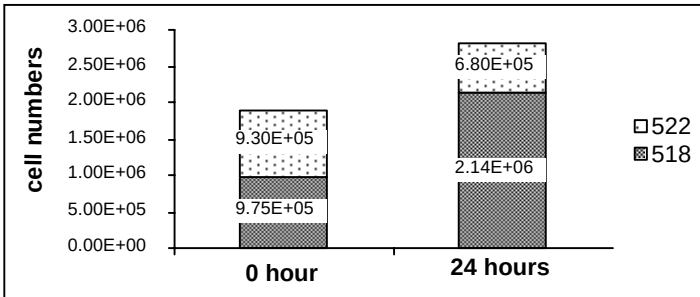

Figure 12. Relative ratio of B518 and B522 cocultures after starvation for 24 Hours

Then a final concentration of 1% and 10% PYTG nutrient was added to the starved cocultures. After 24 hours of nutrient supply, the number of viable cells of each bacterial strain was obtained by serial dilutions (Figure 13). With low nutrient supply (1% PTYG),

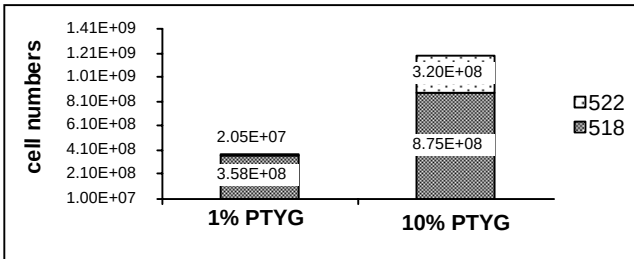

Figure 13. Effect of nutrient supplies on starved B518 and B522

the number of organic degrader B522 represents less than 6% in the final bacterial populations. On the other hand, with high nutrient supply (10% PTYG), the number of organic degrader (B522) still maintains about 27% of the total populations. This result indicates that the fraction of biodegrader is drastically decreased if low nutrient supply is used for enhancing bioremediation when there is a fast-growing bacterial strain in the microbial communities, such as the B518 in our case. Apparently, B518 depletes the nutrient at a faster pace. However, further experiments are required to support this hypothesis.

6. RELEVANCE, IMPACT AND TECHNOLOGY TRANSFER:

a. How does this new scientific knowledge focus on critical DOE environmental management problems?

To investigate the tolerance of soil bacteria at environmental contaminants that occur at DOE site is the focus of this project. Organic solvent and radiation are two of the major problems at DOE site. Using a molecular biology approach, we have gained some insight into how soil bacteria can tolerate organic solvent and radiation. We have found that catalase and superoxide dismutase are essential for the extreme radiation resistance of *Deinococcus radiodurans*. We have found that *pspA* is required for solvent resistance. Thus, we may be able to enhance the solvent or radiation resistance of microbes with biodegradative capabilities by over-expressing these genes. Furthermore, the growth response study of starved subsurface bacteria to limiting nutrient is relevant to the current approach of biostimulation.

b. How will the new scientific knowledge that is generated by this project improve technologies and cleanup approaches to significantly reduce future costs, schedules, and risks and meet DOE compliance requirements?

The solvent inducible genes or starvation inducible genes that are identified in this study may be useful for future development as molecular probes to monitor the process of bioremediation. The different growth responses of different bacteria to biostimulation suggest the need to understand the physiology of soil bacteria before the implementation of biostimulation strategy.

c. To what extent does the new scientific knowledge bridge the gap between broad fundamental research that has wide-ranging applications and the timeliness to meet needs-driven applied technology development?

Natural bioremediation and engineered bioremediation are two currently cost-effective corrective action for addressing organopollutant in the environment. We have investigated the capabilities of an indigenous bacterium, *Sphingomonas aromaticivorans*, for natural bioremediation. By completely sequencing the large plasmid pNL1 carried by *S. aromaticivorans*, we have identified genes involved the catabolic activities associated with the degradation of various aromatic compounds. Furthermore, we have also identified several starvation inducible genes, and developed a genetic system for the *Sphingomonas aromaticivorans*. More importantly, our data showed that biostimulation of biodegraders in a microbial consortium requires a better understanding of the physiology of other non-biodegrader competitive microbes. This new scientific knowledge allows us to further explore the capabilities of *Sphingomonas* species in natural bioremediation at subsurface environment.

On the other hand, using *D. radiodurans* as a model microbe for engineered bioremediation, we have developed a genetic method to analyze its extreme resistance to environmental stresses. The identification of starvation inducible genes involved in glycogen and fatty acid metabolism in *D. radiodurans* suggests that *D. radiodurans* may store up glycogen and fatty acids as reserve, which suggest that *D. radiodurans* can survive in environmental conditions where nutrient is scanty. Moreover, we have identified several stress related genes such *pspA* (required for solvent tolerance), *catA* and *sodA* (required for oxidative and radiation resistance). This new knowledge will allow us to develop new strategy to enhance the solvent, oxidative or radiation resistance of *D. radiodurans* or other microbes for enhanced bioremediation.

d. What is the project's impact on individuals, laboratories, departments, and institutions? Will results be used? If so, how will they be used, by whom, and when?

At PNNL, this project has broadened the molecular biology capabilities such as DNA sequencing, cloning, gene construction, and bacterial genetics in the Molecular Biosciences Department. Dr. Michael Daly at USUHS is currently investigating the mutants, the catalase and superoxide dismutase knockouts (Markillie et al., 1999) constructed in this project, for the understanding of oxidative stress. Dr. Susan Varnum, a previous postdoc on this project, is currently hired as a staff scientist at PNNL. Several undergraduates have been involved in this project. One of them, Preston Hradecky, is now working in a biotech company, while others may pursue graduate studies.

e. Are larger scale trials warranted? What difference has the project made? Now that the project is complete, what new capacity, equipment or expertise has been developed?

The ultimate goal of this project is to devise a better strategy of natural bioremediation through biostimulation, and a better scenario to engineer *D. radiodurans* for bioremediation at mixed waste sites. We have identified several stress inducible genes, and have established genetic methods to deliver foreign genes into both *S. aromaticivorans* and *D. radiodurans*. Since the complete genome of *D. radiodurans* has been determined, it is now possible to identify all stress inducible genes in the whole genome using microarray technology. By identifying more stress-inducible genes, it will be relatively easy to discover the promoter structure that responds to an environmental stress. On the other hand, the complete sequence of the pNL1 catabolic plasmid allows us to investigate later gene transfer of the catabolic plasmid in microbial consortium. Further analysis of the underlying mechanism that controls the differential response of subsurface bacteria to nutrient supply is important for optimizing biostimulation strategy.

f. How have the scientific capabilities of collaborating scientists been improved?

The project involved both molecular biology and analytical biochemistry, which has broadened the knowledge base of both groups.

g. How has this research advanced our understanding in the area?

The paper on the complete DNA sequence of the catabolic plasmid pNL1 from *S. aromaticivorans* has been cited 30 times since it was published in March 1999 (Romine et al., 1999). The knockout methods developed for *D. radiodurans* are being used for functional

analysis of other genes. Catalase and superoxide dismutase mutants constructed in this project are being used by other lab for further studying of oxidative stress in *D. radiodurans*. The importance of *sodA* in radiation resistance support the discovery of superoxide dismutase in cyanobacteria as a protective enzyme during cycles of desiccation and rehydration (Shirkey et al., 2000).

h. What additional scientific or other hurdles must be overcome before the results of this project can be successfully applied to DOE Environmental Management problems?

This work identified stress-inducible genes from two soil bacteria, *Deinococcus radiodurans* and *Sphingomonas aromaticivorans*. Promoters from these genes will be useful for expressing biremediating genes in engineered bacteria. However, the promoter structure of these genes need to be defined more precisely and genetically before they can be utilized effectively. Furthermore, an expanded study of nutrient response of starved microbe consortium instead of just a two subsurface bacteria to various dose of nutrient supply will be necessary to devise an effective biostimulation and bioremediation scenario.

i. Have any other government agencies or private enterprises expressed interest in the project? Please provide contact information.

Dr. Linda Chrisey (chrisel@anr.navy.mil; 703-696-4504) at Office of Naval Research has indicated some interest in the determining whether a similar pNL1 catabolic plasmid exists in marine *Sphingomonas* species or not. Moreover, the underlying mechanisms that determine the later gene transfer of pNL1 plasmid among *Sphingomonas* species or other species are another area of interest.

7. PROJECT PRODUCTIVITY:

Did the project accomplish all of the proposed goals?

Yes. The project has accomplished all the proposed goals except the engineered *Deinococcus radiodurans* strain was not able to degrade TCE. Stress-inducible genes were identified and cloned from both *S. aromaticivorans* and *D. radiodurans*. We have established genetic methods to engineer both *Deinococcus radiodurans* and *S. aromaticivorans.* We have gained knowledge in the growth response of starved subsurface bacteria to limiting nutrients.

8. PERSONNEL SUPPORTED:

Kwong-Kwok Wong, Ph.D. (35%) Principal investigator
Susan Varnum, Ph.D. Postdoc (50%)
Meng Markillie, Technician (100%)
William Chrisler, Technician (40%)
Rita Cheng, Technician (10%)
Preston B. Hradecky, UCSU, Colorado, Summer student (8 months)
Bradley R. Cleveland, University of Washington, Summer student (5 months)
Graduate student: Jonathan L. Sebat, University of Idaho (3 months)

9. PUBLICATIONS:

Published in peer-reviewed journals and books (briefly describe the published results).

1. Romine, M., L.C. Stillwell, **K.K. Wong**, S. J. Thurston, E.C. Sisk, J. D. Saffer, and J. K. Fredrickson. 1999. Complete sequence of a 184.5-kb catabolism plasmid from *Sphingomonas aromaticivorans* strain F199. Journal of Bacteriology 181:1585-1602.
   - A catabolic plasmid pNL1 was completely sequenced, and many biodegradative genes were identified within the DNA sequences.

2. Markillie, L.M., S. Varnum , P. Hradecky, and **K.K. Wong**. 1999. Targeted mutagenesis by duplication insertion in the radioresistant bacterium *Deinococcus radiodrans*: radiation sensitivities of catalase (*katA*) and superoxide dismutase (*sodA*) mutants. Journal of Bacteriology 181:666-669.

   - A general genetic method was developed to inactivate genes in *Deinococcus radiodurans*. Catalase and superoxide dismutase were shown to be important for extreme radiation, and oxidative resistance of *Deinococcus radiodurans*.

3. **Wong, K.K.**, L.M. Markillie, and J.D. Saffer. 1997. A novel method for producing partial restriction digeston of DNA fragments by PCR with 5-methyl-dCTP. Nucleic Acids Res. 25:4169-4171.
   - A molecular method was developed to generate molecular markers for identifying variation in gene sequence.

4. **Wong, K.K.**, L. C. Stillwell, C. A. Dockery, and J.D. Saffer. 1996. Use of tagged random hexamer amplification (THRA) to clone and sequence minute quantities of DNA - application to a 180 kb plasmid isolated from *Sphingomonas* F199. Nucleic Acids Res. 24: 3778-3783.
   - A sequencing method was developed to obtain sequences from minute quantities of large plasmid DNA.

10. INTERACTIONS::

Results from this projects have been presented at several conferences and workshops organized by American Society of Microbiology, DOE and EMSL. The following are the presentations at the corresponding conferences and workshop.

Markillie LM, Chrisler WB, and Wong KK. 1988. Functional analysis of *Deinococcus radiodurans* genome by targeted mutagenesis. ASM Conference on Small Genomes, Lake Arrowhead, CA, September 20-24, 1998.

Wong KK. 1988. Genetic Analysis of stress responses in soil bacteria for enhanced bioremediation of mixed contaminants. Environmental Management Science Program Workshop. Chicago, Illinois, July 27-30, 1998.

Wong KK. 2000. Genetic analysis of stress response of *Deinococcus radiodurans*. Environmental Molecular Sciences Symposia - the Sciences of radiation-resistant *organism Deinococcus radiodurans*. Environmental Molecular Sciences Laboratory, Richland,

Washington, June 23rd, 2000.

Collaborations. Jerome J. Kukor, Ph.D. at Rutgers University, has provided the gene construct (toluene 3-monooxygenase) which has been engineered into *Deinococcus radiodurans*. However, no TCE degradation activity was detected in the engineered strains. Dr. Kukor has also analyzed the TCE degradation capability of a few *Sphingomonas* species.

11. TRANSITIONS

This project is a fundamental research to investigate the stress responses of soil bacteria. The knowledge will serve as basis for further evaluating biostimulation strategy as well as for developing genetically engineered bacteria.

12. PATENTS: NONE**.**

13. FUTURE WORK:

What remains to be done ?

1. Although *Sphingomonas* strains are able to degrade several aromatic compounds, they are relatively sensitive to the toxicity of organic solvent. With the genetic system that we have developed for *S. aromaticivorans*, it is possible to enhance the solvent resistance by overexpressing solvent inducible gene in *S. aromaticivorans* such as *pspA* and *robA* genes.
2. Since we have obtained the complete sequence of the catabolic plasmid pNL1, it is possible now to investigate of effect of biostimulation on catabolic plasmid transfer or stability among *Sphingomonas* strains in a microbial consortium.
3. Promoter structure of stress inducible genes in *Deinococcus radiodurans* needs to be define more precisely before they can be effectively used for expressing foreign genes in *D. radiodurans*.

Will the project lead to future work?

We have applied for a renewal of the project. Although reviewers' comments are encouraging, the proposal was not selected for support.

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