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

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Progress Report

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Research Objective

To provide increased knowledge of stress responses of indigenous microbes at contaminated sites as well as using microbes as molecular probes for monitoring the performance and effectiveness of bioremediation, we propose to identify the stress-inducible genes and promoters from two soil bacteria, *Deinococcus radiodurans* and *Sphingomonas* F199. These organisms represent two phylogenetically distinct groups of soil bacteria, each of which has specific features of interest for bioremediation. *D. radiodurans* exhibits high resistance to external stress; F199 is a deep subsurface (Savannah River Site) organism with unique degradative capabilities.

Research Statement

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, and should contribute to environmental management and restoration actions that would reduce the cost and time required to achieve DOE's cleanup goals.

Stress-inducible genes identified in this project can also 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 strategies. In addition, stress-inducible regulatory elements identified in this project will be useful for creating genetically engineered microorganisms able to degrade hazardous wastes under stress conditions at contaminated sites.

Research Progress

Research Performed

Analysis of Starvation Stress on Gene Expression

Our initial effort has been directed toward identifying starvation-induced genes and their promoters using ribonucleic acid (RNA) arbitrarily primed polymerase chain reaction (RAP-PCR) methodology. The first task in this approach was to identify appropriate starvation conditions for *D. radiodurans*. Cultures of *D. radiodurans* were grown to saturation in a minimal media (Raj, et al. 1960), harvested, washed, and suspended to a density of 1×10^{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 1. Putative differentially identified bands were cut out of the gels, cloned into the pCR-Blunt vector (InVitrogen), and sequenced using an Applied Biosystems 373 automated ribosomal RNA (rRNA) sequencer. Three of the putative clones were identified as either 16S or 23S rRNA. Two clones share sequence homology with open reading frames (ORFs) of unknown function. Northern analysis, a standard method used to analyze levels of RNA, is currently underway to verify the differential expression of these two clones. Sequencing of the sixth identified clone is being pursued.

Figure 1. Identification of Starvation-Inducible Genes From *Deinococcus Radiodurans* Using RAP-PCR.

Starvation culturing condition has also been worked out for *Sphingomonas aromaticivorans* FI99. RNAs were isolated from cells that have been starved for 1 day, 4 days, and 21 days. Six different arbitrary primers have been screened and seven putative differentially expressed genes have been identified. Three of them are novel but the rest are either 16S or 23S artifacts. An example of the RNA fingerprint is shown in Figure 2.

Analysis of Other Stresses on Gene Expression

The effect of toluene and peroxide on the viability of both *D. radiodurans* and *S. aromaticivorans* FI99 were studied. Preliminary studies indicated that starved or stationary cells of *D. radiodurans* can tolerate toluene at a level of up to 50% (V/V) for a period of 1 hour. However, after 12 hours incubation with 12% toluene, the cell titer decreased by 4 orders of magnitude. On the other hand, exponentially growing cells are highly sensitive to toluene and were killed completely within 1.5 hours. Genetic response to toluene stress at sublethal levels

is being pursued. The physiological response to peroxide stress has been studied; findings indicate that the level of resistance was growth phase-dependent (Figure 3). Efforts are currently underway to identify genes whose expressions are growth regulated or induced during late log phase (Figure 4).

Figure 2. Identification of Starvation-Inducible Genes From *Sphingomonas Aromaticivorans* Using RAP-PCR.

Figure 3. Peroxide Resistance of *D. Radiodurans* at Different Growth Phase.

Figure 4. Identification of Genes Whose Expression is Growth-Phase-Dependent by RAP-PCR. Arrows indicate genes that are growth-phase regulated.

Methodology Development

To further improve the RNA fingerprinting methodology, a 16S and 23S subtraction step is being developed that will eliminate the cloning of 16S and 23S from background amplification. Besides the RAP-PCR technology, several new approaches are being pursued to identify stress-induced promoters. One of these approaches uses a promoter probe plasmid for promoter fusion experiments. A promoter probe plasmid has been constructed based on a *Deinococcus* cloning vector that contains a promoter-less chloramphenicol reporter gene. A *Deinococcus radiodurans* promoter library has been constructed with this plasmid and promoter clones will be screened for the expression of chloramphenicol resistance under different stress-induced environments. A promoter library has been constructed from *Sphingomonas* using a broadhost range promoter probe plasmid pRKZ1- μ m (supplied by Ronald Olsen) using *lacZ* reporter gene. Current efforts are aimed at establishing an effective transformation system in both *D. radiodurans* and *S. aromaticivorans* F199. Dr. Ronald Olsen, University of Michigan, will provide several plasmid vectors that include the clone containing toluene 3-monooxygenase for establishing a genetic system in these bacteria. Furthermore, a set of 96 arbitrary primers, which are available from another unrelated sequencing project, are being screened for generating more informative fingerprints. DNA fragments and probes are currently being designed to define stress responses of genes involved in contaminant metabolism, plasmid replication, and plasmid transfer in *Sphingomonas aromaticivorans* F199.

Summary of Findings

- Two putative novel genes that expressed at starvation condition from *Deinococcus radiodurans* were cloned and sequenced.
- Three putative novel genes that expressed at starvation condition from *Sphingomonas aromaticivorans* F199 were cloned and sequenced.
- Findings indicate that starved or stationary cells of *Deinococcus radiodurans* are more resistant to toluene stress than exponentially growing cells.
- It has been shown that the high resistance to hydrogen peroxide (up to 100mM) of *Deinococcus radiodurans* is growth-phase-dependent.

The isolated starvation genes from both *S. aromaticivorans* and *D. radiodurans* will be used to develop gene expression systems to express toluene 3-monoxygenase in the corresponding bacteria. Based on our findings, stationary cells of the to-be-engineered bacteria will be evaluated for their degradative ability to trichloroethylene (TCE) and toluene in pure culture study. In addition, we will test whether hydrogen peroxide will create a niche for *D. radiodurans* when the bacteria is inoculated to soil samples with an established microbial community. Because of its high level of hydrogen peroxide resistance, it is expected that *D. radiodurans* could be established in any new environment when applied in environmental soil samples with an established microbial community.

Reference

Raj, H.D., F.L. Duryee, A.M., Deeney, C.H., Wang, A.W., Anderson, and P.R. Elliker, 1960. "Utilization of Carbohydrates and Amino Acids by Micrococcus Radiodurans." *Canadian Journal of Microbiology* **6**: 289-298.