Genetic analysis of stress responses in soil bacteria for enhanced bioremedation of mixed contaminants

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Introduction

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.

Enhanced *in situ* removal of hazardous wastes by stimulating growth of indigenous bacteria with nutrients has been demonstrated. However, how much and how often to apply these supplements has been determined empirically. As a result, a controlled, and reproducible 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.

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 possesses 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. 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.

Objectives

- to understand stress responses of *Deinococcus radiodurans (model for genetic engineered bacteria)*
- to identify and use of stress inducible genes to direct the expression of biodegradative genes cloned from other bacteria into *Deinococcus radiodurans* R1
- to identify stress inducible genes from *Sphingomonas aromaticivorans* F199 (model for indigenous bacteria)
- to understand stress responses of *S. aromaticivorans* F199 as regard to the catabolic plasmid pNL1(180 kb, completely sequenced)

Approaches

Identification of stress-inducible genes:

- RNA fingerprinting by arbitrarily primed PCR
- Analysis of genome sequence

Development of genetic systems:

- duplication insertion
- plasmid transformation
- transposon mutagenesis

Sphingomonas

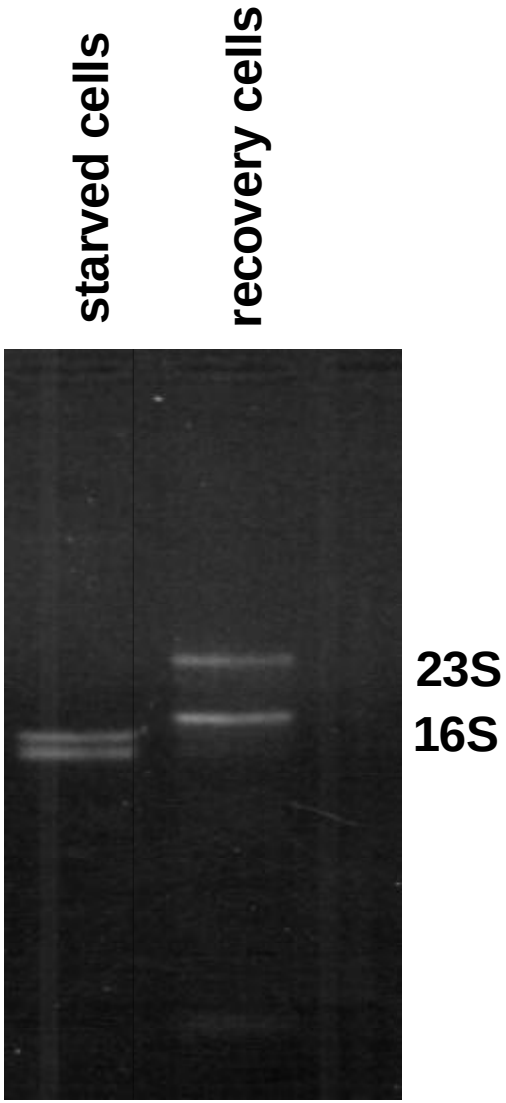
- **Ubiquitous in soil, water and sediments.**
- **Several strains were isolated from deep subsurface at DOE's Savannah River sites.**
- **Can degrade a wide range of contaminants: dibenzofuran, hexachlorocylcohexane, chlorinated biphenyls, naphthalene, xylene, and polyaromatic compounds.**
- **Presence of catabolic plasmids**

Deinococcus radiosurans

Extreme resistant to environmental insults:

- **genotoxic chemicals.**
- **oxidative agents.**
- **high level of ionizing radiation.**
- **UV radiation.**
- **desiccation.**

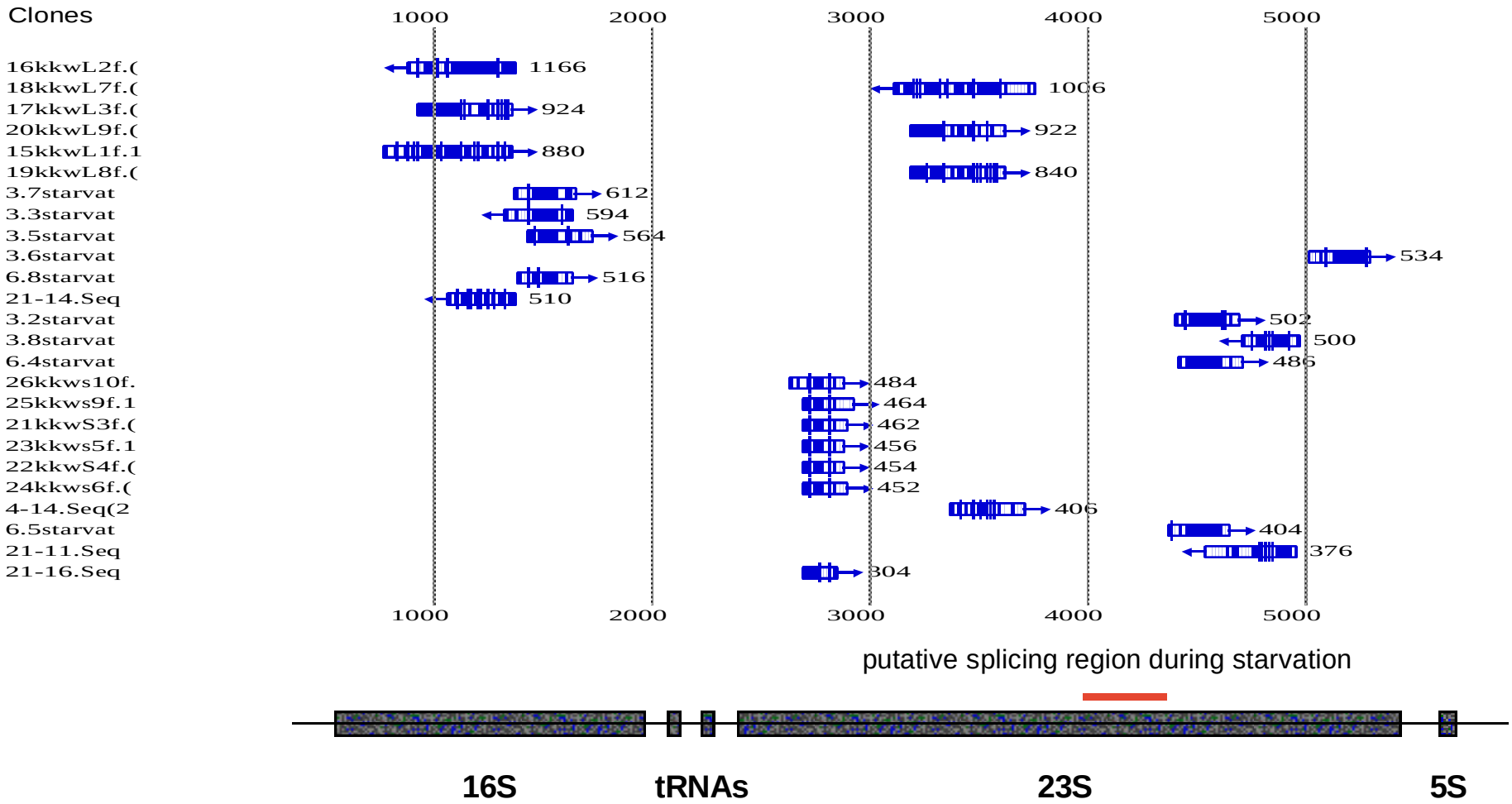
Analysis of total RNA extracted from starved culture and recovery culture



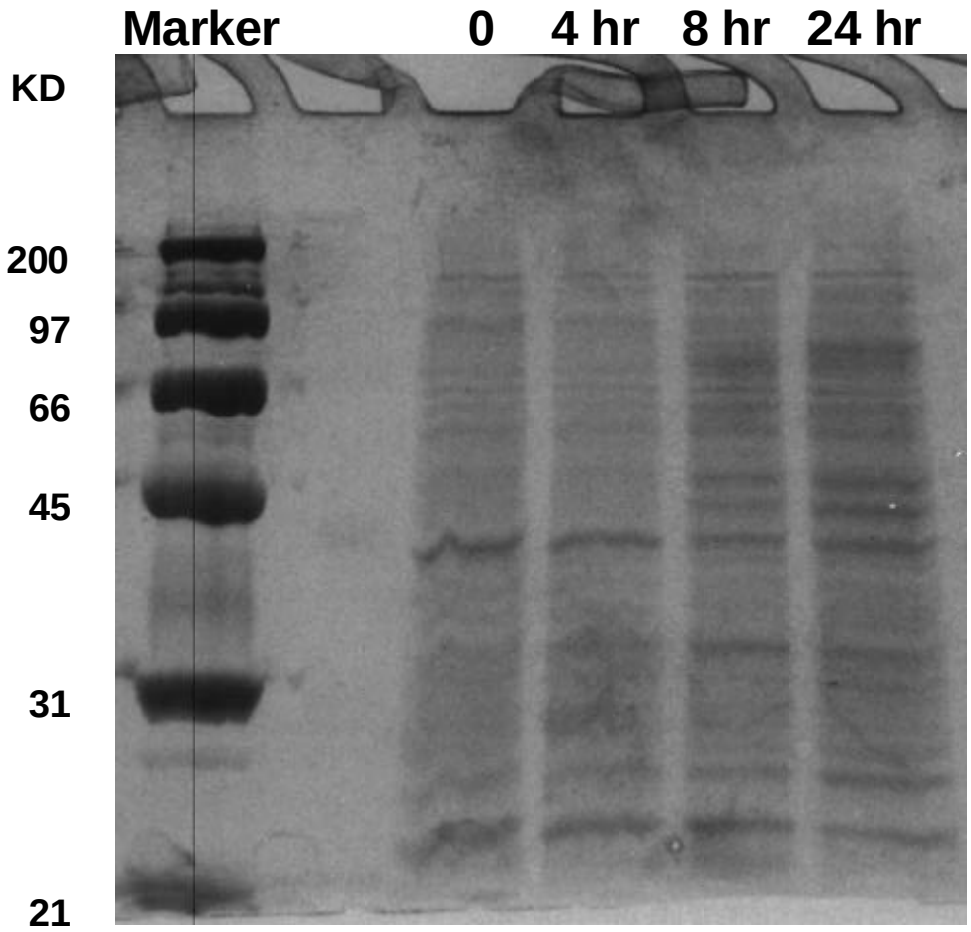
Analysis of total RNA isolated from starved cells and recovery cells (24 hours after nutrient was added to starved cells), we found that the size of rRNA molecules (both 16S and 23S rRNA) decreased in sizes. It is possible that rRNA has undergone fragmentation under starvation condition. Currently, we are trying to determine the sequences of the 16S and 23 rRNA from both starved cells and recovery cells. By comparing the rRNA sequences of starved cells and recovery cells, we might be able to develop oligonucle-otide probes useful for monitoring the number of recovery cells which are metabolic active cells during bioremediation.

Preliminary analysis suggested that the 5' end of 16S may be prccessed and the central part of 23S rRNA may be processed.

Alignment of randomly amplified 16S and 23S rRNA fragments from RNA extracted from starved *Sphingomonas aromaticivorans to Pseudomonas* rrn operon.



Total protein analysis of 10 month starved *S. aromaticivorans* culture after nutrient addition

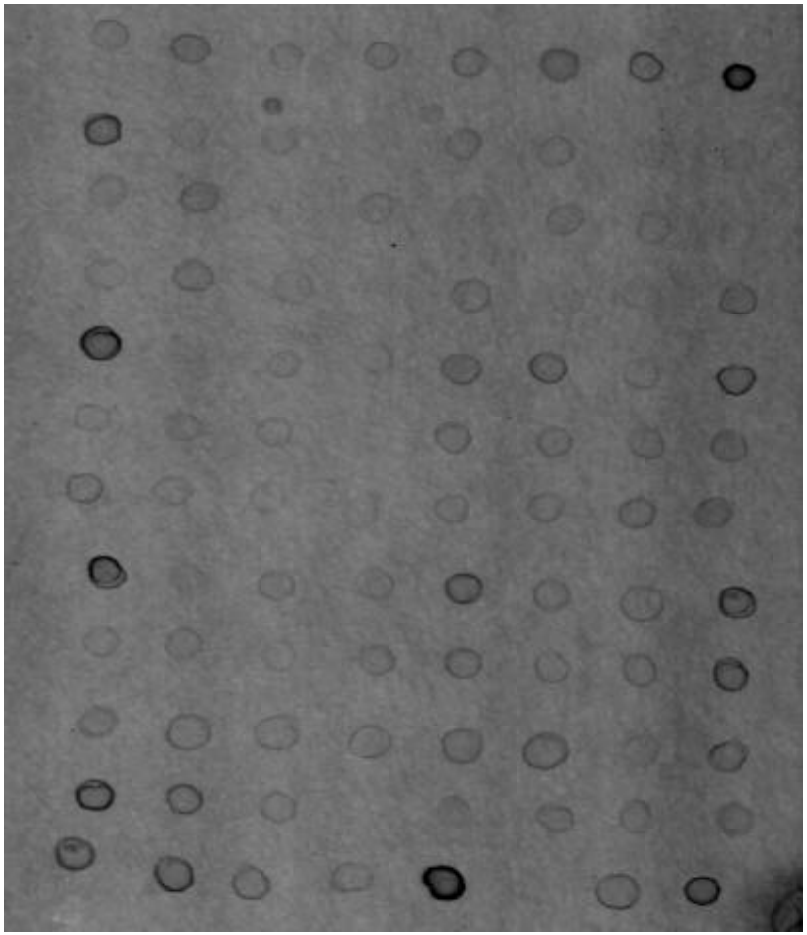


Insufficient nutrient in the environment is one of the reasons that limit the degradative ability of the bacteria. Enhanced *in situ* removal of hazardous wastes by stimulating growth of indigenous bacteria with nutrients has been demonstrated. However, the effect of adding nutrient has to be monitored. Using *S. aromaticivorans* as a model, we investigated the effects of adding nutrient to starved cells in terms of protein expression, rRNA, and gene expression pattern on catabolic plasmid pNL1.

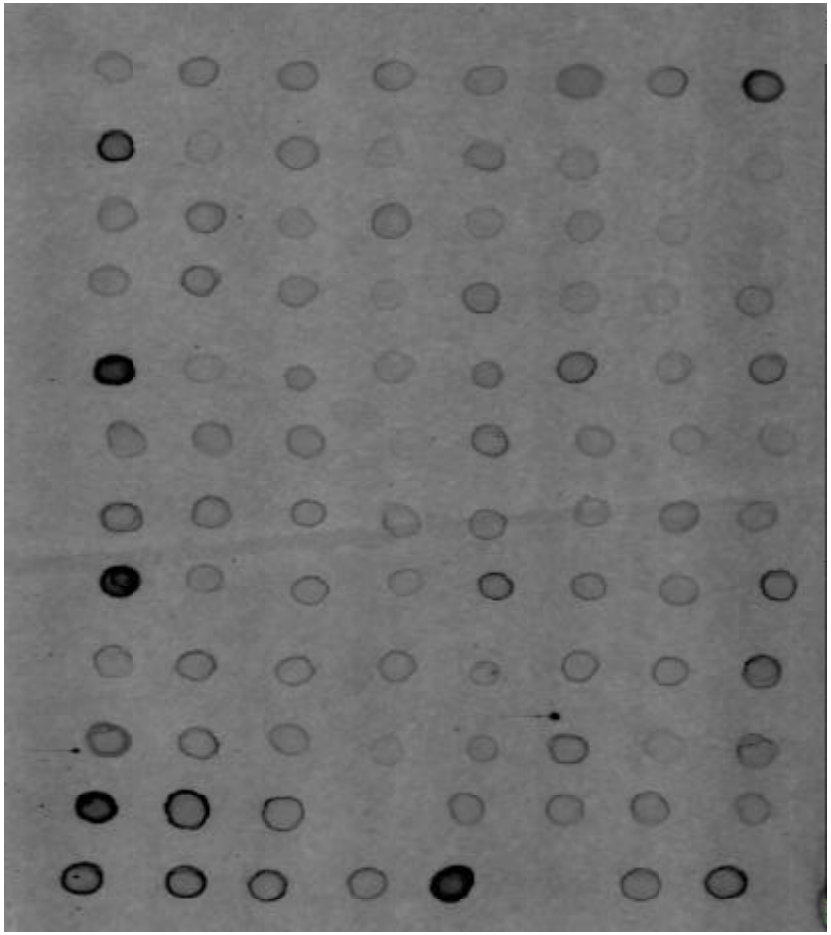
S. aromaticivorans cells have been starved in reconstituted well water without carbon and nitrogen sources for 9 months. Nutrient (TGY medium) was added to the starved cell, and proteins were extracted from the cell culture after 4, 8 and 24 hours. Extracted proteins were analyzed using a SDS-PAGE (Fig. 1). It was found that only a few major proteins were present in starved cells, and there was not much detectable changes 4 hours after nutrient was added to the starved cells. However, several prominent proteins appeared 8 hours later. This result suggests that there is a lag time before starved cells response to nutrient supplement. These identified proteins may be useful for monitoring the bioremediation process during biostimulating with nutrient supplements.

Identification of genes on the catabolic plasmid pNL1 that response to nutrient addition

Starved cells

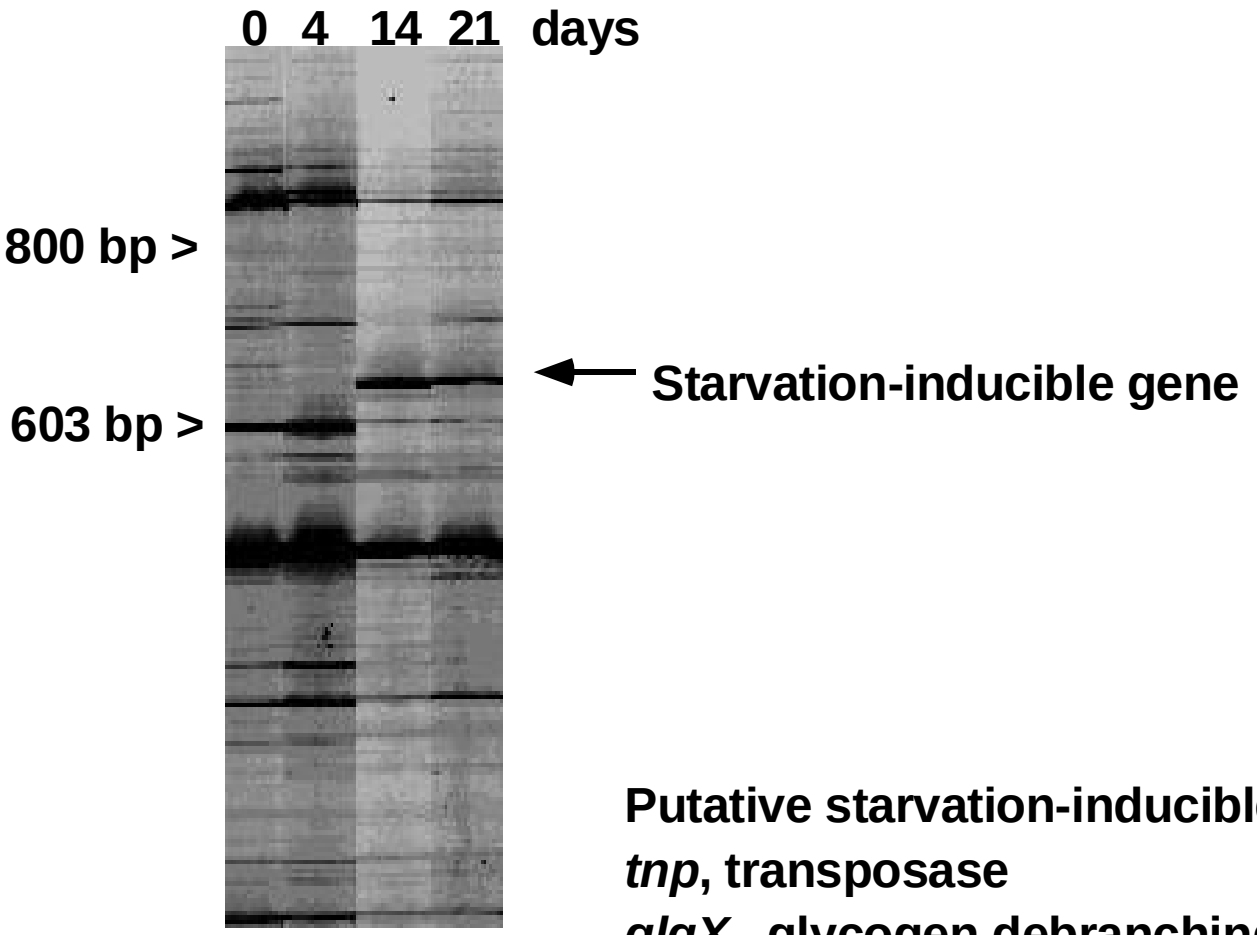


recovery cells



S. aromaticivorans carries a catabolic plasmid pNL1, which encodes genes for the degradation of a variety of aromatic compounds such as naphthalene, biphenyl and xylene. A plasmid library of 1 to 2 kb fragments, derived from sonicated pNL1 DNA, were used for making a dot blot. Flourescein-labeled cDNA probes, derived from both starved and recovery cells, were used for hybridization with the dot blots containing the plasmid library. Fig. 3 shows that a few genes on the catabolic plasmid pNL1 is still relatively active in starved cells. The activities of those starvation active genes respond more robust to the nutrient supplement. Apparently, most of the catabolic genes were not activated by the supplement of nutrient. This result suggests that nutrient supplement, although stimulate the growth of the bacteria, does not necessarily stimulate the biodegradative activities of the bacteria. Alternative strategies need to be developed for stimulating the biodegradative activities of indigenous bacteria.

Identification of starvation-inducible genes by RNA fingerprinting



Putative starvation-inducible genes:

***tnp*, transposase**

***glgX*, glycogen debranching enzyme**

***hbd*, 3-hydroxybutyryl coenzyme A dehydrogenase**

Putative stress inducible genes identified by analysis of *Deinococcus radiodurans* R1 genome sequences by blastx search:

katA, (3.4e-257), catalase A inducible by peroxide and stationary phase.

ftsK, (6e-91), cell division gene, regulator of usp (universal stress inducible protein)

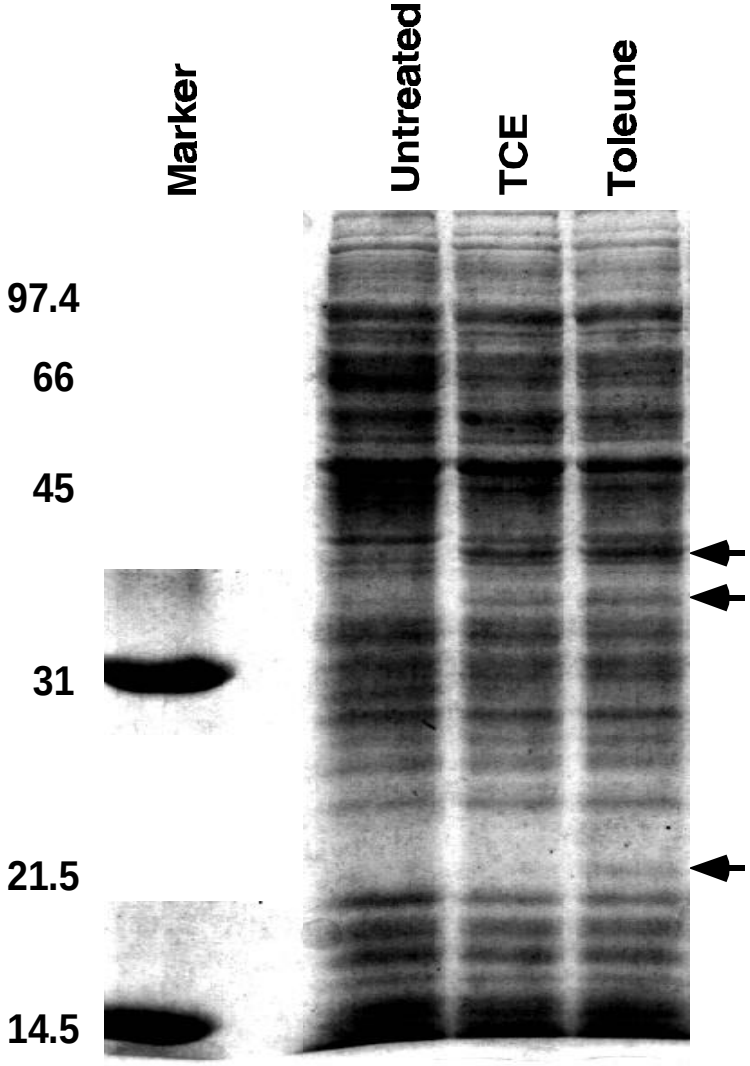
ahpF, (4e-29), alkyl hydroperoxide reductase, induced by peroxide.

pspA, (5e-19), phage stress protein, induced by starvation and solvent stress.

cspD, (1e-11), cold shock protein, induced by low temperature and starvation.

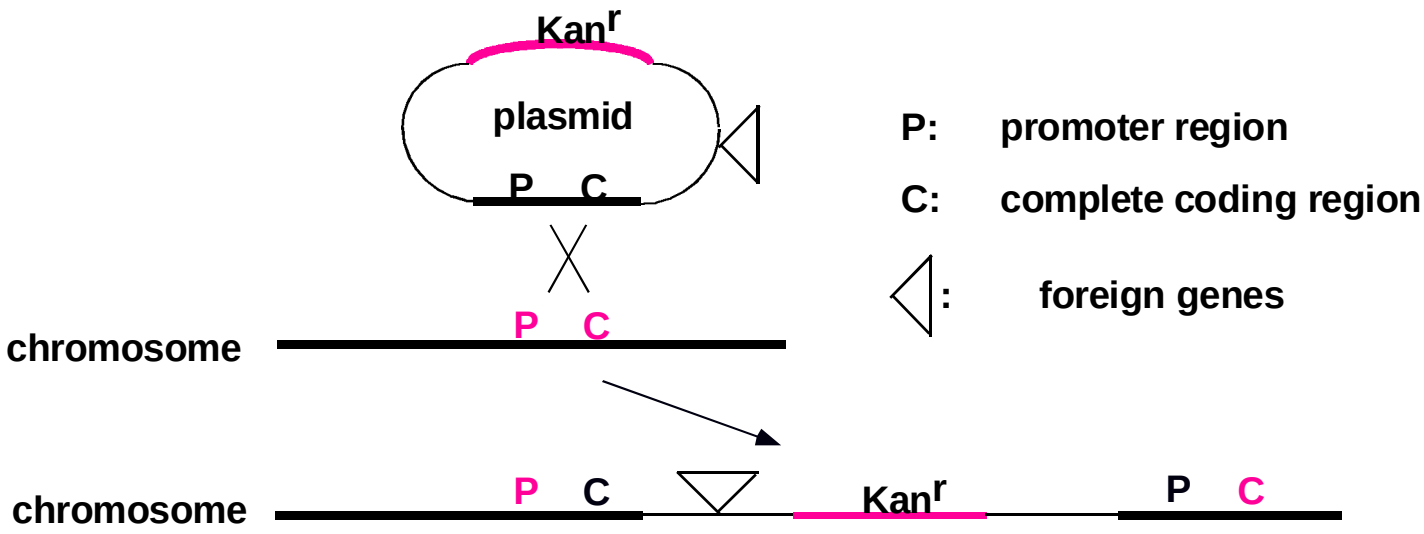
pexB, (7e-10), core starvation protein.

Identification of solvent stress inducible proteins in *Deinococcus radiodurans*



Genetic systems in *Deinococcus radiodurans* R1

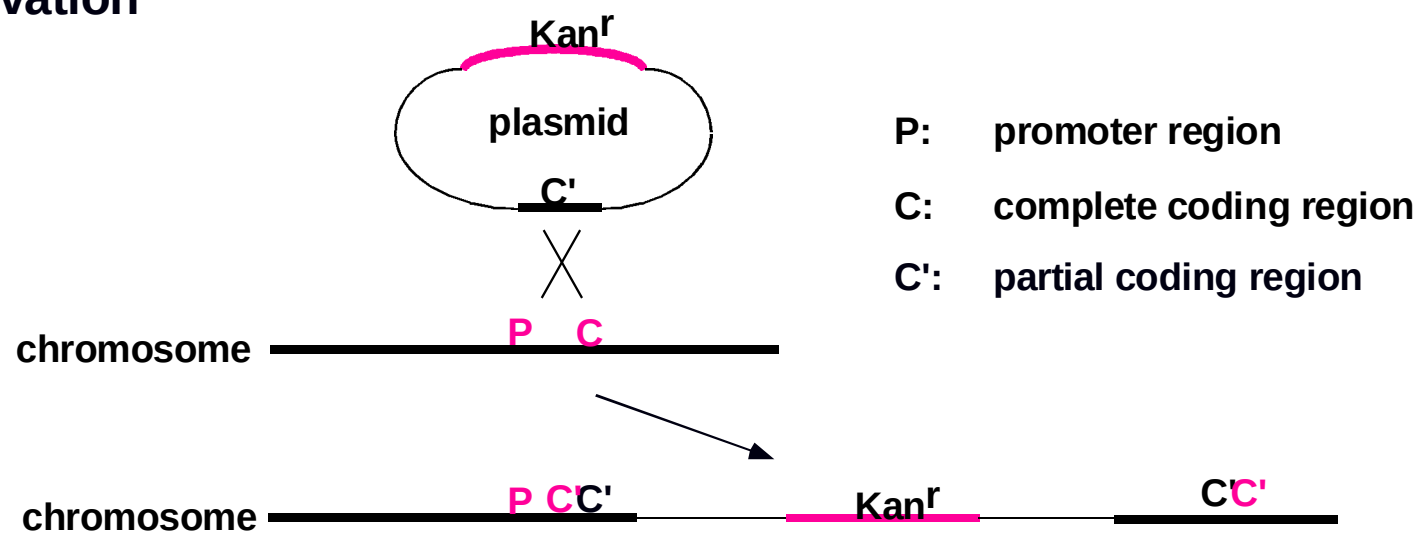
Duplication insertion



*we have used catalase gene to insert toluene-3 -monoxygenase into *Deinococcus radiodurans* genome

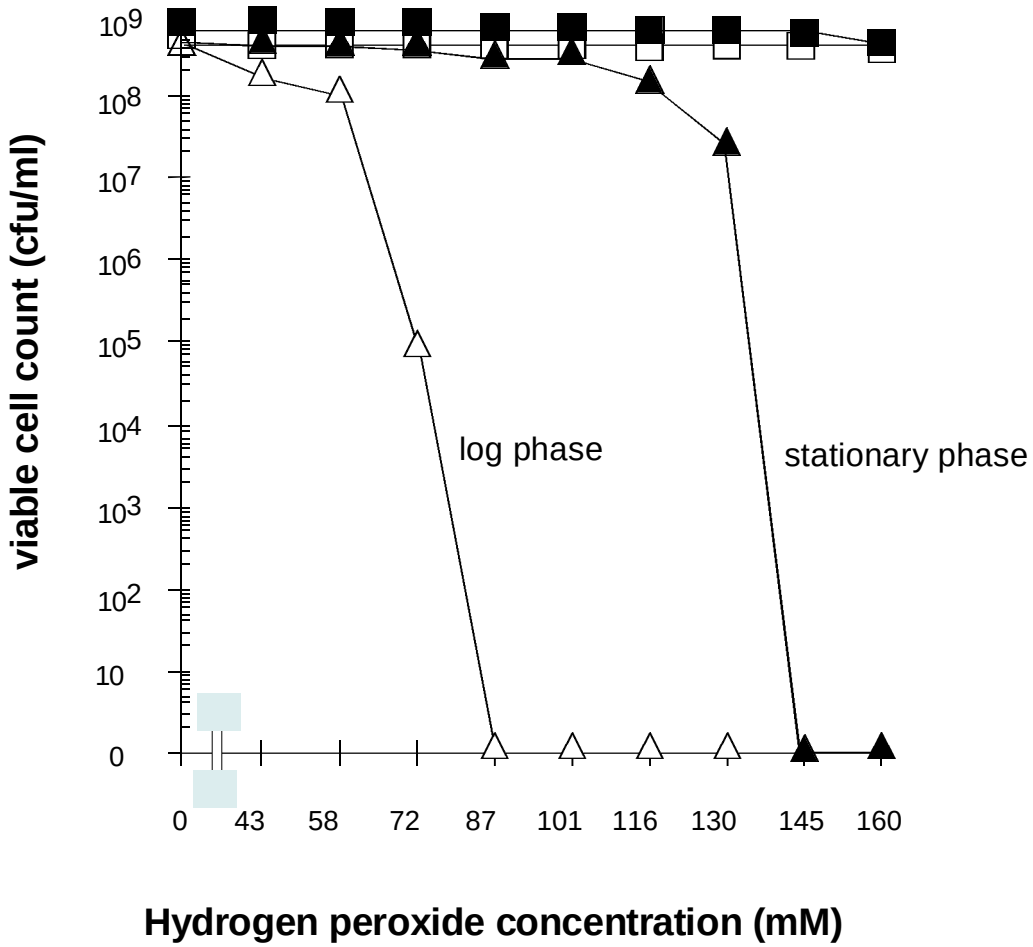
Genetic systems in *Deinococcus radiodurans* R1

Duplication inactivation



Partial coding region is duplicated but the complete coding region is interupted by the plasmid DNA insertion.

Hydrogen peroxide sensitivity of *katA* mutant



Summary

1. We have identified several starvation inducible genes from *Sphingomonas aromaticivorans* and *Deinococcus radiodurans* for the expression of toluene 3 monooxygenase.

2. 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.

3. We have observed a solvent stress response in *D. radiodurans* and identified several solvent inducible proteins.

4. Adding nutrient to starved *S. aromaticivorans* did not stimulate the expression of biodegradative genes.

5. 23S rRNA undergoes fragmentation when *S. aromaticivorans* is starved.

6. We have established genetic methods to deliver foreign genes into *S. aromaticivorans* and *D. radiodurans*.

Planned Activities

1. We will determine the complete sequences of rRNA of starved cells and recovery cells. Obtained sequences will be useful for the development of oligonucleotide probes for monitoring the physiological state of soil bacteria.

2. We have been concentrated on the studies of starvation responds of soil bacteria. We will shift our focus on the stress responses to trichloroethylene, a major contaminant at DOE's contaminant sites.

3. Soil bacteria with enhanced metal and solvent resistance will be more desirable, at mixed-contaminant sites with relatively higher contaminant level. It has been demonstrated that over-expressing *robA* or *pspA* genes in *E. coli* can enhance its resistance to metal and organic solvent. We will attempt to over-expression these genes in both *S. aromaticivorans* and *D. radiodurans*.

4. Further analysis of all *Deinococcus* strains expressing Tbu, including Northern analysis, timing of expression and analysis of these strains degradative ability for trichloroethylene and toluene in pure culture studies.