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FINAL REPORT
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ROLE OF STARVATION GENES IN THE SURVIVAL OF DEEP
SUBSURFACE BACTERIAL COMMUNITIES

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INTRODUCTION

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The investigation dealt with several aspects of subsurface bacterial survival and their nature, and involved close collaboration between the P.I. and Co-P.I.s and their co-workers.

A. The starvation genes -- their molecular characterization and physiological role. Mutants of *Pseudomonas putida*, a common environmental bacterium with counterparts in the subsurface, were isolated by transposon mutagenesis. These mutants were highly sensitive to starvation stress. Reporter gene fusions also showed that these genes were starvation genes since they were induced several fold when the cultures were starved. Since the regulatory regions (promoters) of starvation genes are of interest in bioremediation and in experiments designed to understand the roles of starvation genes in the maintenance of microbial community structure, the promoter of one of these genes (*pstarv1*, contained in strain MK107) was characterized in detail. Quite unexpectedly, it turned out to be regulated by the alternate sigma factor, sigma 54. This was established by a detailed analysis of the sequence of the promoter, the upstream region, and the mRNA product of the promoter and the structural gene.

B. Role of *pstarv1* in biofilms. As a preliminary to these studies, the growth characteristics of *Pseudomonas putida* MK1 and MK107 were compared for cells growing in batch cultures or as an attached monolayer in microstat cultures. The growth rate of the deletion mutant MK107 was similar to the wild type strain in either complex or defined media and both in batch culture and microstat culture (Table 1). It was noteworthy that both MK1 and MK107 grew at a reduced rate when attached in the defined media tested (M9 amended with either glucose or acetate). The 60% increase in doubling time was unexpected and is currently under investigation.

Table 1. Growth characteristics of *Pseudomonas putida* MK1 and MK107 in suspension cultures and attached to a surface

Culture	Batch Culture		Microstat	
	<u>MK1</u>	<u>MK107</u>	<u>MK1</u>	<u>MK107</u>
<u>Medium</u>				
LB	44	46	39 ± 3	39 ± 3
Glucose (M9)	83	98	99 ± 8	108 ± 11
Acetate (M9)	99	114	152 ± 10	148 ± 12

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Biofilms were grown as pure culture of MK107 or with this bacterium as a member of a heterogeneous natural microbial community. A microstat was used and a fluorogenic substrate, fluorescein di- β -D-galactopyranoside (FDG) was used to detect starvation gene expression within the biofilm, using confocal laser microscopy.

The objective of this study was to evaluate the response of two strains of *Pseudomonas putida* to substrate concentration gradients, and in particular the expression of starvation genes under low nutrient conditions by mutant MK107. The microstat was used to simulate natural conditions by creating response surfaces containing nutrient concentrations varying in a continuum from eutrophic to oligotrophic conditions. A number of modifications to the microstat were necessary to meet the requirements of this study. It was also necessary to demonstrate that the selected *Pseudomonas* strains are capable of surface colonization. These studies indeed indicated that both the MK1 and MK107 strains of form biofilms, both on the gel surface in the microstat, and the glass attachment surface in flow cells. Subsequent experiments with MK107 demonstrated that it reacted to response surfaces, created by concentration gradients of C vs P, and C vs N, by forming biofilms up to 20 μ m thick at the high concentration end of the response surface. A notable decrease in biomass was observed when moving towards a lower concentration of any one of C, P, or N. Image analyses of these response surfaces revealed a biomass coverage of the surface which varied from >55% at high nutrient concentration to <1% at distances greater than 15 mm away from the nutrient source. FDG was used for the detection of starvation gene expression by MK107 under complete or semi-starvation conditions. The results obtained in both microstat and flow cell experiments suggested a delay in the onset of starvation gene expression in biofilms compared to suspended cells. This suggests that the impact of starvation on individual cells is somehow mitigated when they are part of a biofilm compared to when they are present as single cells.

Another finding pointing to the dampening of the starvation stress on cells in a biofilm came from the finding that the starvation-gene induced cells represented less than 20% of the total population. This was the case even after prolonged periods of carbon depletion. Ratiometric analyses of FDG- cleavage based fluorescence versus biofilm biomass of pure culture biofilms indicated that the starvation gene induction occurred in <20% of the total population. Thus, interactions occurring within a community somehow dampen the impact of starvation. Exactly how this is achieved, remains to be determined.

As expected, there was an increase in the *starv1* gene expression with time after reduction of carbon source concentration in the influent medium, both in pure-culture as well as heterogeneous biofilms. Up to 5 times more starvation-gene expression occurred in cells closest to the attachment surface; while not definitely known, it remains possible that this increase occurred because the *pstarv1* promoter responds also to the "stress" of attachment. Strain MK107 could tolerate higher concentrations of chlorinated hydrocarbons when cultured in a heterogeneous biofilm community, than when cultured in a community consisting of MK107 alone. This finding has direct bearing on the DOE problem of heavy contamination with hydrocarbon, heavy metal and nuclear waste, since it indicates that cultivation of the remediating microbes in a biofilm is conducive to their resistance to environmental insults.

Another intriguing finding was that the expression of the starvation gene appeared to occur at the time when the cells neared division. This may mean a link between the starvation gene expression and the cell division cycle. This phenomenon will be investigated in detail in future proposals and projects.

Homologues of the gene *starv1* of surface *P. putida* in the subsurface counterparts of this bacterium. To select subsurface strains phylogenetically related to MK1, the complete 16S ribosomal RNA gene sequence was determined for *Pseudomonas putida* MK1. This sequence was aligned to published rRNA sequences and compared to sequences of the 16S rRNA gene as they are determined by the Deep Subsurface Culture Collection. Dr. Schmidt, using the rRNA homology studies determined that a subsurface isolate, Zat 47, bears close phylogenetic relationship with *P. putida*. We used Southern hybridization to locate the *starv1*

gene in Zato 47. A strong cross-reacting band was found. The homologous DNA of Zato is being sequenced to determine its homology with the *starv1* gene of the surface *P. putida*.

Starvation survival of Zato was also investigated. It had a distinctly different pattern of starvation than the surface *P. putida*. At the onset of starvation, it greatly reduced its size and exhibited a 4-5-fold increase in number. It was also more tolerant of starvation than the surface *P. putida*.

Selection of oligotrophic subsurface bacteria. This was done both using biofilms and selection as suspension cultures in a mono-culture chemostat. Core samples from the Cerro Negro deep subsurface site were used. Tryptic soy broth (TSB) was used as a complex growth medium at concentrations varying from 0.1 to 20 g/l. The organisms isolated from the site material were not inhibited by the high nutrient concentrations, and the highest numbers of colony forming units were encountered on plates containing 20 g/l TSB. At least four different colony and cell morphotypes could be distinguished from these cores on agar plates and in flow cells, respectively.

Enrichments in the chemostats gave truly intriguing results. Several samples were analyzed; a typical outcome was that with sample CNAR 16-425. Aseptically collected rock was crushed and suspended in sterile water. The suspension served as inoculum. Inoculation directly on 1/4th strength Luria Broth plates produced very little growth. Batch inoculation in the same medium produced almost a pure culture of cocci. In chemostats run using the same medium at dilution rate of 0.06 h^{-1} , selection led to appearance of cell shapes and colony morphologies not seen initially at all, and in the end to a stable population consisting of two members. Some cell shapes were seen transiently only and could not be isolated. But five cultures each giving a different cohort of cell shape and colony morphology were obtained in pure culture. The succession of population as nutrient limitation got established in the chemostat was instructive. After one volume change (ca. 24 h) two types of bacillus shapes, one long and slender, the other stubbier, and vibrio shapes along with some spores were seen. The slender bacillus disappeared at 96 h (4 vol. changes), but reappeared at 168 h (7 vol changes). At 96 h a coccus shaped bacterium also became a sizable part of the population, but rapidly disappeared. At 144 h slender rods thinner than any seen up to this point became dominant, but then disappeared. In the end, the slender and stubbier bacilli became the stable populations. All these morphologically different forms gave different colony morphologies.

Clearly, if tested only by conventional enrichments, all the forms seen, except for the cocci would not have been detected. If the experiments had been combined with rRNA analysis, all the other bacteria (except the coccus) would have been classified as viable but nonculturable. Another point is worth noting. The succession of populations occurred only under nutrient limited conditions. This suggests that this succession became possible by microbial interactions that can occur only under nutrient limited conditions. These results high light the importance of using conditions resembling those of the natural habitat in selecting microorganisms from nature.

Relationship between subsurface bacteria. The most significant findings of these studies were:

- Based on ribosomal RNA sequences, it was determined that a majority of iron-reducing bacteria from subsurface environments are closely related to one another, and form a evolutionary cohesive group within the delta proteobacteria. An oligonucleotide probe was designed that is specific for this group of bacteria and tested both by hybridization with RNA extracted from subsurface environments and as a PCR amplification primer with DNA extracted from a variety of soil and sedimentary environments.
- A collection of arthrobacters have been isolated from surface soils and it was determined that the isolates contain nearly identical 16S rRNA sequences as compared to subsurface arthrobacter isolates. This collection of surface and subsurface isolates permits a comparison

of genome structure between surface and subsurface populations. Cultures of the surface and subsurface isolates have been sent to Dr. Robert Miller for analysis of the *recA* gene. Comparative studies of this collection of surface and subsurface isolates permits the question of the origins of the subsurface populations to be addressed.

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