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EFFECT OF NUTRIENT LIMITATION ON
GENOMIC REARRANGEMENTS IN
PROKARYOTES

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Final Report

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Abstract.

During the total agreement period, more than 200 unique heterotrophic aerobic bacteria were isolated from samples of deep subsurface sediments retrieved from depths of 149.2 and 178.9 m of a vertical bore hole at Cerro Negro (CNV1). All the isolates were characterized phylogenetically and deposited to SMCC. Several clones belonging to a family currently believed to be of strictly marine origin (*Microscilla*) were found among the isolates. The bacteria might be the descendants of those present in marine environment at the time of deposition. This is an important conclusion for the entire deep subsurface "Microbial Origins" project. We determined diversity of populations of prokaryotic genomes of independent bacterial clones isolated from both surface soil, and subsurface sediments. The degree of diversity for deep subsurface isolates was exceedingly high, much higher than that observed for surface soil isolates. We have found that the genomes of some subsurface isolates are significantly more plastic than those for strains isolated from surface environments. The most intriguing discovery was isolation and cultivation of populations of non-plateable novel ultra small bacteria, which are phylogenetically more close to chloroplasts of higher plants, than any other existing bacteria including Cyanobacteria.

The first goal of the project was isolation of a number of bacterial strains from deep subsurface sediments. We isolated heterotrophic bacteria from samples of deep subsurface sediments retrieved from depths of 149.2 and 178.9 m of a vertical bore hole at Cerro Negro (CNV1). Only a small portion (about 0.04%) of viable bacteria observed in the sediment could be grown on solid nutrient media frequently used for cultivation of environmental bacterial isolates. Comparison of different nutrient media during the course of the experiment revealed that using R2A synthetic medium always led to a larger number of colonies with a better variety of bacteria grown. This medium, therefore, might be recommended as the medium of choice for isolation of bacteria from deep subsurface sediments. In total, more than 200 bacterial isolates were obtained and cleaned till homogeneity. Most of these isolates were deposited into SMCC (Subsurface Microbial Culture Collection, supervised by Dr. D.L.Balkwill) and are available for scientific community.

One objective of the research was to determine diversity of populations of prokaryotic genomes when organisms are grown under various conditions. We have examined the genome structure of independent clones of a single bacterial species isolated from surface soil, and subsurface sediments.

From an agricultural surface soil, 164 morphologically and phenotypically distinct clones were isolated. Based on substrate utilization and membrane fatty acid profiles, as well as on 16S rDNA analysis, these organisms were identified as *Variovorax paradoxus*. Variation among the clones was assessed using DNA fingerprints, restriction length polymorphism (RFLP) of genomic DNA, plasmid profiles, and growth rates. Both fingerprinting and pulse-field gel electrophoresis (PFGE) of restricted genomic DNA showed there were 14 distinct genotypes. The number, sizes and organization of plasmids also varied among these clones. These data suggested that significant genetic diversity can exist in local populations of soil bacteria, and that the methods used can be used to readily characterize differences in genome organization. These findings were reported at Gordon Conference, 1995, at the Annual Conference of the NSF Center for Microbial Ecology, 1995. It has been partially published in Appl. Environ. Microbiology, 1997, the second part is in preparation for publication.

The species diversity and genetic variation within bacterial populations from samples recovered from 178.9 m was determined based on comparison of colony and cell morphology, fatty acid profiles (MIDI, Inc.), Rep-PCR fingerprinting using different primers, and partial sequencing of their 16S rDNA. Based on the data obtained, the isolates were divided into several groups on species level. Within each bacterial group, only a few isolates shared the same patterns of Rep-PCR fragments, and only two pairs of isolates were identical on the level of partial sequence of their 16S rDNA. The degree of diversity was exceedingly high, and much higher than that observed for surface soil isolates. Given the density of the plateable cells in the sediment as low as 1000 cells/g (which suggests long distances between the cells) and very low concentration of bio-available carbon source and water, there may be minimal interaction and competition among the populations. This raises the question of

whether this assemblage of microorganisms should be considered a microbial "community". The extent of diversity found might reflect adaptation specialization to an extremely adverse environment that has occurred during the 100 million years since the sediment was deposited. The data were reported at the Annual Conferences of the NSF Center for Microbial Ecology, 1995, and 1996, at the Annual Meeting of American Society for Microbiology in 1997, at Midwest Conference on Molecular Microbiology, 1996, and at the International Conference of Soil Ecology Society (Manhattan, KS, 1997). The data were partially published in Journal of Industrial Microbiology in 1996.

Interestingly, among the bacterial strains we have isolated from the deep subsurface sediments, there were several clones belonging to a family currently believed to be of strictly marine origin (*Microscilla*). The bore hole site was set in the middle of a desert, and the sediment had been deposited 100 Million years ago from a shallow marine environment. This suggests that some of the bacteria recovered from the sample may be indeed descendants of the bacteria existed in the ancient ocean at the time of deposition. This is an important suggestion for the entire deep subsurface microbiology project. The finding was reported at the Annual Meeting of American Society for Microbiology in 1996, and published in Journal of Industrial Microbiology in 1996.

Another interesting finding was that enormously high percentage of the bacteria isolated from the deep subsurface sample contained large and super-large plasmids (of size more than 100 kb). This is unusual for surface soil isolates and is often attributed to bacteria with advanced ability to utilize xenobiotics in highly oligotrophic environments. The results were discussed at the Annual Meeting of American Society for Microbiology in 1996, and are in preparation for publication.

To address the question of frequency of genomic rearrangements among the bacteria isolated from soil surface and deep subsurface, two sets of experiments were set. One of these included growth of two different subsurface isolates in diffusion gradient chambers with a steady state gradient (from 0 to 6 mM) of acetate (as sole carbon source). Samples taken across the gradient after some period of incubation were analyzed by Rep-PCR fingerprinting. The data indicated the genetic diversity was substantially higher among clones from low nutrient environments, than among those from high nutrient environments. The other experiment was a simple evolution experiment done in triplicate with six subsurface isolates and four surface strains. Each line of isolates was transferred every ten generations for at least 50 transfers in both minimal and high nutritional media. The genetic diversity was measured by fingerprinting the genome using Rep-PCR. The results showed that the genomes of some subsurface isolates are significantly more plastic than those for strains isolated from surface environments. All these data are quite intriguing and rise many questions relevant to the "Origins Program". One is whether this is a characteristic unique to subsurface organisms? Is this a strategy elaborated by bacteria to survive these strictly oligotrophic conditions existing in deep subsurface? Could the mutation frequency be regulated by nutrient availability? The findings were reported at the

International Symposium on Microbial Ecology (ISME-7, Brazil, 1994), and at the Jacques Monod Conference on Evolutionary Genetics and Adaptation (France, 1995). The data are in preparation for publication.

The most exciting discovery made in the course of the three years work with the subsurface samples is that we managed to isolate and cultivate a population of non-plateable ultra-bacteria, which are phylogenetically more close to chloroplasts of higher plants, than any other existing bacteria including Cyanobacteria. Axenic cultures of several of these populations have been obtained by dilution culture technique. These bacteria are growing very slowly at highly diluted liquid nutrient media, and cannot be grown on any solid media tried to date. Morphologically, they appeared to be very small rods (100x20 nm). Any attempts to confirm that the bacteria are capable to photosynthesize failed yet. Nevertheless, analysis of their 16S rDNA sequences revealed these are clustered deeply within the Chloroplasts of higher plants. Their prokaryotic nature of these microorganisms was proved by hybridization experiment with either fluorescently labeled universal prokaryotic, specific to chloroplasts, or eukaryotic probes (the only probe unable to hybridize to these cells appeared to be eukaryotic). All this suggests that the organisms may represent microbial species with deep evolutionary roots and might be the descendants of those present in marine environment at the time of deposition. The discovery was reported at the Annual Conferences of the NSF Center for Microbial Ecology, 1995, and 1996, Midwest Conference on Molecular Microbiology, 1995, Midwest Ecology and Evolution Conference, 1996, at the Annual Meetings of American Society for Microbiology in 1996, and 1997. The data are in preparation for publication.

To conclude, the data obtained are directly relevant to Research Goal 1 as outlined in the Origins of Microorganisms in Deep Subsurface Environments Research Implementation Plan, specifically Research Priorities 1.1.2, 1.1.3, 1.2.1, 1.2.1, and 1.2.4. Although we have somewhat deviated from the research plan as put forth in the proposal, we feel that the course of research we had followed is more aligned with the central questions posed in the Research Implementation Plan. It has been our impression from previous correspondence from SSP that we could and should pursue investigations that hold promise to yield findings important to the entire program. We have done so. This, of course, means that not all specific objectives outlined in the original proposal were thoroughly addressed during this grant period. Hopefully, our research will be viewed in the broad context of its contribution to the Origins Program and not narrowly in terms of the objectives set forth initially.

Dr. Igor V. Zlatkin

List of Publications issued during the total agreement period.

Dunbar J., Zlatkin I., Forney L. (1995) Variation in genome organization among *Variovorax paradoxus* clones isolated from soil. In: Center for Microbial Ecology research findings. Spring 1995. p. 58-60.

Zlatkin I.V., Forney L.J. (1995) Viable oligobacteria from a deep subsurface sediment. In: Center for Microbial Ecology research findings. Spring 1995. p. 177-179.

Zlatkin I.V., Schneider G.M., deBruijn F.J., Forney L.J. (1995) Diversity of microbial populations from a deep subsurface environment. In: Center for Microbial Ecology research findings. Spring 1995. p. 180-182.

Zlatkin I.V., Marsh T.L., Forney L.J. (1996) Chloroplast-like bacteria from a deep-subsurface sediment. In: Abstracts, 16th annual Midwest Ecology & Evolution Conference, 12-14 April, 1996, Abstract #22, p.27

Tamura K., Zlatkin I.V., Kamagata Y., Tiedje J.M., Forney L.J. (1996) Transfer of a 2,4-D catabolic gene from *Variovorax* sp. HW1 isolated from Hawaiian pristine soil. In: Abstracts, 96th General meeting of the American Society for Microbiology. Abstract #G-411, p. 458.

Zlatkin I.V., Forney L.J. (1996) Marine bacteria cultivated from a terrestrial deep subsurface sediment. In: Abstracts, 96th General meeting of the American Society for Microbiology. Abstract #N-61, p. 332.

Zlatkin I.V., Marsh T.L., Forney L.J. (1996) Isolation and characterization of free living bacteria from a deep subsurface environment that are phylogenetically related to contemporary chloroplasts. In: Forum on environmental remediation & environmental toxicology and microbial ecology forum, 19-20 September, 1996. p. 124-125.

Zlatkin I.V., Forney L.J. (1996) High microbial diversity within paguate sandstone from the deep subsurface. In: Forum on environmental remediation & environmental toxicology and microbial ecology forum, 19-20 September, 1996. p. 126-127.

Zlatkin I.V., Schneider M., deBruijn F.J., Forney L.J. (1996) Diversity among bacteria isolated from the deep subsurface. *J. Industr. Microbiology*. 17: 219-227.

Zlatkin I.V., Forney L.J. (1997) Changes in the structure of the Microbial community in sediment retrieved from the deep subsurface during storage at 4°C. In: Abstracts, 97th General meeting of the American Society for Microbiology. Abstract #N-132, p. 403.

Zlatkin I.V., Forney L.J. (1997) Are there microbial communities of aerobic heterotrophic bacteria in the deep subsurface? In: Abstracts, Soil Ecology Society International Conference, 27-30 May, 1997. p. 67

Dunbar J., White S., Forney L. (1997) Genetic diversity through the looking glass: effect of enrichment bias. *Appl. Environ. Microbiology* 63:1326-1331.

Zlatkin I.V., Roberts M.S., Marsh T.L., Forney L.J. (1998) Novel bacteria from late cretaceous deep subsurface sediments are phylogenetically related to the chloroplasts of higher plants. In: Abstracts. International Symposium on Microbial Ecology, ISME-8.

Dunbar J., Zlatkin I., Forney L. Genetic variation in a localized population of *Variovorax paradoxus*. (In preparation for "Appl. Environ. Microbiol.")

Zlatkin I.V., Roberts M.S., Marsh T.L., Forney L.J. (1998) Isolation and preliminary characterization of free-living bacteria from the deep subsurface that are phylogenetically related to chloroplasts of higher plants. (In preparation for "Science").