DOE/ER/60478--2

DE90 014624

Final Progress Report

for

MICROSCOPICAL AND CULTURAL EXAMINATION OF SUBSURFACE MICROORGANISMS

U. S. Department of Energy Contract No. DE-FG05-86ER60478
(July 20, 1986-October 31, 1989)

to

Florida State University
(Dr. David L. Balkwill, PI)

DOE Report No. DOE/ER/60478--2

Submitted: February 20, 1990

DETAILED SUMMARY OF TECHNICAL ACCOMPLISHMENTS

Following is a summary of our major technical accomplishments during the entire funding period (7/20/86-10/31/89) of DOE Grant No. DE-FG05-86ER60478 to Florida State University. The more detailed scientific results of our efforts are described in four peer-reviewed publications, as well as in manuscripts (to be submitted for publication) that are still in preparation (see below).

A. Analysis of Samples from the Savannah River Site

Microbiological analyses were performed on 60 samples of topsoil and subsurface sediments (to depths of 1,500 feet below land surface) from DOE's Savannah River Site (SRS). Forty-five of these samples were received in June and July, 1986; the remaining samples were received in July and August, 1988. The detailed analytical procedures are described in the original proposal and subsequent supplemental/continuation proposals. Specific tasks that were completed with the use of these procedures are listed below:

1. Viable Counts

The numbers of viable, aerobic, chemoheterotrophic microorganisms in each SRS sample were determined with two types of viable-count analyses: plate counts and most-probable-number (MPN) determinations. The plate counts were performed with a variety of nutrient media and subsequent incubation temperatures (total of 11 plate counts per sample), in order to enumerate aerobic, chemoheterotrophic microorganisms with different nutrient requirements and optimum growth conditions. All of the counts were done in triplicate (for statistical purposes):

Media:	Temperature(s):
PTYG (peptone-tryptone-yeast extract-glucose)	$4^{o}C$, $23^{o}C$, $55^{o}C$
PTYG after heat-shock at $80^{o}C$	$23^{o}C$
1% PTYG (1:100 dilution of PTYG)	$4^{o}C$, $23^{o}C$, $55^{o}C$
1% PTYG after heat-shock at $80^{o}C$	$23^{o}C$
BHI (brain-heart infusion)	$23^{o}C$, $37^{o}C$
Agar-water medium	$23^{o}C$

The MPN determinations were performed for two reasons: (i) to corroborate the general results of the plate counts and (ii) to count aerobic, chemoheterotrophic microorganisms capable of degrading specific carbon sources. As with the plate counts, the MPN determinations were carried out with several nutrient media and incubation temperatures (total of 11 MPN determinations per sample):

Media:	Temperature(s):
PTYG	$23^{o}C$, $55^{o}C$
1% PTYG	$23^{o}C$, $55^{o}C$
BHI	$37^{o}C$
Mineral salts-glucose	$23^{o}C$
Mineral salts-citrate	$23^{o}C$
Mineral salts-acetate	$23^{o}C$
Mineral salts-sucrose	$23^{o}C$
Mineral salts-lactose	$23^{o}C$
Mineral salts-mannitol	$23^{o}C$

2. Determination of Microbial Diversity

Microbial diversity (the number of distinct types of aerobic, chemoheterotrophic microorganisms) in each of the SRS samples was determined by analysis of the microbial colonies that appeared on the nutrient plates used for viable counts (plate counts; above). Each of the colonies was described with respect to 16 visible morphological characteristics, and the resulting colony descriptions were compared to determine the number of distinct types on each set of plates. This analysis was completed for all of the nutrient media and incubation temperatures listed (for plate counts) above, except for the agar-water medium, on which the colonies did not develop enough to facilitate accurate description (total of 10 analyses per sample).

3. Light Microscopical Examination of Samples

All of the SRS samples were analyzed with a light microscopic technique (known as the PVP flotation procedure) that was developed in this laboratory and was designed to obtain information on: (i) *in situ* microbial cell morphological traits, which can reflect the nutritional status of the microorganisms; (ii) microbial diversity (based on comparison of cell morphological traits); (iii) the presence of microbial microcolonies *in situ*, an indication of active growth; and (iv) the presence of distinctive microbial types that have unique metabolic capabilities. The flotation procedure (see original grant proposal and supporting references) was performed at least twice on each SRS sample, after which more than 2,500 photomicrographs were taken to establish a permanent record of the results. The data reported in published papers (below) were obtained by an extensive visual analysis of these micrographs.

B. Analysis of Microbial Cultures Isolated from SRS Samples

Pure cultures of aerobic, chemoheterotrophic microorganisms (mostly bacteria) were isolated from all of the SRS samples and examined with morphological and physiological methods. The cultures were also preserved (in frozen form), in order to develop a DOE Subsurface Microbial Culture Collection (SMCC), which is currently housed in our laboratory at Florida State University. Some of the culture isolation and culture analysis activities were funded by a grant from the Savannah River Laboratory and by a different DOE grant (DE-FG05-88ER60685). The following summary lists only the tasks that were accomplished with funding from DOE Grant No. DE-FG05-86ER60478 (*i.e.*; the grant for which this final report has been prepared).

1. Isolation and Preservation of Cultures

We attempted to isolate each of the distinct colony types noted during the analysis of colony types growing on nutrient plates for enumeration studies (above). As a result, approximately 4,100 distinct microbial strains were successfully isolated from the 60 SRS samples and preserved in frozen form. Two frozen stocks were prepared for each isolate (one for storage in a freezer at -75^{o}C and the other for storage under liquid nitrogen at -196^{o}C).

2. Analysis of Cell Morphological Traits

All of the microbial cultures isolated from the first 45 SRS samples (the samples received in 1986) on PTYG and 1% PTYG media at 23^{o}C (total of approximately 1,200 cultures) were examined by light microscopy (of stained preparations), to determine their basic cell morphological characteristics. Six specific morphological traits (most of which

will be of use during identification of the cultures in the future) were recorded for each of the cultures examined.

3. Analysis of Physiological Traits

All of the microbial cultures isolated from the first 45 SRS samples (the samples received in 1986) on PTYG and 1% PTYG media at 23°C (total of approximately 1,200 cultures) were analyzed with the API Rapid NFT testing system. This system allowed us to determine the presence or absence of 21 specific physiological characteristics for each of the cultures tested. The resulting data were then used for comparison of samples from different depths and different drilling sites (below).

C. Data Handling

1. Development of a Digitized Data Base

All of the data from analyses of samples and isolated microbial cultures (a total of more than 100,000 data points) were entered into a digitized data base, in order to facilitate subsequent data reduction and analysis, as well as to facilitate the transfer of data to DOE and other DOE investigators. Extensive quality assurance measures were also performed, in order to ensure the accuracy and completeness of the data base.

2. Data Analysis

The data (in the digitized data base) were analyzed extensively, in order to obtain additional information on microbial diversity, metabolic traits, and possible identities. Of particular interest was the comparison of data for samples from different geological formations and/or different drilling sites at the SRS. This analysis indicated that different microbial strains were present in different samples, a finding that has important implications with respect the possible use of subsurface microorganisms for the bioremediation of groundwater contaminants (see attached publications).

3. Provision of Data to DOE and Other PIs

Various forms of data were provided to DOE officials, SRS officials, and the other principal investigators throughout the duration of the grant. A complete set of data (in digital form) was provided to Dr. Robert Meglen, at the University of Colorado. This data set was used (by Dr. Meglen) to look for correlations between subsurface microbiological traits and the subsurface physical/chemical environmental factors.

D. Cooperative Investigations

1. Los Alamos National Laboratory

In 1987, we participated in cooperative research with scientists at the Los Alamos National Laboratory. The purpose of this project was to evaluate phase differential scattering (PDS) technology, a laser-based technique developed at Los Alamos, as a tool for the rapid identification and characterization of subsurface microorganisms. It was hoped that such a tool could be used for direct examination of subsurface samples, thereby alleviating the need for tedious isolation and analysis procedures. Unfortunately, our preliminary experiments indicated that the PDS system in place at that time was not able to distinguish between subsurface microbial isolates that had very similar cell morphological traits. This was discouraging because many of the isolates from the SRS samples do have

similar morphological traits. In addition, the sensitivity of the PDS technique was too low to permit the direct detection of microbial cells in sediment samples themselves. Scientists at Los Alamos speculated that these limitations might be overcome by adding a far more powerful laser source to their PDS instrument, but there were no funds available for such an expensive undertaking. Consequently, we decided to discontinue our PDS experiments and redirect our efforts to a more thorough physiological characterization of the microbial strains from the SRS (as described above).

2. Pacific Northwest Laboratory

In 1988 and 1989, we participated in a cooperative research project with scientists at the Pacific Northwest Laboratory. The objectives of this project (commonly known as GEMHEX) were: (i) to better define the extent to which the composition and metabolic traits of the subsurface microbial community vary on a relatively small (*i.e.*, cm) scale, and (ii) to relate such variations to specific chemical and physical parameters of the subsurface environment. The specific GEMHEX tasks that were accomplished in our laboratory are summarized as follows:

* The aerobic, chemoheterotrophic microorganisms in 25 SRS core-segment samples were enumerated by plate counting (only one nutrient medium, at one incubation temperature).
* The diversity of aerobic chemoheterotrophs in all 25 samples was determined by analysis of colony types appearing on the enumeration plates (as described for the other SRS samples, above).
* Approximately 250 distinct strains of aerobic, chemoheterotrophic microorganisms (mostly bacteria) were isolated from the GEMHEX core samples and preserved in the SMCC.
* All of the isolated GEMHEX cultures were analyzed with the API Rapid NFT System, in order to determine the presence or absence of 21 specific physiological traits for each culture.
* Roughly 100 of the GEMHEX cultures were also analyzed with the Biolog GN testing system, which determined the presence or absence of 95 specific physiological traits for each culture.
* All data were entered into a digitized data base, analyzed extensively, and forwarded to scientists at Pacific Northwest Laboratory.

All work on the GEMHEX project was completed shortly before the termination of the grant. It is anticipated that the results will be described in two joint publications with scientists at Pacific Northwest Laboratory (see below).

PERSONNEL AND PROFESSIONAL TRAINING

Most of the research was performed with the help of paid technicians, in order to: (i) ensure that subjective analytical tasks (such as the description of colony morphological traits) were performed consistently throughout the entire project period, (ii) maximize the efficiency of the laboratory, and (iii) facilitate the establishment of a more comprehensive quality assurance/quality control system. Nevertheless, one doctoral student (K. P. Stim) worked on portions of the research and successfully completed her degree program during the last funding period of the grant. In addition, two undergraduate students successfully completed Junior-Senior Thesis projects (independent study projects that involve writing and defending a small thesis), and three other undergraduates completed less extensive independent-study projects related to the grant. No post-doctoral fellows were involved in the research.

PRESENTATIONS OF FUNDED RESEARCH

The results of the funded research have been presented by the PI or his co-workers at several national and international meetings and symposia. Following is a list of presentations given up to the time this report was prepared:

1. "Diversity of Prokaryotic and Eukaryotic Populations in Deep Aquifers" - Annual Meeting of the American Society for Microbiology, Miami Beach, Florida, 1988.

2. "Distribution and Characterization of Bacteria in Deep Aquifers" - U. S. Department of Energy Conference on Mobility of Colloidal Particles in the Subsurface: Chemistry and Hydrology of Colloid-Aquifer Interactions, Manteo, North Carolina, 1988.

3. "Diversity and Activities of Microorganisms in Deep Aquifers" - Deutsche Forschungsgemeinschaft Abschlusskolloquium DFG-Schwerpunkt: Hydrogeochemische Vorgange im Wasserkreislau in der ungesattigten und gesattigten Zone, Kiel, Federal Republic of Germany, 1989.

4. "Association of Bacteria with Mineral Surfaces in Aquifer Sediments" - Annual Meeting of the American Society for Microbiology, New Orleans, Lousiana, 1989.

5. "Phylogenetic Study of Bacteria from Deep Aquifer Sediments" - Annual Meeting of the American Society for Microbiology, New Orleans, Louisiana, 1989.

6. "Isolation and Characterization of Chemoheterotrophic Bacteria from the Deep Subsurface" - Annual Meeting of the Society for Industrial Microbiology, Seattle, Washington, 1989.

7. "Microbiology of Deep Aquifers" - International Conference on the Microbiology of Sanitary Landfills, Oregon Graduate Center, Beaverton, Oregon, 1989.

8. "Density and Distribution of Aerobic, Chemoheterotrophic Bacteria in Deep Southeast Coastal Plain Sediments at the Savannah River Site" - First International Symposium on Microbiology of the Deep Subsurface, Orlando, Florida, 1990.

9. "Attached and Unattached Bacterial Populations in Deep Aquifer Sediments from a Site in South Carolina" - First International Symposium on Microbiology of the Deep Subsurface, Orlando, Florida, 1990.

The PI also presented findings from the funded research in seminars at the following universities and national laboratories:

1. Rensselaer Polytechnic Institute, Department of Biology (September, 1986).
2. Pacific Northwest Laboratory (September, 1987).
3. Idaho National Engineering Laboratory (September, 1987).
4. Florida State University, Department of Biological Science (October, 1987).
5. Pacific Northwest Laboratory (November, 1988).
6. Idaho National Engineering Laboratory (November, 1988).
7. Dalhousie University, Department of Biology (September, 1989).
8. Savannah River Laboratory (October, 1989).

PUBLICATIONS

To date, the activities funded by the grant have led to 10 published abstracts and four published, refereed papers (copies attached):

A. Published Abstracts

1. Balkwill, D.L. 1988. Numbers, diversity and physiological traits of chemoheterotrophic bacteria in deep-aquifer sediments. Abst. Annu. Meeting, Amer. Soc. for Microbiol., Miami, Beach, FL.

2. Fredrickson, J., D. Balkwill, J. Zachara, J. McBride, S. Li, and D. Workman. 1989. Microbial distribution and diversity within single formations of Southeast Coastal Plain unconsolidated subsurface sediments. Abst. Annu. Meeting, Amer. Soc. for Microbiol., New Orleans, LA.

3. Stim, K.P., R.H. Reeves, and D.L. Balkwill. 1989. Phylogenetic study of bacteria from deep aquifer sediments. Abst. Annu. Meeting, Amer. Soc. for Microbiol., New Orleans, LA.

4. Balkwill, D.L. 1989. Isolation and characterization of chemoheterotrophic bacteria from the deep subsurface. Abst. Annu. Meeting, Soc. for Industrial Microbiol., Seattle, WA.

5. Stahl, D.A., R. Key, and D.L. Balkwill. 1990. Phylogenetic diversity among subsurface microorganisms. Proc. 1st Int. Symp. on Microbiology of the Deep Subsurface, Orlando, FL.

6. Reeves, J.Y., R.H. Reeves, and D.L. Balkwill. 1990. Restriction endonuclease analysis of deep subsurface bacterial isolates. Proc. 1st Int. Symp. on Microbiology of the Deep Subsurface, Orlando, FL.

7. Stim, K.P., G.R. Drake, S.E. Padgett, and D.L. Balkwill. 1990. 16S ribosomal RNA sequencing analysis of phylogenetic relatedness among aerobic chemoheterotrophic bacteria in deep aquifer sediments from a site in South Carolina. Proc. 1st Int. Symp. on Microbiology of the Deep Subsurface, Orlando, FL.

8. Balkwill, D.L. 1990. Density and distribution of aerobic, chemoheterotrophic bacteria in deep Southeast Coastal Plain sediments at the Savannah River Site. Proc. 1st Int. Symp. on Microbiology of the Deep Subsurface, Orlando, FL.

9. Fredrickson, J., D.L. Balkwill, J. Zachara, F. Brockman, E. Griffin, and S. Li. 1990. Microorganisms in deep cretaceous sediments of the Atlantic Coastal Plain: vertical variations and sampling considerations. Proc. 1st Int. Symp. on Microbiology of the Deep Subsurface, Orlando, FL.

10. Krzanowski, K.M., C.A. Sinn, and D.L. Balkwill. 1990. Attached and unattached bacterial populations in deep aquifer sediments from a site in South Carolina. Proc. 1st Int. Symp. on Microbiology of the Deep Subsurface, Orlando, FL.

B. Refereed Papers (copies attached)

1. Balkwill, D.L. 1990. Deep-aquifer microorganisms. In: D.P. Labeda (ed.), The isolation of microorganisms from nature for biotechnology applications. McGraw-Hill, Yew York, pp. 183-211.

2. Balkwill, D.. 1989. Numbers, diversity, and morphological characteristics of aerobic, chemoheterotrophic bacteria in deep subsurface sediments from a site in South Carolina. Geomicrobiol. J. 7:33-52.

3. Balkwill, D.L., J.K. Fredrickson, and J.M. Thomas. 1989. Vertical and horizontal variations in the physiological diversity of the aerobic chemoheterotrophic bacterial microflora in deep Southeast Coastal Plain sediments. Appl. Environ. Microbiol. 55:1058-1065.

4. Fliermans, C.B., and D.L. Balkwill. 1989. Microbial life in the deep terrestrial subsurface. Bioscience 39:370-377.

Three additional manuscripts are still in preparation. Two of these are being prepared with J. K. Fredrickson and J. M. Zachara at Pacific Northwest Laboratory and will describe results of the GEMHEX project. The remaining manuscript will report findings from the last set of SRS samples (the "fourth-hole" samples taken in 1988).

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