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TENTH QUARTERLY TECHNICAL PROGRESS REPORT

on

MOLECULAR BIOLOGICAL ENHANCEMENT OF COAL BIODESULFURIZATION

Contract No. DE-AC-22-89PC89902

to

U. S. DEPARTMENT OF ENERGY
PITTSBURGH ENERGY TECHNOLOGY CENTER

by

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EXECUTIVE SUMMARY

The following work was accomplished during the 10th Quarterly Report Period by Battelle:

1. The conditions needed to select transformants were examined in detail.

2. Electoporation conditions for efficient transformation of T. ferrooxidans were studied. Increased field strength appears to be needed for efficient transformtion of this organism.

3. The effect of coal chemicals on several strains of T. ferrooxidans was examined. None of these chemicals prevented the growth of these microorganisms.

4. TFI70 was examined for metabolism of dibenzothiophene (DBT) and DBT metabolites. No metabolic activity was detected. TFI92 is being similarly tested.

5. Southern hybridization experiments designed to identify the (conserved) minimal replicon present in Thiobacillus pTFI91 family of plasmids is underway.

TABLE OF CONTENTS

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INTRODUCTION

The U.S. Department of Energy (DOE) is investigating the microbial-mediated release of sulfur from coal as viable industrial process for coal desulfurization. The overall DOE goal is to develop an optimized microbiological process that is cost-efficient, operates under mild conditions and is simple to engineer.

It would be desirable to have improved strains that would be capable of removing both inorganic and organic sulfur from coal. This project is directed toward achieving this goal.

OBJECTIVE

The objective of this project is to produce one or more microorganisms capable of removing the organic and inorganic sulfur in coal. The specific technical objectives of the project are to:

- Clone and characterize the genes encoding the enzymes of the "4S" pathway (sulfoxide/sulfone/sulfonate/sulfate) for release of organic sulfur from coal
- Return multiple copies of genes to the original host to enhance the biodesulfurization activity of that organism
- Transfer this pathway into a fast-growing chemolithotrophic bacterium
- Conduct a batch-mode optimization/analysis of scale-up variables.

This report presents the results of research at Battelle during the 10th Quarterly Report period beginning on September 15, 1991.

EXPERIMENTAL RESULTS

The primary focus of the Battelle-OSU team is the development of a recombinant Thiobacillus. This focus is reflected in the work described below.

Genetic System for Thiobacillus ferrooxidans

In order to construct a recombinant T. ferrooxidans capable of removing both organic and inorganic sulfur from coal a genetic system is needed for this organism. This genetic system will consist of several components: 1) a shuttle vector capable of replication in T. ferrooxidans and an alternative host (Escherichia coli); 2) a means of selecting for the presence and retention of a genetically marked vector in either host; and 3) a method of moving DNA into T. ferrooxidans cells (such as electroporation). We are currently building and defining these components.

Construction of a Thiobacillus Shuttle Vector

To be useful for cloning, the shuttle vector must replicate in both Thiobacillus and E.coli. This can be achieved by finding a vector with an origin of replication functional in both hosts or by the construction of a hybrid vector with two origins. A cloning strategy was designed to test for the function of the Thiobacillus plasmid, pTFI91, origin in E. coli and to result in the construction of a hybrid vector with two origins.

Construction of a series of potential shuttle vectors for Thiobacillus strains using all or portions of pTFI91 was completed (see 9th Quarterly Report). A series of vectors is necessary because the location of the plasmid origin of replication is unknown. Cloning the pTFI91 plasmid by restriction with several enzymes should result in at least one vector with a functional Thiobacillus origin. In E.coli, each of the hybrid vectors tested appear to be dependent on the colE1 origin of replication (from the E. coli derived plasmid) rather than the Thiobacillus plasmid origin, indicating that the Thiobacillus plasmid host range does not include E. coli.

The same series of plasmids are currently being directly tested for replicon activity in Thiobacillus strains using the electroporation technique outlined below.

The broad host range plasmid RSF1010 may be capable of replicating in Thiobacillus. To test this possibility, an RSF1010 plasmid bearing the mercury resistance genes of transposon Tn501 is also being tested for transformation into Thiobacillus strain TFI70.

Characterization of a family of Thiobacillus plasmids

Knowing the location of the Thiobacillus plasmid replicon would reduce the experimental effort anticipated to develop a shuttle vector. One strategy for identifying the Thiobacillus plasmid origin and minimal replicon involves identification of to conserved regions in several related pTFI plasmids. Southern hybridization experiments have greatly improved the understanding of the laboratory strains of Thiobacillus and detected additional plasmids in these strains. Plasmid DNA samples from these strains are being prepared. Seven laboratory strains (TFI29, TFI35, TFI70, TFI85, TFI91, TFI92, and DSM5083) were examined and all were found to contain plasmid sequences. Southern hybridization experiments detect a high degree of similarity among these plasmids and indicate that of the seven strains tested, at least six harbor related plasmids which can be divided into three types based on their restriction patterns. (The plasmid from TFI35 not yet characterized, and may also be of the same group). The similarity of these plasmids define a region of DNA which may contain the minimal replicon for these plasmids, and the presence of non-conserved regions may indicate where sequence insertion or deletion can be tolerated. A detailed examination of these plasmids is being pursued.

Hybridization experiments also indicate that no striking similarity exists between one of these plasmids, pTFI91, and the replication regions of common laboratory plasmids (including incompatibility groups P, Q, N, W, and the Streptomyces plasmid pHN1). The inplication is that the pTFI91 family of

Thiobacillus plasmids may be significantly divergent from common laboratory vectors.

Electroporation of Thiobacillus

Electroporation is most likely the best method for transforming T. ferrooxidans strains. A series of experiments were begun previously to determine the conditions (e.g., field strength [kV/cm]) which will allow electroporation of TFI70 (see 9th Quarterly Report) and TFI92. In the absence of a proven shuttle vector, electroporation is evaluated by measuring cell survival. The electroporation of DNA into a cell is generally associated with a reduction in cell survival of approximately 50%.

Preliminary results (see August report) results showed that was no loss of viability with field strengths less than 15kV/cm. By contrast, the viability of E. coli cells are reduced in this range of field strengths, and E. coli cells are also efficiently transformed at these field strengths.

Higher field strengths available with the BTX instrument at the Ohio State Biotechnology Center were used in subsequent transformation experiments. The field strength was raised to above 31 kV per centimeter, and uresults indicate that cell viability under these conditions is greatly reduced. In addition, the viability of cells is reduced by electroporation at field strengths between 20 and 30 kV per centimeter. At these slightly lower field strengths, the viability of the cells depends not only on the field strength but also on the duration of the discharge pulse. Experimentation has indicated that the BTX electroporation instrument can reliably discharge for up to 2 milliseconds with a field strength of about 22 kV per cm. Higher field strengths, up to 32 kV per cm can be achieved at shorter pulse intervals. At very high field strengths and long pulse durations, the sample is destroyed by electrical arcing in the cuvette. The transformation experiments described below utilize field strengths in the range of 22 to 30 kV per cm and up to 5 msec pulse lengths.

A method was developed to assess the effect of electroporation on cell function that was independent of time consuming cell plating techniques. It is possible to estimate the effect of electroporation on cell metabolism by observing the rate of iron oxidation, "rust formation", by electroporated cells innoculated immediately into liquid TSM media and grown up to 24 hours. Cultures which are not electroporated or subjected to mild electroporation conditions form "rust" precipitate in 6 hours and plate at high efficiency. Cells subjected to harsher electroporation conditions recover more slowly, with the highest field strength conditions reducing "rust formation" even 24 hours after treatment. Plating of cells with reduced rates of rust formation indicates these cultures have reduced numbers of viable cells. The development of this cell function assay aids quick evaluation of electroporation conditions, and helps reduce the reliance on three week cell plating experiments to determine cell viability.

Conditions for Transformant Selection

To select for either antibiotic or mercury resistance the sensitivity of the potential host strain and the level of expected resistance provided by the cloned marker gene must be known. Characterization of the strains TFI70, TFI92 and DSM5083 with respect to sensitivity and resistance was accomplished during this Quarter.

A preliminary characterization of chloramphenicol (CM) and mercury sensitivity of the strains TFI92 and TFI70 was performed. The sensitivity of Thiobacillus to these agents depends on whether the cells are cultured on plates or liquid media, on the number of cells used for inoculation and most importantly, on the strain being tested. As previously discussed, the strain DSM5083 has a higher resistance to mercury than the Thiobacillus strains TFI92 and TFI70, with maximum tolerances reaching up to 4 μg per ml $HgCl_2$ for the DSM5083 but less than 1 μg per ml $HgCl_2$ per ml for the other two strains. The mercury selection media is extremely light sensitive and required the development of methods to handle these plates. Strain TFI70 has been observed to grow on plates containing 400 μg per ml chloramphenicol, while the less resistant TFI92 strain is sensitive to CM concentrations above 100 μg per ml.

These observations have allowed for the definition of the proper range of mercury and CM concentrations to utilize for selection of transformed Thiobacillus. Battelle's strategy is to plate out a series of cell concentrations, after electroporation, on plates containing a range of mercury and antibiotic concentrations.

The selection of E. coli RSF1010/Tn501 transformants is now routinely performed on LB agar plates with up to 20 μg $HgCl_2$ per ml. Mercury resistant Thiobacillus colonies are being selected by plating on TSM media containing mercury concentrations ranging from 0.5 to 2 μg per ml $HgCl_2$. Growth in liquid media containing 0.5 to 4 μg per ml have been used to maintain selective pressure on bacteria initially selected on solid media.

Solid media containing 150 to 250 μg per ml of CM is being used as a selection for Thiobacillus transformants resistant to this antibiotic. No significant background of Thiobacillus colonies has been seen when up to 10^7 electroporated cells were plated. Small Thiobacillus colonies present on CM plates are used to inoculate liquid media containing 50 to 100 μg per ml of CM.

Thiobacillus Transformation Experiments

A series of experiments was performed using selected electroporation conditions, DNA concentrations and plating media in a attempt to demonstrate transformation of Thiobacillus. Plasmids used included the putative chloramphenicol resistant shuttle vectors described previously and a version of the RSF1010 plasmid with genes encoding mercury resistance.

During this reporting period, four potential Thiobacillus / E. coli shuttle vectors were electroporated into the host vector TFI92. The plasmids tested represent those which insert the ColE1 plasmid origin and CM selectable marker into each of four locations in the Thiobacillus plasmid with little or no deletion of the Thiobacillus sequences. For selection of CM resistance, electroporated cells were plated on TSM media containing 150, 200 and 250 μg CM per ml. These concentrations of CM virtually eliminated background (non-

transformed) colony growth. As many as 100 slow growing colonies have been observed in experiments where the shuttle vectors BTL107, BTL201 and BTL305 were electroporated into cells, but 5 to 10 colonies were seen when the plasmid BTL501 or the minus DNA control electroporations were plated.

Potential transformants are being tested for the presence of recombinant plasmid by restriction analysis, by Southern hybridization and by transformation of competent E. coli with Thiobacillus DNA preparations from the selected colonies.

In addition the electroporation experiment has been repeated with pBTL plasmid DNA samples prepared from dam$^-$, dcm$^-$ E. coli cells to reduce the possibility of Thiobacillus host restriction of methylated DNA (by analogy to the E. coli mcr restriction system)

The analysis of DNA samples prepared from TFI92 indicate that there is a plasmid already present in the strain. This plasmid is similar, if not identical to the pTFI91 plasmid which was used to create the pBTL shuttle vector series. This complicates the transformation experiment in two ways: first, detection of transformed shuttle vector DNA is difficult in strains bearing a plasmid with a somewhat similar restriction pattern. More importantly however, establishment of a plasmid transformed into a strain with a resident, probably incompatible plasmid is expected to be more difficult than transformation into a strain not bearing a such a plasmid. One possible solution being pursued is to develop a laboratory strain of Thiobacillus which does not have the plasmid.

The broad host range plasmid RSF1010 may be capable of replicating in Thiobacillus. To test this possibility, an RSF1010 plasmid bearing the mercury resistance genes of transposon Tn501 was electroporated into Thiobacillus strain TFI70. Mercury resistant colonies were obtained using TSM media containing 0.5, 1 and 2 μg per ml $HgCl_2$. Individual colonies were cored from the selective plates and cultured in liquid media containing mercury. DNA samples from these colonies have been prepared and are being analyzed.

In these mercury selection experiments there is a constant, low number of Thiobacillus cells which adapt in some way to growth on mercury. After plate selection (on TSM with 0.5 μg per ml $HgCl_2$) these cells reproducibly tolerate up to 2 μg per ml of mercury in liquid media. The presence of mercury tolerant cells in a population of Thiobacillus does limit the sensitivity of our mercury selection for transformed Thiobacillus, with up to one hundred mercury resistant Thiobacillus colonies formed per 10^7 cells plated. The recovery efficiency of mercury adapted cells and their colony size depends in part on the number of cells plated.

Further hybridization experiments with T. ferrooxidans laboratory strains indicate that several of these, including TFI92, DSM5083 and TFI70 may have sequences homologous to the Tn501 encoded mer genes, and so a chromosomal copy of these genes may be responsible for spontaneous mer resistance.

Screening of the DSM5083 Library

In order to develop a useful genetic system for T. ferrooxidans a selectable marker gene is needed. We plan to use the genes encoding mercury resistance from either Tn501 or from Thiobacillus strain DSM5083. Construction of a genomic library from T. ferrooxidans strain DSM5083 was completed in the previous Quarter.

The DSM5083 mer genes will be identified by their homology to the mer genes carried on Tn501. A probe has been made from a DNA fragment encoding most of the Tn501 mer operon. This fragment was isolated from the plasmid (pΔRSF1010::Tn501), and a probe made from this isolated fragment was used in preliminary experiments to determine the best hybridization conditions for the filter screening experiment. There was strong hybridization (dot blot) of this probe to control DNA samples and to genomic DNA from the DSM5083 strain, but reduced hybridization to negative control samples. Hybridization conditions have been selected which reproducibly detect the presence of mer genes in chromosomal DNA preparations or in single colonies screened on nitrocellulose filters. As a result of the nitrocellulose filter screening, 18 strongly hybridizing colonies were selected and DNA samples from these

clones were prepared. Preliminary restriction analysis indicates that some of these plasmids may contain fragments of the proper, predicted size.

The DSM5083 library was also screened directly for mercury resistance. The clones transferred to microtiter dished were stamped onto selective media containing 10, 15 and 20 μg per ml of $HgCl_2$. Over 40 colonies exhibited some growth in the initial screening of over 3000 clones, but none were found to be reproducibly resistant to these mercury concentrations. Dot blot analysis of these putative mercury resistant clones demonstrated no striking similarity to the Tn501 mer gene.

Effect of Coal Chemicals on T. ferrooxidans

Several strains of T. ferrooxidans were screened for sensitivity to chemicals used in the processing of coal slurries. TFI70 was selected initially as a preferred host from the collection screened, based upon genetic criteria. The work was extended to include the strains TFI29, TFI35, TFI85, TFI91, TFI92 and DSM5083. It was also important to examine the fitness of these strains from a process development standpoint. Each strain of T. ferrooxidans was tested for sensitivity to coal chemicals at levels ranging from 0.1X to 10X of that used in the coal industry (kerosene was tested only with TFI70).

The chemicals used were supplied by PETC and are noted below:

Focculents (Superfloc 16, Superfloc 214, Accoal-Floc 355)

Use Range: 1-10 ppm
Testing Range: 0, 0.1, 1, 10, 100 ppm

Frothers (Aerofroth 65, MIBC)

Use Range: 2-20 ppm
Testing Range: 0, 0.2, 2, 20, 200 ppm

Collector (Kerosene)

Use Range: 10-100 ppm
Testing Range: 0, 1, 10, 100, 1000 ppm

None of the coal chemicals irreversibly inactivated any of the strains, even at the highest concentrations tested. This result indicated that thiobacilli suspended in the bulk fluid of a coal slurry should not be inactivated by coal preparation agents. Further work using coal slurries is needed to determine if these chemicals, which are expected to concentrate on the surface of the coal particles, effects reduced depyritization activity.

DBT Metabolism by Thiobacillus

Selected Thiobacillus strains will be examined for the metabolism of DBT and DBT metabolites. Because of its importance as a potential recombinant host, TFI70 was examined first. Aliquots (10 ml) of pre-grown TFI70 or sterile medium were added to separate tubes containing 100 ppm (final concentration) of DBT, DBT sulfoxide, DBT sulfone, o,o'-biphenol, biphenyl, or 2-phenylphenol. The cultures were analyzed by HPLC for metabolites after approximately one week of incubation at 26° C. No products appeared to be formed, and no obvious loss of the added compound was evident, indicating a lack of metabolism of these chemicals (9th Quarterly Report). TFI92 is

presently being tested in a similar manner for metabolism of DBT-related compounds.

CONCLUSIONS

1. Progress continues toward the isolation of the mer gene from DSM5083. Experiments aimed at identifying mercury resistance genes are underway.

2. Treatment of T. ferrooxidans TFI70 cells by electroporation using high field strengths stuns cell metabolism and reduces cell viability, indicating that we may be in the correct range of field strength to transform cells.

3. Plating conditions for the selection of recombinant T. ferrooxidans have been chosen.

4. Mercury and CM selection of Thiobacillus from transformation experiments yielded some colonies which are resistant to mercury or CM. Analysis of DNA is needed to demonstrate whether these are actual transformants.

5. Direct screening of the E. coli library of Thiobacillus DNA did not yield any mercury resistant clones, perhaps because such genes are not functional in the transformed library. Partial clones of the Thiobacillus mer genes may have been identified by colony hybridization screening. These clones are being further characterized.

6. The Thiobacillus strains used in this study are resistant to above working concentrations of some common coal processing chemicals, indicating that these chemicals may not have a detrimental effect on biodesulfurization of coal.

7. The strains of Thiobacillus used in our laboratory all contain extrachromosomal plasmids. Most, at least 6 of 7 plasmids isolated are of a related type. The comparison of these plasmids by restriction analysis and Southern hybridization should prove useful in locating a minimal replicon for this family of Thiobacillus plasmids.

8. The presence of related plasmids in the laboratory strains may partly complicate the transformation experiment.

FUTURE PLANS

The following work is planned during the next quarterly report period:

- Evaluation and further development of Thiobacillus electroporation methodology.
- Characterization of possible cloned fragments of the mercury resistance gene from DSM5083.
- Determination of the location of pTFI91 minimal replicon using Southern hybridization.
- Evaluation of selected plating conditions for potential T. ferrooxidans transformants.
- Testing of specific plasmid constructs by transformation into T. ferrooxidans.
- Analysis of DNA from mercury and CM resistant Thiobacillus colonies for the presence of plasmid sequences.
- Completion of studies to determine if TFI92 has the ability to metabolize DBT-related compounds.

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