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PITTSBURGH ENERGY TECHNOLOGY CENTER

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March 13, 1992

MOLECULAR BIOLOGICAL ENHANCEMENT OF COAL DESULFURIZATION

for

11TH QUARTERLY TECHNICAL PROGRESS REPORT

PETC TPO

William P. Barnett

U. S. DOE patent clearance is not required prior to publication of this document

Battelle
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ELEVENTH 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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March 13, 1991

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EXECUTIVE SUMMARY

The following work was accomplished during the 11th Quarterly Report Period by Battelle:

1. The plasmid from *Thiobacillus ferrooxidans* strain TFI91 (plasmid pTFI91) was definitively restriction mapped.

2. A region of sequence conserved among a family of *Thiobacillus* plasmids was located on the restriction map of plasmid pTFI91.

3. A potential chloramphenicol sensitive electroporation host (*Thiobacillus cuprinus*) was identified and partially characterized.

4. The *mer* gene from *Thiobacillus ferrooxidans* strain DSM583 was cloned and partially characterized.

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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 original specific technical objectives of the project were 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.

By letter of September 3, 1991, from the Project Manager at Department of Energy, Pittsburgh Energy Technology Center, these objectives of this project were redirected toward finding and developing suitable vectors for *Thiobacillus* strains. All work on bacterial strains from Lehigh University was terminated since they did not contain desulfurization traits represented by the "4S" pathway.

This report presents the results of research at Battelle during the 11th Quarterly Report period beginning on December 14, 1991.

EXPERIMENTAL RESULTS

The primary focus of the Battelle-Ohio State University team is the development of a recombinant strain of *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.

The same series of plasmids are currently being directly tested for replicon activity in *Thiobacillus*.

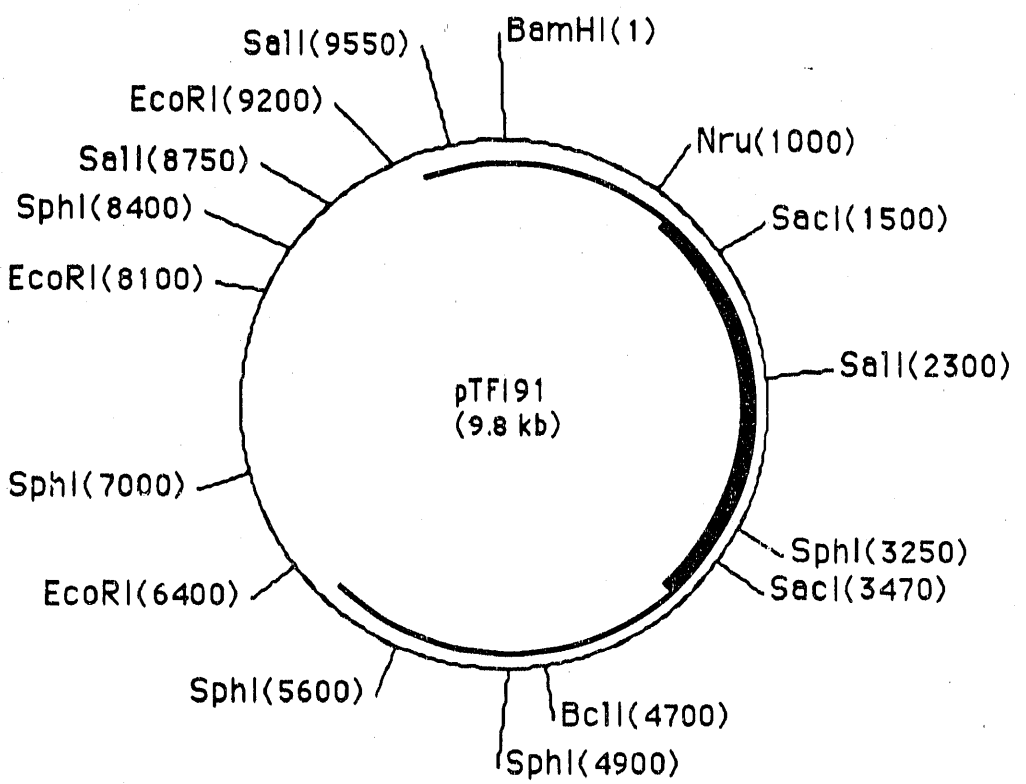
Characterization of a Family of *Thiobacillus* Plasmids

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 conserved regions in several related endogenous 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 DSM583) 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 that can be divided into three types based on their restriction patterns. (The plasmid from TFI35 is not well characterized, and may also be of the same group).

One of our goals for the plasmid study was to establish a detailed restriction map for the 9.8 kilobase pair (kb) plasmid from strain TFI91. A plasmid restriction map of pTFI91 is shown in Figure 1, featuring sixteen restriction sites of seven restriction enzymes. Additional restriction sites are being added to this map. The positions of the restriction sites on this map are accurate to within about 100 base pairs.

The similarity of the *Thiobacillus* plasmids define a region of DNA that 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 to determine the regions of conserved sequence among the *Thiobacillus* plasmids is being pursued. The PTFI91 restriction map also highlights the region found to be conserved with the 20 kb plasmid from *Thiobacillus ferrooxidans* strain DSM583. A restriction map of DSM583 as well as some current restriction gel and Southern blot data will be included in future reports. So far, no similarity between the pTFI plasmid and commonly available broad host range vectors has been documented (including incompatibility groups P, Q, N, W, and the *Streptomyces* plasmid pHN1).



The heavy and thin lines indicate regions of pTFI91 which strongly or moderately cross hydridize with the plasmid from strain DSM 583.

FIGURE 1. RESTRICTION MAP OF pTFI91

The location of conserved sequences in pTFI91 plasmid suggests that, as expected, some of the BTL series plasmid shuttle vectors may be functionally inactive in *Thiobacillus*. The prediction is that cloning of the *E. coli* vector pHSG398 into the Bcl I, BamHI and Nru I sites may insertionally inactivate a conserved sequence. The most promising shuttle vector candidates at this time may be those which contain the intact 7 kb Eco R1 fragment (See July 1991 report for detailed descriptions of these plasmids).

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 percent.

Preliminary results showed that field strengths ranging from about 20 to 30kV/cm were required to significantly reduce the viability of TFI92 cells. By contrast, the viability of *E. coli* cells are reduced with field strengths of less than 15kV/cm, and *E. coli* cells are also efficiently transformed at these field strengths (see 10th Quarterly Report).

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 DSM583 with respect to sensitivity and resistance to chloramphenicol, mercury and ampicillin was accomplished during the 10th Quarter. A preliminary characterization of chloramphenicol (CM) and mercury sensitivity of the strains TFI92 and TFI70 was performed. These studies have been extended to *Thiobacillus cuprinus*.

Thiobacillus cuprinus sensitivity to antibiotics

Thiobacillus cuprinus is capable of growing both chemolithotrophically and heterotrophically, and in a pH range from about 2.5 to 7. These characteristics make *T. cuprinus* an attractive host for transformation experiments. The following data indicate that this strain is very sensitive to chloramphenicol and ampicillin, and tests continue to determine the resistance of this strain to mercury chloride and tetracycline.

Antibiotic sensitivity was assessed by culturing *T. cuprinus* in a simple medium (ATCC medium 64 plus 0.05 percent yeast extract, pH 3.9) and using 10 μl of this culture to inoculate duplicate microtiter wells filled with 200 μl of antibiotic containing media. Table 1 summarizes the concentrations tested.

TABLE 1. Antibiotic Sensitivity Testing for *T. cuprinus*

Antibiotic	Concentration Levels, μg/ml
Ampicillin	0, 2, 5, 10, 20, 50, 100,
Chloramphenicol	0, 5, 10, 20, 50, 100, 200, 300

After three days of incubation at room temperature, the ampicillin wells showed growth up to 5 μg/ml but no growth at 10 μg/ml (cell growth is being measured quantitatively by optical density). The chloramphenicol well series had growth at 0 μg/ml but not at 5 μg/ml. After more than a week, all of the wells showed some signs of very faint turbidity, but not significant growth. In addition, tetracycline and mercury sensitivity was tested in similar assays, with preliminary indications that the strain is sensitive to less than 5 μg per ml of tetracycline or 0.5 μg per ml mercury chloride. The tetracycline and mercury sensitivity tests are being repeated.

Induction of Mercury Resistance in *Thiobacillus* Strains DSM583, TFI70 and TFI92

As described in the January report, experiments where *Thiobacillus* were plated onto TSM plates containing 0.5 μg per ml of $HgCl_2$, some small, variable number of colonies were routinely recovered. *Thiobacillus* from these colonies were resistant to up to 4 μg per ml of $HgCl_2$, indicating that these cells were "adapted" to allow growth in the presence of Hg^{++}. These small colonies represent the background in the transformation experiments. Further experiments indicated that these three strains of *Thiobacillus* can be preadapted to growth on Hg^{++} simply by first culturing them on a sub-lethal dose of Hg^{++}. Characterization of this induction effect is in progress.

Southern Blot Hybridization of *Thiobacillus* Chromosomal DNA

The presence of chromosomal mercury resistance genes was previously detected by Southern blot of total genomic DNA isolated from the *Thiobacillus* strains TFI92, TFI70 and DSM583 (see 10th Quarterly report). Additional *Thiobacillus* strains available in our laboratory as well as 8 new strains from the ATCC service are currently being tested for sensitivity to mercury and for the presence of *mer* genes. The use of the mercury resistant trait as a selectable marker will depend on the identification of a suitable mercury sensitive host.

Screening of the DSM583 Library

It was planned that the genes encoding mercury resistance from either Tn501 or from *T. ferrooxidans* strain DSM583 would be used as a selectable genetic marker for transformation. Cloning of the *Thiobacillus mer* gene from a genomic library, therefore, was a high priority item. A genomic library using DSM583 DNA fragments cut with the enzyme Pst I and cloned into the vector pBluescriptIISK has been constructed and over 3000 individual clones were transferred to nitrocellulose filters.

This Pst I library was screened by filter hybridization to identify any *Thiobacillus mer* genes with homology to the *mer* gene cluster isolated from

the transposable element Tn501. 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.

Cloning of the *Thiobacillus mer* Operon of DSM583

The filter replicas of the library of DSM583 chromosomal DNA described above were screened by hybridization to the Tn501 mer gene probe. Eighteen possible clones were picked in the primary screen, and plasmid DNA was prepared from each. Restriction analysis and Southern blotting identified four possible *mer* gene clones. Plasmids from two of these clones were retransformed into *E. coli*, and the ampicillin resistant transformants were tested for mercury resistance. Preliminary experiments indicated that both of these clones confer a moderate degree of resistance to mercury (up to 2.5 μg per ml in liquid LB media). A more complete characterization (including restriction mapping) of these clones is underway.

CONCLUSIONS

1) Plasmid pTFI91 from *Thiobacillus ferrooxidans* strain TFI91 has been precisely mapped by restriction analysis.

2) Conserved regions of DNA are shared by a family of plasmids isolated from laboratory strains of *Thiobacillus*, and these conserved sequences have been mapped in the pTFI91 plasmid.

3) Conserved regions in the *Thiobacillus* plasmids may indicate the location of functional replicons in these plasmids.

4) *Thiobacillus cuprinus* is sensitive to chloramphenicol and may be a convenient host for electroporation of *Thiobacillus* shuttle vector plasmids.

5) The *mer* gene from *T. ferrooxidans* DSM583 has been cloned. Experiments aimed at specifically identifying mercury resistance genes are underway.

FUTURE PLANS

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

- Evaluation and further development of *Thiobacillus* electroporation methodology.

- Characterization of the cloned mercury resistance gene from *T. ferrooxidans* DSM583.

- Experiments to further define restriction maps and conserved sequences in the family of *Thiobacillus* plasmids.

- Testing of additional *Thiobacillus* strains from the ATCC culture collection for suitable electroporation hosts.

- Evaluation of selected plating conditions for potential *T. ferrooxidans* and *T. cuprinus* transformants.

- Testing of specific plasmid constructs by transformation into *T. ferrooxidans* and *T. cuprinus*.

- Analysis of DNA from putative transformant *Thiobacillus* colonies for the presence of plasmid sequences.

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