MOLECULAR BIOLOGICAL ENHANCEMENT OF COAL BIODESULFURIZATION

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SIXTH QUARTER REPORT (February-April 1990)

Prepared by
**Barbara A. Bielaga
John J. Kilbane**

IGT Project No. 61095

for
**U.S. Department of Energy
Pittsburgh Energy Technology Center**

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EXECUTIVE SUMMARY

Three research topics were addressed during this quarter (February 1990 to April 1990): genetic experiments, biochemical experiments, and experiments with coal.

A small cryptic plasmid has been detected in Rhodococcus rhodochrous strain ATCC 19067 and is being used to construct a cloning vector capable of replicating in Rhodococcus rhodochrous IGTS8. The construction of this cloning vector will provide a tool that will, for the first time, allow certain genetic analyses to be performed using Rhodococcus rhodochrous IGTS8. Accordingly, the successful construction of this cloning vector will be a major breakthrough in the analysis and enhancement of the desulfurization trait of IGTS8. Conjugation experiments with plasmid pRF2 have now been successful with Rhodococcus fasciens, Rhodococcus globulus ATCC 19370, and Rhodococcus rhodochrous ATCC 13808. Transconjugants have not yet been obtained using IGTS8.

Biochemical experiments have been performed that provide compelling evidence for the presence of a modified "4S" desulfurization pathway in IGTS8. Dibenzothiophene (DBT), DBT-sulfoxide, DBT-sulfone, and thianthrene (as well as other organosulfur compounds) are each capable of serving as the sole source of sulfur for the growth of IGTS8. GC/MS and GC/AES analyses have revealed that the only detectable metabolite produced by IGTS8 from either DBT, DBT-sulfoxide, or DBT-sulfone is 2-hydroxybiphenyl. Moreover, the metabolism of thianthrene by IGTS8 yields 2-hydroxydiphenylsulfone. This biochemical work is an important contribution to the project as it is the first documentation ever of a microbial pathway for the sulfur-specific metabolism of organosulfur compounds. Biochemical investigations have also been performed to examine the range of organosulfur compounds that can be used as carbon and/or sulfur sources by IGTS8 and related Rhodococcus rhodochrous strains. IGTS8 is unique in its ability to utilize most organosulfur compounds as sulfur sources and only one organosulfur compound (1,3-propanedithiol) appears to be utilized as both a carbon and a sulfur source by IGTS8. Furthermore, some organosulfur compounds have been found to be inhibitory to IGTS8 but not to other bacteria such as Escherichia coli or Enterobacter species. This inhibition phenomenon may be related to the desulfurization ability of IGTS8 and will be investigated further as it could

potentially be used to develop an assay for the presence or absence of the desulfurization trait. Additionally, biochemical experiments were performed in which the proteins of IGTS8 were examined. These initial experiments have revealed several differences in the protein contents of IGTS8 as compared with a desulfurization-deficient mutant of IGTS8.

Experiments with coal were begun during this quarter. The kinetics of organic sulfur removal from an Illinois No. 6 coal using IGTS7 and IGTS8 will be determined. These experiments are intended to establish a baseline to which derivatives of IGTS8 improved by molecular biology can be compared.

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OVERALL OBJECTIVE

The overall objective of this project is to use Molecular Genetics to develop strains of bacteria with enhanced ability to remove sulfur from coal, and to obtain data that will allow the performance and economics of a coal biodesulfurization process to be predicted.

WORK PLANNED FOR THE CURRENT QUARTER

The work planned for the current quarter (February 1990 to April 1990) includes the following tasks:

- Developing techniques for the genetic analysis of Rhodococcus rhodochrous IGTS8; particularly electroporation and conjugation
- Include Rhodococcus strains other than IGTS8 in conjugation and electroporation experiments, and examine these strains for the presence of plasmids
- Perform further biochemical analyses of IGTS8.

WORK PERFORMED DURING THE CURRENT QUARTER

Development of Genetic Techniques

The development of genetic techniques to introduce DNA into IGTS8 and their subsequent use to study the desulfurization trait of this microorganism continues to be the highest research priority. Genetic tools and procedures for use in Rhodococcus rhodochrous are not currently available for many of the experiments relevant to this project. Therefore, the development of genetic tools and procedures is crucial to the success of this project and is currently the focus of most of our research. The key limitation in our ability to perform genetic analyses of IGTS8 at this time is the lack of a cloning vector, or any plasmid at all, that is capable of replicating in IGTS8 in particular and Rhodococcus rhodochrous in general.

The entire collection of Rhodococcus cultures in our possession has been examined by a variety of DNA isolation procedures for the presence of small plasmids that might be used to construct a cloning vector capable of replicating in IGTS8. A potentially suitable plasmid has been found in Rhodococcus rhodochrous ATCC 19067 and Figure 1 contains a reproduction of a DNA electrophoresis gel that illustrates the presence of this plasmid. The plasmid, designated pKGB1, possesses no known genetic markers so there is no way to select for the presence of pKGB1. That plus the lack of an optimized method for the isolation of DNA from Rhodococcus rhodochrous has made it difficult to purify pKGB1. Thus far we have been unsuccessful in obtaining significant quantities of pKGB1, DNA, and indeed, many of our plasmid purification experiments have failed to yield any pKGB1 DNA whatsoever. Nonetheless we have succeeded in partially characterizing pKGB1 inasmuch as it is approximately 9 kilobasepairs and possesses cleavage sites for the restriction enzymes EcoR1, Bcl1, and Xho1. The focus of current research on this topic is to ligate pKGB1 to pBR325. This will result in a composite replicon that possesses several selectable genetic markers contributed by pBR325 (resistance to tetracycline, chloramphenicol, and ampicillin) as well as a variety of known restriction endonuclease cleavage sites. This composite replicon should be capable of replicating in Rhodococcus rhodochrous, and should be an extremely useful tool in the genetic analysis of IGTS8.

Conjugation experiments with the Rhodococcus fasciens plasmid pKF2 are now routinely successful using R. fasciens strains as recipients and have

Figure 1. DNA ELECTROPHORESIS GEL SHOWING A PLASMID DETECTED IN A
RHODOCOCCUS RHODOCHROUS CULTURE

recently been successful with R. rhodochrous ATCC 13808, and with R. globulus ATCC 19370. Other Rhodococcus strains, including IGTS8, have so far been unable to acquire pRF2 in conjugation experiments. However, now that strains of R. rhodochrous and R. globulus that possess pRF2 exist it may be that one of these strains can successfully be used as donors of pRF2 in conjugations with IGTS8. If pRF2 can be successfully introduced into IGTS8, it is hoped that the plasmid may be useful as a means to introduce transposable elements into IGTS8, to mobilize nonconjugal plasmids (such as cloning vectors), and/or to mediate the transfer of chromosomally-encoded DNA.

Biochemical Analyses

Biochemical research during this quarter has included metabolic pathway studies, investigations with a wide range of organosulfur compounds, and preliminary analyses of the proteins of IGTS8.

The pathway of DBT biodegradation that results in the specific removal of sulfur from the molecule has been hypothesized to occur via the "4S" pathway DBT/DBT sulfoxide/DBT sulfone/DBT sulfonate/dihydroxybiphenyl plus sulfate. The biochemical pathway of desulfurization possessed by IGTS8 has now been definitively established to be a variant of the "4S" pathway resulting in the conversion of DBT to 2-hydroxybiphenyl.

Experiments performed during the past quarter examined the kinetics of utilization of DBT, DBT sulfoxide, and DBT sulfone in the IGT Sulfur Bioavailability Assay and the products of bacterial (IGTS8) metabolism of each of these compounds as determined by GC/MS. Additionally the metabolites of thianthrene produced by IGTS8 were also investigated by GC/MS and GC/AES (Atomic Emission Spectroscopy).

The IGT Sulfur Bioavailability Assay reveals that DBT, DBT sulfoxide, DBT sulfone and thianthrene can all be successfully used by IGTS8 as the sole source of sulfur for growth; however, the kinetics of utilization vary. DBT and DBT sulfone are utilized more readily than is DBT sulfoxide which exhibits a minor but reproducible delay in utilization by IGTS8. Moreover cultures grown with DBT sulfoxide never quite reach the same optical density as cultures grown with DBT or DBT sulfone irregardless how long the cultures are incubated. This may be inconsistent with the hypothesis that sulfur-specific metabolism of DBT produces DBT sulfoxide and DBT sulfone as intermediate

products in the pathway of DBT desulfurization. On the other hand, GC/MS studies show that the ONLY metabolites detected in IGTS8 cultures grown with either DBT, DBT sulfoxide, or DBT sulfone is monohydroxybiphenyl. Moreover, the yield of monohydroxybiphenyl from each of these three compounds is equal (at equivalent cell densities), and corresponds to the minimum sulfur (sulfate) requirement for bacterial growth, approximately 0.2 mM.

Thianthrene (C_{12}, H_8, S_2) is also utilized by IGTS8 as the sole source of sulfur and the resultant metabolites have been investigated by GC/MS and GC/AES. Atomic emission spectroscopy is a state of the art analytical technique that allows the empirical formulas of unknown compounds to be determined unequivocally and also allows unknown compounds to be accurately quantified. Both of these capabilities using a GC/AES are far superior compared to what can be obtained using GC/MS. Our investigations have revealed that only a single thianthrene metabolite is detectable by our analytical techniques using IGTS8 cultures, and the structure of that metabolite has now been accurately determined. The mass spectrum of this compound is shown in Figure 2. The structure tentatively proposed for this metabolite indicates that some hydrogen bonding and electron sharing is apparently taking place leading to the structures pictured in Figure 3. This/these structures are thermodynamically stable and may pose steric hindrance to further metabolism by IGTS8 which would explain why this metabolite accumulates in IGTS8 cultures. Again, the quantity of this metabolite that is observed corresponds to the minimum sulfur requirement for bacterial growth, approximately 0.2 mM in the original aqueous culture supernatant. This presumptive metabolite of thianthrene produced by IGTS8 is entirely consistent with a variant of the "4S" pathway of desulfurization resulting in the formation of monohydroxybiphenyl from the metabolism of DBT. Taken altogether then, the fact that DBT sulfoxide and DBT sulfone are both metabolized to yield monohydroxybiphenyl and that thianthrene may be metabolized by a pathway that yields a sulfone intermediate, strongly suggests that IGTS8 possesses a variant of the "4S" pathway. This biochemical work is an important contribution to the project, and it is the first documentation ever of a microbial pathway for the sulfur-specific metabolism of organosulfur compounds.

Figure 2. MASS SPECTRUM OF A METABOLITE OF THIANTHRENE PRODUCED BY IGTS8

Figure 3. PROPOSED STRUCTURE OF A METABOLITE OF
THIANTHRENE PRODUCED BY IGTS8

A new experimental approach was initiated during the past quarter in an effort to produce a derivative of IGTS8 that has enhanced expression of carbon-sulfur bond cleaving enzymes. That approach involves the use of organosulfur compounds as sole carbon sources as well as sole sulfur sources for growth. This strategy was avoided previously in this project during attempts to isolate bacteria that possess specificity/preference for the cleavage of carbon-sulfur bonds because the literature demonstrated that this approach uniformly led to the isolation of bacteria that possessed the Kodama pathway, or the carbon-destructive pathway of DBT metabolism. Instead, we employed organosulfur compounds as sulfur sources and provided alternative sources of readily metabolizable carbon sources. However, now that we already possess an organism, IGTS8, that produces enzymes with a specificity for the cleavage of carbon-sulfur bonds, the strategy of using organosulfur compounds as sole sources of both carbon and sulfur has potential advantages. The goal continues to be to produce bacteria/enzymes that will specifically remove organically bound sulfur from coal while leaving the calorific value/carbon content of the coal intact. The use of some organosulfur compounds, that are not typically found in coal, as both carbon and sulfur sources for IGTS8 may be a good strategy to obtain increased levels of expression of carbon-sulfur bond cleaving enzymes. Since carbon compounds are used by bacteria both as energy sources and as sources of carbon for incorporation into biological compounds, a growing bacteria requires vastly greater quantities of carbon than sulfur. Therefore, if an organosulfur compound serves not only as the sole sulfur source but also as the sole source of carbon and energy then much greater quantities of the compound must be metabolized in order to support bacterial growth. Accordingly, if the initial step in the metabolism of such compounds occurs by the cleavage of carbon-sulfur bonds then the use of certain organosulfur compounds as sole sources of sulfur, carbon, and energy for the growth of IGTS8 may result in derivative cultures that express higher levels of desulfurization enzymes.

The organosulfur compounds that have been tested as carbon sources are 1,3-dithiane, 2-trimethylsilyl-1,3-dithiane, 1,4-dithiane, 1,4-dithiane-2,5-diol, trans-1,2-dithiane-4,5-diol, 1,5-dithiacylooctan-3-ol, 1,4,7-trithiacyclononane, trithiane, 1,3-propanedithiol, benzyldisulfide, phenyldisulfide, 2-nitrophenyldisulfide, 3-nitrophenyldisulfide,

4-nitrophenyldisulfide, 2-aminophenyl disulfide, and 4-aminophenyldisulfide. IGTS8 is routinely grown using glycerol or ethanol as the carbon source, so it was thought that the cleavage of carbon-sulfur bonds in the compounds listed above would result in the liberation of metabolites containing from one to four consecutive carbon atoms that might then serve as carbon and energy sources for IGTS8. Preliminary testing shows that all of the compounds listed above except 1,3-dithiane, 2-trimethylsilyl-1,3-dithiane, 1,4,7-trithiacyclononane, 3-nitrophenyldisulfide, 2-aminophenyldisulfide, and 4-aminophenyldisulfide can serve as sulfur sources for IGTS8, yet only one of these compounds (1,3-propanedithiol) has been observed to serve as a carbon and energy source. These results are confusing. It is not clear how the majority of these organosulfur compounds can serve effectively as sulfur sources, yet apparently only one can serve as a carbon source. Moreover, the reason the 1,2-dithiane and 1,4-dithiane compounds can serve as sulfur sources for IGTS8 but 1,3-dithiane compounds can't is not apparent. These observations will be confirmed by further testing.

These studies with a variety of organosulfur compounds has also led to the observation that some organosulfur compounds may inhibit the growth of IGTS8, but are not toxic to other microorganisms, such as *Enterobacter* cultures. This observation is preliminary and must be confirmed and extended; however, the data in hand suggests that these organosulfur compounds may specifically inhibit IGTS8 rather than being generally toxic to microorganisms. Therefore this inhibition may be due to the desulfurization enzymes of IGTS8 transforming these compounds into toxic metabolites. Future work will examine the toxicity of these compounds with strains of *Rhodococcus rhodochrous* in addition to IGTS8 to determine if the observed toxicity is related to the desulfurization trait.

Analysis of proteins possessed by IGTS8 was begun during the past month with the aim being to identify desulfurization enzymes. Figure 4 illustrates results obtained with various concentrations of two cell cultures: IGTS8 and a desulfurization mutant of IGTS8. The two gels pictured are protein extracts of bacterial cultures electrophoresed on 10% acrylamide gels and stained with coomassie blue dye. Gel 149-1 represents samples obtained from sonicated cells and gel 149-2 contains samples obtained by lysozyme treatment of bacterial cells. There are no obvious/conclusive differences in the protein bands seen

149-1

149-2

Legend: Gel 149-1 contains samples produced by the sonication of bacterial cells. Lane 1 is a Molecular Weight Standard. Lanes 2, 4, and 6 are derived from various concentrations of dsz⁺ IGTS8 cells. Lanes 3, 5, and 7 are derived from various concentrations of dsz⁻ IGTS8 cells. Gel 149-2 contains samples produced by lysozyme treatment of bacterial cells. Lane 1 is a Molecular Weight Standard. Lanes 2, 4, and 6 are derived from various concentrations of dsz⁺ IGTS8 cells. Lanes 3, 5, and 7 are derived from various concentrations of dsz⁻ IGTS8 cells. Lane 8 is a lysozyme control sample.

Figure 4. ANALYSIS OF PROTEINS DERIVED FROM dsz⁺ AND dsz⁻ IGTS8

on gel 149-1 when IGTS8 and the mutant culture are compared; however, gel 149-2 shows several differences. First of all, the intensity of the bands contributed by the lysozyme control varies between the IGTS8 and the mutant samples, which gives the appearance of greater differences between the samples than really exists. When lysozyme-derived bands are ignored there are still at least three protein bands that appear to differ between the IGTS8 and the mutant samples even when variations in sample concentration are taken into account. These three bands are indicated by arrows in Figure 4. These observations are encouraging and may allow us to identify desulfurization enzymes, but these results must be repeated before their significance can be appreciated.

In related experiments cells were obtained from three cell cultures: a desulfurization mutant of IGTS8, IGTS8 grown in the presence of excess sulfate, and IGTS8 grown with DBT as the sole source of sulfur. These cell populations were sonicated and then electrophoresed on a 12% acrylamide gel and stained with coomassie blue dye. Visual inspection of the gel shown in Figure 5 will reveal several differences in the protein band patterns between the various samples. Several other protein gels have also been run using various concentrations of acrylamide and using two different stains to visualize the protein bands; however it is not possible at this time to clearly identify a desulfurization-related protein.

Experiments With Coal

During this quarter, work with coal was begun to provide kinetics and mass balance data on the removal of organic sulfur from coal using IGTS7 and IGTS8. This data is intended to serve as a baseline with which to compare improved bacterial cultures resulting from this project.

Prior to this project biodesulfurization results were obtained with an Illinois #6 coal that is naturally low in pyritic sulfur. The previous coal biodesulfurization results were also obtained using a mixed bacterial culture IGTS7. The limitation of those previous results was that the coal was part of an experiment designed to develop bacterial cultures and not designed for coal treatment. Subsequently, neither kinetics nor mass balance data was obtained. In the current coal biodesulfurization tests the same batch of Illinois #6 coal that was used previously will again be used so that additional variables are not introduced into these experiments.

Figure 5. ANALYSIS OF PROTEINS DERIVED FROM INDUCED,
UNINDUCED, AND MUTANT POPULATIONS OF IGTS8

Legend: The sample in lane 1 is derived from a dsz⁻ mutant of IGTS8, lane 2 is derived from IGTS8 cells grown with an excess of sulfate, lane 3 is derived from IGTS8 cells grown with DBT as the sole source of sulfur, and lane 4 is a molecular weight standard.

The coal will first be ground to 200 mesh to increase the available surface area, and will then be Soxhlet extracted with water to remove leachable sulfate. The coal will then be used in biodesulfurization experiments testing four conditions: 1) IGTS7, 2) IGTS8, 3) sterile control, and 4) a non-sterile control. The time points that will be examined will be 0, 1 growth cycle (about two weeks), 1 month, 2 months, and 3 months. The experiments will be performed by requiring bacteria to grow using coal as their sole source of sulfur. Sulfur-free mineral salts medium containing 50 mM glucose and/or glycerol will be used. The maximum amount of any organosulfur compound desulfurized by IGT's cultures corresponds to the concentration needed to satisfy the bacteria's growth requirement (about 0.2 mM sulfate). The sulfur requirement for growth and the cell density of IGTS8 that can be obtained in sulfur-free BSM is such that to desulfurize 1 gram of coal containing 2% organic sulfur a minimum of 6 liters of bacterial culture must be grown at the expense of the sulfur in that coal. This is inefficient and is precisely why the current DOE program is focused on improving the efficiency of biodesulfurization using molecular genetics. Nonetheless there is no good alternative at this time. If desulfurization-competent cells are pregrown, concentrated, and used to desulfurize coal an even greater amount of bacterial cells are required to achieve desulfurization (see the fifth quarterly report). Moreover, the desulfurization trait of IGTS8 is inducible; therefore, the conditions needed to achieve desulfurization of coal are exacting. The desulfurization trait of IGTS8 (IGTS7) is induced by physiological stress due to sulfur starvation and the presence of readily metabolizable sulfur such as sulfate leached from coal or sulfur compounds liberated by dead/lysed bacteria are preferentially metabolized and prevent the removal of organically-bound sulfur from coal. The mere presence of IGTS7 and IGTS8 in an experiment does not in and of itself constitute a sufficient condition for desulfurization. These tests will be performed under conditions that allow for the maximum expression of the desulfurization trait.

The analysis of coal samples will be performed using IGT's modification of the ASTM procedure in which the coal sample is split into two portions. A total sulfur determination is performed on one portion while the other portion is progressively submitted to chemical treatments to determine the sulfate, pyritic, and the non-pyritic sulfide content followed by a total sulfur determination of the coal residue. One gram samples of coal are sufficient

for all of these analyses. Significant results will subsequently be confirmed using other analytical techniques such as elemental analysis, the electron microbeam method for the direct determination of organic sulfur, EXAFS and/or EXANES. Alternatively, some biotreated coal samples may be analyzed to determine their calorific value, or subjected to other forms of analysis. Future tests of biodesulfurization may also be performed using other coal such as the microbially depyritized coal samples generated by Louisiana State University.

WORK PLANNED FOR THE NEXT QUARTER

The work planned for the next quarter (May 1990 to July 1990) will include:

- Construct a cloning vector that can be used in Rhodococcus rhodochrous IGTS8 from the small cryptic plasmid found in Rhodococcus rhodochrous ATCC 19067
- Develop techniques for the genetic analysis of IGTS8
- Continue biochemical experiments, particularly those that may allow the identification of desulfurization-related enzymes
- Continue experiments with coal to determine the kinetics of organic sulfur removal.

GLOSSARY

Auxotroph -- A bacterial strain that contains a mutation in one or more genes involved in the synthesis of amino acids or vitamins. Such strains require that specific amino acids or vitamins are present in a bacterial growth medium to grow, because, unlike other bacteria (prototrophs), they lack the ability to synthesize a particular amino acid or vitamin.

Clone -- An identical copy. Clones can be entire organisms such as identical twins, or molecular clones in which a single gene (or even a portion of a gene) is copied/reproduced. Molecular cloning often involves obtaining a fragment of chromosomal DNA that encodes a desired gene and ligating/fusing that DNA fragment to a plasmid. Because plasmids are capable of replication it will not only replicate itself, but also the newly inserted/cloned fragment of chromosomal DNA.

Conjugation -- Is the self-directed transfer of plasmid DNA from one bacterium to another.

Gene -- Is a segment of DNA that is involved in producing a polypeptide chain.

Ligation -- Is the formation of a phosphodiester bond to link to adjacent bases separated by a nick in one strand of a double-stranded DNA molecule.

Phage -- (Bacteriophage) is a bacterial virus.

Plasmid -- An extrachromosomal circular molecule of DNA capable of replication, often being present in multiple copies per cell. Plasmids are several orders of magnitude smaller than chromosomes and encode auxiliary functions. The chromosome encodes for all of the functions that allow a bacterial cell to reproduce and be identified as a particular species of microorganism; whereas, plasmids are not present in all bacteria and encode for specialized functions such as resistance to antibiotics. Plasmids can not only be inherited from one generation to the next, but can often also be transferred to other bacteria of the same or related species. This and the fact that plasmid DNA can be purified from bacterial cells with relative ease make plasmids useful tools in research involving bacterial genetics. The name of a plasmid always starts with a lower case letter p, for example pBR325.

Restriction Endonucleases (enzymes) -- Recognize specific short sequences of DNA and cleave the double stranded molecule, most often yielding DNA fragments with short single-stranded ends. The various restriction enzymes are named by abbreviating the name of the species of origin followed by a number, that is, *ECO* RI.

Transconjugant -- A bacterium that has acquired DNA by the process of conjugation.

Transduction -- Refers to the transfer of a gene from one bacterium to another by a phage.

Transformant -- A bacterium that has acquired DNA by the process of transformation.

Transformation of Bacteria -- Describes the acquisition of new genetic markers by incorporation of added DNA.

Transposon -- Is a DNA sequences able to replicate and insert a copy of itself at a new location in the genome. Transposons are named with the prefix Tn, followed by a number, that is, Tn 917.

Vector -- A general term referring to genetic elements (plasmids, viruses, transposons, and others) that contain or can be constructed to contain a DNA fragment of interest. Vectors are sometimes referred to as vehicles.

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