FINAL TECHNICAL REPORT

September 1, 1991 through August 31, 1992

Project Title: Genetic Engineering of Sulfur-Degrading *Sulfolobus*
ICCI Project Number: 91-1/2.3A-6
Principal Investigator: Nancy W.Y. Ho
Laboratory of Renewable Resources Engineering
Purdue University
1295 Potter Building
West Lafayette, IN 47907-1295

Project Monitor: Dr. Dan D. Banerjee, CRSC

ABSTRACT

It is generally agreed that an effective microbial process would be most economical for the removal of organic sulfur and the finely dispersed inorganic sulfur in coal, particularly the former. However, past experience has taught us that some microorganisms may have the enzymes to degrade organic sulfur but themselves are not suitable to be cultured under the conditions best for coal desulfurization. On the other hand, some microorganisms can grow very well under the conditions most suitable for coal desulfurization but do not contain the enzymes most suitable for coal desulfurization.

In this "genetic recombinant" era, we believe that we can adopt a new approach to solve this problem. In this approach, a microorganism that can grow vigorously under the conditions most suitable for coal desulfurization will be chosen as the host for actively carrying out the desulfurization process. An effective gene cloning system is expected to be established for the chosen species so that heterologous and homologous genes encoding most of the effective enzymes for coal desulfurization can be cloned on the high copy-number plasmid or plasmid-like molecules. As a result, the sulfur-degrading enzymes can be overproduced and an effective and economical biodesulfurization process for both organic and inorganic sulfur in coal can be developed.

Species of *Sulfolobus* are microorganisms that can be actively cultured under the conditions most favorable for coal desulfurization. The objective of this project is to develop an effective gene cloning system for some of the *Sulfolobus* species.

One important element for the establishment of a gene cloning system for a microorganism is to have a selection mechanism. Up to now, we have developed three selection mechanisms for the establishment of a gene transfer system for the *Sulfolobus* species. In this year, we also constructed the best cloning vectors for the *Sulfolobus* species.

EXECUTIVE SUMMARY

In order to develop a successful microbial process for the removal of organic sulfur in coal, it is necessary to develop stable strains which can remove organic sulfur from coal at fast rates and can also withstand the harsh conditions favorable for desulfurization of coal. After years of intensive searching, no such ideal microorganism has been found. Taking advantage of the recent advances in recombinant DNA and gene cloning techniques, a more practical approach is to combine the properties of several diverse bacterial strains to construct by design a hybrid species which has most of the desired properties if not all of them, for the removal of organic sulfur from coal. More specifically, if a host which can grow very well under the conditions best for coal desulfurization and has an effective gene cloning system allowing it to stably amplify foreign and native genes and overproduce high levels of the enzymes encoding by the cloned genes, such an organism may be the best candidate for the development of a cost-effective microbial process for the removal of organic sulfur from coal.

Sulfolobus species are known to be able to grow very well under the conditions most suitable for coal desulfurization. Our recent results showed that the development of an effective gene cloning system mediated by high copy-number plasmids allowing the overproduction of numerous folds of foreign and native enzymes are within reach. The major objective of our research for this project is to continue our effort for the development of an effective gene cloning system for the *Sulfolobus* species. Our approach to completing this objective is to insert a kanamycin-resistance gene (Km^R), encoding a heat-stable enzyme that inactivates the antibiotic kanamycin, into a piece of *Sulfolobus* DNA which contain a replication origin. We have identified such a piece of DNA and constructed several cloning vectors which theoretically should be able to be stably maintained in *Sulfolobus*. The vectors are also high copy-number in nature.

Another objective of our research for this project is to reinvestigate the possible use of the sulfur moiety in model compound DBT as its sulfur source by some of the *Sulfolobus* species. Our results seem to imply that some of the *Sulfolobus* species can be induced to use DBT as its sulfur source transiently, but not long lasting. In addition, we have also developed the necessary techniques to culture *Sulfolobus* on solid plates. We have also successfully electroporated *Sulfolobus* species.

OBJECTIVE

The objectives of the proposed research is to first establish a plasmid-mediated genetic transformation system for the selected *Sulfolobus* species. With establishment of such a genetic transformation system for these microorganisms, they may be genetically engineered to be able to effectively remove organic sulfur from coal.

INTRODUCTION AND BACKGROUND

It is generally agreed that the best cost-effective process for coal cleaning is a combined physical microbial process which uses physical methods to first remove the coarsely disseminated large particles of pyrite, followed by using a microbial process to remove both organic sulfur and finely disseminated inorganic sulfur (pyrite). However, past experience has taught us that some microorganisms may have the enzymes to degrade organic sulfur but themselves are not suitable to be cultured under the conditions best for coal desulfurization. On the other hand, some microorganisms can grow very well under the conditions most suitable for coal desulfurization but do not contain the enzymes most suitable for coal desulfurization.

In this "recombinant" era, we believe that we can adopt a new approach to solve this problem. In this approach, a microorganism that can grow vigorously under the conditions most suitable for coal desulfurization will be chosen as the host for actively carrying out the desulfurization process. An effective gene cloning system is expected to be established for the chosen species so that heterologous and homologous genes encoding most effective enzymes for coal desulfurization can be cloned on the high copy-number cloning vectors. As a result, the sulfur-degrading enzymes can be overproduced and an effective and economical biodesulfurization process for both organic and inorganic sulfur in coal can be developed.

Sulfolobus species can grow very well under the conditions most suitable for coal desulfurization. Our recent results showed that it is highly possible to develop an effective gene cloning system for this species. Our approach to develop a gene cloning system for the *Sulfolobus* species is to identify a piece of DNA containing a replication origin and to convert the DNA into a cloning vector.

For development of a gene cloning system for a microorganism, it also requires establishing at least one selection mechanism. So far we have established at least three different selection mechanisms for the development of a genetic transformation systems for the *Sulfolobus* species. One such system is to use a kanamycin resistance gene. Another is to use the *Sulfolobus* genes for tryptophan synthesis.

For the isolation of the DNA fragment containing the desired replication origin, we have to develop a pro-vector. It is a 1 kb DNA fragment. In order to ensure that the isolated DNA fragment have the desired features we not only had to clone the DNA fragment but we also had to sequence it.

EXPERIMENTAL PROCEDURES

DNA Sequencing

pKS-SF was used to transform *E. coli* strain CJ236. Amp resistant colonies were selected, cultured, in LB-Amp medium, and infected with a helper phage to produce single-stranded DNA. The resulting single-stranded DNA was used as the template for DNA sequencing. Proper oligonucleotides were synthesized and used as the primers to obtain the desired sequence data.

Culturing the Sulfolobus Species with DBT

DBT was added to the regular cultural medium for *Sulfolobus* to replace the sulfate ion or to replace both the sulfate ion and the carbon source.

RESULTS AND DISCUSSION

DNA Sequence Analysis of the 1 kb Provector DNA Fragment

We sequenced more than 80% of the 1 kb provector genes cloned on pKS-SF. The DNA sequence information is essential for determining where to insert the Km^R gene. From the nucleotide sequence, we find the best site for the insertion of the Km^R gene. Insertion of Km^R at the selected site will make it possible to express the antibiotic gene with a strong promoter. However, the recombinant DNA fragment resulted from the insertion of the Km^R gene at the strong promoter may not contain sufficient 5' non-coding sequence (less than 250 bp) homologous to the DNA fragment that contains the replication origin. The latter is the genetic process dependent on for transferring the heterologous genes to the DNA fragment in vivo. Thus, we selected a second site insertion of the Km^R. Insertion of Km^R at the second site will give the resulting recombinant provector a better chance to be incorporated into the DNA fragment by homologous recombination. However, at the second site, the inserted Km^R is placed under a weaker promoter.

Construction of Recombinant Provectors for the Sulfolobus Species

We have completed the construction of four *Sulfolobus* provectors. In two of them, the Km^R is expressed by the entire genetic elements for *Sulfolobus* species gene expression. The Km^R gene was expressed only by the *Sulfolobus* promoters in the other two provectors.

Isolation of Genes Encoding Enzymes for Tryptophan Biosynthesis in Sulfolobus Species

E. coli tryptophan mutants were obtained from various labs. A *Sulfolobus* gene bank was also prepared. This gene bank was used to transform the various *E. coli* tryptophan mutants. We found that at least two *Sulfolobus* genes for its tryptophan synthesis can be isolated by this approach.

CONCLUSION

We have constructed four vectors for the transformation of *Sulfolobus* species. We also have provided an alternative selection mechanism in case the bacterial Km^R cannot be expressed in the selected *Sulfolobus* species.

Genetic transformation systems have been developed for several thermophilic microorganisms. Genetic transformation systems have also been developed for some of the acidophilic microorganisms. Thus, it should not be too difficult to develop such a system for the *Sulfolobus* species.

Publications

The following papers which have acknowledged or will acknowledge the support by ICCI/DOE have been submitted or are in preparation for publication.

1. Ho, N.W.Y. and Brainard, A., "Sensitivity of *Sulfolobus acidocaldarius* to 15-Fluorouracil and the development of a resistant strain of the latter species to the pyrimidine analog," resubmitted to Fuel (1992).
2. Ho, N.W.Y., "Potential selection mechanisms for the establishment of a genetic transformation system for *Sulfolobus acidocaldarius* and related species," presented at the Second International Symposium on Biological Processing of Coal, and has been published in the Proceedings of the Symposium.
3. Ho, N.W.Y., Chen, Z. D., and Brainard, A., "Isolation and sequence analysis of a *Sulfolobus acidocaldarius* promoter fragment capable of functioning in *E. coli*," submitted to Energy and Fuel (1992).
4. Ho, N.W.Y., and Chen, Z. D., "Functional Expression of Km^R with *Sulfolobus* promoters in *E. coli*," manuscript in preparation.