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TECHNICAL REPORT

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Project Title: Genetic Engineering of Sulfur-Degrading *Sulfolobus*

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ABSTRACT

Recent studies have shown that some microorganisms can play a significant role in removing the sulfur compounds from coal. *Sulfolobus acidocaldarius* is one such microorganism. Some microorganisms can remove only organic sulfur from coal, other can remove only inorganic sulfur from coal, but *S. acidocaldarius* seems to be able to remove both the organic and the inorganic sulfur from coal. Furthermore, *S. acidocaldarius* has been shown to be able to use the sulfur and carbon derived from coal as its sole carbon and energy source for growth. These properties make this microorganism unique for coal desulfurization. This project is aimed at applying recombinant DNA techniques to improve the capability of *S. acidocaldarius* for coal desulfurization, which includes making it the host for housing foreign genes that encode the most effective enzymes for coal desulfurization. Since there is no established vectors and procedures for introducing vectors into *S. acidocaldarius* and related microorganisms, our immediate goal is to establish a gene cloning system for this species.

During the present quarter, we have studied a few systems which can be used as the potential selection mechanism for the selection of the desired transformants. In addition, during this quarter, we also analyzed the extracellular proteins from *S. acidocaldarius* as well as other potential organic sulfur removing species. Furthermore, we also managed to obtain most strains and plasmids that are needed for this work.

In the next quarter, our efforts will largely be concentrated on the isolation of the double stranded virus from *Sulfolobus* sp B12 which was obtained from Prof. Zillig of Max-Planck-Institut fur Biochemie, West Germany. During the next quarter, we will also try to isolate other plasmids and cellular DNA that we may need for this work. Preliminary construction of cloning vectors for *S. acidocaldarius* may also begin.

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EXECUTIVE SUMMARY

In order to establish a transformation system for the introduction of plasmids into *S. acidocaldarius*, it is necessary to establish a selection mechanism to differentiate the cells which have acquired the plasmid and those which have not. There are several ways to develop such a system. The most convenient way is to choose a chemical compound such as an antibiotic which can inhibit the growth of the particular strain. Furthermore, there is a gene, preferably already being cloned and stored on a plasmid which can produce a protein, making the particular strain resistant to the particular chemical. We have identified that there are three chemical compounds, designated as compound A, B, and C which are potentially useful to serve as the selective agent to establish a transformation system for *S. acidocaldarius*. In this period we have studied the sensitivity of *S. acidocaldarius* towards these three compounds.

We found that all three compounds can inhibit the growth of *S. acidocaldarius*. However, the degree of growth inhibition on *S. acidocaldarius* caused by these agents are different. The results for testing the sensitivity of *Sulfolobus* towards these three compounds are summarized in Table 1.

According to these results, all three compounds can be used as the selective agents. However, Compound #2 and Compound #3 are better than Compound #1. The resistance gene for Compound #2 has been cloned and we already have the cloned gene on hand. There is no cloned resistance gene for Compound #3, but it can be isolated and we are already in the process of isolating such a gene.

Proteins present in the culture medium of *Sulfolobus, E. coli*, yeast *Pichia stipitis* and another leading organic-sulfur degrading strain were isolated and subjected to acrylamide gel electrophoresis analysis. *E. coli* and yeast are used as controls. It is known that *E. coli* contains numerous secreted proteins and yeast *Pichia stipitis* does not contain much secreted proteins. We found that *Sulfolobus* secretes numerous proteins into its medium, but the leading organic sulfur-degrading species does not contain hardly any secreted proteins.

Table 1.

Sulfolobus Cultures(5-10ml)	OD600		
	2 days	4 days	6 days
blank(medium alone)	0	0	0
1(none)	0.053	0.047	0.03
2(25µ1A)	0.003	0.011	0.017
3(50µ1A)	0.002	0.002	0.013
4(100µ1A)	0.002	0.002	0.007
5(200µ1A)	0.001	0.001	0.004
6(25 µ1B)	0.003	0.003	0.008
7(50µ1B)	0.001	0.001	0.001
8(100µ1B)	0.001	0.001	0.003
9(200µ1B)	0.001	0.001	0.001
10(2µ1C)	-	-	-
11(4µ1C)	-	-	-
12(8µ1C)	-	-	-
13(10µ1)	-	-	-
14(none)	0.052	0.045	0.033

Concentration of A: 0.5 mg/ml
Concentration of B: 0.5 gm/ml
Concentration of C: 25.0 gm/ml

OBJECTIVE

The objectives of the proposed research is to first establish a plasmid-mediated genetic transformation system for the sulfur degrading *Sulfolobus*, and then to clone and overexpress the genes encoding the organic-sulfur-degrading enzymes from *S. acidocaldarius* as well as from other microorganisms, to develop a *Sulfolobus*-based microbial process for the removal of both organic and inorganic 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 which can remove both organic sulfur and finely disseminated inorganic sulfur (pyrite). Among the sulfur degrading microorganisms only *S. acidocaldarius* can remove both organic sulfur and inorganic sulfur from coal. Also the *Sulfolobus* species has been shown to be a facultative autotroph which can be cultured on coal and use the organic sulfur compounds released from coal as their sole carbon and energy source (24). Furthermore, *S. acidocaldarius* can tolerate strong acidic conditions and requires high temperature for growth. These properties make this species an ideal microorganism for the development of an effective microbial process for the desulfurization of Illinois coal, since it is known that all Illinois coal cannot be sufficiently cleaned to comply with the federal standards by using physical cleaning methods alone.

In order to make *S. acidocaldarius* to become a host for overproduction of sulfur degrading enzymes originally from this or other bacteria, it must have an established DNA-mediated transformation system for amplifying genes or transferring foreign genes into this microorganism. Currently no such system has been established for *S. acidocaldarius*. Hence, we proposed to establish such a system for this species.

EXPERIMENTAL PROCEDURES

Since we have proposed to use antibiotic geneticin as the selective agent for the establishment of a genetic transformation system for *S. acidocaldarius*, it is necessary to test whether this organism is sensitive to geneticin. In this period, we have studied the sensitivity of *S. acidocaldarius* to geneticin as well as to other antibiotics and compounds which are potentially useful as the selective agents for the establishment of a genetic transformation system for *S. acidocaldarius*.

In addition, we also studied the proteins secreted by *S. acidocaldarius* and a few other species potentially useful for the removal of organic sulfur from coal.

RESULTS AND DISCUSSION

In order to establish a plasmid-mediated genetic transformation system for *S. acidocaldarius*, one of the essential elements is to establish an effective selection mechanism to differentiate transformants from non-transformants. A selection mechanism usually contains a compound present in the cultural medium, which is either toxic to or non-utilizable by the nontransformed ordinary host cells, and a gene cloned on a plasmid,

which produces a protein product capable of modifying the toxic or non-utilizable compound and making it nontoxic to or utilizable by the host cells. Thus any cells which have acquired the plasmid will be able to grow on the cultural medium containing the compound.

Initially we proposed to use kanamycin resistance gene (Km^R) and antibiotic geneticin as the selection mechanism to differentiate the transformants form the non-transformants. In this period, we found that not only is *S. acidocaldarius* sensitive to geneticin (designated as compound #1) as we expected, it is also highly sensitive to two other compounds (designated compound #2 and #3) and there are gene products available to inactivate all three compounds. Therefore, not only one but there are three selection systems potentially useful for the development of a plasmid-mediated genetic transformation system for *S. acidocaldarius*. These results are reported here in Table 1.

CONCLUSION AND RECOMMENDATION(S)

Due to the fact that we have identified and developed three selection systems potentially useful for the development of selection system for *S. acidocaldarius*, the opportunity to develop an effective plasmid mediated transformation system for this organism has been greatly enhanced. Although there are genes available to inactivate all three compounds, two of them are *E. coli* bacterial antibiotic resistance genes. These *E. coli* genes may not function in *S. acidocaldarius*. Therefore, we should try to isolate a strong promoter from *S. acidocaldarius*, best that such promoter can also be functionally expressed in *E. coli*, to be used for the expression of the foreign genes in *S. acidocaldarius*. Such a promoter will also be useful for the expression of sulfur degrading genes from other microorganisms in *S. acidocaldarius*.