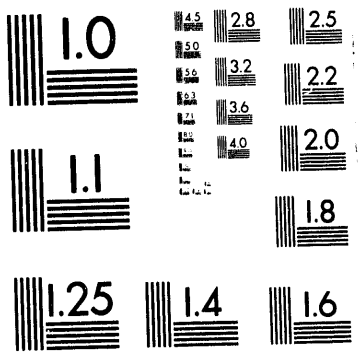
1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6

1 of 1

FINAL TECHNICAL REPORT
September 1, 1992 through August 31, 1993

Project Title: **MICROBIAL STRAIN IMPROVEMENT FOR ORGANOSULFUR REMOVAL FROM COAL** DE-FC22-92PC92521

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Project Manager: Dr. Ken Ho, ICCI

ABSTRACT

IGT has developed a microbial culture of Rhodococcus rhodochrous, designated as IGTS8, that is capable of specifically cleaving carbon-sulfur bonds in a range of organosulfur model compounds and is capable of removing organic sulfur from coal and petroleum without significantly sacrificing the calorific value of the fuel. Although IGTS8 possesses the ability to specifically remove organic sulfur from coal, a major research need is to develop improved strains of microorganisms that possess higher levels of desulfurization activity and therefore will permit more favorable biodesulfurization process conditions: faster rates, more complete removal, and smaller reactor size. Strain improvement is the single most important aspect to the development of a practical coal biodesulfurization process and accordingly is the focus of research in this project.

During the past year, significant progress was made toward improving the biodesulfurization capabilities of Rhodococcus rhodochrous IGTS8. Our main objective was to identify and characterize strong promoters of IGTS8. The DNA sequencing of the promoter region and chloramphenicol resistance gene of pRF2, as well as six mutant promoters, was determined. The 16S structural gene of IGTS8 was isolated and used to identify the putative promoter of this gene. Four promoter probe vectors were constructed and are currently being used to analyze the strength of Rhodococcus promoters: from the IGTS8 genome, mutants of promoters from the chloramphenicol resistance gene of pRF2, the promoter from the 16S RNA gene, and various strong inducible promoters.

EXECUTIVE SUMMARY

The objective of this project is to develop derivatives of Rhodococcus rhodochrous IGTS8 that possess enhanced levels of desulfurization activity and to evaluate the ability of improved cultures to remove organic sulfur from coal.

The approach taken in this project to achieve that objective is to use genetic engineering/recombinant DNA techniques to produce strains of IGTS8 with improved desulfurization activity. IGTS8 has been shown to be capable of specifically cleaving carbon-sulfur bonds in a range of organosulfur model compounds and in coal. The challenge now is to develop a practical, economical coal biodesulfurization process. It is not only desirable, but absolutely essential, that improved derivatives of IGTS8 with greatly enhanced levels of desulfurization activity should be developed in order to allow briefer treatment times, more complete removal, smaller reactor volumes, and more favorable coal-to-bacterial culture ratios in the biodesulfurization of coal.

For the past year, the focus of research was on the identification and characterization of Rhodococcus promoters. The plan was to obtain microbial cultures with enhanced levels of desulfurization activity ultimately by replacing the promoter/regulatory sequences of the desulfurization genes with stronger promoters capable of achieving high levels of transcription/ expression.

We have compiled a database containing all of the published DNA sequences that are known or suspected to encode promoters in Rhodococcus species and have taken steps toward establishing guidelines for the identification of Rhodococcus promoters. The data that are being compiled include ribosomal binding sites, transcriptional start sites, and particularly DNA sequences upstream from transcriptional start sites. Computer comparisons will be made to aid in finding the formula for locating Rhodococcus promoters.

The rules for genetic expression in Rhodococcus differ significantly from those of more well-characterized microorganisms such as E. coli and B. subtilis. We plan to better characterize the genetic expression in Rhodococcus through the isolation and characterization of many different Rhodococcus promoters.

The promoter controlling the expression of the chloramphenicol resistance gene of pRF2 was chosen for thorough analysis because chloramphenicol resistance is a selectable marker, and because mutant candidates that may contain altered promoters can be readily isolated.

The DNA sequence of the promoter and structural region of the chloramphenicol resistance gene of pRF2 was determined. Six of the Cm mutant promoters, designated pCP8, pCP11, pCP12, pCP14, pCP17 and pCP18, have been sequenced in both the forward and reverse directions. Several base pair changes have been noted when these sequences were compared to the original sequence, and these changes are being evaluated.

Four promoter probe vectors have been constructed and one commercially purchased vector has also been utilized for studying promoters in IGTS8. These vectors are called pRCM1, pRCAT1, pRCAT2, pRCAT3, and pKK232 (Pharmacia, NJ). Each of these vectors contain structural genes encoding chloramphenicol resistance, but lack a promoter to express these resistance genes. One or more unique restriction sites is present on each vector just proximal to the resistance genes, allowing the insertion of DNA fragments that can be assessed for the presence of promoters by selection for those derivatives that express chloramphenicol resistance. Promoter probe vectors are being used to identify promoters capable of functioning at higher levels in Rhodococcus rhodochrous than those that have currently been characterized, and these strong promoters will subsequently be characterized to determine their DNA sequences and transcriptional start sites and will then be used to express the desulfurization genes.

Ribosomes are among the most abundant components of bacterial cells. The promoter for the 16S ribosomal RNA gene is a good candidate for a strong promoter since it must allow for many copies of 16S RNA to be transcribed, especially in log phase cultures. Moreover, straightforward methods are available to isolate this promoter. While the 16S RNA gene of Rhodococcus rhodochrous IGTS8 has not been cloned prior to this work, 16S RNA genes from dozens of other bacterial species have been cloned and sequenced. Although the promoters for the 16S RNA gene vary considerably, the structural gene is highly conserved between prokaryotic species. Small primers from both the 5' and 3' ends have been synthesized and recognized as universal. These primers were used in conjunction with polymerase chain reaction (PCR) technology to amplify and clone the structural portion of the 16S ribosomal gene of R. rhodochrous IGTS8. Then colony hybridization was used to isolate the promoter region of the 16S RNA gene. This DNA fragment is being analyzed in three ways: primer extension combined with DNA sequencing to determine the size of the RNA transcript and transcriptional start site; ligation into pRCAT3 for analysis of CAT activity; and ligation of this fragment with the desulfurization genes. Analysis of this promoter will continue in the next fiscal year.

A strategy for the isolation of strong inducible promoters is currently being studied through a technique called subtractive RNA hybridization. Subtractive RNA hybridization allows specific genes in a microorganism to be identified through a multi-step process. Inducible promoters are desirable for the reason that the expression of some genes/proteins at high levels may cause cell death. When using an inducible promoter, cells may be grown to stationary phase under normal conditions, then as the inducer is added the genes are expressed and cell death is no longer a concern. The search for inducible promoters for IGTS8 genes related to the utilization of urea and guanidine hydrochloride is in progress and will continue in the next fiscal year.

OBJECTIVES

The objective of this research is to develop improved derivatives of Rhodococcus rhodochrous IGTS8 with greatly enhanced levels of desulfurization activity and to evaluate the ability of improved cultures to remove organic sulfur from coal. Specifically, recombinant DNA techniques will be used to identify DNA fragments that encode desulfurization genes which will be subsequently manipulated to achieve enhanced expression of these genes.

INTRODUCTION AND BACKGROUND

There are numerous physical, chemical, and microbiological techniques that can effectively remove inorganic sulfur from coal prior to combustion. Moreover, there are physical and chemical techniques for pyrite/ash removal that have been successfully commercialized and are routinely employed in the coal industry. While there are technologies capable of removing organic sulfur from coal prior to combustion, no commercially viable technology currently exists. The removal of organically bound sulfur from coal by physical/chemical techniques requires harsh conditions as compared with microbiologi-cal techniques; therefore a microbiological approach may result in more favorable economics than currently available technologies.

Unfortunately, with nearly all Illinois coals, physical cleaning alone will not produce a coal that complies (if burned without additional sulfur removal from the combustion gases) with New Source Performance Standards (NSPS). To remove the additional organic sulfur, another method such as microbiological treatment that breaks the chemical bonds binding the sulfur to the carbon must be developed. A coal cleaning strategy employing only physical or chemical cleaning methods to remove as much of the pyrite and other mineral matter as economically possible will be unlikely to produce a product capable of meeting emission standards. A separate step such as microbial cleaning will likely be needed that focuses on the removal of the organic sulfur. A microbial culture, Rhodococcus rhodochrous IGTS8, has been identified that is capable of selectively cleaving carbon-sulfur bonds in model compounds and in coal; however, the level of expression of desulfurization enzymes by IGTS8 is low. Improved bacterial cultures are needed if a practical coal biodesulfurization process is to be developed. The goal of this project is to use recombinant DNA techniques to develop microbial cultures with enhanced desulfurization abilities.

EXPERIMENTAL PROCEDURES

Restriction digests were performed at 37°C for 1 hour at a concentration of at least 1 enzyme unit per microgram of DNA (Gibco BRL, CA). Dephosphorylation of vectors was performed with HK phosphatase (Epicenter Technologies, WI) as instructed. Ligation was accomplished using T4 DNA ligase (Gibco BRL, CA). Southern hybridization and colony hybridization were performed by the published methods in Perbal, A Practical Guide to Cloning, pp. 424-428 and 422-423. DNA probes were labelled using ^{32}P dCTP and a nick translation kit (Amersham, IL). Polymerase Chain Reaction (PCR) was accomplished using published methods and apparatus of Perkin Elmer, CT. Sequencing reactions were carried out using the Sanger chain termination method with Sequenase™ 2.0 (USB, OH). Sequencing gels were run on a 7M urea, 6% Long Ranger™ gel (AT Biochem, PA) in 1XTBE buffer for 2 hours at 1500 volts with the Sequigen (Bio-Rad Laboratories, CA) sequencing apparatus. DNA sequences were computer analyzed with the PC Gene program (Intelligentics, Inc., CA).

RNA was isolated by a modification of the method of Chomczymski, P. et al. (1987) in Anal. Biochem. Vol. 162, pp. 156-157. Vanadyl ribonucleoside complexes were purchased from Gibco BRL, MD and Trisolv™ was purchased from Biolecx Laboratories, Inc., TX. Primers for primer extension and RNA for subtractive hybridization were labelled with gamma ^{32}P dATP (Amersham, IL) and T4 polynucleotide kinase (Promega, WI). Primer extension experiments were performed using the methods of Ausubel et al., Current Protocols in Molecular Biology, pp. 4.8.1-4.8.3; Sambrook et al., Molecular Cloning A Laboratory Manual, pp. 7.79-7.83; and Forsman, M. et al. in Molecular Microbiology (1989), Vol. 3(10), pp. 1425-1432. Avian-reverse transcriptase was purchased from Promega, WI. Subtractive hybridization experiments were carried out according to the method of Apte, S. K. et al. in Plant Molecular Biology (1990), Vol. 15, pp. 723-733.

RESULTS AND DISCUSSION

We continue to examine various methodologies to isolate improved promoters that will eventually be used to achieve enhanced expression of the desulfurization gene(s) of IGTS8. The methods employed include the construction of promoter probe vectors suitable for isolating Rhodococcus promoters, sequencing the promoter region and chloramphenicol gene of pRF2, isolation of mutants capable of growing on high levels of chloramphenicol, subcloning the mutant promoters for this gene and sequencing them. We have also isolated a putative promoter for the 16S RNA gene of Rhodococcus rhodochrous IGTS8 and are simultaneously analyzing this DNA fragment while searching for strong inducible promoters in IGTS8.

Characterization of the relative strength of the native desulfurization promoter was the first step toward promoter evaluations. However, before this experiment could be done, a good procedure for the isolation of large amounts of Rhodococcus rhodochrous IGTS8 RNA was needed. This was a difficult task as long incubations are required to lyse Rhodococcus cells and RNA is notoriously unstable. During the extended period required for the lysis of IGTS8 cells, RNase is steadily released into the environment, thus degrading the RNA. The successful isolation of large amounts of undegraded RNA from IGTS8 has been completed. Northern blot experiments indicate that the relative abundance and/or stability of mRNA from cloned desulfurization genes is apparently low as no clear mRNA transcripts were identified. This was expected based on the relatively low specific desulfurization activity of IGTS8 cells. RNA hybridization experiments did detect two possible transcripts of approximately 2.6 Kilobases (Kb) and 1.2 Kb of the chloramphenicol resistance gene originally found on the Rhodococcus fasciens plasmid pRF2. These results suggest that mRNA transcripts for this chloramphenicol resistance gene are present at relatively high levels and/or have high stability in IGTS8. Therefore, the promoter of this chloramphenicol resistance gene may be useful in obtaining increased levels of expression of desulfurization genes.

Experiments were performed to determine the mechanism of resistance encoded by the chloramphenicol resistance gene obtained from pRF2. Standard spectrophotometric assays used to monitor chloramphenicol acetyltransferase (CAT) enzymatic activity using Rhodococcus rhodochrous IGTS8 containing pRF29 (which contains the Cm gene from pRF2) and E. coli containing pBR325 (which contains a 1-cm gene known to possess a CAT enzyme). Table 1 clearly illus-

trates that the Cm gene of pRF2 does not appear to encode the CAT enzyme. Similar results were obtained when lysed cell extracts were examined. To further examine the mechanism of resistance encoded by the Cm gene of pRF2 growth inhibition assays were performed using spent culture medium resulting from the growth of R. rhodocrous/pRF29, R. erythropolis/pRF29, E. coli/pBR325, and B. subtilis/pBD64 (another plasmid known to encode for a CAT enzyme) in nutrient broth containing 30 micrograms of chloramphenicol per ml. After each culture had grown to stationary phase, the cells were removed by centrifugation followed by filtration through 0.45 micron filters. These filtered supernatants were then added to top agar containing either of two chloramphenicol sensitive E. coli strains: LE392 or DH5a. These top agars were then spread onto nutrient agar plates and incubated at 37°C O/N. These tests are designed to determine if chloramphenicol is altered (acetylated), degraded, or left unaltered by chloramphenicol resistant cultures. Acetylation or degradation of chloramphenicol will destroy its antibiotic activity and allow the growth of chloramphenicol sensitive strains in the presence of spent culture medium, whereas unaltered chloramphenicol, resulting from cultures having altered permeability to chloramphenicol, will yield spent culture medium that still inhibits sensitive bacteria. The results of growth inhibition assays are shown in Table 2 which indicate that the mechanism of resistance encoded by the Cm gene of pRF2 is probably altered membrane permeability.

Southern blot experiments have indicated that the Cm gene of pRF2 does not hybridize with pBR325, pBD64, or with pGR71 which is consistent with the hypothesis that the Cm gene of pRF2 does not encode a CAT enzyme. Since the chloramphenicol gene of pRF2 has a mechanism of resistance that involves the bacterial membrane as does the desulfurization genes, this promoter was chosen for a more thorough investigation.

The promoter controlling the expression of the chloramphenicol resistance gene of pRF2 was chosen for thorough analysis because chloramphenicol resistance is a selectable marker, and because mutant candidates that may contain altered promoters can be readily isolated. The DNA sequence of the promoter and structural region of the chloramphenicol resistance gene of pRF2 was determined (Figure 1). [While this work was in progress, the sequence of this gene was independently determined by other researchers, confirming data reported here (Desomer et al., Molecular Microbiol. 6:2377-2385, 1992).] The important elements of this sequence are the XbaI site indicated by the number 1, the farthest boundary of the

suspected promoter region indicated by the number 2, the two NlaIII sites indicated by the numbers 3 and 4, the transcriptional initiation point indicated by the number 5, the HindIII site indicated by the number 6, the translational initiation point indicated by the number 7, the EcoRI site indicated by the number 8, and the site at which translation terminates indicated by the number 9. Primer extension studies identified the transcriptional initiation point (no. 5), and subcloning experiments indicated the farthest boundary of the suspected promoter region (no. 2). This then defines a 50 bp region suspected of encoding the promoter. An examination of this 50 bp region using PC-GENE software (Intelligenetics Corp.) and libraries of published DNA sequences indicates that this Rhodococcus promoter does not possess sequences normally associated with promoters of other more well characterized microorganisms such as E. coli or B. subtilis, nor does this 50 bp region show obvious similarities to the limited number of published sequences of genes derived from members of the genus Rhodococcus. What this means then is that while a potentially useful promoter capable of functioning in Rhodococcus has been localized to a 50 bp region, the sequence/structural features important for the functioning of Rhodococcus promoters is unknown.

Mutant colonies capable of growth in the presence of 400 to 500 microorganisms of chloramphenicol (Cm) per milliliter (mL) were obtained (the normal level of resistance is 25 to 50 micrograms/mL). These mutants may very well contain altered/improved promoters. The promoter regions of 20 of these high level chloramphenicol resistant mutants have been subcloned and DNA sequencing is in progress. The promoter region can be excised as a 70 bp NlaIII fragment or as a 700 bp XbaI-HindIII fragment and cloned into pUC19 for subsequent sequencing. The status of the analysis of these 20 promoter mutant candidates is summarized in Table 3.

The 70 bp NLAIII fragment contains the 50 bp region suspected of encoding the promoter and little else; however, in the event that sequences beyond those contained in this region are important for the functioning of the Cm resistance promoter, then an examination of the larger XbaI-HindIII fragment may allow the identification of other regulatory sequences. Six of the Cm mutant promoters, pCP8, pCP11, pCP12, pCP14, pCP17 and pCP18 have been sequenced in both the forward and reverse directions. Several base pair changes were noted when the sequences were compared to the original sequence. These changes occur in pCP12, pCP17, and pCP18 (Figure 2). The base change in pCP12 occurs at position 20 where

a C has been replaced with an A, in pCP17 a C at position 7 was replaced with a G and there is a C missing at position 59. The mutation in pCP18 the bases TA at positions 56-57 have been reversed to AT. The differences between these mutants and the original can be seen in Figure 2. These base changes may contribute to promoter strength in allowing Rhodococcus strains to have increased resistance to chloramphenicol.

To begin the process of transcription, RNA polymerase must recognize specific binding sequences on the DNA template. These recognition sequences are called promoters. Organisms such as E. coli and B. subtilis have been extensively studied, and from comparisons between promoters, rules have been made to find promoter sequences. In E. coli, two common sequences of 6 nucleotides occur at 10 bases and 35 bases upstream of the transcriptional start site. Consensus sequences were determined by comparison and most E. coli promoters differ by only a few base changes from the consensus sequence. However, other E. coli promoters have been found that recognize different consensus sequences which occur at 12 and 24 bases upstream of the transcriptional start site, and the consensus sequences found for promoters in B. subtilis follow altogether different but consistent rules.

Rhodococcus promoters do not seem to follow the rules determined for E. coli and B. subtilis. Rhodococcus promoters have not been as extensively studied as E. coli and B. subtilis. We have been collecting DNA sequence data of Rhodococcus promoters and suspected promoter regions. This information should allow the determination of promoter sequences more easily than at present. Sequences have been collected from the EMBL GenBank and recent publications of Rhodococcus species and other Gram positive species which are related, such as microorganisms from the genus Corynebacteria, Lactococcus, Streptococcus, Streptomyces, Arthrobacter, and Brevibacteria. Through the study of Rhodococcus promoter sequences, rules for the identification of promoters will be determined. The data that are being compiled include ribosomal binding sites, transcriptional start sites, and particularly DNA sequences upstream from transcriptional start sites. Computer comparisons will be made to aid in finding the formula for locating Rhodococcus promoters. Once the promoters have been identified, site specific mutagenesis would be a logical next step. It will be important to be able to recognize how base changes within promoters effect promoter strength to analyze mutants and find a strong promoter for the desulfurization genes.

The chloramphenicol resistance gene of pRF2 probably does not encode a chloramphenicol acetyltransferase enzyme like the majority of Cm resistance determinants. The mechanism of resistance appears to be altered membrane permeability. Since the desulfurization enzymes are also thought to function within the bacterial membrane, then structural features of the Cm resistance gene/protein may be useful in understanding the expression, transport, and functioning of desulfurization genes/proteins.

Four promoter probe vectors have been constructed and one commercially purchased vector has also been utilized for studying promoters in IGTS8. These vectors are called pRCM1, pRCATA1, pRCAT2, pRCAT3, and pKK232 (Pharmacia, NJ). Each of these vectors contain structural genes encoding chloramphenicol resistance, but lack a promoter to express these resistance genes. One or more unique restriction sites is present on each vector just proximal to the resistance genes, allowing the insertion of DNA fragments that can be assessed for the presence of promoters by selection for those derivatives that express chloramphenicol resistance. Except for pRCM1, which has the Cm gene from pRF29 whose mechanism of resistance is related to permeability rather than acetylation of chloramphenicol, the promoter probe vectors can be used to measure the relative strength of the promoter inserts through the chloramphenicol acetyl transferase (CAT) assay which is a convenient, well documented assay. Several Cm resistant colonies have been isolated using these vectors. These derivatives have been found to contain inserts of R. rhodochrous IGTS8 chromosome ranging in size from about 100 to 2000 bp and providing levels of chloramphenicol resistance ranging from 25-200 ug/mL. Figure 4 shows the maps of the promoter probe vectors constructed at IGT and Figure 5 shows the commercially purchased vector pKK232.

The search for strong promoters in Rhodococcus would not be complete without looking for known promoter regions that may be stronger than the native desulfurization promoter. Ribosomes are among the most abundant components of bacterial cells. The promoter for the 16S ribosomal RNA gene is a good candidate for a strong promoter since it must allow for many copies of 16S RNA to be transcribed, especially in log phase cultures. Moreover, straightforward methods are available to isolate this promoter. While the 16S RNA gene of Rhodococcus rhodocrous IGTS8 has not been cloned prior to this work, 16S RNA genes from dozens of other bacterial species have been cloned and sequenced. Although the promoters for the 16S RNA gene vary considerably, the structural gene is highly conserved between prokaryotic

species. Small primers from both the 5' and 3' ends have been synthesized and recognized as universal. These primers were used in conjunction with polymerase chain reaction (PCR) technology to isolate the structural portion of the 16S ribosomal gene of R. rhodochrous IGTS8. The primers used to amplify the 16S RNA structural gene and a plasmid containing this cloned 1.5 Kb gene designated pEBC2, is shown in Figure 6.

In order to find the Rhodococcus promoter for this gene, partially digested genomic DNA from R. rhodochrous IGTS8 was analyzed through Southern hybridization to radiolabelled pEBC2. Hybridization occurred at 4.5 Kb+3.2 Kb EcoRI digested chromosomal fragments, a 4.0 Kb Bam HI fragment, and a 3.0 Kb HindIII fragment (Figure 7). Chromosomal DNA fragments corresponding to the sizes that hybridized were ligated into the vector pUC19. Then colony hybridization was used to isolate the promoter region of the 16S RNA gene.

A 1.5 Kb EcoRI fragment was identified as a candidate likely to contain the 16S RNA promoter. This clone was designated p16SPRO. This DNA fragment is being analyzed in three ways: primer extension combined with DNA sequencing to determine the size of the RNA transcript and transcriptional start site; ligation into pRCAT3 for analysis of CAT activity, and ligation of this fragment with the desulfurization genes. Analysis of this promoter will continue in the next fiscal year.

A strategy for the isolation of strong inducible promoters is currently being studied through a technique called subtractive RNA hybridization. Subtractive RNA hybridization allows the specific microorganism to be identified through a multi-step procedure. Genomic DNA is immobilized on nitrocellulose by Southern blotting. Total RNA of uninduced cells is used to bind to sites that are not specific for the gene of interest, then the DNA is hybridized to radiolabelled total RNA isolated from cells induced for the genes of interest. A flow chart depicting subt active RNA hybridization is shown in Figure 8. Inducible promoters are desirable for the reason that the expression of some genes/proteins at high levels may cause cell death. When using an inducible promoter, cells may be grown to stationary phase under normal conditions, then as the inducer is added the genes are expressed and cell death is no longer a concern. The search for inducible promoters for IGTS8 genes associated with the utilization of urea and guanidine hydrochloride is in progress and will continue in the next fiscal year.

Table 1. SPECTROPHOTOMETRIC ASSAY FOR CHLORAMPHENICOL ACETYLTRANSFERASE

A 412 nm (background subtracted)

Time, min.	E. coli control	E. coli/ pBR325	R.r. Control	R.r.pRF29
5	0.013	0.900	0.015	0.015
10	0.012	0.995	0.010	0.005
15	0.000	0.970	0.001	0.003

Table 2. GROWTH OF CHLORAMPHENICOL SENSITIVE CULTURES IN THE PRESENCE OF FILTERED SUPERNATANTS DERIVED FROM CHLORAMPHENICOL RESISTANT CULTURES

	Bacterial Growth	
Source of Supernatant	LE392	DH5α
Cm Control	-	-
E.c./pBR325	+	+
R.r./pRF29	-	-
B.s./pBD64	+	+

Figure 1. DNA SEQUENCE OF THE CHLORAMPHENICOL RESISTANCE GENE OF pRF2

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BASE  COUNT    458  a  813  c  817  g  628  t                    ORIGIN

   1  t[1]ctagagcgg acgcagggcg cgccggcctc gttcgcattt gctgctctcg cccgcccgca
  61  gcgcggttgc atgctgcaat ggtggaccga actggattcg tgcaccgacg tctctgtgtg
 121  atgcccgacg ctgcgcgatc cgtctacgtg ctcgacttcg gcccagatgg cgttctgtat
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 421  ccctgcaata gctctgtagg agctttacga cacggaactg tgccttcgct gggtgctctc
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2521  tcgtcgctga aggctactca ggcaaggaag tagcgcgtgc actcggaatg tcaccgaaaa
2581  ccgttgagac gcatatccaa cgaattttcg accggtacgg cgttcggaat cgtgcgggtg
2641  ttgtagcgag gtggatggct agtaagcacc tcgcggcgag ctgatagagc acagctgtgt
2701  ccggccatac ggatcc
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Table 3. STATUS OF THE ANALYSIS OF THE PROMOTER REGION OF HIGH LEVEL CHLORAMPHENICOL RESISTANT MUTANTS

Strain	Isolated From	XbaI-HindIII Fragments	NLAIII Fragment
CP1	CW22/pIGTS3	+	-
CP2	CW22/pIGTS3	+	+
CP3	CW22/pIGTS3	+	+
CP4	CW22/pIGTS3	+	+
CP5	CW22/pIGTS3	+	+
CP6	CW22/pIGTS3	+	+
CP7	CW22/pIGTS3	+	+
CP8	CW22/pIGTS3	+	+
CP9	CW22/pIGTS3	+	+
CP10	IGTS8/pRF29	+	+
CP11	IGTS8/pRF29	+	+
CP12	IGTS8/pRF29	+	+
CP13	IGTS8/pRF29	+	+
CP14	IGTS8/pRF29	+	+
CP15	IGTS8/pRF29	+	+
CP16	IGTS8/pRF29	-	-
CP17	IGTS8/pRF29	+	-
CP18	IGTS8/pRF29	+	-
CP19	IGTS8/pRF29	+	-
CP20	Cw22/pTOXI1	+	-

Figure 3. DNA SEQUENCE COMPARISON OF NLAIII FRAGMENTS

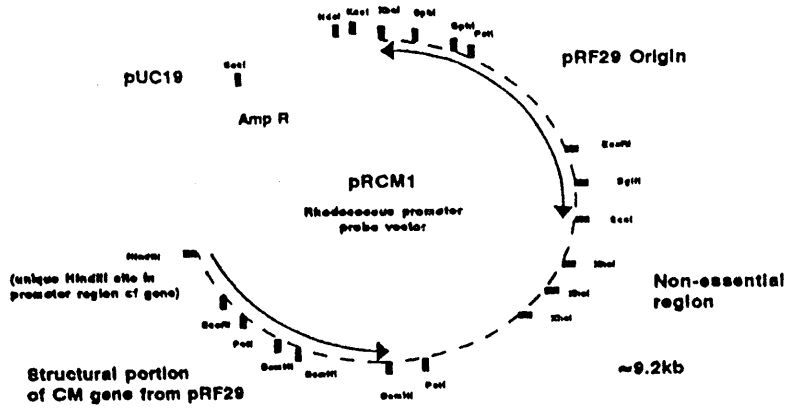
The alignment was done on 7 Nucleic acid sequences.
Character to show that a position in the alignment is perfectly conserved: '*'
Character to show that a position is well conserved: '.'

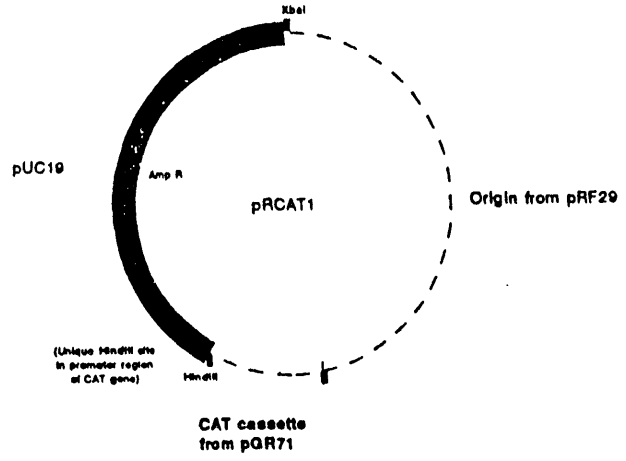
Alignment

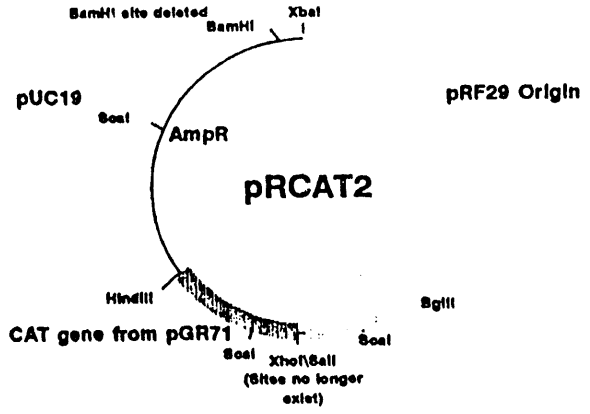
```
NLA3        TGCCGTCTGGGATACGCGACGCCCCGAATCGGTTGGACAATCACATCCGA    50
CP12        TGC-GTCTGGGATACGCGAAGCCCCGAATCGGTTGGACAATCACATCCGA    49
CP17        TGCCGTGTGGGATACGCGACGCCCCGAATCGGTTGGACAATCACATCCGA    50
CP18        TGC-GTCTGGGATACGCGACGCCCCGAATCGGTTGGACAATCACATCCGA    49
CP8         ---------GGATACGCGACGCCCCGAATCGGTTGGACAATCACATCCGA    41
CP14        -----------ATACGCGACGCCCCGAATCGGTTGGACAATCACATCCGA    39
CP11        TGC-GTCTGGGATACGCGACGCCCCGAATCGGTTGGACAATCACATCCGA    49
                     ******** **********************************

NLA3        CATTGCTAACTTCGATGACATGTCCAGCC      79
CP12        CATTGCTAACTTCGATGACATG--CAA--      74
CP17        CATTGCTAA-TTCGATGACATG--CAA--      74
CP18        CATTGCATACTTCGATG----------        66
CP8         CATTGCTAACTTCGATGACATG-------      63
CP14        CATTGCTAACTTCGATGACATG-CCT---      64
CP11        CATTGCTAA-------------------       58
            ******..*
```

Figure 4. MAPS OF PROMOTER PROBE VECTORS







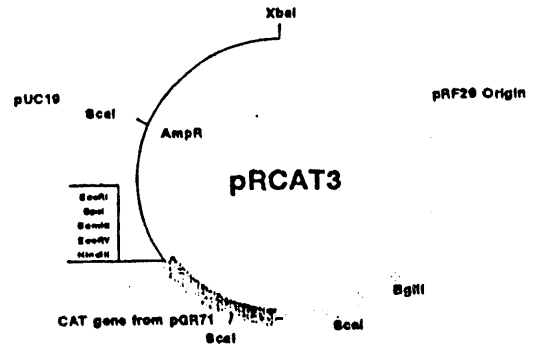


Figure 5. MAP OF PROMOTER PROBE VECTOR pKK232

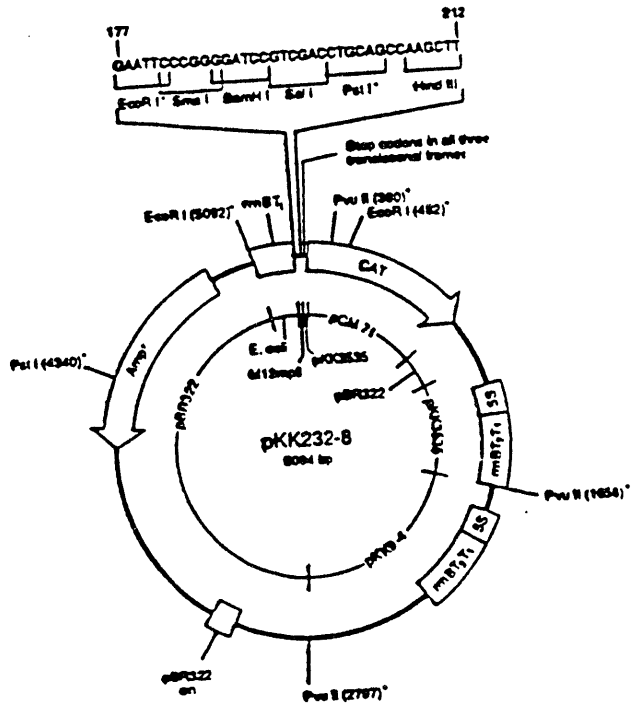


Figure 6. DNA SEQUENCE OF 5 PRIME AND 3 PRIME UNIVERSAL PRIMERS FOR THE 16S RNA GENE AND MAP OF pEBC2

Primer A

5' CCGAATTCGTCGACAGAGTTTGATCCTGGCTAG 3'

Primer B

5' CCCGGGATCCAAGCTTAGAAAGGAGGTGATCCA 3'

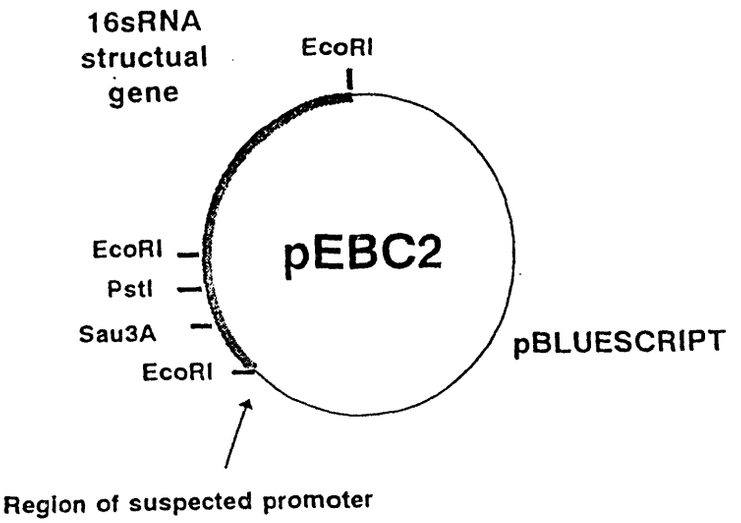


Figure 7. SOUTHERN HYBRIDIZATION OF RHODOCOCCUS CHROMOSOME WITH pEBC2

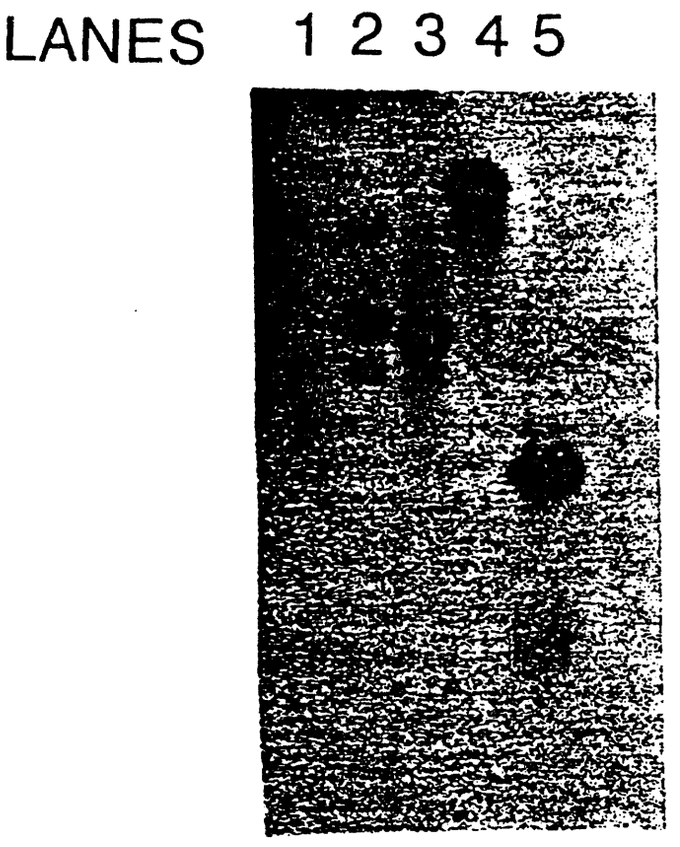
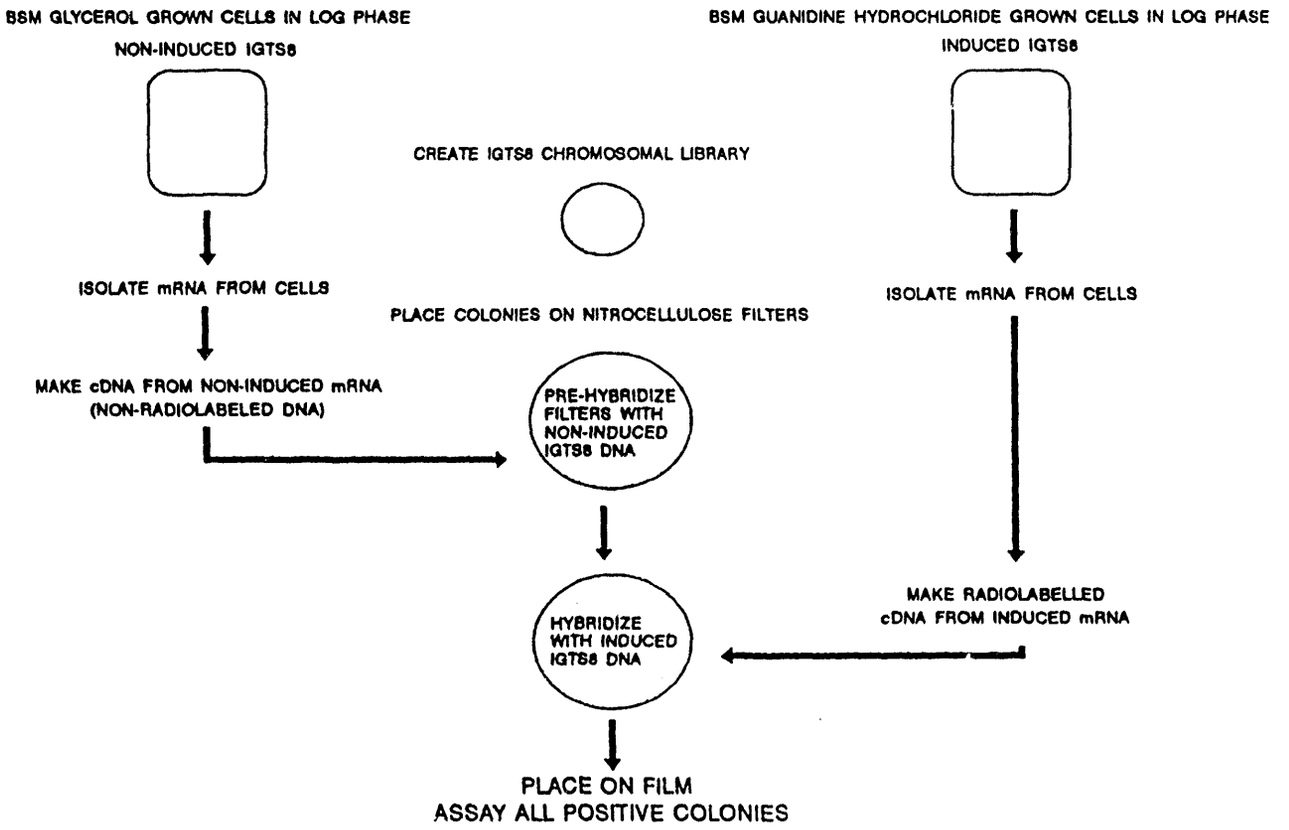


Figure 8. SUBTRACTIVE RNA HYBRIDIZATION FLOWCHART



CONCLUSIONS AND RECOMMENDATIONS

The isolation and characterization of Rhodococcus promoters is proceeding extremely well. This work should continue so as to identify particularly strong Rhodococcus promoters and to understand the sequence requirements for gene expression in Rhodococcus so that this information can be used to construct improved promoters which will ultimately be used to express the desulfurization genes of IGTS8.

PROJECT MANAGEMENT REPORT
June 1, 1993 Through August 31, 1993

Project Title:	**MICROBIAL STRAIN IMPROVEMENT FOR ORGANOSULFUR REMOVAL FROM COAL**
Principal Investigator:	Dr. John J. Kilbane II Institute of Gas Technology
Project Manager:	Dr. Ken Ho, ICCI

COMMENTS

The project was as scheduled.

11.

EXPENDITURES - EXHIBIT B

Cummulative Projected and Estimated Actual Expenditures by Quarter

Quarter *	Types of Cost	Direct Labor	Materials and Supplies	Travel	Major Equipment	Other Direct Costs	Indirect Costs	Total
Sept. 1, 1992 to Nov. 30, 1992	Projected	8,000	5,000	-	-	-	15,508	28,508
	Estimated Actual	9,000	750	-	-	-	15,980	25,730
Sept. 1, 1992 to Feb. 28, 1993	Projected	18,000	9,000	-	-	-	34,420	61,420
	Estimated Actual	14,000	2,500	-	-	-	25,151	41,651
Sept. 1, 1992 to May 31, 1993	Projected	30,000	10,000	410	-	-	56,402	96,812
	Estimated Actual	24,500	9,000	-	-	-	45,031	78,531
Sept. 1, 1992 to Aug. 31, 1993	Projected	36,789	10,800	410	-	-	68,839	116,838*
	Estimated Actual	37,048	11,880	160	-	-	67,750	116,838

* Cumulative by quarter

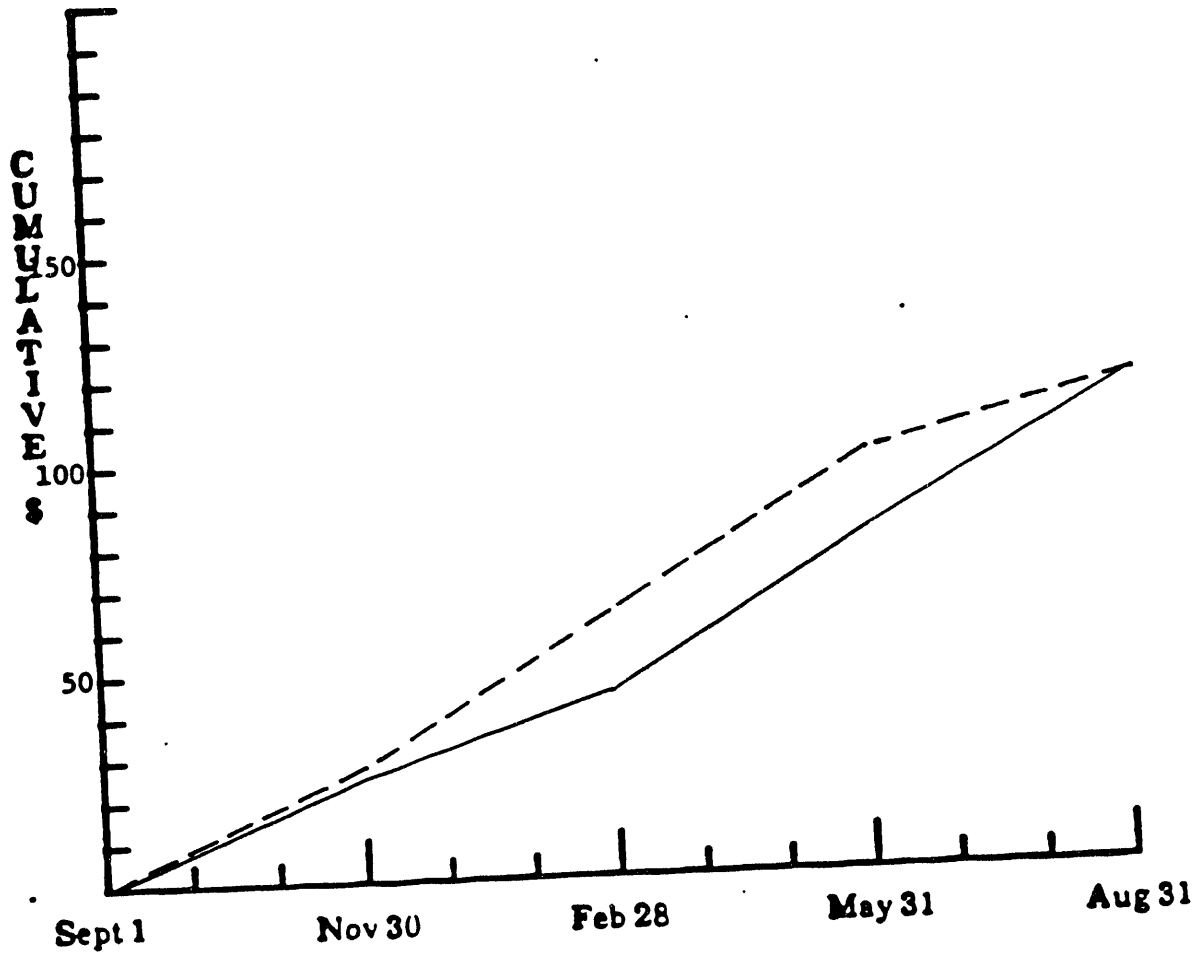
* Requested from CRSC

IGT Project 40312

CUMMULATIVE COSTS BY QUARTER - EXHIBIT C

(IN THOUSANDS)

"Microbial Strain Improvement for Organosulfur Removal from Coal"

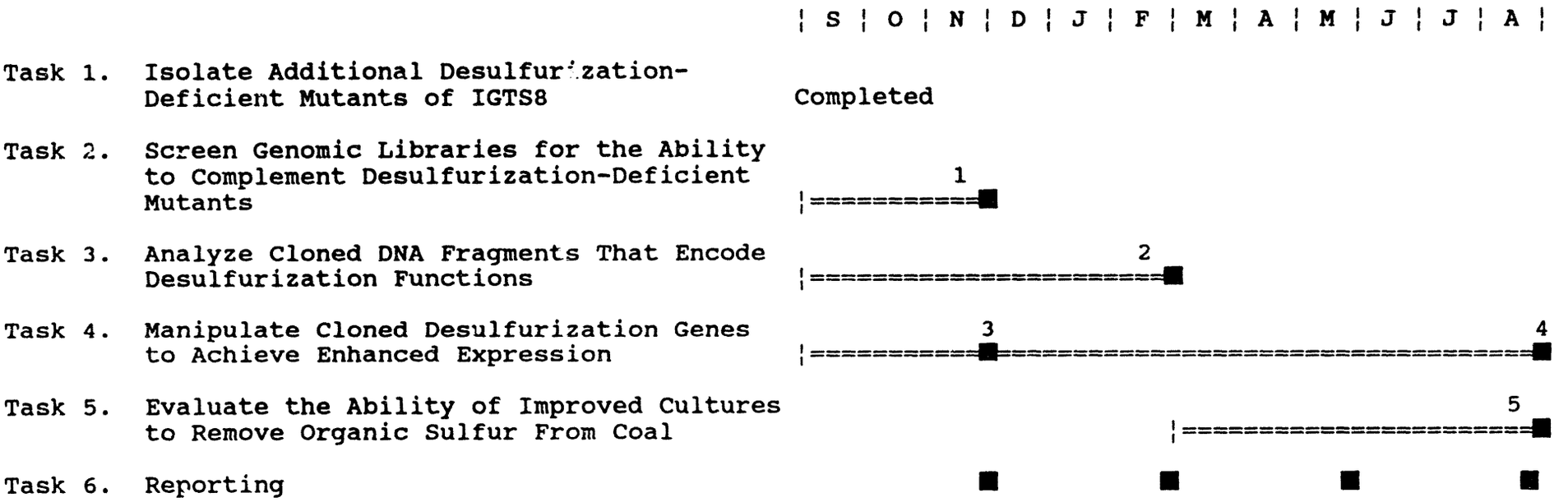


Months and Quarters

0 = Projected Expenditures ----------

Δ = Actual Expenditures ——————

Total CRSC Award $ 116,838



X = Milestone
Q = Quarterly Report
F = Final Report
■ = Completed Milestone
1 = Screening of genomic libraries is completed.
2 = Analysis of all cloned desulfurization genes is completed.
3 = The effect of supplying IGTS8 with multiple copies of at least some desulfurization genes is evaluated.
4 = Manipulation of cloned genes is completed.
5 = Evaluation of improved cultures in coal biodesulfurization experiments is completed.

DATE
FILMED
4/12/94
END