

## FINAL TECHNICAL REPORT

September 1, 1990 through August 31, 1991

Project Title: Genetic Engineering of Sulfur-Degrading *Sulfolobus*  
ICCI Project Number: 89-2/P.1.1-6  
Principal Investigator: Nancy W.Y. Ho  
Laboratory of Renewable Resources Engineering  
1295 Potter Building  
Purdue University  
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. In particular, one of the *Sulfolobus* species, *S. shibatae* B12, contains a double-stranded DNA virus which might be easily converted into a gene cloning system. The objective of this project is to develop an effective gene cloning system for some of the *Sulfolobus* species, particularly *S. acidocaldarius* and *S. shibatae*.

One important element for the establishment of a gene cloning system for a microorganism is to have a selection mechanism. In the first quarter, we established three potential selection mechanisms for the establishment of a gene transfer system for the two selected *Sulfolobus* species.

For the remaining quarters, our efforts were devoted to the construction of potential cloning vectors and development of transformation protocols for the establishment of an effective transformation system for the selected *Sulfolobus* species.

## EXECUTIVE SUMMARY

In order to make *Sulfolobus* species an ideal microorganism for the removal of organic sulfur in coal, an effective gene transfer system must be established for these species. To establish a plasmid mediated transformation for the selected *Sulfolobus* species it is essential to construct a plasmid which can be selected and also replicated in the host cell. The plasmid should be an *E. coli-Sulfolobus* shuttle vector so that its preparation can be carried out in *E. coli*. A plasmid as such has to contain two essential elements. One is a segment of DNA which can serve as a replication origin so that the plasmid can be replicated autonomously in the hosts. The other is a selectable gene which allows the host cells containing this plasmid to be differentiated from the rest cells under special conditions so that the transformed cells can be easily identified.

In the first quarter, we have identified that at least three systems are potentially useful as the selection mechanisms for the establishment of a transformation system for both *S. acidocaldarius* and *S. shibatae* B12.

In the second quarter, our major accomplishments include the successful isolation of the double-stranded DNA virus from *S. shibatae* B12 (previously known as *Sulfolobus* B12). This virus will serve as the plasmid or part of this virus will serve as the replication origin of a plasmid vector for the establishment of the genetic transformation system. Not only have we successfully isolated the viral DNA, but we have also developed an improved method for the isolation and purification of the viral DNA without the presence of contaminating chromosomal DNA. The viral DNA was linearized by the restriction endonuclease BamH1 and digested into four fragments by the restriction endonuclease EcoRI as previously reported. Furthermore, the BamH1 fragment and each of the EcoRI fragments of the B12 viral DNA were cloned in plasmid pUC19.

In the second quarter, we have also carried out extensive studies on the stability of the B12 viral DNA. We found that it is remarkably stable. For instance, it is still present in the host cells in high copies after more than 10 transfers. Hence, it will provide a natural gene cloning system for *S. shibatae* B12. In this case, the DNA should first be integrated into the viral DNA which is still located on the chromosome, followed by inducing the virus with low dosages of uv to release the virus from the chromosome. The double-stranded DNA of the virus then can be replicated numerous times to overproduce large amounts of the desired protein products encoded by the genes cloned on the viral DNA.

In this third quarter, our efforts have largely been concentrated on: (i) the construction of potential vectors suitable for the genetic transformation of either *S. acidocaldarius* or *S. shibatae* B12; (ii) isolation of *Sulfolobus* promoters functioning in *E. coli*; and (iii) experimenting with various reported techniques for culturing thermophiles on solid medium.

The potential cloning vectors for *Sulfolobus* species are constructed by two different approaches. One approach is to construct a bank of vectors which are potentially capable of autonomously replicating in the host cells. We have constructed two such banks. These vectors can be used to transform both *S. acidocaldarius* and *S. shibatae* B12 but they are most suitable for *S. acidocaldarius* and possibly also for other *Sulfolobus* species that do not contain the viral DNA.

The second approach is to construct *E. coli* plasmids which are capable of integrating into *S. shibatae* B12 viral DNA by homologous recombination. Such an *E. coli* plasmid should contain a viral DNA fragment with unique restriction sites to facilitate the insertion of selection marker or other genes desired to clone. After the selectable gene is integrated into the viral DNA, the virus is then released from the chromosome by uv induction and serves as a high copy-number vector. The integrative method is only suitable for *S. shibatae* B12.

One potential problem for the above described vectors is that intact *E. coli* promoters for *Hyg*<sup>R</sup> and *Km*<sup>R</sup> might not be expressed in *Sulfolobus*. In order to overcome this potential difficulty, an effort was made to explore the possibility of the isolation of *Sulfolobus* promoters which can also function as promoters in *E. coli*. These promoters not only will be useful for the expression of the selection markers such as *Km*<sup>R</sup> and *Hyg*<sup>R</sup>, but also will be extremely useful for the expression of other heterologous genes. Our results showed that such promoters can be isolated from *Sulfolobus* DNA.

In the last quarter of this funding year, we have analyzed the nucleotide sequence of one of the promoters. Furthermore, DNA has been isolated from 5FU resistant strain. This DNA and wild-type *S. acidocaldarius* and *S. shibatae* B12 as the hosts are being used to establish the ideal conditions for genetic transformation of the selected *Sulfolobus* species. We planned first trying to use 5FUR<sup>R</sup> DNA for the transformation of *S. acidocaldarius* to 5FU resistant by integrative transformation. This work is currently in progress.

Some of the above described results have been presented at the Second International Symposium on Biological Processing of Coal, May 1-3, 1991, San Diego, CA.

## 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 *Sulfolobus*- 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 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 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. In particular, one of the *Sulfolobus* species, *S. shibatae* B12, contains a double-stranded DNA virus which might be easily converted into a gene cloning system. The objective of this project is to develop an effective gene cloning system for some of the *Sulfolobus* species.

During the first two quarters of this year, we have shown that selection mechanisms are available for the establishment of a gene transfer system for at least two of the *Sulfolobus* species; *S. acidocaldarius* and *S. shibatae* B12.

In this third quarter, the construction of various cloning vectors suitable for genetic transformation of either a *S. acidocaldarius* and *S. shibatae* B12 has been carried out. Furthermore, *Sulfolobus* promoters which can also function as promoters in *E. coli* were also isolated.

In the last quarter, the nucleotide sequence of one of the *Sulfolobus* promoters which can also function in *E. coli* was analyzed. The chromosomal DNA was also isolated from the 5FU<sup>R</sup> of *S. acidocaldarius*. Furthermore, experimentation of the ideal conditions for the introduction of chromosomal DNA or plasmid DNA into the *Sulfolobus* cells has also begun.

## EXPERIMENTAL PROCEDURES

Standard procedures were used to isolate plasmid DNA, to ligate DNA, to transform *E. coli* strains and to carry other recombinant processes. DNA isolated from *S. acidocaldarius* and *S. shibatae* was used for the construction of banks for the isolation of *Sulfolobus* promoters. pLG83 is a plasmid containing a promoterless hygromycin B resistance gene. This plasmid was used to construct *Sulfolobus* promoters banks. *E. coli* containing pLG83 cannot be cultured in medium with 400 mg/ml. Colonies that can grow on medium containing 400 mg/ml hygromycin B were selected for possibly containing a *Sulfolobus* promoter which also functions in *E. coli*.

DNA sequence analysis was carried out by generating single-stranded DNA with the phagemid and sequencing the resulting single-stranded DNA with the dideoxy chain termination technique.

### Sensitivity of *S. acidocaldarius* to G418, Hyg, and 5FU

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 is 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, Hyg and 5FU are better than G418.

### Sensitivity of *S. B12* to G418, Hyg, and 5FU

We found that *S. B12* is very sensitive to Hyg but only moderately sensitive to G418 and 5FU. Our results are shown in Table 2.

### Development of 5FU Resistant Strain of *S. acidocaldarius*

If a 5FU resistant strain can be isolated, 5FU and its resistant gene might be an excellent selection system for *S. acidocaldarius*. Since 5FU<sup>R</sup> gene is a native gene of the host, there should not be a problem for its expression in the host. Furthermore, it is also heat stable. Hence, the isolation of such a strain is highly desirable. We found that wild-type *S. acidocaldarius* could be induced to become resistant to 5FU. This was accomplished by inoculating the wild-type strain into *Sulfolobus* medium (1 to 10 dilution) containing 5FU (25 mg/ml). A resistant strain was obtained by prolonged incubation in the presence of 5FU (25 mg/ml). The resistant strain appears to be stable and it still retains its full resistance to 5FU after numerous transfers in the presence or in the absence of 5FU.

### Construction of Potential Cloning Vectors for *S. acidocaldarius* and *S. B12*

Besides requiring the presence of a selectable marker, a cloning vector for a special host also must contain a replication origin. The replication origin of the double-stranded DNA virus of *S. B12* should be the most ideal replication origin for the construction of the cloning vectors for the *Sulfolobus* species. The *S. B12* viral DNA was isolated by the procedure described in the literature, with modification as shown in Figure 1.

The potential cloning vectors for *Sulfolobus* species are constructed by two different approaches. One approach is to construct a bank of vectors which are potentially capable of autonomously replicating in the host cells. Such vectors consist of *E. coli* plasmids containing either *Km*<sup>R</sup> or *Hyg*<sup>R</sup> and they also contain a *S. B12* viral DNA fragment from

digestion of the viral DNA by a suitable restriction enzyme. Transformation of *Sulfolobus* cells with the bank of plasmids should be able to be accomplished by electroporation or other techniques. Cells resistant to G418 or Hyg will be isolated from liquid culture or from solid plates. The presence of plasmids from the resistant strain will be analyzed either by recovery of the plasmids from the resistant strains or by hybridization. We have constructed two such banks. One is by insertion of Sau3A digested *S. B12* viral DNA into BamH1 linearized pUCKm6 (Figure 2) and the other is by insertion of HaeIII digested *S. B12* viral DNA into the HindIII site (after filling in the ends) of pLG90 (Figure 3). These vectors can be used to transform both *S. acidocaldarius* and *S. B12* but they are most suitable for *S. acidocaldarius* and possibly also for other *Sulfolobus* species that do not contain the viral DNA.

The second approach is to construct *E. coli* plasmids which are capable of integrating into *S. B12* viral DNA by homologous recombination. Such an *E. coli* plasmid should contain a viral DNA fragment with unique restriction sites to facilitate the insertion of selection marker or other genes desired to clone. After the selectable gene is integrated into the viral DNA, the virus is then released from the chromosome by uv induction as reported previously and serves as a high copy-number vector. The integrative method is only suitable for *S. B12* which is the only *Sulfolobus* species containing the viral DNA.

To facilitate the construction of integrative vectors, we first cloned the BamH1 fragment which contains the entire viral DNA and also the four individual EcoRI fragments of the B12 viral DNA into *E. coli* plasmid pUC19 as shown in Figure 4. These cloned fragments provide the source of DNA for the construction of the desired integrative plasmids.

#### Isolation of *Sulfolobus* Promoters Functioning in *E. coli*

One potential problem for the above described system is that intact *E. coli* promoters for *Hyg*<sup>R</sup> and *Km*<sup>R</sup> might not be expressed in *Sulfolobus*. In order to overcome this potential difficulty, an effort was made to explore the possibility of the isolation of *Sulfolobus* promoters which can also function as promoters in *E. coli*. This was carried out by the insertion of Sau3A digested chromosomal DNA from *S. acidocaldarius* and *S. B12* into BamH1 partially digested pLG83 which contains a promoterless *Hyg*<sup>R</sup>, located on a 1.1 kb BamH1 fragment. Transformation of *E. coli* (DH5 $\alpha$ ) with the ligation mixture and selection of the transformants on agar plates containing rich medium with both Ampicillin (50  $\mu$ g/ml) and Hyg (400  $\mu$ g/ml) were carried out. Two large colonies were selected and plasmid DNA were isolated from these colonies. We found that both plasmids isolated from the hyg resistant strains contain inserts as shown in Figure 5. This indicates that *Sulfolobus* promoter fragments can be isolated by the approach described in this report. These promoters not only will be useful for the expression of the selection markers such as *Km*<sup>R</sup> and *Hyg*<sup>R</sup>, but also will be extremely useful for the expression of other heterologous genes. The DNA fragments containing the *Sulfolobus* promoters are being characterized.

#### Analysis of the Nucleotide Sequence of a *S. acidocaldarius* Promoter Fragment Capable of Functioning in *E. coli*

The nucleotide sequence of the *Sulfolobus* promoter cloned in pLG83-PSA1 (Figure 5, Lane 4) was analyzed and the sequence is shown in Figure 6. The sequence does contain the key elements of *Sulfolobus* promoters.

## DISCUSSION

Our results indicated that both *S. acidocaldarius* and *S. B12* are very sensitive to hyg. At the level 400-500 µg/ml, Hyg can totally inhibit the growth of both *S. acidocaldarius* and *S. B12*. However, both of these microorganisms are only inhibited by G418 in the presence of high concentration of this antibiotic. *S. acidocaldarius* was found also highly sensitive to 5FU but *S. B12* is only moderately sensitive to low concentration of 5FU (5 to 10 µg/ml). Currently we are testing the effect of higher concentration of 5FU on *S. B12*. Nevertheless, the hygromycin system seems to be suitable for both these microorganisms.

The drug sensitivity studies and other studies described in this report indicate that the construction of cloning vectors and the development of a transformation system for *S. acidocaldarius* and *S. B12* are within reach.

One possible limitation for the hygromycin system is whether the enzyme hygromycin B phosphotransferase (HPT) encoded by Hyg<sup>R</sup> is heat stable. Nevertheless, with recent advances in site-specific mutagenesis, the heat stability of one enzyme can be modified without too much difficulty. Furthermore, *Km<sup>R</sup>* has been shown to be used as a selection marker for plasmid-mediated transformation of *Bacillus stearothermophilus*, a thermophile and the culture that normally grows at 65°C.

## Publications

The following papers which have acknowledged or will acknowledge the support by CRSC/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," submitted to Appl. Environ. Microbio. (1991).
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 will be 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*," manuscript in preparation.
4. Ho, N.W.Y., and Brainard, A., "Sensitivity of *Sulfolobus* species to genetisin and hygromycin," manuscript in preparation.

Table 1  
Sensitivity of *Sulfolobus acidocaldarius* to Various Chemicals

<i>Sulfolobus acidocaldarius</i> Cultures (5-10 ml) in Medium 1	OD600		
	2 days	4 days	6 days
Blank (medium alone)	0	0	0
1 (control)	0.053	0.047	0.03
2 (25 µl A)	0.003	0.011	0.017
3 (50 µl A)	0.002	0.002	0.013
4 (100 µl A)	0.002	0.002	0.007
5 (200 µl A)	0.001	0.001	0.001
6 (25 µl B)	0.003	0.003	0.008
7 (50 µl B)	0.001	0.001	0.001
8 (100 µl B)	0.001	0.001	0.003
9 (200 µl B)	0.001	0.001	0.001
10 (2 ml C)	-	-	-
11 (4 µl C)	-	-	-
12 (8 µl C)	-	-	-
13 (10 µl)	-	-	-
14 (control)	0.052	0.045	0.033

A: G418, 250 mg/ml

B: Hyg, 400 mg/ml

C: 5FU, 25.0 mg/ml

Medium 1: provided by ATCC



Table 2  
Sensitivity of *Sulfolobus shibatae* B12 to Various Chemicals

<i>Sulfolobus shibatae</i> B12 Cultures (5-10 ml) in Medium 2	OD600	
	2 days	6 days
Blank (medium alone)	0	0
1 (control)	0.457	0.930
2 (25 $\mu$ l A)	0.035	0.017
3 (50 $\mu$ l A)	0.030	0.013
6 (10 $\mu$ l B)	0.032	0.008
7 (20* $\mu$ l B)	0.025	0.001
10 (2 $\mu$ l C)	0.197	0.228
11 (4 $\mu$ l C)	0.178	0.275

A: G418, 250 mg/ml

B: Hyg, 400 mg/ml

C: 5FU, 25.0 mg/ml

Medium 2: provided by W. Zillig, Max-Planck-Institute für Biochemie,  
Martinsried, FRG.

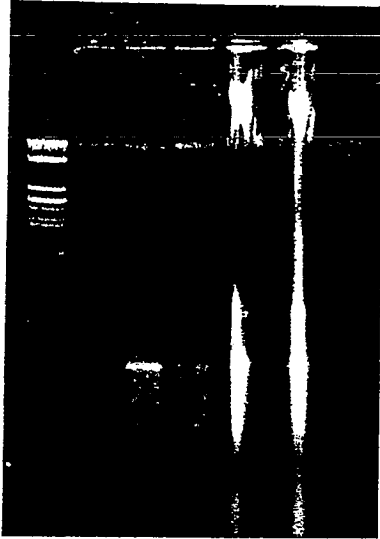


Figure 1  
 Double-stranded B12 viral DNA  
 isolated from *S. shibatae*.  
 Lanes, 2, 3, 4, and 7: DNA  
 isolated by our  
 procedure.  
 Lanes 5 and 7: DNA isolated by  
 the procedure described by  
 Yeats.

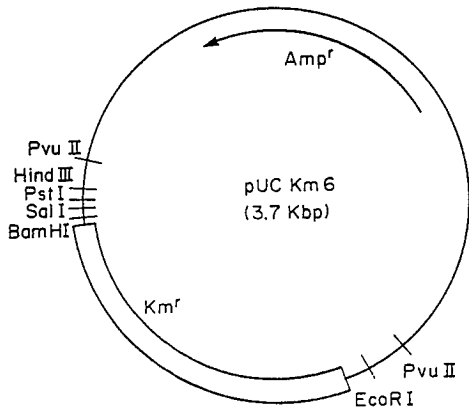


Figure 2 Restriction map of  
 pUCKm6 (Reference 8).

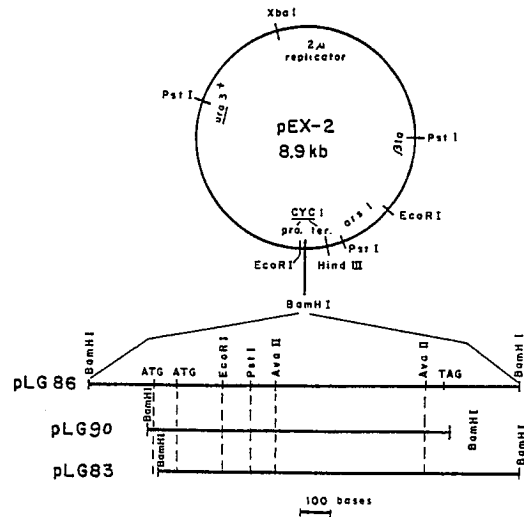


Figure 3. Restriction maps of  
 pLG90 and pLG83 (reference 5, and  
 L. Gritz personal communication).

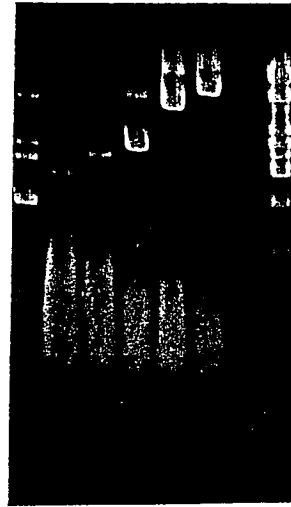
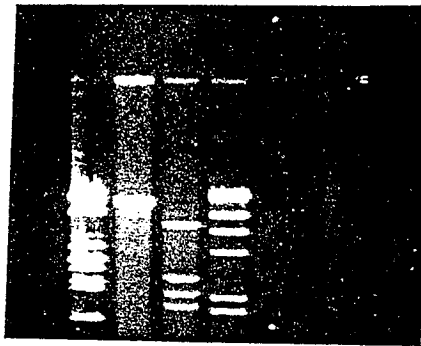


Figure 4. Cloning BamH1 and EcoRI digested B12 viral DNA fragments into pUC19. Left: Restriction digestion of viral DNA. Lane 1 and 4, molecular markers; Lane 2, BamH1 digestion of B12 viral DNA; Lane 3, EcoRI digestion of viral DNA. Right: Normal BamH1 and EcoRI fragments of B12 viral DNA cloned into plasmid pUC19. Lane 1, pUC19; Lane 2, pUC19-B12 E1; Lane 3, pUC19-B12E2; Lane 4, pUC19-B12E8; Lane 5, pUC19-B12E7; and Lane 6, pUC19-B12 Bam2.

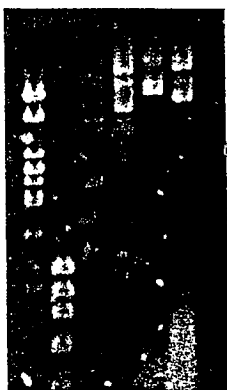


Figure 5. Plasmids containing *Sulfolobus* promoters. Lanes 1 and 2, molecular markers; Lane 3, pLG83; Lanes 4 and 5, plasmids isolated from hygromycin (400  $\mu$ l/ml) resident colonies. Both are larger than pLG83, indicating that they contain inserts.

**Nucleotide Sequence of a *S. acidocaldarius* Promoter Fragment  
Capable of Functioning in *E. coli***

10	20	30	40	50	60
AAAAGAGSTAABGAAGAGATGCATCAATCTAAACTCTTAATTACAATGATCGTGTAGCTAT					
70	80	90	100	110	120
AATAAGCCC GTAAGAACCGCTCCAGCAAATATCTGATATAGTTGAATCAATAATAGGTAA					
130	140	150	160	170	180
AGCGTTTTATAGAGTAACTTAAAGATAGAAAAGSTCGAAATTTTGGATTTTCAATTATAA					
190	200	210	220	230	240
TTATATAAATGAGTATATGTGGAATTTGCTTTTTGGAAAGTAAAAAATTATCAGATTATAA					
250	260	270	280	290	300
AATAAAGGTTCTTCCTTTTACATCTAACTTTTTCTTGGCATATATGTTAGAATCGATATC					
310	320	330	340	350	360
TATATCTGATTACTCTTACCTGAGCTTGTTCACCCCTACAGTATAATTGATACGACAAT					
370	380	390	400	410	420
CATATTCATACCACTATATTCGACTCCTATTCAAGAGATAGAATGTTTCTCCAAAATTGA					
430	440	450	460	470	480
CTATAATAAAATCAAAATTTAAGGATGA <u>ACTTTTCAATACTATATTTGATTGGGTTAACCA</u>					
490	500	510	520	530	540
GCAGTTGAGGGAAAATTATGAGCTTTTTCATACATACTTTAATA <u>GATCCCGGCCTGAAC</u>					
					ProGluL

TCACCGC  
euThr

Figure 6.