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Cloning and Expression of a cDNA for a Human Purine Biosynthetic Enzyme

Presented to the faculty of Lycoming College in partial fulfillment of the requirements for Departmental Honors in Biology

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February 16, 2005

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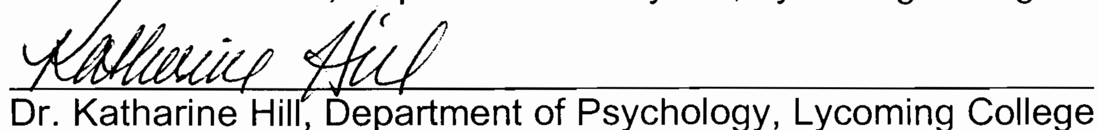

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Introduction

The purine biosynthetic pathway is a 14 step pathway that ultimately converts 5-phosphoribosyl 1-pyrophosphate (PRPP) to AMP and GMP (Appendix A-1). AMP and GMP are two of the bases that compose DNA and RNA. The purine pathway is conserved among most organisms, because the production of purines is necessary for energy metabolism and the synthesis and repair of DNA and RNA.

This study focuses on the fourth step of the purine biosynthetic pathway, the conversion of formylglycinamide ribonucleotide (FGAR) to formylglycinamidine ribonucleotide (FGAM) by FGAR amidotransferase (FGARAT), also referred to as FGAM synthase. In this step, an amino group is transferred from glutamine, and then the terminal phosphoanhydride bond breaks to enolize the carbonyl group of FGAR to produce FGAM (Figure 1 and 2). Significant differences exist between the human FGARAT and FGARAT in many bacteria; exploiting those differences could lead to a new antimicrobial agent.

Figure 1: Diagram illustrating the reaction of FGAR to FGAM, by FGAR amidotransferase (Anand et al, 2004).

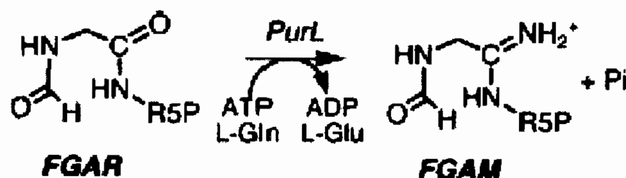
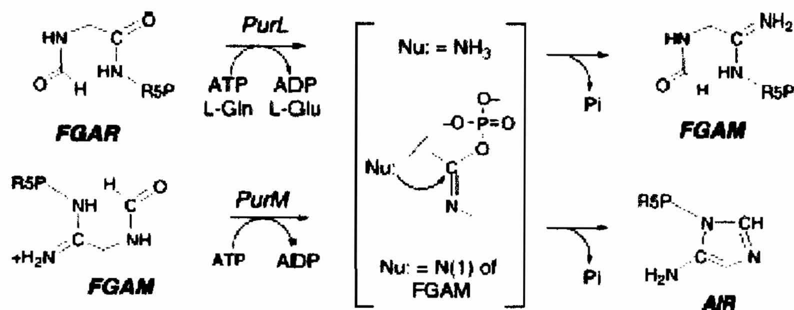


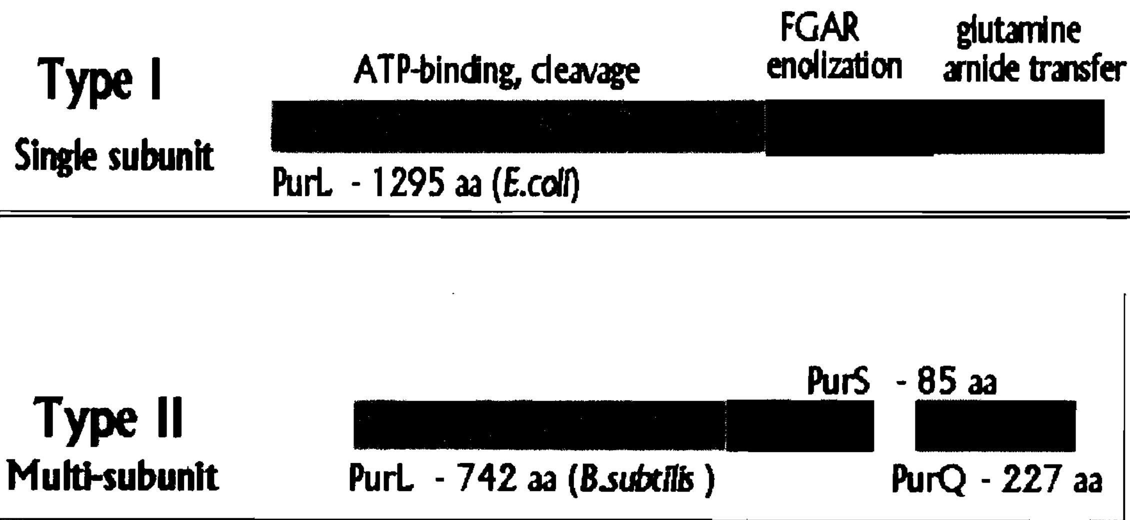
Figure 2. FGAR to FGAM showing the intermediates (Hoskins et al; 2004).



FGARAT is on chromosome 17 (Patterson et al, 1999). It was the last purine biosynthetic enzyme to be crystallized (Anand et al, 2004). The crystallization will allow for further characterization. Recent studies have proposed that FGARAT should be added to the list ATP-requiring enzymes (Anand et al, 2004). Figure1 shows that the conversion of ATP to ADP is necessary to form the product. Also, FGARAT is a relatively large protein with a molecular weight of 146 kiloDaltons. A proposed function for FGARAT is as a metabolon, or scaffold protein of the purine pathway. Scaffold proteins stabilize the high energy compounds involved in an enzymatically catalyzed reaction. The size of FGARAT and the instability of reaction intermediates involved suggest that it acts as a scaffold for other steps in this pathway (Anand et al, 2004).

The gene that encodes FGARAT is purL. There are three different types of FGARAT: Type 1 and Type 2 and a recently identified heterodimeric enzyme.

Figure 3: A comparison of Type 1 and Type 2 FGAR amidotransferases.



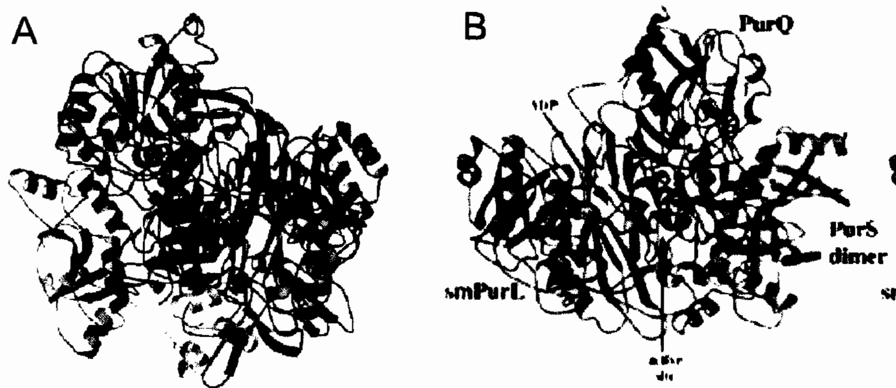
Type 1 is the single subunit protein that is found in Humans and β and γ proteobacteria (or Gram-negative bacteria). Within the single subunit there are 3 domains, the N-terminal domain, the FGAM synthase domain, and the glutaminase domain. The FGAM synthase domain contains the FGAR binding

site, and thus is responsible for the nucleophilic attack by ammonia to the ATP-activated FGAR amide oxygen (Figure 2) (Hoskins et al; 2004)

Type 2 is a three subunit protein, and is found in Archaea and most other bacteria (Gram-positive bacteria), including *Staphylococcus aureus.* The three subunits are encoded by the genes purL, purS, and purQ. PurL is homologous to the FGAM synthase domain in Type1. PurQ has the same function as the glutaminase domain. PurS is homologous to the N-terminal domain of single subunit purL, but does not have a known function. There is a third type of FGARAT that is a two subunit, heterodimeric protein that is found in δ proteobacteria (Sehi and Newman, unpublished); this type will not be focused on in this study.

The structural difference between Type 1 and Type 2 is the target for inhibition. Protein sequence alignment show significant homology between Types 1 and 2, but also significant differences. Figure 5 show cartoon comparisons of Type 1 FGARAT from *Salmonella typhimurium* and Type 2 FGARAT from *Bacillus Subtilis.*

Figure 5: Comparison between Type 1 *Salmonella typhimurium* (A) and Type 2 *Bacillus Subtilis* (B) Protein Structures (Hoskins et al; 2004) (Anand et al, 2004).



Biochemical studies require large amounts of purified protein in its active form. Our lab accomplishes this by using two vector systems, TOPO pCR 2.1 TA

cloning system (Invitrogen) and pET expression system (Novagen). pCR2.1 is a plasmid that is specially engineered to have high ligation rates when inserting a PCR product. PCR, or polymerase chain reaction, is used to amplify a DNA sequence. During the process of DNA amplification through PCR, a single deoxyadenosine (A) is added to each end of the DNA fragment by Taq polymerase. The pCR2.1 vector is a circular DNA plasmid that has an opening flanked by sticky ends with one deoxythimidine (T) at each side of the opening of the plasmid. The T's and A's are bound together by topoisomerases that are covalently bound to the pCR2.1 vector. This process for T-A insertion with topoisomerases has over 95% efficiency and is also is easier because it does not require an additional restriction site (Invitrogen, 2001). pCR 2.1 is plasmid that is not involved in protein expression. Its purpose is to maintain the target gene for insertion into protein expression vectors. The TOPO system also has the lacZ gene incorporated into the plasmid for blue-white screening. The lacZ gene encodes the β-galactosidase enzyme. The cells are grown in the presence of X-gal, the substrate for. The reaction between the enzyme and x-gal is a blue product. If the clone is inserted into the plasmid correctly the lacZ gene will be separated from its promoter and lacZ is not expressed. If the β-galactosidase enzyme is not present, the cells will remain white in color. pCR 2.1 map found in Appendix A-2.

The pET vector protein expression system facilitates the expression of a target protein. The vector is engineered to have a multiple cloning site with cut sites for various restriction enzymes to easily insert the gene of interest. pET vectors utilize the T7 RNA polymerase available in engineered strains of *E. coli*. These strains have a lac promoter which activates expression of T7 RNA polymerase when in the presence of lactose or a structurally similar compound such as IPTG. The pET vector contains a T7 RNA polymerase promoter upstream of the cloning site, so the production of T7 RNA polymerase by the cell activates the expression of the target protein. Therefore, induction of the cells carrying the pET vector with IPTG results in production of the target protein. Diagram of pET expression system found in Appendix A-3.

The pET vector system also facilitates protein purification by incorporating a 6x Histidine tag (His-tag) into the C-terminus of the protein. The His-Tag is highlighted yellow in figure 6. The his-tag has affinity to nickel ions, so that the target protein can be separated from other bacterial proteins. There are several protocols developed for nickel affinity purification. The method used in this study is magnetic nickel agarose bead affinity purification, specifically using His-Bind Magnetic Agarose Beads (Novagen, 2004.) Nickel ions are bound to a magnetic agarose bead. The proteins with a his-tag will bind to the nickel ions attached to the bead. A strong magnet can be used to separate the beads from the other proteins extracted from the cell. The proteins are washed from the beads using elution buffers. The results can be analyzed on SDS-PAGE verify the size of the protein.

Figure 6: FGARAT fusion protein produced from Hs purL. FGARAT has a total of 1358 residues and molecular weight (MW) of 146 kDa.

```
atgggcagcagccatcatcatcatcatcacagcagcggcctggtg
M  G  S  S  H  H  H  H  H  H  S  S  G  
Ccgcgcggcagccatatgtccccagtccttcacttctatgtt...
               H     S  P  V  L  H  F  Y  V

...aactggaccctggaagggagctgctga
   N  W  T  L  E  G  S  C  
```

His-tag

of proteins that affect cell growth and viability, which stabilizes the pET recombinants. Lysozyme and tRNA genes are on the same plasmid, which is resistant to chloramphenicol. Chloramphenicol resistance allows the use of double-selective media to select for cells carrying both the pET plasmid and the plasmid carrying lysozyme and tRNA genes.

The expression and purification of FGAR amidotransferase is necessary for future biochemical studies to exploit the differences in the FGARAT enzymes of S. aureus and H. sapiens. The goal of this study is purify soluble protein extract of human FGAR amidotransferase. Sufficient protein was expressed and purified, but the protein proved to be insoluble. Future studies will attempt to solubilize the protein for purification and use in biochemical studies.

Methods

Ligation of purL insert into pET 15, 19, and 28a and transformation into competent cells

The HspurL pCR 2.1 TOPO clone (Leader, 2001) was digested with restriction enzymes to isolate the purL gene. The digest reaction (40ul) included 20ul purified plasmid, 4ul 10x buffer (NEB), 5ul NdeI (NEB), 3.5ul XhoI (NEB), 3.5 BglII (NEB), and 4ul 10xBSA. The reaction incubated for 2 hours at 37°C. The three bands created by the digest were separated by gel electrophoresis. The band at 4.0kbp was cut out from the gel and purified using Qiaquick gel extraction kit (Qiagen).

The pET vectors, 15, 19, and 28a, were digested with NdeI and XhoI to open the plasmid for insertion of the cDNA cut with identical enzymes. The digest reaction (40ul/vector) included 15ul purified plasmid, 4ul 10x buffer (NEB), 4ul NdeI (NEB), 4ul XhoI (NEB), 4ul 10xBSA, and 9ul dH2O. The reaction incubated at 37°C for 2hours. To finish preparing the vectors for ligation, the enzymes were inactivated by heating for 65°C for 20min.

The purL insert was ligated into the pET vectors using DNA ligase (NEB) that requires a minimum of one hour at room temperature incubation time, though longer incubation time is acceptable. One ligation reaction was prepared for each pET vector (15, 19, 28a); each 10ul reaction included 1ul ligase, 1ul ligase buffer, 3ul purified purL plasmid, and 5ul pET vector. The reactions were incubated at room temperature (25°C) for 1hour. A control ligation was conducted using a reaction containing only pET vector without the insert. The 10ul reaction mixture contains 6ul purified plasmid vector (15b, 19b, 28a), 1ul 10x ligase buffer, and 1ul ligase.

The ligation mixtures were transformed into NovaBlue competent cells according to the protocol accompanying the cells (Novagen, 2004). 2ul of ligation reaction was mixed with one 50ul tube of competent cells. The cells were incubated on ice for a minimum of 5 minutes. The cells were then heat shocked for exactly 30seconds at 42°C and cooled on ice for 5min. To ensure individual colonies, 25ul of pET 15 and pET 19 were plated onto LB plates selecting for

arnpicillin resistance. For the Kanimycin resistant plasmid pET28a, 250ul of SOC growth medium was added to the transformation tube. After a 30min incubation at 37°C, 100ul of the transformation was plated on LBkan. The plates were incubated overnight at 37°C.

Analysis of purL ligation into pET vectors

Plasmids were isolated from four or five colonies from each vector, pET 15, 19,28a, to be analyzed. The Qiaprep spin miniprep kit was used to purify the plasmid from NovaBlue competent *E. coli* cells. The plasmids were run on a 0.8% agarose gel to identify the colonies where the purL insert was ligated into the pET vector. The positive lane where the vector included the insert was digested with enzymes to verify the presence of the Hs purL insert. To ensure that the purL gene correctly inserted into the pET 15 plasmid, the plasmid was digested with HindIII (NEB) to check the orientation. A second reaction with the ligation restriction sites, NdeI and XhoI (NEB), confirmed the size of the insert. A small scale digest (10ul) was completed using 3ul purified plasmid, 1ul of each enzyme, 1ul 10x buffer (NEB), and dH20 to a final volume of 10ul. The reaction incubated at 37°C for 1-2 hours. The restriction enzyme digestions were analyzed using gel electrophoresis on a 0.8% agarose gel.

General Procedure for Protein Expression

Two LB flasks were inoculated for each *E. coli* strain and/or construct with previously prepared overnight culture. The cultures incubated for 2-3 hours at 37°C while shaking, until the cultures reached an OD_{600} 0.5 to 1.0. When the cells reached the required cell concentration, 100mM IPTG was added to a final concentration of 1mM to one culture per *E. coli* strain and/or construct. The cultures with IPTG were considered induced (I) and the cultures lacking IPTG were considered uninduced (U). The time zero OD_{600} was recorded for each flask using a light spectrophotometer. Then, the cultures incubated while shaking at room temperature (25°C). 1-5mL samples were taken at pre-determined time points. When each sample was taken, the OD600 was recorded

and the cells were centrifuged at maximum speed for 3-5 minutes to pellet the cells and the supernatant was removed. The pellet was stored at -20°C until further analysis.

The pelleted cells were resuspended in 150ul BugBuster (Novagen, 2004) per OD_{600} per mL of sample. BugBuster solubilizes the cells to extract the proteins. To reduce viscosity, 1ul Benzonase (Novagen) per 1mL BugBuster was added to each sample and incubated at 37°C for 1hr. Benzonase is an endonuclease that degrades any form of DNA or RNA to lower the viscosity of the protein extract. For the crude sample, 100ul was transferred from each sample into a microcentrifuge tube. 3xSDS was added to a final concentration of 1xSDS to each tube and heated at 95°C for 20min. To analyze the protein, 10ul of each sample was loaded onto a 4-15% Tris SDS-PAGE gel (Biorad) and run at 150 volts for approximately 45min. The gel was stained overnight with Coomassie Blue Stain and destained for 4-6 hours with 10% acetic acid, 30% methanol, and dH2O (Sambruch and Russel, 2001). The Kaleidoscope Pre-stained protein Standard (BioRad) served as the marker.

To separate the protein extracts into soluble and insoluble fractions, the extracts in BugBuster were centrifuged at 14,000rpm for 10min. The supernatant (soluble extract) was transferred to another microcentrifuge tube. The pellet was resuspended in the same volume of BugBuster. The soluble and insoluble samples were included on a SDS-PAGE gel with the crude samples for comparison.

Protein Induction with purL pET15 in BL21(DE3) E. coli strain

Four 5mL tubes were inoculated with overnight cultures. Ampicillin (Amp) selected for the pET plasmid. Two tubes are designated induced and uninduced for the purL pET 15 plasmid and two tubes for the pET 15 only plasmid. 1mL samples were taken after 8hrs and analyzed using SDS-PAGE.

Purification of FGARAT fusion protein by Magnetic Nickel Affinity Agarose Beads

1mL cell extracts of an 8hr induction of Hs purL in pET15 in BL21(DE3) were lysed with BPERII (Pierce, 2002). The beads were washed to prepare for binding. 50ul of slurry, 25ul His-Bind Magnetic Agarose Beads (Novagen) and 25ul buffers, was washed four times with 200ul of 1xBinding buffer. The beads were collected for each was in the Magnetight Rack (Novagen). After the fourth wash the beads were resuspended in 50ml of 1xBinding buffer. Next, the equilibrated beads were combined with the 1ml protein extract. The mixture incubated at room temperature for 5 minutes, inverting often to mix. The beads were separated from the extract in the magnetic rack. To remove unbound proteins from the beads, the beads were washed with 200ul of 0.5x Wash buffer three times (Sambrook and Russell, 2001). The washes were saved for analysis. To elute the Hs purL His-tag fusion protein from the magnetic beead, 200ul 0.5x Elution buffer (Sambrook and Russell, 2001) was added to the beads and incubated at room temperature for five minutes. A second and third elution was completed with 100ul of 0.5x elution buffer and incubated at room temperature for 5 minutes. The crude extract, post-binding extract, and the three elutions were analyzed on SDS-PAGE.

48hour induction with purL pET 15 in Rosetta E. coli Strain

Four flasks of 125 ml LB selecting with Chloramphenicol (Cam) and Ampicillin (Arnp) were inoculated with overnight cultures. Two flasks were inoculated with purL pET 15 plasmid in Rosetta and the other two with the control pET15 plasmid in Rosetta. Following the same protocal previously stated in the general procedure for protein expression, 5mL samples were removed every 8hrs during the 48 hour time period. The solubility of the protein was determined using the BugBuster protocol.

Small scale induction for 8hrs

Four 5ml LB tubes were inoculated; two inoculated with pET 15 in Rosetta and two tubes with purL in Rosetta strain. A sample was taken at 8hrs after induction with IPTG at room temperature. The samples were prepared and

induction with IPTG at room temperature. The samples were prepared and analyzed using SDS-PAGE gel electrophoresis on the same day to help the protein become soluble.

Protein induction at 25°C, and 15°C over an eight hour time period.

Four 50ml LB flasks were inoculated with ovemight culture. Two flasks were inoculated with purL in Rosetta strain and two flasks with pET 15 in Rosetta strain. The flasks incubated at 37°C till OD600 was reached. The contents of each flask was divided to create the induced and uninduced samples. The induction continued to proceed at 25°C and 15°C. 5mL samples were obtained at 1hr, 2hr, 4hr, 6hr, and 8hr. The general induction protocol was followed.

Results and Discussion

pCR 2.1 Clone and Preparing Insert for Ligation

The orientation of the PurL insert is shown in Figure 7 in the TOPO pCR 2.1 TA vector. The purL cDNA is approximately 4.0 kbps, which is approximately the same size as the 3.9 kps pCR 2.1 vector. In order to distinguish between the pCR 2.1 vector and the purL insert, the vector was cut a third time, resulting in three fragments (figure 8). The results of the pCR2.1 clone digests shows a single band at the expected size of 5.5kbps (figure 9).

Figure 7: Map of Hs purL in pCR 2.1 Clone

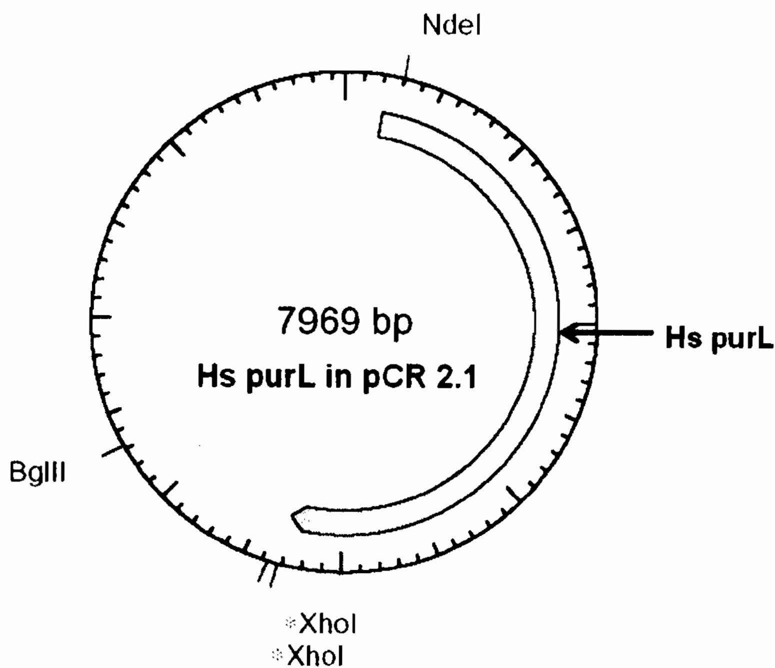


Figure 8: Hs purL in pCR 2.1 digested by Bgl11, Nde1, and Xho1 and separated by gel electrophoresis. Arrow indicates Nde/Xho1 Hs purL fragment that was cloned into a pET vector.

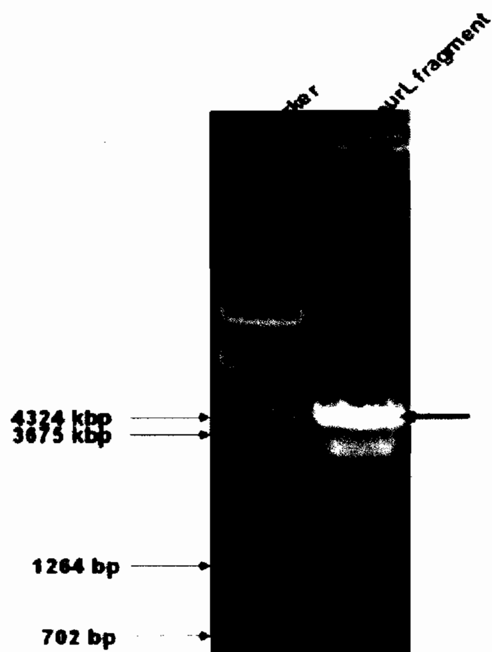
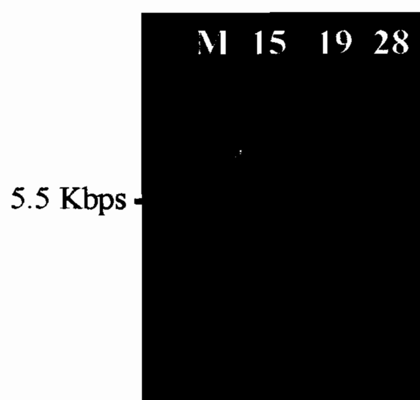


Figure 9. pET vectors (15, 19, and 28a) digested with Nde/XhoI and analyzed by gel electrophoresis.



Cloning of PurL gene into pET 15b

10-20 Colonies were formed on each plate after transformation of, pET 15, 19, and 28 ligated with Hs purL into *E. coli* BL21(DE3). The electrophoresis of the plasmid preps from the three plates showed one positive plasmid (Figure 10).

The two different sized bands of DNA ran at 5.5kbps and 9.5kbps. The band at 5.5kbps is the only pET vector. The band at 9.5kbps is the pET vector with the Hs purL insert. The positive clone in pET 15 is approximately 4kbps above the expected band of 5.5kbp. Figure 11 is a map of Hs purL in pET 15, which shows the restriction sites used to verify the insertion of Hs purL.

The restriction digests to verify the presence of Hs purL were also positive (Figure 12). The HindIII lane has two bands at 7.0 kbps and 3.0kbps showing that the insert is in the correct orientation. A backward oriented insert would have bands at 9kbp and 700bps. The results of the restriction enzyme digest with NdeI and XhoI showed a band at 4.0 kbps is at the expected size of the purL fragment. The uncut control lane shows the unlinearized plasmid preparation (Figure 12).

Figure 10: Results of plasmid preparations from transformants. The green arrow shows the positive ligation of Hs purL in pET 15 at approximately 9.7kbps. The DNA ladder marker is labeled M.

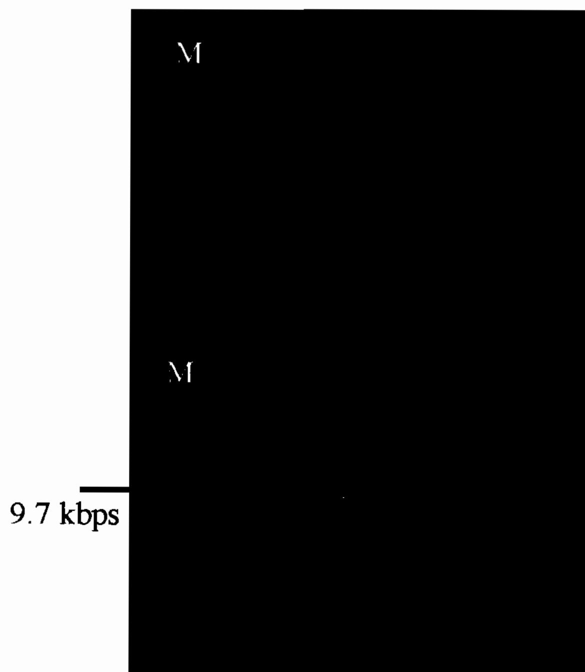


Figure 11. Map of Hs purL in pET 15 clone

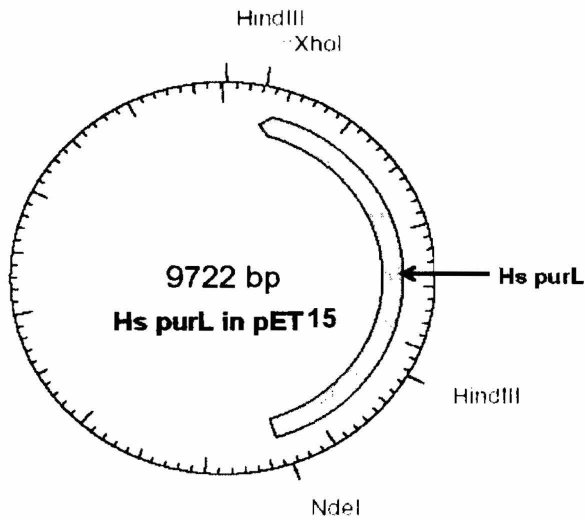
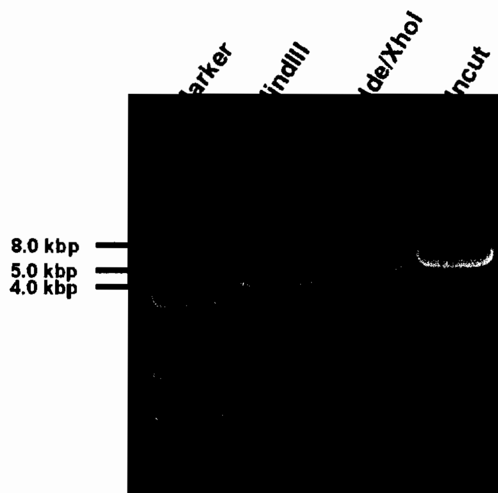


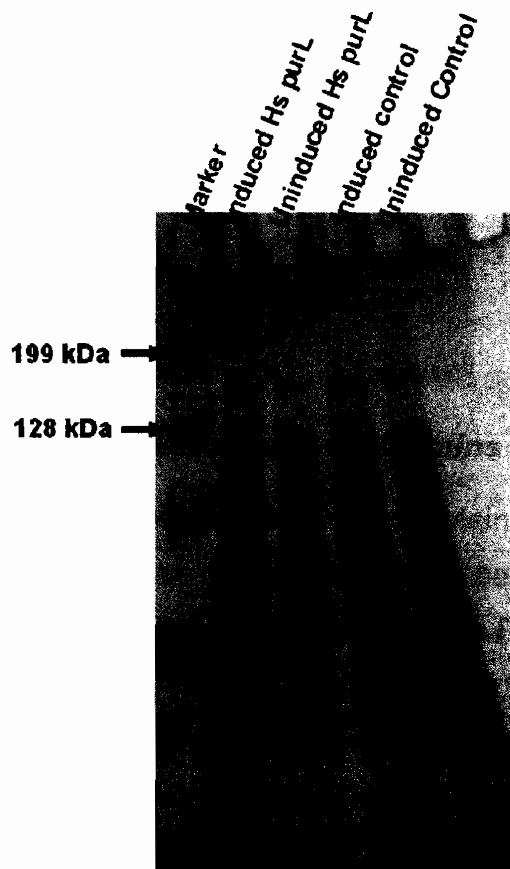
Figure 12. Analysis of Hs purL in pET 15 clone by restriction enzyme digestion. The arrow at 4.0 bps shows Hs purL in the Nde/XhoI Lane.



Protein Expression and Purification of Hs purL pET15b in BL21 (DE3)

The SDS-PAGE gel showed a band at the expected size of purL at approximately MW 146 kDa in the induced Hs purL lane (Figure 13). The band is confirmed to be Hs purL, because the band is not in the uninduced Hs purL lane or either control lane. The band was not present in the uninduced lane, because IPTG was not added to the cells, so the pET vector plasmid was not activated to express the target protein. The control lane was BL21(DE3) transformed with pET 15 plasmid not containing the purL insert. As expected, the two lanes with the pET 15 control did not have a band for Hs purL.

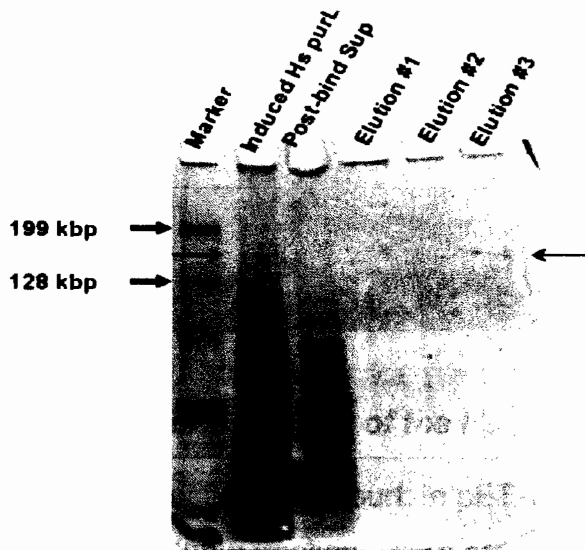
Figure 13: Cells were incubated at 24°C for 24hrs in presence (induced) or absence (uninduced) of IPTG. Proteins were separated by SDS-PAGE on a 4-20% gradient gel. Arrow indicates Hs purL fusion protein.



Affinity Chromatography

The band in the induced Hs purL lane between the 199 kDa and 128 kDa protein standards is at approximately 146kDa, the size of Hs purL (Figure 14). The second lane is the post-binding supernatant in which the Hs purL His-tag fusion proteins have been removed by binding with the nickel magnetic agarose beads. In elution lane #3, there is a Hs purL band at 146kDa. The presence of a functioning his-tag on the Hs purL protein shows that the pET plasmid with Hs purL is functioning properly.

Figure 14: Affinity purification of Hs purL fusion protein. The proteins were separated by SDS-PAGE on a 4-20% gradient gel. The arrows indicate the Hs purL fusion protein.

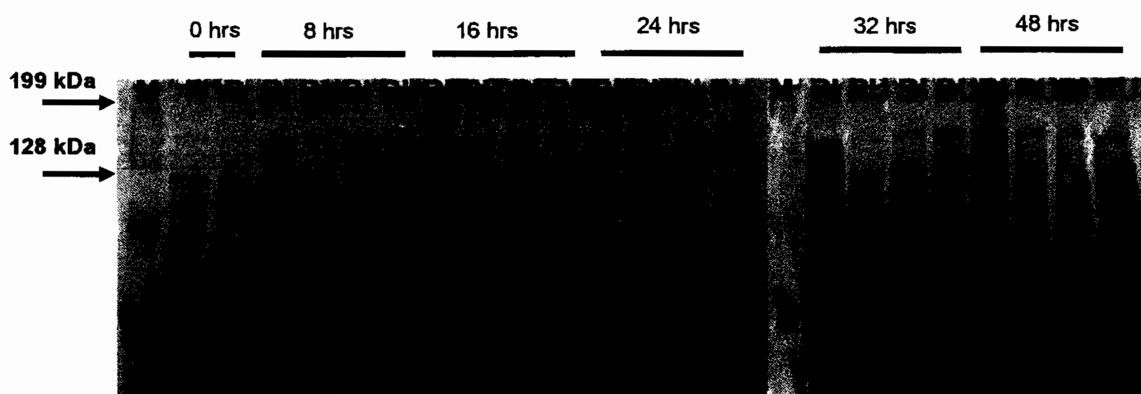


Expression comparison of E. coli strains transformed with Hs purL pET 15b.

For biochemical studies, more protein than what is shown in Figure14 is required. To increase expression, the Hs purL pET15 plasmid was transformed into the Rosetta *E. coli* strain. As shown in Figure 15, the expression level of Hs purL was increased 100 fold. Lane M is the Kaleidoscope Protein standard. Lanes designated B are the BL21 (DE3) *E. coli* strain. Lanes designated R are the Hs purL plasmid in Rosetta strain. The lane with Rosetta induced (RI)

extracts show the Hs purL high expression levels. The uninduced Rosetta lane (RU) shows no protein expression. The BL21(DE3) comparison lanes (BI and BU) for each time point do not have any significant protein expression. The Hs purL protein expression levels remained constant throughout the experiment. The constant levels show that the ideal protein expression time maybe below 8hrs. To have the highest efficiency protein isolation, the greatest proportion of target protein to background protein levels is required.

Figure 15: IPTG induction of Hs purL pET 15 in Rosetta Strain (R) in parallel with BL21 (DE3) (B). Induced lanes (I) and uninduced lanes (U) are shown for each time point. Arrow indicates Hs purL fusion protein.



Determination of Solubility of the Hs purL protein.

A 8hr induction of Hs purL in pET 15 in Rosetta was completed and the soluble and insoluble fractions were prepared. Figure 16 shows that the FGARAT protein was insoluble. A soluble protein is required because soluble proteins are presumed to be biochemically active. If the protein is folded incorrectly agglutination can occur causing the protein to become insoluble. Also the cell can react to the foreign protein by packaging it into inclusion bodies. It is not surprising that FGARAT is insoluble due to its size. Four killibase pairs is a very large protein to express in a bacterial strain. One way to improve solubility is to vary the temperature. Lower temperatures can be conducive to protein

folding and result soluble protein. Figure 17 shows the result of an induction at 15°C over an 8 hour time. There was no FGARAT bands visible even at the longest time point of 8-hours. The protein extracts showed that the cells must take longer to produce proteins at the lower temperature. Figure 18 shows the room temperature (RT) induction conducted simultaneously with the 15°C induction. The four, six, and eight hour time points show the insoluble FGARAT protein. The earlier time points at one and three hours show no visible FGARAT protein. The minumum incubation time at room temperature is 4-hours to have significant protein expression for analyzation.

Figure 16: 8-hour induction of Hs purL in pET 15 in Rosetta *E. coli.* The arrow shows the size of FGARAT and the corresponding insoluble protein. The lanes are Crude Extract (C), Soluble (S), Insoluble (I), and Crude Uninduced (CU).

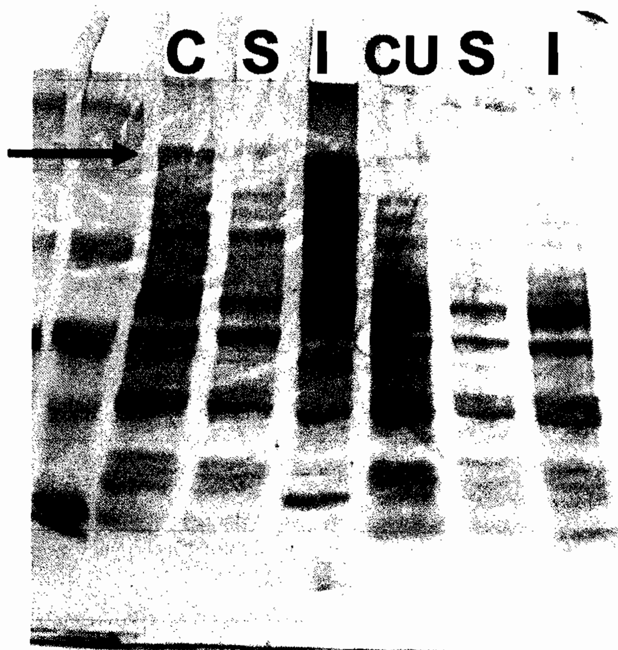


Figure 17. IPTG induction of Hs purL fusion protein at 15°C with time pints between 0 and 8 hours. The arrow indicates the presumed location of the Hs purL fusion protein. Lane C indicates the induced crude extract with all cell proteins. Lane CU indicated the uninduced control sample. Lane S indicates the soluble proteins in the induced extract. Lane I indicates the insoluble proteins in the induced exstract. The lane order remains the same throughout the gel.

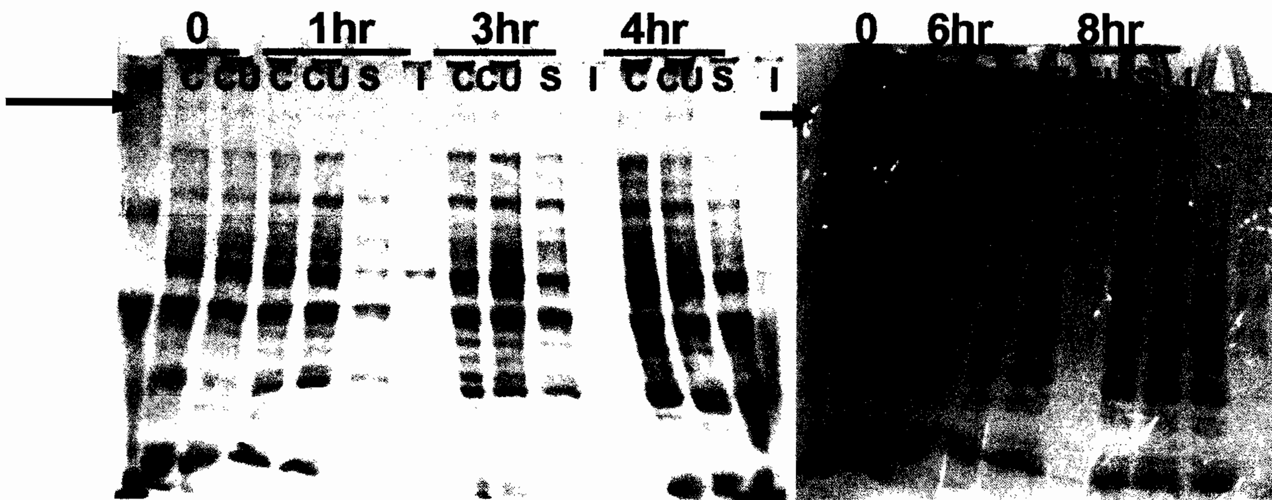
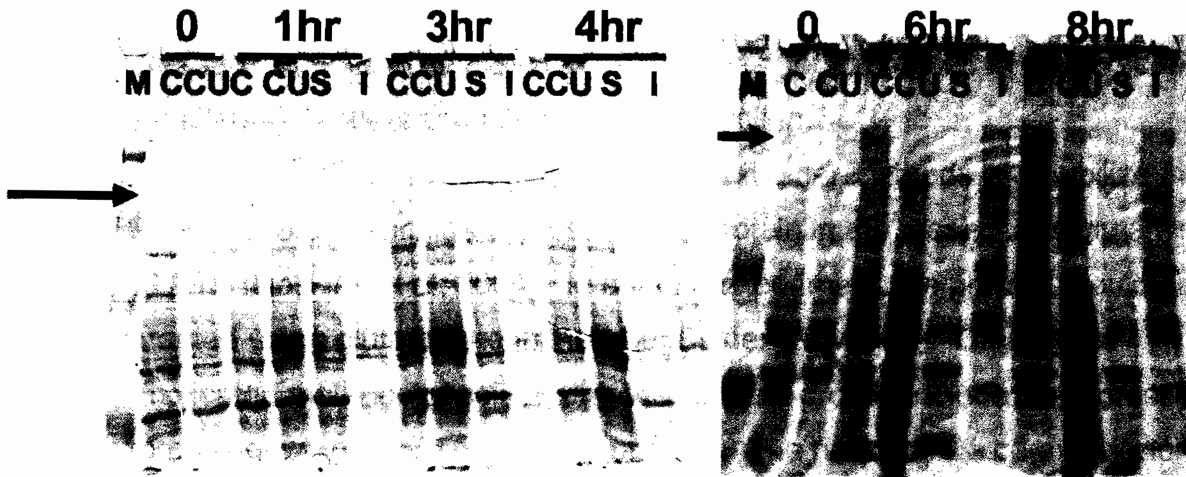


Figure 18. IPTG induction of Hs purL fusion protein at 15°C with time points between 0 and 8 hours. The arrow indicates the presumed location of the Hs purL fusion protein. The lanes are noted as in Figure 17.



Conclusions

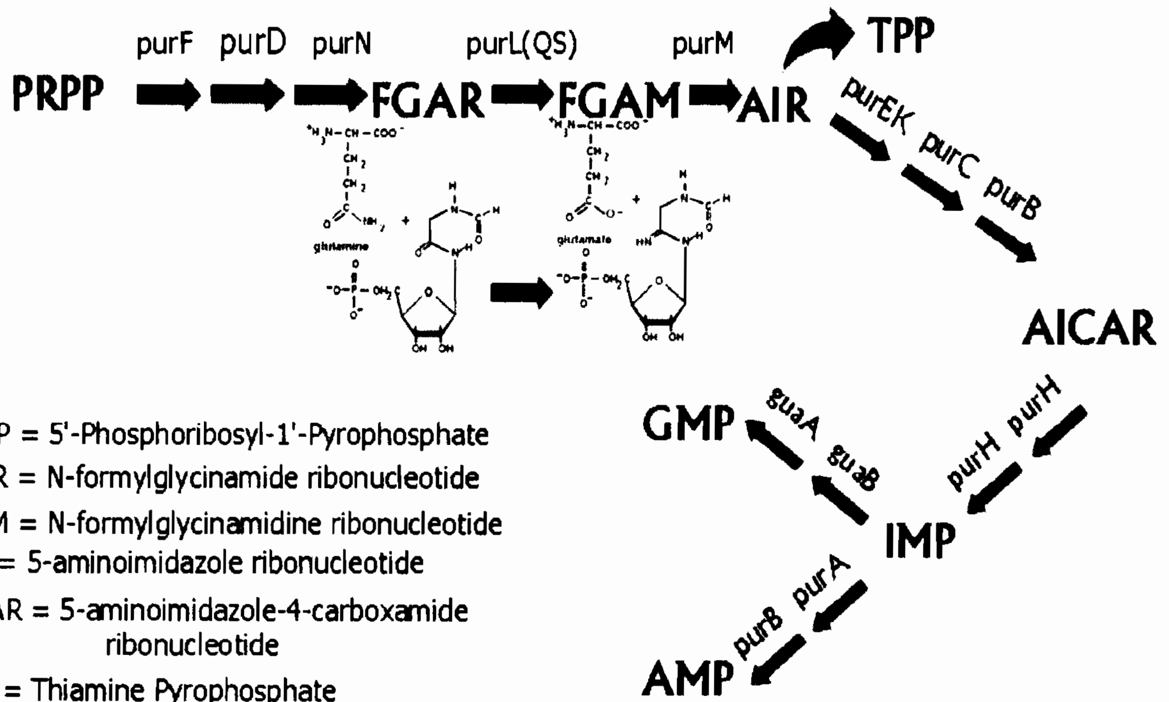
Hs purL cDNA was successfully ligated into the pET 15 expression vector. FGARAT was expressed and purified in BL21 (DE3). With the help of the engineered Rosetta *E. coli* strain, the expression levels of FGARAT were increased 100 fold. The FGARAT protein was not expressed in a soluble form after altering temperature and length of induction.

Recent studies published in 2004 used other methods to solubilize the FGARAT protein, such as expressing the protein in the presence of glutamine and ATP (Hoskins et al; 2004). In a future experiment, the proteins could be extracted using glutamine-enriched BugBuster (Novagen). Other more complicated methods of solubilizing the Hs purL protein include co-expression, expressing two or more proteins in one cell. The need for the co-expression could be linked to the scaffold protein qualities of FGARAT.

Biochemical studies can begin once the purification of FGARAT in an active soluble form is complete. The purification of the multi-subunit S. aureus enzyme is also necessary to begin biochemical studies. The biochemical studies will test for inhibitors of the S. aureus Type II multisubunit FGARAT by enzyme assays. The human FGARAT will act as a control for the enzyme assays, because a successful antimicrobial agent requires the human FGARAT to be unaffected by the inhibitor. In the future, these biochemical studies may lead to an antimicrobial agent that will inhibit the increasing number of antibiotic-resistant S. aureus strains.

Appendix A

Figure 1: 14-step Purine Biosynthetic Pathway



PRPP = 5'-Phosphoribosyl-1'-Pyrophosphate
FGAR = N-formylglycinamide ribonucleotide
FGAM = N-formylglycinamidine ribonucleotide
AIR = 5-aminoimidazole ribonucleotide
AICAR = 5-aminoimidazole-4-carboxamide ribonucleotide
TPP = Thiamine Pyrophosphate

Figure 2. TOPA TA cloning plasmid; pCR 2.1

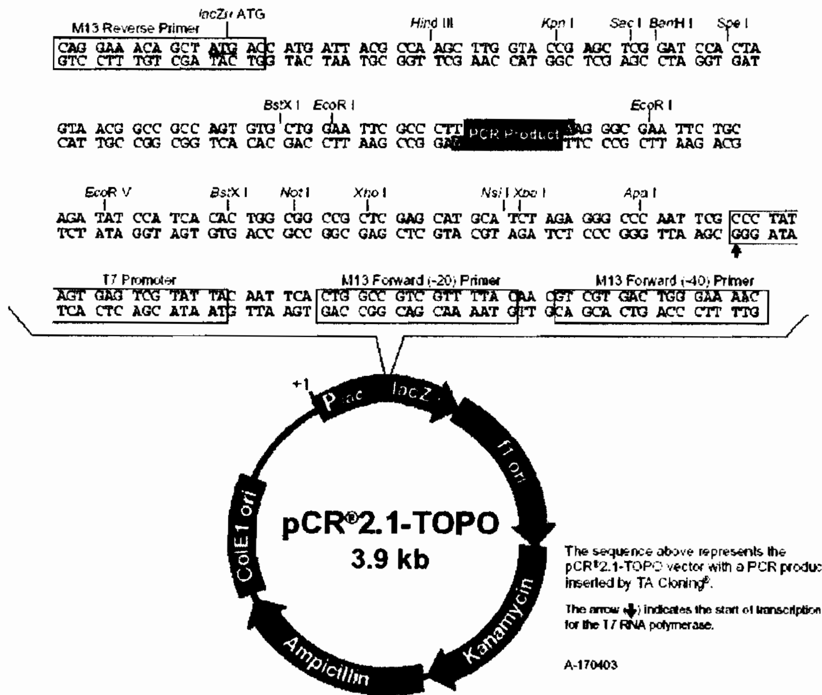


Figure 3: pET Expression System

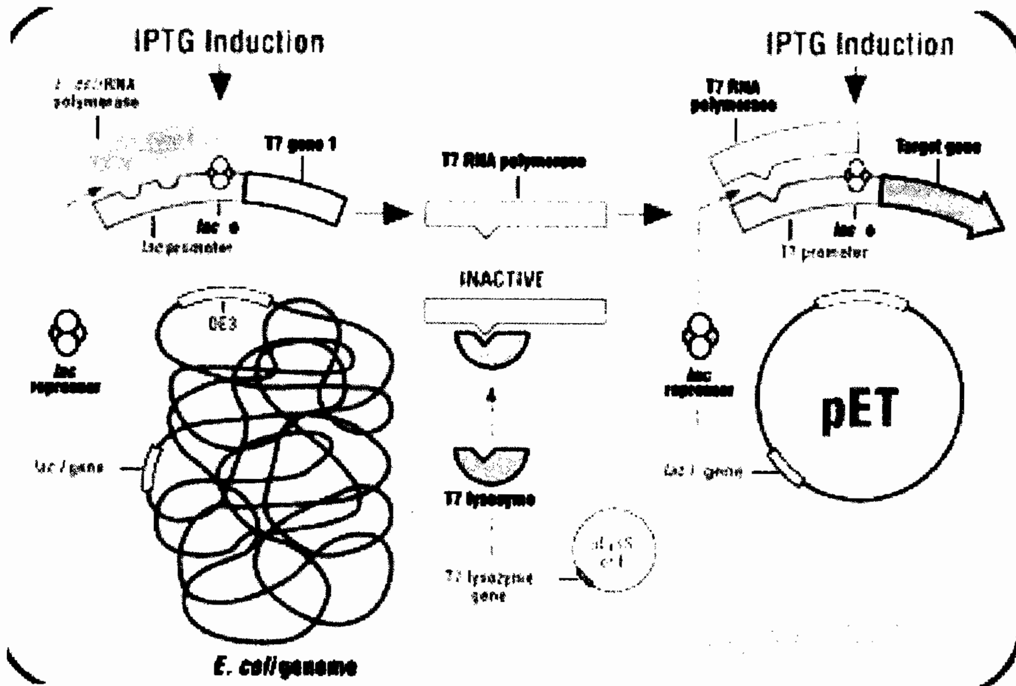
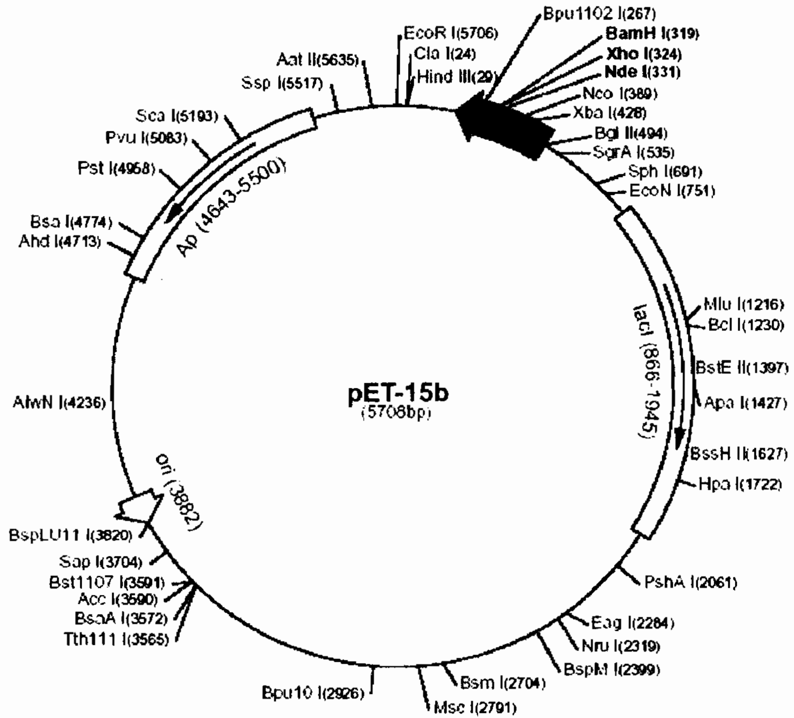
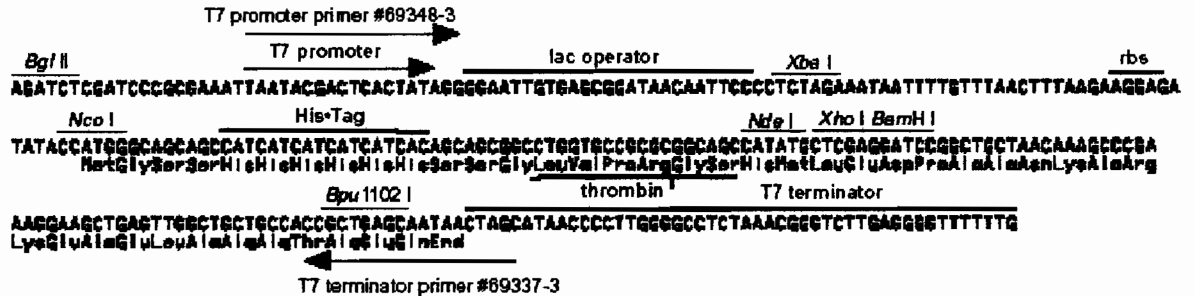


Figure 4: pET 15 Map

pET-15b sequence landmarks	
T7 promoter	463-479
T7 transcription start	452
His•Tag coding sequence	362-380
Multiple cloning sites (*Nde* I - *Bam*H I)	319-335
T7 terminator	213-259
lacI coding sequence	(866-1945)
pBR322 origin	3882
bla coding sequence	4643-5500





pET-15b cloning/expression region

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