The Cloning, Overexpression and Purification of the Human FGAR Amidotransferase Protein

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

By
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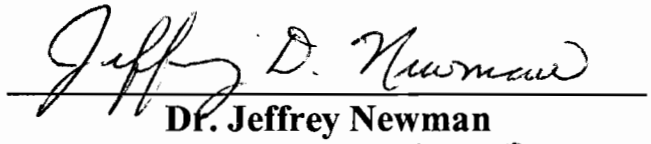
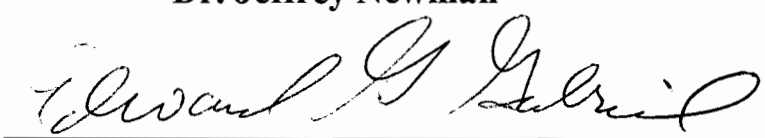
April 17th, 2002



Dr. Jeffrey Newman



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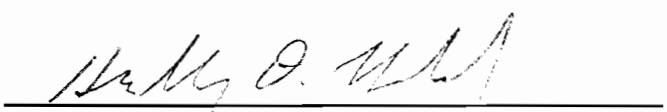

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Abstract

The fourth step of the purine biosynthetic pathway consists of the conversion of phosphoribosyl-formylglycinamide (FGAR) into phosphoribosyl-formylglycineamidine (FGAM) by the enzyme FGAR Amidotransferase (FGARAT). This enzyme is unique because there are two different types. Type I is found in Eukaryotes and ß and γ Proteobacteria. Type II is present in Archaea and most Bacteria. *Staphylococcus aureus* is a bacterium with the Type II enzyme. Each year this bacterium is developing new resistances to current antibiotics. This mutant bacterium can therefore only be stopped with novel antibiotics. Because of the two different forms of the FGAR Amidotransferase enzyme, the fourth step of the purine biosynthetic pathway is an optimal location to develop an inhibitor-type antibiotic that will block only the bacterium's ability to synthesize DNA. To begin this study, the Type I enzyme must be expressed. The Type I enzyme is encoded by a single gene, *Pur*L. In this particular project, two systems, both Invitrogen's TOPO TA® Cloning and Novagen's pET Expression systems, were used to clone the *Homo sapiens Pur*L gene. Following development of the clone, expression of the protein was examined. An optimal expression time and temperature for the production of the FGAR Amidotransferase protein has yet to be determined.

Introduction

A 14-step pathway precedes the production of AMP and GMP. This *de novo* purine biosynthetic pathway, seen in Figure 1, begins with 5-phosphoribosyl 1-pyrophosphate (PRPP). Following 10 reaction steps, inosine monophosphate (IMP) is formed. From IMP a branching occurs in the pathway in which AMP and GMP are ultimately generated (Zalkin and Dixon 1992). Most organisms have the ability to produce purines in this manner. The complete pathway and all its components are found in Appendix A, Figure 1.

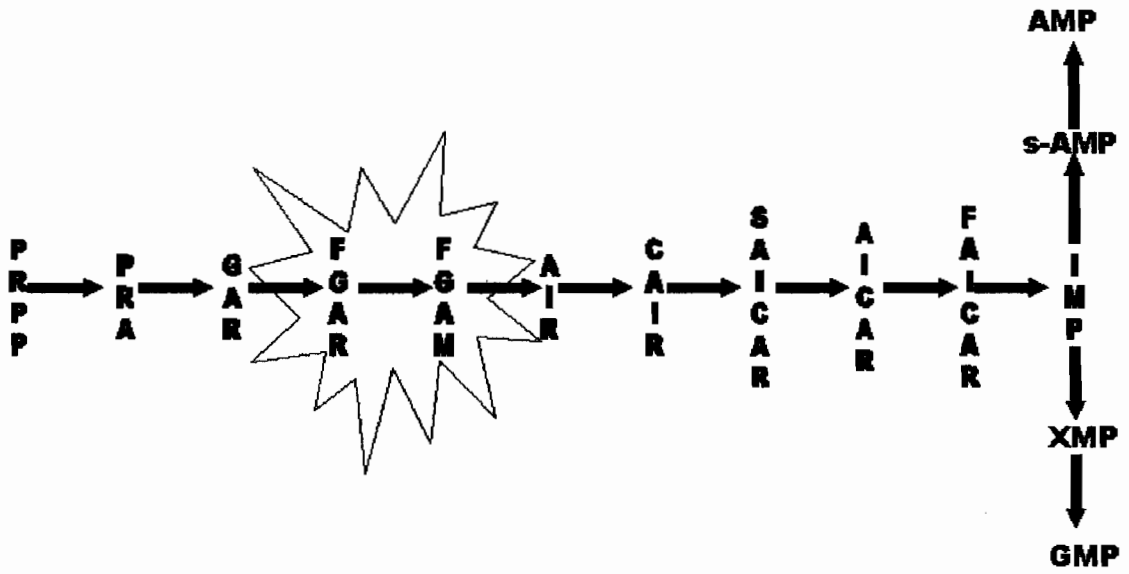

Figure 1. *The Purine Biosynthetic Pathway.*

Regulation of all genes involved in the pathway, except *Pur*A (found in the 11th step of the pathway to make AMP), is based on coregulation by the *Pur*R regulatory gene and responds to purine base levels in what is known as the *pur* regulon (Zalkin and Dixon 1992). To aid in the study of the *pur* regulon all the genes of the purine biosynthetic

pathway needed to be cloned. The *E.coli Pur*F gene, which encodes for the enzyme PRPP amidotransferase in the first step, was the first bacterial gene of the purine biosynthetic pathway to be cloned for this purpose (Tso *et al.* 1982). To date, the clones and structures for all enzymes in the bacterial pathway are available, with the exception of the *Pur*L structure (Kappock *et al.* 2000).

The fourth step of the pathway is catalyzed by one of two different types of enzymes, Type I and Type II. During the fourth step phosphoribosyl-formylglycinamide (FGAR) is converted to phosphoribosyl-formylglycineamidine (FGAM), as shown in Figure 2. FGAR amidotransferase (FGARAT) (also referred to as FGAM synthase), which is encoded by the gene *Pur*L, is the enzyme of this step. This involves the transfer of an amino group from glutamine, the cleavage of ATP's terminal phosphoanhydride bond, and the enolization of the carbonyl group of FGAR to produce FGAM, ADP, P_i, and glutamate (Schendel *et al.* 1989). The mechanism for the reaction can be seen in Appendix A, Figure 2.

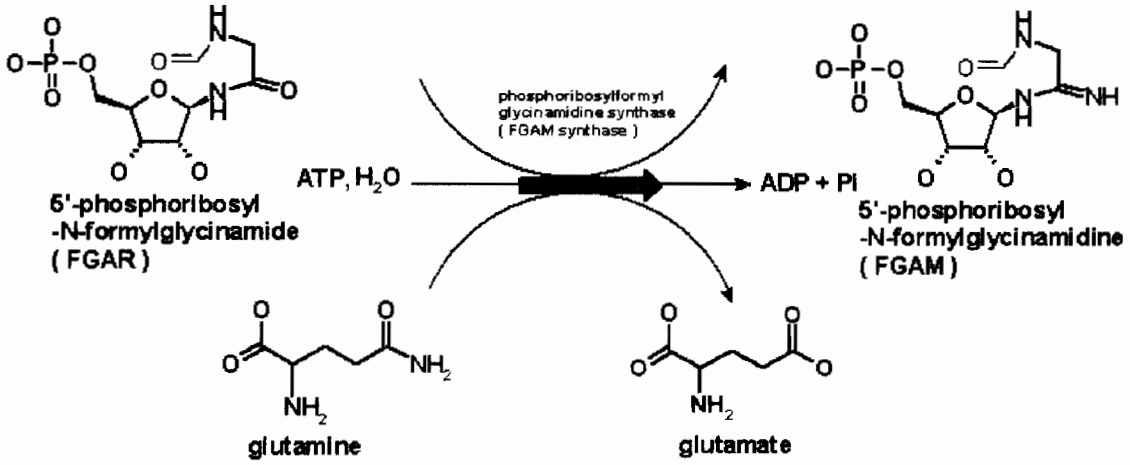

Figure 2. *The FGAR to FGAM reaction. (Marchesini 2001).*

The Type I enzyme consists of a single polypeptide; *Pur*L (see Figure 3 below). This is found in eukaryotes and the β and ϒ proteobacteria. The enzyme was first purified from *Salmonella typhimurium* (French *et al.* 1963). Other purification work has been completed on chicken liver (Mizobuchi and Buchanan 1968, Buchanan *et al.* 1978, and Schendel and Stubbe 1986), Erlich ascites tumor cells (Chu and Henderson 1972) and *E.coli* (Schendel *et al.* 1989). Patterson *et al.* (1999) isolated a human genomic clone of the gene and also analyzed the *Drosophila melanogaster, Caenorhabditis elegans, Schizosaccharomyces pombe, Saccharomyces cerevisiae,* and *Haemophilus influenzae* enzyme sequences.

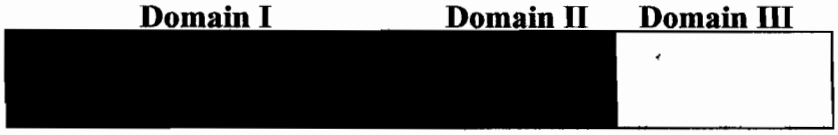

Figure 3. *The Type I FGAR Amidotransferase enzyme (PurL) is a single subunit.*

The Type II enzyme has three subunits, encoded by the *Pur*L, *Pur*S, and *Pur*Q genes (see Figure 4 below). It is present in Archaea and most Bacteria. Ebbole and Zalkin first purified these genes from *Bacillus subtilis* in 1987. They revealed a *Pur*L composition of two polypeptides, encoded by the genes *Pur*Q and *Pur*L. Newman (1992) identified a 3rd subunit in the same region of the other genes. Since this subunit was a purine auxotroph (Newman 1994) and had no other known homologs (Newman 1992), it was predicted to be a component of FGAR Amidotransferase. Saxild and Nygaard (2000) later did more work on the predicted third subunit of the enzyme, and called it the *Pur*S gene. In 1999, Patterson *et al.* found that the Eubacteria, *Aquifex*

aeolicus, Bacillus subtilis, Lactobacillus casei, Mycobacterium leprae, Mycobacterium tuberculosis, and Synechocystis, and Archaebacteri, *Archaeoglobus fulgidus, Methanobacterium thermoautotrophicum, and Methanococcus jannaschii* all have the Type II enzyme as well.

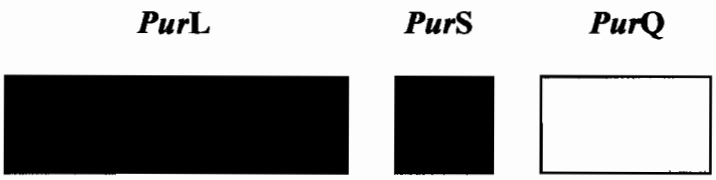

Figure 4. *The Type II FGAR Amidotransferase enzyme has three separate subunits, PurL, PurQ, and PurS.*

FGARAT is a glutamine amidotransferase. Consistent with the three common properties of glutamine amidotransferases outlined by Zalkin (1985), it has the capacity to use NH_3 instead of glutamine, exhibited by *Schendel et al.* in 1989. It also has a glutaminase activity and has selective inactivation of glutamine-dependent activity (Zalkin 1985). Ebbole and Zalkin (1987) later noted the three conserved regions found to be essential for a glutamine amidotransferase. They were a cysteine at 1180, a histidine at 1310, and a glutamate at 1312, all which are present in FGARAT (Patterson *et al.* 1999) (Fredericks and Marzo 2000).

The Type I enzyme can be broken down into three domains which correlate with different steps in the FGAR to FGAM conversion (see Figure 3). Domain I is about 791

amino acids long and is at the N-terminal region of the protein. This domain is responsible for cleaving ATP (Sampei and Mizobuchi 1989). Domain III is involved in the transfer of the amide nitrogen of glutamine in the reaction. It is located at the C-terminal region and is approximately 254 amino acids long (Sampei and Mizobuchi 1989). Finally, Domain II, located between Domains I and III, is responsible for the enolization of the carbonyl oxygen of FGAR. Domain II, which is approximately 249 amino acids long, therefore contains the FGAR binding site (Sampei and Mizobuchi 1989).

Two subunits of the Type II enzyme are homologous to two domains of the Type I enzyme. *Pur*Q is homologous to the C-terminal Domain III, of the Type I enzyme. The two sequences are 28% identical (Sampei and Mizobuchi 1989). *Pur*L is homologous to the N-terminal domain, Domain I and II, of the Type I enzyme. Domain I surpasses *Pur*L by 246 amino acids though. In analysis, without the extra amino acids, the two enzymes are 29% identical (Sampei and Mizobuchi 1989). *Pur*S, just recently named by Saxild and Nygaard (2000), is a necessary component of the Type II enzyme, but its function has not been defined. Newman (1992) and Saxild and Nygaard (2000) suggested that it acts as a linker of the catalytic subunits or plays a role in catalysis.

Since the fourth step of the purine biosynthetic pathway is now known to exhibit two different forms of the FGARAT enzyme in Eukaryotes and most bacteria, it has therefore become an ideal target for antibiotic development. An inhibitor of the Type II enzyme would block production of purines and prevent growth of most bacteria, while not thwarting synthesis of eukaryotic AMP and GMP. This type of antibiotic development is known as a "target-based technology" approach (Rosamond and Allsop

2000). This is a more selective and sensitive system, which has been developed over the last ten years, mainly because of the development of genome projects. Almost all current antibiotics have come from other, semi-rational optimization programs that have been based on compounds and natural products identified by whole-cell, antimicrobial screening (Rosamond and Allsop 2000).

Today, many strains of bacteria in the clinic have developed resistances that reduce or avoid an antibiotic's action. One that has developed many different drug resistances over the years is *Staphylococcus aureus. S. aureus* infections can range from minor skin irritations to life-threatening deep infections such as pneumonia, endocarditis, meningitis, postoperative wound infections, septicemia, and toxic shock syndrome (Balaban *et al.* 1998). *S. aureus* is a gram-positive bacterium with the Type II enzyme. Virulence factors produced by *S. aureus* are the main cause for *S. aureus* diseases. The synthesis of the virulence factor is controlled by RNAIII, a regulatory RNA molecule that is encoded by the *agr* locus (Balaban *et al.* 1998). Normal *S. aureus* infections are treated with a cephalosporin, cloxacillin, dicloxacillin, Vascomycin, or Penicillin G or V (Prescott *et al.* 1996). Many documented cases of drug resistances to these specific drugs have been found around the world. In 1997 a study done by Witte *et al.* found that the prevalence of methicillin-resistant *S. aureus* (MRSA) is 8.7% in central Europe and Germany. This was up from 1.7% in 1990. In Southern Europe the incidence of MR*SA* is greater than 30%. In the United States the frequency of this resistance is 29% of all *S. aureus* infections (Panlilio *et al.* 1992).

To identify a possible inhibitor of the *S. aureus* FGARAT enzyme, functionality of the enzyme must be studied. To do this, purified proteins and substrate are needed.

All subunits of the *S. aureus* enzyme (*Pur*L, *Pur*S, and *Pur*Q), the human *Pur*L protein and the FGAR substrate are needed. Purified proteins can be obtained through cloning, expression and purification of the desired gene. The substrate, FGAR, must be synthetically developed.

In our laboratory, two systems are used in the development of the purified *Pur*L, *Pur*S, and *Pur*Q proteins. Invitrogen's TOPO TA Cloning® kit allows for a highly efficient cloning of the desired PCR product into a plasmid vector. From here, large-scale preparations of the clone can be used to enter into Novagen's pET system. Expression of the target gene is eased by the system's strong expression and purification capabilities.

The TOPO TA Cloning® system (Invitrogen, Carlsbad, CA) is based on two principles. The vector supplied includes a single 3'-thymidine residue overhang. With this overhang, efficient ligation of PCR product into the vector is produced due to the nontemplate-dependent terminal transferase activity of *Taq* polymerase during PCR. In PCR, *Taq* polymerase adds a single deoxyadenosine (A) to the 3' ends of PCR products (Invitrogen, Carlsbad, CA).

Another novelty of this system is its speed, due to the removal of the commonly used ligation step. In general, breaking and rejoining DNA strands in a step-wise fashion with covalent protein-DNA intermediates is a function of DNA topoisomerases (Shuman 1991). *Vaccinia* DNA topoisomerase is sequence specific for a 5'-CCCTT where it will bind to duplex DNA and cleave the phosphodiester backbone of one strand (Shuman 1991). During the reaction a covalent bond is formed between the 3'-phosphate of the incised strand and a tyrosyl residue (Tyr-274) of the protein (topoisomerase) to conserve

bond energy (Shuman 1994). From there the phospho-tyrosyl bond between the DNA and topoisomerase is attacked by the 5'-hydroxyl of the originally cleaved strand. This releases the topoisomerase (See Figure 5). Shuman (1994) therefore suggests a recombinant molecule is created and proposes its use in a novel approach to molecular cloning. In Invitrogen's TOPO TA Cloning® system, Topoisomerase I is covalently bound to the vector. Both the 3'-thymidine overhangs and the Topoisomerase I effectively enable an efficient cloning reaction to proceed.

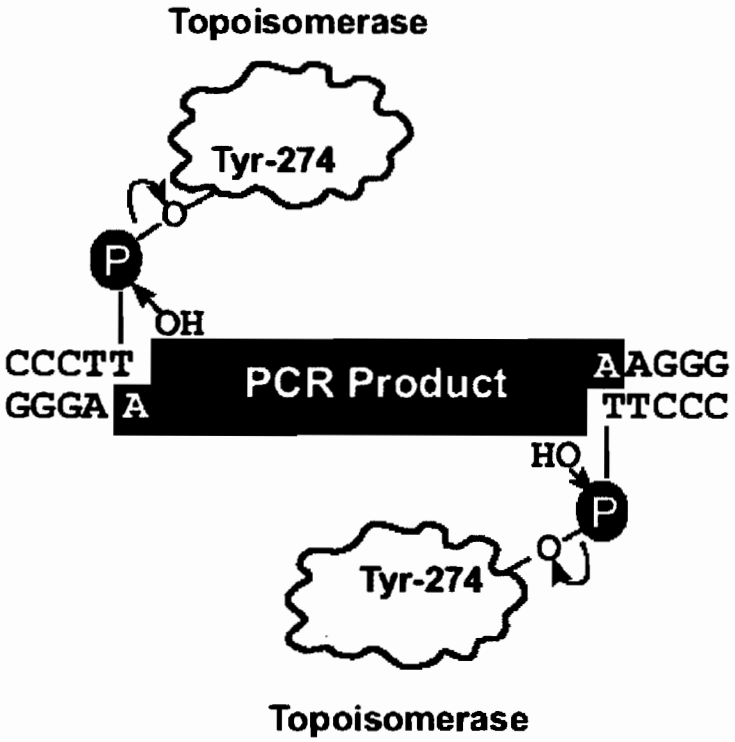

Figure 5. *The reaction of Topoisomerase I with the vector and PCR Product (Invitrogen 2001).*

Transfer of the gene into the Novagen pET system is needed for protein expression once the clone is mass-produced from the Invitrogen system. The Novagen pET system provides strong transcription and translation signals and purification tags. The key to the system is the activity of Bacteriophage T7 RNA polymerase.

Bacteriophage T7 RNA polymerase has specificity for its own promoters. This promoter sequence is not found in *E. coli* and is long enough that it is unlikely to occur by chance in DNA (Studier and Moffatt 1986). T7 RNA polymerase's high rate of transcription actually interferes with the host cell's RNA polymerase and will reduce expression of genes under the control of other host promoters (Studier and Moffatt 1986). Because of the advantage Bacteriophage T7 RNA polymerase has, Novagen developed host strains containing λDE3 lysogen. The λDE3 lysogen is so called because it is a lysogen of Bacteriophage DE3, a λ derivative with the immunity region of phage 21, and has a DNA fragment with the *lac*I gene, the *lac* UV5 promoter and, most importantly, the T7 RNA polymerase gene (Studier and Moffatt 1986). The *lac* UV5 promoter regulates transcription of the T7 RNA polymerase gene and is inducible by isopropyl-β-D-thiogalactopyranoside (IPTG). Another feature of the host strains are pLys plasmids. Because the Bacteriophage T7 RNA polymerase is so active, even a low basal level of expression in an uninduced cell can allow relatively toxic target genes to be produced. Studier (1991) found that T7 lysozyme, after cutting a bond in the host cell wall, could bind and inhibit T7 RNA polymerase. This therefore reduces its ability to transcribe genes in uninduced cells. PLys plasmids code for T7 lysozyme (Novagen cat 2001) (See Figure 6 below).

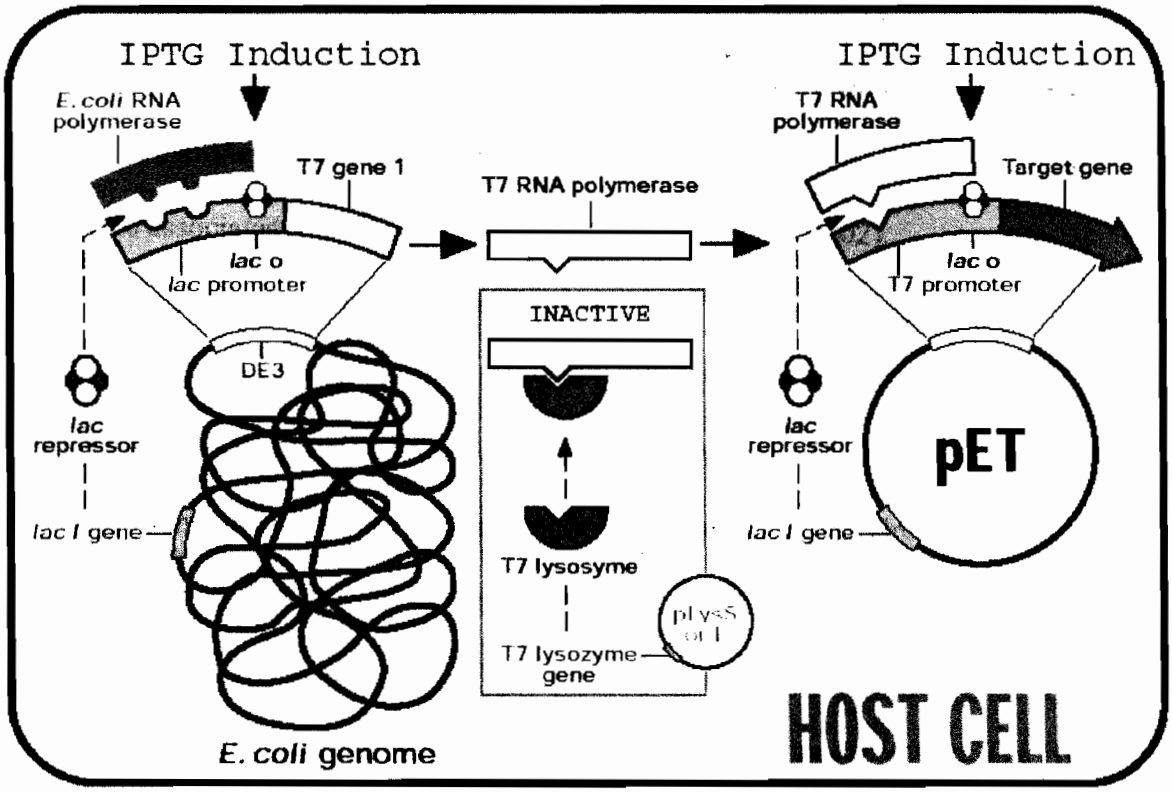

Figure 6. *The specificity of the Novagen pET system for expression of a target gene (Novagen 1999).*

Host strains in the Novagen system can also contain a T7 *lac* promoter to aid in establishing plasmids of λDE3 lysogens. As stated previously, the basal levels of toxic target gene products in uninduced cells can inhibit growth of λDE3 lysogens. The T7 *lac* promoter is just another way to regulate their expression prior to induction. Many host strains are available with the pET system; the most widely used is BL21 and its derivatives (Novagen 1999).

Gibco BRL Life Technologies (Carlsbad, CA) also developed a host strain derived from BL21. One of their novel strains is BL21-SI. These cells are salt-inducible. Their expression is regulated by osmolarity and is under the control of the

*pro*U promoter. They control expression more tightly than other BL21 cells. They also carry an *end*A1 mutation to increase plasmid yield. Another reason for use of these cells is the fact that the salt concentration is titratable which helps with dealing with solubility problems associated with overexpression (Life Technologies 2002).

Novagen developed pET vectors based on the design of Studier and colleagues. Two types were produced, transcription and translation vectors. Transcription vectors are used when the target gene already contains an ATG start codon and prokaryotic ribosome-binding site built into its sequence. Translation vectors already contain the translation signals. All vectors contain either an ampicillin or kanamycin resistance gene for use as a selective marker. Also, most contain a fusion tag next to the cloning site to aid in detection and purification of the target protein following expression. The His-Tag® sequence is commonly found in pET vectors. It encodes for a sequence of His residues. Once expression is induced by IPTG, the His-Tag® can then be used to ease purification using nickel ion affinity column chromatography. When protein containing the His-Tag® is washed over the nickel ion column, the target protein will bind to the column. Unwanted proteins can then be washed away. The target protein can then be eluted from the column, by using imidazole or a slight pH change. Purification is complete following removal of the His-Tag® with a specific protease.

This project involves the use of both the TOPO TA Cloning® and the Novagen pET system to produce a purified *Homo sapiens* FGAR amidotransferase (*Pur*L) protein. Following PCR production of the target gene, which is 4,018 bp long, cloning into the TOPO TA® vector, pCR-2.1, (vector map and cloning site sequence is seen in Appendix A, Figures 5 and 6) was completed. The target gene was then cloned into pET-15b

(Novagen 2001). PET-15b is a translation vector containing an ampicillin resistance site, His-Tag®, T7 promoter, and a thrombin cleavage site (Vector map and cloning site sequence is seen in Appendix A, Figures 3 and 4). The host expression strains used were BL21 (DE3) and BL21-SI. Expression of the target protein was induced by IPTG or NaCl. Following expression and purification of all the *S. aureus* and *Homo sapiens'* proteins involved in the fourth step of the purine biosynthetic pathway, enzyme assays can be conducted to identify an inhibitor of *S. aureus* FGAR amidotransferase.

Materials and Methods

PCR

A cDNA clone (HH00072), received from Takahiro Nagase at the Kazusa DNA Research Institute, Kisarazu, Chiba, Japan, was used to amplify the desired *Pur*L gene (KIAA 0361) (Patterson 1999). Polymerase Chain Reaction was used, with primers designed by Mr. Andrew Cardillo (2001). The sequences were as follows, HsFGARATstartNdeI: 5'-CCTTGTCATATGTCCCCAGTCCTTCACTTCT-3' and HsFGARATstopXhoI: 5'-TATATTCTCGAGTCAGCAGCTCCCTTCCA-3'. A 50µl reaction consisted of 1 µL DNA, 7.5 µL HsFGARATstartNde1 (100pmole/µl), 7.5 µL HsFGARATstopXho1 (100pmole/µl), 5.0 µL Ex-*Taq* Buffer (TaKaRa, Madison, WI), 5.0 µL dNTPs (TaKaRa, Madison, WI), 24 µL dH2O, and 0.2 µL Ex-*Taq* polymerase (TaKaRa, Madison, WI). The HYBAID Omn-E Thermocycler (Franklin, MA) was used. 30 cycles each consisted of 1 minute of 94°C, 1 minute of 55°C, and 2 minutes of 72°C. The final cycle was 1 minute of 94°C, 1 minute of 55°C, and 10 minutes of 72°C.

Cloning Reaction and Transformation into TOPO TA® vector

The TOPO TA Cloning® Kit, Version N, from Invitrogen (Carlsbad, CA) was used to clone and transform the PCR Product into the pCR2.1-TOPO® vector. The cloning reaction consisted of 4 µL of fresh PCR product, 1 µl Salt Solution (1.2 M NaCl and 0.06 M $MgCl_2$, and 1 µl pCR2.1-TOPO® vector. This reaction was incubated for 10 minutes at room temperature.

Transformation into One Shot® Chemically Competent TOP10F' *E.coli* from Invitrogen was completed using the TOPO TA Cloning® Kit (Invitrogen Instruction Manual 2001). The transformants were selected on LB AMP Plates with 40 µl IPTG (100 mg/mL) and 50 µl X-gal (20mg/mL) at 37°C overnight. Clones containing inserts were identified by α – complementation (Sambrook and Russell 2001).

Plasmid Purification and Analysis

The plasmid miniprep-CTAB method protocol (Del Sal *et al.* 1989) was used to purify the plasmid from the *E.coli* cells. Restriction enzyme digestion with HindIII (New England Biolabs, Beverly, MA) was used to analyze the direction of gene insertion into the pCR2.1 TOPO® vector. Small-scale (10µl) digests were conducted using 5 µL purified plasmid, 0.5 µL enzyme (HindIII) (20 kU/mL), 1.0 10X µL buffer (New England Biolabs, Beverly, MA), and 3.5 µL distilled water. The reaction was incubated at 37°C for 1 hour. Agarose gel electrophoresis revealed the direction of the insert.

Large-scale Preparation of Purified Plasmids

The QIAGEN Plasmid Midi Prep Purification Kit from QIAGEN (Valencia, CA) was used to yield 250 µL of purified plasmid. Restriction enzyme digestion with HindIII and agarose gel electrophoresis was again utilized to analyze results.

Isolation of *Pur*L gene from pCR2.1

Enzymes were used in a single digestion to isolate the *Pur*L gene from the pCR2.1® vector. A large scale, 45µL, digest consisted of 30µL purified plasmid DNA,

4.5 μL 10X Buffer 2 (New England Biolabs, Beverly, MA), 3.5 μL NdeI (20 kU/mL) (New England Biolabs, Beverly, MA), 3.5 μL BamHI (20 kU/mL) (New England Biolabs, Beverly, MA), and 3.5 μL XbaI (New England Biolabs, Beverly, MA). This was incubated at 37°C for 1.5 hours. Agarose gel electophoresis was conducted. The QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) was used to purify the *Pur*L fragment from the gel.

PET vector Digestion

Previously purified pET vectors (Novagen, Madison, WI) were digested (45 μL, large-scale) with NdeI and BamHI at 37°C for 1.5 hours in preparation for ligation. Gel extraction using the QIAquick Gel Extraction Kit was conducted following agarose gel electrophoresis.

Ligation of *Pur*L into pET 15b and Transformation into Supercompetent cells

A ligation reaction was created using 1.0 μL ligase (25 kU/mL) (TaKaRa, Madison, WI), 1.0 10X μL ligase buffer (TaKaRa, Madison, WI), 2.0 μL purified *Pur*L, and 6.0 μL pET-15b. The reaction took place for 1 hour at room temperature.

Transformation into TOP10F' Supercompetent cells (Invitrogen, Carlsbad, CA) was conducted. 2 μL of the ligation reaction was placed in a vial of TOP10F' cells. The cells and ligation plasmid mixture was incubated on ice for 5 minutes and heat shocked for 30 seconds at 42°C. The mixture was then immediately placed on ice while 250 μL of SOC medium was added. The new culture was then shaken horizontally at 37°C for 1

hour. The reaction was then plated onto a LB Kan plate with 40 μL IPTG and 50μL X-gal and incubated overnight at 37°C.

Purification and Analysis of Product

A colony was chosen and its plasmid was purified using the QIAprep Spin Miniprep Kit by QIAGEN (Valencia, CA). A small-scale digestion with HindIII was completed. Agarose gel electrophoresis of cut and uncut plasmids was used to analyze the products. QIAGEN Plasmid Midi Preps were done to provide 250μL of purified plasmid.

Transformation into Expression Cells

The freshly purified product was transformed into both BL21 (DE3) (Novagen, Madison, WI) and BL21-SI (Life Technologies, Carlsbad, CA) cells for protein expression. Competent BL21 (DE3) cells were first prepared following the protocol in Sambrook and Russell (2001). BL21-SI cells were bought as competent cells. Their transformation protocol was found at invitrogen.com. The pET-15b-*Pur*L, pET-15b control and pMYB5 (New England BioLabs, Beverly, MA) control vectors were transformed into the competent BL21 (DE3) cells, again using the protocol in Sambrook and Russell (2001) or the invitogen.com protocol for the BL21-SI cells. The bacteria were plated onto a LB Amp plate for BL21 (DE3) and a LBON Amp Plate for BL21-SI.

Purification and Analysis of Products

QIAPREP Spin Miniprep Kit purification was then conducted on selected clones. Digestion with HindIII and agarose gel electrophoresis was used to check for presence of the gene insertion in the plasmid.

Expression of Proteins (BL21 (DE3))

Freshly prepared cell cultures containing the pET-15b-PurL, pET-15b and pMYB5 plasmids were used to inoculate 2 mL of LB Amp (50 μL of the cell culture). This was grown overnight at 37°C. 50 μL of this culture was then grown for 2 hours at 37°C in 5 mL LB Amp media. Following this 2-hour incubation, 1 mL was removed and 1mM IPTG was added to the remaining culture. This was incubated at 30°C. 1 mL samples were removed at several time periods for a period of 6 hours. The 1 mL samples were all centrifuged for 1 minute in a microcentrifuge (Eppendorf Centrifuge 3415C). They were then resuspended in 100 μL of 1X SDS Loading buffer (50mM Tris-Cl, 100mM dithiothreitol, 2% (w/v) SDS, 0.1 % bromophenol blue, 10% (w/v) glycerol). The samples were incubated for 3 minutes at 100°C prior to loading onto a SDS-PAGE gel (Gradipore Pre Cast Polyacrylamide Electrophoresis Gels 4-20% iGels (10 sample wells), Frenchs Forest, Australia). The BIO-RAD Protein Kaleidoscope Prestained Standard was used as a marker. SDS-PAGE gels were run for approximately 2 hours at 150 V in a Bio-Rad Mini-PROTEAN 3 Electrophoresis cell (Hercules, CA). They were stained for 1.5 hours with Coomassie Brilliant Blue (FisherScientific, Fair Lawn, NJ) and destained in 50% methanol and 10% glacial acetic acid overnight.

Expression of Proteins (BL21-SI)

Expression of proteins using BL21-SI cells was done in the same manner as above except the IPTG induction was replaced with a treatment of 0.3 M NaCl. Also, LB Amp media was replaced with LBON Amp media (LBON media is LB Amp media without NaCl).

Results and Discussion

Cloning into TOPO TA® Vector

Eukaryotic DNA differs from prokaryotic DNA in that it contains introns and exons. During transcription of eukaryotic DNA the introns are removed and exons are spliced together, along with other modifications, to form mRNA. This mRNA contains an intact open reading frame (ORF), which is translated into the desired protein. To clone the ORF, RNA can be isolated from humans. Before cloning can be completed though, it must be transcribed back into a cDNA sequence through Reverse Transcriptase PCR (RT-PCR). In this project, a cDNA clone of the gene was received from Takahiro Nagase, who is currently conducting research in this area at the Kazusa DNA Research Institute, Kisarazu, Japan.

Past experiences in our laboratory with the cloning of the Human *Pur*L gene have failed. Previous work attempted to clone the product directly into a Novagen pET vector. The *Pur*L gene is very large, approximately 4,018 base pairs (partial sequence found in Appendix B). In the past, PCR was not providing sufficient quantity of the product. In this study, cloning into the pCR ®2.1-TOPO® vector was first completed. The theory behind this initial subcloning was to provide a larger amount of the gene, which could then be ligated into the pET vectors. The pET vectors were necessary for further protein expression studies.

The initial step in the TOPO® TA Cloning procedure was PCR. The primers were designed to include an NdeI site incorporating the start codon and an XhoI site after the stop codon. These two restriction sites are found in the cloning sites of several pET

vectors, this would allow for ligation into these pET vectors. The PCR results are found in Figure 7 below.

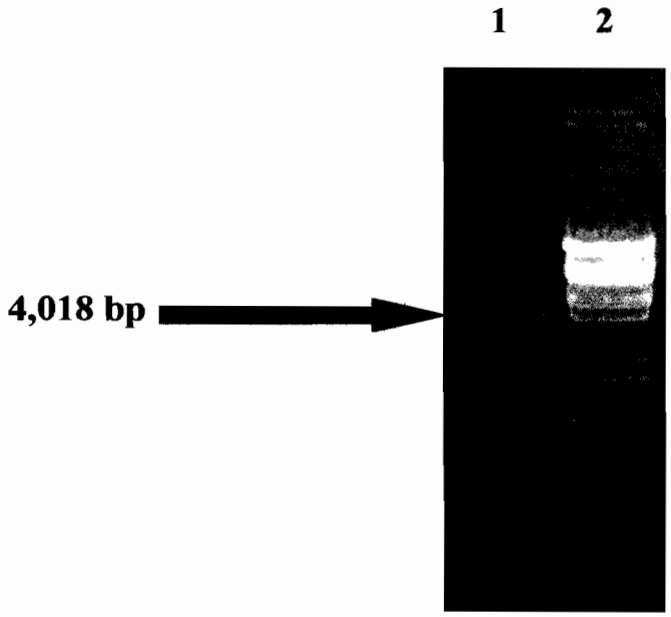

Figure 7. Polymerase Chain Reaction results following purification of the Human PurL gene. Lane 1 is the PCR product. Lane 2 is the λBstEII marker.

The TOPO® TA Cloning Kit was used to clone the purified *Pur*L gene. As stated previously, the Invitrogen kit was efficient because of the TA technology and Topoisomerase I. Another beneficial step to this procedure was transformation into chemically competent cells. The TOP10F' strain of One Shot® Chemically Competent *E. coli* was chosen to increase the efficiency of cloning and plasmid propagation. With the One Shot® cells there is a 10^8/μg yield of transformants vs. the 10^6/μg yield that is found through use of normally prepared competent cells. This particular strain has many genotypic features. *Hsd*R allows for efficient transformation of unmethylated DNA from PCR amplifications. *Lac*ZΔM15 allows for blue/white screening following IPTG

induction of recombinants. The elimination of non-specific Endonuclease I digestion provides for cleaner DNA preps through the gene *end*A1. *rec*A1 reduces occurrence of non-specific recombination in cloned DNA. Finally, an F' episome in the cell has a tetracycline resistance gene and allows for isolation of single-stranded DNA from vectors with f1 origins of replication, which is in pCR ®2.1-TOPO.® There is also a *lac*I^{q} repressor in the F' episome for inducible expression from, among others, the *lac* promoters using IPTG (Invitrogen 2002).

Following cloning and transformation into the TOP10F' cells, 6 white colonies were chosen and purified. HindIII, with restriction site 5'-AAGCTT-3', was chosen to analyze the direction of the *Pur*L insertion into pCR ®2.1-TOPO.® HindIII sites were found at 229-234 base pairs into the pCR ®2.1-TOPO® vector and 1,102 base pairs into the *Pur*L gene (seen in Appendix B). If the gene was inserted in the 5' to 3' direction, with the start primer located at 295 base pairs into the recombinant plasmid, digestion with HindIII would reveal two products, both a 1,168 and a 6,750 base pair fragment (see Figure 8 below). If the gene was inserted in the opposite direction, the two products would be 2,982 and 4,936 base pairs. Following DNA agarose gel electrophoresis, 4 of the 6 plasmids were purified and contained the inserted *Pur*L gene (see gel below, Figure 9). Three were in the 3' to 5' direction (stop primer was located at 295 base pairs into the recombinant plasmid) and one was in the 5' to 3' direction.

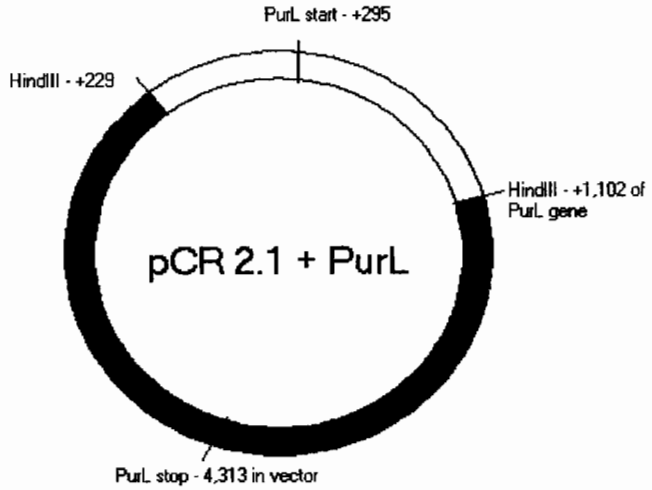

Figure 8. Vector Map containing HindIII sites in pCR-2.1 + PurL in the 5' to 3' direction. The two fragments are in yellow and blue.

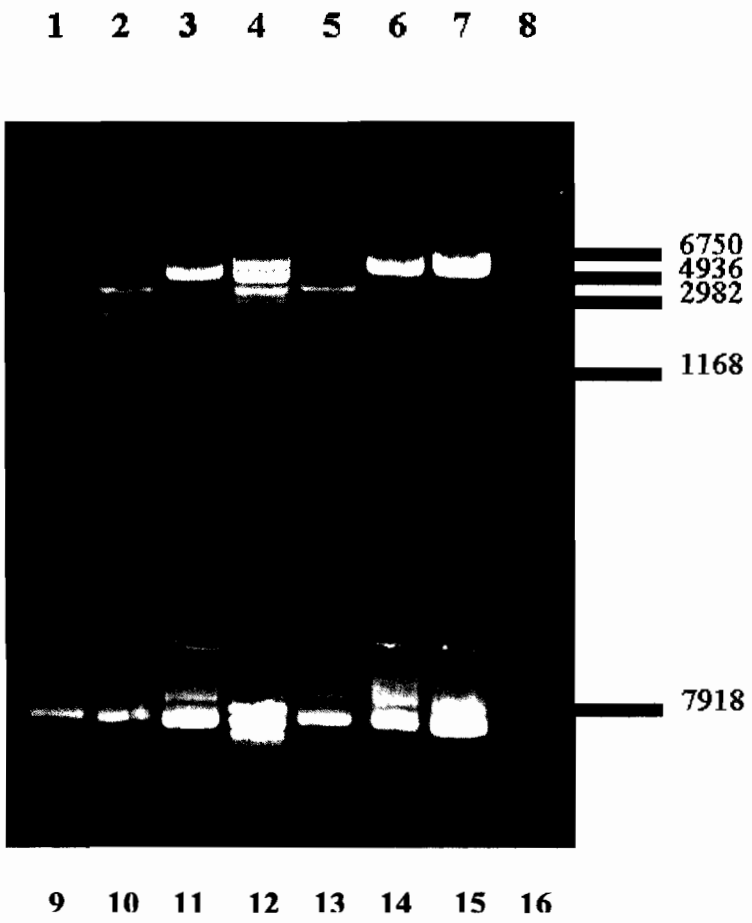

Figure 9. Agarose gel electrophoresis of purified MiniPrep DNA (PurL in pCR 2.1) and digestions of these products with HindIII. Lanes 4 and 12 are the λBstEII marker. Lanes 9-16 are 6 selected colonies following Plasmid MiniPrep-CTAB method purification. The size, 7,918 bp corresponds to the pCR2.1 vector + PurL gene. Lanes 1-8 are the corresponding Mini-Preps digested with HindIII. Three are in the 3' to 5" direction and one is in the 5' to 3' direction.

After MidiPreps to produce large quantities of the clone, the *Pur*L gene had to be isolated from the pCR ®2.1-TOPO® vector. It could then be reinserted into a pET vector for protein expression. A normal isolation would consist of a two enzyme digestion using the enzymes in the start and stop primers, NdeI and XhoI. Following this, gel extraction purification would complete the procedure. Unfortunatly the pCR ®2.1-TOPO® vector was approximately the same size as the *Pur*L gene. pCR ®2.1-TOPO® is

3,900 bp and the *Pur*L gene is 4,018 bp. Running this digestion through gel electrophoresis would not separate the two fragments, which is necessary for gel extraction purification.

The new approach was then to choose 3 enzymes, 2 that isolated the gene and one to cut the vector into 2 pieces and create overhangs incompatible with subsequent cloning steps. The enzymes chosen could only be in these sites of the recombinant plasmid or it would break apart the gene. The enzymes chosen were NdeI, XbaI, and BamHI. NdeI was found only in the start primer of the gene. XbaI was found only 50 bp after the inserted PCR product in the vector. These two enzymes would isolate the gene when the insert was in the forward direction. BamHI was only at 253 bp of pCR ®2.1-TOPO.® The three enzymes together would provide 3 fragments, a 41 bp of vector, a 3,809 bp fragment also of vector, and a 4,068 bp fragment, which contains the *Pur*L gene (see Figure 10 below). Again, the fragments of 3,809 and 4,068 bps were very similar, but the hope was that they would not re-ligate due to the differences in their overhangs produced by the three different enzymes. The gel results are found in Figure 11 below.

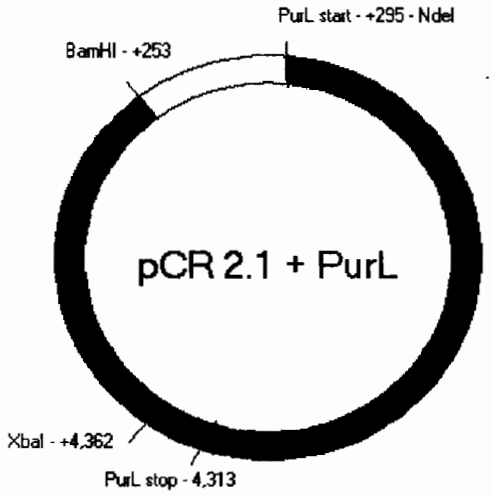

Figure 10. ***Vector map containing XbaI, NdeI, and BamHI sites in pCR2.1+PurL in the 5' to 3' direction. The three segments are shaded in various colors.***

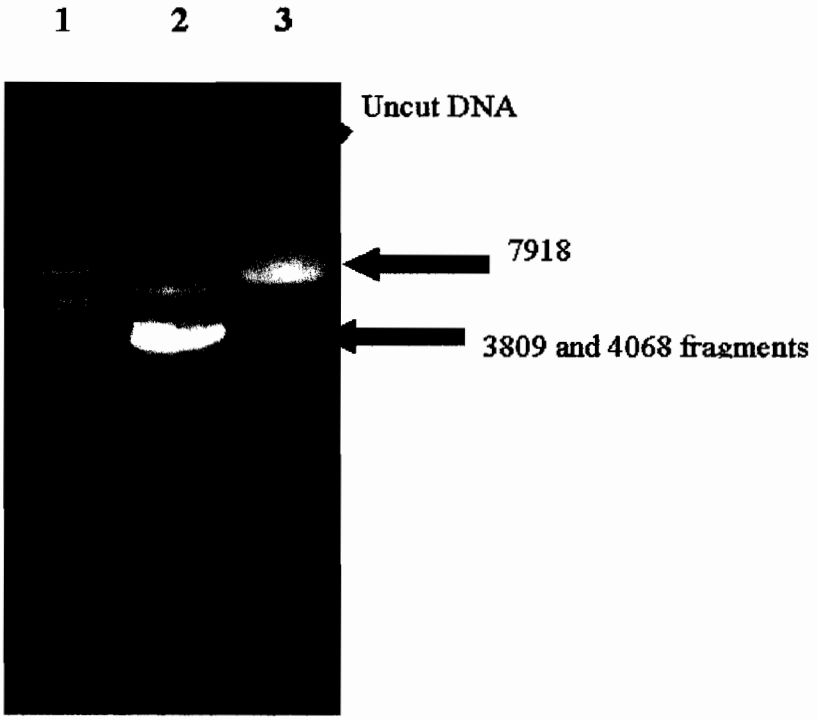

Figure 11. Agarose Gel Electrophoresis following digestion of pET-15b-PurL recombinant plasmid with XbaI, BamHI, and NdeI. Lane 1 is the λBstEII marker. Lane 2 is the digested plasmid. Lane 3 is the uncut pET-15b-PurL recombinant plasmid.

PET-15b + *Pur*L Cloning

The pET-15b vector was cut with NdeI and XbaI to allow for future ligation of the isolated *Pur*L gene. The digested pET-15b was run through gel electrophoresis and gel purified. The results are seen in Figure 12 below. A 5,703 bp fragment was found, the size of the pET-15b vector.

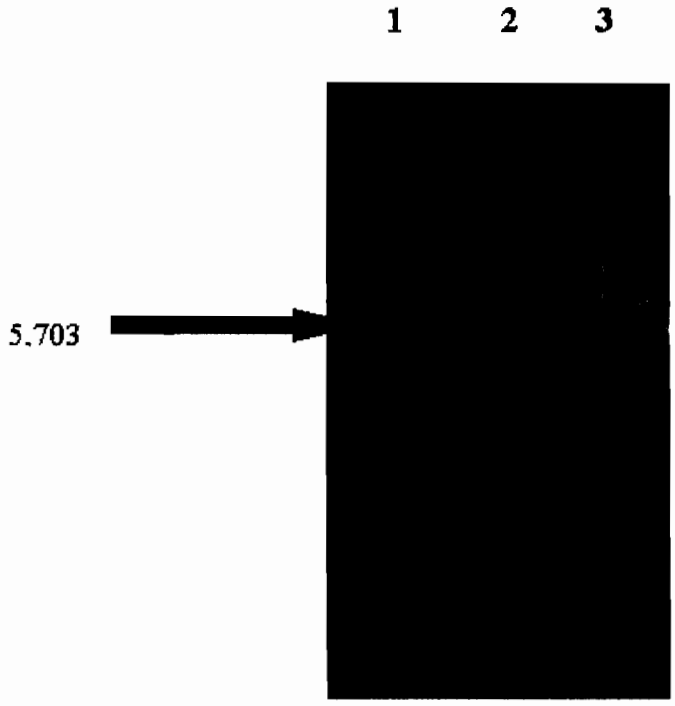

Figure 12. Agarose Gel Electrophoresis following the digestion of the pET-15b vector. Lane 1 is uncut pET-15b. Lane 2 is pET-15b digested with NdeI and XbaI. Lane 3 is the λBstEII marker.

Following ligation of the *Pur*L gene into the pET-15b vector and transformation of the clone into TOP10F' cells one white colony was found on the pET-15b-*Pur*L plate. This one colony was isolated and purified. Then, to ensure an inserted gene was present,

and to determine the gene orientation, a digest with HindIII was again conducted. HindIII is found at 29 bp in the pET-15b vector and 1,102 bp in the *Pur*L gene. Two fragments should have been created, depending on the direction of the insert. The digestion was successful (see Figure 14). The *Pur*L gene was inserted into pET-15b. It was found to be in the forward direction because the digestion revealed fragments at 1,393 and 8,333 bp, this was as expected.

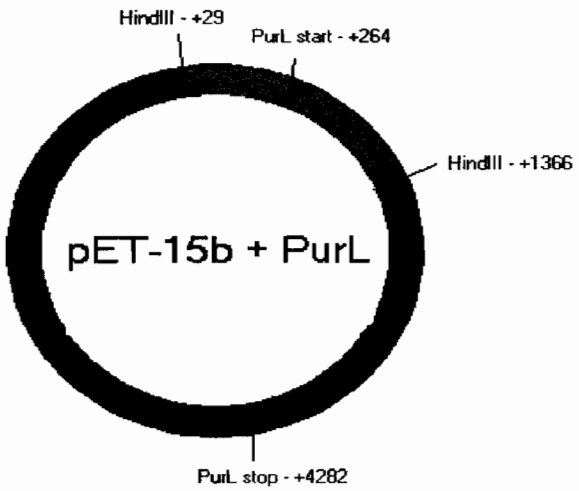

Figure 13. Vector map containing HindIII sites in the pET-15b-PurL recombinant plasmid. The two fragments of the plasmid when the gene is inserted in the 5' to 3' direction are shaded in orange and blue.

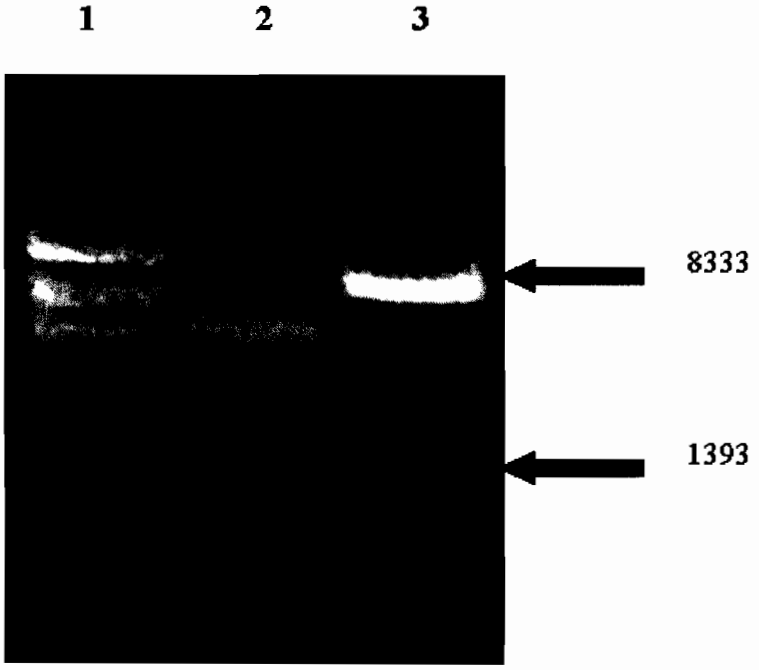

Figure 14. Agarose gel electrophoresis of QIAGEN MiniPrep purified plasmid DNA (pET-15b-PurL). Lane 1 is the λBstEII marker. Lane 2 is purified pET-15b-PurL. Lane 3 is the HindIII digested pET-15b-PurL.

Finally, the pET-15b clone had to be retransformed into expression cells. The TOP10F' cells it previously was transformed into could not have been used for the pET expression. They do not contain a promoter for induction; therefore the Novagen BL21 (DE3) and Life Technologies' BL21-SI strains were used. A final digest of the purified plasmid was again completed to confirm transformation.

Protein Expression in BL21 (DE3) cells

Expression of protein in BL21 (DE3) cells was induced by isopropyl-β-D-thiogalactopyranoside (IPTG). When IPTG is added it induces the T7 RNA polymerase in the BL21 (DE3) cells. The T7 RNA polymerase then can transcribe the *Pur*L gene in the pET-15b vector. 1 mM IPTG was added to three growing cultures of BL21 (DE3) cells. One contained the *Pur*L-pET-15b plasmid, one the pET-15b plasmid and one contained the pMYB5 plasmid. From there the optimal expression temperatures and times needed to be found. Examining protein samples at several time periods and changing temperatures following IPTG induction could identify the optimal temperature. In this project, an induction temperature of 30°C was used and different time periods up to 6 hours of induction were examined. 30°C was used because incubation at this temperature has led to soluble, active proteins without accumulation of inclusion bodies (Novagen 1999).

Following isolation of the proteins from the cells, a SDS-PAGE gel was run. Figure 15 show the pET-15b protein 0,1, 2, 4, and 6 hours following induction. It also shows the first three time intervals of the pET-15b-*Pur*L culture, 0, 1, and 2 hours after induction. PET-15b was used as a control to compare to the culture containing *Pur*L. The pET-15b-*Pur*L protein should have been expressed at approximately 140 kDa. Any difference at that area of the gel between the control protein and an added band in the *Pur*L protein would have indicated correct expression of *Pur*L. Using the Bio-Rad Kaleidoscope Prestained Standard, sizes could be determined. As evidenced by the gels below, no difference was found between the *Pur*L-pET-15b and the pET-15b crude extract.

pMYB5 was also used as a protein control. This was used to ensure proper induction. pMYB5 has been proven to work in this lab in the past. Therefore we could compare the results of this induction with prior data. The fusion protein encoded by pMYB5 was not expressed as found previously (Boob 2001). Therefore it was deduced that the induction with BL21 (DE3) cells was not working properly. This could have been for numerous reasons. The cells could have been old and mishandled or the IPTG stock could have been mislabeled or at the wrong concentration. The pMYB5 expression data is shown in Figure 16 below.

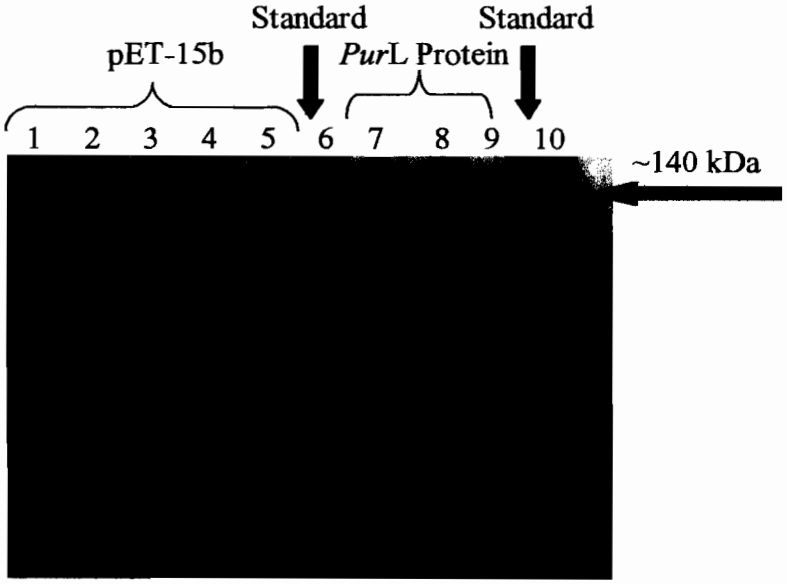

Figure 15. SDS-PAGE gel following Coomassie Brilliant Blue staining. Lanes 1-5 show pET-15b in BL21 (DE3) as a control 0, 1, 2, 4 and 6 hours after induction with IPTG. Lanes 6 and 10 are the BIO-RAD Kaleidoscope Prestained Standard. Lanes 7-9 are the PurL-pET-15b plasmid in BL21 (DE3) 0, 1, and 2 hours after induction with IPTG.

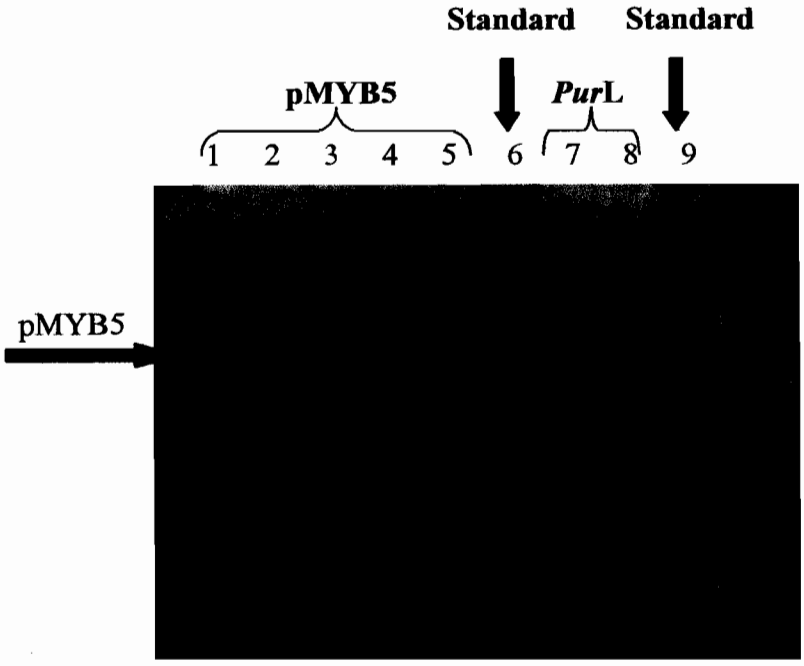

Figure 16. SDS-PAGE gel of pMYB5 and pET-15b-PurL protein in BL21 (DE3). Lanes 1-5 are pMYB5 0, 1, 2, 4, and 6 hours after induction with IPTG. Lanes 6 and 9 are the BIO-RAD Kaleidoscope Prestained Standard. Lanes 7 and 8 are pET-15b-PurL at 4 and 6 hours after induction with IPTG.

Protein Expression in BL21-SI cells

Since BL21 (DE3) cells were not yielding proper pMYB5 induction, BL21-SI cells were used. The difference with these cells is that expression of T7 polymerase is induced with NaCl instead of IPTG. Also, an increase in plasmid yield was claimed by the manufacturer (Life Technologies). The expression was completed in exactly the same way as the BL21 (DE3) cells, except for the replacement of 1 mM IPTG with 0.3 M NaCl. Also, a new media, LBON was used for cell growth because it lacked NaCl as one of its components. The expression yielded approximately the same results as the BL21 (DE3) expression though. The protein gels are seen in Figures 17 and 18 below. Again,

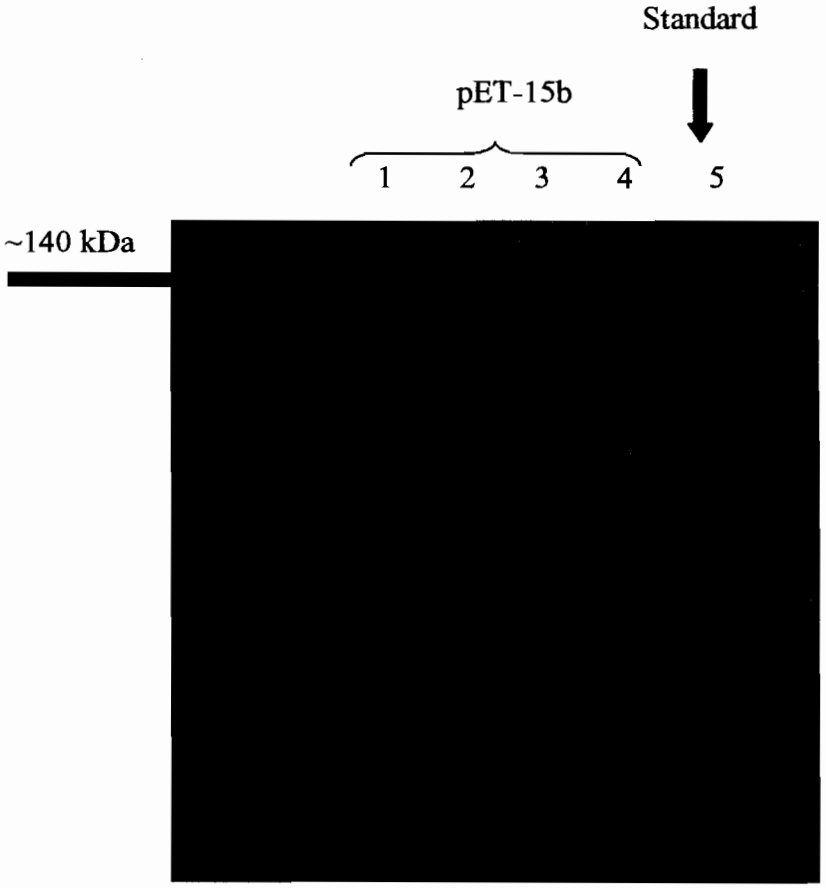

Figure 17. SDS-PAGE gel of pET-15b protein following induction with 0.3 M NaCl. Lanes 1-4 are the pET-15b protein 0, 1, 3, and 5 hours following induction. Lane 5 is the Bio-Rad Kaleidoscope Prestained Standard.

These results lead to several new possibilities, but unfortunately time constraints ended the project at this point. The fact that no difference was found between the pET-15b control and the *Pur*L-pET-15b protein in either the BL21 (DE3) cells or the BL21-SI cells could mean that the gene is not present in the plasmid. Since plasmid purification and gel electrophoresis after the gene was inserted in the vector yielded the expected results, one could assume that the gene was inserted. To further examine this particular problem, sequencing of the plasmid can be done. If the gene is shown to be inserted, one must further examine the sequence and make sure it has all the correct components. A

mutation might have produced an insert that cannot be expressed. Both cell strains were grown at 37°C and induced at 30°C. Different temperatures than these could be tried during expression of protein. Once a protein is found for the pET-15b-*Pur*L plasmid, various time periods and temperatures can be examined to find the optimal expression time and temperature. Also, various concentrations of IPTG and NaCl can be experimented with. Following production of a soluble, expressed protein, purification using a nickel ion affinity column can be completed to obtain the FGAR Amidotransferase protein.

This project resulted in the completed cloning of the human *Pur*L cDNA into both pCR-2.1 and pET-15b. These recombinant plasmids were transformed into BL21 (DE3) and BL21-SI cells for expression. The optimal expression temperatures and times were not found during this past year. Various expressions were examined, though. Since every protein requires a different expression protocol, developing one specific for a particular protein is a time-consuming task. The work completed here will be of value to the future researchers.

Following completion of the purification of the human FGAR Amidotransferase protein, as well as the *Pur*S, *Pur*L and *Pur*Q subunits of *Staphylococcus aureus* and the development of the FGAR substrate, enzyme assays can begin. The enzyme assays will be set up to examine an inhibitor of the Type II FGAR Amidotransferase enzyme. This will provide insight into the ultimate development of an antibiotic to combat the increasingly mutant *Staphylococcus aureus*.

Appendix A

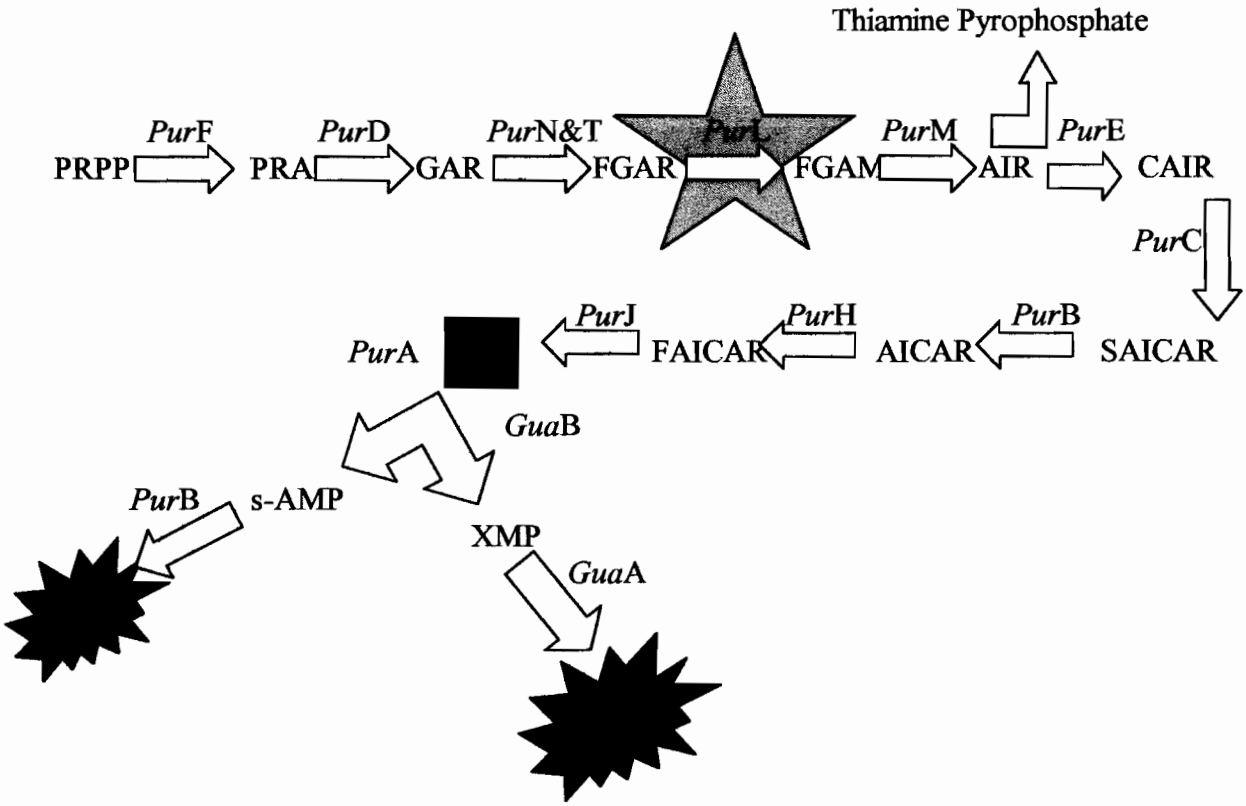

Figure 1. ***THE PURINE BIOSYNTHETIC PATHWAY***

Abbreviations:
PRPP: phosphoribosylpyrophosphate
PRA: phosphoribosylamine
GAR: glycinamide ribonucleotide
FGAR: formylglycinamide ribonucleotide
FGAM: formylglycinamidine ribonucleotide
AIR: aminoimidazole ribonucleotide
CAIR: carboxyamide ribonucleotide
FAICAIR: formamidoimidazolecarboxamide ribonucleotide
IMP: inosine monophosphate
XMP: xanothosine monophosphate
GMP: guanosine monophosphate
AMP: adenosine monophosphate

Genes encode for:
*Pur*F: PRPP amidotransferase

*Pur*D: GAR synthetase
*Pur*N&T: GAR formyltransferase
*Pur*L: FGAR amidotransferase
*Pur*M: AIR synthetase
*Pur*E: AIR carboxylase
*Pur*C: SAICAR synthetase
*Pur*B: adenylosuccinate lyase
*Pur*H: AICAR formyltransferase
*Pur*J: IMP cyclohydrolase
*Pur*A: adenylosuccinate synthetase
*Gua*B: IMP dehydrogenase
*Gua*A: guanosine monophosphate synthetase

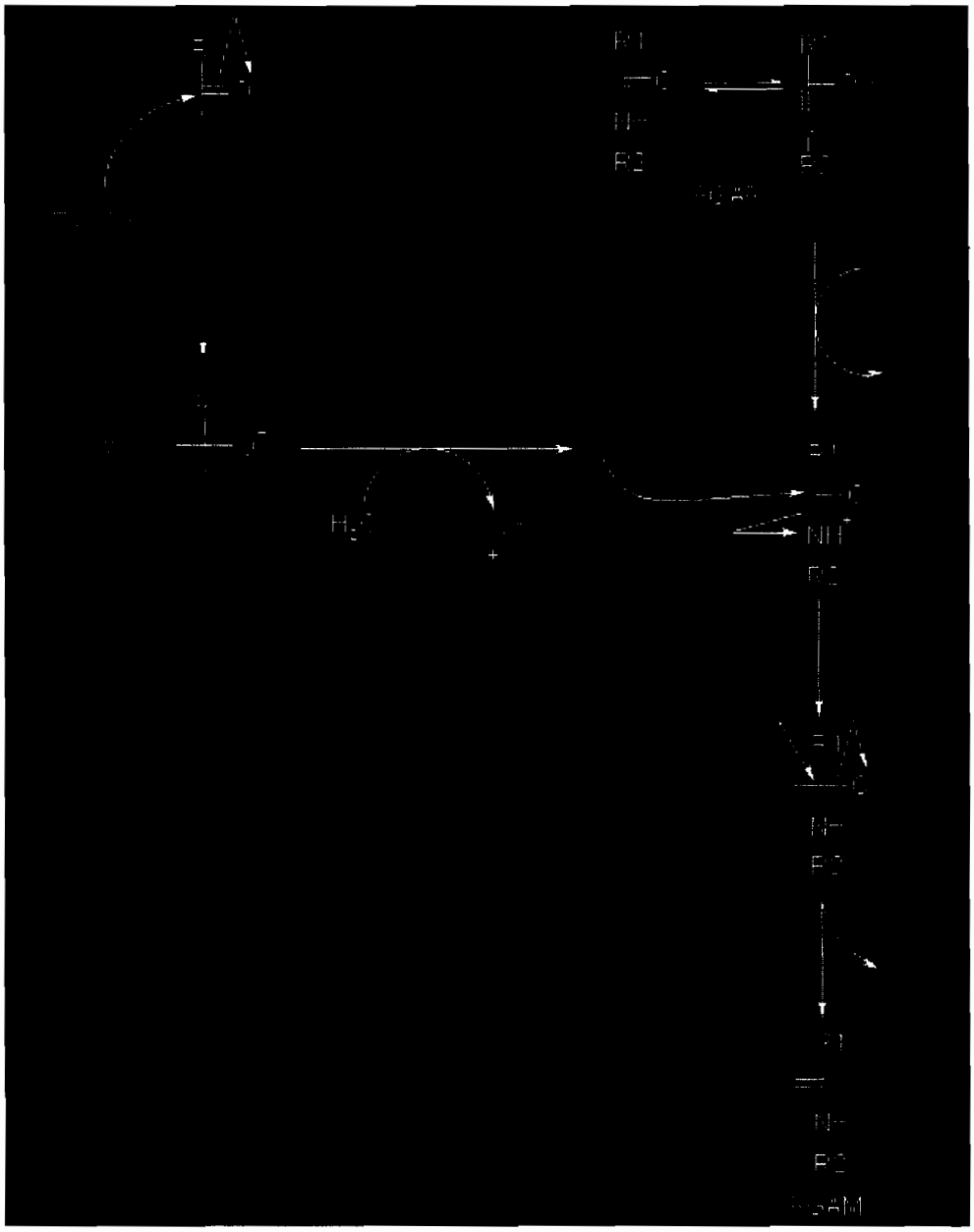

Figure 2. *The mechanism for the conversion of FGAR to FGAM at the fourth step in the Purine Biosynthetic Pathway (Marchesini 2001).*

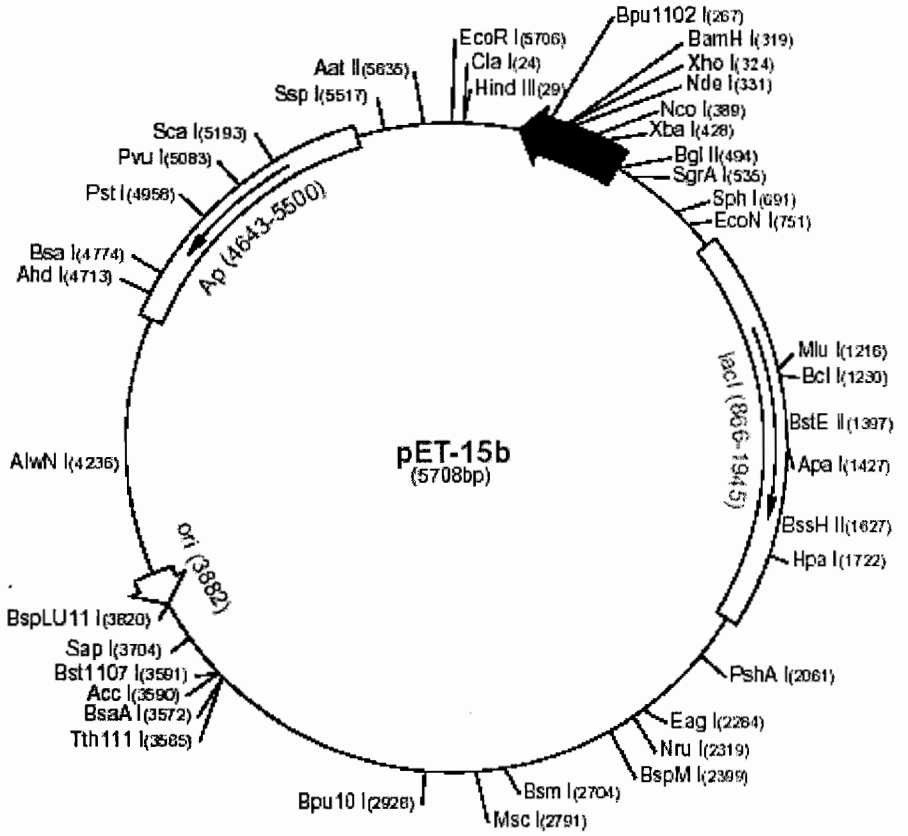

Figure 3. *The pET 15b vector from Novagen's PET expression system (Novagen 1999).*

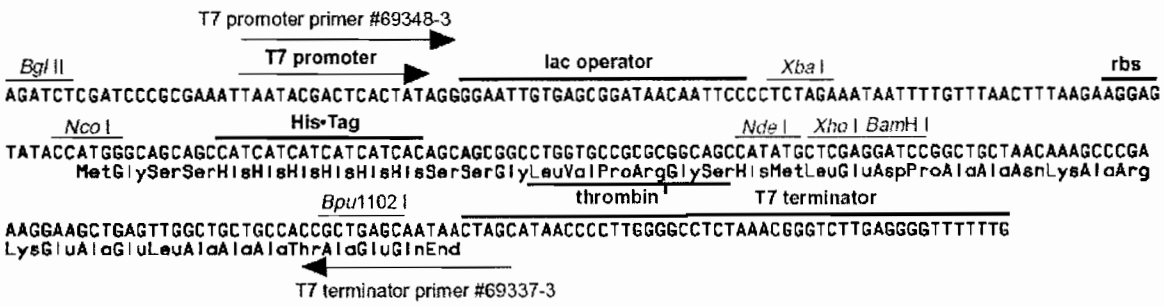

Figure 4. *The area around the cloning site for the pET 15b vector of Novagen (Novagen 1999).*

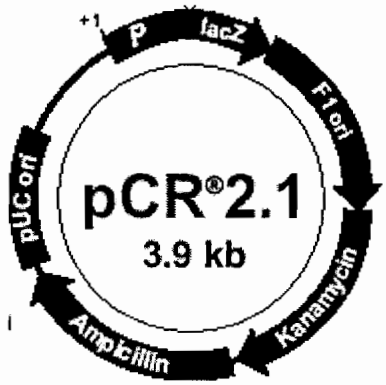

Figure 5. *The pCR® 2.1 TOPO® vector from Invitrogen.*

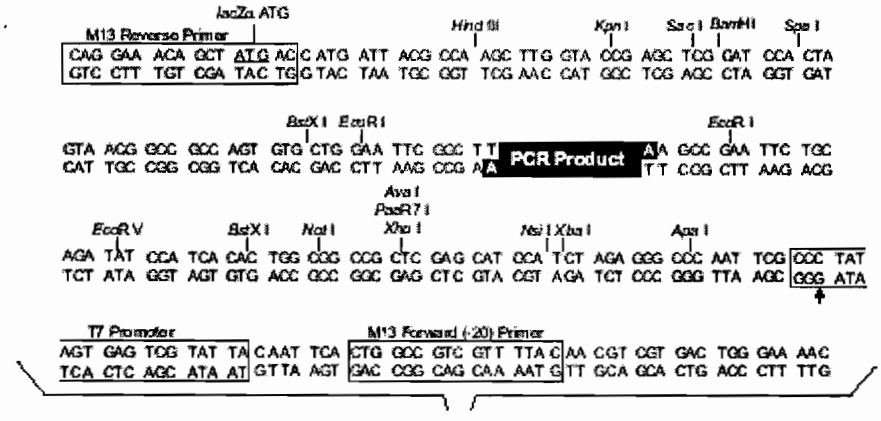

Figure 6. *The sequence and sites around the cloning site of the pCR® 2.1 TOPO® vector from Invitrogen.*

Appendix B

Partial Human *Pur*L ORF

```
human purL ORF
        1         11        21        31        41        51        61
      1 atgtccccagtccttcacttctatgttcgtccctctggccatgaggggcagcctctggacacactcgga
        tacaggggtcaggaagtgaagatacaagcagggagaccggtactcccccgtcggagacctgtgtgagcct

     71 ggaaactgcaagggaaactgccagagctgcagggcgtcgagactgaactgtgctacaacgtgaactggac
        cctttgacgttccctttgacggtctcgacgtcccgcagctctgacttgacacgatgttgcacttgacctg

    141 agctgaggccctccccagtgctgaggagacaaagaagctgatgtggctgtttggttgccccttactgctg
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    211 gatgatgttgctcgggagtcctggctccttcctggctccaatgacctgctgctggaggtcgggcccaggc
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    281 tgaacttctccaccccaacatccaccaacatcgtgtcagtgtgccgcgccactgggctggggcctgtgga
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    351 tcgtgtggagaccacccggcgctaccggctctcgtttgcccacccccgtcagctgaggtggaagccatt
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    421 gctctggctaccctgcacgaccggatgacagagcagcacttcccccatcccatccagagtttctcccctg
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    631 cagcggaacccgagcactgtggaggcctttgacttggcgcagtccaatagcgagcacagccgacactggt
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        caggctaaggatgccggactcctggggtgtgccggttcggcgaaggtcgttgtcgttcccgactctgtac
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```
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      aacagaagtgtcgtctctgagtgttgaaagggtgtcctcatacggggaaatcaccacgttggtgaccgtg

 981  agggggccggattcgagatgtccagtgcacaggccgcggggcccacgtggtggctggcactgccggctat
      tcccccggcctaagctctacaggtcacgtgtccggcgccccgggtgcaccaccgaccgtgacggccgata

                                                          HindIII
1051  tgctttggaaatctgcatattccaggttacaatctgccctgggaggatctaagcttccagtatcctggga
      acgaaacctttagacgtataaggtccaatgttagacgggaccctcctagattcgaaggtcataggaccct

1121  attttgcccggcccctggaggttgccattgaagccagtaatggagcttctgactatggcaacaagtttgg
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```

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