Expression and Purification
of *Homo sapiens* FGAR Amidotransferase

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The Expression and Purification of *Homo sapiens* Formylglycinamide Ribonucleotide (FGAR) Amidotransferase

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Abstract:

In order to stop proliferation of *Staphylococcus aureus*, a human pathogen, this research lab's goal is to exploit the species-specific structural differences in N-formylglycinamide ribonucl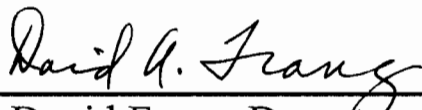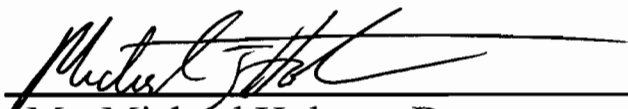eotide (FGAR) amidotransferase allowing for selective inhibition of purine biosynthesis in *S. aureus*. My research will focus on the expression and purification of *H. sapiens* FGAR amidotransferase, the control enzyme for subsequent pharmacological studies.

The *H. sapiens* FGAR amidotransferase gene was cloned into the Novagen pET 15b expression vector; restriction enzyme digestion confirmed the ligation. SDS-PAGE analysis revealed less than optimal expression of the *H. sapiens* gene in initially transformed bacterial strains, probably due to species-specific codon preferences. Therefore, the vector was transformed into a strain engineered with *H. sapiens* transfer RNA molecules that should allow for human codon recognition, and ultimately, better target protein expression.

After careful analysis of the pET15b vector map and the previous research results, it was determined that the *H. sapiens* purL gene was inserted into the pET15b vector backwards and outside of the cloning region of the pET15b expression vector. This improper orientation of the insert absolutely inhibits any potential target protein expression. A poor choice of restriction enzymes for insert excision from the pCR2.1 TOPO vector, chosen in previous experiments, made the proper ligation of the insert within the pET15b vector impossible.

Unfortunately, time restraints prohibit the proper ligation of the *H. sapiens* purL insert into another pET expression vector. Future studies should focus on ligating the insert within the pCR2.1 TOPO vector and then, into a pET expression vector, using the Xho I and Nde I restriction sites as ligation insertion points. Afterwards, the research focus should shift toward optimizing target protein expression and fusion-protein purification, via nickel-ion affinity chromatography, to produce an aliquot of purified *Homo sapiens* FGAR amidotransferase enzyme.

Introduction:

Background Information:

Purines are essential biological molecules that play integral roles in many important cellular functions including, but not limited to: DNA and RNA synthesis, cellular energy metabolism and second messenger systems (Patterson et al., 1999). *De novo* purine biosynthesis is a fourteen-step process that leads to the eventual production of either guanylate (GMP) or adenylate (AMP) (Berg et al, 2002). The initial purine nucleotide inosinate (IMP) is generated in ten steps from the original substrate 5-phosphoribosyl 1-pyrophosphate (PRPP). After the production of IMP, the pathway splits to produce GMP or AMP. Virtually all free-living organisms are capable of synthesizing purines in this manner (Patterson et al., 1999).

The fourth step of the *de novo* purine biosynthetic pathway is catalyzed by the enzyme phosphoribosylformylglycineamide amidotransferase (FGARAT). This enzyme is nearly unique in higher eukaryotes in that it has only a single function, as do only two other enzymes in the eukaryotic pathway leading up to the generation of IMP (Barnes et al., 1994). All other enzymes have multiple functions. It converts phosphoribosyl-formylglycinamide (FGAR) to phosphoribosyl-formylglycineamidine (FGAM). The FGAR amidotransferase enzyme catalyzes the irreversible transfer of an amide group from glutamine to FGAR, converting it into FGAM; this reaction is dependent upon ATP (Patterson et al., 1999; Barnes et al., 1994). FGARAT exists in two different forms, type I and type II.

The two enzyme types differ structurally in the number of polypeptides necessary for the unique enzymatic activity of FGARAT (see figure below). The first type exists as

a single gene purL and is translated into a single polypeptide. This single polypeptide

form of purL consists of three domains. The type I form of FGARAT exists in all

eukaryotes, including *Homo sapiens*, and β and γ proteobacteria (Patterson et al., 1999).

The second type of FGARAT consists of three polypeptides, all encoded by different

genes: purL, purS and purQ. The type II form of FGARAT exists in eubacteria outside

the β and γ proteobacteria group and in all archaebacteria studied to date, including

Staphylococcus aureus (Patterson et al., 1999).
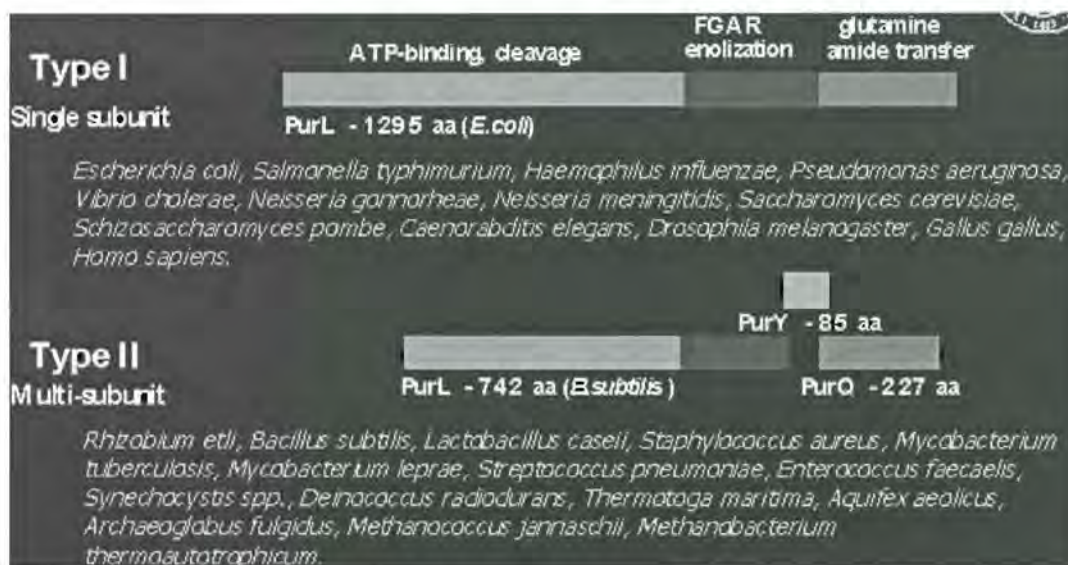
Figure 1. The two different forms of the FGAR amidotransferase enzyme. The type I form, which exists in *H. sapiens*, consists of a single subunit divided into three different domains. The type II form, which exists in *S. aureus*, consists of three different polypeptides.

Regardless of the structural differences between the two types of FGAR

amidotransferase enzymes, sequence homology studies indicate that both types work in

an identical fashion. The N-terminal ATP-binding domain and the C-terminal glutamine

amide transfer domain in the type I enzyme appear to be homologous to the purL and

purQ subunits of the type II enzyme, respectively (Patterson et al., 1999; Batra et al.,

2002). Recent research has also indicated that the purS subunit of the type II enzyme acts

to hold the purL and purQ subunits together to ensure enzymatic activity (Batra et al., 2002). Also, the molecular mass of FGAR amidotransferase appears to be highly similar among species having the type I enzyme, indicating a possible evolutionary relationship (Barnes et al., 1994).

The structural differences between the *Homo sapiens* and prokaryotic FGAR amidotransferase enzymes suggests the possibility of exploiting the purine biosynthetic pathway as a means of controlling such bacterial growth, without harming their human hosts. This type of therapy is known as "target-based technology" (Rosamond and Allsop, 2000). As mentioned earlier, purines are fundamental molecules in many biochemical processes, and any interference with the biosynthetic pathways to produce them would mean death for the organism. The structural differences between many bacterial and human FGARAT enzymes might allow for the development of certain compounds that would interfere with the purine biosynthetic pathway at the fourth step in bacteria, but not in humans. This would thereby kill the invading species, while allowing the human's pathways to function normally.

Staphylococcus aureus is a human pathogen that causes a wide range of symptoms, from minimal to severe (van Wamel, 2002). Many times, the overuse of an antibiotic selects for bacteria that are antibiotic resistant (Sefton, 2002). *S. aureus's* resistance to methicillin is a major concern not only to hospitals, but also to the general public (Sefton, 2002). In the United States, twenty-nine percent of all *staphylococcus aureus* infections are resistant to methicillin (Panlilio et al., 1992). FGAR amidotransferase is a good target protein for antibiotic development because it is different

in *S. aureus* than it is in humans; therefore, its activity can be stopped in bacteria, inhibiting the essential purine synthesis, without hurting humans.

pET Expression System:

The expression system used in the following experiments is Novagen's pET expression system (See Figure 2). Very generally, this system utilizes an IPTG-inducible T7 RNA polymerase to control expression of the target protein ligated within the pET vector (Novagen, 2001). More specifically, this expression system is most often used in conjunction with a DE3 lysogen bacterial host to obtain the most effective results.

DE3 lysogen bacteria contain the T7 RNA polymerase gene within the bacterial host genome (Novagen, 2001). The *lacI* gene, also located within the bacterial host genome, codes for the repressor protein that inhibits expression of the T7 RNA polymerase under normal conditions (Novagen, 2001). Expression of this gene is controlled by the *lac*UV5 promoter and is IPTG-inducible. When induced with IPTG, the repressor's activity is inhibited and the T7 RNA polymerase gene is translated into the functional enzyme. Although this promoter system is efficient for the controlled expression of most proteins, there is some protein expression in the uninduced state (Novagen, 2001).

In order for more stringent control of target protein expression, host strains are available which contain the T7 lysozyme gene, pLys (Novagen, 2001). T7 lysozyme inhibits T7 RNA polymerase, thereby stopping target protein expression in the uninduced state (Novagen, 2001). The bacterial expression strains used in these experiments contained the pLysS gene, which codes for smaller amounts of T7 lysozyme versus the

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pLysE gene, which codes for far more T7 lysozyme and therefore allows for much more

stringent control of protein expression.
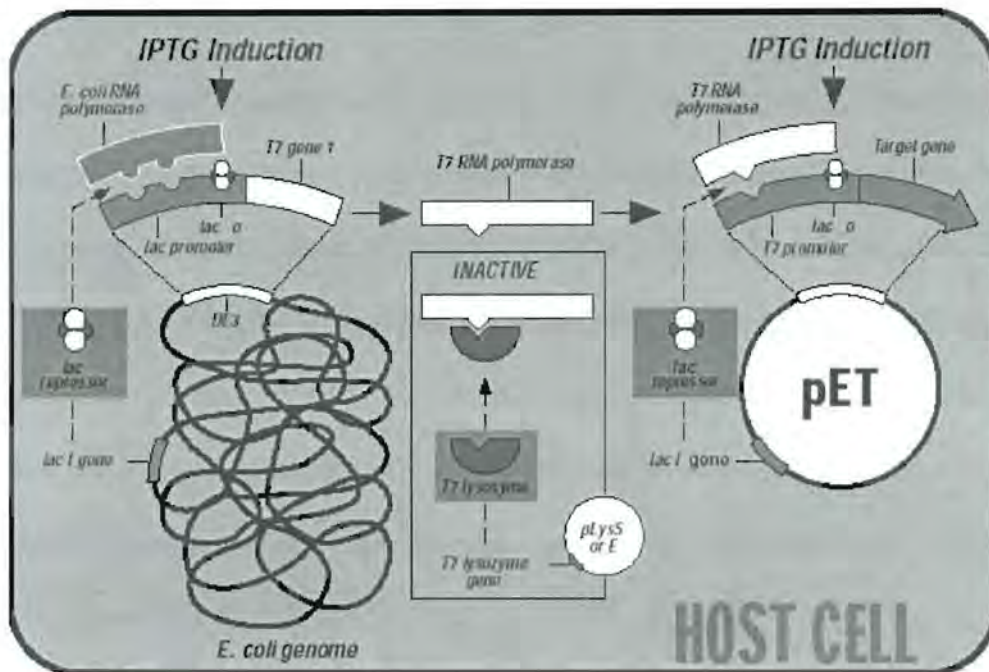
Figure 2. Schematic of the pET expression system. (Novagen, 2003)

The specific pET vector used in the following experiments was the pET15b

expression vector (see Appendix, Figure 1). This particular pET expression vector was

also engineered with an IPTG-inducible T7*lac* promoter region for increased stringency

of target protein expression (Novagen, 2001). The pET15b expression vector contains a

lac operator region downstream from the T7*lac* promoter. Under uninduced conditions,

the *lac* repressor is bound to the *lac* operator and therefore inhibits the activity of any T7

RNA polymerase that may have been accidentally translated by the host during the basal

period (Novagen, 2001). The vector also contains its own *lac*I gene to ensure that

enough repressor is made to successfully inhibit T7 RNA polymerase activity on the

plasmid (Novagen, 2001). This second IPTG-inducible *lac* promoter system is merely another way that the pET expression system controls target protein expression.

Target Fusion Protein Purification Via Metal Chelate Affinity Chromatography:
The pET15b expression vector used in this research has also been engineered with a fusion tag for convenient purification of the target protein. A string of six histidines followed by a thrombin cleavage site is located upstream of the cloning site at the N-terminus (Novagen, 1999). The 6x His-Tag® is translated as part of the target protein and serves as an efficient and extremely popular means of isolating the target protein during metal chelation chromatography purification (Novagen, 2001; Pierce Biotechnology Inc., 2002).

Polyhistidine tags, such as the 6x His-Tag® found in Novagen's pET15b expression vector, bind tightly to a nickel ion charged resin (Sambrook & Russel, 2001). A procedure known as metal chelate affinity chromatography has exploited this fact, allowing for effective and efficient purification of recombinant proteins containing such a fusion tag. The B-PER 6xHis Spin Purification Kit (Pierce Biotechnology, 2002) and Ni-NTA Magenetic Agarose Beads (Quiagen, 2001) were used to facilitate target protein purification. In the former kit, the fusion proteins associate themselves with nickel chelated agarose beads and in the latter, they associate themselves with nickel chelated magnetic beads. In both cases, the targeted fusion protein is effectively isolated from the rest of the soluble proteins in the extract (Pierce Biotechnology, 2002; Quiagen, 2001).

During these metal chelate affinity chromatography purification procedures, miscellaneous proteins without the fusion tag can be washed away while only the

recombinant target protein containing the polyhistidine tag will bind to the immobilized nickel ion beads (Pierce Biotechnology Inc., 2002). After the contaminant proteins have been removed, the target protein is easily eluted by a chelator such as imidazole (Sambrook & Russel, 2001). Imidazole competes with the fusion protein for binding sites on the nickel ion charged resin causing the polyhistidine tag to disassociate itself from the charged resin, eluting the target protein in the process. Subsequently, the polyhistidine tag can be removed from the target protein at a protease cleavage site such as the thrombin site encoded by the pET15b expression vector (Novagen, 1999; Novagen, 2001; Sambrook & Russel, 2001).

Materials and Methods:

Restriction Enzyme Digestion of Purified Cloned Plasmid Preparation, pET 15b:

A small scale restriction enzyme digestion (10 μL) with NdeI and XbaI was performed using 3 μL purified plasmid, 1.0 μL NdeI enzyme, 1.0 μL XbaI enzyme, 1.0 μL Buffer 2 (New England Biolabs), 1.0 μL 10x BSA (New England Biolabs) and 3.0 μL dH$_2$0. The reaction was incubated at 37°C for 3.5 hours. Agarose gel (0.8%) electrophoresis was used for restriction enzyme digestion analysis.

Transformation Purified Plasmid Preparation into Bacterial Cell Lines for Target Protein Expression:

Competent cells of BL21(DE3) (Novagen), BL21(SI) (Life Technologies) and ER2566 (New England Biolabs) were prepared and subsequently transformed with an aliquot of the purified purL clone in pET15b. The competent cells for all three of the previously mentioned bacterial strains were prepared in the following manner:

Two 2.5 mL tubes of LB liquid media were inoculated with a single colony of either BL21(DE3) or ER2566 and incubated at 37°C overnight with shaking; one 2.5 mL tube of LB liquid media (without NaCl) was inoculated a single colony of BL21(SI) and incubated at 37°C overnight with shaking. (The BL21(SI) bacterial expressions strain is always inoculated into LB liquid media not containing NaCl and is always plated on LB agar plates without NaCl. (For future reference, simply assume this if it is not stated in the text).

Three new tubes of LB liquid media were each inoculated with 100 μL of a different overnight liquid culture. The tubes were incubated, with shaking, at 37°C for

2.5 hours, until the liquid was slight turbid. The cultures were then centrifuged for five minutes in a cold centrifuge, and the supernatant was decanted. The cells were resuspended in 5 mL of ice cold $CaCl_2$ (0.1M) and placed on ice for 30 minutes. The cells were then again centrifuged in a cold centrifuge for 5 minutes, and the supernatant was decanted. The cells were resuspended in 0.5 mL ice cold $CaCl_2$ (0.1M) and placed on ice.

One-half microliter of each of the three following purified plasmid preparations were aliqoted into three different 1.5 mL microfuge tubes: pET15b (Novagen), the purL clone in pET 15b (Leader, 2002), or pMYB5 (New England Biolabs). (The pET15b and pMYB5 were used as negative and positive controls, respectively). Fifty microliters of each of the three prepared competent cells mixtures was aliquoted into the nine tubes containing the DNA, mixed gently and incubated on ice for 30 minutes. The cells were then heat shocked in a 42°C heating block for two minutes, had 1 mL of LB liquid media added to each tube, was mixed by inversion and then incubated at 37°C for 1-2 hours.

The contents of each of the nine tubes were plated onto individual LB agar plates containing ampicillin (100 μg/mL). Plates were incubated at 37°C overnight.

General Induction of Target Protein Expression in all Three Expression Strains and Analysis of Results:

Three 3 mL tubes of LB liquid media were supplemented with ampicillin (100 μg/mL) and inoculated with a single colony of each of the three different transformations into the BL21(DE3) expression strain; this was repeated for the three different transformations into both the ER2566 and BL21(SI) expression strains. All nine inoculated medias were incubated at 37°C overnight, with shaking.

Eighteen new tubes of 2.5 mL LB liquid media were inoculated with 100 μL of each overnight culture (two tubes per overnight culture) and ampicillin antibiotic (100 μg/mL). The new cultures were then incubated at 37°C for 1-2 hours until they reached an OD_{600} value between 0.5 and 1.0.

Once the appropriate OD_{600} value was obtained, one tube of each pair was induced with 25μL IPTG to a final concentration of 1mM. Also, NaCl was added to a final concentration of 0.3M in the induced tubes containing BL21(SI) transformants. All tubes were incubated at 37°C, with shaking for 3.5 hours. Afterward, the OD_{600} readings for each of the induced and uninduced tubes were taken. The induction conditions were those recommended by <u>Molecular Cloning: A Laboratory Manual</u> (Sambrook & Russel, 2001).

One mL of the solution in each of tubes was aliquoted into 1.5 mL microfuge tubes; the tubes were centrifuged at maximum speed in a tabletop microcentrifuge. The pellets were resuspended in 100 μL 1X SDS gel-loading buffer[*] for every one OD_{600} unit and heated to 100°C for three minutes (Sambrook & Russel, 2001). Samples were then tap-spun in a tabletop microcentrifuge and analyzed by SDS-PAGE on a 4-20% Tris-HCl BioRad ReadyGel at 200V. (All SDS-PAGE protein gels were stained in Coomassie Brilliant Blue (FisherScientific) stain and destained in 50% methanol and 10% acetic acid. For future reference, simply assume this if it is not stated in the text). The Protein Kaleidoscope Prestained Standard (BioRad) was used as a marker.

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[*] 50mM Tris-HCl, 100mM dithiothreitol, 2%(w/v) SDS, 0.1% bromophenol blue, 10%(w/v) glycerol)

Optimization Experiments for Induction of Target Protein Expression in BL21(DE3) Bacterial Expression Strain, Separation of Different Protein Fractions and Analysis of Results:

Three tubes of LB liquid media treated with ampicillin antibiotic (100μg/mL) were inoculated with BL21(DE3) transformants containing the negative control (pET15b), the purL clone in pET15b and the positive control (pMYB5). The tubes were incubated overnight at 37°C, with shaking.

An induced and uninduced tube for each overnight culture was prepared for each induction temperature variable (15°C, 23°C and 30°C) by adding 100 μL of the overnight culture to two new tubes containing 2.5 mL LB liquid media. All 18 tubes were then incubated at 37°C, with shaking, until the OD_{600} value was between 0.5 and 1.0.

For each different induction temperature and cell culture, the tube assigned to be induced was supplemented with IPTG to a final concentration of 1mM. The tubes were then incubated, with shaking, at their predetermined induction temperatures.

Once the tubes had been induced for three hours, the OD_{600} values of each tube were taken. One milliliter of each sample was aliquoted into a 1.5mL microfuge tube and centrifuged at maximum speed in a tabletop microcentrifuge; the pellets were frozen at -20°C. The remaining cultures were then allowed to continue incubating at their predetermined induction temperatures. This procedure was repeated again after the tubes had been incubating for a total of eight hours. The general induction guidelines were taken from Molecular Cloning: A Laboratory Manual (Sambrook & Russel, 2001).

Two hundred microliters of BPER-I (Pierce Biotechnology, Inc.) was added to each frozen pellet for each of its OD units. Fifty microliters of each sample were aliquoted into 0.5 mL microfuge tubes as the crude extracts. The remaining solutions

were centrifuged on a tabletop microcentrifuge for five minutes at maximum speed. The supernatants were placed in 1.5 mL tubes and saved as the soluble extracts. The remaining pellets were resuspended in volumes of BPER-I equal to the volumes of the soluble extracts; these solutions were saved as the insoluble extracts.

After all the extracts were prepared, 3x SDS loading buffer was added in a volume equal to one-half the volume of each extract. The solutions were heated to 95°C in heating block for 15 minutes, tap-spun on a microcentrifuge and analyzed by SDS-PAGE on a 4-20% Tris-HCl BioRad ReadyGel at 200V.

Transformation of purL Clone, Negative Control and Positive Control into Novagen's Rosetta-Blue™(DE3)pLysS Expression Cell Line:

Competent cells were prepared as indicated above in media containing tetracycline (12.5μg/mL) and chloramphenicol (34μg/mL) antibiotics in place of ampicillin antibiotic. Transformation mixtures were plated on LB agar plates containing ampicillin (100μg/mL), tetracycline (12.5μg/mL) and chloramphenicol (34μg/mL). (The Rosetta-Blue™(DE3)pLysS bacterial expression strain is always inoculated into LB liquid media and plated on LB agar plates containing ampicillin (100μg/mL), chloramphenicol (34μg/mL) and tetracycline (12.5μg/mL). (For future reference, simply assume this if it is not stated differently in the text).

General Induction of Target Protein Expression and Analysis of Results:
Three overnight cultures using a single colony from each of the transformations in Novagen's Rosetta-Blue™ (DE3)pLysS expression strain. One hundred microliters of

each overnight culture was used to inoculate two new 2.5mL tubes of LB liquid media (representing the induced and uninduced samples). The new six inoculations were incubated at 37°C until the OD_{600} value was between 0.5 and 1.0. One tube of each pair was induced with IPTG to a final concentration of 1mM; all six tubes were incubated at 37°C, with shaking, overnight. The OD_{600} values were taken for each sample after the induction.

One milliliter of each 2.5mL culture was transferred to a 1.5mL microfuge tube and centrifuged at maximum speed for one minute. The supernatants were decanted and the pellets were resuspended in 100μL 1x SDS loading buffer per one OD unit. The crude solutions were heated at 95°C in a heating block for three minutes, briefly centrifuged and then analyzed by SDS-PAGE on a 4-20% Tris-HCl BioRad Ready Gel at 200V. The general guidelines for the induction were taken from Sambrook and Russel's Molecular Cloning: A Laboratory Manual (2001).

Optimization Experiments for Induction of Target Protein Expression in Novagen's Rosetta-Blue™ (DE3)pLysS Bacterial Expression Strain, Separation of Different Protein Fractions and Analysis of Results:

Induction experiments were performed as previously described using the transformed Rosetta-Blue™(DE3)pLysS strains for the negative control, positive control and purL clone plasmid preparations. The inductions were performed according to general guidelines stated in Sambrook and Russel's Molecular Cloning: A Laboratory Manual (2001). The inductions were performed at both 37°C and 23°C, for both three- and eight-hour time intervals.

After the cultures were induced for three hours, the OD_{600} values were recorded, and one milliliter of each culture (induced and not induced) was transferred to a 1.5mL microfuge tube. The tubes were centrifuged at maximum speed in a tabletop microcentrifuge for one minute, supernatants were decanted and all pellets were frozen at -20°C. The remaining cultures were then allowed to incubate at their respective temperatures. After a total of eight hours of induction time, the OD_{600} values were recorded, and one milliliter of each culture was transferred to a 1.5mL microcentrifuge tube. The tubes were centrifuged in a tabletop microcentrifuge at maximum speed for one minute, supernatants were decanted and all pellets were frozen at -20°C.

Each pellet was resuspended in $200\mu L$ of BPER-I for every one OD unit of the respective sample culture. A $50\mu L$ aliquot of each resuspension was transferred to a 1.0mL microcentrifuge tube as the crude extract. The remainder of each suspension was then centrifuged in a tabletop microcentrifuge at maximum speed for five minutes. The supernatant was transferred to a 1.5mL microfuge tube and saved as the soluble extract. The remaining pellet was resuspended in a volume of BPER-I equal to the volume of the soluble extract and saved as the insoluble extract.

To all the samples, 3x SDS loading dye was added in a volume equal to one half the total volume of each sample. All samples were heated at 95°C in a heating block, tap-spun in a tabletop microcentrifuge and analyzed by SDS-PAGE on a 4-15% Tris-HCl BioRad ReadyGel at 200V.

Large-scale Induction of Target Protein Expression, Separation of Different Protein Fractions and Analysis of Results:

The general protocol followed for this large-scale expression of the target protein was derived from Sambrook and Russel's Molecular Cloning: A Laboratory Manual (2001). Fifty milliliters of LB containing ampicillin ($100\mu g$/mL) was inoculated with a single transformed colony of Rosetta-Blue™ (DE3)pLysS containg HspurL. Two 50 mL flasks of LB liquid media containing ampicillin ($100\mu g$/mL) were each individually inoculated with single transformed colonies of Rosetta-Blue™ (DE3)pLysS containing either the positive or negative controls. The cultures were incubated overnight at 37°C, with shaking. One milliliter of the control overnight cultures was used to inoculate two 5mL flasks of LB liquid media containing ampicillin ($100\mu g$/mL). The entire overnight culture of Rosetta-Blue™ (DE3)pLysS transformed with HspurL was used to inoculate a prewarmed 500mL flask of LB liquid media containing ampicillin antibiotic ($100\mu g$/mL). The new cultures were incubated at 37°C, with shaking, until the OD_{600} values were between 0.5 and 1.0.

All three cultures were induced with IPTG to a final concentration of 1mM and incubated at room temperature, with shaking, for eight hours. The entire experimental induction was centrifuged in a large centrifuge for fifteen minutes at 8,000 rpm; 1mL of each control induction was centrifuged in a tabletop microcentrifuge for one minute at maximum speed.

The experimental cell pellet was resuspended in BPER-II (Pierce Biotechnology, Inc.) at a volume equal to $50\mu L$ for every one milliliter of culture centrifuged. A $50\mu L$ aliquot of the resuspension was transferred to a 0.5mL microfuge tube and saved at -20°C as the crude extract. One milliliter of the resuspension was transferred to a 1.5mL

microfuge tube and centrifuged in a tabletop microcentrifuge for five minutes at

maximum speed. The supernatant was transferred to a 1.5mL microfuge tube and saved

at -20°C as the soluble extract. The remaining cell pellet was resuspended in a volume of

BPER-II equal to the volume of the soluble extract and saved at -20°C as the insoluble

extract. The remainder of the original large-scale resuspension was stored at -20°C.

SDS loading dye (3x) was added to the crude and soluble samples at a volume

equal to one-half the volume of the extracts. The samples were heated at 95°C for 15

minutes and analyzed by SDS-PAGE on a 4-15% Tris-HCl BioRad ReadyGel, run at

200V.

Purification of the Target Fusion Protein by Nickel Ion Affinity Column Chromatography:

Target protein purification was completed using the standard protocol and solutions in the

B-PER 6XHis Spin Purification Kit Instruction Manual (Pierce Biotechnology, 2002).

Purification was performed on the remainder of the large-scale induction resuspension

(previously stored at -20°C). The remaining resuspension was thawed and centrifuged at

approximately 14,000 rpm in a clinical centrifuge for fifteen minutes. The supernatant

was transferred to a capped 15mL tube containing 1mL of nickel-chelated agarose (Pierce

Biotechnology, 2002) and shook on ice for ten minutes. The tube was then spun down in

a cold centrifuge at maximum speed for five minutes. The supernatant was removed and

the agarose resin was resuspended with 250μL of wash buffer (Pierce Biotechnology,

2002). The remaining solution was transferred to a provided spin column and centrifuged

at maximum speed for two minutes in a tabletop microcentrifuge. The collection tube

was changed and 500μL of wash buffer was added to the spin column. The tube was

incubated at room temperature for five minutes and then centrifuged at maximum speed

for two minutes in a tabletop microcentrifuge. The eluted solution was saved as the first

wash. This procedure was repeated for three more additional washes, and the collection

tube was changed for each wash and saved as such.

The collection tube was changed again after the last wash and $500\mu L$ of elution

buffer (Pierce Biotechnology, 2002) was added to the spin column. The spin column was

incubated at room temperature for five minutes and then centrifuged at maximum speed

for two minutes. The eluted solution was saved as the first elution. This procedure was

repeated for a total of four elutions, and the collection tube was changed in between each

and saved.

Twenty microliters of each wash and elution samples were aliquoted to an 0.5mL

microfuge tube. Ten microliters of 3x SDS sample loading buffer was added to each of

the samples, and each was heated to 95°C for approximately 15 minutes. The samples

were analyzed by SDS-PAGE on a 4-15% Tris-HCl BioRad Ready Gel at 200V.

***Plasmid Preparation of the HspurL cloned pET 15b vector from a Rosetta-Blue™
(DE3)pLysS Transformant:***

A plasmid preparation of the *Hs*purL cloned pET 15b vector was prepared by following

the standard midi-prep protocol in Qiagen's Plasmid Purification Handbook (2002). A

restriction enzyme digestion, using the same restriction enzymes as noted previously, was

done to confirm the presence of the *Hs*purL plasmid within the pETvector.

Optimized Experiment for Induction of Target Protein Expression in Novagen's Rosetta-Blue™ (DE3)pLysS Bacterial Expression Strain and Analysis of Results:

The same culture that was used in the plasmid preparation was also used in a small-scale induction experiment to decrease the number of variables possibly affecting protein expression results. Small-scale induction experiments were performed as previously described using the transformed Rosetta-Blue™(DE3)pLysS strains for the negative control, positive control and purL clone in pET15b plasmid preparations. The inductions were performed according to general guidelines stated in Sambrook and Russel's Molecular Cloning: A Laboratory Manual (2001). The inductions were performed at 23°C, for eight hours.

After eight hours of induction time, the OD_{600} values were recorded, and one milliliter of each culture was transferred to a 1.5mL microcentrifuge tube. The tubes were centrifuged in a tabletop microcentrifuge at maximum speed for one minute, supernatants were decanted and all pellets were frozen at -20°C.

The pellets were resuspended in $100\mu L$ 1X SDS loading buffer per one OD unit. The crude solutions were heated at 95°C in a heating block for three minutes, tap-spun in a microcentrifuge for one minute and then analyzed by SDS-PAGE on a 4-20% Tris-HCl BioRad Ready Gel at 200V.

Large-Scale Induction of Target Protein Expression, Separation of Different Protein Fractions and Analysis of Results:

The general protocol followed for this large-scale expression of the target protein was derived from Sambrook and Russel's Molecular Cloning: A Laboratory Manual (2001). Three milliliters of LB liquid media containing ampicillin ($100\mu g/mL$), chlorampehnicol

($34\mu g/mL$) and tetracycline ($12.5\mu g/mL$) was inoculated with a single transformed colony of Rosetta-Blue™ (DE3)pLysS containing *Hs*purL. The tube was incubated at 37°C overnight, with shaking.

The next morning, the entire 3mL culture was used to inoculate a 70mL flask of LB liquid media containing ampicillin ($100\mu g/mL$), chlorampehnicol ($34\mu g/mL$) and tetracycline ($12.5\mu g/mL$). The flask was incubated at 37°C, with shaking, until the OD_{600} value was between 0.5 and 1.0.

After the appropriate OD_{600} value was reached, the flask was induced with IPTG to a final concentration of 1mM. The flask was incubated at room temperature, with shaking, for eight hours.

After the induction time period had elapsed, the culture was centrifuged in a large centrifuge for 15 minutes at ~8,000 rpm and the pellets were frozen at -20°C overnight. The cell pellet was resuspended in BPER-II at a volume equal to $50\mu L$ for every one milliliter of culture centrifuged. A $50\mu L$ aliquot of the resuspension was transferred to a 0.5mL microfuge tube and saved at -20°C as the crude extract. One milliliter of the resuspension was transferred to a 1.5mL microfuge tube and centrifuged in a tabletop microcentrifuge for five minutes at maximum speed. The supernatant was transferred to a 1.5mL microfuge tube and saved at -20°C as the soluble extract. The remaining cell pellet was resuspended in a volume of BPER-II equal to the volume of the soluble extract and saved at -20°C as the insoluble extract. The remainder of the original large-scale resuspension was stored at -20°C.

SDS loading dye (3x) was added to the crude and soluble samples at a volume equal to one-half the volume of the extracts. The samples were heated at 95°C for 15

minutes and analyzed by SDS-PAGE on a 4-15% Tris-HCl BioRad ReadyGel, run at 200V.

Purification of the Target Fusion Protein using Ni-NTA Magnetic Agarose Beads and Nickel Ion Affinity Resin Chromatography:

Target protein purification was completed using the sixth protocol in the Ni-NTA Magnetic Agarose Beads Handbook (Qiagen, 2001). Wash and elution buffers were taken from the B-PER 6XHis Spin Purification Kit (Pierce Biotechnology, 2002).

Purification was performed on 1mL of the remainder of the large-scale induction resuspension. Two-hundred microliters of the 5% Ni-NTA Magnetic Agarose Bead suspension (Qiagen, 2001) was added to the resuspension. The solution was mixed gently on a shaker for 30 minutes at room temperature. The tube was placed on the magenetic separator for one minute, and the supernatant was removed. This supernatant was saved as the post-binding supernatant. The tube was removed from the magnet, and the magnetic beads were gently resuspended in $500\mu L$ wash buffer (Pierce Biotechnology, 2002). The tube was placed back on the magnetic separator for one minute and then the supernatant was removed and saved as the first wash. This washing procedure was repeated two additional times, and each wash supernatant was saved.

After the third wash, the tube was removed from the magnetic separator, and the magnetic beads were gently resuspended in $100\mu L$ of elution buffer (Pierce Biotechnology, 2002). The tube was placed back on the magnetic separator for one minute and then the supernatant was removed and saved as the first elution. This procedure was repeated three additional times, and each elution supernatant was saved.

Ten microliters of each wash and elution samples were aliquoted to a 0.5mL microfuge tube. Five microliters of 3x SDS sample loading buffer was added to each sample and heated for approximately 15 minutes at 95°C. The samples were analyzed by SDS-PAGE on a 4-20% Tris-HCl BioRad Ready Gel at 200V.

Results and Discussion:

Restriction Enzyme Digestion of Purified Cloned Plasmid Preparation, pET 15b and Transformation of the Purified Plasmid Preparations into Bacterial Cell Lines for Target Protein Expression:

The restriction enzyme digestion with Xba I and Nde I confirmed the presence of the

purL insert within the pET15b plasmid preparation previously prepared by Leader (2002)

(See Figure 1a).

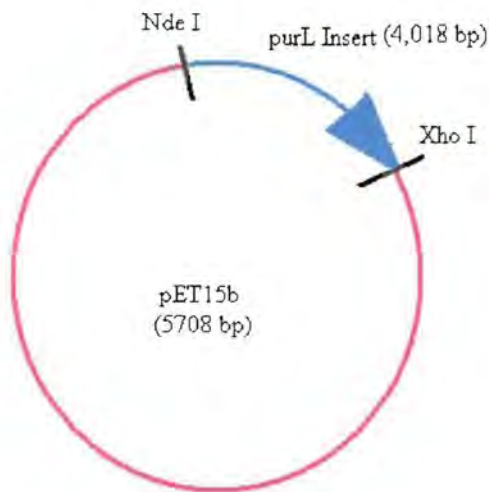**Figure 1a.** A representation of the purL insert within the pET15b expression vector

Lane four in Figure 1b shows the purL insert at approximately 4,018 base pairs,

the expected size. The second and third lanes show the control restriction enzyme

digestions using only NdeI restriction enzyme and only XbaI restriction enzyme,

respectively. The controls resulted in a linearized cloned pET15b vector, as was

expected.

Upon confirmation that the plasmid preparation (Leader, 2002) contained the purL insert, transformation experiments were performed as noted in the methods. All attempted transformations were successful.
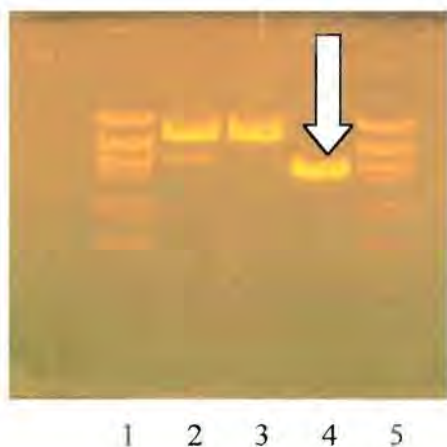

Figure 1b.
Agarose Gel Electrophoresis following digestion of the pET15b vector containing the purL insert.

1 2 3 4 5

General Induction of Target Protein Expression in all Three Expression Strains and Analysis of Results:

Unfortunately, the BL21(SI) expression strain did not culture very well in the pre-induced state and therefore, the only BL21(SI) transformants used in this first induction experiment contained the pET15b negative control plasmid.

Figure 2a illustrates the expression results for the BL21(DE3) and BL21(SI) cell lines four hours after IPTG-induction. Lanes 1, 3 and 5 contain the protein extracts from the induced BL21(DE3) cultures in the following order: pET15b (negative control), purL in pET15b (experimental target protein), and pMYB5 (positive control). Lanes 2, 4 and 6 contain the protein extracts from the uninduced BL21(DE3) cultures in the same order.

The induction results indicated that there was possible target protein expression at approximately 140 kiloDaltons in the BL21(DE3) host expression strain upon induction with IPTG (See Figure 2a, lane 3). Also, there was no target protein expression in any of

the uninduced cultures, as expected (See Figure 2a, lanes 2, 4 and 6). However, there was no expression of the positive control at approximately 97kD, indicating that perhaps IPTG-induction did not occur properly (See lane 5 in Figure 2a).

There was no apparent expression of the target protein in any of the other IPTG-induced cell lines. Although the positive control did not appear to express itself as it should have, the fact that there was potential target protein expression in the induced BL21(DE3) culture invited further experimentation on that specific expression strain. Therefore, the BL21(DE3) expression cell line was determined to be the most promising for future experiments.
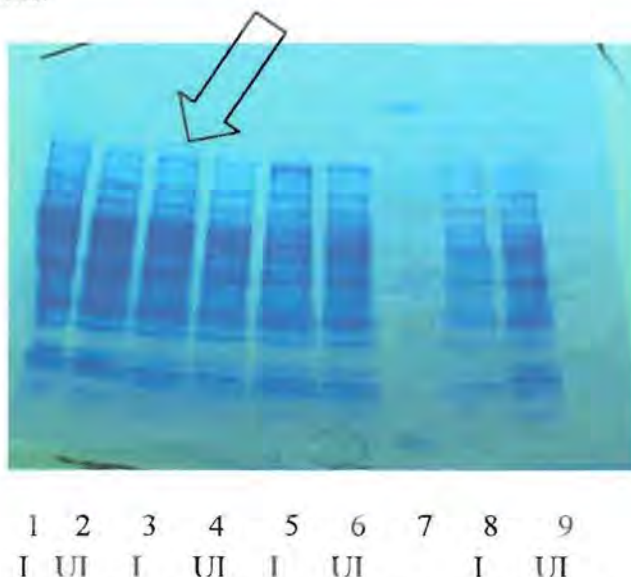

<table>
<tr><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td><td>7</td><td>8</td><td>9</td></tr>
<tr><td>I</td><td>UI</td><td>I</td><td>UI</td><td>I</td><td>UI</td><td></td><td>I</td><td>UI</td></tr>
</table>

Figure 2a. SDS-PAGE gel following Coomassie Brilliant Blue staining. Lanes 1-6 contain the crude protein extracts from the BL21(DE3) cell line four hours after IPTG-induction. Lanes 8-9 contain the crude protein extracts from the BL21(SI) cell line four hours after IPTG-induction. In lane 3, there appears to be target protein expression in the BL21(DE3) host upon IPTG-induction at approximately 140 kD.

Optimization Experiments for Induction of Target Protein Expression in BL21(DE3) Bacterial Expression Strain, Separation of Different Protein Fractions and Analysis of Results:

There appeared to be no target protein expression in any of the inductions, regardless of induction time or temperature. Also, there appeared to be no expression of the positive control protein. Previous research regarding the expression of the control protein in the

BL21(DE3) cell line had similar results (Leader, 2002). Since the BL21(DE3) cells were not properly expressing the positive control protein, another protein expression cell line, Novagen's Rosetta-Blue™(DE3), was acquired.

The Rosetta-Blue™(DE3) expression strain was chosen because it is genetically engineered to contain *Homo sapiens* transfer RNA (tRNA) genes, which prefer the codons most prevalent in human mRNA. The genetic code is degenerate in the sense that more than one codon codes for the same amino acid (Rodolphe & Mathe, 1999). As a result of evolution, different species have genetic sequences that preferentially code for one particular codon over another in messenger RNA (Kane, 1995). These codon preferences are unique to a species, and are reflected in the relative abundance of the various tRNAs within their cells (Bagnoli & Lio, 1995; Deng, 1997). Such inconsistent codon preferences can cause inefficient translation when attempting to express a eukaryotic protein in an *E. coli* host (Wang et al., 1994).
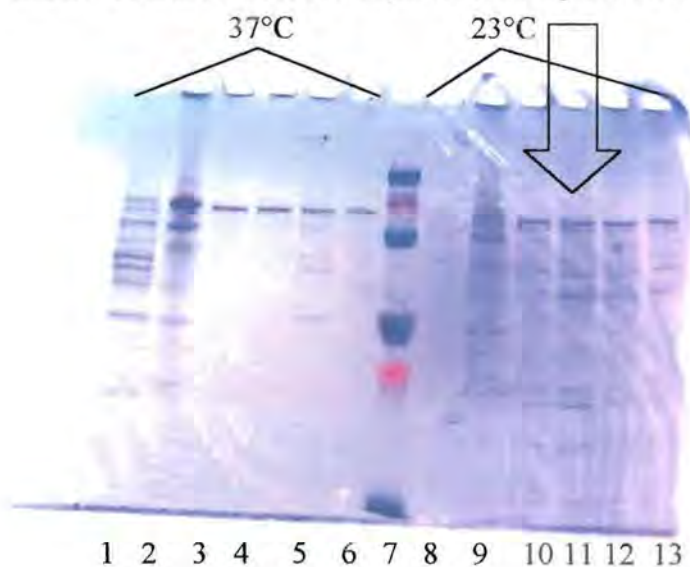
Specific codons that are extremely prevalent in human genes such as AGG, AGA, AUA, CUA, CCC and GGA are far rarer in *E. coli* (Xu et al., 2001; Novagen, 2002; Wang et al., 1994; Hu et al., 1996.) The transfer RNAs associated with these rare *E. coli* codons are also among their rarest produced in the cell (He et al., 1996). Therefore, when human codon preferences show up in *E. coli* mRNA sequences, there may not be enough of the rare t-RNAs to assemble the protein, resulting in stalled expression (Xu et al., 2001).

We utilized Novagen's Rosetta-Blue™(DE3)pLysS expression strain for the subsequent experiments. This strain has been genetically engineered to contain the genes responsible for the production of the tRNAs associated with the preferred human codons

rare in *E. coli* (Novagen, 2002). Also, this particular strain contains the pLysS plasmid, which contains the T7 lysozyme gene (Novagen, 2002). As was mentioned earlier, T7 lysozyme inhibits T7 RNA polymerase production in the uninduced state and provides another mechanism to control target gene expression within the host (Novagen, 2001).

Optimization Experiments for Induction of Target Protein Expression in Novagen's Rosetta-Blue™ (DE3)pLysS Bacterial Expression Strain, Separation of Different Protein Fractions and Analysis of Results:

Figure 3a illustrates the crude extract expression results eight hours after IPTG-induction. Lanes 1-6 contain the crude protein extracts from the cultures incubated at 37°C. Lanes 1, 3 and 5 contain the uninduced crude extracts in the following order: pMYB5 (positive control), purL in pET15b (experimental target protein), and pET15b (negative control). Lanes 2, 4 and 6 contain the induced crude extracts in the same order. Lanes 8-13 contain the crude protein extracts from the cultures incubated at room temperature (23°C). Lanes 8, 10 and 12 contain the uninduced crude extracts in the following order: pMYB5 (positive control), purl in pET15b (experimental target protein), and pET15b (negative control). Lanes 9, 11 and 13 contain the induced crude extracts in the same order. This SDS-PAGE analysis revealed possible expression of the target protein in lane 11 at approximately 140 kD.

Figure 3a. SDS-PAGE gel following Coomassie Brilliant Blue staining. Lane 11 shows slight possible expression of the target protein at ~ 140 kD in the crude extract.

To further analyze the observed band, the crude and soluble extracts of the cultures transformed with purL were analyzed using SDS-PAGE. Figure 3b shows the results of this analysis.

Lanes 1-6 contain the crude and soluble extracts incubated at room temperature. Lanes 8-13 contain the crude and soluble extracts incubated at 37°C. The first and second lanes contain the soluble extracts of the induced and uninduced positive control cultures, respectively. There appears to be expression of the positive control at approximately 97 kD, as was expected. Lanes 3 and 4 contain the soluble and crude extracts of the induced experimental cultures. SDS-PAGE analysis shows expression of a protein at approximately 140 kD, which is the appropriate size for the target protein. Lanes 5 and 6 contain the soluble and crude extracts of the uninduced experimental cultures. There appears to be no protein expression of approximately 140 kD in either of these uninduced lanes, as was expected. The apparent optimal conditions for expressing the target protein were determined as being an 8-hour, room temperature induction period with a final IPTG concentration of 1mM.
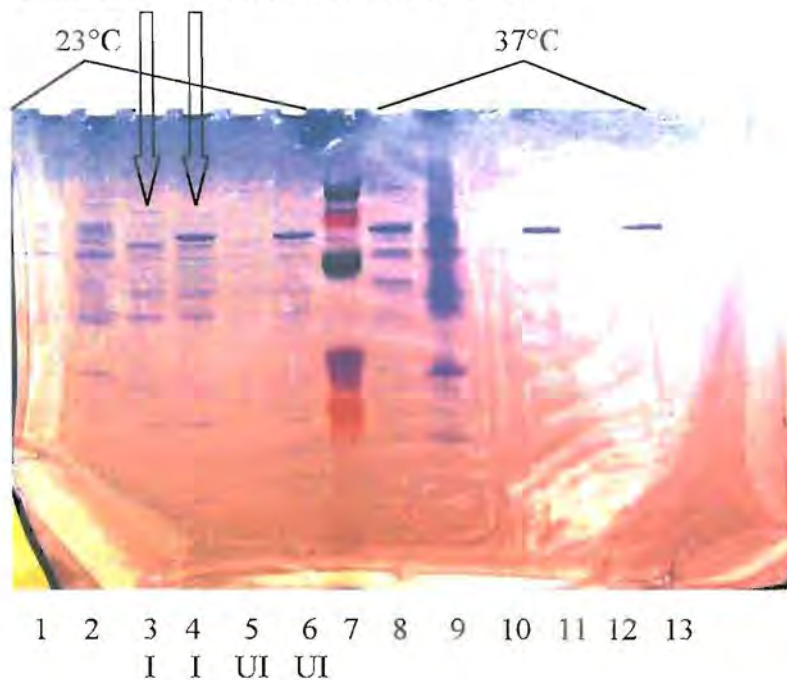

Figure 3b. SDS-PAGE gel following Coomassie Brilliant Blue Staining. Lanes 3 and 4 show expression of the target protein at approximately 140 kD.

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Large-scale Induction of Target Protein Expression, Separation of Different Protein Fractions, and Purification of the Target Fusion Protein by Nickel Ion Affinity Column Chromatography:

The large-scale induction of the target protein yielded apparent low-level expression of

the positive control and the target protein (in both soluble and crude extracts). Figure 4

illustrates the results of the large-scale induction.

Figure 4. SDS-PAGE gel following Coomassie Brilliant Blue Staining. Lane 3 contains the crude extract of the induced experimental culture after an 8-hour induction period. Lanes 4 and 7 contain the soluble extracts of the induced experimental culture after an 8-hour induction period.

1 2 3   4 5 6 7
      I   I I I I

Analysis of the nickel-ion affinity column chromatography purification showed

no evidence of the target protein in any of the elutions or washes.

Plasmid Preparation of the HspurL cloned pET 15b vector from a Rosetta-Blue™ (DE3)pLysS Transformant, Optimized Experiment for Induction of Target Protein Expression and Analysis of Results:

SDS-PAGE analysis indicated that there was possible expression of the target protein, at

approximately 140kD, eight hours after induction with IPTG at room temperature.

Large-Scale Induction of the Target Protein Expression, Separation of Different
Protein Fractions and Purification of the Target Fusion Protein using Ni-NTA
Magnetic Agarose Beads and Nickel Ion Affinity Resin Chromatography:

Figure 5 illustrates the large-scale induction results of the purL cloned bacteria eight

hours after induction with IPTG at room temperature. The first lane contains the

insoluble extract. Lanes 2 and 3 contain the crude extract. The fourth lane contains the

soluble extract.

There appears to be possible target protein expression in the crude protein

extracts. Also, a very faint band appears at approximately 140kD in lane 4, the soluble

extract.
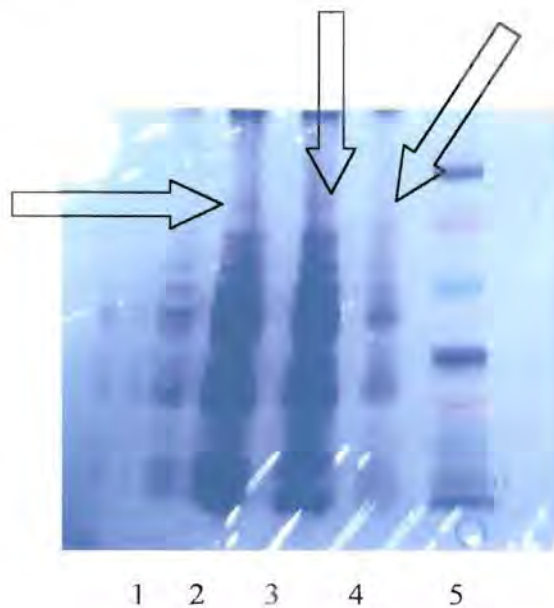
Figure 5. SDS-PAGE gel following Coomassie Brilliant Blue staining. Large-scale induction results show that there is possible expression of the target protein in the crude protein extract (lanes 2 and 3).

1   2   3   4   5

However, despite the apparent protein expression in the crude and soluble

extracts, SDS-PAGE analysis of the nickel-ion affinity resin chromatography purification

showed no evidence of the target protein in any of the elutions or washes.

Due to the lack of sufficient target protein expression in any of the induction experiments, it was not surprising that target protein could be purified from the soluble extract via either purification method. However, the lack of sufficient protein expression was confusing.

Restriction digests had confirmed that the pET15b vector contained the purL gene. The last rounds of general and optimized induction experiments were performed on a bacterial culture known to have been successfully transformed with the pET vector containing the *Hs*purL insert. And, as stated previously, the Rosetta-Blue™(DE3)pLysS bacterial expression strain was genetically engineered to combat the most likely potential inhibitor of the target protein expression. Therefore, the lack of sufficient target protein expression alerted us to the fact that perhaps there was another problem inhibiting expression of the *Hs*purL gene, besides codon bias.

Our close inspection of the previous student's research (Leader, 2002) prompted analysis of the structure of the pET15b vector. Previous research regarding this project included incorporation of an Nde I restriction site into the start codon for the *Hs*purL gene and the incorporation of an Xho I site into the stop codon for the gene (Leader, 2002). Also, previous research experiments ligated the *Hs*purL insert into a pCR2.1 TOPO vector (Invitrogen) and then, subsequently, into the pET15b expression vector. The ligation of the insert into the expression vector had to be done so that the orientation of the insert would allow for the sequence to be translated into a protein. Leader verified that the pET15b clone, which the current research was based upon, contained the insert in the proper orientation (Leader, 2002). However, analysis of the pET15b schematic revealed that the previous researcher was mistaken in her analysis of the insert's

orientation in the pET15b vector. As a result, we discovered another factor possibly prohibiting the expression of the target gene.

Previous research included the ligation of the *Hs*purL insert into a pCR2.1 TOPO vector prior to its ligation within the pET15b vector. This was done because prior experiments showed that direct ligation of the insert into a pET vector was more difficult. Leader predicted that subcloning the insert into the pCR2.1 TOPO vector would produce greater amount of the target gene that would then be used for ligations into the pET15b vector (Leader, 2002).

However, when it was time for the insert to be taken out of the pCR2.1 TOPO vector and gel-purified for future ligation into a pET expression vector, it was discovered that the pCR2.1 vector and the *Hs*purL insert were approximately the same size and would not allow for a precise gel-purification (Leader, 2002). Therefore, the cloned pCR2.1 TOPO vector was cut with three restriction enzymes in order to extract the insert from the vector and cleanly gel-purify it: Nde I, Xba I and Bam HI (Leader, 2002). The thought was that the restriction enzymes Nde I and Xba I would cut slightly more than the entire insert out of the pCR2.1 TOPO vector, and the Bam HI digest would cut the remaining vector in half. In total, this triple enzyme digestion was supposed to allow for a better distinction between the insert and the vector to be seen in a DNA gel electrophoresis analysis of such a restriction enzyme digestion (Leader, 2002). In preparation for ligation of the *Hs*purL insert into the pET15b vector, Leader also cut the pET15b vector with Nde I and Xba I, as well (Leader, 2002).

At first, this appears to be very standard procedure. However, upon closer inspection of the pET15b vector map (see Appendix, Figure 1), it was discovered that

cutting the vector with Nde I and Xba I would not produce the desired fusion-tagged protein (See Figure 6). Also, it ensured that the *Hs*purL insert would be oriented in the reverse direction, thus not allowing for target protein expression (See Figure 7).

Figure 6. The sequence below is for the T7 expression region in the pET15b expression vector. It is apparent how the 6x-histidine tag was eliminated from the sequence when the pET vector was cut with Xba I and Nde I restriction enzymes in preparation for insert ligation.

pET-15b cloning/expression region
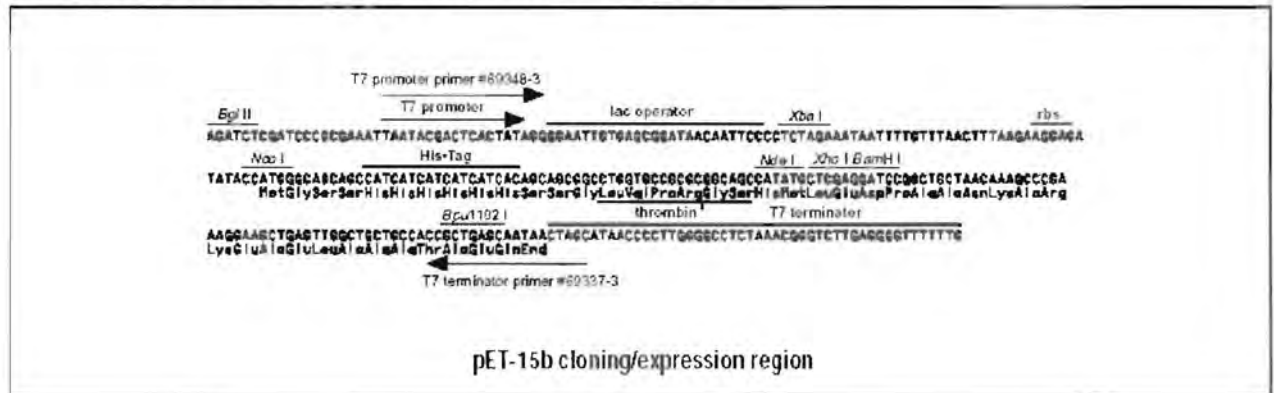
(Novagen, 1999)
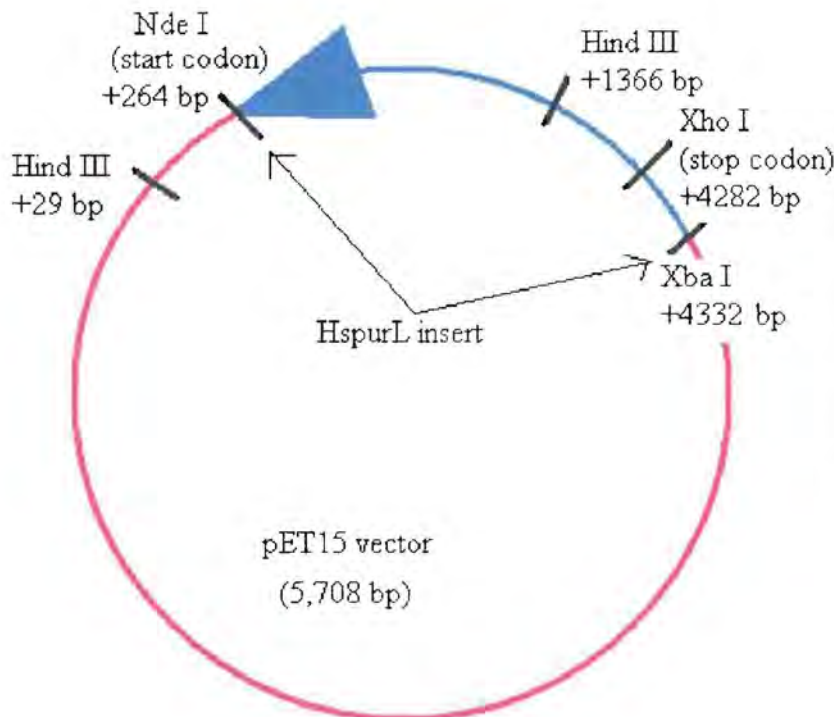

Figure 7. The pET15b vector and the *Hs*purL insert were both cut with Nde I and Xba I to allow for subsequent ligation reactions (Leader, 2002). However, this put the insert in the reverse orientation, thus preventing target protein expression.

Nde I (start codon) +264 bp
Hind III +1366 bp
Xho I (stop codon) +4282 bp
Hind III +29 bp
Xba I +4332 bp
HspurL insert
pET15 vector (5,708 bp)

There is no way for the target protein to be expressed with the orientation that the *Hs*purL gene currently has within the pET15b vector (See Figure 7). Therefore, the 140 kD fragment often seen was either another protein or an artifact, but never the desired target protein.

Previous research cut the *Hs*purL insert out of the pCR 2.1 TOPO vector with Xba I and Nde I to allow for a better distinction between it and the vector during subsequent DNA gel purification of the insert (Leader, 2002). The pET15b vector was then cut with Xba I and Nde I in preparation of the HspurL insert ligation (Leader, 2002). However, the Xba I site is not within the cloning region of the pET vector's T7 expression region. Using Xba I and Nde I as the ligation sites for the insert into the pET15b vector immediately negated target protein expression.

The T7 expression region on the pET15b vector is reversed, such that the T7 promoter is downstream of the Xba I site, which is downstream of the start codon. Therefore, the T7 expression region essentially runs in reverse order to express the target protein: from a downstream promoter region to an upstream stop codon. The pET vector contains the *Hs*purL insert in an orientation that has the Xho I site and the stop codon located near the downstream Xba I site and the promoter region. The start codon and Nde I site are located upstream from the promoter region, Xba I site and Xho I site with its incorporated stop codon. However, since expression in this particular system runs from the downstream promoter region to the upstream stop codon, the Xho I site containing the stop codon is reached before the Nde I site containing the start codon. Therefore, target protein expression is stopped before it ever really has a chance to begin.

It is believed that the *Hs*purL insert should have been excised from the pCR2.1

vector with Nde I, Xho I and Bam HI. This combination of restriction enzymes would

have not only allowed for a clear distinction between the insert and the vector for gel

purification procedures, but would have also allowed the insert to be ligated into the

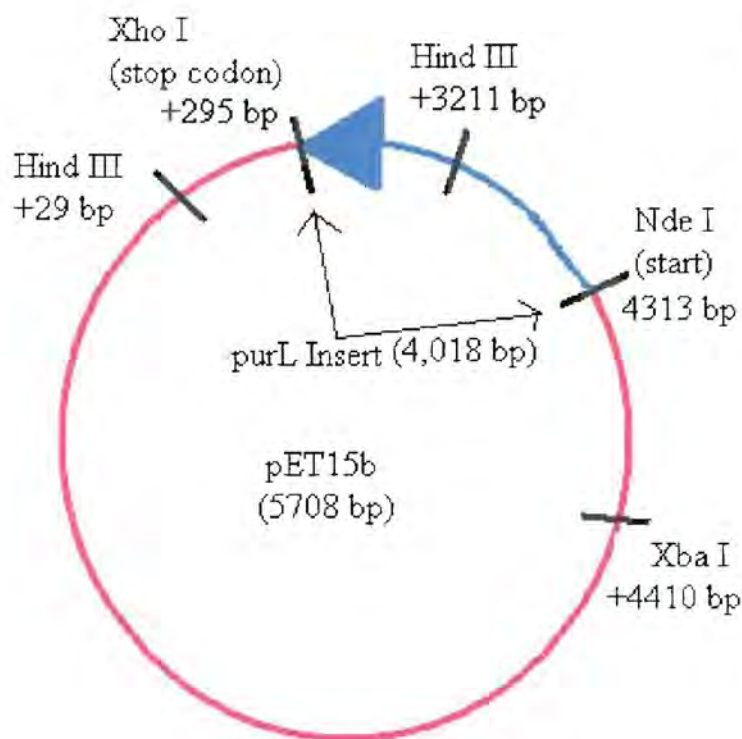pET15b vector within its cloning region (See Figure 8).

Figure 8. This diagram shows the cloned pET15b vector with ligation of the insert at Nde I and Xho I, as suggested above. Target protein expression would begin at the promoter region, which is downstream (on this diagram) from the Xba I site to the stop codon at the Xho I site.

However, regardless of the fact that the Xba I site is not located within the

pET15b cloning region and therefore, immediately oriented the insert in the wrong

direction, the choice to use Xba I for ligation of the *Hs*purL insert into the pET vector

was poor for another reason, as well.

The Xba I restriction site is located approximately 50 base pairs downstream of

the 6x-Histidine tag, which is located downstream from the Nde I restriction site (See

Figure 6). In order to ligate the *Hs*purL insert between the Xba I and Nde I restriction sites, the sequence coding for the fusion tag would be excised out of the to-be translated sequence. Therefore, even if there was a way for the target protein to be expressed with its reversed orientation within the pET15b vector, there would be no way to purify the protein product via nickel-ion affinity chromatography, as was previously planned.

It has been determined that the *Hs*purL insert is actually oriented in the wrong direction within the pET15b expression vector. Such an orientation would absolutely prohibit any target protein expression. As a result of the backwards orientation of the insert, translation of the target protein would not be able to begin because the stop codon would be reached prior to the start codon; target protein expression would, in a sense, stop before it had begun.

Future research must focus on cloning the *Hs*purL insert into the pCR2.1 TOPO vector again. Once this has been successfully completed, the insert must be extracted for subsequent ligation into a pET expression vector. However, due to the fact that the pCR2.1 vector and the *Hs*purL insert are of very similar size, the insert cannot be extracted from the pCR2.1 vector with Nde I and Xho I, alone. Instead, the cloned vector should be digested with Nde I, Xho I and Bam HI to allow for gel purification of the insert.

The pET15b expression vector (or another similar one) can then be cut with Xho I and Nde I to allow for ligation of the *Hs*purL insert. This will prohibit the automatic ligation of the insert in the reverse orientation which was inescapable with the insert's ligation between the Xba I and Nde I sites in the pET15 vector. Also, ligation of the insert into the pET vector between Xho I and Nde I will allow for the 6x-histidine tag to

remain on the translated target protein. Restriction enzyme digestion should then be done, using Hind III, to verify whether or not the clones contain the *Hs*purL insert in the proper orientation (Leader, 2002).

Unfortunately, time constraints on this project prohibit the previously suggested experiments for future research. Nonetheless, this research will prove invaluable to future researchers in this lab who may attempt to express and purify the Homo sapiens FGAR amidotransferase enzyme. Most likely, the improper orientation of the HspurL insert, due to a bad choice of ligation sites within the pET vector, is the primary problem regarding the lack of target protein expression.

Conclusion:

It has been recently determined that the *Homo sapiens* purL insert was cloned backwards into the pET15b expression vector system used for target protein expression. Previous improper analysis of the pET15b vector map allowed for such an error in the previous research that this current study was built upon. Unfortunately time restraints prohibit the re-cloning of the *Hs*purL insert, in the proper orientation, into another pET expression vector.

This work has proved to be invaluable to other researchers, regardless of the fact that a purified protein product has yet to be yielded. Future work should focus on cloning the *Hs*purL insert into the pCR2.1 TOPO vector, excision of the insert with Nde I, Bam HI and Xho I, and gel purification of the insert. Then the insert must be cloned once again into a pET expression vector that has been cut with Nde I and Xho I in preparation for insert ligation. After the *Hs*purL insert has been successfully cloned into a pET expression vector, target protein expression induction experiments will have to be conducted as described previously in this work. Once optimal conditions for the target protein expression is determined, purification of the *Homo sapiens* form of FGAR amidotransferase should be completed, using either of the nickel-ion affinity chromatography procedures described previously in the text.
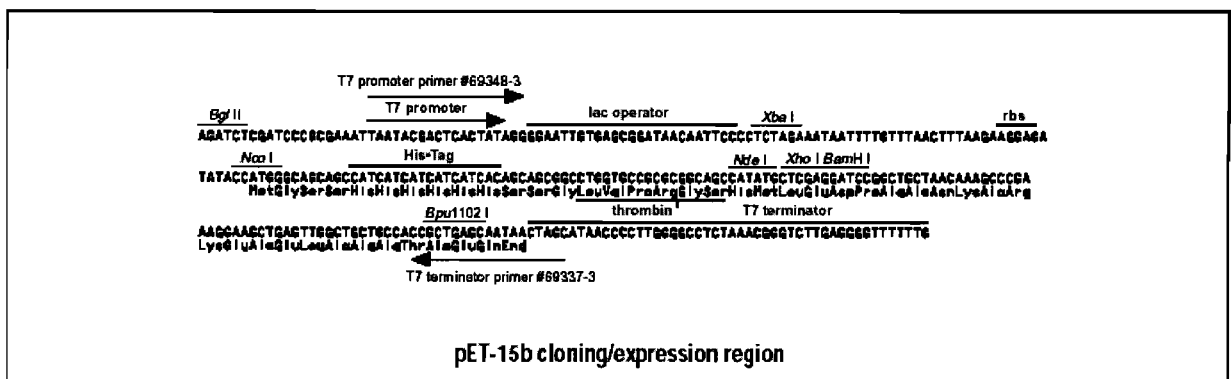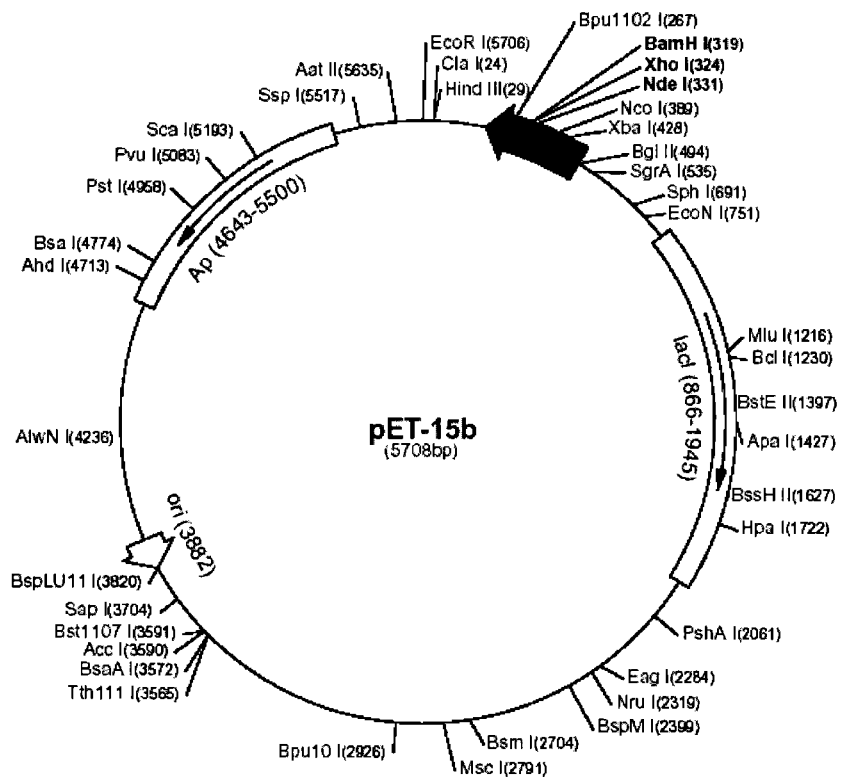
Appendix:

Figure 1:

pET-15b Vector

The pET-15b vector (Cat. No. 69661-3) carries an N-terminal His•Tag® sequence followed by a thrombin site and three cloning sites. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below.

pET-15b sequence landmarks

T7 promoter	463-479
T7 transcription start	452
His•Tag coding sequence	362-380
Multiple cloning sites	
(NdeI - BamH I)	319-335
T7 terminator	213-259
lacI coding sequence	(866-1945)
pBR322 origin	3882
bla coding sequence	4643-5500

pET-15b (5708bp)

Restriction sites: EcoR I(5706), Cla I(24), Hind III(29), Aat II(5635), Ssp I(5517), Sca I(5193), Pvu I(5083), Pst I(4958), Bsa I(4774), Ahd I(4713), Ap (4643-5500), Bpu1102 I(267), BamH I(319), Xho I(324), Nde I(331), Nco I(389), Xba I(428), Bgl II(494), SgrA I(535), Sph I(691), EcoN I(751), Mlu I(1216), Bcl I(1230), BstE II(1397), Apa I(1427), BssH II(1627), Hpa I(1722), lacI (866-1945), PshA I(2061), Eag I(2284), Nru I(2319), BspM I(2399), Bsm I(2704), Msc I(2791), Bpu10 I(2926), ori (3882), BspLU11 I(3820), Sap I(3704), Bst1107 I(3591), Acc I(3590), BsaA I(3572), Tth111 I(3565), AlwN I(4236)

pET-15b cloning/expression region

Novagen, 2001

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