Chapter 4

PROGRESS TOWARDS AN L,F-TRANSFERASE SELECTIVE FOR A NON-CANONICAL AMINO ACID

The work in this chapter was designed in collaboration with Kostya Piatkov and Tae Hyeon Yoo.

Abstract

Post-translational modifications of proteins present opportunities for chemists to subvert natural cellular processes for the introduction of chemical handles with which targets can be assayed, tracked or identified. The *E. coli* enzyme leucyl, phenylalanyl-tRNA protein transferase (L,F-transferase) is known to append a leucine or phenylalanine residue to the N-termini of proteins bearing arginine or lysine residues at that position. The permissivity of the enzyme *in vitro* has been demonstrated using both chemically and enzymatically aminoacylated tRNA substrates to deliver non-canonical amino acids for the modification of purified proteins and peptides. However, modification with non-natural amino acids has not yet been achieved *in vivo*. The high intracellular concentrations of leucyl- and phenylalanyl-tRNA as well as the preference of the L,F-transferase for its natural aminoacyl-tRNA substrates hinders the *in vivo* introduction of non-canonical amino acids. In order to overcome these limitations, a transferase that is selective for a non-canonical amino acid over its natural substrates, leucine and phenylalanine, must be designed. An experimental strategy using negative selection for antibiotic resistance and a positive screen using flow cytometry has been designed to identify transferase mutants with increased affinity towards the non-canonical amino acid *p*-ethynylphenylalanine. Conditions for synthesis of a saturation mutagenesis library of transferase mutants have been optimized and the strains necessary for the negative screening have been created.

4.1 Introduction

Proteins are modified post-translationally in many different ways. These modifications signal changes in protein activity and contribute to the diversity of protein activity in a cell at any given time. Phosphorylation, methylation, biotinylation, and proteolysis are some of the common post-translational events. These fundamental chemical changes in protein structure represent exciting territory where chemistry can be used to elucidate biological processes.[1-4] Futhermore, the inherent ability of enzymes to effect these changes can be exploited and modified to allow the introduction of novel moieties into proteins both to study biological processes and to create new bioconjugates.

As part of the N-end rule pathway in *E. coli*, the L,F-transferase post-translationally targets its protein substrates for degradation by the ClpAP protease through the N-terminal addition of leucine or phenylalanine.[5] Native protein substrates of the L,F-transferase in the N-end rule pathway have not yet been identified. Ideally, a method by which the L,F-transferase modifies its protein substrates with a reactive and bioorthogonal amino acid would allow the enrichment and isolation of its very low-concentration substrates through chemical tagging and affinity chromatography. The method of bio-orthogonal non-canonical amino acid tagging (BONCAT) has been demonstrated as a tool for the proteomic identification of newly synthesized proteins in eukaryotic cells using the ribosomal introduction of reactive non-canonical amino acids.[6] The extension of this technology to elucidate the *E. coli* N-end rule pathway necessitates the implementation of an *in vivo* system that allows L,F-transferase-mediated attachment of non-canonical amino acids onto N-end rule pathway substrates. The sensitivity of the method is dependent on the development of two separate components: a

mutant L,F-transferase selective for Etf and a tRNA that is translationally inactive. In order to identify a variant of the L,F-transferase that is selective for Etf, a library of cells containing mutant transferases will be screened first for the lack of activity towards leucine or phenylalanine and then selected for positive activity towards Etf. The identification of a translationally inactive tRNA will be the focus of future work in the laboratory. Moreover, the development an *in vivo* system will also allow the production of N-terminally modified proteins for bioconjugate applications, such as protein therapeutics.[7]

4.1.1 Selection Design

Overview

The L,F-transferase has been shown to utilize a variety of natural amino acids, as well as non-canonical amino acids *in vitro*.[8-10] However, the affinity of the transferase for its primary leucyl-tRNA substrate and the relatively high concentration of leucyl-tRNA in *E. coli* inhibits the use of non-canonical amino acids by the L,F-transferase *in vivo*. In order to circumvent this issue, we intend to create a transferase that transfers *p*-ethynylphenylalanine (Etf) from a *Saccharomyces cerevisiae* $tRNA^{Phe}$ better than the natural substrates, leucine and phenylalanine. We are interested in both increasing affinity of the transferase towards Etf and decreasing its affinity towards its natural substrates. In order to address both of those goals, a two-part selection process has been designed and is illustrated in Figure 4-1. We will take advantage of the N-end rule pathway in which the L,F-transferase participates for both steps of the selection method. A mutant *S. cerevisiae* PheRS capable of charging Etf has been developed for the site

specific introduction of *p*-bromophenylalanine and other tryptophan analogs into proteins in *E. coli*.[11, 12] This synthetase and its cognate $tRNA^{Phe}$ will be used to deliver Etf to the transferase during the selection process.

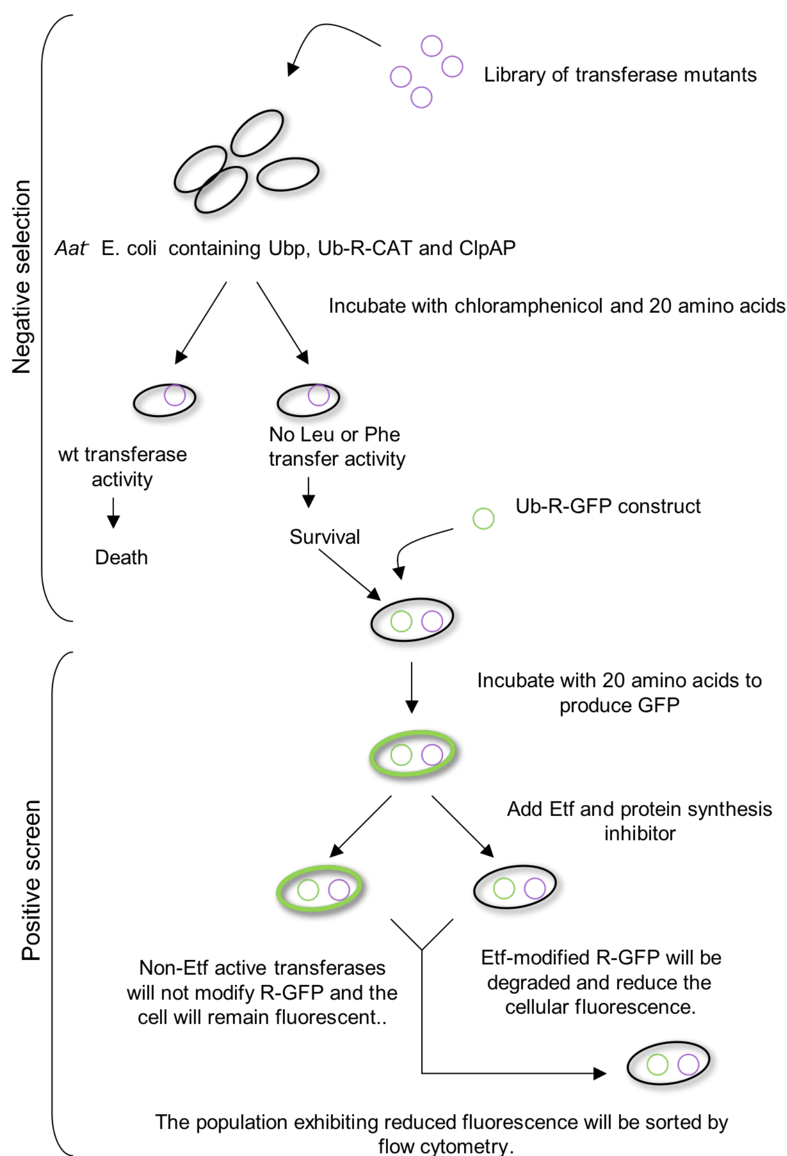

Figure 4-1. Design strategy for the identification of Etf-specific transferase mutants.

Negative selection against wild-type transferase activity

The first part of the strategy is a negative selection against the transfer of the natural aminoacyl-tRNA substrates to a reporter protein conferring antibiotic resistance, chloramphenicol acetyl-transferase (CAT). This reporter protein will be produced as a ubiquitin fusion to allow generation of an arginine N-terminal residue using the ubiquitin protease method pioneered by Varshavsky and coworkers.[5, 13] The ubiquitin protease will remove the N-terminal ubiquitin from the fusion protein, releasing an arginyl-CAT (R-CAT) protein. When active L,F-transferase is present, R-CAT will be tagged for degradation, and the cell will be sensitive to chloramphenicol (Figure 4-1). Without active transferase, the R-CAT construct will be stable and the cell will survive exposure to chloramphenicol. The Ub-R-CAT construct has been introduced into the genome of the *E. coli* strain KPS54, and complimentary strains containing negative and positive control constructs Ub-F-CAT and Ub-DR-CAT (KPS53 and KPS55, respectively) were also obtained from the Varshavsky laboratory. Ub-F-CAT will always be degraded by ClpAP while Ub-DR-CAT will never be degraded regardless of the activity of the L,F-transferase. The ubiquitin protease from *Saccharomyces cerevisiae* has also been inserted into the genome, under control of a strong constitutive promoter. The wild-type L,F-transferase gene, *aat*, was removed from the genome of the strain KPS53 by chromosomal recombination to produce a host strain for identification of transferase mutants. This negative selection step will remove wild-type and mutant transferases that will transfer leucine and phenylalanine (Figure 4-1).

Positive screen for Etf transfer

After the negative selection, surviving cells will be transformed with a low-copy number plasmid containing a constitutively expressed ubiquitin-arginine-green fluorescent protein fusion (Ub-R-GFP). This screen relies on degradation of the R-GFP substrate protein after modification by Etf. Cells whose preliminary GFP fluorescence is reduced through the expression of mutant transferases and Ub-R-GFP will be separated from the highly fluorescent population by flow cytometry (Figure 4-1). A variant of wild-type GFP, GFP6, was used to allow greater susceptibility to proteolysis at 37°C.[12, 14] Two control constructs encoding fusion proteins will also be produced. One control results in a stabilized GFP (Ub-DFR-GFP) that will never be degraded by the N-end rule pathway and conversely, the other control produces a destabilized GFP (UB-FR-GFP) that will always be degraded.

The chaperone protein that upregulates ClpAP activity and physically interacts with N-end rule substrate degrons is ClpS, which has been shown to have degenerate recognition for N-terminal phenylalanine, leucine, tryptophan, and to a lesser extent, tyrosine.[15, 16] Based on the similarity of the ClpS recognition with the L,F-transferase aminoacyl-tRNA recognition, we assume that transfer of Etf to the N-terminus of R-GFP will lead to its recognition by ClpS and subsequent degradation by ClpAP. Addition of a protein synthesis inhibitor at the same time as Etf will reduce the alteration of cellular fluorescence through the ribosomal incorporation of Etf into the Ub-R-GFP construct.

4.1.2 Design of mutant L,F-transferase libary

In a survey of single-alanine mutants of the L,F-transferase for leucine and phenylalanine activity, the T194A mutation was shown to have reduced leucine transfer while retaining activity towards phenylalanine.[17] This mutant L,F-transferase was chosen to serve as the base construct for the generation of a saturation mutagenesis library due to its inherent selectivity towards phenylalanine. The four residues selected for mutation were chosen through examination of the crystal structure of the L,F-transferase complexed with the aminoacyl analog puromycin (RSCB file 2DPS).[17] A 5 Å radius from the methoxyphenyl ring was used to identify potential mutation sites. Residues identified as participating in peptidyl transfer, Q188 and D186, or in protein substrate binding, E156, were not considered for mutation.[18] Four sites, M144, M158, L170, and I185 (Figure 4-2), were chosen due to their proximity to the phenyl ring of the substrate analog and were randomized by PCR gene assembly using degenerate NNK codons.

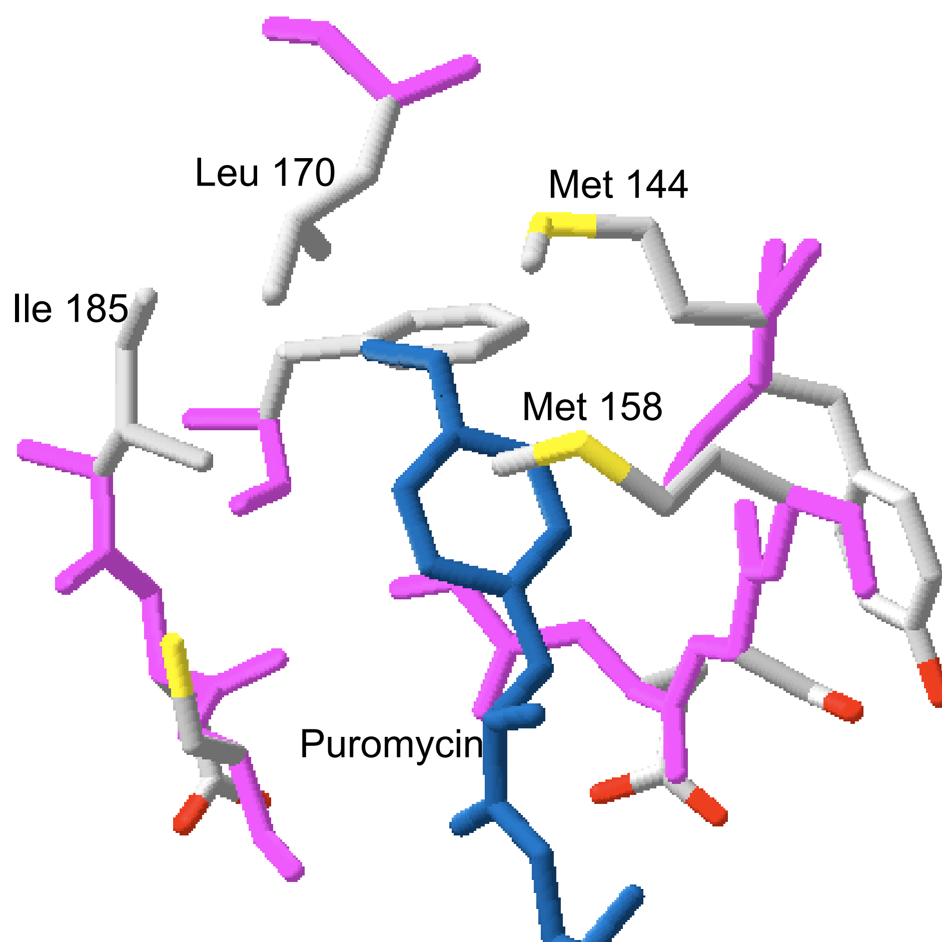

Figure 4-2. Binding pocket of the *E. coli* L,F-transferase with puromycin (blue) bound. Residues within 5 Å of the methoxyphenyl ring of puromycin are shown. The backbone atoms are shown in pink. The labeled residues, M144, M158, L170, and I185, were chosen for saturation mutagenesis to create a library of mutant transferases. Figure produced in Swiss PDB-Viewer.

4.2 Materials and Methods

4.2.1 Materials

p-Ethynylphenylalanine (Etf) was synthesized as previously described.[19] All enzymes were purchased from New England Biolabs. All chemicals were purchased from Sigma-Aldrich and used without further purification. Oligonucleotides were purchased as noted below. DNA sequencing was performed by Laragen, Inc. Vectors pQE70 and pRep4 were purchased from Qiagen.

4.2.2 Introduction of mutant tRNA into pQE9-IK-GFP-yFRS

The *S. cerevisiae* $tRNA^{Phe}$ gene was codon-optimized for *E. coli* and DNAworks was used to create primers for PCR assembly (Table 4-1). The promoter for the *E. coli* $tRNA^{Phe}$ gene, *pheU,* was used as the promoter for the tRNA gene from *S. cerevisiae*. The *pheU* terminator was amplified from genomic DNA using primers pheUtermF and PheUtermR (Table 4-1). After PCR assembly from the six tRNA_prom primers, the full length product was digested with EcoRI and ligated together with the amplified *pheU* terminator. The entire promoter-tRNA-terminator construct was then digested with SacII and ligated into pQE9_GFP6_yFRS_lacI[12] to create pQE9_GFP6_yFRS_ytRNAwt. The orientation of the tRNA insertion was determined through both sequencing and restriction digestion.

4.2.3 Construction of pQE30Aat and pQE70_MetSAat

The *aat* gene was amplified from genomic *E. coli* DNA as described[20] and ligated into pQE30 digested with BamHI to form pQE30Aat. The T194A mutation of *aat*

was inserted by QuickChange™ site-directed mutagenesis using primers AatQC_T194Afor (5'GTC CTT AAC GAT CAC GCT GCA TCG CTT GGT GCC TGC-3') and AatQC_T194Arev (5' GCA GGC ACC AAG CGA TGC AGC GTG ATC GTT AAG GAC-3').

A construct containing a constitutively expressed *aat* gene was created by amplification of the *metG* promoter region from pAJL-20[21] using primers MetSpF and MetSp_AatR and amplification of a T194A *aat* mutant from pQE30Aat using primers MetSp_AatF and AatR_Nhe1, followed by PCR assembly of the two fragments. The full length product was then digested with *AatII* and *NheI* prior to ligation into pQE70 to form pQE70_MetSAat.

4.2.4 Construction of pQE9-MetSAat_yFS_tRNAwt

The final vector to be used in the screening of transferase mutants contains the yeast phenylalanyl-tRNA synthetase, a wild-type yeast phe-tRNA, and a copy of the MetSp_Aat gene construct. The vector described above, pQE9_GFP6_yFRS_tRNAwt, was digested with HpaI and PciI, its ends blunted using the Klenow fragment of DNA polymerase, and ligated back together to remove the copy of *lacIq* and reduce the size of the vector. This modified pQE9_GFP6_yFS_tRNAwt was then digested with AatII and NheI to remove the inducible GFP and to allow the insertion of the AatII / NheI fragment from pQE70_MetSAat, creating the final vector, pQEMetGp_Aat_yFrS_ytRNAwt (Figure 4-3).

4.2.5 PCR assembly of Ub-R-GFP6

The *metG* promoter region was amplified from pAJL-20[21], ubiquitin was amplified from pUbR-βgal[5], and GFP6 was amplified from pQE9_GFP6_lacI_yFrS. The primers used to generate the three fragments are given in Table 4-3. Each fragment was gel-purified and then the whole construct assembled using PCR. The final construct was amplified with primers MetSpF and GFPHindIIIR, digested with AatII and HindIII, and inserted into pQE70. Control constructs MetGp-Ub-FR-GFP6 and MetGp-Ub-DFR-GFP6 will be created with the same method.

4.2.6 Library assembly

A library of *aat* mutants was constructed using saturation mutagenesis of the MetSp_Aat gene construct in pQe70_MetSAat. The primers used for library assembly are given in Table 4-2. Three rounds of PCR were required to create the library due to the close proximity of the four mutation sites. The first round of PCR introduced mutations at residue 158 using primer pairs: PQe3260F / M158NNKF-r and M158NNKF / PQe500 to produce individual fragments which were then assembled and amplified with pair pQe3260F/pQe500R to form the 1.2 Kb construct. A second round of assembly with degenerate primers introduced mutations at residue position 185 using primer pairs PQe3260F / I185NNKF-r and I185NNKF / PQE500R. The resulting mixture was amplified as above and gel purified. The amplified mixture was used as template for the final round of mutagenesis. Degenerate PCR primers were used to add the two final saturation mutagenesis sites in the cassette at positions M144 and L170. Three separate reactions were required, using primer pairs, pQe3260F / M144NNKF-r, M144NNKF /

L170NNKF-r, and L170NNKF / pQe500R. All three fragments were gel purified and used in an assembly PCR to make the complete 1.2 Kbp construct. This amplified construct was purified and digested with *AatII* and *NheI* for ligation into pQeMetGp_Aat_yFRS_ytRNAwt.

4.2.7 Strain production

Strains KPRC1, KPRC2, and KPRC3 were constructed by removing the *aat* gene from strains KPS53, KPS54, and KPS55, respectively, using the Datsenko and Wanner chromosomal recombination method.[22] Primers used for the removal of *aat* were AatpKDlongF(5'GCTATTATTGAACGATCCGACTTGCGTGGAGTTTTGCGGTCAGTGTAGGCT GGAGCTGCTTC-3') and AatpKDR (5'-CCGCAATTATAACACTCTGGGGAGAAATGTGCCGAA AACATTCCATATGAATATCCTCCTTA-3').

Table 4-1. Primers for assembly of the yeast tRNA gene cassette

Sequence Name	Sequence (5'-3')
tRNAprom1F	GAA CCG CGG CGA TTT GCC GCA ATC TTA A
PheUtermF	CAC AGA ATT CGC ACC AAA TTC ATA TAA ACG G
PheUtermR	GAG TTC CGC GGA CGA CAT TTC ACG TCA G
T7_ytRNA_F	TAA TAC GAC TCA CTA TAG CGG ATT TAG C
yTRNA_R	TGG TGC GAA TTC TGT GG
tRNAprom5F	GAT TAC GGT TTA ATG CGC CCC GTT GTG CGG ATT TAG CTC AGT TGG
tRNAprom6R_whole	ATC TTC AGT CTG GCG CTC TCC CAA CTG AGC TAA ATC CGC ACA ACG
tRNAprom2R	TCA GTA AAA GCG ATT CAA CTG CTT AAG ATT GCG GCA AAT CGC
tRNAprom3F	GCA GTT GAA TCG CTT TTA CTG AAA TTA GGT TGA CGA GAT GTG CA
tRNAprom4R	GGG CGC ATT AAA CCG TAA TCT GCA CAT CTC GTC AAC CTA ATT

Table 4-2. Primers for the synthesis of the transferase library

Name	Sequence (5'-3')
MetSpF	GGA TCC GAC GTC CTA GCT CTA GAG ACG
MetS_AatF	GAA GTA ATG CCT ACT ATG CGC CTG GTT C
MetS_AatF-r	GAA CCA GGC GCA TAG TAG GCA TTA CTT C
Aat_NheR	GAA TTC GCT AGC TCA TTC TTG TGG
M158NNKF	CTA TTT TGT GGC GAG TCC NNK TTC AGC CGG ATG GG
M158NNKF-r	CCC ATC CGG CTG AAM NNG GAC TCG CCA CAA AAT AG
M144NNKF	GAG CTT GTC GGC GGT NNK TAC GGC GTG GCC CAG
M144NNKF-r	CTG GGC CAC GCC GTA MNN ACC GCC GAC AAG CTC
L170NNKF	GCG TCT AAA ACG GCG NNK CTG GTA TTC TGT GAG
L170NNKF-r	CTC ACA GAA TAC CAG MNN CGC CGT TTT AGA CGC
I185NNKF	CAT GGC GGT AAG CTT NNK GAC TGC CAG GTC C
I185NNKF-r	GGA CCT GGC AGT CMN NAA GCT TAC CGC CAT G
pQe3260F	GGG TTA TTG TCT CAT GAG CG
PQe500R	ATT CCG GAT GAG CAT TCA TCA G

Table 4-3. Primers for the assembly of MetGp-Ub-GFP constructs

Name	Sequence (5′-3′)
MetSp-UbF	GAA GTA ATG CCT ACT ATG CAA ATT TTC GTC
MetSp-UbF-r	GAC GAA AAT TTG CAT AGT AGG CAT TAC TTC
UB-R-linkF-rev	CAC GCC GGT CAC CAG TTC TTC GCC TTT GCT ACG ACC ACC TCT TAG CCT TAG C
R-link-GFP-F	CGT AGC AAA GGC GAA GAA CTG GTG ACC GGC GTG ATG AGT AAA GGA G
GFP_HindIII-F-rev	GCT AAT TAA GCT TGG CTG CAG
Ub-DFR-linkF	GCT AAG GCT AAG AGG TGG TGA TTT TCG TAG CAA AGG CGA AGA ACT GGT GAC CGG CGT G
Ub-DFR-link-rev	CAC GCC GGT CAC CAG TTC TTC GCC TTT GCT ACG AAA ATC ACC ACC TCT TAG CCT TAG C
Ub-FR-linkF	GCT AAG GCT AAG AGG TGG TTT TCG TAG CAA AGG CGA AGA ACT GGT GAC CGG CGT G
Ub-FR-link-rev	CAC GCC GGT CAC CAG TTC TTC GCC TTT GCT ACG AAA ACC ACC TCT TAG CCT TAG C
pQeNheR	CCA AGC TAG CTT GGA TTC TCA CC
MetSpNheF	GGA TCC GCT AGC CTA GCT CTA GAG ACG

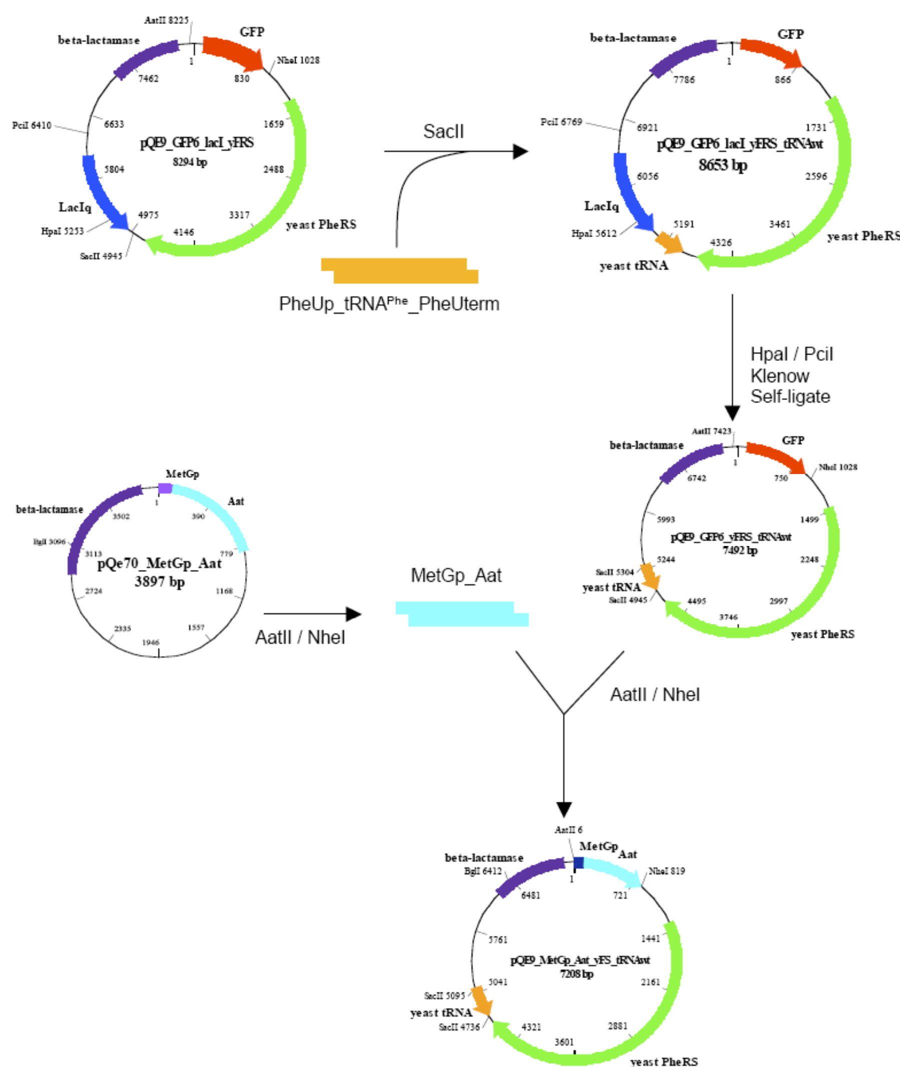

Figure 4-3. Cloning diagram for the final vector. Yeast tRNAPhe is assembled with the pheU promoter and terminator from *E. coli* and inserted into the SacII site of pQE9_GFP6_lacI_yFRS to create pQE9_GFP6_lacI_yFRS_tRNAwt. The *lacI* gene was removed by the digestion of pQE9_GFP6_lacI_yFRS_tRNAwt with HpaI and PciI, followed by blunting with Klenow, and finally, self-ligated to form pQE9_GF6_yFRS_tRNAwt. MetGp_Aat from pQE70_MetGp_Aat was inserted between the AatII and NheI sites of pQE9_GFP6_yFRS_tRNAwt to form the final vector, pQE9_MetGpAat_yFRS_tRNAwt.

4.3 Results

4.3.1 Production of aat^{-} *stains*

E. coli strains containing Ub-CAT fusion proteins and ubiquitin protease were obtained from Kostya Piatkov in the Varskavsky laboratory (Caltech). The three strains, KPS53, KPS54, and KPS55 contain UB-F-CAT, UB-R-CAT, and UB-DR-CAT, respectively. The *aat* gene of each of the strains was removed by chromosomal recombination as verified by single colony PCR.[22] The genotype of the KPS54 derivative was further verified by the stabilization of R-CAT due to the removal of the *aat* gene.

4.3.2 Creation of the final vector, pQEMetSAat_yFS_ytRNAwt

The final construct for the selection process must contain the transferase gene under a constitutive promoter, the T415G mutant yeast PheRS gene, and a wild-type yeast tRNA^{Phe} gene. First, the *S. cerevisiae* tRNA^{Phe} was assembled with the *E. coli* tRNA^{Phe} promoter, *pheUp*, and then ligated to the *pheU* terminator to form the tRNA cassette. This 300bp cassette was then inserted into a SacI site in a vector containing the yeast PheRS to form pQE9_GFP6_yFRS_ytRNAwt.

The natural promoter for the *E. coli* MetRS, *metGp* was chosen to control the transferase gene due to its constitutive expression and strength.[23] The *metGp* promoter and the T194A mutant L,F-transferase were PCR-amplified and ligated into pQE70 at the AatII and NheI restriction sites to form pQE70_MetSAat (Figure 4-2). The MetGp_Aat cassette was inserted into pQE9_GFP6_yFRS_ytRNAwt in place of the inducible GFP6 gene at the *AatII* and *NheI* restriction sites.

4.3.3 Assembly of the MetGp-Ub-R-GFP cassette

For the positive screen, a constitutively expressed GFP6 will be used to identify mutant transferases that will efficiently charge Etf. The GFP construct was created by individual amplification of the *metG* promoter, ubiquitin and GFP6 before assembly of the final cassette. A linker region between the ubiquitin and the GFP was inserted to ensure that the N-terminus of GFP was accessible for modification by the L,F-transferase.[15] The 900 bp cassette, shown in Figure 4-4a, was inserted into pQE70 with AatII and HindIII. In order to insert the cassette into pREP4, the metG*p*-Ub-R-GFP construct will be amplified with primers pQMetSNheF and pQeNheR for insertion at the single NheI site of pREP4.

4.3.4 Library Synthesis

The library of mutant L,F-transferases was produced through three rounds of mutation introduction by assembly PCR. The three rounds were required by the proximity of the four chosen mutation sites, which are each approximately 45 base pairs apart. The first round of PCR randomizes position 158 in the gene and the second round randomizes position 185. In the final round, three PCR reactions result in the addition of two mutation sites at position 144 and position 170. Figure 4-4b shows the result of amplification after round 2 of the mutagenesis. Once created, the library was digested with *AatII* and *NheI*, and test ligations performed with similarly digested vector pQeMetSAat_yFRS_ytRNAwt. The best yield of transformants arose from a 1:4 ratio of vector to insert concentration.

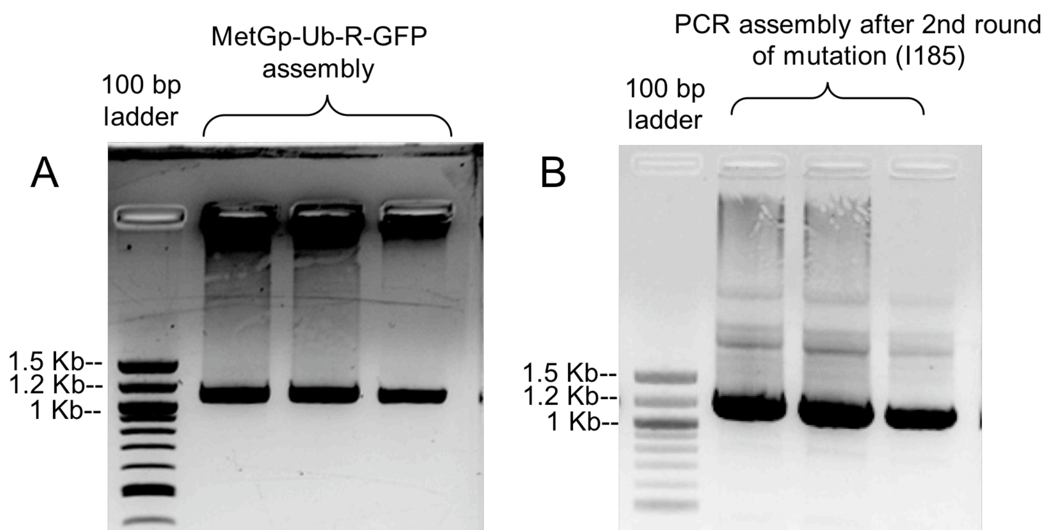

Figure 4-4. PCR assembly of MetGp-Ub-R-GFP and the transferase library. A) Assembled MetGp-Ub-R-GFP cassette is shown on a 1.5% agarose gel. The expected 1.1 Kb product is observed. B) Amplification of the transferase gene after the insertion of mutations at a second location, residue I185. The 1.2 Kb product will be used as template for the introduction of the last two randomized codons.

4.4 Conclusions

The strains and vectors required for the identification of a mutant transferase selective for *p*-ethynylphenylalanine have been synthesized. The ubiquitin-arginine-GFP fusion cassette has been created and will be inserted into pREP4. The two control cassettes, UB-FR-GFP and UB-DFR-GFP must also be assembled and placed in pREP4. Conditions for the ligation of the library into the final vector have been tested and an optimal vector to insert ratio has been identified. Large-scale ligation of the library into the final vector can now be undertaken. The range of chloramphenicol resistance for the

three host strains must be determined before proceeding to the first negative selection. Despite a few minor cloning steps left for control constructs, the library has been assembled and will soon be ready for the selection of *p*-ethynylphenylalanine-specific mutant transferases. Once a selective transferase is identified, it can be used for the identification of natural protein substrates of the L,F-transferase-mediated N-end rule pathway in *E. coli*.

4.5 References

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