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Investigations into the Molecular Mechanisms of Bacterial Pathogen-Host Interactions: Construction of a Dual Plasmid System for Incorporation of Unnatural Amino Acids into *Pseudomonas syringae* pv. tomato DC3000

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“Investigations into the Molecular Mechanisms of Bacterial Pathogen-Host Interactions:
Construction of a Dual Plasmid System for Incorporation of Unnatural Amino Acids into

Pseudomonas syringae pv. tomato DC3000”

by

Scott D. Raber

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Under the Supervision of Professor Jiantao Guo

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Investigations into the Molecular Mechanisms of Bacterial Pathogen-Host Interactions:
Construction of Dual Plasmid System for Incorporation of Unnatural Amino Acids into

Pseudomonas syringae pv. tomato DC3000

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University of Nebraska, 2015

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A dual plasmid system for the incorporation of unnatural amino acids into plant pathogen, *Pseudomonas syringae* pv. tomato DC3000, has been designed. This invention is expected to allow (a) mutations of proteins synthesized by the bacterium, *P. syringae* pv. tomato DC3000, that can capture molecular targets, especially for such modified proteins secreted by the phytopathogen into the host plant cells of *A. thaliana* and *S. lycopersicum*, (b) expression of biological probes in the bacterial species to monitor changes in redox, nutritional, and other small molecule states over pre-, post- and *in situ* disease stages, and (c) secretion of such biological probes into an infection host organism to monitor intracellular changes of redox, nutritional, and small molecule states in infected host. This plasmid system will allow proteins to feature unnatural amino acids in substitution for selected original residues by site-specific alteration of the protein sequence to the TAG stop codon for recognition exogenous and orthogonal TAG-suppressing tRNA that has been charged with the unnatural amino acid by its cognate tRNA synthetase (RS). Because the suppressor tRNA and its counterpart synthetase are designed to only interact with each other, proteins expressed by the bacterial species containing this orthogonal tRNA/RS pair will enable *in vivo* production of proteins featuring specialized disease-based monitoring capabilities. In addition, this plasmid system is expected to be compatible not only with other pathovars of the *P. syringae* genus, but also other phytopathogens and soil

bacteria, thereby significantly enhancing the researcher's ability to understand the role and response of these microorganisms within their environment.

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I wish to give further recognition to the troves of scientific and popular literature that offer insight, inspiration and off-hand thinking, for without this information there would be very slow progress when one is investigating and inventing something new.

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LIST OF ABBREVIATIONS

DC, DC3000	<i>P. syringae</i> pv. tomato DC3000
GFP	green fluorescent protein
Pyl	pyrrolysine; pyrrolysyl
Tyr	tyrosine; tyrosyl
RS	tRNA synthetase
UAA	unnatural amino acid
NAA	natural amino acid
BpF	<i>p</i> -benzoyl- <i>L</i> -phenylalanine
AzF	<i>p</i> -azido- <i>L</i> -phenylalanine
<i>Mb.</i>	<i>Methanosarcina barkeri</i>
<i>Mj.</i>	<i>Methanocaldococcus jannaschii</i>
LB	lysogeny broth
KB-T	modified King's B medium with tryptone
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
RT	room temperature
Sec	seconds
Min	minutes
Hrs.	hours
Tet	tetracycline
Gen	gentamycin
Kan	kanamycin
Cm	chloramphenicol
<i>v</i>	vector
<i>i</i>	insert

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Chapter 1 – Introduction and Specific Aims

1.1 General Introduction

Understanding host-pathogen interactions at a molecular level requires ability to identify which proteins or small molecules are involved in the communication within and between the two species. Within the host, for instance, signal pathways for normal growth will become altered once a stress – whether physical, biological, or chemical – has affected the organism. The response to stress must allow the host species to maintain its normal processes while altering the local or systemic regions to remedy and repair the self. An entity that directs all of its resources to stress response and does not maintain normal survival processes will likely suffer and die. Likewise, the pathogen species must keep its own normal processes functioning while it attempts invasion of the host and with the host's response for immunity, the pathogen also becomes stressed. Indeed, disease events could be seen as an aggressive form of communication where both entities share a region of immediate interaction, or front line, behind which both species must organize their secondary preparations and, as the altercation continues, sustain their own bodies in the event that the battle should end. Immediately, the host or pathogen must begin repair and subsequent growth, allowing for reproduction and, if necessary, further defense (or offense) against additional forces. It is not surprising, then, that approaching the disease event with the perspective of warfare should yield tactics (i.e. espionage) for understanding the behavior of the players involved and, especially, their respective mechanisms for battle.

It has been shown (*Hueck, 1998; Ronald, 2010*) that in bacterial diseases of animals or plants, all classes of biological molecules (e.g. DNA and RNA, proteins, lipid membranes, polysaccharides, and various signaling molecules) are involved in this hostile tête-à-tête.

Therefore, the need to understand the chromosomal, transcript, and protein-based responses are important for recognizing the mechanisms of disease, especially, interactive partners in signaling.

Many pathogens that infect animal or plant hosts are able to manipulate their host's cellular activity and structure by secretion of proteins and small molecules into the host cell bodies. The plant pathogen, *Pseudomonas syringae*, secretes proteins through a Type III secretion system – a needle-like apparatus constructed outside the bacteria that creates a portal into the host cell (*Figure 1*). This system allows a direct extracellular tunnel between the bacterial cytosol and the host cytosol such that any secreted proteins, called effector proteins, can be transported quickly into the host cell body and exert effects to modify the invaded species. Effector proteins secreted by this system features a secretion-specific terminus peptide sequence to help the bacterial body properly transport and export the protein into the extracellular apparatus. It is noted that in virulent bacterial species there are different types of secretion systems, classified as Type I, II, III, IV, V, a Type VI system (which secretes into both bacterial and eukaryotic cells) and even a Gram-positive Type VII system (*Tseng, 2009; Russell, 2014*). These secretion methods are different than the more common mechanisms in both pathogenic and non-pathogenic species, such as the Sec-dependent (*Pugsley, 1993*) and Twin-arginine translocation system (*Bronstein, 2005*). Most systems feature an export apparatus for proteins or small molecules to promote survival of the bacterial pathogen in its new host environment. The Type IV system, for instance, allows the *Agrobacterium tumefaciens* to export its *Ti* (tumor-inducing) plasmid into the stalk of the host-plant body, allowing for DNA-based alteration of the host (*Gelvin, 2003*); this method, incidentally, has been exploited to impart resistance in tomato plants from the tobacco mosaic virus, showing that a pathogen's tool-set can also be a useful tool for humans. The Type III system mechanism can likewise be exploited as a useful tool.

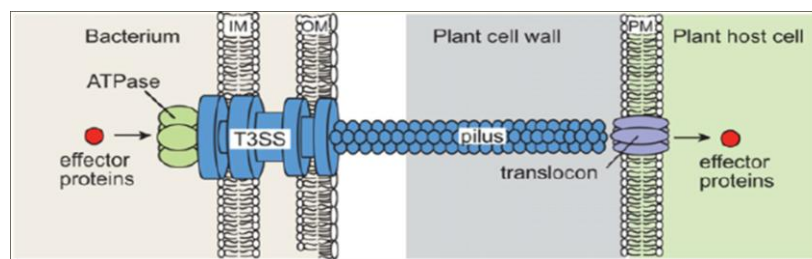


Figure 1: Illustration showing constructed Type III secretion system extending from bacterial pathogen into plant host cell wall. Also depicted is a generic effector protein (*red circle*) passing from invader into infected organism (*adapted from Buttner, 2009*).

One particular strain of the *P. syringae* family is *P. syringae* pv. tomato DC3000 (DC3000), is capable of infecting both its natural host, the tomato plant, and the model plant *Arabidopsis thaliana*. The natural host suffers from bacterial black speck (*Hueck, 1998*), which causes lesions on leaves and fruits of the tomato plant and resulting in lower harvest and storage (*Cuppels, 1986*), while *A. thaliana* infection produces chlorosis, wilting, and subsequent death (*Katagiri, 2002*). Like many other pathogens, DC3000 constructs an effector secretion apparatus after infiltrating the host body and exports macromolecules into the host cell cytosol. The DC3000 Type III secretion system is recorded to secrete at least 40 proteins (*Cunnac, 2009*), including several that aide in building the pathway from pathogen to host (e.g. harpin proteins), and some of which work in concert with host native machinery to shift the host's awareness and growth phase. A number of secreted effectors require post-translational modification from host proteins before being able to cause their intended effects.

Currently, there are many effector proteins with unknown targets and it is therefore important to identify the host substrates for these proteins. Understanding the target of each effector will help to uncover the dynamics of the molecular basis of disease as caused by

DC3000. To aid in understanding this basis, the ability to capture a target *in situ* will be of greatest benefit, as some protein targets may not be expressed except during the infection process. However, it is also useful to pinpoint effector proteins in their post-infection location while still contained within the host cell as this would also shed light on the disease process. A salient methodology to allow for both methods of analysis, as well as studying pathogen-specific events leading to and during infection, involves the incorporation of unnatural amino acids (UAA) into those effector proteins, thereby allowing them to become tools for researchers to study the pathogen-host system in finer detail. For example, covalent binding of an effector to its target(s) through use of a crosslinking amino acid (e.g. phenylalanine derivatives featuring benzophenone or azido substituents; *Figure 2*), would produce an extractable complex from the lysate of the host-pathogen cellular mixture. The effector-target complex can be examined to reveal the protein (or small molecule) counterpart to the bacterial secreted protein. In addition, other amino acids with different functionality could be used for study, as has been shown in recent literature (*Wang, 2003; Ryu, 2006; Liu, 2010; Coin, 2011*).

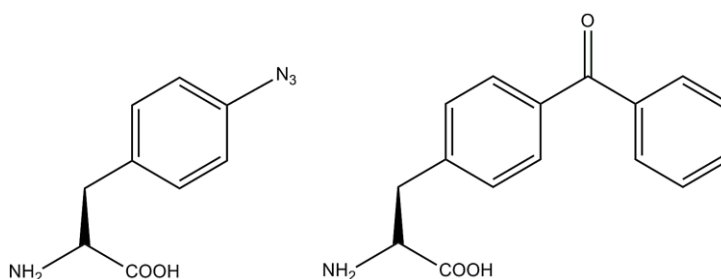


Figure 2: Crosslinking phenylalanine derivatives, *p*-azido-*L*-phenylalanine (AzF; *left*) and *p*-benzoyl-*L*-phenylalanine (BpF; *right*).

The general methodology for producing proteins with unnatural amino acids was developed by the group of Peter G. Schultz in the early 1990's (for review: *Liu, 2010*). The process allows for *in vivo* production of peptides and proteins using uniquely interacting tRNA and tRNA synthetase (RS) pairs, thereby allowing synthesis of polypeptides featuring unnatural (or alternative) amino acids inserted at the point of an introduced stop codon. The TAG stop codon is the most frequently used premature stopping site due to its low frequency in most chromosomes (*Povolotskaya, 2012*). The DNA sequence for the protein of study is altered by site-directed mutagenesis to change, for example, a tyrosine residue's TAT codon to TAG, and attempted translation from mRNA transcript for this mutated protein would produce a truncated (and generally inactive) protein. Selection of the amino acid sequence for modification to a stop codon is dictated by the residue's location in the protein's tertiary structure – to allow for proximity of a target protein – as well as the residue's role in native protein folding. Altering a proline residue to BpF, for instance, would likely reduce chance of proper protein folding even though the surface position of the residue would be the same for protein-protein contact, as proline generally provides for a specific kink in the molecule's folded structure.

After successful codon mutation, a pre-selected TAG-specific exogenous tRNA and its corresponding RS, are then expressed in the organism that produces the mutant protein's nucleotide sequence. Upon recognition of the TAG stop codon by this tRNA, premature polypeptide termination is not engaged, but instead the RS-specific UAA is charged to the exogenous tRNA and the polypeptide sequence is translated to the full-length protein (*Figure 3*).

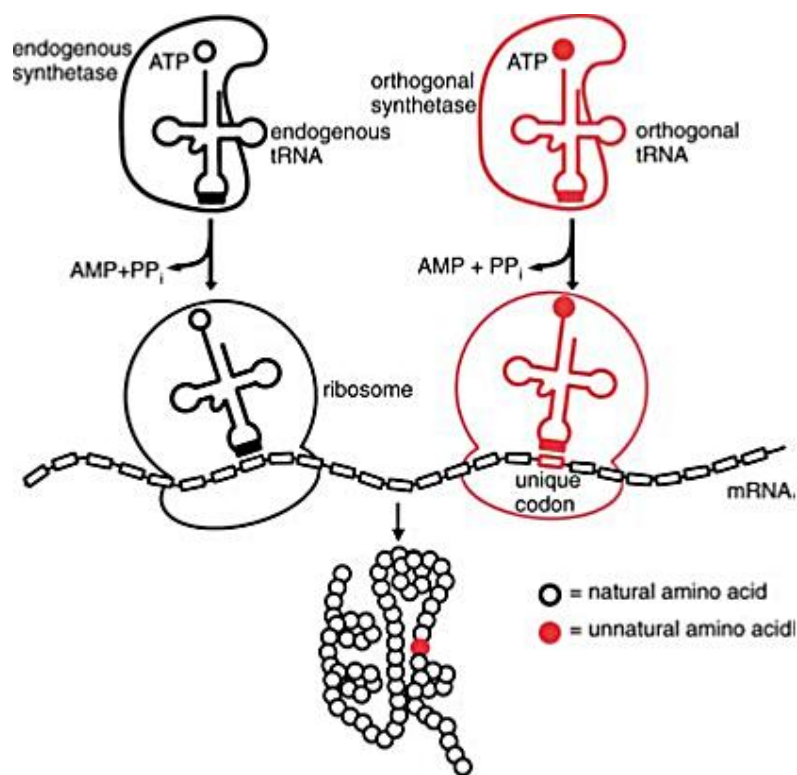


Figure 3: Illustration to show the effect of orthogonal tRNA and synthetase pair for *in vivo* incorporation of an unnatural amino acid (*shown as red circle*) into protein during translation. Left-hand molecular species (*black*) are the native machinery in the organism, while the right-hand species (*red*) are the exogenous and orthogonal tRNA and corresponding synthetase (*retrieved from Wang, 2005*).

1.2 Project Overview and Specific Aims

With UAA-incorporation technology, modified forms of effector proteins can be synthesized within the pathogen and secreted directly into the host cell. This method of *in vivo* capture helps to ensure proper location and targeting of effector proteins. In the event an effector's target is not produced at a high concentration except during the disease state, pathogen-

initiation of infection would promote more available target amount for access and capture. A two-plasmid system has thus been developed to allow for the incorporation of unnatural amino acids into the plant pathogen, *Pseudomonas syringae* pv. tomato DC3000, with the intent of capturing effector targets *in planta*.

A pair of plasmids is used to individually control the transcription of the exogenous tRNA and protein of interest, and, separately, the corresponding synthetase. Both the tRNA and synthetase must be unable to interact with the native DC3000 protein synthesis machinery and so two sets of plasmid pairs were selected from Archaea, as these are not expected to interact with eubacterial machinery (Yanagisawa, 2008; Wang, 2010): *Methanosarcina* spp. which utilize a pyrrolysine-tRNA/synthetase to naturally suppress TAG codons, and the anti-codon mutated tyrosyl-tRNA and mutated synthetase from *Methanocaldococcus jannaschii*. For both sets of plasmid pairs, the tRNA and GFP reporter protein are both on the same plasmids, pBBR1MCS3 (Kovach, 1995), and the synthetase is expressed from pML123 (Labes, 1990). Both of these parent vectors were provided by James Alfano (University of Nebraska-Lincoln).

The pBBR1MCS3 vector (Tet^R; Figure 4) was chosen for housing a GFPuv reporter protein and the exogenous tRNA. The reporter protein shows fluorescence when fully folded and a mutation at position, N149, to TAG stop codon (GAU → TAG) yields premature termination of the translated peptide sequence and thus would produce no full-length or fluorescing protein.

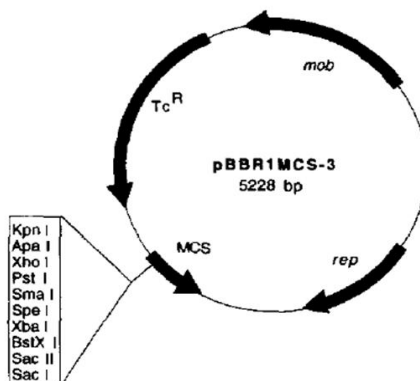


Figure 4: Plasmid map for pBBR1MCS3 vector for tRNA, GFPuv constructs. {Kovach, 1995}

The pML123 vector (Gen^R; Figure 5) from pML10 family is used as the synthetase vector for which several promoters have been positioned upstream the multiple cloning site (Table II, Labes, 1990). Vector pML123 (and pML122) possesses neomycin acetyltransferase promoter and terminator regions to yield strong promotion in soil bacteria, but not *E. coli*. Therefore, pML123 was modified to feature *tac* and *T5* promoters and their corresponding ribosome binding sites in this study, expecting moderate expression in both *E. coli* and *P. syringae* DC3000.

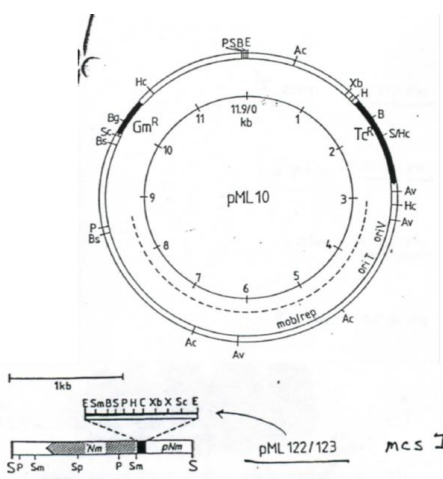


Figure 5: Plasmid map for pML123 vector for synthetase constructs. {Labes, 1990}

1.2.1 *Methanosarcina barkeri* Pyl-tRNA^{CUA} and *Methanosarcina mazei* Pyl-tRNA Synthetase

Several *Methanosarcina* spp. utilize an unusual amino acid, pyrrolysine, and possess unique tRNA and synthetase pairs for incorporation of this amino acid in their biological framework. The native anticodon arm (CUA) for the pyrrolysyl-tRNA recognizes the codon, TAG (Srinivasan, 2002).

This thesis study employs the pyrrolysyl-tRNA (Pyl-tRNA) to recognize the TAG stop codon and a cognate synthetase developed (Yanagisawa, 2008) for incorporation of unnatural amino acids, including a boc-protected lysine, which can be inserted into proteins of study. Investigations involved use of the wild-type cognate pyrrolysine-tRNA synthetase (PylRS), a mutant with higher affinity for boc-lysine (PylRS Y348F), and a mutant evolved to better recognize natural amino acids (PylRS NAA, or PylRS^{NAA}). Pyl-tRNA/RS pair was the first studied in *P. syringae* DC3000, to investigate the tRNA transcription under native DC3000 *tRNA-met-3* promoter and terminator regions. This tRNA expression was the first step in successful

incorporation of UAAs. In addition, this project also utilizes different promoters for the synthetase plasmid (*tac* and *T5*) in attempt to enhance RS expression in *P. syringae* DC3000.

***1.2.2 Methanocaldococcus jannaschii* Tyrosyl-tRNA^{CUA} and Evolved *M. jannaschii* Synthetase**

A dual plasmid system was also designed to feature *M. jannaschii* Tyr-tRNA^{CUA} and cognate synthetase, by which the wild-type tyrosyl-tRNA synthetase (TyrRS WT), and two evolved RS specific for either *p*-benzoyl-*L*-phenylalanine (BpF) or *p*-azido-*L*-phenylalanine (AzF) were analyzed separately. Both the BpF and AzF amino acid derivatives of phenylalanine have been shown to yield successful covalent capture of protein targets (*Chin, 2002; Chin, 2002*).

Further study involved modifying the *M. jannaschii* Tyr-tRNA promoter sequences to alter product transcription. The prokaryotic promoters, *lpp*, an upstream promoter for the *E. coli* outer membrane lipoprotein (*Inouye, 1985*), and *proK*, featured in the *E. coli* prolyl-tRNA codon (*Ryu, 2006*) were chosen for this procedure. Our laboratory possessed vectors which contained the Tyr-tRNA already downstream the *lpp* or *proK* promoters, as well as all tRNA/RS pairs.

Although successful expression of the full-length mutant GFPuv N149TAG can be detected by fluorescence, it is beneficial to confirm reporter protein expression by visualization of proteins by electrophoretic and Western blot membrane development methods. These methods were employed for additional confirmation of achieving unnatural amino acid incorporation via the present invention.

Results and methods for constructing a two plasmid system for successful incorporation of unnatural amino acids into *P. syringae* pv. tomato DC3000 are provided in this thesis.

Chapter 2. Investigation of Promoters for Transcription of Exogenous tRNA and tRNA Synthetase in *Pseudomonas syringae* pv. tomato DC3000.

2.1 Identifying a Native *P. syringae* DC3000 Promoter for Exogenous tRNA

Incorporation of unnatural amino acids into living species requires the addition of an exogenous tRNA and corresponding synthetase that have a high affinity for the amino acid to be incorporated but have little or no affinity to natural amino acids. This selectivity is first acquired by modification of the tRNA and synthetase structures by site-directed mutagenesis and evolution of these molecular species during growth assays in presence of the unnatural amino acid.

However, fine-tuning the tRNA and synthetase is only useful insofar as the molecules can be used within the species of study. Effective expression of these components *in vivo* is required to install unnatural amino acids into proteins of study and for all these processes there must be promotion of the tRNA and synthetase transcripts (1) at an effective rate to meet the demands of the ribosomal machinery synthesizing the nascent peptide chain for any proteins of study, (2) at a level low enough to not create toxic results from these exogenous molecules, (3) as a stable form of tRNA or synthetase that can exist long enough within the cell to allow for the first case, above. Case (2) is especially important if during the selection and evolution process the tRNA or synthetase still interact with endogenous protein translation machinery. Finally, there must be a method for observing the efficacy of tRNA and synthetase production and activity, i.e. Northern blot, protein-based electrophoresis, or reporter protein.

To enable incorporation of unnatural amino acids into *P. syringae* DC3000 by use of exogenous tRNA, the host organism must promote transcription of the tRNA for expression without degradation of the gene product before it is cleaved to maturity. Therefore, this project

required designing a tRNA gene cassette having 5' and 3' regions naturally found in *P. syringae* DC3000.

One promoter sequence expected to be effective in *P. syringae* DC3000 is that for tRNA genes. Initiator tRNA (*Met*) promoters are shown to yield high transcript products for pathogenic bacteria, such as *Mycobacterium tuberculosis* (Vasanthakrishna, 1997) and such promoters have been used to express exogenous tRNA in that same species (Wang, 2010). Although, *P. syringae* DC3000 is not an animal pathogen, it can be assumed that native initiator tRNA promoter and terminal regions would be similarly effective for this plant pathogen. Because stable RNA *in vivo* require (a) a consensus upstream sequence that is regulated to produce an appropriate supply of transcript and (b) the downstream poly(A) tail to provide for recognizable precursor-tRNA that can be processed into the active unit, the expression of an exogenous tRNA within *P. syringae* DC3000 should be successful when using the 5' and 3' regions from native tRNA genes. In addition, control studies in *E. coli* using *P. syringae* DC3000 5' and 3' regions of the initiator tRNA are expected to yield functional products, although likely at a lower level than for the native organism.

Using the *E. coli* gene *metY* as the proto-type for an initiator tRNA in DC3000, nucleotide BLAST of the *E. coli* DH1 methionyl-tRNA, *metY* (NC_017638.1), against the DC3000 genome showed a 93% identity with *tRNA-met-5* (NC_004578.1). It was expected that the native 5' and 3' flanking regions would be sufficient for transcription and processing of any exogenous tRNA. However, as is shown in *Figure 7*, the *met-5* initiator tRNA appears downstream to *tRNA-leucyl-4*, making possible difficulty ensuring the promoter region could be easily amplified via PCR without contamination of unwanted DNA. Therefore, the flanking regions of another methionyl-tRNA, *tRNA-met-3*, from *P. syringae* DC3000 was chosen (*Jiantao*

Guo, UNL) for amplification and tRNA cassette construction. This process was performed using PCR was used to amplify the 5' and 3' regions of *P. syringae* DC3000 *tRNA-met-3* from genomic DNA and these regions were used to flank the *M. barkeri Pyl-tRNA* gene via overlapping PCR. This tRNA cassette was then inserted into the vector containing the GFPuv reporter protein sequence.

```

Score = 120 bits (132), Expect = 2e-23
Identities = 75/81 (93%), Gaps = 0/81 (0%)
Strand=Plus/Plus

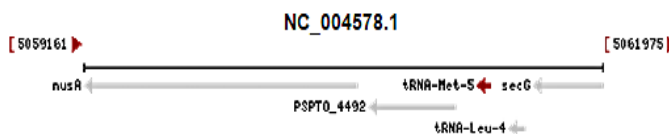
Query 5061287  AAAC TGGT TGC GGGGG CTGG ATT TGA ACC AAC GAC CTT CGG GTT ATG AGC CCC GAC GAG CT 5061346
           |||
Sbjct 3296115  AAAG TGGT TGC GGGGG CCG GAT T TGA ACC GAC GAT CTT CGG GTT ATG AGC CCC GAC GAG CT 3296174

Query 5061347  ACCA AACT GCT CCAC CCC GCG 5061367
           |||
Sbjct 3296175  ACCA GGCT GCT CCAC CCC GCG 3296195

```

Figure 6: Nucleotide BLAST showing 93% homology between *E. coli* DH1 (Subject) and *P. syringae* DC3000 (Query); (<http://www.ncbi.nlm.nih.gov/>)

Sequence: NC_004578.1 (5061291..5061367, complement)



Genomic Sequence: NC_004578.1

[Go to ref](#)

Go to nucleotide: [Graphic](#)

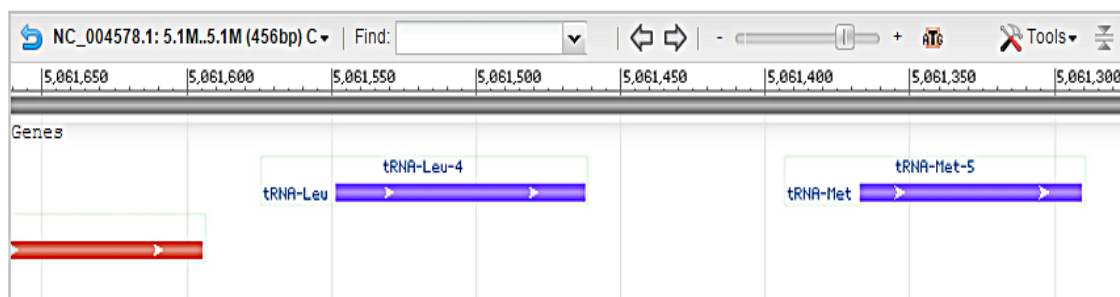


Figure 7: Reference location of *P. syringae* DC3000 *tRNA-Met-5* within genetic region as found within NCBI information site. Image shows *leucyl-tRNA* upstream in same locus and so *tRNA-met-5* was later substituted for *tRNA-met-3*.

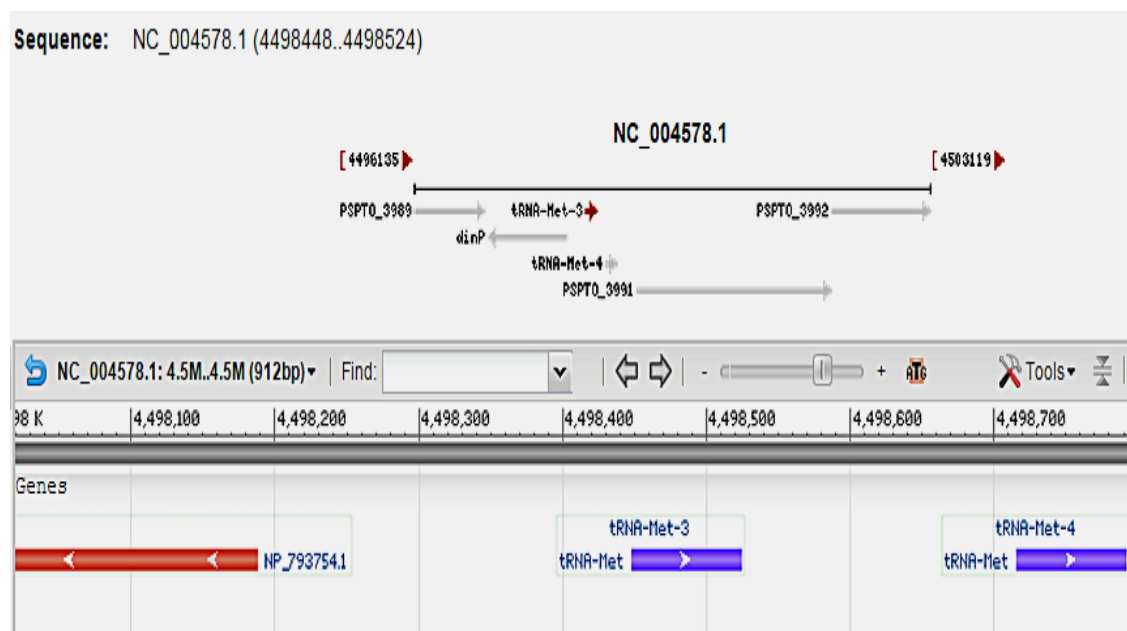


Figure 8: Reference location of *P. syringae* DC3000 *tRNA-Met-3* within the genetic region as found within NCBI information site.

2.2 Developing *tRNA*, *GFPuv* and *Synthetase Plasmids* for Testing *tRNA* Promotion

Plasmids containing the GFP_{uv} reporter protein as wild-type or N149TAG mutant were prepared and, for mutant GFP_{uv}, the Pyl-tRNA gene cassette featuring *P. syringae* DC3000 *tRNA-met-3* promoter region was prepared. The *tRNA-met-3* terminator region provided difficulty in cassette design and so the Pyl-tRNA, upon initial amplification from house vector, was amplified with a designed poly(A) tail to allow for proper *in vivo* processing. It was not observed that strains featuring only the mutant GFP_{uv} N149TAG and any suppressor tRNA give fluorescence results to suggest DC3000 houses TAG stop codon suppressor machinery. This

result is not surprising given the low frequency of TAG in the *P. syringae* DC3000 genome, as shown in *Table 1*, below (Nakamura, 2000). Indeed, it was not expected that any TAG suppression native to DC3000 would cause significant false positive results in studies involving biological probes or crosslinking amino acids, as strains induced with the necessary UAA at 1.0 mM or greater concentrations should promote higher UAA-incorporated mutant proteins rather than natural amino acid positioning in the TAG stop codon site.

The following results are organized to reflect the methods in Chapter 4. Plasmid construct images are shown, followed by initial cell-based fluorescence assays for strains featuring that construct.

Pseudomonas syringae pv. *tomato* str. DC3000 [gbbct]: 5616 CDS's (1855630 codons)

fields: [triplet] [frequency: per thousand] ([number])

UUU 13.0 (24036)	UCU 3.8 (7044)	UAU 9.2 (16997)	UGU 2.5 (4584)
UUC 23.2 (43122)	UCC 9.5 (17703)	UAC 16.3 (30291)	UGC 7.3 (13601)
UUA 2.2 (4173)	UCA 4.5 (8310)	UAA 0.8 (1459)	UGA 1.9 (3506)
UUG 18.0 (33343)	UCG 14.4 (26654)	UAG 0.4 (651)	UGG 13.9 (25795)
CUU 8.8 (16294)	CCU 7.8 (14466)	CAU 9.3 (17209)	CGU 15.5 (28672)
CUC 14.8 (27469)	CCC 10.3 (19027)	CAC 13.6 (25203)	CGC 30.4 (56423)
CUA 2.3 (4261)	CCA 5.9 (10956)	CAA 12.6 (23407)	CGA 4.4 (8216)
CUG 67.4 (125106)	CCG 23.9 (44309)	CAG 32.9 (61112)	CGG 8.4 (15504)
AUU 14.4 (26735)	ACU 5.9 (11000)	AAU 9.5 (17666)	AGU 6.6 (12318)
AUC 32.3 (59851)	ACC 27.3 (50617)	AAC 22.3 (41384)	AGC 22.6 (41855)
AUA 2.9 (5394)	ACA 5.0 (9228)	AAA 15.7 (29051)	AGA 2.0 (3731)
AUG 23.3 (43283)	ACG 12.8 (23792)	AAG 21.8 (40461)	AGG 2.8 (5163)
GUU 10.0 (18519)	GCU 13.7 (25510)	GAU 20.9 (38824)	GGU 17.6 (32575)
GUC 24.1 (44720)	GCC 43.4 (80492)	GAC 33.5 (62138)	GGC 44.5 (82643)
GUA 6.3 (11640)	GCA 16.3 (30329)	GAA 30.5 (56516)	GGA 3.9 (7291)
GUG 30.6 (56694)	GCG 32.5 (60304)	GAG 25.8 (47821)	GGG 10.3 (19182)

Coding GC 59.27% 1st letter GC 63.20% 2nd letter GC 43.16% 3rd letter GC 71.44%

Table 1: Codon analysis frequency in *P. syringae* pv. tomato DC3000. Stop codon frequency is highlighted by red border in upper right-hand corner of table. {Nakamura, 2000 ; retrieved directly from: <http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=223283> (June 6, 2014)}

2.2.1 pBBR1MCS3-GPFuv N149TAG and pBBR1MCS3-GFPuv WT

Reporter protein plasmids were constructed and tested for fluorescence in *E. coli* Genehog, *E. coli* NEB5 α , and DC3000, as single transformants or in presence of exogenous synthetase. Fluorescence data is shown in subsequent sections.

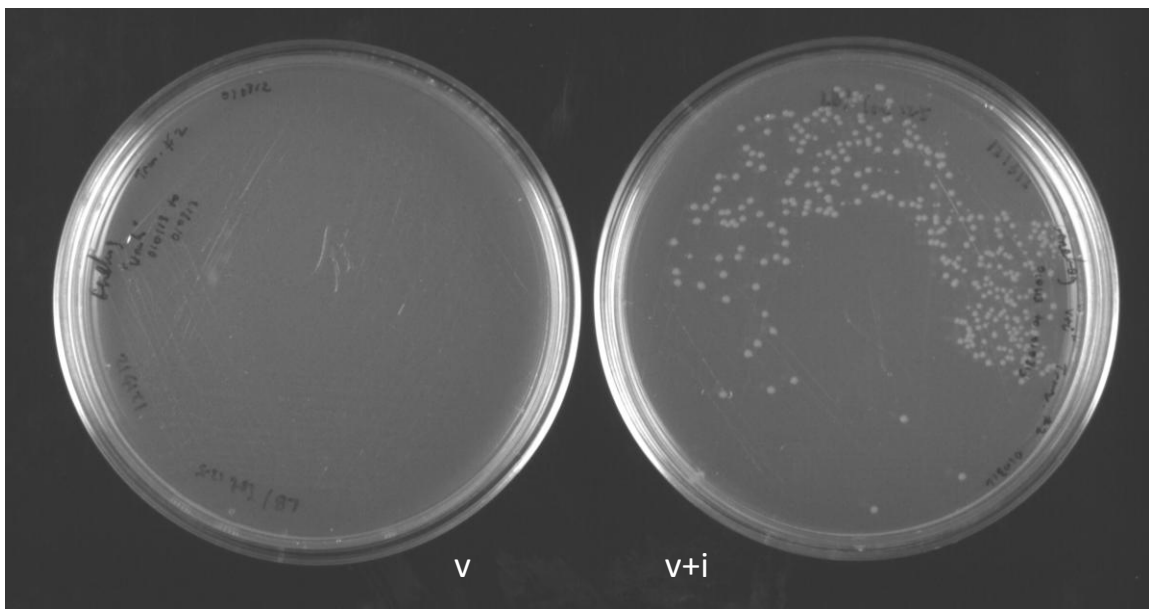


Figure 9: Plated Genehog cells for ligation product from pBBR1MCS (*v-only*; *left*) with GFPuvN149TAG cassette insert (*v+i*; *right*). Results are similar for GFPuv WT ligation.

2.2.2 *pBBR1MCS3-GFPuv N149TAG-Mb. Pyl-tRNA (pSR6.067)*

Use of pBBR1MCS3-GFPuv N149TAG for construction of pSR6.067 containing the Pyl-tRNA allowed for transformation of Genehog and DC3000 wild-type cells. Cells were inoculated to liquid culture and grown in their optimal environments, induced, and later analyzed for fluorescence of cells pellets or culture liquid supernatant.

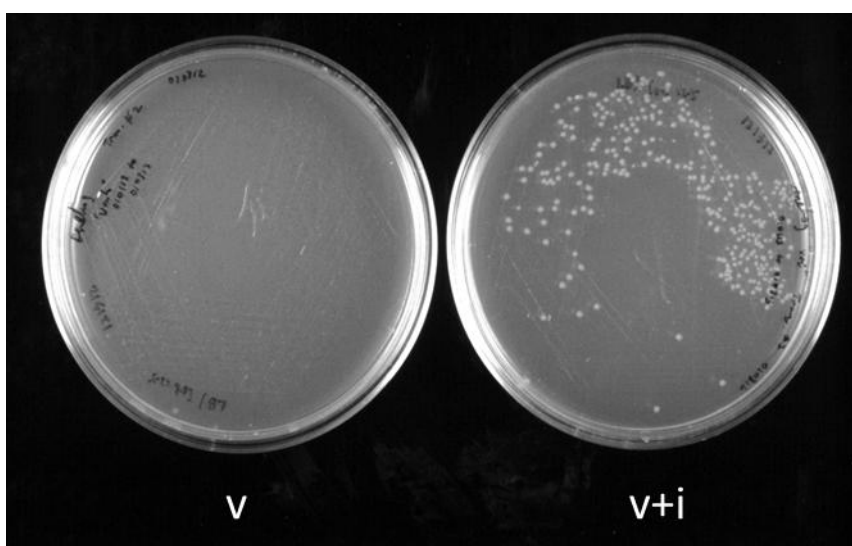


Figure 10: Genehog cells on LB/Tet¹⁰ agar plates for pSR6.067 ligation product. (*Abbrev.: v, vector only control; v+i, vector + Pyl-tRNA insert.*)

2.2.3 *pML123-tac-PylRS WT*

Construction of the synthetase-containing vector involved several steps, including an intermediate plasmid to create a promoter cassette featuring the *tac* promoter, as vector, pML123 contains the strongly-promoting neomycin promoter and as the *tac* promoter is useful in both *E. coli* and *P. syringae* (Labes, 1990), using this latter promoter offered a good positive control during plasmid development in both strains. Vector was co-transformed into Genehog and

DC3000 cells along with either pBBR1MCS-GFPuvN149TAG only, or pSR6.067 (Pyl-tRNA, GFPuvN149TAG). Co-transformed cells were analyzed for fluorescence of cell pellets and liquid culture suspensions.

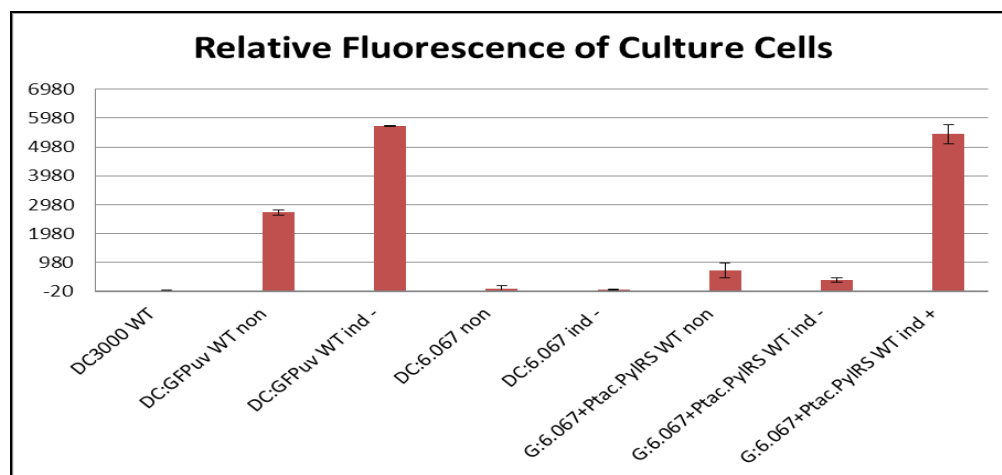
Transformation of DC3000 (DC), by electroporation or Genehog cells (G), by heat shock, with pSR6.067 was successful and subsequent transformation of DC:pSR6.067 with pML123-PylRS (electroporation) or G:pSR6.067 chemical competent cells with pML123-PylRS allowed for inoculation into liquid media and induction of Pyl-tRNA/RS machinery with 0.3 mM IPTG and 5 mM Boc-lysine.

Fluorescence experiments (*Table 2; Graph 1*) show fluorescence only for induced Genehog cells (OD_{600} 0.05) with added boc-lysine (5 mM). Repeated attempts at incubating induced DC3000 cells at room temperature (RT) or 30 °C did not result in fluorescence.

Nevertheless, it was apparent the Pyl-tRNA gene cassette featuring the DC3000 promoter region functioned properly, as shown by the high fluorescence in Genehog cells under induction with Boc-lysine added. With this noted success of the tRNA cassette functionality, efforts turned to investigate the exogenous synthetase activity in *P. syringae* DC3000 -- by substituting with mutant synthetase (PylRS Y348F and PylRS^{NAA}) and altering promoter regions in the pML123 vector from *tac* to *T5*.

Strain:Plasmid	OD ₆₀₀	OD _{fluor}	RFU	Dev.	Reported	+/-
DC3000 WT	4.00	0.05	-11.125	37.76029	-11.1	37.8
DC:GFPuv WT non	13.2	0.05	2710	84.59314	2710	84.6
DC:GFPuv WT ind -	13.5	0.05	5698.333	16.19671	5700	16.2
DC:6.067 non	4.20	0.05	79	84.59314	79.0	84.6
DC:6.067 ind -	4.70	0.05	35.66667	16.19671	35.7	16.2
G:6.067+Ptac.PylRS WT non	1.40	0.05	700	255.9727	700	256
G:6.067+Ptac.PylRS WT ind -	1.40	0.05	379.3333	77.51344	380	77.5
G:6.067+Ptac.PylRS WT ind +	1.60	0.05	5417	323.9182	5420	324

Table 2: Single trial test for fluorescence of GFPuv (WT or N149TAG) in DC3000 or Genehog cells with all samples incubated for 19 hours and densities normalized to OD₆₀₀ 0.05 for analysis. For ease of comparison, “Reported RFU +/- RFU” shown in right-hand columns. *DC3000 WT, wild-type strain DC3000; DC3000:pBBR-GFPuv WT non-induced (non) and 0.3 mM IPTG induced (ind-) DC3000 transformed with pSR6.067 (listed as DC:6.067); G:6.067+Ptac.PylRS WT, Genehog transformed with pSR6.067 and pML123.tac.PylRS WT feat. non, ind-, and IPTG + 5 mM boc-lysine (ind+).*



Graph 1: Counterpart for Table 1, showing relative fluorescence of cells for DC3000 wild-type (*blank*), and DC3000, Genehog transformed strains as non-induced (*non*), IPTG induced (*ind-*) and IPTG + 5 mM boc-lysine (*ind+*). (Abbrev. as DC, DC3000; GFPuv WT, pBBR1MCS3-GFPuv WT; 6.067, pSR6.067; G, Genehog; Ptac.PylRS WT, pML123.tac.PylRS WT. Error bars reflect consistency being multiple measurements for same samples to denote precision of scanning with SynergyH1 reader).

Strain:Plasmid	OD ₆₀₀	OD _{fluor}	RFU
DC3000 WT	4.0	0.05	666
DC:6.067:Ptac.PylRS WT non	5.7	0.05	353
DC:6.067:Ptac.PylRS WT ind-	5.8	0.05	448
DC:6.067:Ptac.PylRS WT ind+	4.9	0.05	263

Table 3: Single trial fluorescence of DC3000 with pSR6.067, pML123.tac.PylRS WT in comparison to DC3000 wild-type (WT) cells. Abbrev. DC, DC3000; 6.067, pSR6.067; Ptac.PylRS WT, pML123.tac.PylRS WT; non, non-induced; ind-, IPTG induced; ind+, IPTG induced + boc-lysine. Single scan for suspensions normalized to 0.05 OD₆₀₀. DC3000 WT considered as blank; correction not done to show positive RFU values.

2.3.1 Analysis of DC3000:pSR6.067 featuring pML123-tac.PylRS Y348F

PCR amplification of PylRS Y348F from pBK vector resulted in digestible product that was prepared as for PylRS WT to produce

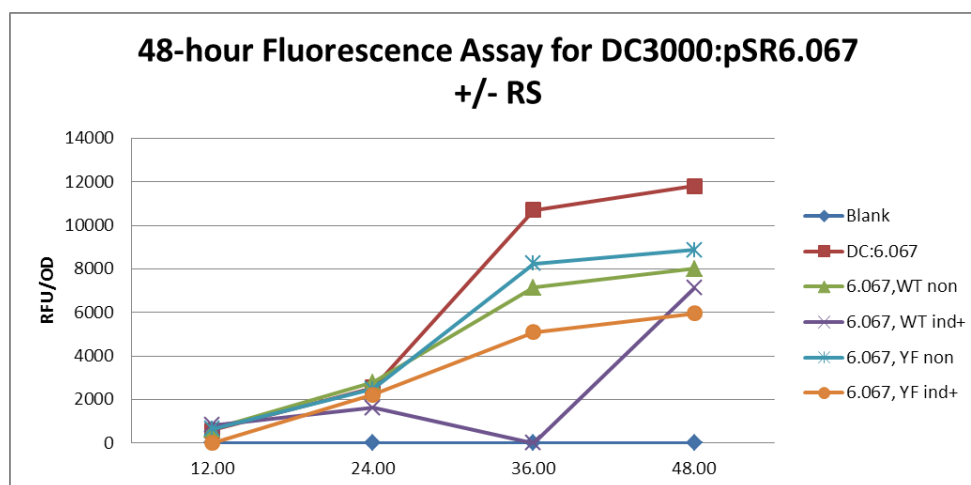
Sample	OD _{fluor}	RFU	RFU/OD
Blank	N/A	0	0
G:6.067:YF non	0.1	37.5	375
G:6.067:YF ind	0.1	1329.5	13295
DC:6.067:YF non	0.1	-10	N/A
DC:6.067:YF ind	0.1	-1.5	N/A
DC:6.067 only	0.1	40	400

Table 4: Initial test for GFPuv mutants from Genehog (G) or DC3000 (DC) cells containing pSR6.067 (6.067) and pML123.tac-PylRS Y348F (YF). PBS blank, non-induced (non) and induced (ind) with IPTG and 5 mM boc-lysine.

Sample	12 hrs. PI				24 hrs. PI			
	OD _{fluor}	RFU	RFU/OD	Reported	OD _{fluor}	RFU	RFU/OD	Reported
Blank	N/A	0	N/A	0	N/A	0	N/A	0
DC:6.067	1.1375	682.5	600.0	600.0	1.1285	2888.5	2559.592	2559.6
6.067, WT non	1.074	692.5	644.7858	644.8	0.99	2812.5	2840.909	2800
6.067, WT ind+	0.8315	694	834.6362	835	0.9385	1530.5	1630.794	1630.8
6.067, YF non	1.106	723	653.7071	654	1.093	2715.5	2484.446	2485
6.067, YF ind+	0.758	1442	1902.375	1900.	0.759	1695.5	2233.86	2234

Sample	36 hrs. PI				48 hrs. PI			
	OD _{fluor}	RFU	RFU/OD	Reported	OD _{fluor}	RFU	RFU/OD	Reported
Blank	N/A	0	N/A	0		0	N/A	0
DC:6.067	0.9555	10231.5	10708.01	10710	0.767	9078.5	11836.38	11800
6.067, WT non	0.866	6190	7147.806	7150	0.552	4426.5	8019.022	8020
6.067, WT ind+	0.7735	4332	5600.517	5600.	0.6795	4839.5	7122.149	7122
6.067, YF non	0.9255	7633	8247.434	8247.4	0.852	7565	8879.108	8880
6.067, YF ind+	0.62	3164.5	5104.032	5100	0.5555	3314.5	5966.697	5966.7

Table 5: RFU/OD values for 48-hour time course of non-induced (non) and induced (IPTG and boc-lysine; ind+) DC3000 cells containing pSR6.067 (6.067) with pML123.tac-PylRS (WT or Y348F). Abbrev: P.I., hours post induction; WT, pML123.tac.PylRS WT; YF, pML123.tac.PylRS Y348F; DC:6.067 (DC3000:pSR6.067 only).



Graph 2: RFU/OD values for samples shown in *Table 5*. No strains featured higher fluorescence than Pyl-tRNA, GFPuv n149TAG, suggesting the synthetase promoter was at fault *Abbrev. 6.067, pSR6.067; WT, pML123.tac.PylRS WT; YF, pML123.tac.PylRS Y348F. .*

A useful method for detecting successful of the dual plasmid system requires reproducible fluorescence increase over non-induced (*non*) cells for those samples induced with both IPTG and the unnatural amino acid (*ind+*). These “*ind +*” cell pellets should also always show higher fluorescence than IPTG-only induced (*ind -*) pellets. There was no attempt at analyzing fluorescent of pellets for DC3000:pBBR1MCS3 strain; the wild-type DC3000 and transformed strains featuring reporter proteins were suitable controls for this project.

Cell-based fluorescence assay for DC3000:6.067:PylRS (WT, Y348F) showed the pyrrolysine tRNA/RS system worked well in *E. coli*, but it was considered to change the synthetase promoter from *tac* to *T5* to better understand lack of success in DC3000. After changing promoters, values of fluorescence dependent on adding boc-lysine to the culture significantly increased over non-induced cultures. However, analysis of samples induced with IPTG only also produced significantly high fluorescence. Because of this, it appeared that either the Pyl-tRNA or the PylRS was interacting with DC3000 native translational machinery.

After the wild-type PylRS did not show the expected high fluorescence in DC3000, it was decided to construct the synthetase vector for the DC3000 system using the Tyr348Phe pyrrolysine synthetase mutant (PylRS Y348F), with intent that the mutant would provide brighter fluorescence for the GFPuv reporter in induced strains due to higher affinity for the Boc-lysine amino acid (*Yanagisawa, 2008*).

Initial test of GFPuv mutants from Genehog and DC3000 featuring pSR6.067 and pML123.*tac*.PylRS Y368F are shown in *Table 2*. Incidentally, Genehog-expressed GFPuv N149-boc-lysine fluorescence was not as intense as expected and a second test was run for induced DC3000 cells harboring either the PylRS Y368F mutant or a second pML123.*tac*.PylRS WT (PylRS #2) plasmid, but results for DC3000 were similar (*data not shown*).

2.3.2 pML123-*tac*-PylRS NAA Mutant

The Pyl-tRNA/RS system within *E. coli* Genehog cells gave expected increased fluorescence for cultures induced and charged with Boc-lysine, but this trend was not observed in *P. syringae* DC3000. Considerations for this inconsistency included (a) a mutation in *tac*, RBS, or PylRS DNA sequence in the pML123 plasmid, (b) inability for DC3000 to take up/use Boc-lysine, or (c) poor efficiency of *tac* promoter for synthetase production in DC3000.

To examine the first possibility, sequencing of the pML123-PylRS vector with primers to recognize *tac* promoter site. Results showed no mutation in the promoter, RBS sites, or the upstream sequence for PylRS.

To examine if DC3000 had trouble importing or utilizing Boc-lysine, we attempted to use a library of PylRS variants prepared in our lab (*Tong Ju, University of Nebraska-Lincoln*) to select for a synthetase that could recognize natural amino acids (NAA). Use of a PylRS^{NAA} would still allow for interaction with the Pyl-tRNA but charge the tRNA with natural amino acids to produce a full-length and fluorescing GFPuv N149-NAA protein.

This selection process required the use of *E. coli* cells containing the pREP-PylT plasmid (*Neumann, 2008*) featuring a T7 RNA polymerase and a chloramphenicol acetyltransferase (CAT) that both possess TAG stop codons within the peptide sequence -- two stop codons in the

polymerase, one in the resistance protein -- as well as the suppressor *Mb. Pyl-tRNA^{CUA}* for PylRS interaction. Properly suppressed TAG codons allow for full-length synthesis of the polymerase which will recognize the plasmid-based T7 promoter region preceding the plasmid's wild-type GFPuv reporter protein. Expression of full-length CAT from the plasmid yields chloramphenicol resistance with increasing TAG suppression due to effective synthetase charging of natural amino acids to the suppressor tRNA.

The following results feature plated cells related to the evolution of PylRS Y348F synthetase with increased selectivity for natural amino acids (PylRS^{NAA}).

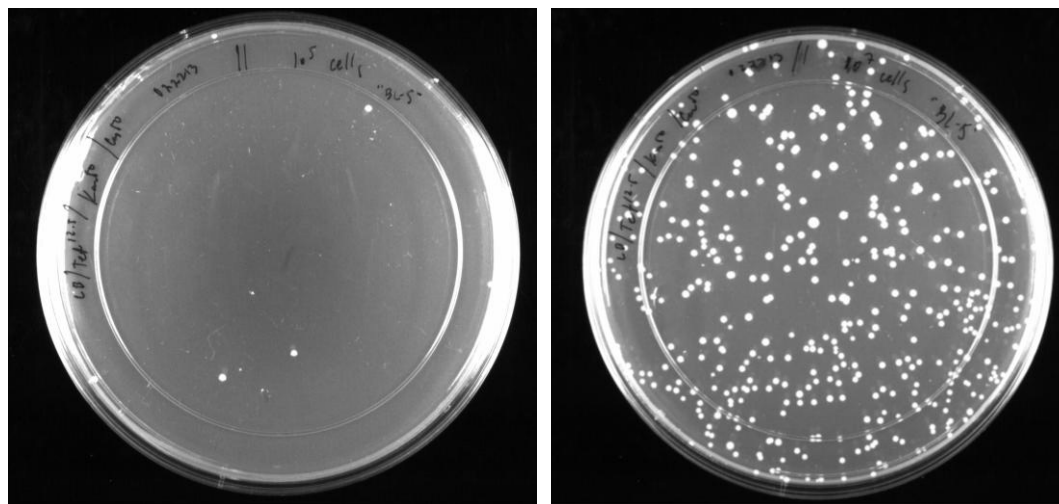


Figure 11: *BL-5* library plates for 10^5 and 10^7 cells prepared from glycerol stock. Two colonies from the 10^7 plate were chosen for purify PylRS^{NAA}, a synthetase to recognize natural amino acids.

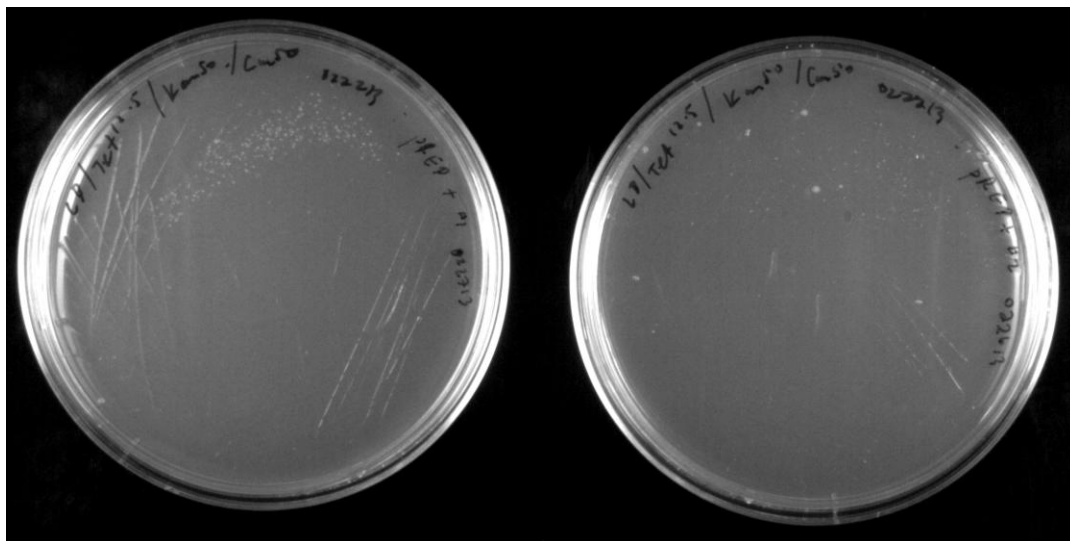


Figure 12a: Plated *E. coli* pREP-PylT cells (see below) for transformants prepared from plasmids purified from initial *BL-5* plating.

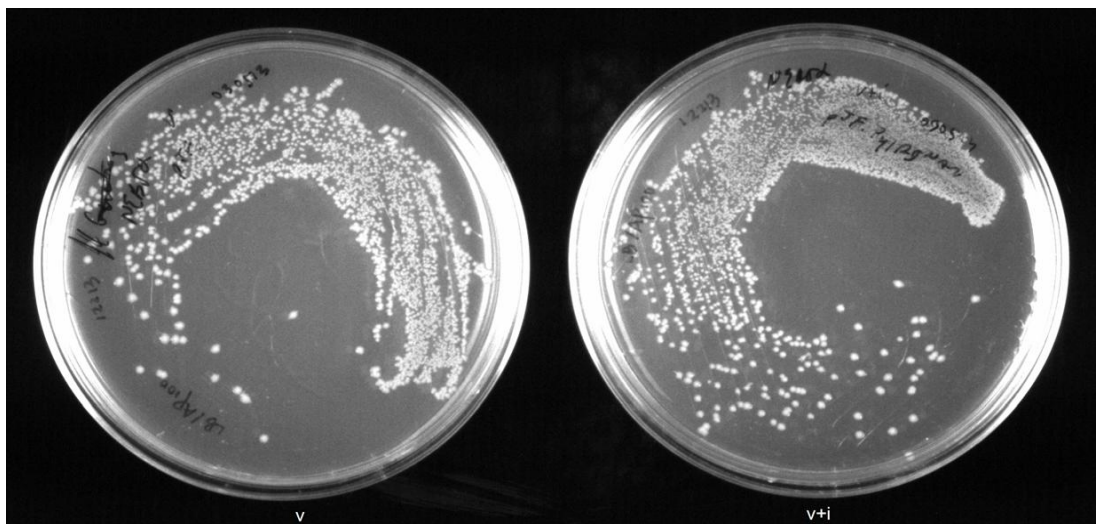


Figure 12b: Transformed Genehog cells from ligation of digested pJF118EH-mod1 (*v-only*; left) and digested PylRS^{NAA} insert (*v+i*; right).

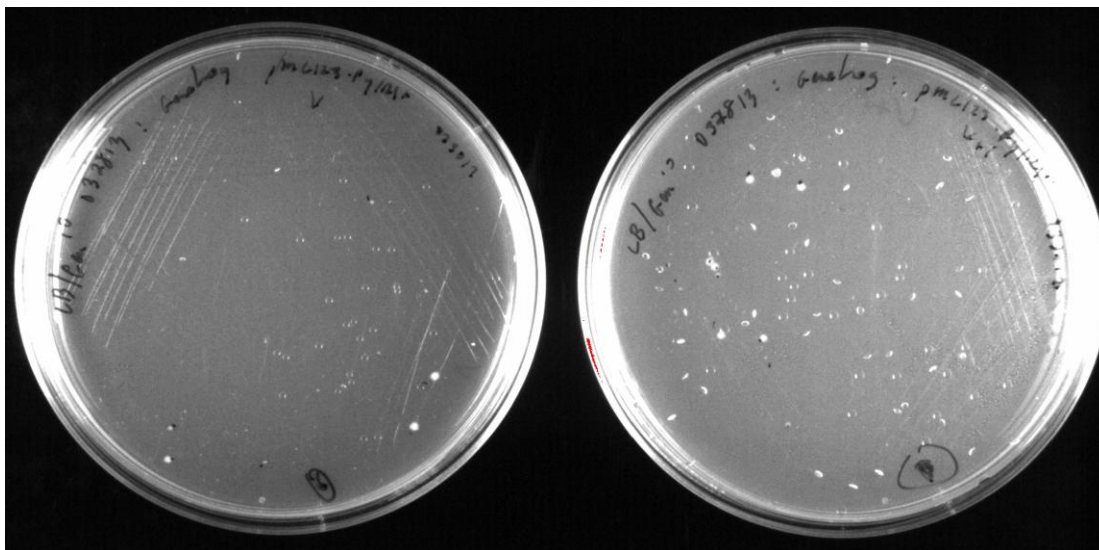


Figure 13: DNA gel for Genehog plated cells from ligation of digested pML123 (*v-only*; *left*) and PylRS^{NAA} insert (*v+i*; *right*). Ligation efficiency for *v*:*v+i* was 6:15 colony forming units.

As shown in *Figure 11*, a single-round selection yielded viable colonies in presence of chloramphenicol (50 $\mu\text{g}/\text{mL}$), but recombination, transformation, and growth under IPTG produced no significant GFPuvN149-NAA fluorescence in DC3000 cells (*data not shown*). Therefore, it was decided that the synthetase promoter should be switched from *tac* to the same *T5* promoter cassette as used with the GFPuv reporter proteins.

As a final note on use of Boc-lysine in DC3000, there has been little research into the effective uptake of unnatural amino acids into *P. syringae* and different bacterial species are likely to have subtle differences in their specific amino acid import/transport machinery. A survey of the KEGG database shows such differences between DC3000 (*left*) and *E. coli* DH10B (*right*) strains as shown in *Figure 14*. The ArgT protein (*red border*; *left column*; *white box*) may be tantamount for uptake of Boc-lysine in DC3000, for example, given its categorized role in

transport of lysine, arginine, and ornithine – all polyamine amino acids. However, other transporter proteins (e.g. HisM) may be suspect to recognition of Boc-lysine, in this case.

A supplemental study of any unnatural amino acid uptake in DC3000, would involve addition of the amino acid to mid-log phase DC3000 cells in culture media followed by collecting of supernatant over given time-points for analysis by LC-MS/MS. This method could be easily complemented by introduction of exogenous or overexpressed native transporter proteins into the host, followed by growth under similar conditions for analysis. To note, however, in the event an organism of study does not need or cannot use the amino acid, there is always a chance of excretion of the molecule back into the supernatant. In the case of Boc-lysine, if effective uptake was accompanied by ineffective use, the amino acid could be expected to be released back into the environment in total or degraded for recycling of useable functional groups and waste release.

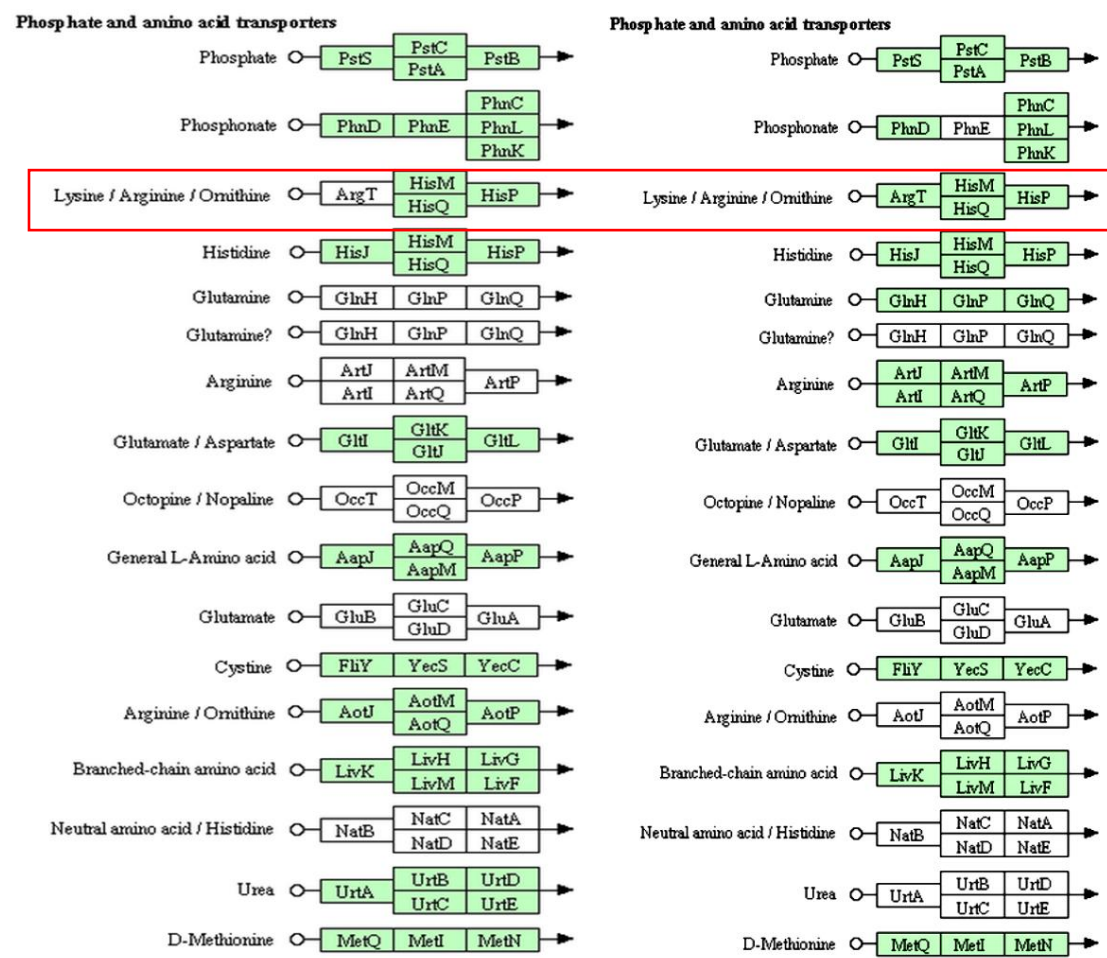


Figure 14: Comparison of phosphate and amino acid transporters between *P. syringae* pv tomato DC3000 (left) and *E. coli* DH10B (right). Blocks highlighted in green are known pathway nodes found in that organism. For link to the *P. syringae* pv. tomato DC3000 KEGG site:

{http://www.genome.jp/kegg-bin/show_pathway?pst02010}

2.4 pBBR1MCS3.T5-PylRS WT, pML123.T5-PylRS WT, and pML123.T5-PylRS Y348F

To enhance expression of the synthetase, PylRS (WT or Y34F), an alternative promoter was selected. It was seen that the GFPuv WT was well expressed in both *E. coli* and DC3000 cells, so it was decided to use the T5 promoter and ribosome binding site (RBS) for increasing

synthetase production. As the *T5.RBS.GFPuv* WT (or N149TAG) cassette was initially cut from a pLei-based vector, substitution of the reporter protein for either synthetase allowed for direct insertion of *T5.RBS.PylRS* (WT or Y348F) from pLei vector into plasmids pML123 and also pBBR1MCS3. Vector pBBR1MCS3 had shown successful expression (as per fluorescence) of GFPuv WT from DC3000 so this construct was created for possible troubleshooting of synthetase expression. That is, if there had been a problem with the new pML123.*T5* constructs, the pBBR1MCS3.*T5* series would have served as an additional experimental control.

Shown below in *Figures 15, 16* are the plates for *E. coli* cells transformed with ligation products at first and second stages of experimental design. Fluorescence results are shown in *Table 6*.

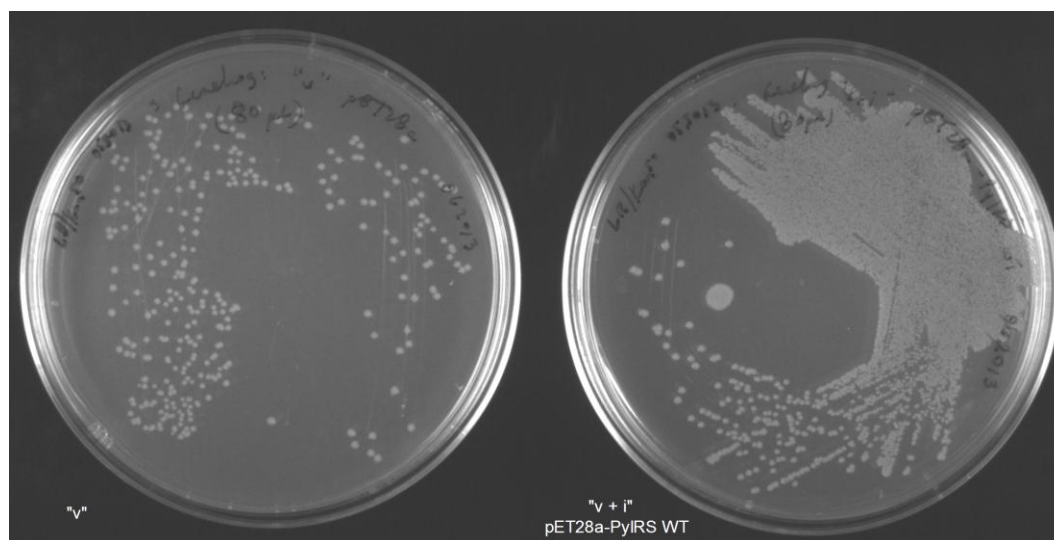


Figure 15: Plated Genehog cells for ligation of pET28a (*v-only*; *left*) with PylRS WT insert (*v + i*; *right*). Plates for PylRS Y348F were similar; vector sample used was same for both.

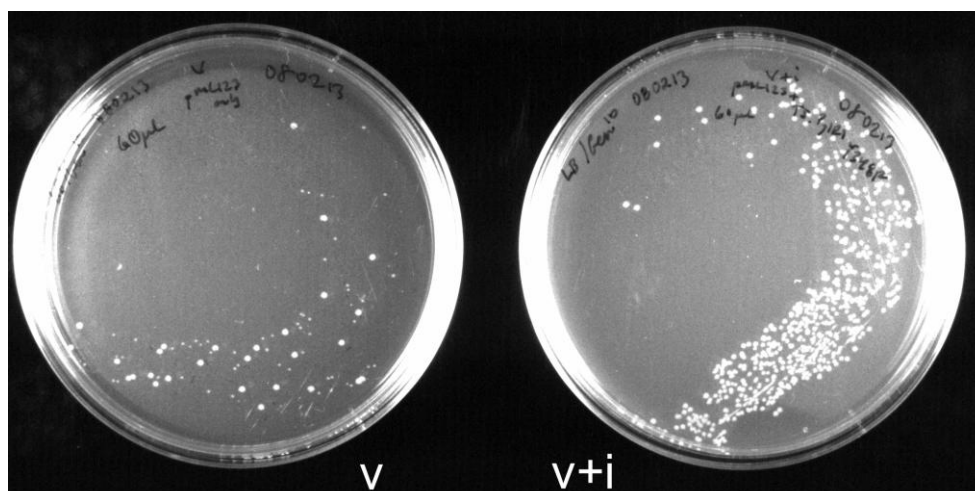


Figure 16: Plated Genehog cells for ligation of pML123 (*v-only*; *left*) with PylRS Y348F insert (*v* + *i*; *right*). Plates for PylRS WT ligation and transformation utilized same vector sample.

Sample	20 hrs. 30 °C, 16 hrs. 4 °C				43 hrs. 30 °C, 16 hrs. 4 °C			
	RFU	OD _{fluor}	RFU/OD	Reported	RFU	OD _{fluor}	RFU/OD	Reported
Blank	0	N/A	N/A	0	0	N/A	0	0
KB-T/Tet10	670.	N/A	N/A	670.	670.	N/A	N/A	670.
DC WT	1218.75	0.569	2141.916	2140	N/D	N/A	N/D	N/A
6.067:PylRS WT non	1092.50	0.246	4441.057	4440	1773.25	0.297	5970.539	5970
6.067:PylRS WT ind-	1159.75	0.174	6665.23	6670	4251.75	0.327	13002.29	13000
6.067:PylRS WT ind+	829.50	0.332	2498.494	2500	4292.75	0.506	8483.696	8480
6.067:PylRS YF non	1041.00	0.069	15086.96	15100	1460.75	0.05	29215	29200
6.067:PylRS YF ind-	1486.25	0.119	12489.5	12500	906.50	0.039	23243.59	23200
6.067:PylRS YF ind+	5472.00	0.298	18362.42	18400	2872.00	0.236	12169.49	12200

Table 6: Comparison of DC3000 cells featuring pSR6.067 (6.067) in presence of either pML.T5.RBS.PylRS WT (*PylRS WT*) or pML.T5.RBS.PylRS Y348F (*PylRS YF*). 20-hour and 43-hour time-points were grown post-induction (0.5 mM IPTG; 5 mM boc-lysine) at 30 °C, 250 rpm and then set into 4 °C for 16 hours. Results of induction with amino acid (*ind+*) against induction without amino acid (*ind-*) showed that over 43-hour period, fluorescence of (*ind+*) was lower than that for (*ind-*). These unexpected results suggest the host strain translation machinery

may be working with plasmid-based machinery to incorporate natural amino acids into TAG location of GFPuv N149 reporter protein.

Substitution of the synthetase promoter to the *T5* promoter cassette gave increased fluorescence of the GFPuv reporter protein, but it was realized that a significant increase over non-induced cultures was apparent (*Table 6*) without the addition of boc-lysine unnatural amino acid. These results are not the expected $non < ind- < ind+$ RFU/OD for transformed *P. syringae* DC3000 cultures and, therefore, it was decided that this Pyl-tRNA/RS system would not be suitable for selectively producing mutant proteins in future experiments. There was no further attempt to understanding the interactions of exogenous tRNA or synthetase. An alternative tRNA/RS pair, from *Methanocaldococcus jannaschii*, was next studied.

For further analysis, however, it was decided to attempt purification of any full-length GFPuvN149 mutants that may be produced within DC3000 cells under induction alongside Boc-lysine. Purifications and electrophoretic analyses were performed in concert with *P. syringae* DC3000 cultures featuring the *M. jannaschii* tRNA/RS dual plasmid system; results are therefore shown in Chapter 3.

Chapter 3: Construction and Use of Dual Plasmid System Featuring *M. jannaschii* Tyrosyl-tRNA^{CUA} and pML123.T5. *Mj.* BpFRS (pSR8.010, pSR8.017)

A different tRNA/RS pair was introduced into *P. syringae* pv. tomato DC3000 from the Archaea, *Methanocaldococcus jannaschii*. The tRNA anticodon arm has been modified to recognize the TAG stop codon; the corresponding synthetase for this Tyr-tRNA^{CUA} has been evolved in separate experiments to select for specific unnatural amino acids. Different synthetase proteins, including the wild-type TyrRS and the evolved species, BpFRS and AzFRS that specific for the crosslinking amino acids, BpF and AzF, resp., were studied in presence of the tRNA to effect full-length translation of the GFPuv reporter protein featuring UAA at position N149TAG. The full-length GFPuvN149-UAA mutant produces fluorescence when properly folded and also contains a C-terminal 6xHis tag to allow for purification by nickel affinity column.

3.1 Construction of Vector, pSR8.010, Containing *M. jannaschii* tRNA and GFPuvN149TAG

The vector containing the GFPuvN149TAG reporter protein, pBBR1MCS3-GFPuv N149TAG, was modified to include the *M. jannaschii* Tyr-tRNA^{CUA} gene cassette featuring the *P. syringae* DC3000 *tRNA-met-3* promoter region and the poly(A) tail as in Chapter 2. Analyses of fluorescence in DC3000:pSR8.010 (8.010) as non-induced or IPTG-induced against wild-type DC3000 culture were performed against DC3000 wild-type cells at 2-days (*Table 7*), and 5 days (*Table 8*) against DC:6.067 (Pyl-tRNA). In this experiment IPTG induction serves only to give expression of the GFPuvN149TAG truncated protein. In all cases, there was a level of background fluorescence for the induced cells. As shown in *Table 9*, the fluorescence for DC:8.010 cells (1460 RFU/OD) was higher than for DC:6.067 (513 RFU/OD) under IPTG induction, suggesting that either the *M. jannaschii* tRNA was more stable, more highly expressed,

or better at supplying natural amino acids to the TAG codon site than the *M. barkeri* tRNA. Nevertheless, because the GFPuvN149-based fluorescence was generally lower under IPTG-induction (Table 7), and fluorescence measurements for DC3000 cells featuring *only* the GFPuvN149TAG construct (Table 9; *GFP non, ind*) were also higher than wild-type DC3000 cells, it was decided to pursue studies involving vector pSR8.010 in presence of the synthetase proteins.

Sample	RFU	Dev.	RFU/OD	Dev.	Reported	Dev.
Blank	-0.052593	0.152029	--	--	-0.05	0.15
DC WT	--	--	478.2639	37.97862	478	38
DC:pSR8.010 non	--	--	2045.848	453.1645	2050	453
DC:pSR8.010 ind-	--	--	1839.58	437.5054	1840	438

Table 7: Average of three trials for cell fluorescence of DC3000:pSR8.010, featuring the *M. jannaschii* Tyr-tRNA^{CUA}, GFPuv N149TAG. Cells were incubated 44-48 hours P.I. with 0.5 mM IPTG working concentration (*ind-*) or no IPTG (*non*), followed by 20 hours incubation at 4 °C. Wild-type DC3000 is shown as reference; OD₆₀₀ values used for calculating RFU/OD were between 0.1 and 1.2, as measured by 10x dilution of cell suspension prior to dilution for assay.

DC3000 pSR8.010 120/18 Samples (120 hrs 30 °C, 18 hrs 4 °C)					
	OD (final)	RFU	Dev.	RFU/OD	Reported
PBS	N/A	0.333333	53.850	N/A	0.333
WT	1.10	1019	13.697	926.3636	926
8.010 non	1.00	2870.5	37.217	2870.5	2870
8.010 ind-	1.10	2556	37.202	2323.636	2320

Table 8: Single trial fluorescence test of 120-hour P.I. DC3000:pSR8.010 (8.010) after induction of mid-log cultures with 0.5 mM IPTG (*ind-*), incubation at 30 °C, 250 rpm, followed by 18 hours at 4 °C. OD₆₀₀ values were obtained at 10x dilution of culture sample and back-calculated for RFU/OD results. DC3000 wild-type (WT) is shown for comparison.

Sample	OD ₆₀₀	RFU	Dev.	RFU/OD	Reported
Blank	N/A	0	12.910	N/A	0
WT	7.10	5615.333	36.501	790.892	791
GFP non	11.50	11144	67.735	969.0435	969
GFP ind	12.60	14684	199.610	1165.397	1170
8.010 non	5.30	6241	75.974	1177.547	1180
8.010 ind	7.20	10516.33	141.924	1460.602	1460
6.067 non	24.40	23759.33	299.604	973.7432	974
6.067 ind	26.00	13344.67	190.768	513.2564	513

Table 9: Single trial fluorescence test for DC3000 containing pBBR1MCS3-GFPuv N149TAG only (*GFP*), pSR8.010 (*8.010*), or pSR6.067 (*6.067*) against wild-type (*WT*) strain. Sample OD₆₀₀ values were obtained at 10x culture suspension and then back-calculated for RFU/OD results. Deviation (*Dev.*) in emission results are for triplicates of same sample in microplate wells or quadruplet for PBS blank. Samples were either induced (*ind-*) with 0.5 mM IPTG or non-induced (*non*).

3.2 DC3000:pSR8.017; Analysis of DC3000 Harboring pSR8.010 and pSR8.017 Plasmids

Plasmid pSR8.017 was designed using the *T5* promoter region for expression of *M. jannaschii* tyrosyl-synthetase evolved to charge BpF onto the cognate tRNA^{CUA}. Plasmid pSR8.017 (pML123.*T5*.BpFRS) then used to co-transform DC3000:pSR8.010 for growth in liquid media in presence of IPTG +/- 0.1 mM BpF for fluorescence analysis (*Table 11*).

Induction under influence of BpF produced higher fluorescence (*ind+*; 2620 RFU/OD) than without BpF (*ind-*; 2020 RFU/OD) or non-induced cells (*non*; 1840 RFU/OD). This experiment shows the two plasmid system works as expected for the *M. jannaschii* Tyr-tRNA^{CUA}/BpFRS pair in *P. syringae* DC3000 and full-length GFPuvN149-BpF was later purified

by affinity chromatography and fractions were analyzed by SDS-PAGE and Western blot (Figures 26, 27, respectively).

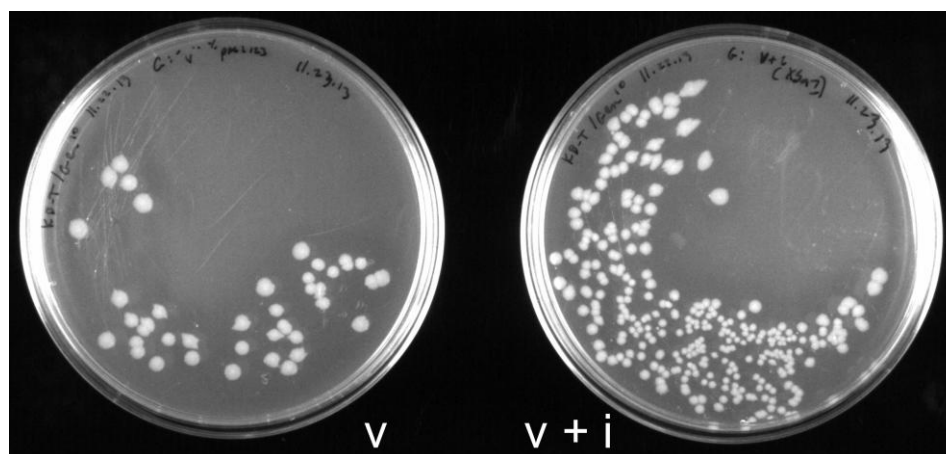


Figure 17: Genehog cells featuring ligation product for pML123 (*v-only*; left) and BpFRS insert (*v+i*; right) to show efficiency of DNA recombination reaction. Cells were plated to KB-T/Gen¹⁰ agar plates.

Sample	RFU	Dev.	RFU/OD	Dev.	Reported	Dev.
Blank	-0.02333	0.202704	--	--	-0.05	0.15
DC WT	--	--	469.8958	49.64479	470.	49.7
DC:pSR8.017 non	--	--	468.0476	21.28055	468	21.3
DC:pSR8.017 ind-	--	--	427.9028	100.5467	428	101

Table 10: Average of duplicate trials for cell fluorescence of DC3000:pSR8.017 (BpFRS-only) induced with 0.5 mM IPTG (*ind-*) or non-induced (*non*) to show strains maintain native fluorescence. Samples were analyzed in concert with wild-type DC3000 strain (*DC WT*).

Sample	RFU	Dev.	RFU/OD	Dev.	Reported	Dev.	
Blank	Blank	-0.04667	0.143965	--	--	-0.05	0.14
WT	WT	--	--	568.2639	173.9573	568	174
8+8 non	8+8 non	--	--	1844.667	638.193	1840	638
8+8 ind-	8+8 ind-	--	--	2020.419	618.8777	2020	619
8+8 ind+	8+8 ind+	--	--	2617.432	546.4868	2620	547

Table 11: Average of fluorescence data for three trials for DC3000:pSR8.010:pSR8.017 (8+8). All cells were induced with 0.5 mM IPTG, where applicable, and with 1 mM BpF (*ind+*) or without (*ind-*) or non-induced (*non*). Cell cultures were grown for 44-48 hours post-induction. Cell densities were either between 0.8 and 1.2 OD₆₀₀ or normalized to OD₆₀₀ 0.1 for analysis.

Because DC3000 transformants featuring BpF-specific synthetase (BpFRS) exhibited higher fluorescence of cultures induced with both IPTG and 1 mM BpF (*ind+*) over IPTG-only induced (*ind-*) and non-induced (*non*) cultures (Table 11), additional confirmation was obtained by the induction and growth of 28 mL DC3000:pSR8.010:pSR8.017 cultures for 48 hours at 30 °C, 250 rpm and subsequent purification of full-length GFPuv N149BpF mutant protein. These results are shown in SDS-PAGE (Figure 23) and Western blot detection images (Figure 24).

3.3.1 *pML123.T5-TyrRS WT (pSR8.037) and pML123.T5-AzFRS (pSR8.044)*

Further investigation into the capability of this dual plasmid system included growth of DC3000:pSR8.010 strain subsequently transformed with plasmids containing *M. jannaschii* wild-type Tyr-RS (pSR8.037) or the evolved synthetase, AzFRS (pSR8.044), which recognizes the *p*-azido-phenylalanine crosslinking amino acid.

Constructs were prepared from in-house plasmids as above for the BpFRS and transformation into *P. syringae* DC3000 and *E. coli* Genehog cells yielded viable colonies for liquid culture growth at 36 or 48 hours. Results shown in the following tables include initial fluorescence measurements of 36-hour cultures of DC3000 with tRNA and wild-type TyrRS (Table 12), multiple-trial experiments to compare fluorescence for TyrRS and AzFRS in 36-hour cultures of DC3000 (Table 13), and 48-hour DC3000 cultures expressing AzFRS alongside tRNA (Table 14). Complimentary fluorescent studies were done in *E. coli* Genehog cells transformed

with tRNA and BpFRS or AzFRS to compare effectiveness of the two evolved synthetase proteins (Table 15).

DC3000 36/48 Samples (36 hrs 30 °C; 48 hrs 4 °C)					
	RFU	Dev.	OD	RFU/OD	Reported
PBS	-0.11111111	7.149204	N/A	N/A	N/A
DC WT	42.6666667	11.0227	0.10	426.67	427
TyrRS non	175.222222	11.3002	0.10	1752.22	1750
Tyr -	268.166667	13.80459	0.10	2681.67	2680

Table 12: Single trial fluorescence readings for DC3000 cell pellets featuring pSR8.010 and pSR8.037 as non-induced (*TyrRS non*) and IPTG-induced (*Tyr-*) over 36-hour period. Deviation (*Dev.*) is shown for multiple analysis of same culture sample in microplate wells. As samples were normalized to $OD_{600} = 0.1$ for analysis.

Sample	RFU	Dev.	RFU/OD	Dev.	Reported	Dev.
Blank	0	0.111111	--	--	0	0.11
DC WT	--	--	508.8889	252.1317	509	252
Tyr non	--	--	2697.407	25.10062	2700	25.1
Tyr ind -	--	--	2304.074	423.2915	2300	423
AzF non	--	--	2294.815	253.4024	2290	253
AzF ind -	--	--	2345.185	364.2027	2350	364
AzF ind +	--	--	1760.37	176.192	1760	176

Table 13: Average of fluorescence readings over three trials for DC3000:pSR8.010:pSR8.044 (AzFRS) cultured over 36 hours P.I. in concert with DC3000 wild-type (*DC WT*), as well as DC3000:pSR8.010:pSR8.037 (*Tyr*). Cultures for transformed strains were either non-induced (*non*), IPTG induced (*ind-*) or induced by IPTG and 1 mM AzF (*ind+*).

Sample	OD_{600}	OD_{fluor}	RFU	Dev.	RFU/OD	Reported
Blank	N/A	N/A	0	7.483	N/A	0
DC 48	4.40	0.10	76.500	12.951	765	765
010+AzF 48 n	1.50	0.10	271.917	28.468	2719.167	2720
010+AzF 48 -	2.30	0.10	233.917	11.859	2339.167	2340
010+AzF 48 +	1.60	0.10	192.917	13.201	1929.167	1930

Table 14: Single trial fluorescence reading for DC3000:pSR8.010:pSR8.044 (*010+AzFRS*) cultured over 48 hours P.I. in concert with DC3000 WT. Cells cultures were either non-induced (*n*), IPTG induced (-) or induced by IPTG and 1 mM AzF (+). Deviation (*Dev.*) is shown for multiple analysis of same culture sample in microplate wells.

Genehog 8+8 (T5.AzF or BpF RS), 19 hours 37 °C, 25 hours 4 °C					
Sample	RFU	Dev.	OD	RFU/OD	Reported
PBS	0.111	26.770	N/A	N/A	N/A
AzF non	36.556	7.468	0.10	365.556	370
AzF ind -	39.222	15.619	0.10	392.222	390
AzF ind +	68.444	8.904	0.10	684.444	680
BpF non	65.000	37.620	0.10	650.000	650
BpF ind -	41.222	17.563	0.10	412.222	410
BpF ind +	117.444	25.006	0.10	1174.444	1200

Table 15: Fluorescence analysis of Genehog cells expressing pSR8.010 and pML123 plasmids containing BpF and AzF synthetase shown as non-induced (*non*), IPTG-induced (*ind-*) or IPTG and UAA induced (*ind+*). Test suggests functional tRNA/RS pairs in Genehog strain as per increased fluorescence with *ind+* > *ind-*.

Initially, DC3000: pSR8.010: pSR8.044 (*AzFRS*) strains did not show significant fluorescence in cells or in supernatant fractions (*Table 13*). In fact, cells induced with IPTG in presence of showed lower RFU/OD values than non-induced or IPTG-induced cultures. It was considered that because *p*-azido-*L*-phenylalanine contains an azide bond able to undergo light-induced reduction to *p*-amino-*L*-tyrosine, *AzFRS* could not to charge this degradation product to the cognate tRNA. To confirm this possibility an additional growth test was performed with all flasks wrapped in aluminum foil for the duration of the experiment. As expected, *Table 16* shows transformed DC3000 cultures in presence of both IPTG and AzF produced nearly two-fold the fluorescence of IPTG-only or non-induced cultures. Therefore, it is shown that the dual plasmid

system using the modified *M. jannaschii* Tyr-tRNA^{CUA} in concert with the corresponding AzF-charging synthetase yields successful incorporation of *p*-azido-*L*-phenylalanine to give full-length fluorescing GFPuvN149-AzF. There was no attempt at purifying GFPuvN149-AzF for visualization on SDS-PAGE or by Western blot detection from this final aluminum foil covering experiment. Future studies with AzF should allow for foil or other light-blocking covering.

Sample	OD _{fluor}	RFU	Dev.	RFU/OD	Reported	Dev.
Blank	N/A	0.333333	9.626353	N/A	0.333	9.63
Non	0.1	54.5	9.949874	545	545	9.95
Ind-	0.1	64.5	14.59795	645	645	14.6
Ind+	0.1	125.2	17.25399	1252	1250	17.3

Table 16: Duplicate DC3000:pSR8.010:pSR8.044 cultures were foil-covered and grown for 42 hours at 30°C before setting at 4°C for an additional 20 hours. Cultures were diluted to OD₆₀₀ 0.1 for fluorescence analysis of non-induced (*non*), IPTG-induced (*ind-*), and IPTG with AzF (*ind+*).

This thesis has shown that the unnatural amino acids, BpF and AzF (*Figure 18*), can be utilized *in vivo* by *P. syringae* DC3000 containing an exogenous tRNA/RS pair to suppress the TAG stop codon and produce full-length reporter proteins. It was noted that the levels of fluorescence were not as high as expected in DC3000 and so the GFPuvN149TAG plasmid (pSR8.010) and the plasmid pair including the wild-type TyrRS in *E. coli* NEB5α F' cells was performed to attempt further characterizing of the this system's effectiveness. NEB5α F' cells (*New England Biolabs*) contain suppressor tRNA, supE44, are able produce full length GFPuvN149TAG proteins with an installed glutamine (*Singaravelan, 2010*). Although results showed expected increased fluorescence (*Table 17, Table 18*) they also confirmed that the system was producing lower fluorescence in all organisms. It was decided to alter the promoter sequences for the tRNA^{CUA} in attempt to increase the productivity of this dual plasmid system.

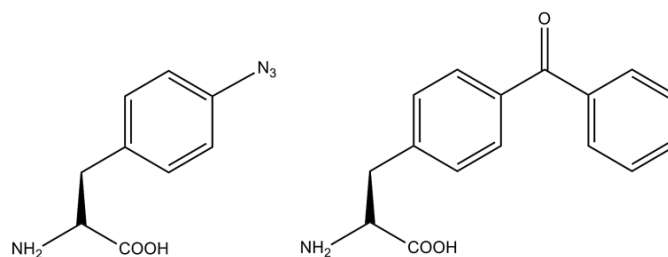


Figure 18: Crosslinking phenylalanine derivatives investigated in this study for incorporation into TAG stop codon sites by *M. jannaschii* Tyr-tRNA in presence of evolved cognate *M. jannaschii* TyrRS synthetase: AzF (left) or BpF (right).

NEB5 α F' with pSR8.010 only (19 hours 37 °C, 25 hours 4 °C)					
Sample	RFU	Dev.	OD600	RFU/OD	Reported
PBS	2.778	6.671	N/A	N/A	N/A
non	12.000	5.585	0.10	120.000	120
ind -	23.556	10.277	0.10	235.556	240

Table 17: Fluorescence analysis of NEB5 α F' cells expressing pSR8.010. Test shows functionality of GFPuv N149TAG under IPTG-induced (*T5*) promotion for fluorescence of *ind-* > *non*.

NEB5 α F' :pSR8.010:pSR8.037 (19 hours 37 °C, 25 hours 4 °C)					
Sample	RFU	Dev.	OD600	RFU/OD	Reported
PBS	0.222	7.190	N/A	N/A	N/A
8+8 (Tyr) non	160.667	8.958	0.10	1606.667	1600
8+8 (Tyr) ind -	292.333	15.827	0.10	2923.333	2900

Table 18: Fluorescence analysis of NEB5 α F' cells expressing pSR8.010 and pSR8.037. Test shows successful working of dual plasmid system that includes TyrRS WT (8+8 (Tyr)) as fluorescence for IPTG-induced (*ind-*) is greater than non-induced (*non*).

3.4 New *Mj.* Tyrosyl-tRNA Promoters: *lpp* and *proK*

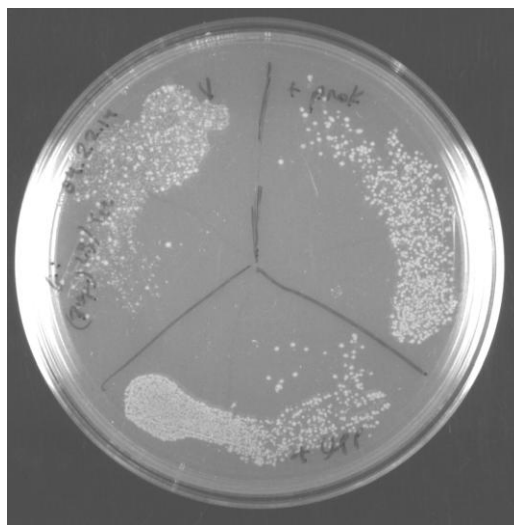


Figure 19: DNA gel for plated cells of pBBR-GFPuvN149 (*v*; *top left*) with either *lpp* cassette (*+lpp*; *bottom*) or *proK* cassette (*+proK*; *top-right*).

A single trial time-point experiment was performed to test if there was increased transcription of Tyr-tRNA and therefore heightened fluorescence of full-length GFPuv N149BpF mutant protein. Cell-based fluorescence results did not show that promoter, *proK*, gave any higher RFU for samples induced by IPTG and BpF. After the first trial, no more studies into the *proK* promoter cassette were performed. Initial results for the *lpp* promotion cassette showed promise and subsequent trials were performed to give varying results. It was noted that when cells fluoresced greater for IPTG and UAA-induced samples these values seemed slightly higher than for the *tRNA-met-3* promoter-based plasmids. A higher amount of protein was therefore purified from 28 mL cultures to compare the *tRNA-met-3* cassette and the *lpp* cassette.

Sample	72 hrs 30 oC, 20 hrs 4 oC				96 hrs. 30 oC, 20 hrs. 4 oC				120 hrs. 30 oC, 20 hrs. 4 oC			
	RFU/OD	Dev.	Reported	Dev.	RFU/OD	Dev.	Reported	Dev.	RFU/OD	Dev.	Reported	Dev.
Blank	--	--	-0.26	0.11	--	--	0.00	0.11	--	--	0.00	0.11
DC WT	2218.889	--	2220	--	3826.667	--	3830	--	5585.556	--	5590	--
DC:lpp:BpF non	1887.037	767.866	1890	768	3184.097	11.490	3180	11.5	2942.546	936.065	2940	936
DC:lpp:BpF ind-	2019.506	250.543	2020	251	3354.653	220.283	3350	220	5026.944	956.558	5030	957
DC:lpp:BpF ind+	3128.796	1026.156	3130	1030	4362.583	1264.071	4360	1260	3349.753	454.469	3350	455

Table 19: Average fluorescence of cells over 4 trials for DC3000:pSR8.052.1:pSR8.017

(*DC:lpp:BpF*) against wild-type DC3000 (*DC WT*). Cultures for transformed cells were either non-induced (*non*), IPTG-induced (*ind-*), or induced with IPTG and 1 mM BpF (*ind+*). Although two samples showed increasing fluorescence in the order of *ind+* > *ind-* > *non*, the other two cultures did not showed *ind-* > *ind+*.

Based on the fluorescence results, combined with lack of detection by Western blot (*Figure 27*), it is noted that the *lpp* promoter cassette did not augment GFPuvN149 expression. In fact, the only tRNA promoter system to give successful incorporation of BpF into GFPuvN149 is for the native *P. syringae* DC3000 as found in vector pSR8.010 (*Figure 26, 27, 28*).

3.5.1 Investigating Secretion of Full-length GFPuv N149 Mutants in Liquid Culture

During a replicate trial for DC3000:6.067 (Pyl-tRNA, GFPuv N149TAG), there appeared a greenish-glow in the liquid cultures after cell pellet collection, after a period of 120 hours. This fluorescence suggested there was significant full-length GFPuv in the culture supernatant that could be seen by eyesight. In presence of 365 nm light, even non-induced liquid sample featured a more intense green fluorescence. Therefore, it was decided to test other strains with tRNA and GFPuv N149TAG only, as well as DC3000 wild-type and DC3000 expressing the GFPuv WT. Subsequent observance of GFPuv WT fluorescence in the culture supernatant lead to culture

other DC3000 strains to see if cell-pellet based fluorescence results could be supplemented by any full-length GFPuv N149 mutants lost to the culture broth.

Attempts to normalize relative fluorescence of sample supernatants failed due to (a) inconsistent homogeneity of collected time-points which could not always be remedied by pipetting of solution, and (b) negative values of A280 (mg/mL) readings against KB-T as a blank for various time-points. Eventually, as no samples except the GFPuv WT cultures provided His-tagged proteins under Western blot detection, the inability to normalize liquid culture samples is not seen as a severe problem in this study.

However, it was regarded that wild-type DC3000 secretes small molecules, especially siderophores such as pyoverdinin (*Jones, 2007*), and other molecular bodies that possess fluorescence. To account for this, liquid cultures of wild-type DC3000 samples at given time-points were analyzed for fluorescence. Finally, a series of analyses for KB-T broth with added tetracycline at 10 $\mu\text{g/mL}$ acts as an additional corrective factor for the supernatant cultures with GFPuv (WT or N149TAG) and tRNA vector.

To observe if full-length GFPuv N149 mutants were expressed and secreted into culture milieu, samples were analyzed over several days post-induction. All time-points were taken from the same initial induced culture. Fluorescent analysis featured multiple reads on same sample as load to two or three wells in microplate. These triplicates were necessary as incident irradiation of GFPuv causes quenching and reduced fluorescence in second and later exposures. Culture supernatant samples were excited at 390 nm with emission detection at 510 nm as for cells.

To detect any full-length GFPuv mutants in the liquid supernatant, cultures were induced and incubated from 48 to 120 hours. Liquid culture supernatant fractions were analyzed by

fluorescence assay. Tetracycline in KB-T was additionally analyzed during intermittent fluorescence reads to obtain average emission for antibiotic at 510 nm of 670. +/- 239 RFU; this value is not featured in correction unless otherwise noted.

Interestingly, there was a consistency for IPTG-only induced cultures showing higher supernatant fluorescence than IPTG with UAA fluorescence for strains, DC3000:pSR6.067 +/- PylRS (WT or Y348F) and DC3000:pSR8.010 +/- RS (TyrRS WT, BpFRS or AzFRS), as well as strains featuring alternative tRNA promoters, *lpp* and *proK*. To further confirm no GFPuvN149 mutants were being secreted into the liquid culture, Western blot detection against 6xHis antibody was performed and the resulting images are shown in *Figures 21-25*.

3.5.2 Purification of GFPuv WT from DC3000 Supernatant

GFPuv WT has been expressed in DC3000 and was secreted into the media. GFPuv WT was purified from combined supernatant fractions (48, 72, 96-hours P.I.) and visualized on SDS-PAGE gel (*Figure 20*). Fractions featuring GFPuv WT were combined, concentrated, and later used as loading control for GFPuvN149 mutant analysis in transformed strains.

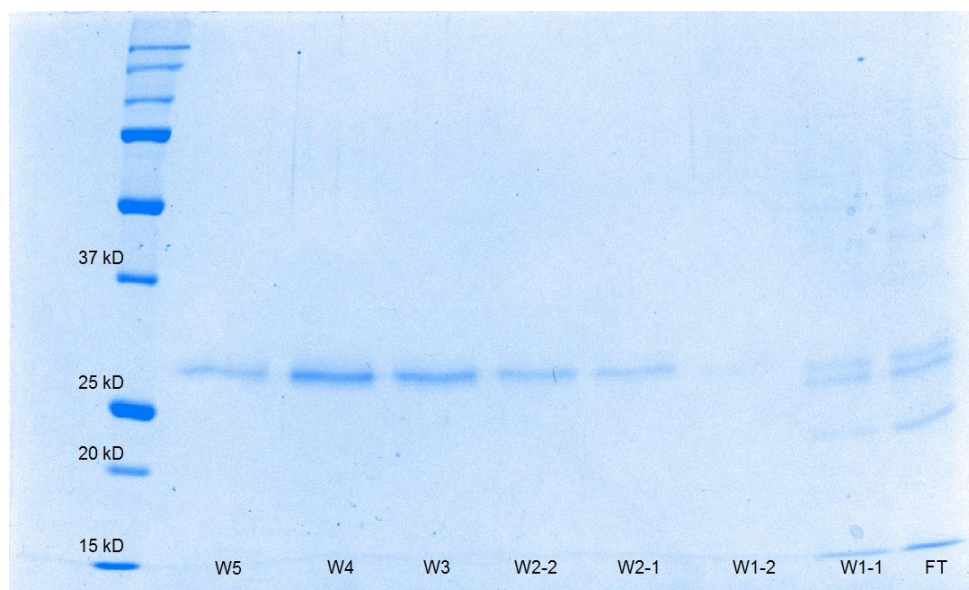


Figure 20: SDS-PAGE results for GFPuv WT purification from DC3000 liquid media. Protein began significant elution at 50 mM imidazole (W2-1) showing an expected 27.8 kD band, equivalent to full-length GFPuv-6xHis. (Lanes annotated as left to right: 1, empty; 2, protein ladder; 3, 500 mM imidazole wash fraction; 4, 100 mM imidazole wash fraction; 5, 74 mM imidazole wash fraction; 6, 50 mM imidazole wash fraction part 2; 7, 50 mM imidazole wash fraction part 1; 8, 25 mM imidazole wash fraction part 2; 9, 25 mM imidazole wash fraction part 1; 10, 3x flow-through (FT) fraction).

3.5.3 GFPuv WT Western Blot Analysis

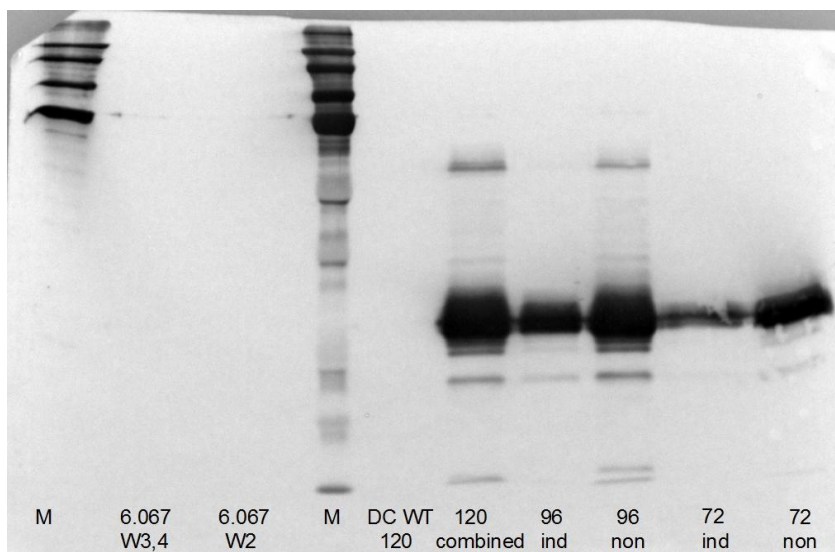


Figure 21: Western blot membrane showing GFPuv products recognized by 6xHis antibody. Time-points are for hours post-induction in DC3000 culture expressing either GFPuv WT, GFPuv N149 TAG along with *Mb. Pyl-tRNA* (6.067), or none (WT). GFPuv WT-6xHis is 27.8 kD in size and is the prominent band in the five right-hand lanes. No His-tagged GFPuv N149 mutant was seen in combined wash fractions from 6.067. (Lanes can be annotated as left to right: 1, pre-stained protein ladder; 2, 6.067 concentrated purification fraction 3 and 4; 3, 6.067 concentrated purification fraction 50 mM imidazole; 4, pre-stained protein ladder; 5, DC3000 WT 120-hour culture supernatant; 6, combined 120-hour DC3000:GFPuv WT supernatant; 7, DC3000:GFPuv WT 96-hour IPTG-induced supernatant; 8, DC3000:GFPuv WT 96-hour non-induced supernatant; 9, DC3000:GFPuv WT 72-hour IPTG-induced supernatant; 10, DC3000:GFPuv WT 72-hour non-induced supernatant).

3.6.1 Western Blot for 6.067

As supplement to previous fluorescence studies, *Figure 21*, lanes 2 and 3, shows concentrated fractions of purified supernatants from DC3000:6.067 96-hour induced and incubated cultures did not reveal 6xHis-tagged protein.

3.6.2 Medium-scale (25 mL) DC3000:6.067 Purification

It was plausible that a higher cell density of DC3000:pSR6.067 IPTG-induced culture could produce a significant amount of full-length GFPuvN149 mutants. Purification and concentration of 25 mL supernatant for 96-hour incubated IPTG-induced DC3000:6.067 was performed and examined by Western blot. However, there was no detected full-length GFPuv N149 mutant, suggesting that the original liquid culture fluorescence observation was not GFPuvN149 dependent.

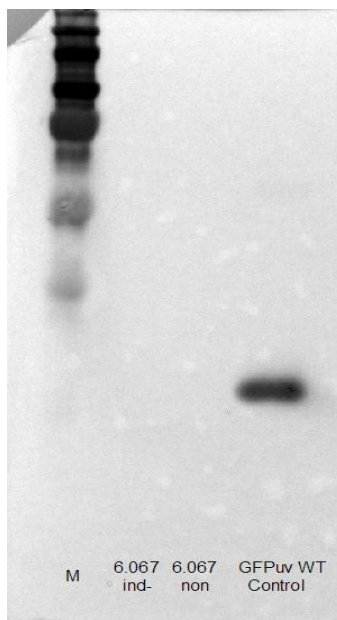


Figure 22: Western blot for detecting full-length GFPuv mutant in DC3000:6.067 purification fractions alongside GFPuv WT loading control (27.8 kD). *Lane order, left to right: 1, pre-stained marker; 2, DC3000:pSR6.067 25 mL ind- purification fraction; 3, DC3000:pSR6.067 non-induced fraction; 4, GFPuv WT control.*

There was no further investigation into the fluorescence of the DC3000:pSR6.067 strain and it was therefore assumed that the intensity of green tint to the liquid cultures that had inspired this supernatant-based inquiry was due solely to small molecules produced by DC3000 in the KB-T broth. It was still necessary to examine others cultures for GFPuvN149 in culture supernatant.

3.7 Western Blot for 96-hour Culture Liquids Media

Because it was seen that wild-type GFPuv was produced in cells and even secreted in high amounts into the liquid media, it was considered that any full-length GFPuv N149-UAA could also have been secreted into the milieu and therefore the cell-based fluorescence screening would not accurately represent the level of mutant GFPuv expression. It was necessary to grow DC3000 strains as induced +/- unnatural amino acids over the same time-course for the wild-type GFPuv-expressing strain. All cultures were prepared as above and grown for 5 days, as mentioned above. Liquid media samples at 96- and 120-hour time-points were analyzed by Western blot. However, there was no indication that any samples besides the DC3000:GFPuv WT expression strain secreted any full-length GFPuv N149 mutants. It should be noted that the AzFRS transformants (pSR8.010:pSR8.044) were not wrapped in foil for this experiment.

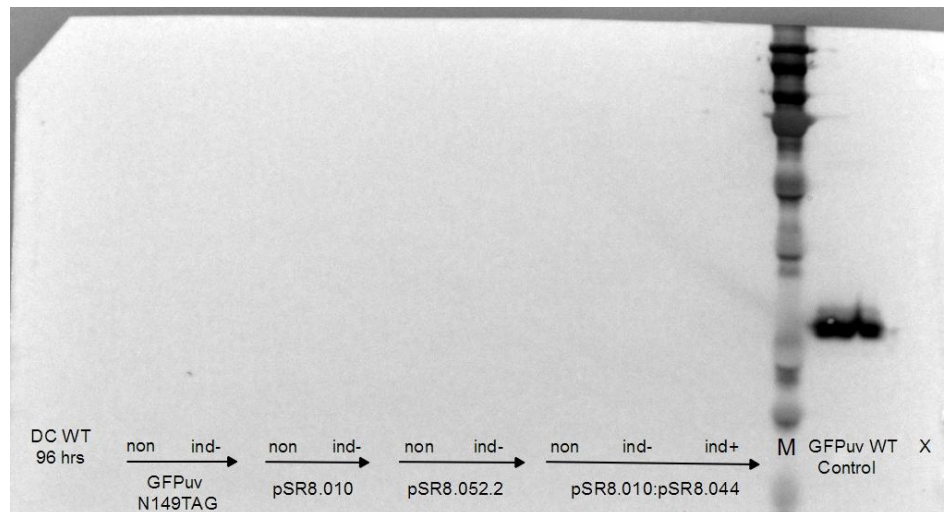


Figure 23: Western blot for nitrocellulose membrane showing select 96-hour post-induction samples from DC3000 strains. Only the pre-stained protein ladder and the GFPuv WT load control (27.8 kD) feature apparent bands, suggesting there was no full-length GFPuv N149 mutants being secreted into the culture. *Loading pattern (left to right): 1, DC3000 WT (DC WT) 96-hour culture broth; 2, DC3000:pBBR1MCS3-GFPuv N149TAG non-induced; 3, DC3000:pBBR1MCS3-GFPuv N149TAG ind- (IPTG induced); 4, DC3000:pSR8.010 non-induced; 5, DC3000:pSR8.010 ind- (IPTG induced); 6, DC3000:pSR8.052.2 non-induced; 7, DC3000:pSR8.052.2 ind- (IPTG induced); 8, DC3000:pSR8.010:pSR8.044 non-induced; 9, ind- (IPTG only); 10, ind+ (IPTG + 1 mM AzF); 11, pre-stained protein ladder; 12, GFPuv WT control; 13, empty.*

3.8 Western Blot for DC3000:pSR8.010: pSR8.017 and DC3000:pSR8.052.1: pSR8.017

Fluorescence assays routinely showed expected results of increased fluorescence with unnatural amino acid addition of *p*-benzoyl-*L*-phenylalanine (BpF). It was therefore necessary to ensure that no full-length GFPuv N149BpF mutants were being secreted into the liquid culture. Western blot detection of 96- and 120- hour cultures did not show any 6xHis-tagged proteins in those fractions, as compared with the loading control, GFPuv WT-6xHis which was purified directly from DC3000:GFPuv WT supernatant fractions. This confirms that is no full-length GFPuv N149BpF mutant being secreted. It is possible that there is either (a) truncated GFPuv N149TAG polypeptides being secreted, or (b) full-length GFPuv N149 mutants with a cleaved 6xHis-tag, but these are both unlikely as (a) truncated GFPuv would have to be formed in very high amounts before being secreted and (b) the probability of selective removal of the C-terminal His-tag would still suggest that supernatant fluorescence values would still be very high even if protein could not be detected by Western blot.

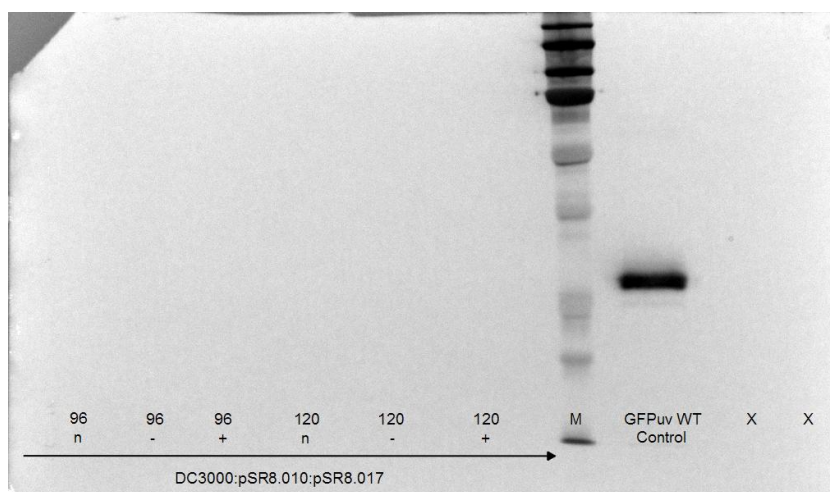


Figure 24: Western blot nitrocellulose membrane showing results from DC3000:pSR8.010:pSR8.017 non-induced (n), IPTG-induced (-) and IPTG-induced with 1 mM

BpF (+) in 96 and 120-hour culture supernatants. Only the GFPuv WT load marker (27.8 kD) and the pre-stained protein ladder feature any band appearance, suggesting there was no full-length GFPuv N149 mutants being secreted into the culture. (*Lane order, left to right: 1, 96 hrs. non-induced; 2, 96 hrs. IPTG-induced; 3, 96 hrs. IPTG and BpF induced; 4, 120 hrs. non-induced; 5, 120 hrs. IPTG-induced; 6, 120 hrs. IPTG and BpF induced; 7, pre-stained ladder; 8, GFPuv WT control; 9, empty lane; 10, empty lane.*)

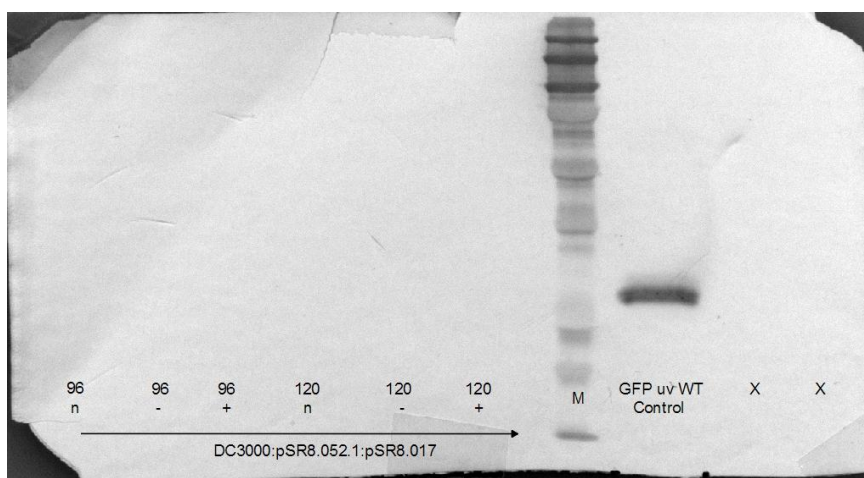


Figure 25: Western blot nitrocellulose membrane showing results from DC3000;pSR8.052.1;pSR8.017 non-induced (non), IPTG-induced (ind -) and IPTG-induced with 1 mM BpF (ind +) in 96 and 120-hour culture supernatants. Only the GFPuv WT load marker (27.8 kD) and the pre-stained protein ladder feature any band appearance, suggesting there was no full-length GFPuv N149 mutants being secreted into the culture. (*Lane order, left to right: 1, 96 hrs. non-induced; 2, 96 hrs. IPTG-induced; 3, 96 hrs. IPTG and BpF induced; 4, 120 hrs. non-induced; 5, 120 hrs. IPTG-induced; 6, 120 hrs. IPTG and BpF induced; 7, pre-stained ladder; 8, GFPuv WT control; 9, empty lane; 10, empty lane. Position of lanes shown on membrane are approximate for samples without apparent bands.*)

3.9 Mid-scale Culture for DC:pSR8.010:BpFRS, DC:8.052.1: BpFRS

Purification of GFPuv N149BpF protein from 50-hour incubated induced DC3000 cultures featuring the evolved BpFRS and the corresponding *M. jannaschii* Tyr-tRNA with either the *tRNA-met-3* or the *lpp* promoter cassette was performed.

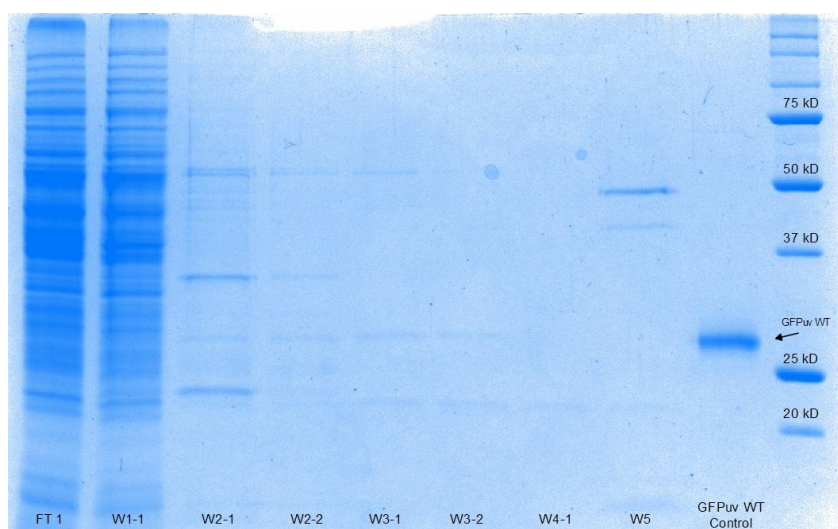


Figure 26: 15% SDS-PAGE gel showing purification fractions of GFPuv N149BpF from DC3000:pSR8.010:pSR8.017 featuring *tRNA-met-3* promoter. Full-length GFPuv N149 mutant is shown in lanes for W2-1, W2-2, W3-1, and W3-2. Lane order, left to right: 1, 3x flow-through; 2, W1-1 (25 mM imidazole); 3, W2-1 (50 mM imidazole); 4, W2-2; 5, W3-1 (75 mM imidazole); 6, W3-2; 7, W4-1 (100 mM imidazole); 8, W5 (500 mM imidazole); 9, GFPuv WT control; 10, unstained protein ladder.

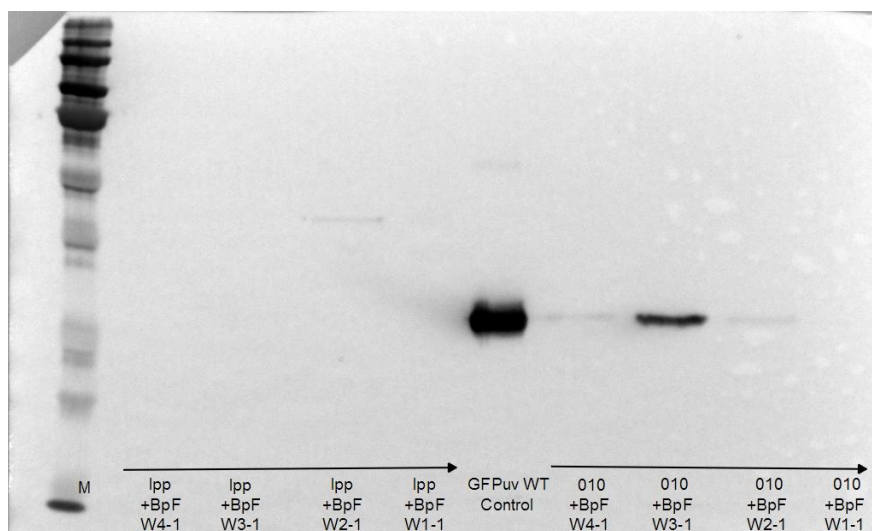


Figure 27: Western blot nitrocellulose membrane showing purification fractions of GFPuv N149BpF from DC3000:pSR8.010:pSR8.017 (010+BpF) and DC3000:pSR8.052.1:pSR8.017 (lpp+BpF). Full-length GFPuv (WT or N149BpF) features 6xHis-tag and is appears for GFPuv WT control (27.8 kD) and 010+BpF wash fractions 2, 3, and 4. Lane order, left to right: 1, pre-stained ladder; 2, lpp+BpF W4-1; 3, lpp+BpF W3-1; 4, lpp+BpF W2-1; 5, lpp+BpF W1-1; 6, GFPuv WT control; 7, 010+BpF W4-1; 8, 010+BpF W3-1; 9, 010+BpF W2-1; 10, 010+BpF W1-1.

Expression of GFPuvN149BpF from *P. syringae* DC3000 has been shown in both fluorescence studies and protein purification (Table 11, Figure 27). In addition, fluorescence data from Table 16 shows that GFPuvN149AzF full-length protein can be expressed in DC3000. Therefore, it is reasonable to suspect other unnatural amino acids – recognized by other evolved *M. jannaschii* tyrosyl-synthetase – could be incorporated in *P. syringae* DC3000 using this system. These results are proof of the success of the present invention for incorporation of unnatural amino acids in the plant pathogen

3.10 Further Increasing the Efficiency of Working Parts

It should be possible to increase expression of the synthetase in plasmid pML123 by substituting for the *T5* promoter and terminator regions with the neomycin promoter and terminator sequences to flank the synthetase insert. It was shown by *Labes, et al.* in 1990 that the neomycin promoting cassette yields very high protein expression for β -galactose in soil bacteria and lower expression levels in *E. coli* (Table 20). This could be suitable for studies involving molecular probes, although effector studies may require weaker promotion.

Plasmid (promoter)	<i>E. coli</i>		<i>R. meliloti</i>		<i>R. leguminosarum</i>		<i>P. putida</i>	
	β Gal	Nm	β Gal	Nm	β Gal	Nm	β Gal	Nm
pML122 <i>lac</i> (pNM)	1400	R	21800	R	13900	R	16300	R
pML130 <i>lac</i> (<i>plac</i>)	2000	R	9050	R	6250	R	9800	R
pML132 <i>lac</i> (<i>tac</i>)	11300	R	2850	R	1150	R	2950	R
pML140 <i>lac</i> (<i>pS1</i>)	40	S	3300	R	1200	R	3350	R
without plasmids	16	S	110	S	130	S	150	S

Table 20: β Gal activities and Nm^R gene expression of vectors carrying the *lacZ* and Nm^R genes in Gram- hosts. Literature reference featured neomycin (Nm) at 100 μ g/mL working concentration; β Gal units are as Miller units. (*R, resistant; S, susceptible; adapted from Labes, 1990; Table II*).

Another promoter to considerably enhance the expression of biomolecules (reporter, synthetase, and even effector proteins) for this UAA incorporation system is the *Amaranthus hybridus* chloroplast promoter, *PpsbA*, as found in plasmid pDSK-GFPuv (*Wang, 2007*). This plasmid was designed for mobility and attachment assays of phytopathogens in planta, especially for the use of mutated GFPuv proteins as biological probes in plant pathogens and their hosts. Indeed, pathogen-expressed probe/effector fused to a Type III secretion signal would be greatly benefitted by use of such a strong promoter. As *PpsbA* is constitutive, in planta studies of

infection may have to be optimized against for severe overexpression of particularly active effector proteins – a dead specimen is not as good for study as one surviving.

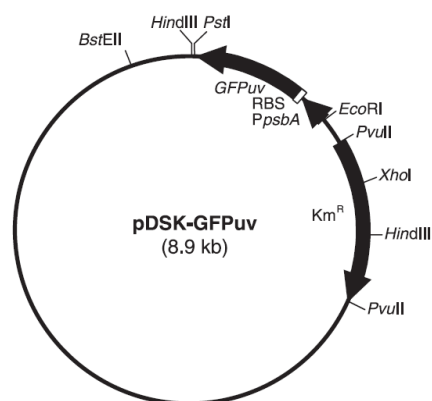


Figure 28: Plasmid map of pDSK-GFPuv featuring GFPuv under promotion of *psbA* chloroplast promoter (PpsbA) and *T7 gene10* ribosome binding site (RBS). {Retrieved from Wang, 2007}

3.11 Secretion of GFPuv WT from DC3000, Revisited

One unexpected result of this study shows that overexpression of the wild-type GFPuv protein in DC3000 produces a GFPuv-dependent fluorescent supernatant that emits differently than any siderophore-based fluorescence produced by the strain. This process appeared even within 48 hours of GFPuv expression in the bacteria, suggesting the GFPuv was likely due to protein secreted from live bacteria, whether than being the residual molecular species from culture-contained burst cells. As the live bacteria pellets also showed fluorescence, it is likely there is a maximal expression amount that can exist within the organism before subsequent secretion of full-length protein into the culture broth. It is also reasonable to suspect the GFPuv content of the liquid, just as in the cells, was not as aggregation and was fully-folded, as non-folded and aggregated forms of GFP (and most likely GFPuv) do not fluoresce (Heim, 1994;

Tanudji, 2002). This secretion event was likely to keep the intracellular compartments from bursting under increasing cellular stress at longer time-points. Incidentally, it was also observed (*data not shown*) that liquid culture supernatants for DC3000:GFPuv WT showed increased fluorescence over DC3000 wild-type in LB media – a richer nutrient broth that would therefore negate any mineral deficiencies for the bacterial strain and thereby making repressing the usual secretion of metal-chelating siderophores. This occurred even after 1.5 hours post-induction (*data not shown*), and further supports the possibility that GFPuv was not being released by dead bacterial cells. It has not been determined by what method these GFPuv WT proteins were being secreted, although it can be assumed the process does not involve virulence-based Type III secretion system (T3SS) as the GFPuv WT protein did not feature a T3SS signal motif, nor did the GFPuv WT contain any other secretion-related peptide signals. Further insight into this process would require very strong expression of the GFPuv N149-BpF mutant, with which one could instigate UV-induced crosslinking of the mutant protein to covalently attach to its exporting protein partner(s). Additionally, if a high concentration of expressed proteins, such as antibiotic resistance proteins not needed for environmental survival, were to be present at high levels in the supernatant, then there may be further interest in using *P. syringae* DC3000 as a lower temperature organism for production of biomolecules for research or educational resources. An example experiment would include transformation of DC3000 cells with a plasmid containing both kanamycin and gentamycin acetyltransferase, but for the transformants to be grown only in gentamycin liquid stock. Presumably, protein electrophoresis or more sensitive blotting techniques (e.g. for kanamycin fused to a 6xHis-tag and induced by IPTG) would quantify any supernatant-localized secreted proteins.

3.12 Possible Role of P. syringae pv. tomato DC3000 as Resource for Primary, Junior, and Secondary Education

Given the easy acquisition of GFPuv from the culture media, it is also likely that other proteins over expressed in DC3000 may be secreted into the liquid media, thus allowing a nearly continuous supply of expressed protein in the supernatant that could be purified by various methods. This supernatant-extraction process could be considered especially useful in laboratories lacking sufficient lysis methods – including primary, middle, and secondary school science labs. Indeed, this would also allow for such small laboratories experiments to have no need of toxic chemicals such as phenol and chloroform, caustic or heavy denaturing agents, or sonication or pressurized-lysis instruments. It has not been observed that strains of *E. coli* K-12 cells expressing GFPuv will secrete this full-length protein into their liquid culture without specialized secretion signals (Mergulhao, 2005) or selective permeation (Penna, 2002). DC3000, therefore, can be considered a less time-involved species for producing some biological products.

Consequently, because *P. syringae* pv. tomato DC3000 is not a human pathogen and is not suited for epiphytic survival, and because the species can grow at a range of temperatures, from 4 °C to 30 °C, room temperature cultures can be incubated on older model shakers and even on sterilized bench, allowing financially-burdened or frugal laboratories to study microbiological techniques with little need of expensive incubation chambers. Protein purification would be facile for younger students as this particular strain expressed a GFPuv WT with a terminal 6xHis-tag. After collecting supernatant samples, students could gain practice of purifying proteins and build an understanding of useful analytical techniques. For GFPuv, specifically, subsequent study of the fluorescence due to proper protein folding at differing pH levels, temperatures or in differing chaotropic agents, such as urea or other amines will help the students understand how

protein activity (analogizing enzymes to fluorescent proteins) is dependent upon specific environmental factors. Finally, should one possess strains that can produce inexpensive mutant GFPuv biological probes, this would allow students to use such tools to understand more of the complex chemistry occurring in biological systems. Regardless, these characteristics – for wild-type GFPuv or mutants – also enable students to study the bacteria and the plant host (e.g. *S. lycopersicum* or *A. thaliana*) interactions, for both the tomato plant and the *Arabidopsis* can be easily grown in a make-shift, moderately sterilized laboratory area.

3.13 Expanding the Usefulness of the Present Invention

Although the direct feature of this plasmid system relates to using the benzophenone and azido derivatives of phenylalanine, other unnatural amino acids can be incorporated given the properly-evolved synthetase. In literature to date, there have been a large number of UAAs constructed, any of which could ideally be featured in formed mutated proteins – proteins that could not only capture host cell targets or partners within the bacteria itself (e.g. transcription factors or scaffold and transporter proteins), but also for study of cellular dynamics, such as free-flow of molecular species *in situ* or redox-related changes using a reporter protein capable of losing fluorescence, quantitatively, in presence of hydrogen peroxide, hydrogen sulfide, ionic mineral concentrations (Fang, 2012; Niu, 2013), nitric oxide, or other signaling molecules. In addition, *in situ* studies are not limited to using a specialized form of the GFPuv protein as a biological probe. This current study was developed with the intent of incorporation crosslinking amino acids, notable BpF and AzF, into secreted effector proteins and capturing their host targets during the natural infection process of the *P. syringae* pv. tomato DC3000 phytopathogen and *A. thaliana* or *S. lycopersicum*. However, this thesis work can be extended to other organisms.

There are over 50 pathovars of *P. syringae*, alone (Xin, 2013), and it is expected that the present dual plasmid system is applicable direct study of these pathovars with their individual hosts. This would be especially beneficial for species such as bean pathovar, *P. syringae* pv. *syringae* B728a, which is a model pathogen for study of epiphytes' sensing of the extra-host environment (UV, wind, temperature). With an alteration of either the tRNA or the synthetase species, this UAA-incorporation system can be used for study in other plant pathogenic bacteria. The two parent plasmids used in this system are characteristically broad-host range bacterial expression plasmids. Notable prokaryotes for study include *Erwinia* spp., *Xanthomonas* spp., *Rhizobium* spp. (Hueck, 1998), or agriculturally-beneficial bacteria, such as *Lysobacter enzymogenes* (Wang, 2013), and even *Agrobacterium tumefaciens* – peaking interest in both pathogen-based and plant transformation studies. UAA-based modifications of *L. enzymogenes* proteins should be easily accomplished by substituting the tRNA vector, pBBR1MCS3 (Tet^R) with its chloramphenicol resistance marker counterpart, pBBR1MCS (Kovak, 1995), as this bacteria is resistant to common antibiotics, excepting gentamycin and chloramphenicol (Wang, 2013). And certainly, successful use *A. tumefaciens* to transform plant species with UAA-incorporating machinery would open a large field of biotechnological study.

Incorporation of UAAs into any of the above bacteria, for example, requires (a) no significant TAG stop codon suppression tRNA native to the system -- although concerns on this point are minimal (Povolotskaya, 2012), (b) that there is no suppression of other stop codons if they are used less frequently than TAG, (c) a suitable pair of exogenous tRNA/RS, for example, *M. barkeri*, or *M. jannaschii*, or other biological species (Chatterjee, 2012), orthogonal to the host organism's native translation machinery, (d) a species for study that is capable utilize the selected unnatural amino acid, and (e) promoting cassette(s) optimal for yielding satisfactory expression

of all exogenous molecules without causing unnecessary toxicity to the bacterium or, if applicable, the later infected host. For most requirements the methods will be similar to what has been described herein. This thesis can therefore be a starting guide to produce a set of optimized working components for the incorporation of unnatural amino acids for study of intracellular and extracellular environmental events such as bacterial-host molecular interactions and this basis of disease.

This thesis presents the methods and resulting success for incorporation unnatural amino acids into the model plant pathogen, *Pseudomonas syringae* pv tomato DC3000 – a task that had not yet been performed – and thus produces a new tool for researchers to study the molecular mechanisms involved in bacterial pathogen-host interactions.

Chapter 4. Materials and Methods

4.1 Common Instruments and Hardware

Nanowater was provided from Barnstead Nanopure (*Thermo Scientific*); Isotemp 2320 water bath, Nanodrop 2000, Precision incubation chambers, and MaxQ 8000 incubation shakers (*Thermo Fisher*); digital analytical balances (*Mettler Toledo*); Manual single-channel pipettes (*Rainin; Mettler Toledo*); pipet tips, polystyrene plates, analog vortex mixer, Digital Heat Block (250 V), and hot plate/stirrer, 50 mL conical tubes, and electroporation cuvettes were purchased from *VWR*; culture tubes, PCR tubes (0.25 mL), laboratory glassware, and additional 50 mL conical tubes from *Fisherbrand*; Mastercycler thermocycler, Mini Spin Plus centrifuge, Centrifuge 5424, and Electroporator 2510 (*Eppendorf*); Allegra X6-R bench-top model (*Beckman-Coulter*); Axygen 0.6 mL and 1.5 mL centrifuge tubes (*Corning*); sonication of cells performed with Model 120 Sonic Dismembrator (*Fisher Scientific*); and all images for DNA gels, SDS-PAGE gels, agar plates, and Western blot colorimetric development of membranes were obtained by GelDoc XR+ imaging system with ImageLab software (*BioRad*).

4.2 Bacterial Cell Preparations

4.2.1 Preparation of Liquid and Solid Culture Media: LB and KB-T

E. coli cells required use of lysogeny broth (LB) as prepared with LB broth media (*Teknova*). LB/agar plates were prepared with additional of 15.0 g/L agar (*Criterion by Hardy*) and, following autoclave sterilization of LB/agar mixture, appropriate working concentration of antibiotics.

P. syringae pv. tomato DC3000 cells required use of modified King's B medium with tryptone substituted for peptone. The modified medium contains 9 mM K_2HPO_4 , 1.6 mM $MgSO_4 \cdot 7H_2O$, 1% v/v glycerol, pH 7.0, with added 2% w/w tryptone (*Bacto*; *BD*). KB-T agar plates were prepared with additional of 18.0 g/L agar and, after autoclave sterilization, appropriate working concentration of antibiotics.

It can be noted that *P. syringae* pv. tomato DC3000 is naturally resistant to both rifampin (as per the genotype) and also chloramphenicol, presumably at concentrations less than 17 $\mu\text{g/mL}$. However, growth of wild-type strain was done without addition of any antibiotics.

All culture media reagents were purchased from Sigma-Aldrich unless otherwise stated. $MgSO_4 \cdot 7H_2O$ was purchased from EMD Millipore. All nanowater was autoclaved before use.

4.2.2 Preparation of Chemical Competent Cells

Chemical competent cells were prepared in CCMB media and store at $-80\text{ }^\circ\text{C}$ until use. CCMB media components: { $CaCl_2 \cdot 2H_2O$ (80 mM), $MnCl_2 \cdot 4H_2O$ (20 mM), $MgCl_2 \cdot 6H_2O$ (10 mM), potassium acetate (10 mM), glycerol (10% v/v), pH 6.4}; media was sterilized through 0.2 μm filter (*VWR*) and stored at $4\text{ }^\circ\text{C}$. Reagents were purchased from *Sigma Aldrich* ($CaCl_2 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$, glycerol) and *Fisher* ($MgCl_2 \cdot 6H_2O$ and potassium acetate).

Chemical competent cells were prepared as follows: overnight inoculants were grown at $37\text{ }^\circ\text{C}$, 250 rpm before subculture into 100 mL LB. Subcultures were incubated to OD_{600} 0.4 to 0.6, then cells were collected over two rounds of centrifugation {5000 x g, 10 min}. Broth was decanted and cells were suspended in 1 mL CCMB media, collected as above, and recovered in 4 mL CCMB. Aliquots were prepared for storage in 1.5 mL or 0.6 mL tubes. In some experiments, liquid ratios were fixed but volume was reduced.

4.2.3 Transformation of Cells

All transformations for *E. coli* strains, Genehog, NEB5 α (*New England Biolabs*), or NEB5 α F' (*Wei Niu, University of Nebraska-Lincoln*), were performed via heat shock method for chemical-competent cells stored at -80 °C. Plasmid DNA was added at 10%, or less, cell volumes and set on ice for 10 minutes before incubated at 37 °C for 30 seconds, with return to ice for 1-2 minutes. Heat shocked cells were recovered with either 500 μ L lysogeny broth for Genehog and NEB5 α transformations featuring Pyl-tRNA/RS system, or 10x initial cell volume of LB (for Genehog cells featuring *Mj.* plasmids). Recovered cells were incubated at 37 °C, 250 rpm for 40-60 minutes in MaxQ 8000 shaker and then plated to pre-warmed (37 °C) LB/agar plates with appropriate antibiotics and plated cells incubated in 37 °C for 1 or 2 days in incubation chambers to obtain medium-sized colonies suitable for inoculation.

All *P. syringae* pv. tomato DC3000 transformations were performed via electroporation using Electroporator 2510. Previous to electroporation, cells were swabbed from 30 °C-warmed plates (1-60 days old) and suspended in 1 mL of cold (4 °C) 1 M HEPES, 10% glycerol (pH 6.8) by vortexing on high and centrifugation at 4 °C, 16 200 x g, 1 minute. Cells were washed an additional two times in cold 1 mL HEPES/glycerol buffer, each, and final pellets were suspended by pipette in 60 μ L on ice. Plasmid DNA was added at 4 μ L for 60 μ L washed cells and this suspension was transferred to pre-chilled electroporation cuvette and kept on ice for 2 minutes before 2500 V pulsing to obtain time constant values between 1.4 and 5.4, depending on cell density in 60 μ L suspension. Immediately after pulse, 400 μ L of cold KB-T broth was added to cuvette to recover cells, after which the volume was transferred to original 1.5 mL tube and set into 30 °C, 250 rpm for one hour of incubation in MaxQ 8000 shaker. Incubated cells were plated to pre-warmed (30 °C) KB-T/agar plates at 150 μ L cells per plate and set for 1-3 days in 30 °C

Precision chamber. It can be noted that for any time constant in the range given, plated cells would generally grow well over an average 2-day period and plates featuring *P. syringae* DC3000 transformants will continue to grow in 4 °C refrigerator up to 6-months if there is ample solid agar media for growth; colonies on these plates, however, will be yellowish-green in color, thick in biofilm, and sticky.

Working concentrations of antibiotics included ampicillin (100 µg/mL), chloramphenicol (34 µg/mL), kanamycin (50 µg/mL), tetracycline (10 µg/mL), and gentamycin (10 µg/mL). All antibiotic solutions (as mg/mL) were stored at -20 °C until needed and returned to freezer after use. Ampicillin, chloramphenicol, and tetracycline were supplied by *Calbiochem*; kanamycin sulfate purchased from *IBI Scientific*; gentamycin was purchased from *Teknova*.

4.3 Recombinant DNA Methods

4.3.1 Plasmid DNA Miniprep

All plasmid DNA was purified from overnight cell cultures following manufacturer's protocol. Three different miniprep kits were used in house during the duration of this project. For *M. barkeri*-related plasmids, PureYield Plasmid Miniprep Kit (*Promega*) was used, while for *M. jannaschii*-related plasmids, Zyppey Plasmid Miniprep Kit (*Zymo Research*), excluding vector pSR8.017, which was purified using GeneJET Plasmid Miniprep Kit (*Thermo Scientific*). All plasmids were eluted in nanowater and DNA concentration tested at 260 nm with Nanodrop 2000.

4.3.2 PCR Methods

All polymerase chain reaction (PCR) procedures utilized autoclaved nanowater (ddH₂O), KOD Hot Start Polymerase (KOD; *EMD Millipore*) and featured 30 cycle programs for denaturing, annealing, and extension stages of reaction. Tables for reaction setup are shown in Appendix I.

4.4 Preparation of Unnatural Amino Acid Reagent

Amino acids used in this study included *p*-benzoyl-*L*-phenylalanine, *p*-azido-*L*-phenylalanine, and boc-lysine (*Bachem*). The amino acids, *p*-benzoyl-*L*-phenylalanine (BpF) and boc-lysine were stored at room temperature, while *p*-azido-*L*-phenylalanine (AzF) was stored at -20 °C.

Amino acid solutions were prepared in 0.6 mL, or 1.5 mL at a concentration of 14-60 mM in 30% 1 M NaOH in nanowater {e.g. 2 mg BpF, 150 μL 1 M NaOH, 350 μL nanowater for a small scale culture of 1-5 mL}.

4.5 Preparation of Cultures – Growth and Lysis

4.5.1 Culture Growth

Inoculants were prepared from plated cells at 30 °C or 4 °C, or from glycerol stocks (6 μL from -80 °C storage) into KB-T broth with appropriate working concentration of antibiotics (e.g. 3 mL KB-T broth, 3 μL Gen¹⁰ stock, 2.6 μL Tet^{12.5} stock) and grown at 30 °C, 250 rpm for 16-48 hours, until saturation (OD₆₀₀ > 1.2). Subcultures were then prepared in KB-T broth with antibiotics as OD₆₀₀ of 0.4 and grown at 30 °C, 250 rpm to final OD 0.7-1.0. These mid-log OD (optical density) cultures were charged with working concentrations of 0.5 mM IPTG (*Fisher Scientific*).

Where applicable, induction also required 1 mM BpF or AzF solution or 5 mM Boc-lysine solution, followed by addition of 1 M HCl at 1:1 molar volume with NaOH in amino acid solution (e.g. for 150 μ L NaOH in 500 μ L total amino acid solution, addition of total volume to culture requires follow-up with 150 μ L 1 M HCl. Cultures were incubated until time-point collection and set into 4 $^{\circ}$ C refrigerator overnight for 16-20 hours before pelleting cells. Wild-type DC3000 was grown from 4 $^{\circ}$ C plates in KB-T with no antibiotics, followed by subculture to OD₆₀₀ 0.4 at the same time as transformed strains, and incubated for equal durations, including 4 $^{\circ}$ C overnight refrigeration and further sample preparation.

4.5.2 Cell Lysis Methods

Lysis of cells for protein analysis required re-suspension of collected cell pellets (4 $^{\circ}$ C, 5000 x g, 10 min; *Allegra X6-R*) into 2 mL lysis buffer per 50 mL cell culture to allow for sonication of cells. Sonication lysis buffer contained 20 mM Tris-HCl (*Trizma*; *Sigma Aldrich*), 0.5 M NaCl, 10% glycerol and 10 mM imidazole, pH 8.0, unless otherwise noted. Sonication was effected and total protein content was checked at 280 nm with Nanodrop 2000 to note protein concentration after complete bursting of cells.

4.6 Purification of Proteins Featuring C-terminal His-tag

All GFPuv-6xHis-tagged proteins expressed as full-length molecular species were purified using GE Ni²⁺-Sepharose resin (*GE Healthcare*) housed in Econo-Pac Chromatography Columns (*BioRad*). Purification buffers were as for lysis buffer (3.5.2) except with wash #1 -5 containing 25 mM, 50 mM, 75 mM, 100 mM, and 500 mM imidazole, resp. Each washing step involved five column volumes of washing buffer, as per manufacturer's protocol. Protein content in purification wash fractions was tested by Nanodrop 2000 with respective washing buffers (or

lysis buffer for total protein, soluble protein, and flow-through) as blank for mg/mL determination. Where applicable, fractions containing protein of interest were combined and concentrated using either Amicon Ultra-4 Centrifugal Filter Unit or Amicon Ultra 0.5 mL Centrifugal Filter, each with 3000 kilodalton (kD) molecular weight cut-off. All Amicon filters were used according to manufacturer's protocol and centrifugations were performed at 4 °C in Sorvall Legend Micro 21R refrigerated microcentrifuge, for 0.5 mL Filter units, or using Allegra X6-R bench-top centrifuge. For GFPuv proteins purified from DC3000 cultures, no storage buffer was prepared or used, as protein samples were not needed further for activity assays.

4.7 DNA and Protein Analysis (SDS-PAGE) Methods:

4.7.1 Preparation of DNA Electrophoresis Gels

All DNA electrophoresis gels were made in house as 0.8-1.2% agarose (*LE, analytical grade; Promega*) in TAE buffer {200 mM Tris, 100 mM acetic acid, 5 mM EDTA, in nanowater} featuring ethidium bromide (*Promega*) as nuclei acid stain at 0.006-0.008% v/v. DNA gels were solidified on Mini-Gel Caster and DNA gels were run in Mini Sub-Cell GT Cell or Wide Mini-Sub Cell GT Cell powered by PowerPac Basic Power Supply (*BioRad*).

4.7.2 Preparation of SDS-PAGE Gels

Denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using Mini-PROTEAN Tetra Cell electrophoresis chamber and a PowerPac Basic Power Supply (*BioRad*). SDS-PAGE gels were made in house as 15% polyacrylamide resolving gel {25% nanowater, 25% Tris, pH 8.8} with a 10% polyacrylamide stacking gel {55% nanowater, 25% Tris, pH 6.8}. Electrophoresis was performed in presence of Tris/glycine/SDS buffer {124 mM Tris, 960 mM glycine, 17 mM SDS, in nanowater}. All SDS-PAGE protein

samples were loaded to prepared gels after mixing with 1:1 volume with 2x Laemli Buffer (*BioRad*) featuring 5% 2-mercaptoethanol (*Sigma Aldrich*) and boiled for 15 minutes at 91-98 °C. Unstained and pre-stained protein marker ladders (*BioRad*) were used and SDS-PAGE gels were typically run at either a constant 35 mA or constant 200 volts at room temperature. After running SDS-PAGE gels were removed from cassette and rinsed in warmed water on rocker to remove protein sample dye, followed either by staining with Coomassie blue stain {70 mg Brilliant Blue G-250 (*MW* 854.02; *Fisher Scientific*) into 1.0 L nanowater with 3 mL conc. HCl; stir at room temperature for 4-6 hours} or directly used for Western blot.

4.8 Western Blot Protein Detection

SDS-PAGE gels rinsed in warmed water were soaked in cold (4 °C) Western blot transfer buffer {25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3-8.5} and set into transfer cassette against nitrocellulose membrane (*BioRad*). Membrane transfer was performed at constant 100 volts for 1 hour in BioRad Mini Trans-Blot Cell transfer chamber at room temperature (RT) in cold transfer buffer. Transferred membrane were cut for sizing with ethanol-rinsed razor and rinsed twice in 10-15 mL PBS (*BioRad*; 0.5-1.0 NaCl, 0.1-0.25% sodium dihydrogen phosphate) with added 0.1% Tween-20 buffer (*Sigma Aldrich*) with rocking for five minutes at RT. Membrane was then blocked overnight for 16-20 hours at 4 °C, with rocking, in 3% blocking solution (*BioRad*) and then rinsed twice for 5 minutes in 10 mL PBST buffer. Primary antibody, 2 µL mouse αHis-tag (*Pierce prod #MA1-21315; Thermo Scientific*) was added to PBS + 1% BSA (*OmniPure, Calbiochem*) for 1:3000 working dilution and membrane was incubated with rocking for 1-2 hours at RT, followed by five separate washes in 10 mL PBST for five minutes, each. Secondary antibody, GaM-HRP (*BioRad*), was diluted to 1:3000 in PBS + 1% BSA {2 µL into 6 mL solvent} to the membrane and incubated for 1-1.5 hours on

room temperature rocker. Membrane was then again rinsed five times for five minutes in 10 mL PBST, each. Colorimetric detection of 6xHis-tagged protein samples on membrane was performed using Opti-4CN detection (*BioRad*) according to manufacturer's protocol, which involved rocking at RT, covered from light, for 28-30 minutes. All developed membranes were then rinsed twice for 5-10 minutes in nanowater before imaging on GelDoc XR+.

4.9 Vector Construction and Plasmid-related Culture Preparations

Initial GFPuv (WT and N149TAG), GFPuv/Pyl-tRNA and PylRS vector design was developed by Wei Niu (*University of Nebraska-Lincoln*). Plasmids were constructed by the author of this work. Setup and program methods for all PCR reactions are provided in Appendix I. All restriction enzymes were purchased from *New England Biolabs*, unless otherwise noted. All plasmid maps were designed by the author with SnapGene software {<http://www.snapgene.com>}, excluding those from cited reference.

4.9.1 pBBR1MCS3-GPFuv N149TAG and pBBR1MCS3-GFPuv WT

Vector pBBR1MCS3 was used for construction of pBBR1MCS3-GPFuv N149TAG and pBBR1MCS3-GFPuv WT. Parent vector, pBBR1MCS3, was cut with *XbaI* and dephosphorylated for 1 hour with Antarctic Phosphatase (*New England Biolabs*) at 37 °C, followed by heat inactivation of enzyme for 5 minutes at 80 °C. Both GFPuv N149TAG and GFPuv WT (wild-type) were cut from their respective pLei-GFPuv vector (group lab stocks) using *NheI* and *XbaI* to feature a 1.1 kb band, and digestions were followed by 1-hour dephosphorylation as for parent vector. All plasmid digestions were performed at 37 °C for 3 hours and restricted plasmids were purified via gel electrophoresis with recovery via ZymoClean Gel DNA Recover Kit (*Zymo Research*), or directly (pBBR1MCS3) by same Zymo Research column purification kit. Ligation

of restricted vector and insert was performed at room temperature for 1 hour with T4 Hot Start ligase (*Thermo Scientific*) and samples were then set on ice prior to transformation of *E. coli* chemical competent cell via heat shock method, or transformation of DC3000 via electroporation. All pBBR1MCS3 constructs evoke tetracycline resistance to viable transformants.

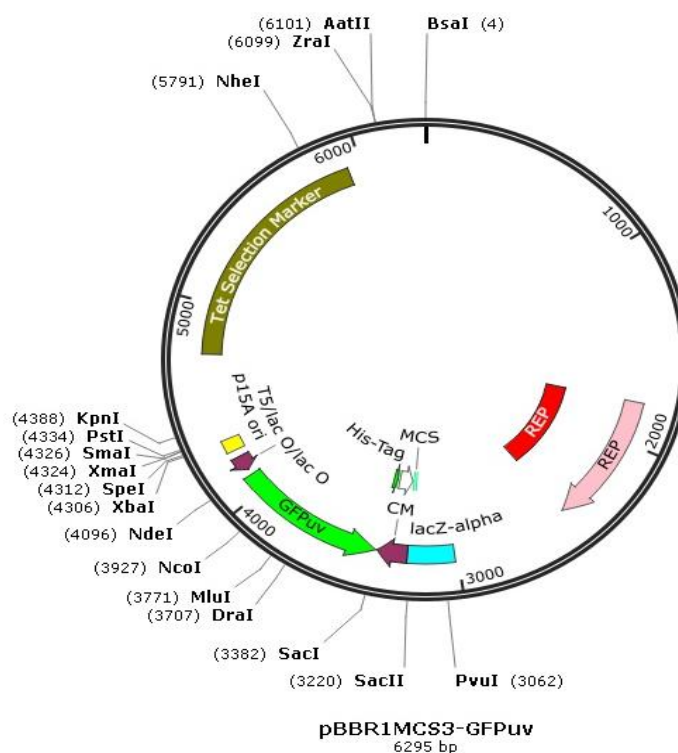


Figure 29: Plasmid map for pBBR1MCS3-GFPuv (WT or N149TAG)

4.9.2 pBBR1MCS3-GFPuv N149TAG-Mb. Pyl-tRNA (pSR6.067)

Design of vector containing the exogenous tRNA utilized a constitutive promoter based on the native *TRNA-met-3* promoter region in DC3000. The upstream region was amplified via polymerase chain reaction (PCR) from chromosomal DNA using Pure-link Genomic DNA Kit (*Invitrogen*) with primers, *tRNA-pBBR-F1-KpnI* (5'-GTG GTA CCG ACC TGA TGG GAT TTT

GCA ATC AAT G-3') and *tRNA-pBBR-R1* (*5'-TCT GTG TGT CGG CAT TCT ACA GAG-3'*), resulting in a 253 bp product which was used after purification (*Zymo Research*) for overlapping PCR with the *M. barkeri* pyrrolysyl-tRNA sequence (150 bp) amplified from plasmid, pLei-PylT (group stock). The Pyl-tRNA primers were, *tRNA-pBBR-F2* (*5'-GAA TGC CGA CAC ACA GAG GAA ACC TGA TCA TGT AGA TC-3'*) and, *New-tRNA-pBBR-R2* (*5'-GAG ACT GCA GCC TTT TTT GAT GTT TGG CGG AAA CCC CGG GAA TCT A-3'*). The underlined regions in primers, *tRNA-pBBR-F1-KpnI* and *New-tRNA-pBBR-R2*, show the enzyme cutting sites, *KpnI* and *PstI*, respectively. Initial overlapping PCR design featured the terminator region of *TRNA-met-3*, rather than a generic poly-A tail, but failure in the annealing stage inspired use of fewer primers pairs. All PCR reaction involved KOD Polymerase (*EMD Millipore*). Overlapping PCR was performed with 15 ng/uL DNA templates and primers, *tRNA-pBBR-F1-KpnI* and *New-tRNA-pBBR-R2* at 69.5 °C annealing temperature to yield product which was digested with *KpnI* and *PstI* and purified by DNA gel electrophoresis and column purified.

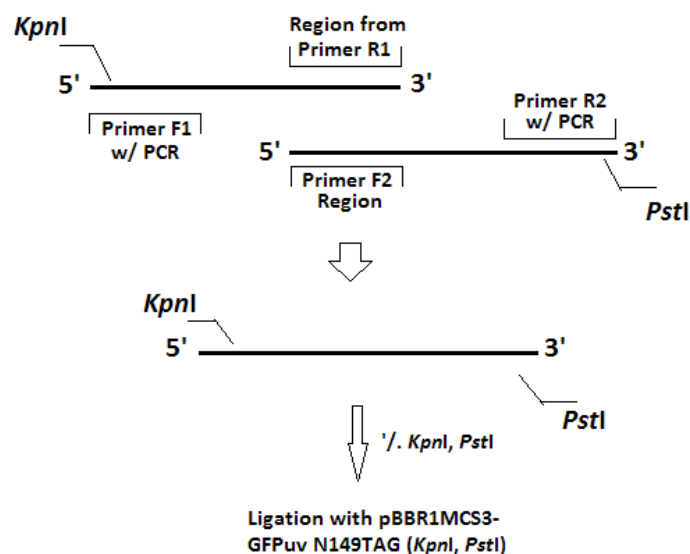


Figure 30: Illustration of overlapping PCR showing regions (R1 and F2) overlapped and primers (F1 and R2) used during final PCR program. Restriction sites, *KpnI* and *PstI* are shown in original fragments and final combined PCR product.

Next, vector pBBR1MCS3:GFPuv N149TAG (*KpnI*, *PstI*) was ligated with prepared Pyl-tRNA fragment (*KpnI*, *PstI*) to obtain purified plasmid with proper insert. This new construct, pSR6.067, contains the GFPuv N149TAG mutant reporter protein, as well as the exogenous Pyl-tRNA flanked by the native DC3000 tRNA-*met-3* promoter (upstream) and a general terminator region. This construct ensures recognition of the exogenous Pyl-tRNA by the DC3000 transcription machinery and promotion of the reporter protein under *T5*-based IPTG induction.

To test for successful ligation, three colonies were picked for separate inoculations of LB broth with added tetracycline, grown 19 hours at 37 °C, 250 rpm, and after cell collection and miniprep, plasmids were digested with *HindIII* for 1 hour at 37 °C before analysis on 1.0% DNA agarose gel.

Plasmid pSR6.067 was transformed into wild-type DC3000 and plated cells were used to inoculate KB-T (Tet¹⁰) media and grown at 30 °C, 250 rpm before subculture and growth to mid-log OD₆₀₀. Induced cultures (IPTG) were examined for cell-based fluorescence.

A repeat trial of DC3000:pSR6.067 fluorescence in cells was performed for a 5-day period to ensure no Pyl-tRNA interaction with DC3000 translation machinery. For this experiment, glycerol stock was used to inoculate KB-T broth with added tetracycline and samples were shaken at 30 °C to saturation, and a 4 mL subculture prepared at OD₆₀₀ 0.4, and induced at mid-log OD₆₀₀. Time-points of 48, 72, 96, and 120-hours post-induction were collected as per 2.9.11 and analyzed as described.

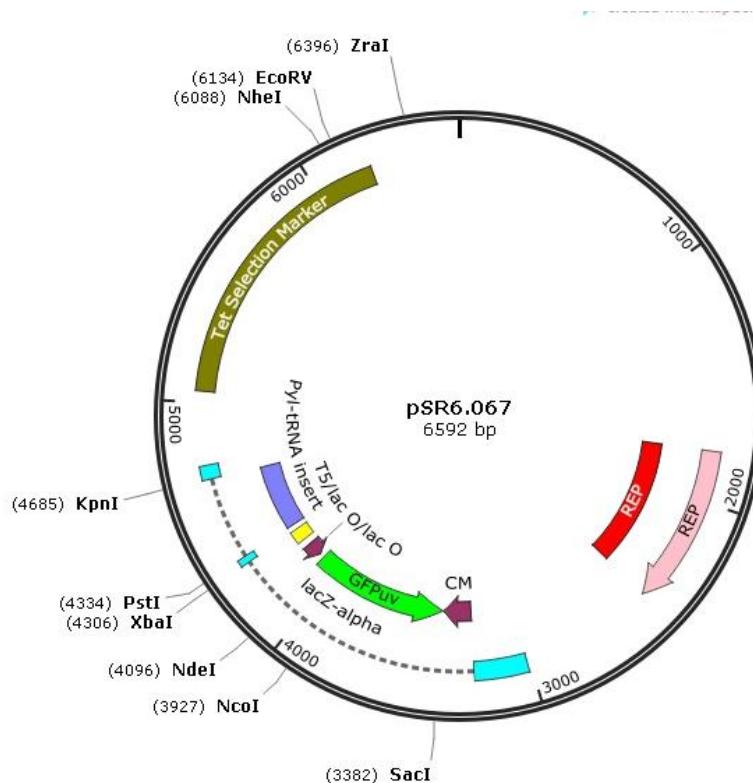


Figure 31: Plasmid map for pSR6.067 (*M. barkeri* Pyl-tRNA; *KpnI*, *PstI*; GFPuv N149TAG).

4.9.3 pML123-tac.PylRS WT

The *M. mazei* pyrrolysine synthetase (PylRS) was PCR amplified using primers, 5'GAG AAT TCA TGG ATA AAA AAC CAT TAG ATG-3' and 5'- GCT CTA GAG CGA AGC GGA ATT AA TTC GCG AAG-3', featuring *EcoRI* and *XbaI* cutting sites (underlined), respectively, from vector pBK-PylRS (Hao, 2011) and purified (Zymo Research) before digestion by *EcoRI* and *XbaI*. Similarly, modified vector pJF118EH-mod1 (Furste, 1986; modification by Wei Niu, University of Nebraska-Lincoln) was digested with *EcoRI* and *XbaI* purified by DNA gel electrophoresis and column purification with ZymoClean Gel DNA Recover Kit (Zymo Research). Ligation of restricted vector and PylRS insert was performed for 2 hours at room

temperature with Hot Start T4 Ligase and ligation product transformed into strain, NEB5 α , followed by plating to LB/ampicillin agar plate.

Plasmid, pJF118EH-PylRS, contains a promoter cassette featuring *tac*.RBS.PylRS which was amplified using primers, *PylRS118toML-B* (5'-GAG GAT CCT TAT CCG GTG CAC CAA TG-3') and *PylRS118toML-H* (5'-CGT TCT GAT TTA ACT GTA TCA GGC TG-3'), and PCR product (1.797 kb) was purified and digested with restriction enzymes, *Bam*HI and *Hind*III, isolated by DNA gel electrophoresis and purified by column purification. In addition, plasmid pML123 was digested with *Bam*HI and *Hind*III and isolated via DNA gel electrophoresis and column purification. Cut vector, pML123, and the PylRS insert were ligated for 4 hours at room temperature, followed by transformation into NEB5 α and plating to LB/gentamycin agar plate.

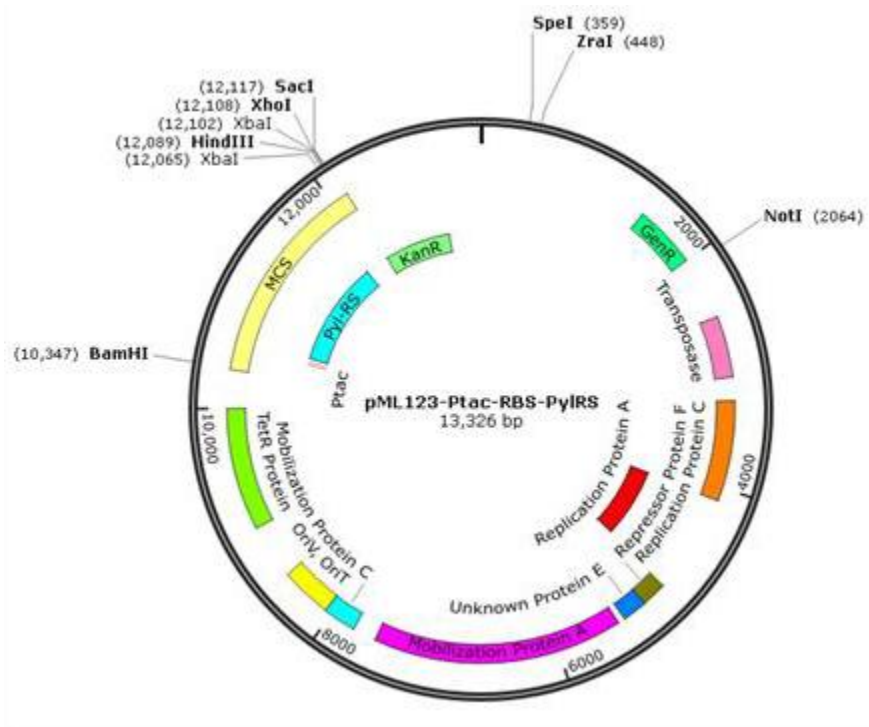


Figure 32: Plasmid map for pML123.tac.PylRS (WT, Y348F, PylRS^{NAA}) into *Bam*HI, *Hind*III.

4.9.4 *pML123-tac.PylRS Y348F*

As for the *tac*-PylRS (2.9.3), the synthetase cassette was PCR amplified from pBK vector using primers, 5'GAG AAT TCA TGG ATA AAA AAC CAT TAG ATG-3' and 5'- GCT CTA GAG CGA AGC GGA ATT AA TTC GCG AAG-3', featuring *EcoRI* and *XbaI* cutting sites (underlined), respectively, from vector pBK-PylRS Y348F (group stock) and digested with *EcoRI* and *XbaI* for ligation into vector pJF118EH-mod1. Subsequent steps for inserting *tac*.PylRS Y348F cassette into pML123 (*BamHI/HindIII*) were also as for wild-type PylRS (3.9.3).

This plasmid featuring the mutated PylRS was co-transformed into Genehog and DC3000 cells along with vector, pSR6.067, for fluorescence analysis of cells induced with IPTG only (ind -) or IPTG and 5 mM boc-lysine (ind +) and grown overnight at resp. incubation temperatures, following overnight incubation without shaking at 4 °C.

For a 48-hour study DC3000 cells containing plasmid pSR6.067 alone, or with pML123.*tac*.PylRS (WT or 368F) were cultured and induced (where applicable) with IPTG and boc-lysine. 1 mL samples were pulled from 30 °C incubated cultures, stored overnight (12 hours) at 4 °C, and then centrifuged {10 min, 16 000 x g, 4 °C} and stored at -80 °C until time of fluorescence reading. Reconstituted cells were washed three times in PBS buffer and examined for RFU, then OD₆₀₀ values were read and RFU/OD calculated for comparison.

4.9.5 *pML123-tac.PylRS-NAA Mutant*

From a PylRS Y348F synthetase library stock in our group labeled, *BL-5*, cells at 10⁷ and 10⁵ dilutions were plated on LB/Tet^{12.5}/Kan⁵⁰/Cm¹⁰⁰ agar plates and mid-sized colonies were used to inoculate liquid LB media with appropriate antibiotic concentrations. Plasmids were purified

and transformed into *E. coli* “pREP-PyIT” cells for positive selection by plating on LB/Tet²⁵/Kan⁵⁰/Cm⁵⁰ plates.

From the *BL-5* library plating, 2 colonies were selected from 10⁷ dilution plating, used to inoculate liquid and streaked to produce 100s of CFU and two were picked for inoculation and plasmid purification. The PylRS Y348F fragment was successfully amplified with PCR primers used for PylRS WT (4.9.3), the PCR product was digested, and ligated into pJF118EH-mod1 to produce pJF-PylRS Y348F. From this pJF-PylRS Y348F, the *tac*.RBS.PylRS Y348F cassette was amplified as before (4.9.3), restricted (*Bam*HI/*Hind*III) and inserted into prepared pML123 (*Bam*HI/*Hind*III). Six colonies of transformants featuring pML123-PylRS Y348F were analyzed and vector pSR7.040.6 was obtained. This vector, containing evolved PylRS Y348F to recognize natural amino acid (NAA) was transformed into DC3000:pSR6.067 or co-transformed with pSR6.067 into Genehog. Inoculations were prepared as before and induced at mid-log OD₆₀₀ with 0.3 mM IPTG and grown at respective temperatures before overnight incubation at 4 °C and cell-based fluorescence analysis.

4.9.6 pBBR1MCS3.T5-PylRS WT, pML123.T5-PylRS WT, and pML123.T5-PylRS Y348F

According to *Figure 32*, shown below, PCR amplification of PylRS (WT or Y368F) from resp. pBK-PylRS initial PCR amplified products of PylRS or PylRS Y348F were digested with *Eco*RI and *Xba*I as before (4.9.3) and inserted into pET28a (*Invitrogen*) within *Eco*RI and *Xba*I sites to produce pET28a-PylRS WT and pET28a-PylRS Y348F. Plasmids were purified by miniprep and digested by *Nde*I and *Sac*I for ligation into the pLei-GFPuvN149TAG vector (*Nde*I, *Sac*I), which had been digested to remove the GFPuv protein, but kept the *T5*.RBS cassette and the 6xHis tag. Ligation was successful and transformation into Genehog c.c. cells allowed for

miniprep and subsequent digestion with *NheI* and *XbaI*. Vectors pBBR1MCS3 and pML123 were both digested with *XbaI* and dephosphorylated with Antarctic phosphatase. Ligation of the *T5.RBS.PylRS* (WT or Y348F) cassette into both vectors was performed for 4 hours at room temperature.

The new synthetase plasmids, pML123.*T5.PylRS* (WT or Y348F) were transformed into DC3000 and Genehog cells for cell-based fluorescence readings. A series of experiments were then conducted to test incorporation in cultures induced with IPTG and boc-lysine at (a) 20- and 42-hours post-induction, (b) 12-hour time-point collection (12-48 hours total), and cultures induced with IPTG and boc-lysine (ind +) or as IPTG-only (ind -).

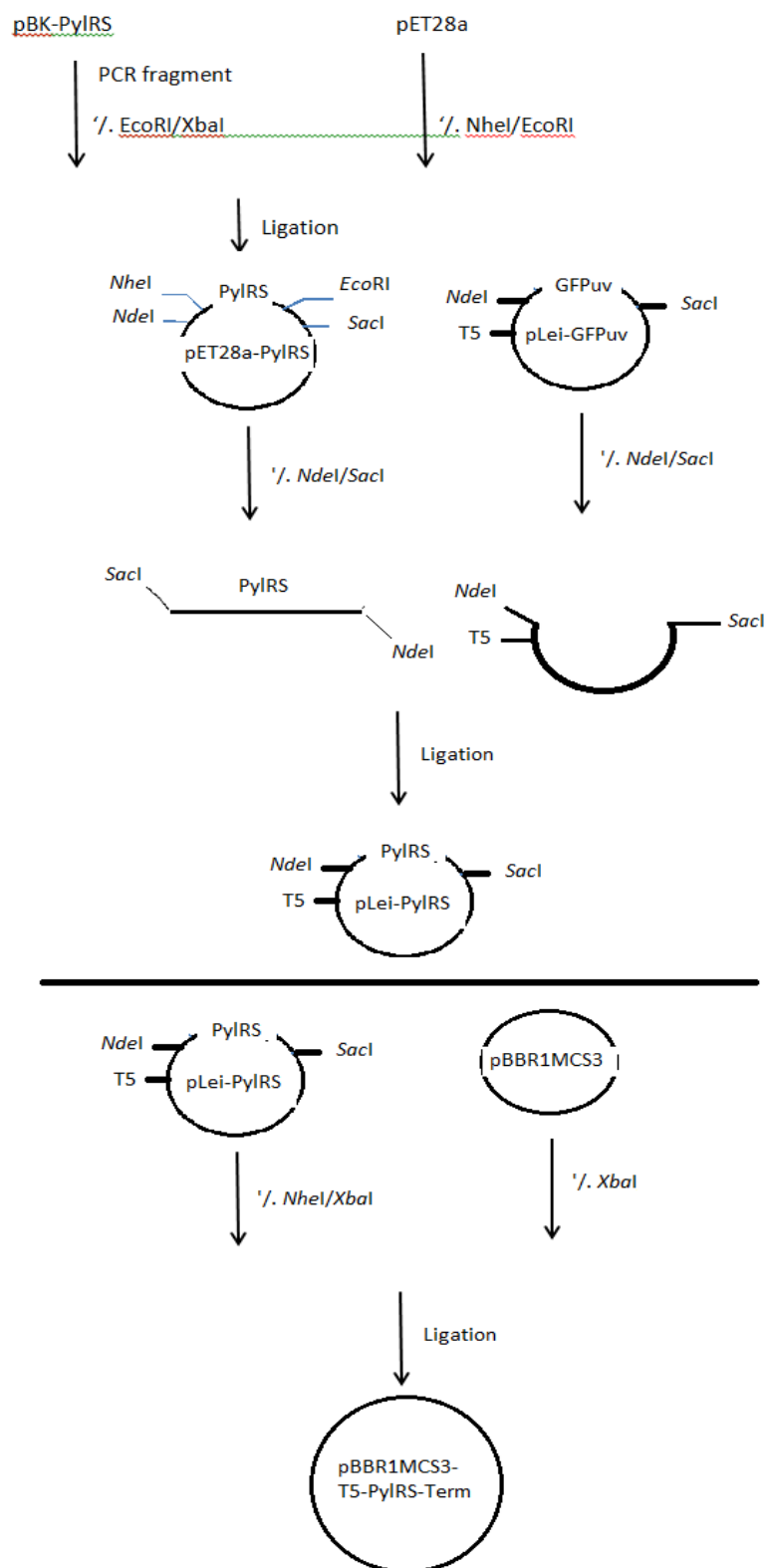


Figure 33: Construction scheme for pBBR1MCS3.T5.PylRS (WT and Y348F) (*shown*) and pML123.T5.PylRS (WT and Y348F) (*not shown*); both feature the same final digestion (*Xba*I).

4.9.7 Introduction of the *M. jannaschii* Tyrosyl-tRNA and RS System

The following details the development of a system for incorporation of unnatural amino acids into the model plant pathogen, *P. syringae* pv. tomato DC3000.

4.9.7.1 Construction of pBBR-GFPuv N149TAG-*M. jannaschii* Tyrosyl-tRNA (pSR8.010)

To construct vector pSR8.010, containing the *M. jannaschii* Tyrosyl-tRNA, it was required to implement overlapping PCR methods similar to the Pyl-tRNA vector (pSR6.067) construction shown in 3.9.2. Primers used for amplifying the Tyr-tRNA from its pLei vector were: *MjtRNA-pBBR-F2* (5'-GAA TGC CGA CAC ACA GAC CGG CGG TAG TTC AGC AGG-3') and *MjtRNA-pBBR-R2* (5'-GAG ACT GCA GCC TTT TTT GAT GTT TGG TCC GGC GGG CCG GAT TTG A-3'). Subsequent overlapping with *TRNA-met-3* promoter region required 15 ng/uL DNA templates and primers, *tRNA-pBBR-F1-KpnI* and *MjtRNA-pBBR-R2* at 69.5 °C annealing temperature. The overlapping PCR product was digested with *KpnI* and *PstI*, isolated by DNA gel electrophoresis and column purified before ligation in *KpnI*, *PstI*-digested pSR6.067. The final construct, pSR8.010 features the Tyr-tRNA^{CUA} from *M. jannaschii* (*Mj.*) as well as the GFPuv N149TAG reporter protein.

DC3000 wild-type cells were transformed with pSR8.010 for inoculation in KB-T culture and incubation with or without addition of 0.5 mM IPTG for cell-based fluorescence test in comparison to DC:6.067 induction with or without addition of 0.5 mM IPTG.

In addition, a single fluorescence test of 120-hour P.I. DC3000 cultures expressing pSR8.010 was performed. Mid-log cultures were induced with 0.5 mM IPTG (*ind*-), and incubate at 30 °C, 250 rpm for 120 hours, followed by 18 hours at 4 °C. 1 mL of collected cells were washed 3x in PBS (500 µL) before analysis. OD₆₀₀ values were obtained at 10x dilution of culture sample and back-calculated for RFU/OD results.

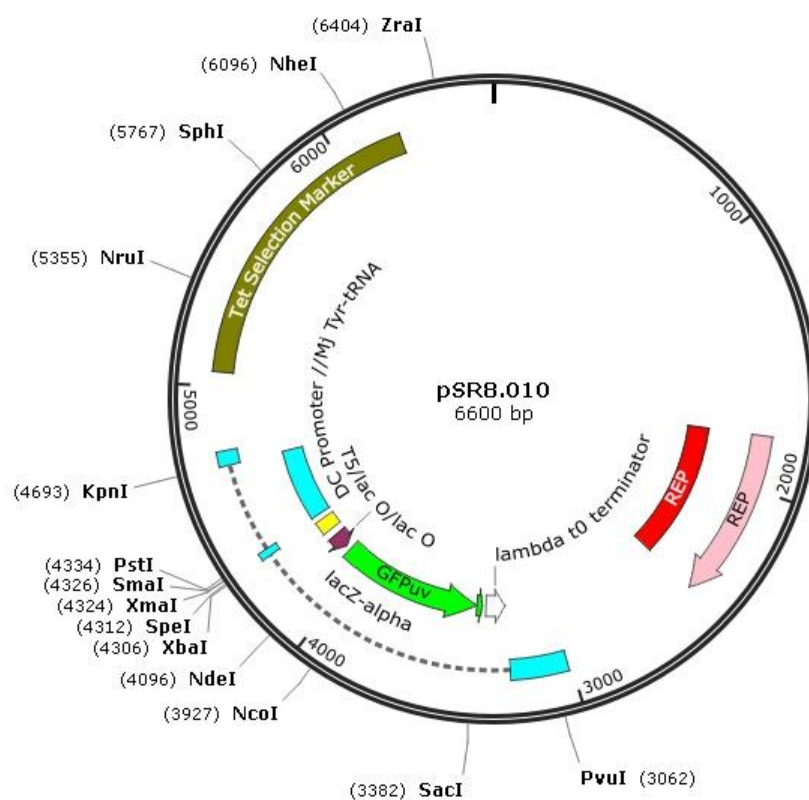


Figure 34: Plasmid map for pSR8.010 (*M. jannaschii* Tyr-tRNA, *KpnI*, *PstI*; GFPuv N149TAG)

4.9.7.2 pML123.T5-BpFRS (pSR8.017)

Mj. Tyr RS evolved to specifically recognize the unnatural amino acid, *p*-benzoyl-*L*-phenylalanine (BpF) was amplified out of plasmid pBK-BpFRS using PCR primers, *R1-YRS-pML123-T5* and *TyRS-lib1-F1-NdeI* (Nanxi Wang, University of Nebraska-Lincoln). The

sequence for primer, *R1-YRS-pML123-T5* (5'-TAT TGA GCT CCT GCA GTT ATA ATC TCT TTC-3'), features a *SacI* cutting site (underlined), while primer, TyRS-lib1-F1-NdeI (5'-TTG AGG AAT CCC ATA TGG ACG A-3') contains an *NdeI* cutting site (underlined). Afterward, the PCR product was digested with *NdeI*, *SacI*, and ligated for 3.75 hours into the intermediate pLei-GFPuv N149TAG-*Mj. Tyr-tRNA^{CUA}*, which had been previously digested (*NdeI*, *SacI*) to remove the GFPuv protein for constructing the *T5.RBS. Mj. Tyr-tRNA^{CUA}* cassette. This cassette was removed from vector (*XbaI*, *NheI*) and inserted into pML123 (*XbaI*) to produce pSR8.017. Plasmid pSR8.017 was used to transform DC3000 cells to yield strains DC3000:pSR8.017 and DC3000:pSR8.010:pSR8.017.

An initial fluorescence test was performed for cells featuring pSR8.010 and pSR8.017. Plated cells were used to inoculate KB-T broth with gentamycin and tetracycline and grown overnight to saturation. Subcultures were prepared and induced at mid-log OD₆₀₀, induced, and were incubated for 36 hours at 30 °C, 250 rpm, then set for 24 hours at 4 °C before collecting 1 mL of each culture {4 °C, 16 000 x g, 8 min} and storage at -80 °C until fluorescence assay preparation. Thawed cultures were resuspended in equal volume of PBS and OD₆₀₀ values were taken at 10x dilution and then back-calculated for RFU/OD values after.

In addition, replicates were performed to average of fluorescence data for three trials for DC3000:pSR8.010:pSR8.017. All cells were prepared as before and incubated post-induction for 44-48 hours followed by 20 hours at 4 °C without shaking. Cell pellets were prepared as described for fluorescence analysis with examined samples either normalized to OD₆₀₀ 0.1 or directly at OD₆₀₀ 0.8 to 1.2.

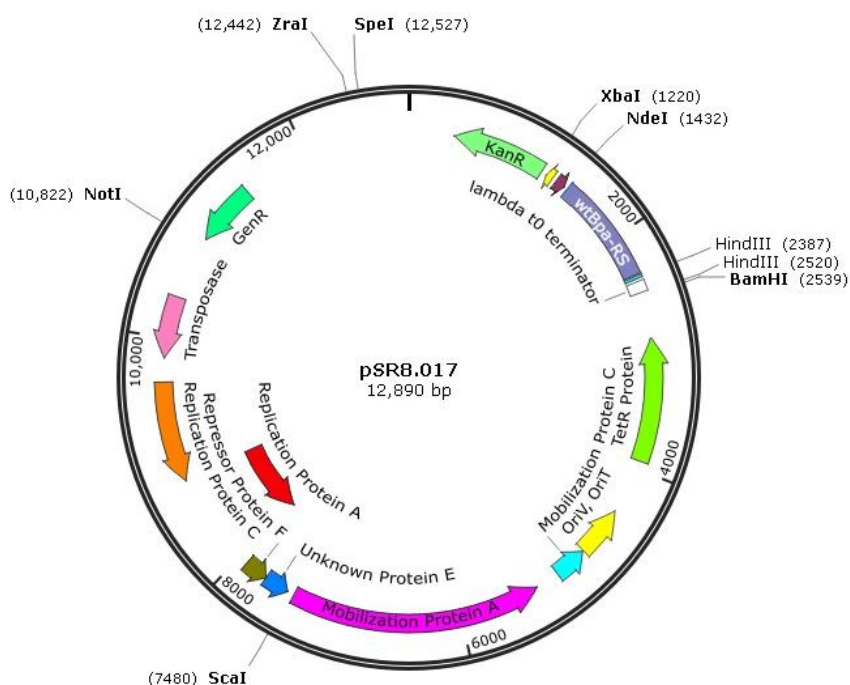


Figure 35: Plasmid map for pSR8.017 (pML123.T5.BpFRS; *XbaI*); pSR8.044 is similar.

4.9.8 pML123.T5-TyrRS WT (pSR8.037)

To help understand reasons for apparent low incorporation of BpF into GFPuv N149TAG present in DC3000, the wild-type tyrosyl-tRNA synthetase (TyrRS WT, or TyrRS) was inserted into the pML123 synthetase vector under promotion of *T5* with the corresponding RBS found in previous *T5* cassettes from pLei vectors: From vector pBK-*Mj*TyrRS (Chin, 2002), the TyrRS was PCR amplified using the same primers as for the BpFRS from its corresponding pBK vector, followed by digestion with *NdeI* and *SacI*. Digested TyrRS fragment was inserted into pLei vector to replace GFPuv protein DNA sequence (*NdeI*, *SacI*) and vector pLei-TyrRS WT was then restricted by *BamHI* and *XhoI* to produce insert fragment for ligation into pML123 (*BamHI*, *XhoI*). It was necessary to use lab-produced strain, Genehog F⁻, featuring an F⁻ fertility plasmid

with gentamycin resistance. Therefore, plasmid miniprep required inoculation into LB media with kanamycin instead of gentamycin – possible because pML123 contains both gentamycin and kanamycin resistance markers. The final product, vector pSR8.037 featured the wild-type TyrRS that is counterpart to the *M. jannaschii* Tyr-tRNA present in vector pSR8.010.

Cultures were inoculated, grown to saturation OD_{600} , and sub-cultured for induction with and without IPTG (0.5 mM working concentration) for growth at 30 °C, 250 rpm. Cultures for fluorescence assays were collected at determined time-points and stored overnight at 4 °C before fluorescence analysis of cell pellets or liquid culture suspensions. An initial 36-hour trial was performed, followed by a series of 44-48 hours induction experiments.

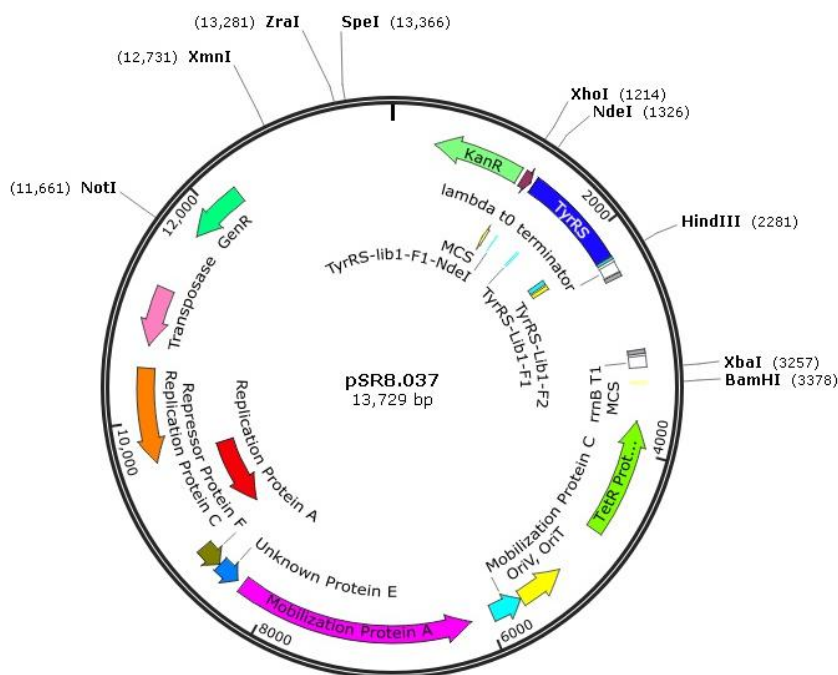


Figure 36: Plasmid map for pSR8.037 (pML123.T5.TyrRS WT; *BamHI*, *XhoI*)

4.9.9 pML123.T5-AzFRS (pSR8.044)

Synthetase vector, pSR8.044, was constructed as follows: AzFRS sequence was PCR amplified from pBK-AzFRS using the same primers used for BpFRS and TyrRS (4.9.7, 4.9.8). As before, PCR product was digested with *NdeI* and *SacI*. Digested AzFRS fragment was inserted into pLei vector to replace GFPuv protein DNA sequence (*NdeI*, *SacI*) and vector pLei-AzF was then restricted with *NheI* and *XbaI* and ligated into pML123 (*XbaI*). The final construct, pSR8.044 is able to selectively recognize *p*-azido-*L*-phenylalanine for charging onto the cognate tyrosyl-tRNA^{CUA} found in pSR8.010.

DC3000 transformants harboring plasmids pSR8.010 and pSR8.044 were prepared and tested for fluorescence of cells after 36 hours of incubation in presence on IPTG with and without addition of 1 mM AzF and with or without covering culture flasks in aluminum foil.

To compliment studies in DC3000, pSR8.010 and corresponding synthetase vectors were transformed into Genehog (BpFRS, AzFRS) or NEB5 α F' competent cells (TyrRS WT). Single colonies were used to inoculate LB broth with appropriate antibiotics and after subculture of saturated inoculants, strains in liquid media were induced with 0.5 mM IPTG working concentration and, where applicable, 1 mM BpF or AzF. No added tyrosine was given to NEB5 α F':pSR8.010:pSR8.037 (TyrRS) cells. Cultures were grown overnight at 37 °C, 250 rpm, then set at 4 °C or 16-20 hours before pelleting, washing in PBS, and fluorescence analysis after dilution to OD 0.1.

4.9.10 New *Mj*. Tyrosyl-tRNA Promoters: *lpp* and *proK*

To attempt an increase in the Tyr-tRNA transcription, two additional promoters were substituted in place of the original *tRNA-met-3* sequence from DC3000.

From vector pLei-GFPuv N149TAG-*Mj* Tyr-tRNA the *lpp.MjtRNA.terminator* cassette featuring the *lpp* promoter and terminator regions flanking the Tyr-tRNA was amplified by PCR using primers, *lpp-tRNA-F-KpnI* (5'-GAG AGA GGG TAC CCG GGA TGT GCT GCA AGG CGA TTA AG-3') and *lpp-tRNA-R-XmaI* (5'-ATA TAT CCC GGG CCC TGG CGC CGC TTC TTT GAG-3') which contain *KpnI* and *XmaI* restriction sites, resp. Digestion of fragment at noted cutting sites allowed for ligation into pBBR1MCS3-GFPuv N149TAG (*KpnI*, *XmaI*) to produce pSR8.052.1. This plasmid was transformed into DC3000 WT and DC:pSR8.017 (pML123-BpFRS) and this strain was further analyzed for cell-based and culture supernatant fluorescence.

Vector pSR8.052.2, implementing the *proK* promoter and terminator (*term.*) regions, was prepared similarly as for the *lpp* promoting cassette. *proK.MjtRNA.term* was PCR amplified from pEvol vector (Young, 2010) using primers, *proK-tRNA-F-KpnI* (5'-GAG AGA GGG TAC CGA GCA CCG GTT TAT TGA CTA CC-3') and *proK-tRNA-R-XmaI* (5'-ATA TAT CCC GGG TTT TTT AAG GCA GTT ATT GGT-3') which feature restriction sites for *KpnI* and *XmaI*, respectively. PCR product was digested with restriction enzymes for the noted cutting sites, isolated by DNA gel electrophoresis and column purification, and ligated into plasmid pBBR1MCS3-GFPuv N149TAG (*KpnI*, *XmaI*) to produce pSR8.052.1. This plasmid was transformed into DC3000 WT and DC:pSR8.017 (pML123-BpFRS) and this strain was further analyzed for cell-based and culture supernatant fluorescence.

DC3000 cells featuring pSR8.017 (BpFRS) were transformed with tRNA plasmids featuring either the *lpp*, or *proK* promoters and plated cells were used for inoculation of KB-T broth with added antibiotics. Cells were grown alongside DC3000:pSR8.010:pSR8.017 for one or two days to saturation, subculture was prepared and then induced at mid-log OD. Induced

cultures were grown for several days with time-points collected at 48, 72 hours. Cell pellets and liquid culture supernatant were analyzed for fluorescence.

Three additional series of DC3000 cultures harboring pSR8.052.1 (*lpp*) and pSR8.017 plasmids were prepared as above and examined for fluorescence of cell pellets over several days.

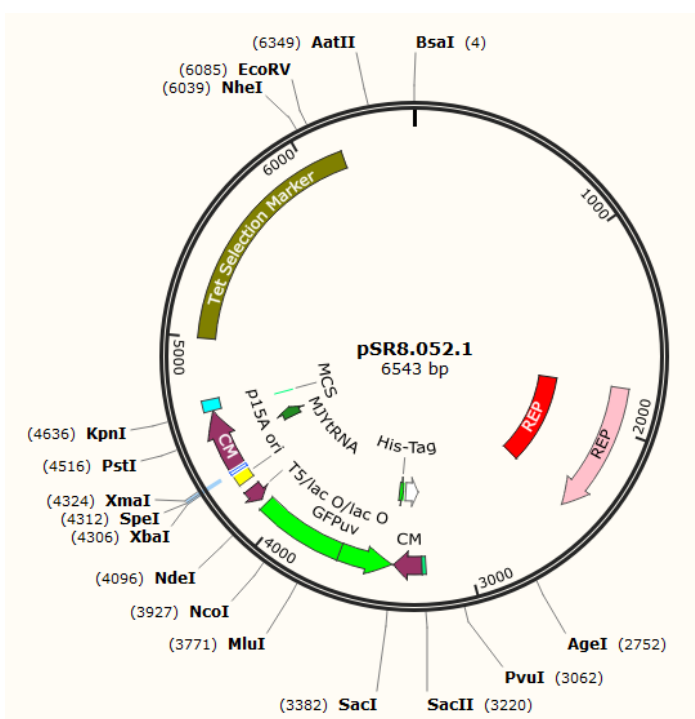


Figure 37: Plasmid map for pSR8.052.1 (*lpp* promoter/*Mj.* Tyr-tRNA; *KpnI*, *XmaI*)

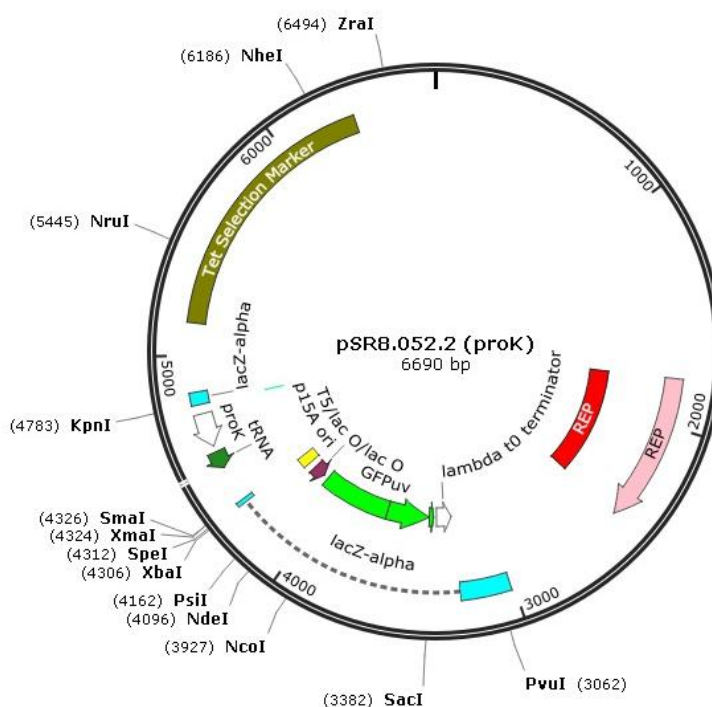


Figure 38: Plasmid map for pSR8.052.2 (*proK* promoter/*Mj.* Tyr-tRNA; *KpnI*, *XmaI*)

4.10 GFPuv Purification from DC3000 Liquid Broth

Inoculants were prepared from plated cells at 30 °C or 4 °C, or from glycerol stocks (6 µL from -80 °C storage) into KB-T broth with appropriate working concentration of antibiotics (e.g. 3 mL KB-T broth, 3 µL Gen¹⁰ stock, 2.6 µL Tet^{12.5} stock) and grown at 30 °C, 250 rpm for 16-48 hours, until saturation ($OD_{600} > 1.2$). Subcultures were then prepared in KB-T broth with antibiotics as OD_{600} of 0.4 and grown at 30 °C, 250 rpm to final OD 0.7-1.0. Induction of mid-log OD cultures featured working concentrations of 0.5 mM IPTG and 1 mM BpF or AzF solution or 5 mM Boc-lysine solution. Cultures were incubated until time-points of 48, 72, 96, and 120-hours post-induction (P.I.) from which 250-300 µL volumes were taken from incubated culture stock and set into 4 °C refrigerator overnight (16-20 hours). Wild-type DC3000 was grown from

4 °C plates in KB-T with no antibiotics, followed by subculture to OD₆₀₀ 0.4 at the same time as transformed strains and incubated for equal durations, including 4 °C overnight refrigeration and further sample preparation.

Cell pellets for all time-points were collected from liquid cultures at 4 °C, 21 100 x g, 15 minutes and supernatants were transferred to 0.6 mL tubes for storage on ice. Pellets were washed in equal volume of PBS solution {10 mM Na₂HPO₄, 140 mM NaCl, pH 7.0} with vortexing, followed by centrifugation at 4 °C, 14 000 x g, 10 minutes for a total of three washes. All collected cell pellets or liquid media for each time-point was stored at -80 °C or 4 °C, respectively, to allow for multiple sample fluorescence analysis on the same day. Liquid media for time-points was kept at -20 °C or -80 °C after fluorescence analysis for later use in Western blot detection.

Samples were loaded to 15% prepared SDS-PAGE gel after boiling at 94 °C for 15.5 minutes in presence of equal volume 2x Laemli buffer. Loaded samples were run in Tris/Glycine buffer at either constant current of 35 mA to 38 mA or constant voltage of 200 V. After electrophoresis, gel was washed in warmed nanowater at room temperature until blue dye on gel had dissipated and then either stained in Coomassie blue staining solution or used directly for Western blot transfer.

4.11 GFPuv WT Western Blot

Liquid media samples from DC3000 expressing GFPuv WT at 72, 96, and 120 hours post-induction (P.I.) were measured for protein concentration (mg/mL) at 280 nm with Nanodrop 2000. Higher concentrated samples were normalized to 25 µg for loading to 15% SDS-PAGE gel and further His-tag detection using Western blot colorimetric method. These time-points were

loaded alongside normalized sample of DC3000 WT secretion (liquid media) at 120 hours P.I. Additionally, 20 μ L volumes of concentrated fraction from DC3000 featuring *M. barkeri* Pyl-tRNA and GFPuv N149TAG mutant (pSR6.067 non-induced and IPTG induced) were loaded to SDS-PAGE gel for comparison under Western blot detection

4.12 Western Blot for 6.067

To determine if DC3000:pSR6.067 cultures contained secreted GFPuvN149-XX, combined purification fractions at wash #2 (50 mM imidazole) and wash #3, 4 (75 mM, 100 mM imidazole) were analyzed against DC3000:GFPuv WT supernatants (4.9.12) and DC WT 120-hours supernatant samples. 280 nm absorbance readings of these fractions did not show appreciable protein concentration, so 20 μ L of each was mixed with equal volume of Laemli loading buffer for sample preparation and loading to SDS-PAGE gel.

4.13 Purification of GFPuv N149-BpF from Medium-scale Culture for DC:6.067

To ensure there is no GFPuv N149TAG-XX mutant being expressed and secreted to the culture supernatant, a 25 mL scale culture was prepared and purification of possible full-length GFPuv N149-XX mutant was attempted.

A 2.5 mL volume of KB-T broth was inoculated with plated cells and grown to saturation in presence of tetracycline and gentamycin. A 1.0 mL portion of the inoculant was added to 25 mL KB-T (with 25 μ L Gen¹⁰ stock, 20 μ L Tet^{12.5} stock) and grown at 30 °C, 250 rpm to OD₆₀₀ of 0.8. Culture was then separated as 2 mL for non-induced and 24 mL for induction with IPTG at 0.5 mM working concentration. Cultures were grown at 30 °C, 250 rpm for 96 hours before setting overnight (20 hours) at 4 °C. 250 μ L of each raw suspension was then prepared for supernatant and cell-based fluorescence analysis, while the remaining portions were centrifuged

for supernatant extraction, and supernatants were frozen at $-20\text{ }^{\circ}\text{C}$ until purification. For original 250 μL aliquots, 30 mg of each induced and non-induced were lysed in Tris/glycerol lysis buffer (pH 8.0) and protein concentration detection with Nanodrop 2000 at 280 nm. 50 μg each total protein portion was then prepared for SDS-PAGE and western blot analysis alongside GFPuv WT supernatant samples from DC3000 (4.9.12).

The supernatant fraction ($\sim 24\text{ mL}$) of the induced DC3000:pSR6.067 culture was thawed overnight at $4\text{ }^{\circ}\text{C}$ and loaded to column with 1 mL Ni^{2+} -Sepharose resin. Flow-through (FT) was reloaded twice before washing with 25 mM imidazole in wash #1, followed by wash buffer series #2-5 (50-500 mM imidazole). Washes #2-5 were combined and concentrated by Amicon Ultra-4 Centrifuge Device to 300 μL prior to storage at $4\text{ }^{\circ}\text{C}$.

4.14 Western Blot for 96-hour Culture Liquids Media

96-hour culture supernatants were analyzed by Western blot protocol to determine if any full-length GFPuv N149 mutant was had been secreted by DC3000. Supernatant samples were examined for protein concentration (mg/mL) with Nanodrop 2000 and normalized to 25 μg total protein before SDS-PAGE sample preparations (Laemli dye, boiling) and loading to gel alongside GFPuv WT loading control. Electrophoresis gel was then transferred to nitrocellulose membrane and membrane was incubated and developed as previously described, but featuring 1.5 hours incubation for both primary and secondary antibodies. Membrane was developed and image was captured.

The 96-hour samples analyzed during this trial included DC3000 WT 96-hrs, GFPuv N149TAG only (non, induced IPTG), pSR8.010 only (non, induced IPTG), pSR8.052.2 (*proK*)

only (non, induced IPTG), pSR8.010:pSR8.037 (non, induced IPTG), and pSR8.010:pSR8.044 (AzFRS; non, induced IPTG, induced IPTG with 1 mM AzF).

4.15 Western Blot for DC:pSR8.010: pSR8.017 (BpF) and DC:pSR8.052.1 (lpp): pSR8.017

To ensure no full-length GFPuv-N149BpF mutants were being secreted into the liquid media, 96-hour and 120-hour supernatant samples were prepared for SDS-PAGE and Western blot detection with 6x-His antibody. Nanodrop 2000 was used to obtain protein concentrations (mg/mL) at 280 nm and samples for loading were prepared at 25 µg total protein based on those 280 nm readings. Samples were loaded to 15% SDS-PAGE gel and later transferred to nitrocellulose membrane. Overnight blocking in 3% blocking buffer was performed at 4 °C, with rocking, and subsequent incubation of the membrane at RT in presence of primary (1.5 hours) and secondary (1.5 hours) antibodies, with a final colorimetric development were all performed. Membrane blot was imaged.

4.16 Purification of GFPuv N149-BpF from Medium-scale Culture for

DC:pSR8.010:pSR8.017 and DC:pSR8.052.1 (lpp):pSR8.017

Medium-scale (28 mL) cultures of DC3000 featuring either pSR8.010 + pSR8.017 (BpFRS) or pSR8.052.1 (*lpp*) + pSR8.017 were prepared as follows: 2.5 mL KB-T was inoculated from glycerol stock (pSR8.010 + pSR8.017) or agar plate stored at 4 °C for 2 months (pSR8.052.1 + pSR8.017) and incubated with antibiotics at 30 °C, 250 rpm overnight. Cultures were then directly added to 25 mL KB-T in 125 mL Erlenmeyer flask with added antibiotics and grown to OD₆₀₀ of 1.0. At this time, 2 mL of each was transferred to culture tube to serve as possible non-induced control, while the remaining fractions were induced with 500 µL of 55.7 mM BpF solution {15 mg BpF, 300 µL 1 M NaOH, 700 µL ddH₂O}, 150 µL 1 M HCl (to

neutralize NaOH}, and 140 μ L 100 mM IPTG (0.5 mM working concentration). All cultures were then incubated for 50 hours at 30 °C, 250 rpm. 500 μ L of initial induced suspensions were transferred to 1.5 mL centrifuge tubes for supernatant and cell pellet fluorescence analysis. The remaining cultures were collected {4 °C, 5000 x g, 50 minutes} and stored at -80 °C until lysis and purification. Lysis was performed in 3 mL (pSR8.010 + BpFRS) or 6 mL (pSR8.052.1 + BpFRS) of lysis buffer {50 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 8.0} with sonication on ice until lysate samples were clear {5 minute rounds on ice as: 10 sec. pulse, 30 sec. pause, 60-65% power; 5 rounds (pSR8.010 + BpFRS) or 7 rounds (pSR8.052.1 + BpFRS) total} and lysates were frozen immediately at -80 °C until centrifugation and lysis supernatant collection {4 °C, 21 000 x g, 30 min}. Select purification fractions of DC:pSR8.010:pSR8.017 were analyzed by SDS-PAGE, while fractions (Wash #1-1, 2-1, 3-1, 4-1) for both induced strains were analyzed by Western blot. Relevant purification fractions from both were analyzed for protein concentration at 280 nm with Nanodrop 2000 with appropriate lysis and wash buffers as blanks. Where applicable, protein samples were normalized to 25 μ g for gel loading.

4.17 Fluorescence Assays

All fluorescence assays were performed using the SynergyH1 microplate reader (*BioTek*) with Costar 96-well black flat-bottom fluorescence plates (#3915; *Corning*). All readings were performed at 19-25 °C ambient temperature with 100x gain, 7.0 mm read height, and excitation at 390 nm with emission at 510 nm. Sample buffer included blanking in phosphate-buffered saline solution (PBS) {10 mM Na₂HPO₄, 140 mM NaCl, pH 7.0}.

4.17.1 Cell Pellets

Pellets of cells were washed three times in equal volume of PBS buffer {4°C, 14 000 x g, 10 minutes each} as preparation for analysis. Cell pellet suspensions were homogenized as best able and liquid-only portions were assayed. Fluorescence readings were performed on cell pellets after testing optical density at 600 nm (OD or OD₆₀₀) at 10x dilution {i.e. 100 µL per 900 µL nanowater} or 20x dilution {i.e. 50 µL sample per 950 µL nanowater}, depending on the turbidity of the culture. Unless otherwise noted, samples were normalized to OD = 0.05 or OD 0.1 before loading to black flat-bottom microplate at volumes of 125 µL sample per well.

Blanking of analyzed samples was performed with PBS only for cell pellets, unless otherwise noted.

4.17.2 Cell Culture Liquid Supernatant

Supernatants for cultures were analyzed as 5 µL liquid media in 120 µL PBS buffer. An additional reading was performed for a solution of either KB-T in PBS or KB-T/Tet¹⁰ in PBS. Over the series of sample readings, values for KB-T/Tet¹⁰ (5 µL solution into 120 µL PBS) were averaged for correction against fluorescence caused by tetracycline in the culture broth.

Blanking of analyzed samples was performed with PBS + KB-T for supernatants.

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APPENDIX I – PCR AMPLIFICATION SETUP AND PROGRAM METHODS

PCR Setup		PCR Method			
Component	Vol. (μ L)				
ddH ₂ O	33.5	95 °C	2 minutes	Activation	* 30 cycles
2 mM dNTPS	5	95 °C	20 seconds	Denaturing	
KOD 10x Buffer	5	64.6 °C	10 seconds	Annealing	
25 mM MgSO ₄	3	70 °C	5.5 seconds	Extension	
10 μ M Primer Mix	1.5	70 °C	7.5 minutes	Termination	
KOD Polymerase	1				
DNA Template	1				

Table 21: PCR Method for *TRNA-met-3* Promoter (DC3000) Amplification

PCR Setup		PCR Method			
Component	Vol. (μ L)				
ddH ₂ O	33.5	95 °C	2 minutes	Activation	* 30 cycles
2 mM dNTPs	5	95 °C	20 seconds	Denaturing	
KOD 10x Buffer	5	56 °C	10 seconds	Annealing	
25 mM MgSO ₄	3	70 °C	5 seconds	Extension	
10 μ M Primer Mix	1.5	70 °C	7.5 minutes	Termination	
KOD Polymerase	1				
DNA Template	1				

Table 22: PCR Method for Pyl-tRNA Amplification

PCR Setup		PCR Method			
Component	Vol. (μ L)				
ddH ₂ O	33.5	95 °C	2 minutes	Activation	* 30 cycles
2 mM dNTPs	5	95 °C	20 seconds	Denaturing	
KOD 10x Buffer	5	69.5 °C	10 seconds	Annealing	
25 mM MgSO ₄	3	70 °C	5 seconds	Extension	
10 μ M Primer Mix	1.5	70 °C	7.5 minutes	Termination	
KOD Polymerase	1				
DNA Template	1				

Table 23: PCR Method for Overlapping PCR of *M. barkeri* Pyr-tRNA with *TRNA-met-3* Promoter Region

PCR Setup		PCR Method			
Component	Vol. (μ L)				
ddH ₂ O	33.5	95 °C	2 minutes	Activation	* 30 cycles
2 mM dNTPs	5	95 °C	20 seconds	Denaturing	
KOD 10x Buffer	5	60.5 °C	10 seconds	Annealing	
25 mM MgSO ₄	3	70 °C	30 seconds	Extension	
10 μ M Primer Mix	1.5	70 °C	7.5 minutes	Termination	
KOD Polymerase	1				
DNA Template	1				

Table 24: PCR Method for PylRS from pBK Amplification

PCR Setup		PCR Method			
Component	Vol. (μL)				
ddH ₂ O	33.5	95 °C	2 minutes	Activation	* 30 cycles
2 mM dNTPs	5	95 °C	20 seconds	Denaturing	
KOD 10x Buffer	5	63.1 °C	10 seconds	Annealing	
25 mM MgSO ₄	3	70 °C	32 seconds	Extension	
10 μM Primer Mix	1.5	70 °C	7.5 minutes	Termination	
KOD Polymerase	1				
DNA Template	1				

Table 25: PCR Method for PylRS from pJF118EH-mod1 Amplification

PCR Setup		PCR Method			
Component	Vol. (μL)				
ddH ₂ O	16.75	95 °C	2 minutes	Activation	* 30 cycles
2 mM dNTPs	2.5	95 °C	20 seconds	Denaturing	
KOD 10x Buffer	2.5	56 °C	10 seconds	Annealing	
25 mM MgSO ₄	1.5	70 °C	5 seconds	Extension	
10 μM Primer Mix	0.75	70 °C	7.5 minutes	Termination	
KOD Polymerase	0.5				
DNA Template	0.5				

Table 26: PCR Method for *M. jannaschii* Tyr-tRNA from pLei-GFPuvN149TAG-*Mj.* Tyr-tRNA Amplification

PCR Setup		PCR Method			
Component	Vol. (μL)				
ddH ₂ O	33.5	95 °C	2 minutes	Activation	* 30 cycles
2 mM dNTPs	5	95 °C	20 seconds	Denaturing	
KOD 10x Buffer	5	69.5 °C	10 seconds	Annealing	
25 mM MgSO ₄	3	70 °C	5 seconds	Extension	
10 μM Primer Mix	1.5	70 °C	7.5 minutes	Termination	
KOD Polymerase	1				
DNA Template	1				

Table 27: Overlapping PCR of *M. jannaschii* Tyr-tRNA with *TRNA-met-3* Promoter Region

PCR Setup		PCR Method			
Component	Vol. (μL)				
ddH ₂ O	16.75	95 °C	2 minutes	Activation	* 30 cycles
2 mM dNTPs	2.5	95 °C	20 seconds	Denaturing	
KOD 10x Buffer	2.5	70 °C	10 seconds	Annealing	
25 mM MgSO ₄	1.5	70 °C	15 seconds	Extension	
10 μM Primer Mix	0.75	70 °C	7.5 minutes	Termination	
KOD Polymerase	0.5				
DNA Template	0.5				

Table 28: PCR Method for Amplification of *lpp/Mj.* tRNA/term Region from pLei vector

PCR Setup		PCR Method			
Component	Vol. (μ L)	95 °C	2 minutes	Activation	* 30 cycles
ddH ₂ O	16.75	95 °C	20 seconds	Denaturing	
2 mM dNTPs	2.5	70 °C	10 seconds	Annealing	
KOD 10x Buffer	2.5	70 °C	15 seconds	Extension	
25 mM MgSO ₄	1.5	70 °C	7.5 minutes	Termination	
10 μ M Primer Mix	0.75				
KOD Polymerase	0.5				
DNA Template	0.5				

Table 29: PCR Method for Amplification of *proK/Mj*. tRNA/term Region from pEvol Vector

APPENDIX II – DNA AGAROSE GEL IMAGES

DNA agarose gels for plasmid constructs, including PCR amplification, overlapping PCR, and ligation insert tests.

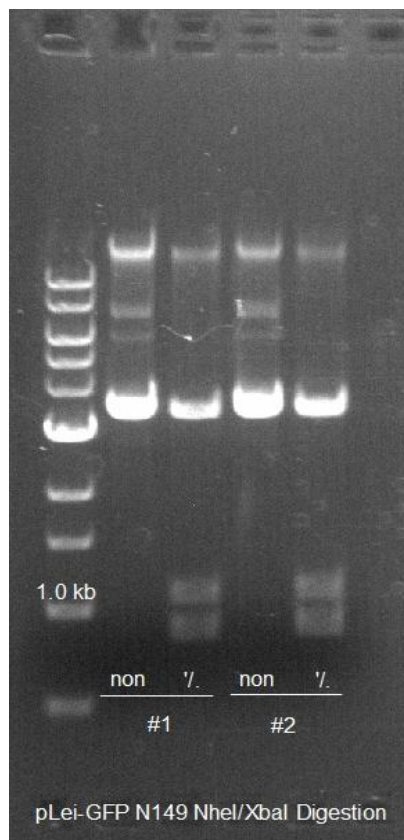


Figure 39: DNA gel for pLei-GFPuv N149TAG small-scale digestion with *NheI* and *XbaI*. In both pLei-GFPuv N149TAG and pLei-GFPuv WT, proper band at 1.1 kb is cut, purified, and ligated to pBBR1MCS3 (*XbaI*). Image shows non-digested (*non*) and digested (*/*) products.

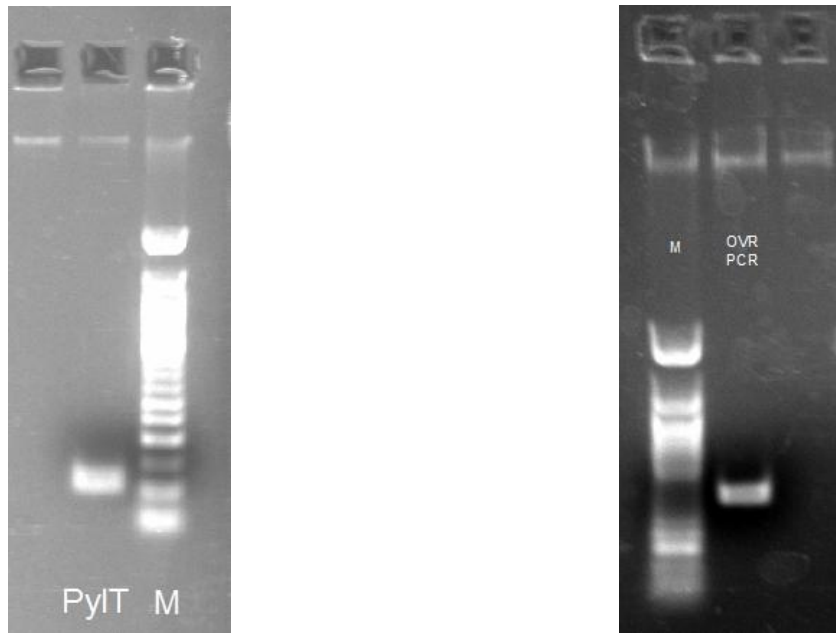


Figure 40: DNA agarose gels showing initial PCR product of Pyl-tRNA (*left*; PylT) and properly-sized overlapping PCR product (*right*) of promoter/tRNA/terminator.

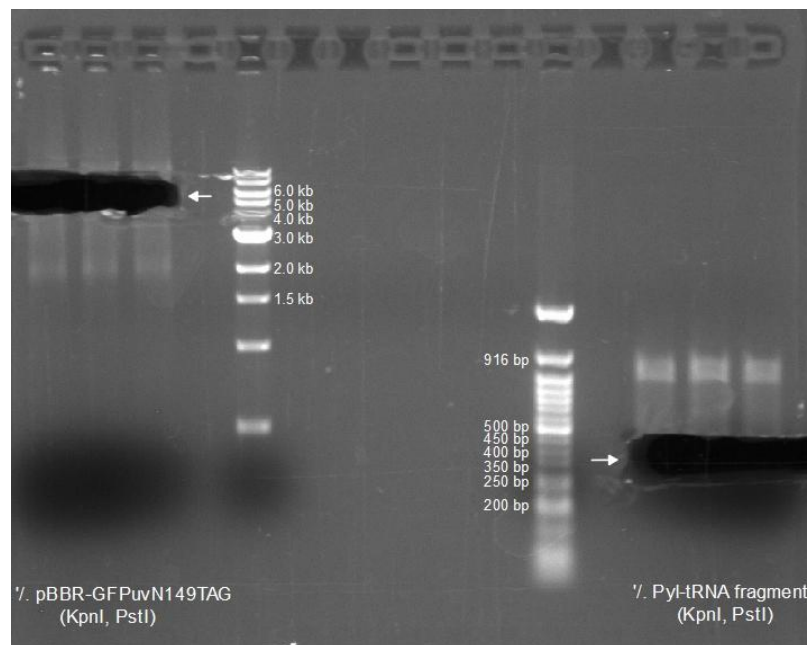


Figure 41: DNA gel for restrictions of vector (pBBR1MCS3-GFPuvN149TAG, *left*) and Pyl-tRNA fragment (*right*).

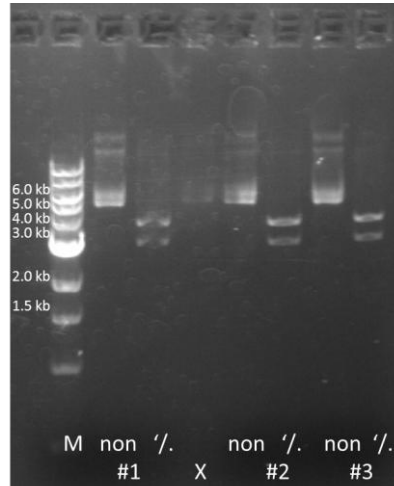


Figure 42: Ligation check for proper Pyl-tRNA insert in pSR6.067 ligation product. *Restriction of plasmids with HindIII to produce two bands (3.66 kb, 2.94 kb) if proper insert, otherwise three bands (3.66 kb, 1.64 kb, 0.995 kb) if no tRNA insert. Lanes (left to right): 1, 1 kb ladder; 2, non-digested sample #1; 3, digested sample #1; 4, empty lane; 5, non-digested sample #2; 6, digested sample #2; 7, non-digested sample #3; 8, digested sample #3.*

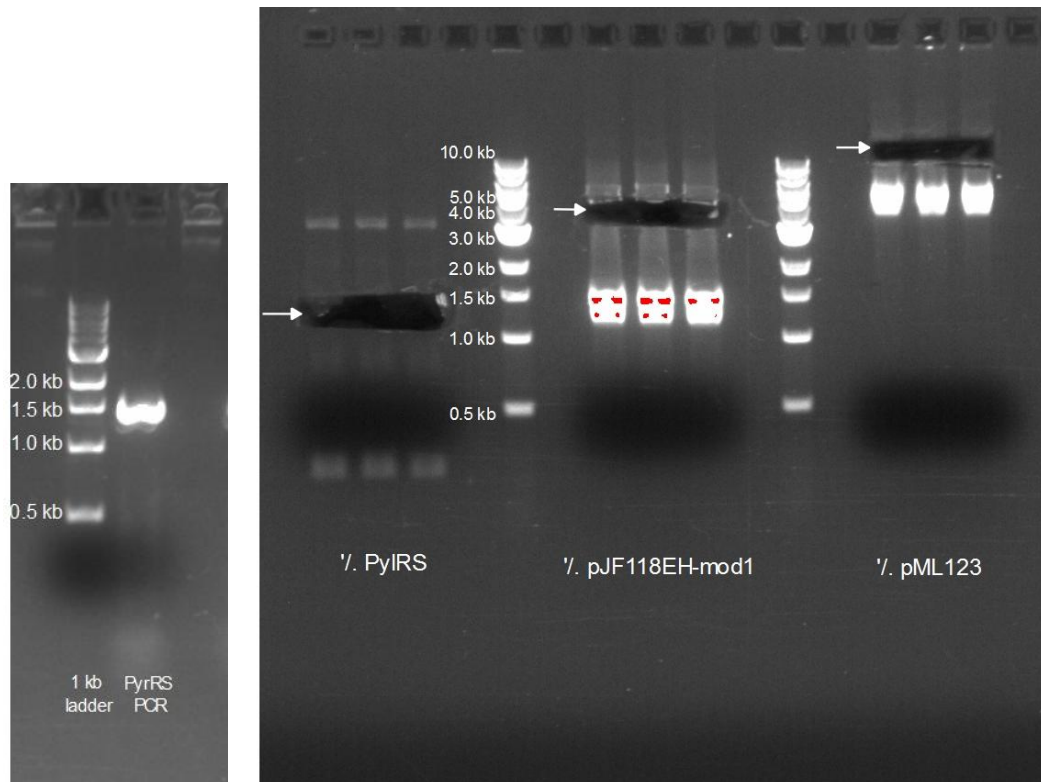


Figure 43: DNA gels showing PCR amplification products for pJF118EH-PylRS WT Construct (*left image*). DNA gels (*right image*) showing digestion products for PylRS PCR fragment (*EcoRI, XbaI*), pJF118EH-mod1 (*EcoRI, XbaI*) and pML123 (*BamHI, HindIII*)

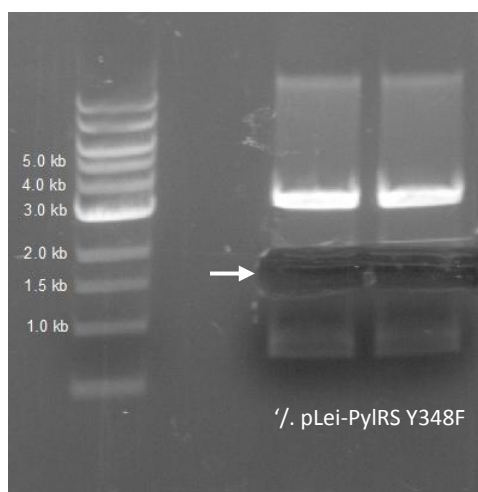


Figure 44: DNA gels showing digestion of pLei-PylRS Y348F (*NheI, XbaI*) for ligation into pML123 (*NheI, XbaI*).

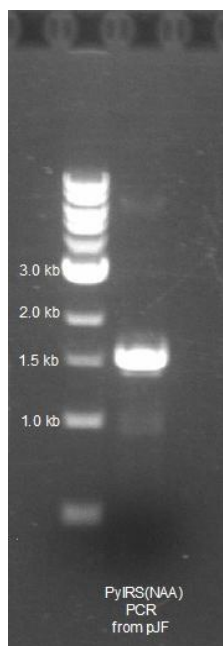


Figure 45: PCR amplification of *tac*.PylRS^{NAA} cassette from pJF118EH-mod1 vector for digestion and insertion into pML123 (*BamHI, HindIII*).

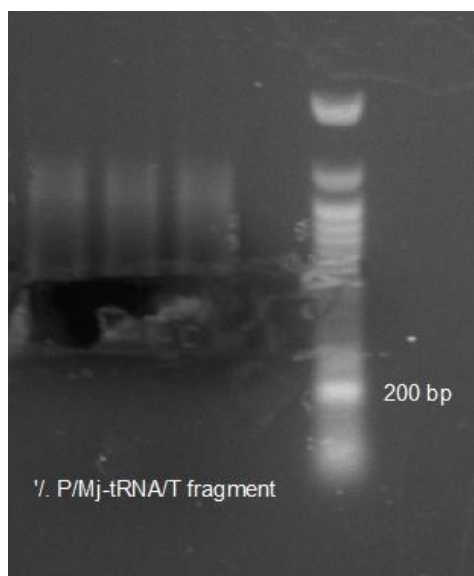


Figure 46: DNA gels showing digestion of *M. jannaschii* Tyr-tRNA promoter//tRNA, terminator overlapping PCR product (*Kpn*I, *Pst*I). Band was excised, purified, and ligated into pBBR1MCS3-GFPuv N149TAG.

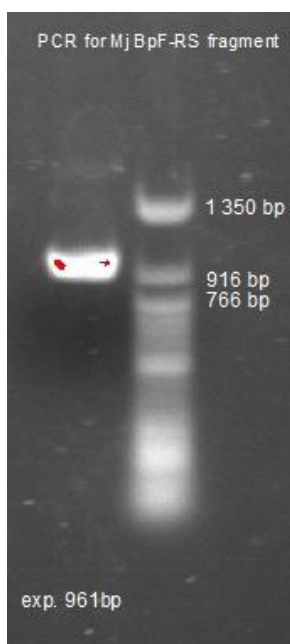


Figure 47: PCR product for amplification of BpFRS from pBK-BpFRS vector.

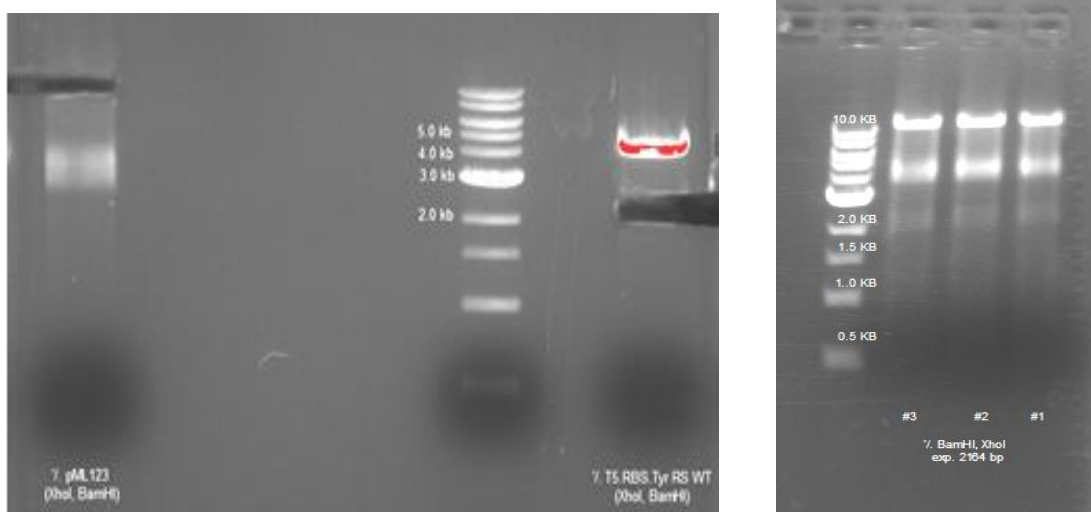


Figure 48: DNA gels (*left image*) showing digestion of pML123 (11.6 kb; *Bam*HI, *Xho*I) and digestion of pLei-TyrRS WT (*Bam*HI, *Xho*I) to obtain *T5.RBS.TyrRS WT* fragment (2.2 kb). Also shown (*right image*) is test for proper insert after ligation of pML123 with *T5.RBS.TyrRS* insert (*expected product is 2.164 kb for Bam*HI, *Xho*I digestion).

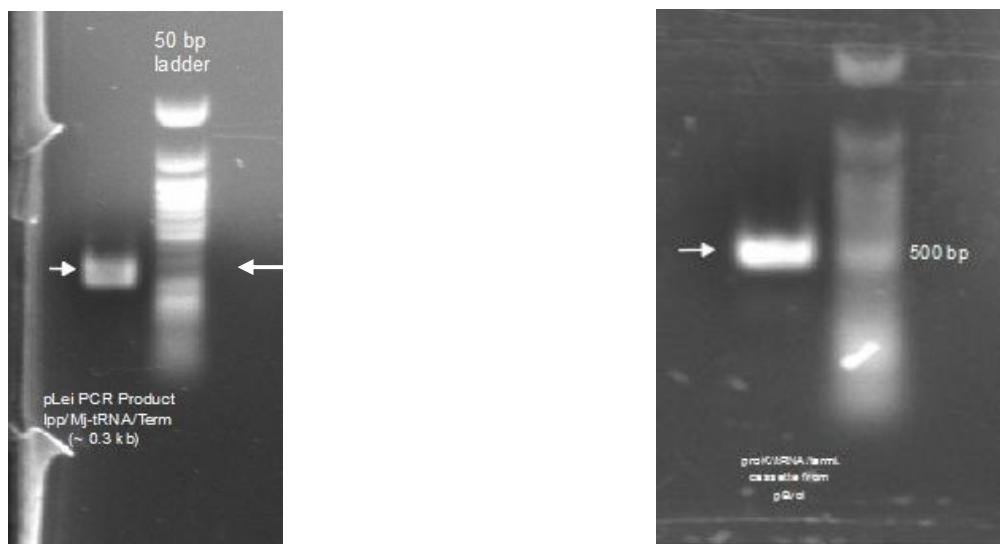


Figure 49: DNA gels showing PCR amplification products for *lpp* (*left*) and *proK* (*right*) promoter//tRNA/terminator from respective parent plasmid.

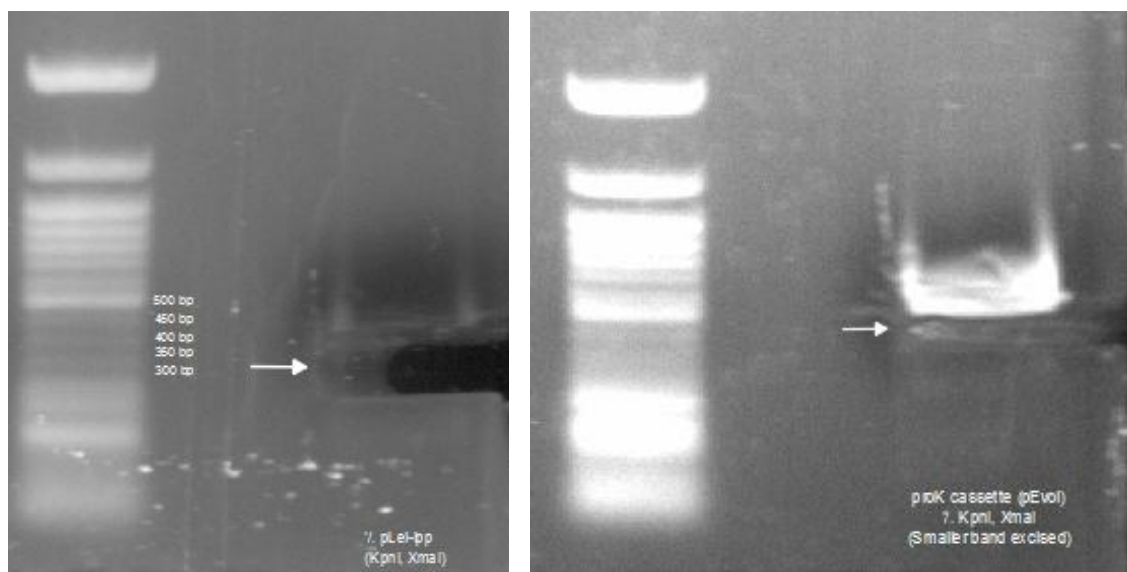


Figure 50: DNA gels showing digestion products of overlapping PCR products (0.3 kb *lpp*, left; 0.5 kb *proK*, right) for insertion into pBBR-GFPuvN149TAG vector.

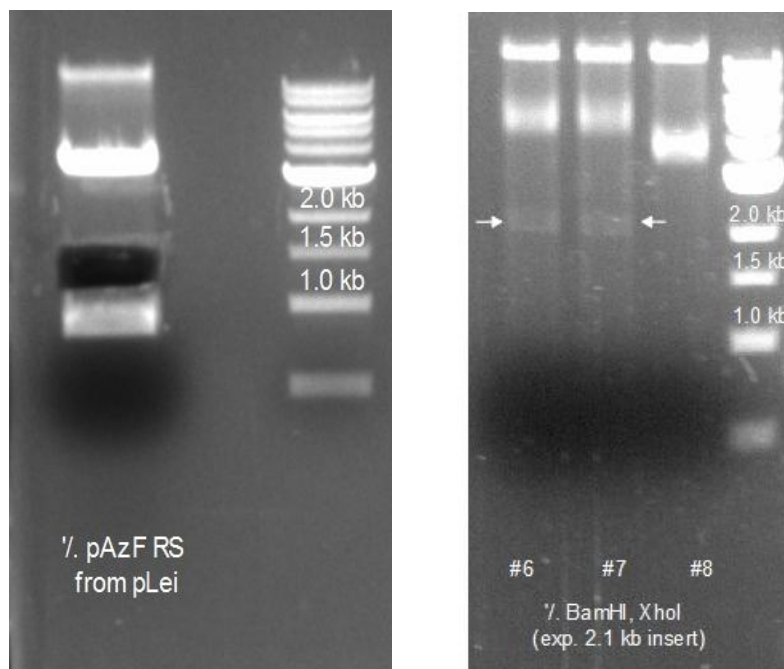


Figure 51: DNA gels showing digestion products for *T5.AzFRS* insert (left image; 1.3 kb from pLei) after ligation into pML123 (*Xba*I). Miniprep from overnight colonies showed 2/8 successful ligation reactions where insert was seen after *Bam*HI, *Xho*I digestion (right image).

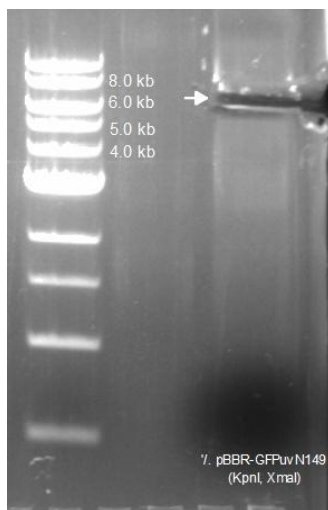


Figure 52: Digestion of vector pBBR1MCS3-GFPuvN149TAG for insertion of *lpp* or *pro* promoter//tRNA/terminator cassette. Reaction was performed overnight, followed by dephosphorylation with Antarctic phosphatase.