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Determining the Fidelity of Protein Synthesis in vivo Utlilizing Bacillus Subtilis ComX Pheromone

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Determining the Fidelity of Protein Synthesis *in vivo* Utlilizing *Bacillus Subtilis* ComX Pheromone

A thesis submitted in partial satisfaction
of the requirements for the degree Master of Science
in Microbiology, Immunology and Molecular Genetics

by

Nicole Seu Fun Yu

2012

ABSTRACT OF THE THESIS

Determining the Fidelity of Protein Synthesis *in vivo*

Utlilizing *Bacillus Subtilis* ComX Pheromone

by

Nicole Seu Fun Yu

Master of Science in Microbiology, Immunology and Molecular Genetics

University of California, Los Angeles, 2012

Professor Beth A. Lazazzera, Chair

Cells maintain fidelity in protein synthesis through various mechanisms including aminoacyl-tRNA synthetases (aaRSs) that are able to discriminate tRNAs and their cognate amino acids. Substrate specificity along with editing functions of aaRSs act to prevent or remove incorrect amino acids charged onto tRNAs. Editing domains are highly conserved and found in both prokaryotes and eukaryotes; knocking out the editing function of an aaRS does not seem to affect cell viability. To determine the biological relevance of the editing domains found on aaRSs, it is essential to determine the contribution of editing to protein fidelity *in vivo*. *Bacillus subtilis* serves as an ideal model to study this phenomenon because it possesses a small gene-enocoded, 10 amino acid pheromone peptide, ComX. ComX is processed, modified, and secreted by *B. subtilis* as a signaling molecule that can easily be

purified. The amino acid sequence of ComX can be altered to some degree while maintaining cellular activity enabling different strains that produce variants of ComX pheromone to be compared for levels of mistranslation. Ultimately future experiments will reveal the impact that editing by an aaRS has on the fidelity of protein synthesis *in vivo* by quantifying the rates of mistranslation in strains of *B. subtilis* that express an editing-defective tRNA synthetase and comparing it to levels of mistranslation to wild type strains.

The thesis of Nicole Seu Fun Yu is approved.

Kenneth Bradley

Robert Gunsalus

Beth A. Lazazzera, Committee Chair

University of California, Los Angeles

2012

Dedication

This work is dedicated to my family. Without your love, support, and patience, it would not have been possible, and for helping me to develop into the person I am today.

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ACKNOWLEDGEMENTS

I would like to thank my advisor Beth Lazazzera for her helpful guidance and support during my research. Her mentorship was invaluable to the completion of this project as she challenged me to grow as a scientist. I would also like to thank my committee members, Kenneth Bradley and Robert Gunsalus for their support and patience.

To my fellow graduate students, Rebecca Terra, Geraldine Briceno and Sara Pirooz, I am thankful for their assistance, encouragement, and friendship. Lastly, I would like to express my appreciation and gratitude to family and senseis for their support and understanding that has provided me the strength to pursue my dreams.

Chapter 1

Introduction

Living cells maintain themselves by carrying out essential functions in response to changing environmental and internal cues. Inadequately functioning proteins would lead to inefficient or absent functions detrimental to cell survival. Maintaining the fidelity of protein synthesis is exceptionally important because properly folded enzymes and proteins are essential for normal cellular functions. There are several important variables that contribute to preserving the fidelity of the genetic code during protein synthesis. Of primary importance is maintenance of the DNA sequence that encodes protein sequences as well as instructions for processing and modifying proteins. In addition to protecting the DNA sequence, reliability of transcription and translation also play important roles in the fidelity of protein synthesis.

Factors that contribute to the fidelity of protein synthesis include DNA replication enzymes, RNA polymerases, tRNA-synthetases and factors like EF-Tu that deliver charged aminoacyl-tRNAs to the ribosome (Ling, 2009). The precise contribution of each of these factors in maintaining the fidelity of protein synthesis *in vivo* is unknown. Overall the multiple factors that cumulatively contribute to the complexity of preserving the fidelity of protein synthesis result in a net error rate of one in every 10^3-10^4 codons (Loftfield, 1972). These measurements were made in the 1970's, by using radio-labeled amino acids to measure amounts of incorrect amino acids incorporated into a protein. The error rate of protein synthesis has not been re-measured using more sensitive and current techniques. A considerable limitation of utilizing radio-labeled amino acids in the assay by Lotfield (1972) is that their method is not as sensitive as using mass spectrometry assays (Lia, 2011). Another current and more robust assay can be used for the observation of amino acid attachment to tRNA (AA-tRNA) synthesis through the use

of tRNA which is ^{32}P-labeled at the terminal C-*p*-A phosphodiester linkage (Wolfson, 2002). This is a method that directly measures the fraction of aminoacylated tRNAs by monitoring amino acid attachment to the 3′-^{32}P-labeled tRNA because it separates AMP and esterified AA-AMP by TLC.

A key way that cells maintain the fidelity of protein synthesis is through discrimination of tRNAs and their cognate amino acids by aminoacyl-tRNA synthetases (aaRSs). AaRSs are vital in the maintenance of the genetic code through correct pairing of amino acids with their corresponding tRNA. AaRSs have duel functionality in their ability to perform aminoacylation and editing functions at separate domains of the enzyme (Figure 1)(Korencic, 2004, Roy, 2004). Aminoacylation occurs in a two-step reaction in the catalytic site (First, 1998). In the first step the amino acid is adenylated; and in the second step the amino acid-adenylate is hydrolyzed so that the amino acid is transferred onto the tRNA (Figure 2). Editing domains serve to remove amino acids from incorrectly charged tRNAs through either the hydrolysis of incorrectly formed amino acid adenylate (aa-AMP) (pre-transfer editing) or the hydrolysis of non-cognate aa-tRNA (post-transfer editing), or for some enzymes a combination of both (Figure 3) (Sarkar, 2011). Substrate discrimination of structurally similar amino acids proves to be more difficult for aaRSs than selecting for larger and more structurally distinct tRNAs. For example the amino acids phenylalanine and tyrosine are chemically identical except for a hydroxyl (-OH) group, and leucine and isoleucine do not differ chemically, but only by positioning of one methyl (-CH3) group. Activation of non-cognate amino acids by aaRSs occurs commonly, occurring sometimes up to 1 in 150 compared to the cognate amino acid (Beuning, 2000, Dock-Bregeon, 2000, Fukai, 2000, Hendrickson, 2002,

Jakubowski, 1992, Kurland, 1992, Mursinna, 2002). This frequency is much greater than the net error rate in translation of about 1 in 10^4, indicating that the inability of aaRSs to discriminate similar amino acids does not compromise the fidelity of translation (Kurland, 1992). Editing compensates for the difficulties of amino acid discrimination. For example isolecucyl-tRNA synthetase (IleRS) activates non-cognate valine only about 200 fold less efficiently than Ile, making a putatively high level of misincorporation of valine instead of Isoleucine by the ribosome during translation (Schmidt, 1995). The activity of the editing domain on IleRS subsequently prevents a high level of valine incorporation by hydrolyzing mischarged amino acid:tRNA pairs (Ling, 2009, Nomanbhoy, 1999).

The editing site is presumed to have the same size discrimination mechanism as the aminoacylation site except that amino acids smaller than the cognate one are accepted and larger cognate amino acids are excluded. Initial discrimination at the aminoacylation site in combination with hydrolysis of bonds formed with non-cognate amino acids in the spatially separate editing site constitutes a double sieve model of editing of an aaRS (Fersht, 1999). This double sieve model presents editing to first occur by having large non-cognate amino acids excluded by size at the aminoacylation site (the first sieve) leaving both cognate and small non-cognate amino acids to be activated. Before release from the aaRS a bond between a tRNA and a small non-cognate amino acid is hydrolyzed by the editing site (the second sieve), that relies on a size discrimination mechanism like the active site except that smaller non-cognate amino acids are accepted and the larger cognate amino acid is excluded. In addition to these 2 mechanisms it has also been proposed that there is re-sampling that is also used as a mechanism for maintaining the

fidelity of protein synthesis (Ling, 2009A). This process of re-sampling allows released mischarged tRNAs to rebind with the aaRS, allowing contact with the editing site to correct mistakes to reduce the rate of error, instead of being shuttled directly by EF-Tu for protein synthesis.

Editing domains are highly conserved and found in both prokaryotes and eukaryotes. There are 20 canonical aaRSs in two highly conserved unrelated structural groups, classes I and II, with 10 aaRSs in each (Eriani, 1990, Cusack, 1997, O'Donoghue, 2003). Division of aaRSs that have certain aa specificities have been almost completely conserved through evolution with the exception of lysyl-tRNA synthetase that belongs to both groups (Ibba, 1997). Structural and functional studies have discovered editing sites for several aaRSs located 35-40 Å away from the aminoacylation site that can be disrupted without affecting aminoacylation activity (Sarkar, 2011). Even though aaRSs are highly conserved knocking out of the editing domains of at least ThrRS, PheRS, and LeuRS in mycoplasma and LeuRS in yeast and human mitochondria has little effect on cell viability except under certain stress conditions (Karkhanis, 2006, Lue, 2005 and Reynolds, 2010). Protein synthesis occurs in the cytoplasm and in organelles in eukaryotes (Dietrich, 1992) and some aaRSs's in organelles lack editing domains such as mitochondrial PheRS without any ill effects, leaving the question of what is the editing domain's biological importance and why is it so highly conserved (Roy, 2005)? In this study, we investigate methods of developing an assay to detect mistranslation *in vivo* and to determine the contribution of aaRSs to the overall rate of editing.

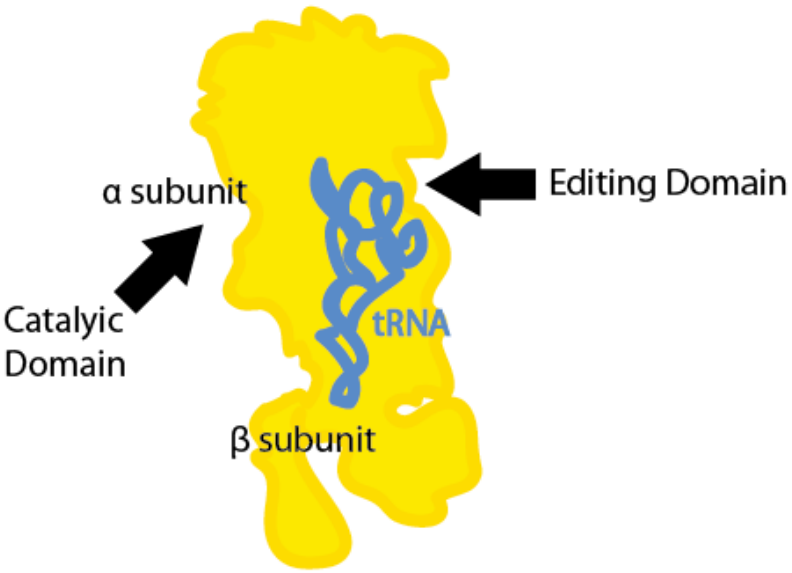

Figure 1. Distinct Aminoacylation and Editing domain. The α domain contains the catalytic site while the ß subunit of PheRS contains the editing site located ~40Å away from each other. Figure modified from Roy et. al 2004.

(1) Aa + ATP + aaRS ↔ aaRS•aa-AMP + Ppi

(2) aaRS•aa-AMP + tRNA ↔ aaRS + aa-tRNA + AMP

Figure 2. Aminoacylation occurs in a two-step reaction. (Aa) amino acid, (aaRS) aminoacyl-tRNA synthetase. In step (1) the amino acid is charged to form an amino acid adenylate intermediate. In step (2) the amino acid adneylate is transferred to the tRNA.

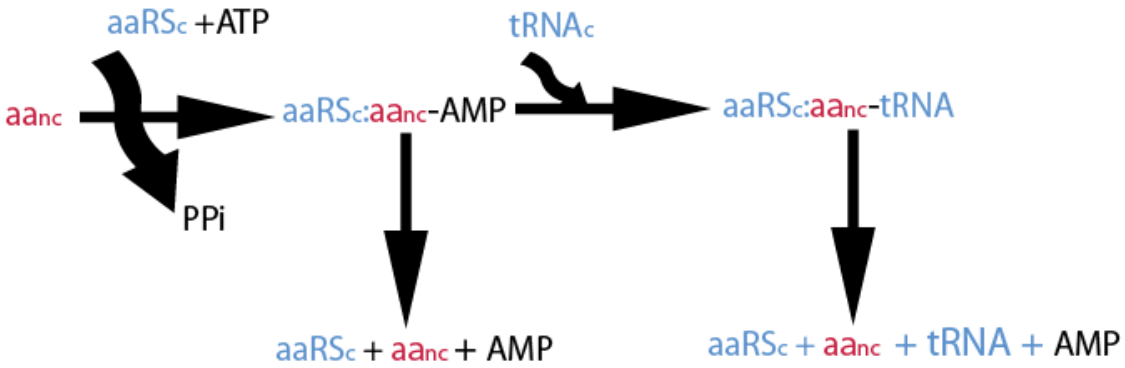

Figure 3. Pre and Post-transfer editing. AaRS editing. Non-cognate amino acid (aanc) is activated and forms a complex with a cognate (c) aaRS. The resulting aa-AMP is either hydrolyzed or transferred to cognate tRNA (tRNAc) and then edited.

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Chapter 2

Bacillus subtilis ComX Phermone is unable to tolerate all hydrophobic single amino acid substitutions

Introduction

The fidelity of protein synthesis is primarily determined by two main events in translation but precise contribution of multiple factors involved in maintaining accuracy of protein synthesis *in vivo* is currently unknown. The main factors in quality control of the synthesis of proteins are the synthesis of cognate amino acid:tRNA pairs by aminoacyl-tRNA synthetases (aaRSs) and the correct pairing of matching aminoacyl-tRNAs (aa-tRNAs) by the ribosome. Even though the precise error rate *in vivo* remains mostly unknown the base rate of error for translation often cited is approximated to be about 1 mistake per 10^4 codons translated in systems ranging from *Escherichia coli* proteins to mammalian hemoglobin (Kramer, 2007). Currently the role that editing functions of aaRSs play are not well understood even though editing by aaRSs does provide a mechanism to protect against amino acid misincorporation. The editing activities of aaRS are nearly universally conserved, but their quality control mechanisms appear to be dispensable for cell viability under standard laboratory growth conditions (Ibba, 1994, Pezo, 2004, Reynolds, 2010, Karkhanis, 2006, Ruan, 2008).

To assess the contribution of editing by aaRSs to protein fidelity *in vivo*, it is essential to be able monitor the levels of mistranslation. The Gram-positive bacterium *Bacillus subtilis* is an ideal model to develop assays for monitoring protein synthesis in a cell. *B. subtilis* naturally secretes a small, 10 amino acid peptide, ComX that can be utilized to measure levels of mistranslation *in vivo* (Figure 1). ComX is processed and modified before being secreted by *B. subtilis* as a signaling molecule where it functions to regulate genetic competence (Hamoen, 2003). ComX peptide variants were produced by site directed mutagenesis resulting in peptides that differed in only one amino acid.

ComX mutants were created in pairs, one with either the native or other selected amino acid and the other with a similar but non-cognate amino acid. For example Figure 2, shows a ComX pair that has similar but non-cognate amino acids. The mutated ComX pheromones that contain similar but non-cognate amino acids can be compared to monitor mistranslation *in vivo*. To evaluate error rates purified pools of ComX from *B. subtilis* that produce each variant will be quantified by mass spectrometry. The purification and expression of ComX by the different variants need to be at comparable levels to ensure that measuring the fidelity of protein synthesis is accurate. It is essential that they be tested to ensure that the ComX produced is properly secreted and processed or we would not be able to see the mistranslated peptide in a pool of purified ComX.

ComX pheromone is structurally composed of a tri-cyclic tryptophan with a hydrophobic farnesyl modification (Figure 1 and 9A). The salient farnesyl or geranyl modification can be used to easily purify ComX pheromone from *B. subtilis* cultured supernatants. ComX naturally acts as an extracellular signaling molecule that activates the transcription factor ComA~P that regulates quorum responses and genetic competence (Figure 3) (Hamoen, 2003). This study shows that not all positions previously shown to tolerate alanine substitutions on the mature ComX pheromone are able to endure other point mutations without loss of cellular activity (Schneider, 2002).

Results

The positions 4, 5, and 10 of *B. subtilis* 168 ComX were chosen for site directed mutagenesis because these positions were either unaffected or produced greater activity than wild type ComX when substituted with an alanine (Schneider, 2002). Position 4 and

5 on ComX was substituted with a phenylalanine (Phe) or a tyrosine (Tyr) because they are similar but not cognate amino acids (Figure 2). Substitutions of Phe or Tyr at position 4 resulted in a loss of activity compared to the wild type as determined by ß-gal assay (Figure 4). The ß-gal assay is an indirect measurement of ComX production as it measures the activation of ComA~P which in turn activates the srfA promoter fused to the lacZ gene (Figure 5). At position 5 there was a loss of activity with a Phe substitution (Figure 4). The aspartic acid residue that is normally at position 10 in ComX was also substituted with a Phe or a Tyr as it was also shown to tolerate the previous hydrophobic substitution with an alanine. Even though positions 4 and 5 did not tolerated Phe or Tyr substitution position 10 initially appeared to tolerate a substitution with either a Phe or a Tyr when whole cells were used to test ComX activity, where cultures were grown to stationary phase and an aliquot of cells was lysed for ß-gal assays (Figures 6 and 7). When testing these same mutants by isolating culture supernatants instead of using cells for ComX, βgal enzymatic assays activity levels above background or negative controls could not be found. Phe and Tyr substitutions were not viable for further experiments because ComX could not be easily isolated from the supernatant and used for comparison of rates of mistranslation, so other non-cognate ComX mutant pairs were created, consisting of one amino acid and the corresponding non-cognate pair.

At position 5 in ComX the naturally occurring threonine was substituted with a similar amino acid, serine. The activity of the substitution of serine for a threonine was similar to that of wild type (Figure 8). At position 10 in ComX I also created a pair of non-cognate ComX sequenes with isoleucine or valine substituting for the endogenous

aspartic acid. The isoleucine and valine substitutions at position 10 had ComX activities that were similar to each other but lower than that of wild type (Figure 8).

Discussion

Mutagenesis of positions 4, 5 and 10 of ComX previously shown to be tolerant to alanine substitutions proved to be intolerant to substitution by larger and more hydrophobic amino acids phenylalanine and tyrosine. Interestingly the Phe or Tyr substitutions at position 10 had cells that produced active ComX confirmed by whole cell activity assays where cells were lysed before being used for ß-gal assays, but when the culture supernatant was separated (cells were left intact an filtered from the media they were grown in), the cell free fraction did not have ComX activity. A putative reason why there was no ComX activity in the supernatant was that changing the last residue of ComX, an aspartic acid to a Phe considerably increased the hydrophobicity of the small signaling molecule. This increased level of hydrophobicity may be enough to potentially cause ComX to become localized and trapped in the membrane of the cell and not be excreted. The ability for these cells to retain cell-cell signaling may be due to protrusion of ComX from the membrane when cells come into contact with one another. Even though the activity of the isoleucine and valine substitutions at position 10 of ComX had lower activity than wild type they still have the potential to be used in future assays comparing rates of mistranslation because they have comparable rates of acitity and can be easily purified from cultured supernatants. As a result of this mutagenesis study it was found that not all positions on ComX pheromone equally tolerate a hydrophobic substitution (Figure 9 and 10). While some positions may be able tolerate an alanine,

substituting a larger hydrophobic amino acid such as phenylalanine results in loss of activity.

Having *B. subtilis* strains that produce ComX with similar but non-cognate amino acids will allow us to see how often non-cognate amino acids become incorporated into peptides and what contribution the editing domain has on quality control correcting mischarged tRNAs before amino acids are incorporated into peptides.

Materials and Methods

Media and Growth Conditions

B. subtilis cells were grown with shaking at 37°C in a defined $S7_{50}$ minimal medium that contained 1% glucose, 0.1% glutamate, and required amino acids at 40 μg/ml (tryptophan and phenylalanine), as described previously (20). When appropriate antibiotics were added at the following concentration: erythromycin (0.5 μg/ml).

Strain Construction

The *B. subtilis* strains were constructed by transformation with chromosomal DNA or plasmids using standard protocols (Bacillus: Cellular and Molecular Biology 2007).

To generate mutants that had similar but non cognate amino acids, we used site-directed mutagenesis on plasmid pTH2 (derivative of phP13, multicopy plasmid in B. subtilis containing *ComQX*) using primers listed in Table 1 below, and confirmed by sequencing using BL 718 5'-GGCTACAGGAGAATATATTGAAACAG-3' internal to ComQ. The plasmids generated from this mutagenesis were transformed into *B. subtilis* strain BAL3356 (*comX srfA-lacZ-neo*). Transformants were plated and streak purified on Erythromycin plates.

Quantifiction of ComX using a biological assay

Before comparing rates of mistranslation it is important to make sure that each ComX variant is equally expressed and purified at comparable quantities. Trifluoroacetic acid (TFA) was added to samples to achieve a final concentration of 20% and a pH of 2. Each sample was passed through 1-ml C18 reverse-phase column (Sep-Pak Plus, Waters), which was previously activated with 30mls 80% acetonitrile (ACN), 0.1% TFA and equilibrated with 10mls 20% ACN, 0.1% aqueous TFA solution. After loading the sample, the column was washed with 3 ml of 20%, ACN containing 0.1% TFA and the ComX peptides were eluted with 80% ACN, 0.1% TFA. 500ul of the eluates were dried in a vacuum centrifuge and re-suspended in 500ul S750 minimal growth medium containing 50 μg ml^{-1} bovine serum albumin. Ten, twofold serial dilutions were made of each sample and these were incubated with a specific *B. subtilis* reporter strain for 60 min at 37°C. The *B. subtilis* reporter strains used for ComX detection do not produce their own edogenous ComX but specifically detect ComX and selectively express a *lacZ* fusion reporter gene in the presence of ComX can be used to measure the pheromones activity (Fig. 7) (Tortosa, 2001). The level of β-galactosidase specific activity for the cells treated with column eluates were compared with the level of activity for the cells treated with eluates from cells that did not produce ComX.

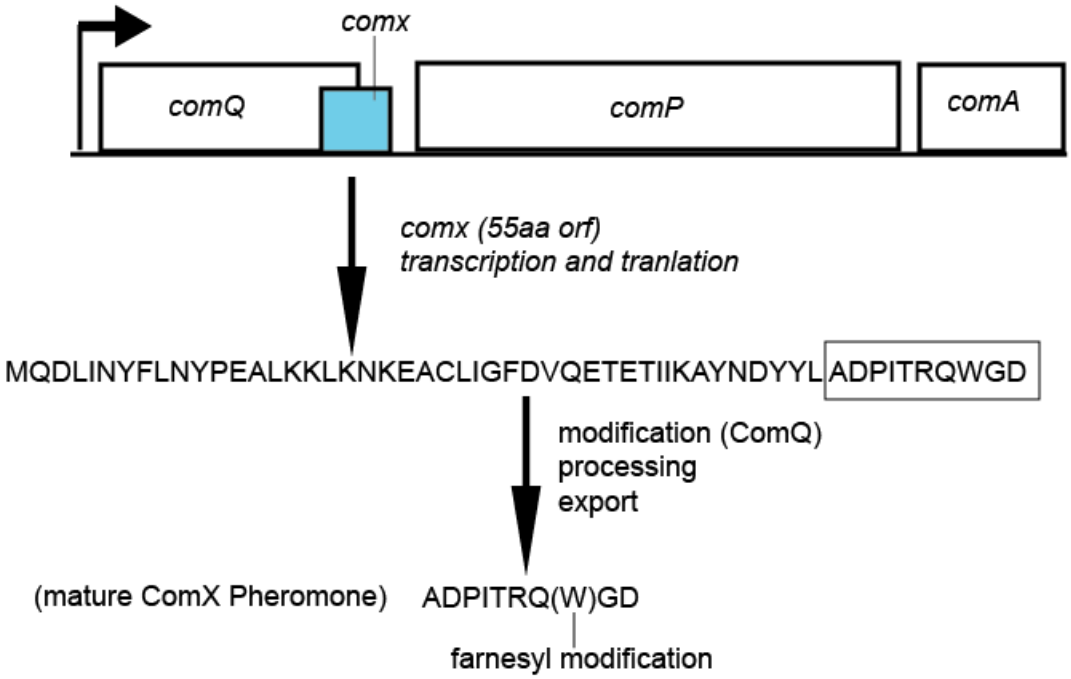


Figure 1. *B. subtilis* gene organization of *comQ, comX*, *comP*, and *comA*. ComX pheromone is produced by B. subtilis, the mature peptide is processed and transported out of the cell.

Wt	**ADPITRQWGD**
D10F	**ADPITRQWGF**
D10Y	**ADPITRQWGY**

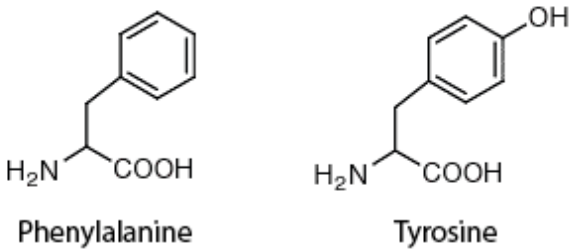


Figure 2. Pair of ComX Pheromones with position 10 substituted with similar but non-cognate amino acids. Wt= wild type, F=phenylalanine, Y= tyrosine. Amino acids shown above where the only difference between phenylalanine and tyrosine is a hydroxyl group (-OH).

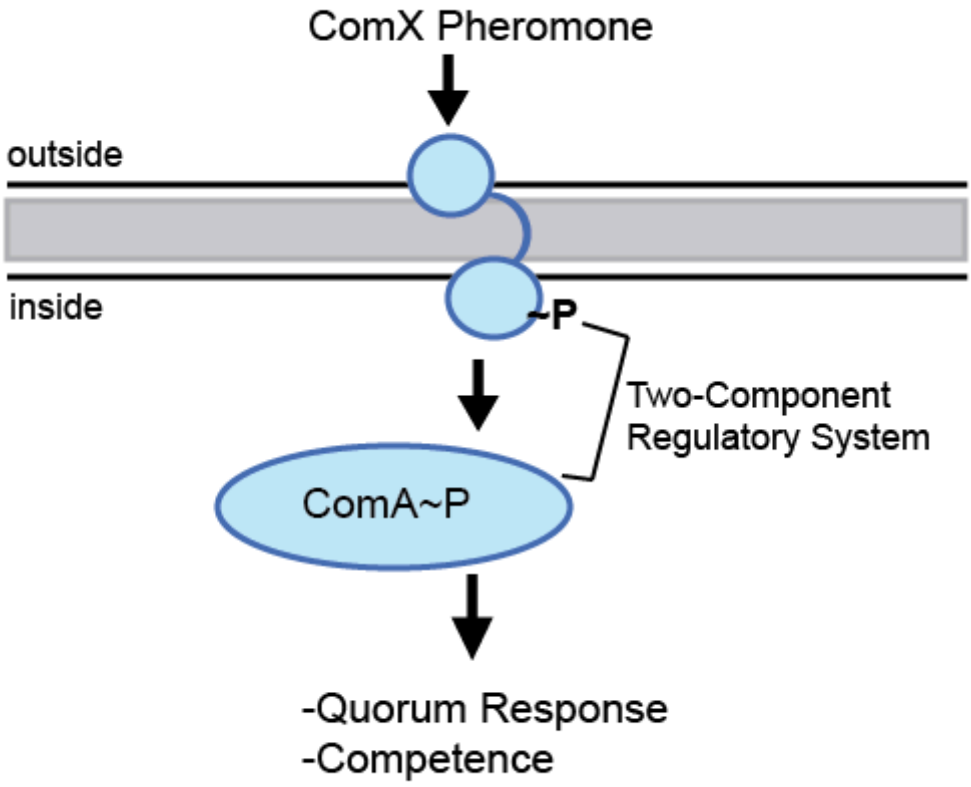


Figure 3. ComX Response in *B. subitilis*. ComX functions as a sensing molecule that modulates changes in gene expression in response to cell density.

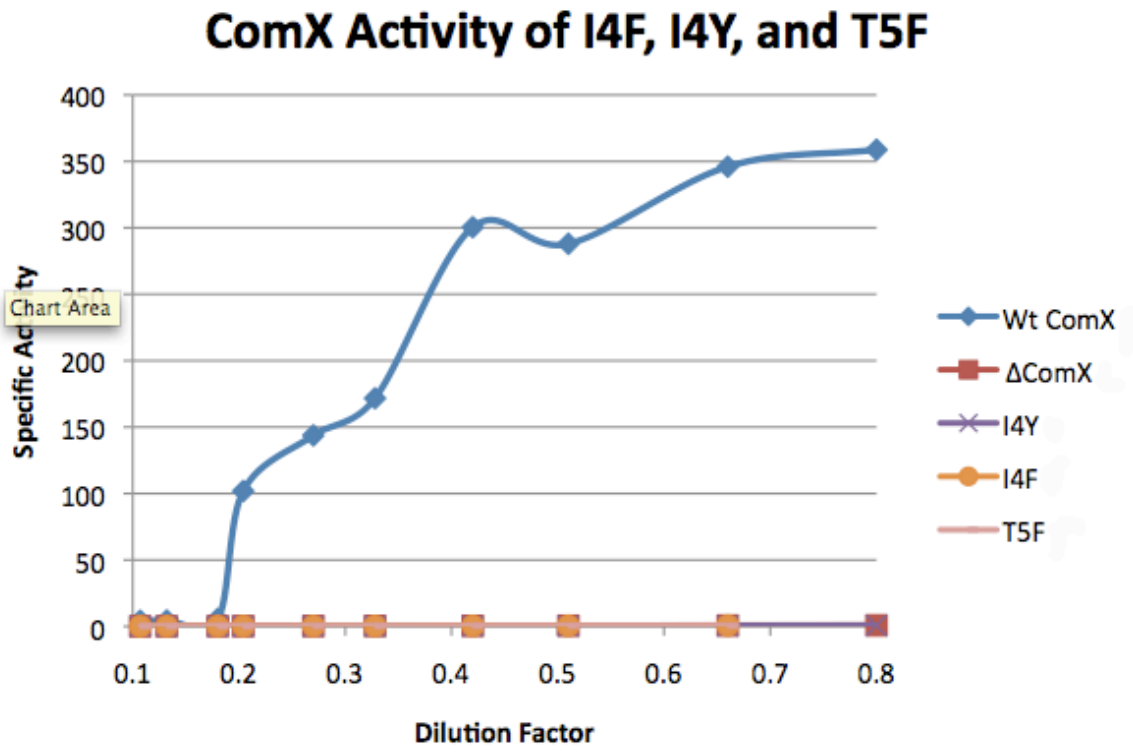


Figure 4. ComX activity of I4F, I4Y and T5F mutants. Substitutions of Phe or Tyr at positon 4 of ComX and Phe at position 5 resulted in loss of cellular activity.

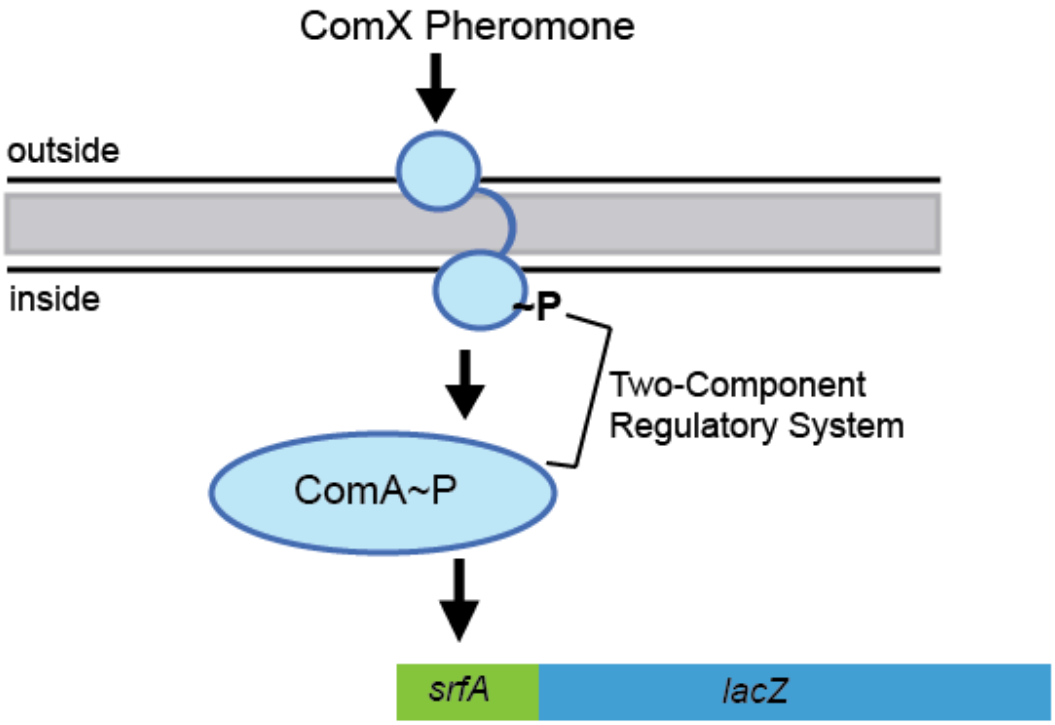


Figure 5. βgal assay to assess production of ComX. ComX that is purified from a producer *B. subtilis* strain is sensed by a reporter strain that does not produce its own endogenous ComX.

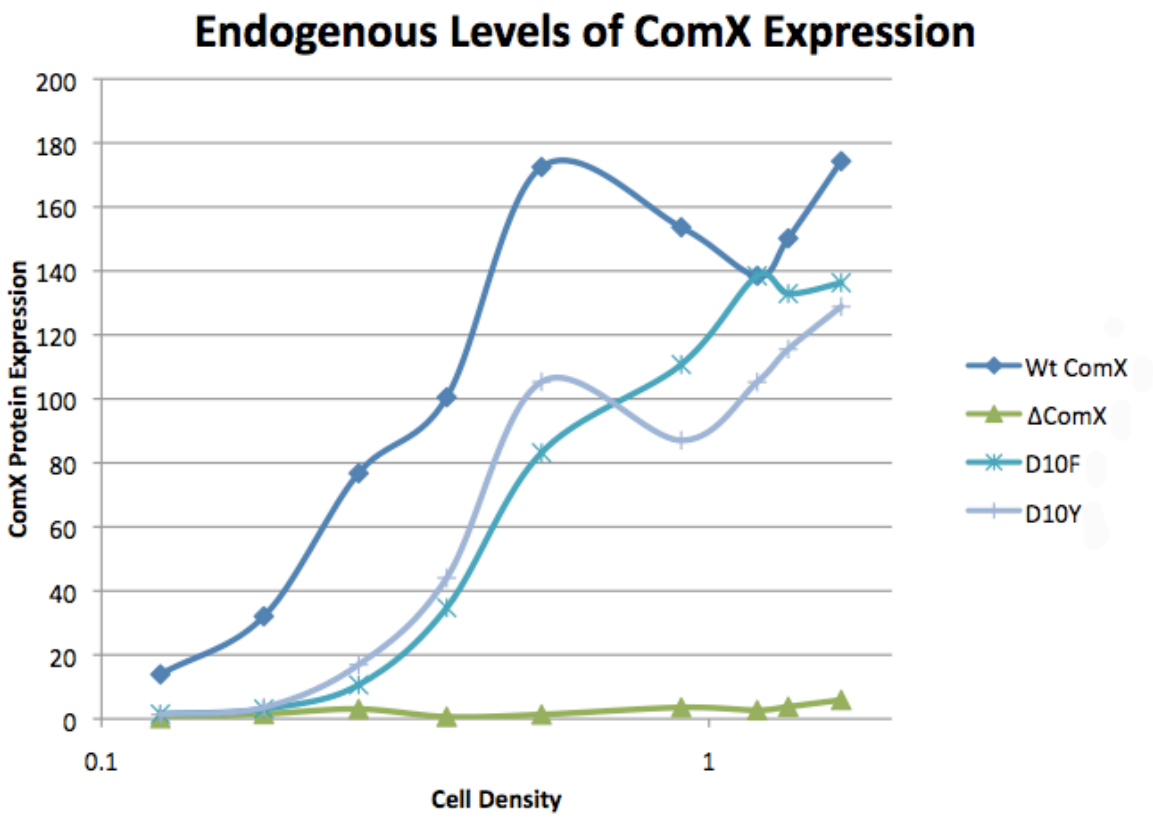


Figure 6. Cell Density (OD600) vs. Protein expression (Specific Activity). As cell density increases, expression of ComX increases. An accumulation of ComX can be seen in wild type (Wt) cells. In the ΔComX there is no increase in gene expression. In the position 10 substitutions in ComX the mutant levels of the Phe and Tyr variants are lower than Wt but do not differ from one another in expression levels.

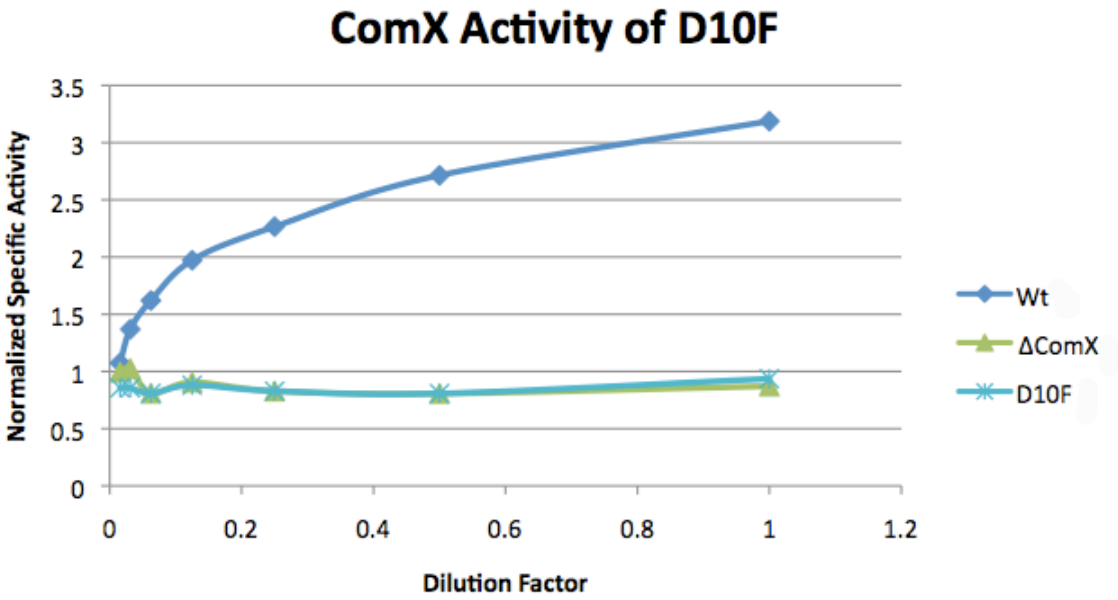


Figure 7. ComX activity of D10F mutant. Compared to wild type or ΔComX the Phe substituted version of ComX did not have cellular activity in the cell free culture supernatants. Specific activity levels normalized to ΔComX.

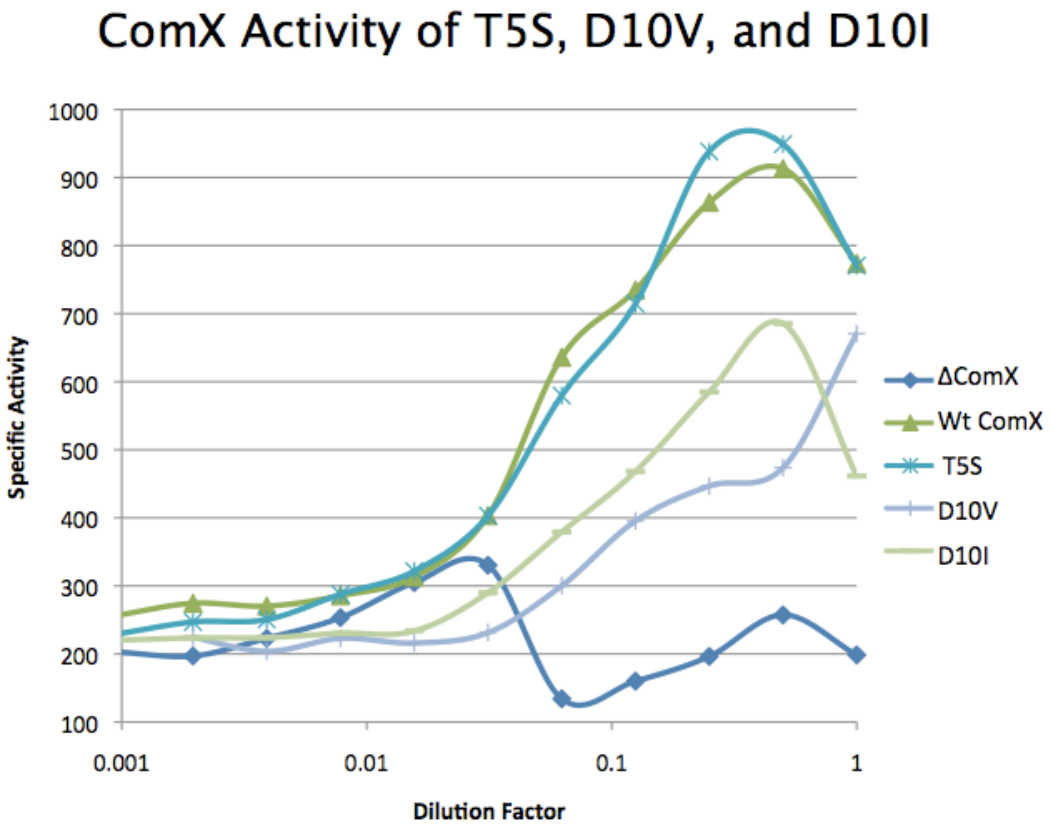
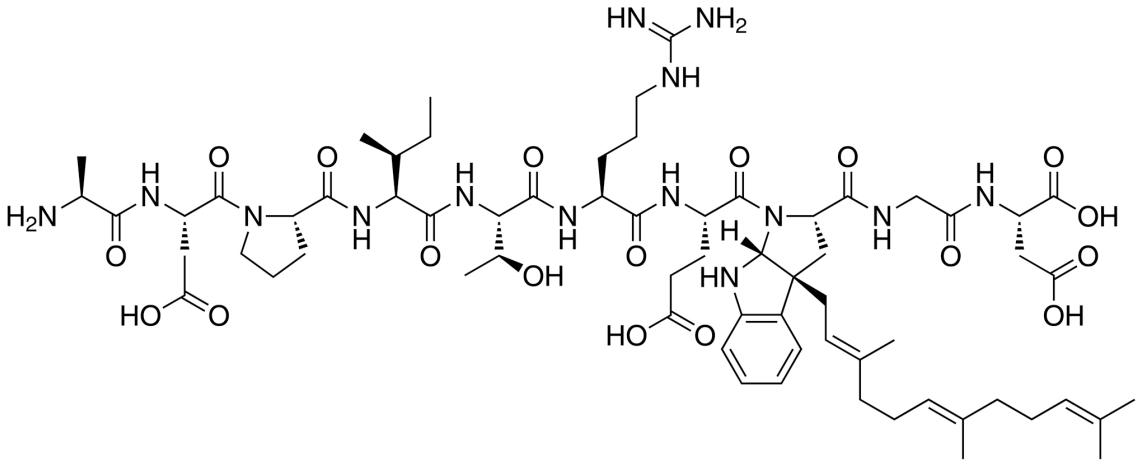
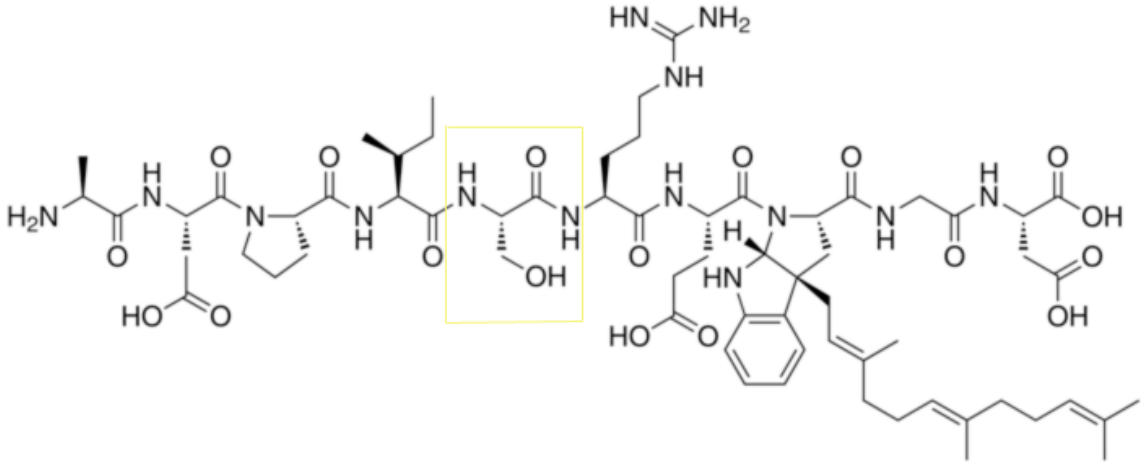


Figure 8. ComX activity of additional position 10 and 5 mutants. Extracellular activity was maintained in the T5S mutant that was similar to wild type. D10I and D10V had similar activity rates to one another.

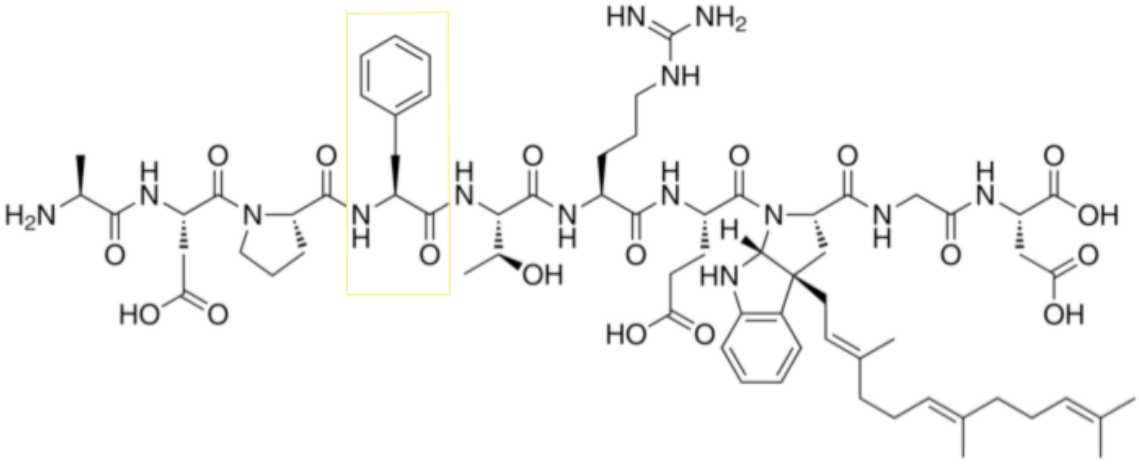
A. Wild type 168 ComX



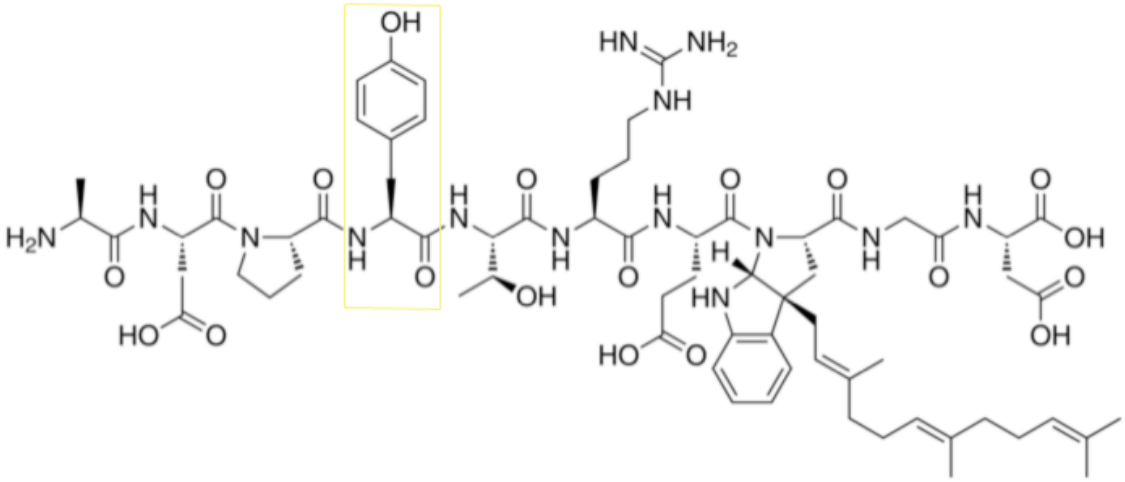
B. T5S



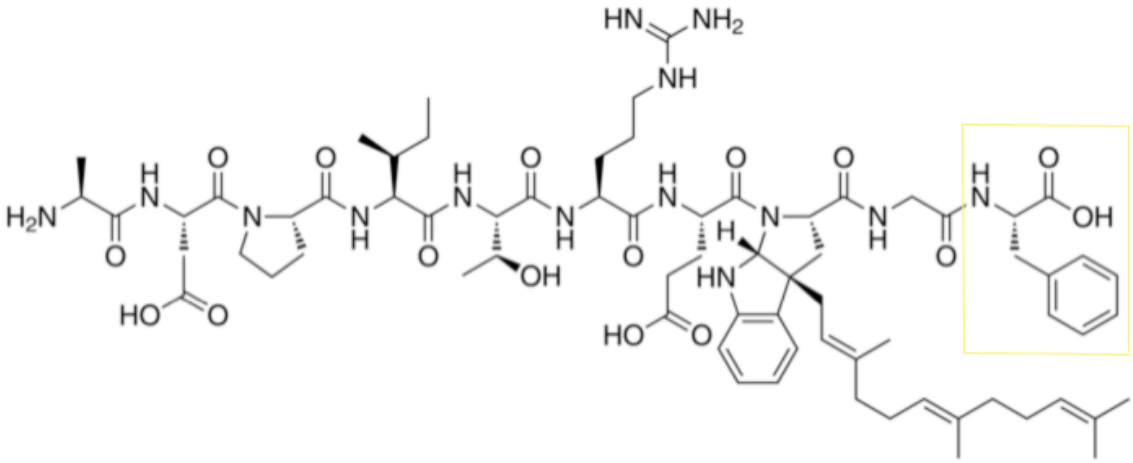
C. I4F



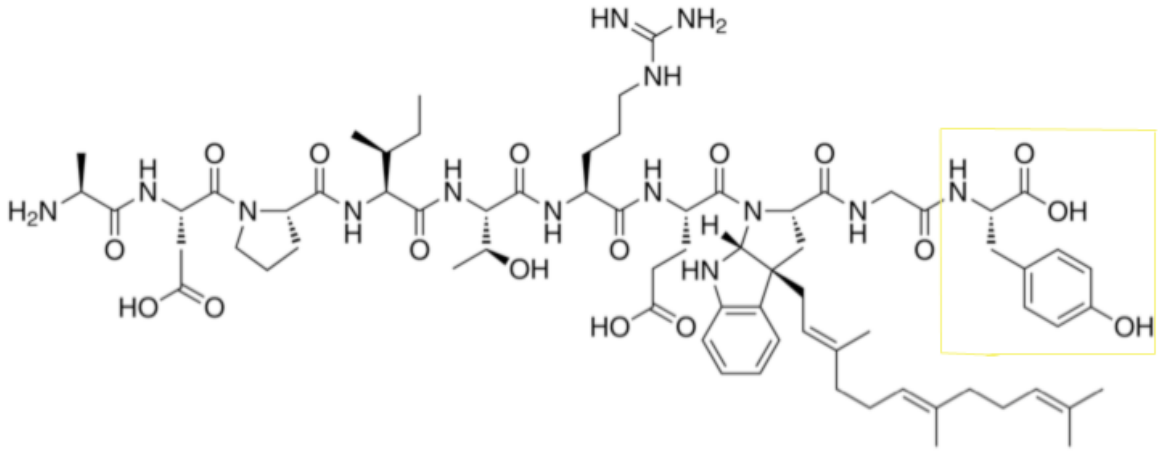
D. I4Y



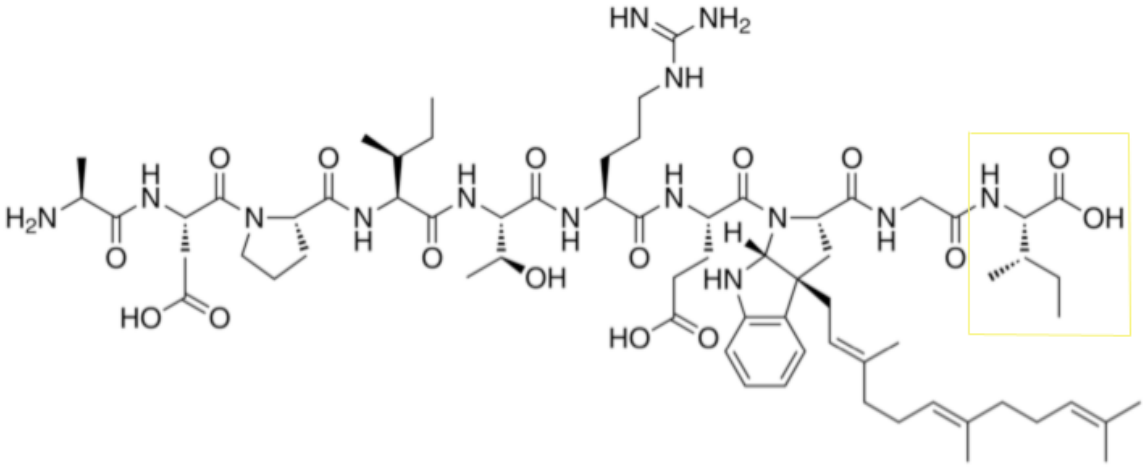
E. D10F



F. D10Y



G. D10I



H. D10V

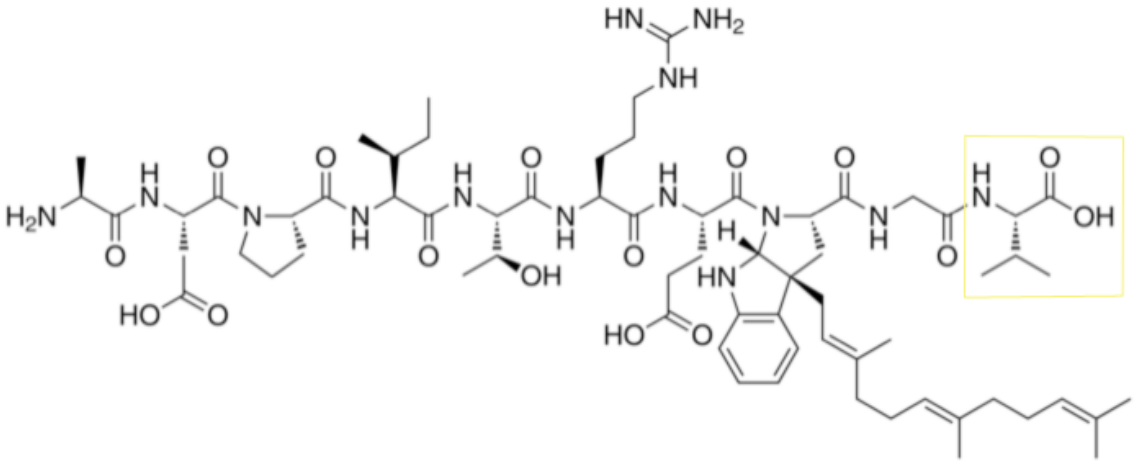


Figure 9. Structure of ComX pheromone and single amino acid subtitutions. (A) Wild type 168 ComX. (B) T5S (C) I4F (D) I4Y (E) D10F (F) D10Y (G) D10I (H) D10V.

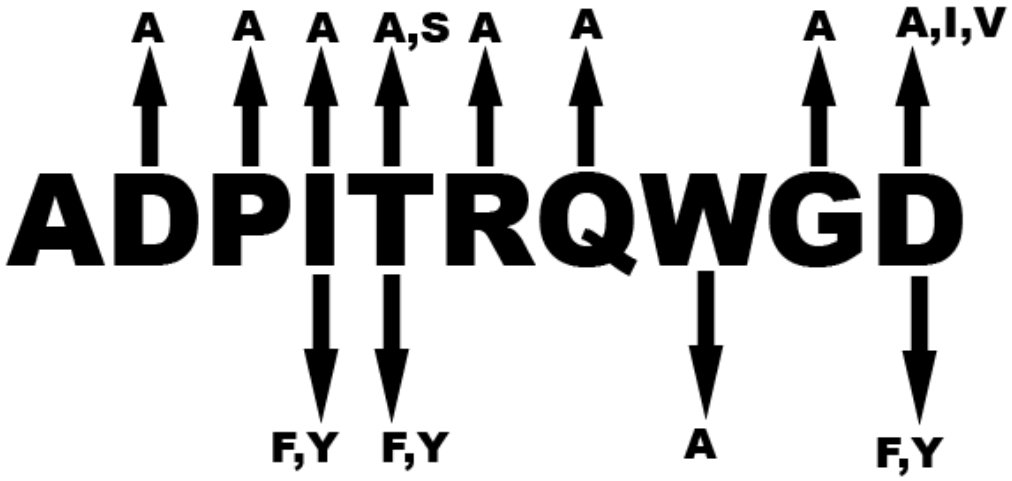


Figure 10. *B. subtilis* 168 ComX extracellular signaling activity by mutants generated by site directed mutagenesis. Up arrows indicate increased, wild type, or comparable levels of activity while downward arrows represent absence of activity. A= alanine, F= phenylalanine, I= isoleucine, V= valine. Summary of data from this study and Schneider et. al (2002).

I4V	5’-ATT ATC TGG CTG ATC CAG TAA CCC GTC AAT GGG GTG ATT AAT AGG-3’
	5’-CCT ATT AAT CAC CCC ATT GAC GGG TTA CTG GAT CAG CCA GAT AAT-3’
T5S	5’-ATC TGG CTG ATC CAA TAA AGC GTC AAT GGG GTG-3’
	5’-CAC CCC ATT GAC GCT TTA TTG GAT CAG CCA GAT-3’
I4F	5'-ATT ATC TGG CTG ATC CAT ACA CCC GTC AAT GGG GTG ATT AAT AGG-3'
	5'-CCT ATT CAC CCC ATT GAC GGG TGT ATG GAT CAG CCA GAT AAT-3'
I4Y	5'-ATC TGG CTG ATC CAT TCA CCC GTC AAT GGG GTG-3'
	5'-CAC CCC ATT GAC GGG TGA ATG GAT CAG CCA GAT-3'
D10F	5'-CCC GTC AAT GGG GTT TCT AAT AGG TGG-3'
	5'-CCA CCT ATT AGA AAC CCC ATT GAC GGG-3'
D10Y	5'-CCC GTC AAT GGG GTT ACT AAT AGG TGG-3'
	5'-CCA CCT ATT AGT AAC CCC ATT GAC GGG-3'
D10I	5’-CCC GTC AAT GGG GTT ATC AAT AGG TGG-3’
	5’-CCA CCT ATT GAT AAC CCC ATT GAC GGG-3’
D10V	5’-CCC GTC AAT GGG GTT GTA AAT AGG TGG-3’
	5’-CCA CCT ATT TAC AAC CCC ATT GAC GGG-3’

Table 2-1. Primers used for ComX site directed mutagenesis.

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Chapter 3

Construction of a PheRS Editing Defective Strain in *Bacillus subtilis*

Introduction

It remains an evolutionary mystery why some aaRSss have editing domains while others do not. Even though aaRSs are indispensable enzymes that work to preserve the genetic code through pairing tRNAs with the correct corresponding amino acids and hydrolyzing incorrectly paired amino acids; this mechanism of removing incorrect amino acids called editing is sometimes dispensable. Previous studies have failed to demonstrate viability defects under standard non-stress growth conditions and knocking out the editing domain of at least a few synthetases such as PheRS, LeuRS, ValRS, AlaRS, and IleRS does not seem to affect cell viability (Karkhanis, 2006, Reynolds, 2010). PheRS serves as an ideal model to investigate the evolutionary significance of editing domains because even though cellular PheRS retains editing functions, they are absent all together in some organelles such as the mitochondria (Roy, 2005). In the event that editing functions were lost within a cell, one would expect there to be detrimental effects on many proteins, however, when PheRS editing is absent in organelles such as yeast mitochondria and in *mycoplasma*, high error rates may be prevented by increased substrate specificity with discrimination against tyrosine (Tyr) (Roy, 2005). This raises the questions of why editing domains are universally conserved through evolution in most organisms and what effects do a variety of different environmental conditions have on cell requirements for translational accuracy?

PheRS occasionally misactivates Tyr instead of phenylalaine (Phe) but it corrects this mistake through editing so that the phenyalanine tRNA-tyrosine pair (Tyr-tRNAphe) is not incorrectly introduced into the genetic code and translated inaccurately (Ling, 2007). A general model has been established for aaRS editing based on either hydrolysis

of misactivated non-cognate amino acids before (pre-transfer) or after (post-transfer) attachment to cognate tRNA. It is now known, that in the case of PheRS and some other aaRSs that post-transfer editing that is critical to maintaining fidelity (Dulic 2010, Minajigi 2010). PheRSs are a class II aaRS composed of 4 subunits with distinct and physically separate aminoacylation and editing domains. Previous studies have shown that PheRS contains separate domains for aminoacylation and editing functions and that the editing domain can be knocked out with out affecting aminoacylation activity *in vitro* (Korencic 2004, Ling 2007).

To assess the contribution of the PheRS editing domain to the fidelity of protein synthesis *in vivo* a *B. subtilis* strain lacking a functional editing domain will be needed. *B. subtilis* serves as an ideal model because it naturally excretes a 10 amino acid peptide, ComX, that is used for cell-cell signaling. *B. subtilis* strains that produce variants of ComX that contain either a Phe or a Tyr exclusively can be used to monitor mistranslation *in vivo*. Phe and Tyr are similar but not cognate amino acids that only differ by a hyrdroxyl group (-OH). Strains that are either wild type or editing defective for *PheST* (phenylalanine tRNA synthetase gene) can have their rates of mistranslation in ComX compared. Editing defective *B. subitilis* that produces ComX with a Phe or a Tyr would be expected to have higher rates of mistranlation, the incorporation of the similar but non-cognate amino acid than the strains that have editing functions.

Homologous recombination will be used to create PheRS editing-defective strain using method of plasmid integration and excision. For this scheme a plasmid that encodes a temperature sensitive origin of replication and erythromycin resistance as a selectable marker and the editing defective version of *pheST* will be integrated by

homologous recombination onto the *B. subtilis* chromosome. These cells will be subjected to counter-selective growth conditions at a low temperature that will stimulate the *B. subitilis* temperature sensitive origin of replication from the inserted plasmid. The plasmid will be excised from the genome by recombination between the wild type and mutant *pheST* alleles. The result will be a mix of cell that will have a normal chromosome structure with one copy of *pheST* that is either the wild type or mutant being split about 50/50 between the two outcomes. The outcomes can be sorted by PCR amplification and sequencing of *pheST*.

In efforts to create an editing deficient PheRS, cloning of the 4kb *pheST* fragment from *B. subtilis* genomic DNA into a vector was an essential first step, so that a point mutation could be introduced into *pheST* to knock out editing but preserve aminoacylation functions. The first method attempted was using a vector for allelic replacement that had been previously described and used in Gram-positive non-transformable bacteria by Arnaud et al 2004. In this method a shuttle vector pMAD that carries a temperature sensitive origin of replication is used to introduce a gene replacement. In a two-step process homologous recombination between a target gene and homologous sequences on pMAD result in the plasmid being transformed into the host chromosome under non-permissive temperatures for pMADs origin of replication (Figure 1). After the integration of pMAD into the host chromosome growth is carried out at a permissive temperature for the sensitive origin of replication producing a second recombination event resulting in half of the cells with the gene of interest replaced.

Results

Initially amplification of the entire 4kb *PheST* from *B. subtilis* genomic DNA by PCR was attempted. The primers were flanked with restriction endonuclease sites (HindIII and BamHI) that resulted in amplified *PheST* with restriction enzyme cut sites at their ends. Attempts were made to ligate cut fragments into a vector with a temperature sensitive replication of origin (pMAD) to incorporate a mutant version of *pheST* by homologous recombination into *B. subtilis*, but the 4kb PheST fragments could not ligated into the pMAD vector successfully. Efforts to clone *pheST* into pJet2.1, pUC19, pBL112 and Pacyc vectors were also unsuccessful. Cloning a full-length *pheST* into multiple vectors may have failed because having its own allele and a copy of *B. subtilis pheST* may be lethal to *E. coli* cells. To test if partial *pheST* was viable in *E. coli* cells a segment containing either most of the 5'end or the 3'end of *pheST* was amplified by PCR for cloning. Even though partial *pheST* could be amplified by PCR it could not be successfully cloned into an *E. coli* vector. Upon a literature investigation, we found that a 12kb fragment containing *B. subtilis pheST* gene was cloned in 1988 (Brakhage, 1989). The 12kb fragment containing *pheST* may have been cloneable because it may contain sequences that detoxify the effects of having a copy of *B. subtilis pheST* in *E. coli*. In an attempt to recreate the previously made clone, primers were designed to encompass the 12kb region, which contained *pheST* and the 5'and 3' end was successfully amplified using primers, but it was not successfully cloned into an *E. coli* vector.

Discussion

From these various and thorough attempts to clone *B. subtilis pheST* it has become evident that there is a lethality associated with the sequence in *E. coli* because numerous efforts to clone it into different vectors were unsuccessful and *B.* subtilis *PheST* has not been cloned in *E. coli* since 1988 (Brakhage, 1989). *pheST* was successfully cloned at that time with an extra flanking region that may be necessary o detoxify the effects of having a copy of the *B. subitilis pheST*. Even though the plasmid integration and excision methods proved unviable, an alternate method using an I-SceI counter selection scheme can be adapted from *Bacillus anthracis* to create an PheRS editing defective strain in *B. subtilis* in future work (Janes, 2006)

Materials and Methods

B. subtilis and *E. coli* strains used in this study are listed below.

1.) pBL112 (*E. coli* MC1061) F'lacIQ LacZM15 Tn10 (Tet), Thr::hyperPspac(erm)

2.) BAL3553 (*B. subtilis* JH642) trpC2, PheA1, amI::pJS34 (srfA-lacZW374 neo) trp and phe aux

3.) BAL2952 (*B. subtilis* JH642) trpC2 pheA1 deltacomX, amyE::srfA-lacZ, pHP13

4.) BAL2508 (*B. subtilis* JH62) trpC2, PheA1, deltacomX, AmyE::srfA-lacZ, pTH2

Amplification of *pheST*

All PCR reactions were carried out under standard protocols as listed by NEB protocol for use of Phusion high fidelity polymerase. For greater specificity 0.2 mM MgCl was added to PCR reactions.

To amplify partial *pheST* two different sets of primers were used. In the first reaction containing the front end of *pheST* BL954 5'-Primer near end of *pheT* with EcoR1 site (5'-GCA TGA ATT CCC ATA TGC TCT CCT TCG TAT AC-3) with BL975 3' Primer near the start of *pheS* with BamHI at end (5'-GAC CGG ATC CGC CTT ACC AGG AGA TT-3') was used. The second set of primers to amplify the back end of PheST were BL974 5'-Primer with EcoR1 site at beginning (5'-GCA TGA ATT CGC GCT TGC GCT TGC TGA TAA AA-3') and BL960 3'primer near the start of PheS 5' with BamHI at the end (5'-GAC CGG ATC CCT GAC TGA GAG AGC ATT TTT AAC-3'). Primers used to amplify full length PheST were primers BL 954 and 960 listed above. Amplified fragments of the appropriate size were isolated by gel extraction by standard protocol provided with Qiagen gel extraction kits.

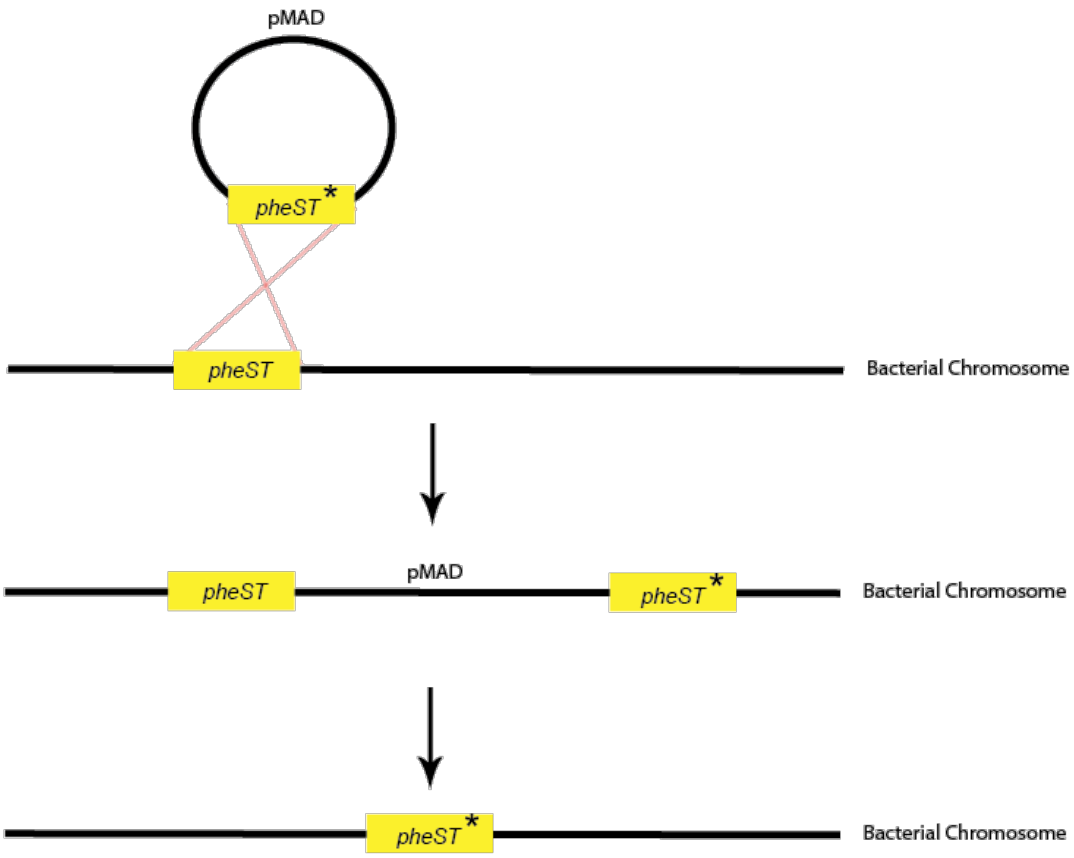

Figure 1. Schematic of homologous recombination to introduce a mutation into *pheST*. A two-step process of homologous recombination between *pheST* and homologous sequences on pMAD resulting in the plasmid being transformed into the host chromosome at non-permissive temperatures for pMADs origin of replication. After the integration of pMAD into the host chromosome growth at a permissive temperature for the origin of replication on pMAD produces a second recombination event resulting in half of the cells with pheST being replaced. *pheST** = editing defective allele, *pheST* = wild type.

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