Modification of Aminoacyl tRNA Synthetase in Order to Incorporate An Unnatural Amino Acid

Grace Burkhart[1], Claire Mammoser[2], Laura Rowe[2]

[1]Valparaiso University, Department of Chemistry, Valparaiso, IN.

Abstract

Proteins allow daily processes in the cell to occur. A protein consists of amino acids. There are twenty natural amino acids coded for in the DNA of organisms. The natural amino acids can be modified to form unnatural amino acids (UAAs). UAAs have useful characteristics when inserted into a protein of a cell, like the ability of fluoresce and the ability to undergo unique reactions. For an UAA to be incorporated into a protein, it must be bound to a transport RNA molecule by an enzyme called aminoacyl tRNA synthetase (aaRS). An existing aaRS was modified in *E. Coli* bacterial cells to incorporate 3-(2-pyridyl)-L-Alanine since it has metal-binding capabilities. Once incorporated, the UAA could act as a sensor for a metal, making it useful to environmental fields. The aaRS was randomly mutated using saturation mutagenesis at sites L32, V65, W108, G158, A159. The cells were run through a positive screen to determine if the mutated aaRS incorporated the UAA into a green fluorescent protein, which glowed if the UAA was inserted. The results of the positive screen showed several mutated aaRSs (2,4,7,8) incorporated 3-(2-pyridyl)-L-Alanine, while other mutants (2, 5, 6, 7, 8, 9) also/instead incorporated the p-cyanophenylalanine amino acid that the original aaRS was designed to select.

Background

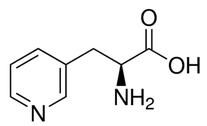


Figure 1. Structure of 3-(2-pyridyl)-L-Alanine (PyAla). Because of its structure, it has the capability to bind metals, specifically Cu^{2+}. (*2*)

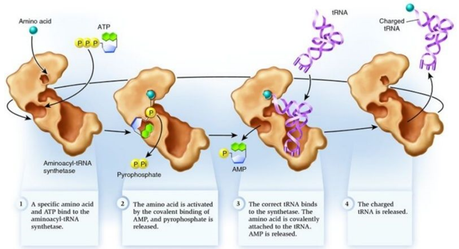


Figure 2. Visualization of the process for how a tRNA molecule gets charged with an amino acid.

The goal of this project is to mutate the aaRS so it will incorporate PyAla using directed evolution. After mutations, the aaRS must undergo two screens to determine its effectiveness. The positive screen tests for PyAla incorporation, and the negative screen tests for PyAla specificity. The positive and negative screen utilize the amber suppression method of unnatural amino acid incorporation, shown in Figure 3. A plasmid coding for a 150TAG mutated green fluorescent protein (sfGFP 150) was inserted into *E. coli* cells containing the mutated aaRS and TAG tRNA plasmid. If a mutated aaRS is successful, the sfGFP 150 will be expressed as a full length protein, causing the cells to fluoresce green.

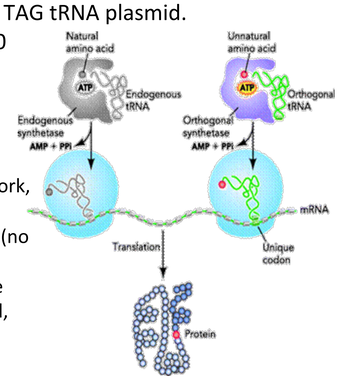


Figure 3. A TAG (stop) codon is inserted into the middle of the DNA sequence (sfGFP) that codes for the protein to be expressed. If the aaRS does not work, and there is no UAA-charged tRNA to override the stop codon, the full length protein is not expressed (no fluorescence). However, if the aaRS does bind the tRNA and UAA together, the TAG stop codon will be overridden, the full length protein will be expressed, and the cells will fluoresce green.

Methods

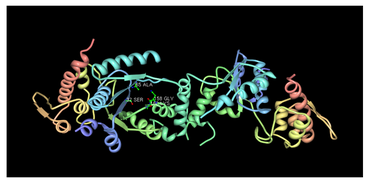
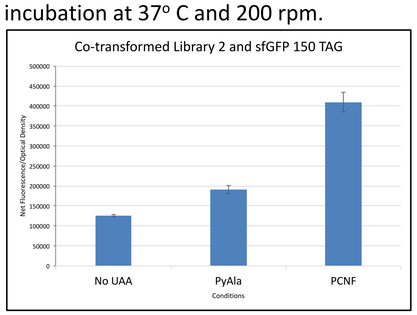


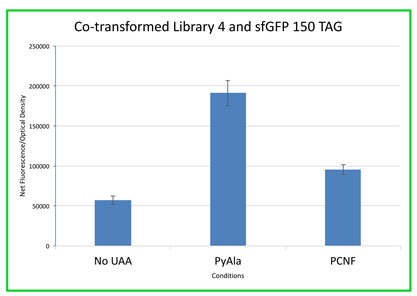
Figure 4. Visual representation of the possible mutation points in the original pULTRA-CNF aaRS enzyme.

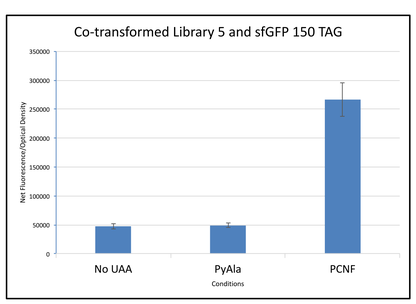
The pULTRA plasmid containing the genes for the p-CNF aaRS and TAG -tRNA was mutated via saturation mutagenesis and degenerate primers for positions L32, V65, W108, G158, and A159 using PCR. (*3*) The mutated plasmids were transformed into *E. coli* cells and grown in spectomycin resistant media. The purified plasmid DNA was restricted, run on agarose gel, and then cells were co-transformed with the plasmid containing the sfGFP 150TAG gene and run through a positive screen. For the positive screen, cells were grown overnight in a culture tube, then 1.5 mL of the culture was added to a flask with 40 mL LB Broth with spectomycin and ampicillin antibiotics for selection. The cells were incubated for 1.5 hours, and PyAla (25 mM) was added to one flask, PCNF (25 mM) was added to the second, and no UAA was added to the third flask.

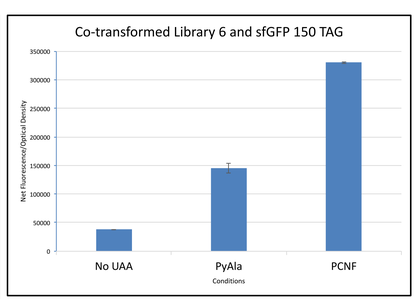
Results and Discussion

The results of the positive screen (Figure 5) on all of the mutated aaRS plasmids. The first bar on the left represents corrected fluorescence of the flask in which there was no UAA, the middle bar the flask with the PyAla only, and the last bar the flask with pCNF only. A fully positive hit should have the middle bar (PyAla) much higher than the other 2 bars. All three flasks had the inducers L-arabinose (0.1%) and IPTG (1 mM) added. Optical density and fluorescence was tested immediately after inducer and UAA addition and then again after overnight incubation at 37° C and 200 rpm.

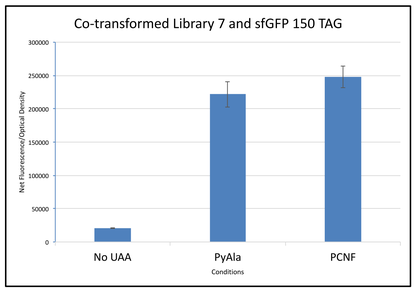


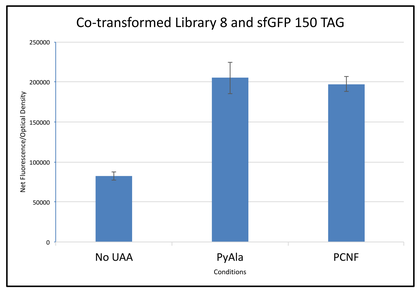






Results and Discussion





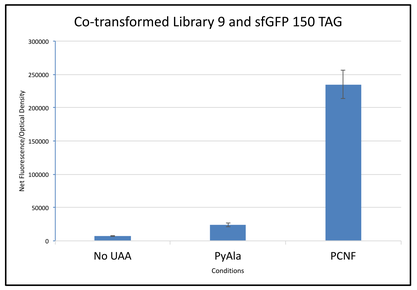
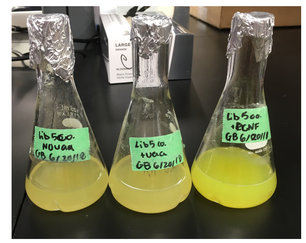


Figure 5. Graphs of the results of the positive screen on the aaRS mutations. The first column shows the control, which has no UAAs added to the broth. The middle column shows the fluorescence of the flask with PyAla added, and the third is with PCNF added. Mutant 4 (green box) indicated is the positive hit.

Figure 6. The three flasks used for the positive screen of co-transformed library plasmid 5. The first flask solution was not glowing green, which is expected, as no UAA was added. The second flask solution that had PyAla added was not glowing, which meant the plasmid failed the positive screen. The third flask solution was glowing, meaning PCNF was incorporated.



Conclusions and Future Work

- Only aaRS mutation 4 solely incorporated the UAA of choice, PyAla, while aaRS mutations 2, 5, 6, 7, 8, and 9 incorporated both PyAla and PCNF, indicating those mutation(s) imparted increased permissivity towards UAA incorporation.
- Future work will include confirming mutation sites with sequencing analysis, expanding the plasmid library via additional saturation mutagenesis, and performing the negative screen on positive hits (4) using a mutated barnase gene containing plasmid.

Acknowledgements

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References

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