

The Effect of EF-P on tRNA Ribosomal P-site Binding

A Senior Thesis

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by

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1. Introduction

1.1 - Bacterial Elongation Factor-P is a Unique Protein with Diverse Academic and Clinical Relevance:

Bacterial elongation factor-P (EF-P) is a universally conserved protein common to all eubacteria. Originally identified as a protein of interest due to its structural similarity in both size and shape to a tRNA, its post-translational modification, and its seemingly essential, but enigmatic role in protein synthesis, EF-P has since become the focus of much scientific inquiry. (1) Initially, a variety of structural and microarray data led researchers to believe that EF-P was responsible for the formation of the first peptide bond in translation. (2) However, subsequent experiments determined that although EF-P is able to increase translation efficiency *in vitro*, it is not vital for ribosome activity. (3) Instead, EF-P's primary function appears to be easing ribosomal stalling that occurs as a result of polyproline stretches in nascent proteins. (4)

Furthermore, proteomic analysis suggested that EF-P may be responsible for the regulation of certain outer membrane proteins, in addition to various virulence factors. For instance, deletion of genes encoding for EF-P or its modification in *Salmonella enterica* resulted in reduced motility, inhibited cell viability and growth, poor growth in low osmolarity conditions, attenuated virulence, and increased susceptibility to antimicrobial compounds. (1)

EF-P possesses homologues in both archaea (aIF5A) and eukarya (eIF5A). Eukaryotic translation initiation factor 5A (eIF5A) employs a unique post-translational modification, hypusine, which is added in a two step system requiring the consecutive action of the enzymes deoxyhypusine synthase and deoxyhypusine hydroxylase. (5) eIF5A is of particular clinical interest because it is a proposed oncogene. In 2001, the overexpression of eIF5A-2 (a selectively expressed eIF5A isoform) was linked to proliferation of various human ovarian cancer tissues, colorectal and ovarian cancer cell lines, and certain transformed mammalian cells. (5) Moreover,

mutations in the eIF5A isoforms were shown to cause somatic defects, disrupt germ cell proliferation, and impair gametogenesis. (6) eIF5A is thought to affect the expression of only a subset of genes and has been shown to affect translation assays *in vitro* by promoting formation of di- and tri- peptides (7).

As such, research into the precise role of EF-P will not only further our understanding of bacterial gene regulation at the translational level, but may also assist in eventual antibiotic research designed to preferentially inhibit microbial virulence in harmful pathogens such as Salmonella. Furthermore, understanding the role of particular EF-P residues in the rate and success of translation will, by extension, illuminate eIF5A's role in rate enhancement of certain nascent proteins in eukarya, and contribute to a medical understanding of the mechanism by which certain cancers proliferate. Such knowledge could pave the way for research dedicated to specialized oncological drug discovery, and consequently have incredible therapeutic value.

1.2 - EF-P Undergoes a Distinctive Modification Pathway to Bind to the Ribosome and Ease the Translation of Certain Nascent Proteins, However the Full Importance of Certain Residues for EF-P's Function Has Not Yet Been Elucidated:

Within the past several years it has come to light that EF-P must undergo a posttranslational modification in order to function intracellularly. The modification pathway occurs in the following three steps: first, the *yjeK* gene product catalyzes the conversion of (S)- α -lysine to (R)- β -lysine. (2) Second, the lysyl tRNA synthetase paralog YjeA (also referred to as PoxA or GenX) activates the (R)- β -lysine and transfers it to the Lys34 residue of EF-P. Lastly, the YfcM protein hydroxylates either the C4(γ) or C5(δ) position of Lys34. (8) The final hydroxylation step is nonessential for EF-P function, however it is hypothesized that hydroxylation allows for additional hydrogen bond stabilization between EF-P and the 3' acceptor stem of a P-site bound initiator tRNA, thereby modulating EF-P activity. (3, 8)

Post modification, EF-P binds the ribosome in a unique position that bridges the E and the P sites, and allows EF-P to interact with the 3' acceptor stem of a P-site bound initiator tRNA. The L1 stalk of the ribosomal 50S subunit then undergoes a dramatic conformational change to allow the L1 ribosomal protein and 23S rRNA to interact with EF-P. (2)

A recent series of scientific articles reveal that once EF-P is properly positioned, its role is to stabilize the ribosome and prevent it from stalling during the synthesis of proteins containing strings of proline residues. (4) Researchers hypothesize that proline's pyrrolidine ring causes steric constraints hindering the proper placement of the amino acid within the peptidyl transferase center, and that in the absence of EF-P, such interactions destabilize ribosome binding and result in sluggish peptide bond formation. Kinetic data suggests that EF-P's presence in the cell can result in over a 100-fold increase in peptide bond formation in the cell. As a result, it is possible that EF-P can be considered a third universal elongation factor, but one that functions in the translation of only a subset of proteins (for example, those containing poly-proline residues). (13, 14)

The fact that past proteomics and microarray data show EF-P and its modification with β -lysine somehow affecting the translation of a subset of genes related to antibiotic resistance, motility, virulence, and osmolarity may be explained by the fact that proline runs exist in many of the nascent proteins that produce these phenotypes (1, 4). For example, EF-P is necessary for the proper function of the Cad-module, a lysine-dependent acid-resistance system that relies upon translation of proteins CadA, CadB, and CadC. The CadC protein, a membrane-integrated sensor and translational regulator that regulates genes of the lysine decarboxylase system in relation to low pH and lysine signals, contains a cluster of prolines that substantially inhibits

ribosomal peptidyltransferase activity in EF-P mutant and deletion strains. (4) If the proline stretch is mutated or deleted, then CadC is synthesized regardless of whether EF-P is present.

Interestingly, another study suggests that *in vivo* depletion of EF-P's eukaryotic homologue eIF5A also results in up to a twofold decrease in protein synthesis when modeled in *S. cerevisiae*. (9) Although this data suggests that all or most yeast mRNAs are undertranslated when eIF5A is absent from the cell (which is not true for the polyproline specific EF-P), it is important to note that the homologue maintains what appears to be a conserved function in translation stabilization.

As discussed, a significant amount of research has been dedicated to characterizing how EF-P is intracellularly modified, as well as how it assists in stabilizing the ribosome during protein synthesis. Both of these functions involve the amino terminal domain of EF-P (also known as Domain I), which not only receives all modifications, but also makes critical positional contacts near the peptidyl transferase center and the aminoacyl acceptor stem of the initiator tRNA. (2)

However, in 2009 *Blaha, et al.* published a structural analysis of EF-P that directed readers' attention to additional contacts made between the Y180 and R183 residues of Domain III and the small ribosomal subunit. These residues were observed to interact with the A1339 and G1338 nucleotides in the 16S RNA of the 30S small ribosomal subunit of EF-P, which are thought to create a 'gate' between the P-site and E-site of the ribosome. They proposed that these interactions could help "prevent premature movement of the initiator tRNA to the E-site" or "enhance the gate [between the E- and the P-sites] and stabilize the fMet-tRNA^{fMet} in the P-site." (2) Likewise, this thesis is interested in the questions of whether these residues are essential for EF-P functionality *in vivo*, and whether they enhance EF-P's function more than just by helping it interact with the ribosomal complex. The purpose of this project is to determine the

effect of mutated EF-P residues on rates and success of ribosomal binding and interactions with the P-site tRNA.

In order to address this question, we conducted site directed mutagenesis upon the Y180 and R183 residues of EF-P, and performed the following three assays: complementation assays to assess the growth phenotypes of the mutants; modification tests in which cell lysates were run on isoelectric focusing gels to determine whether the mutants were still aminoacylated with BLys; and, *fMet* puromycin reactivity assays to indirectly gauge the ability of the mutant EF-P to successfully bind to the ribosome.

2. Materials and Methods

2.1 - Strains, plasmid, and general methods

E. coli BW25113 and *efp* deletion strain was obtained from the Keio collection (Baba 2006).

Plasmid constructs, pTBY11-*efp* and pBAD30-*efp*, were used for expression of EF-P and as templates for site directed mutagenesis. A set of primers containing 30-40 nucleotides each were used to create the three *E. coli* EF-P mutants. The following primers were used to create the two single mutants:

Y180 forward primer: 5' –CGCTCTGGTGAAGCCGTCTCTCGCGTGAAG- 3'; Y180 reverse primer: 5' –CTTCACGCGAGAGACGGCTTCACCAGAGCG- 3'. R183 forward primer: 5' –CGCTCTGGTGAATACGTCTCTGCAGTGAAGTAACTTGAGG – 3'; R183 reverse primer: 5' –CCTCAAGTTACTTCACTGCAGAGACGTATTCACCAGAGCG- 3'.

The following primers were used to create the double mutant: Y180R183 forward primer: 5' –CGCTCTGGTGAAGCCGTCTCTGCAGTGAAGTAACTTGAGG- 3'; Y180R183 reverse primer: 5' -CCTCAAGTTACTTCACTGCAGAGACGGCTTCACCAGAGCG- 3'.

Using *Pfu* Turbo DNA polymerase, site directed mutagenesis reactions were performed in accordance with manufacturer's instructions (QuikChange). Subsequently, PCR products were digested with *DpnI* to eliminate any remaining methylated, non-mutagenized DNA, and used to transform *E. coli* XL1-Blue cells. Each point mutation was confirmed by DNA sequencing.

2.2 - Complementation Assays

Complementation assays were performed in the *efp* deletion strain using the low copy number plasmid-expressed EF-P mutants (pBAD30-Y180, pBAD30-R183, and pBAD30-Y180R183), as well as three controls, including wildtype, $\Delta efp + pBAD30-wtefp$, and $\Delta efp + pBAD30$ -(empty). Overnight cultures of each strain were grown, normalized at A600, and then inoculated into liquid LB media containing 100 μ g/ml ampicillin for selectivity and 1.0% arabinose for induction. Absorbance readings were performed hourly. The EF-P mutants that were unable to complement a slow growth phenotype of the Δefp strain were marked as possibly carrying residues that were essential for functionality and were exposed to further tests.

2.3 - Modification Tests

In order to determine if each EF-P mutant is still being modified *in vivo* by PoxA, protein extract isolated from lysed culture obtained from growth curves of complemented strains described above. Extracts were normalized at A580 and then resolved by isoelectric focusing (IEF), an electrophoretic technique that separates proteins based on their isoelectric point and detects minor changes in the protein due to charge differences. Because the β -lysine post-translational modification introduced an additional positive charge to EF-P, it was expected to focus at a different pH levels in the gel. The IEF gels were then subjected to Western blotting, in

which they were probed using a primary agent containing anti-EF-P polyclonal antibodies, and later an anti-Rabbit-Horseradish Peroxidase secondary antibody and ECL detection substrate (GE healthcare) for visualization.

2.4 - *f*-Met Puromycin Reactivity Assays

In order to conduct *in vitro* experiments, and test each EF-P mutant's affect on the ribosome, the same mutations were cloned and introduced into the EF-P gene on a high-copy expression plasmid pTYB11, which is suitable for protein overproduction. The vector contained a "T7lac" promoter, which was de-repressed by the addition of IPTG. The consequent production of polymerase effectuated plasmid reading and incorporation. Additionally, an incorporated intein affinity tag allowed for isolation upon a chitin column, and cleavage from the product protein by the addition of thiols.

Second, 70S initiation complexes (70SIC) were formed in the following manner: 70S ribosomes (2 uM) were incubated with 2 uM [³⁵S]fMet-tRNA^{fmet}, 4uM poxB mRNA, 2 uM each of IF1, IF2, IF3, and 1uM GTP in polymix buffer (5 mM KPO₄ pH 7.5, 1 mM DTT, 5 mM Mg(OAc)₂, 0.5 mM CaCl₂, 95 mM KCl, 5 mM NH₄Cl, 8 mM putrescine, 1 mM spermidine) for 30 min at 37 °C.

Next, 70SIC (0.2 uM) were incubated with 2 mM purified EF-P protein variants (or BSA/buffer as a negative control) in 1X polymix buffer at 4 °C for 10 minutes. An equal volume of puromycin (0.1 uM final) was added to an equal volume of EF-P bound 70SIC to initiate the reaction. Time points were taken at 0', 20", 40", 1', 2', 4', 8', and 12', and quenched in 2 M KOH. The [³⁵S]fMet-puromycin dipeptide was separated from its unreacted form based upon its polarity using TLC silica gel (running buffer 4:1:1 butanol:acetic acid:H₂O). Finally, the TLC

plates were exposed on phosphor imaging screens for a week before being visualized using STORM software.

3. Results

According to the crystal structure generated by *Blaha et al.*, there exist two residues on EF-P's third domain that interact with the gate between the E- and P- sites of the small ribosomal subunit and may possibly be involved in tRNA positioning or stabilization of the fMet-tRNA_i^{fMet} to prevent premature translocation (Figure 1). We hypothesized that these residues, labeled Y180 and R183, either jointly or severally enhance EF-P's function other than simply by helping it bind to the ribosome. Both residues were mutated according to site-directed mutagenesis to yield two single mutants (Y180, R183), and one double mutant (Y180R183) for analysis. Complementation assays, modification tests, and *fMet* puromycin reactivity assays were subsequently conducted in order to respectively assess the ability of the mutants to grow, and, if they could not, troubleshoot preliminary assays to determine the potential cause of this phenotype.

First, complementation assays were performed in order to determine whether strains with mutated EF-P were able to grow normally (Figure 2). These assays compared wild type *E. coli*, Δefp complemented with a plasmid containing wt-EF-P, Δefp complemented with an empty plasmid, and each of the mutants Y180, R183, and Y180R183. The results of the assay showed a marked difference between strains, indicating that the WT and Δefp complemented with a plasmid containing wt-EF-P (positive control) grew at approximately the same rate, whereas the Δefp complemented with an empty plasmid (negative control) and the three mutants grew much

slower. The Y180, R183, and Y180R183 mutants were not able to rescue the growth phenotype of the deletion strain as seen with the wt EF-P complementation strain, therefore it may be assumed that the mutated residues are necessary for some aspect of EF-P function.

To verify that the mutations did not somehow affect the ability of EF-P to be charged with β -lysine, cell lysates from each complementation strain were run on isoelectric focusing gels to induce pH driven separation of β -lysylated bands (modified) from un- β -lysylated bands (unmodified) (Figure 3). The gels showed that the mutants were being modified when compared to wild type EF-P and the remaining complemented strains.

The controls all functioned as expected, with the exception of the Δefp complemented with a plasmid containing wt-EF-P, which appeared to have been expressed at a level that saturated the modification pathway to the point at which it produced two bands, one of modified, and one of unmodified EF-P. It should be noted that each of the mutants ran at the same level as the known modified WT-EF-P, which demonstrates that they are indeed still being modified by PoxA *in vivo*. However, we expected that the R183 and Y180R183 strains would run slightly lower on the gel due to the basicity of the mutated arginine (now lysine) residue; however, this is not uniformly the case. We hypothesize that this result is due to the action of exoproteases that have cleaved off one or two amino acids on either end of the EF-P sequence.

Finally, *fMet* puromycin reactivity assays were conducted in order to measure the rate of peptide bond formation *in vitro*, which we know correlates to the presence of modified EF-P in the cell. Thus, this experiment served as an indirect measure of the EF-P mutants' ability to bind to the ribosome. These assays compared β -lysylated EF-P, which was generated by *in vitro* aminoacylation, against each of the mutants and a BSA/buffer negative control. Although no quantitative data could be gleaned from this final experiment, the results were conducted in an

internally consistent manner, and qualitative results may be inferred. According to the trials, it appears that much higher puromycin reactivity was induced by the use of β -lysylated EF-P in comparison to the BSA/buffer control or either of the mutants (Figure 4 A-B). Therefore, it appears that the EF-P mutants are not binding to the ribosome, suggesting the conserved residues Y180 and R183 are required for EF-P's interaction with the ribosome.

4. Discussion

4.1 - Implications of Current Results:

Ultimately, the results of this thesis refute our original hypothesis in which Y180 or R183 are not significantly affect ribosomal binding, but mutants would still be unable to complement growth indicating an additional role of these residues in EF-P's function. The data suggests that although the Y180 and R183 residues do not affect EF-P's ability to be post-translationally modified, they are in fact necessary from the perspective of ribosomal binding. It is possible that these particular amino acid contacts are important for anchoring EF-P onto the bridge between the E- and P- sites on the small ribosomal subunit, and provide a means of essential structural stability.

It is interesting to note, however, that these mutations were made on Domain III of EF-P, a domain that is completely absent from EF-P's eukaryotic homologue eIF5A. Therefore, the fact that mutating these residues precludes EF-P from binding to the ribosome presents an opportunity for a novel drug target that would preferentially inhibit bacterial, but not eukaryotic translation. If such an antibiotic were to be crafted, it could either be used alone, or in conjunction with another antibiotic. For example, in the case of Salmonella, either option would

be fruitful, as the former would dramatically decrease that pathogen's virulence, and the latter would employ a two-step approach to inhibit its growth in the host organism.

4.2 - Future Directions:

First, it is necessary to attempt to optimize the *fMet* puromycin reactivity assay. It is possible that optimal data was not obtained during the first course of experimental trials and repeats due to the absence of freshly beta-lysylated EF-P. The use of older radioactive 70S initiation complexes may have contributed to less dramatic observable differences between the positive and negative controls. Alternatively, it is possible that the reacted and unreacted [³⁵S]-*fmet* spots do not show complete separation on the TLC plates. In that case, it would be advisable to either treat the stopped reactions with hydrogen peroxide, or incubate them for 30 minutes at 21 °C before proceeding with TLC spotting. (3)

To further test whether the EF-P mutants are still able to bind the ribosome, the following *in vitro* assays will be performed. First, a polysome profiling assay will be conducted, in which mutated cells are lysed, loaded onto a linear sucrose gradient, and then fractionated by upward displacement. The fractions will then be separated by SDS PAGE, probed with an appropriate antibody, and visualized by Western blotting, in order to determine which components of the lysate—the 30S ribosomal subunit, 70S ribosomal subunit, or polysomes—contain EF-P.

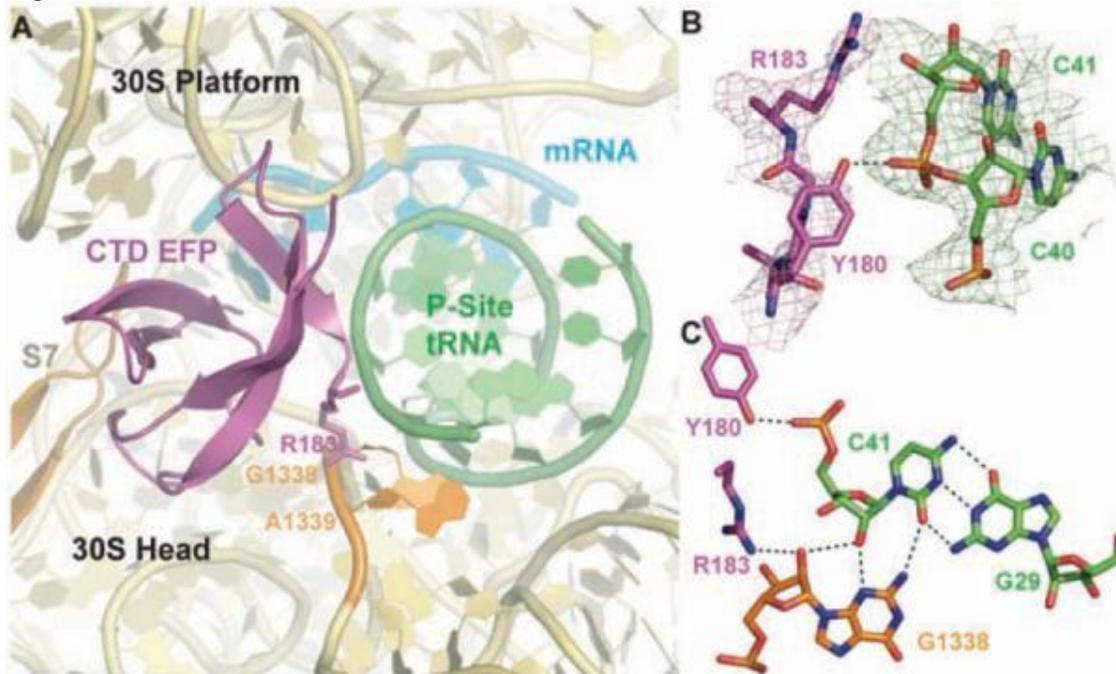
Next, an additional test for ribosome binding will be conducted via a gel shift assay, an electrophoretic technique that separates proteins based on size. In this assay, those EF-P mutants that are able to complex with the larger, less mobile ribosome will move more slowly and be shifted up the gel. Moreover, the affinity of the protein for the ribosome may be calculated using known starting concentrations of protein, ribosome, and the stoichiometry of the complex. If results show EF-P is still functioning normally at the level of aminoacylation and ribosome

binding, it can be assumed that EF-P has an additional function in the ribosome which is being affected by the constructed mutations.

Lastly, purified EF-P variants will be characterized *in vitro* with isolated ribosomes and initiator tRNA via a filter binding assay. These assays will determine EF-P's affect on initiator tRNA-ribosome binding. Structural data suggest EF-P may serve to stabilize the P-site tRNA in the ribosome and thus may directly affect the tRNA's binding. Accordingly, the radiolabeling of formyl-methionine tRNA will allow for visualization of the ribosome-bound tRNA and the free tRNA separated by nitrocellulose filters. First, the assay will be conducted with wild-type EF-P to determine its effect on tRNA-ribosome binding dissociation constants. Next, the assay will be repeated with each of the mutants in a 96 well format, to allow for the addition of varying concentrations of tRNA. It is hypothesized that the dissociation constant of the tRNA for the ribosome will be greater without the aid of the functional EF-P. To further quantify this data, the kinetics of binding will be observed in order to determine the degree to which the mutated residues affect the tRNA binding rate.

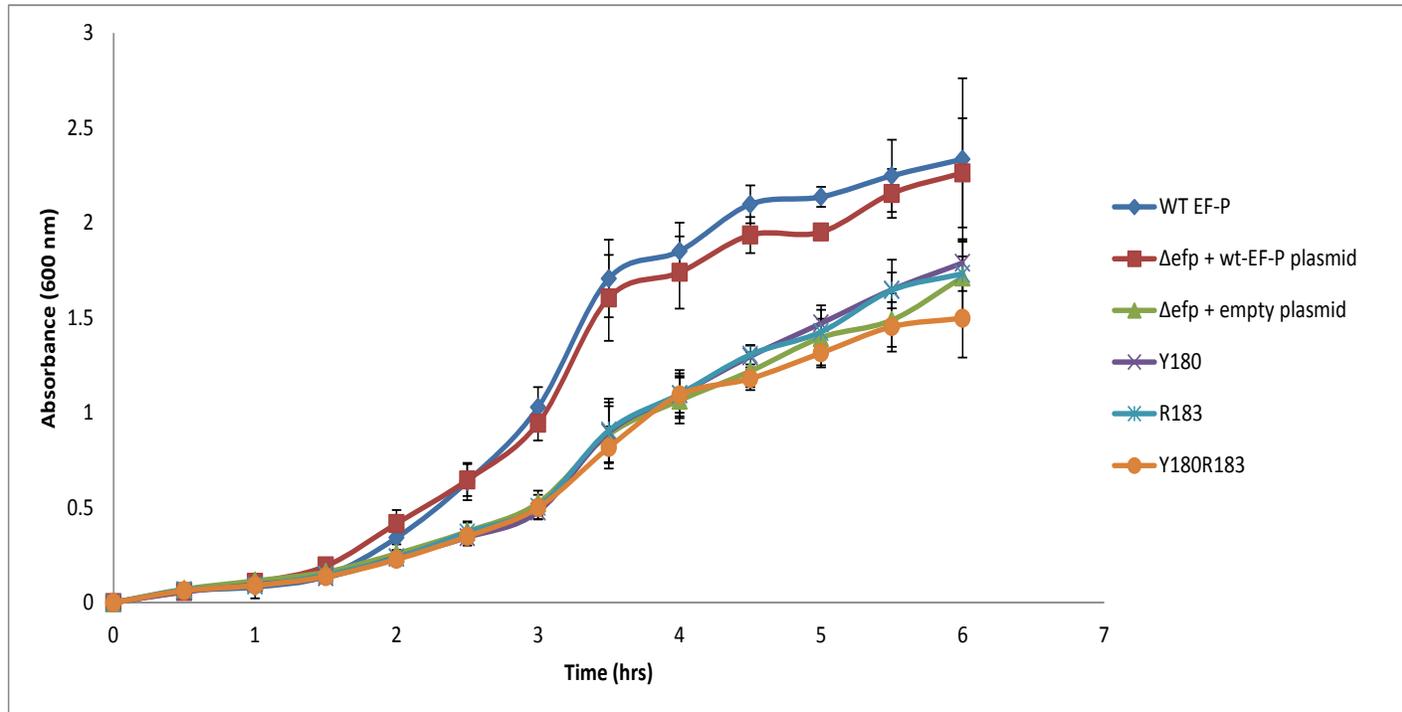
Ultimately, the results of these assays will be useful for characterization and verification of the experimental data that has already been obtained, and can thereby identify which particular amino acid residues are crucial for EF-P's functionality.

Figure 1:



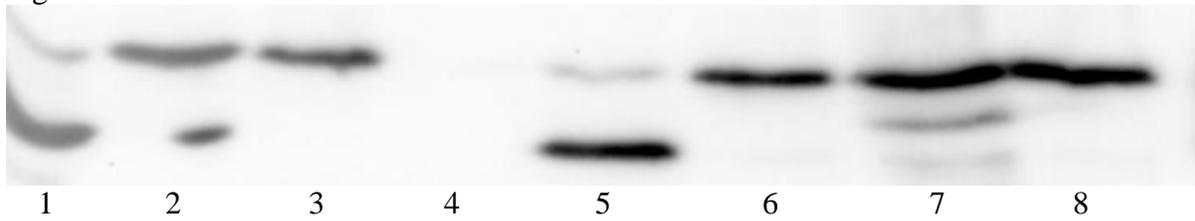
This structure, taken from *Blaha, et al.*, specifically shows the Y180 and R183 residues of EF-P making contacts with the A1339 and G1338 residues of the small subunit of the ribosome. We predicted that these residues performed an essential function and set about conducting site-directed mutagenesis in order to evaluate their necessity.

Figure 2:



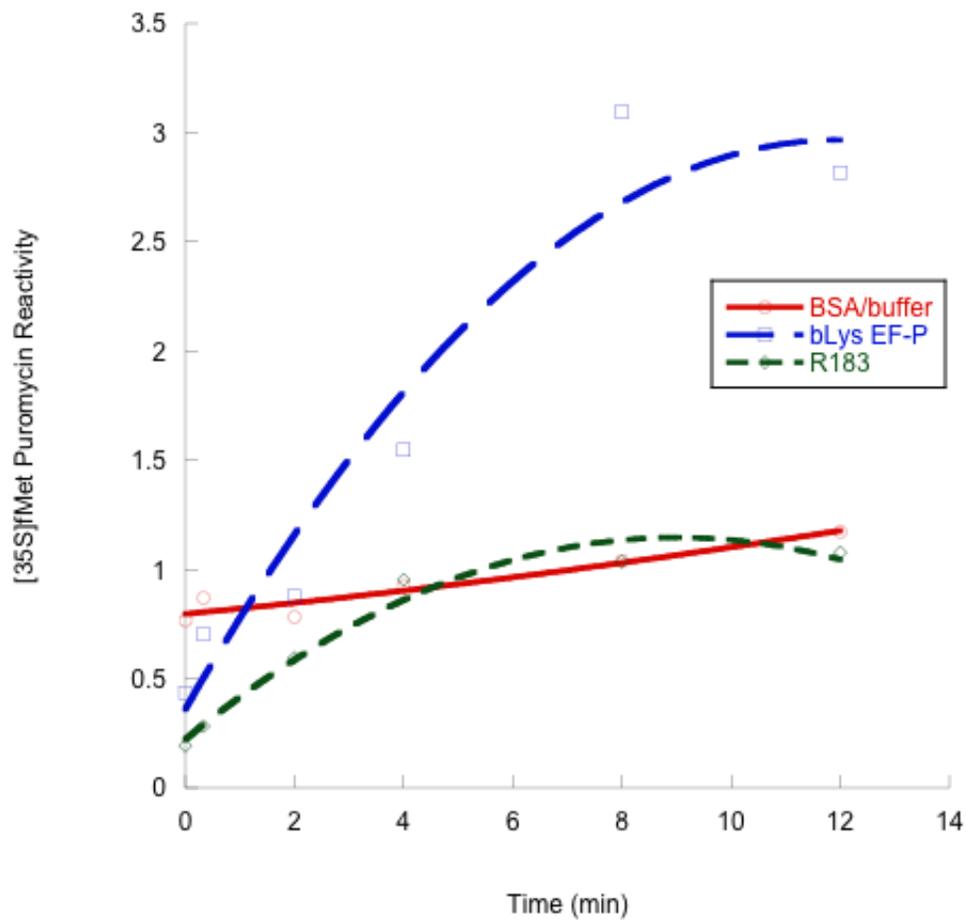
This curve shows the growth rates of wild type EF-P (#72), Δ efp complemented with a plasmid containing wt-EF-P, wild type complemented with an empty plasmid, and each of the mutant strains Y180, R183, and Y180R183. As expected, the positive controls grew normally, but the three mutants and the negative control were unable to complement and rescue the growth phenotype of the deletion strain.

Figure 3:



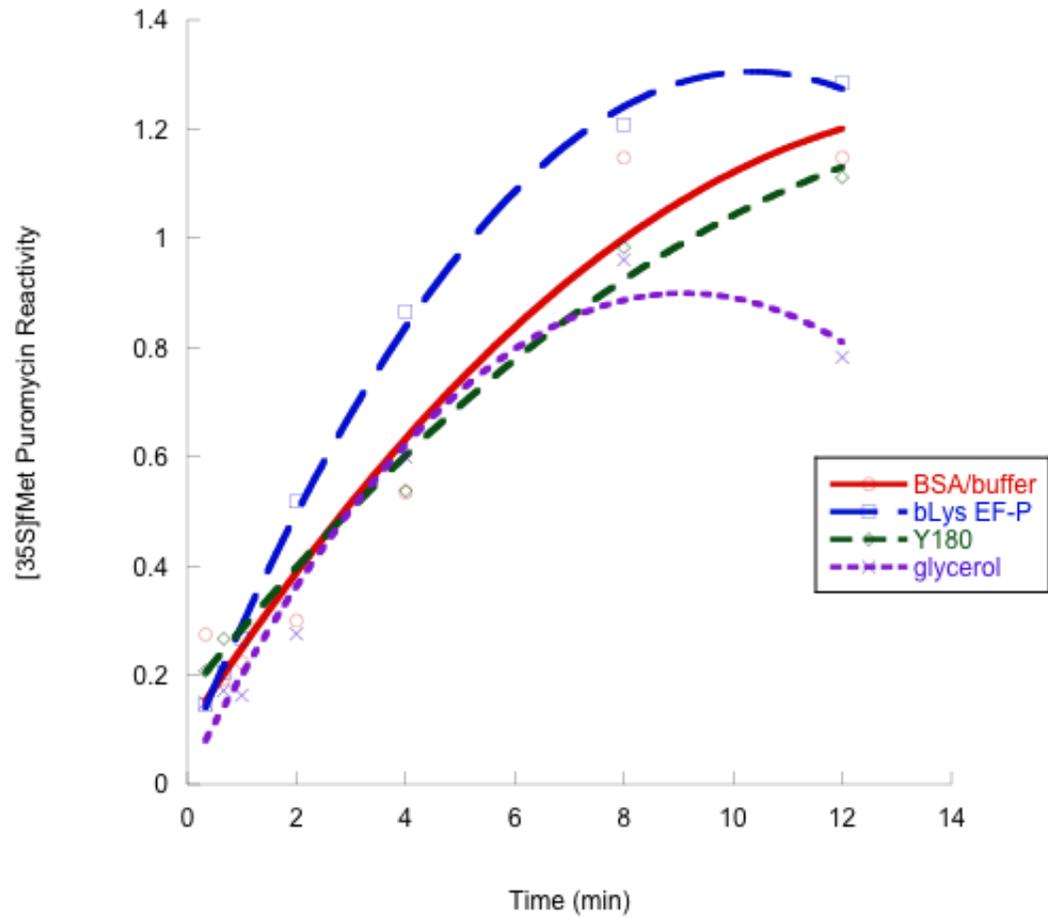
Lane 1: unmodified EF-P; Lane 2: *in vitro* modified EF-P; Lane 3: WT EF-P; Lane 4: Δ efp complemented with an empty plasmid; Lane 5: WT complemented with a plasmid containing wild type EF-P; Lane 6: Y180 mutant; Lane 7: R183 mutant; Lane 8: Y180R183 mutant. Initial tests demonstrate that EF-P is still being modified *in vivo*.

Figure 4A:



A. Shows an accelerated rate of puromycin activity by the β -lysylated EF-P in comparison to the BSA/buffer control or the R183 mutant. This suggests that the mutant EF-P is unable to bind to the ribosome.

Figure 4B:



B. Although this figure does not show as dramatic a difference in rates as Fig. 4A, the β -lysylated EF-P nonetheless exhibits a higher rate of puromycin reactivity than the BSA/buffer control, the glycerol control, or the Y180 mutant strain. This suggests that mutated EF-P is unable to bind to the ribosome.

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