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The role of quality control by the Bacillus subtilis threonyl-tRNA synthetase

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The role of quality control by the *Bacillus subtilis* threonyl-tRNA synthetase

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

By

Alexandria Kathryn Plumer

2013

ABSTRACT OF THE THESIS

The role of quality control by the *Bacillus subtilis* threonyl-tRNA synthetase

By

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Master of Science in Microbiology, Immunology and Molecular Genetics

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Professor: Beth A. Lazazzera, Chair

The threonyl-tRNA synthetase is an enzyme that catalyzes the attachment of the amino acid threonine to the threonyl-tRNA so that protein synthesis at the ribosome can occur. In order to maintain translational fidelity, this tRNA synthetase must employ an editing function that is structurally and functionally separate from this enzyme's aminoacylation capacity. Here, we studied the importance editing function of the *Bacillus subtilis* threonyl synthetase, ThrRS, by making a single amino acid substitution in the separate editing domain. The data in this study suggest that disrupting editing function in the major *B. subtilis* ThrRS, *thrS*, is toxic to the cells under normal growth conditions. A future goal in this project is to study the effect of knocking out the editing function of the minor *B. subtilis* ThrRS, *thrZ*.

The thesis of Alexandria Kathryn Plumer is approved.

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University of California, Los Angeles

2013

DEDICATION

I dedicate this work to my mom, Dr. Kathryn Gardner, M.D., who taught me by example.

Thank you for your sacrifice, empathy and genetics.

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Chapter 1

Introduction

INTRODUCTION

What are aminoacyl-tRNA synthetases?

Aminoacyl-tRNA synthetases are the enzymes that are responsible for attachment of the amino acid to its corresponding tRNA in order to add amino acids to a growing peptide chain in the ribosome [1]. Aminoacyl-tRNA synthetases use a two-step mechanism for the aminoacylation of tRNAs [1]. The first step involves the amino acid and ATP binding at the enzyme's active site, forming an aminoacyl-adenylate [2]. The second step of this reaction is a nucleophilic attack of the intermediate aminoacyl-adenylate by the tRNA, releasing an AMP and an aminoacyl-tRNA [2].

Aminoacyl tRNA synthetases are divided into two classes, which are categorized according to differing sequence motifs [3]. The two classes differ largely in structure, having very different active sites, while maintaining functional similarity, suggesting that the two classes arose by convergent evolution rather than divergent evolution [2, 4, 5]. The active site of class I tRNA synthetases contains a Rossmann fold which is a dinucleotide-binding domain that is absent in class II enzymes [4]. Class I and II tRNA synthetases also differ by the manner in which they bind tRNA [2, 6]. Unlike class I tRNA synthetases, class II enzymes share few unifying structural features [7].

What is editing?

A more recently described function of tRNA synthetases, in addition to their aminoacylation function, is editing [8, 9]. Errors in translation happen at a rate of ~10^{-4}, which is an accumulation of errors in transcription, aminoacyl-tRNA (aa-tRNA)

activation and ribosomal decoding [7]. Some aminoacyl-tRNA synthetases are estimated to misactivate a non-cognate amino acid at about a rate of 10^{-3} [7, 10]. One quality control method that corrects misactivated amino acids is the editing function of aminoacyl-tRNA synthetases [11]. Some aminoacyl-tRNA synthetases have a separate editing domain which can hydrolyze misactivated amino acid:tRNA pairings. Not all tRNA synthetases have editing domains; the editing function seems to become necessary when an aminoacyl-tRNA synthetase cannot achieve sufficient selectivity for the cognate amino acid compared to chemically similar non-cognate amino acid through a single enzymatic step [7]. An example of this is the threonyl-tRNA synthetase, ThrRS, which attaches threonine to its cognate tRNA, but it can also occasionally charge the $tRNA^{Thr}$ with serine [12]. Threonine and serine differ by only a methyl group. The isoleucyl-tRNA synthetase, IleRS, incorrectly adds a valine only 180-fold less frequently than it adds isoleucine [7, 13]. tRNA synthetases that do not have editing functions, like some of the *Mycoplasma* enzymes, make more errors than the homologous *E. coli* tRNA synthetases with a functional editing domain [14].

Editing can occur before or after the incorrect amino acid is attached to the tRNA; processes called pre- and post-transfer editing, respectively [7, 15, 16]. Pre-transfer editing is the hydrolysis of misactivated aminoacyl-AMP intermediate before it is attached to the tRNA; while post-transfer editing is the hydrolysis of incorrect aminoacyl-tRNA [7, 8]. The pre-transfer editing mechanism was described in 1966; when $tRNA^{Ile}$ is added to *E. coli* IleRS, this facilitates the breakdown of Val-AMP [15]. Fersht first elucidated a mechanism for post-transfer editing in 1977 by showing that the *E. coli* isoleucyl tRNA-synthetase (IleRS) hydrolyzes misactivated Val-AMP [8]. Martinis et. al.

showed that in a given enzyme, either pre- or post-transfer editing is dominant [8]. By using the *E. coli* leucyl-tRNA synthetase, LeuRS, she showed that pre- and post- editing are redundant mechanisms. It was demonstrated that when the dominant post-transfer editing pathway was compromised by a substitution that deactivates it, the secondary pathway, pre-transfer editing, is activated [8]. One study substituted an amino acid critical to pre-transfer editing in the IleRS and showed that it could no longer distinguish between isoleucine and noncognate valine in the aminoacylation site. However, this enzyme was still capable of breaking down the mischarged tRNA:amino acid pair via hydrolysis [13]. This finding demonstrates that pre- and post-transfer editing are separate phenomena. It is believed that half of all tRNA synthetases employ editing to discriminate between their cognate and noncognate amino acids [7].

Editing and aminoacylation processes can be separated

The sites of aminoacylation and editing are distinct and are separated in space and functionality [7]. The double-sieve model explains that the aminoacylation and separate editing sites act as a first and second sieve, respectively, to filter out noncognate amino acids [7]. The first sieve acts to discriminate based on size, rejecting large amino acids. The editing site hydrolyzes misactivated amino acids by rejecting the correctly sized or properly hydrophobic amino acids [16, 17].

Class I synthetases use the CPI domain, which is distinct from the aminoacylation site, to perform pre-transfer editing of misacylated tRNAs. Substitution of some of the critical residues in this domain severely decreased the ability of *E. coli* IleRS to hydrolyze an incorrect aminoacylated tRNA without affecting the aminoacylation function significantly [18, 19]. Substitutions in the CP1 domains of *E. coli* ValRS and

LeuRS and *T. thermophilus* IleRS have also been shown not to affect aminoacylation activity [20-22]. These findings demonstrate that editing and aminoacylation are separate processes and operate independently from one another. A crystal structure of the distinct aminoacylation and editing domain in the *E. coli* IleRS was resolved by Nureki et. al., showing that the globular beta-barrel CPI domain of the IleRS protrudes from the aminoacylation domain [18, 19]. When the *E. coli* CPI domain is isolated from the rest of the LeuRS enzyme, it has been shown that this alone can result in hydrolysis of a misactivated tRNA, demonstrating that the CPI domain functions independently [23, 24].

The *E. coli* IleRS has both the pre- and post-transfer editing pathways, mentioned previously. Pre-transfer editing, which is the hydrolysis of misactivated aminoacyl-adenylates (aa-AMPs) before it is transferred to the 3' end of tRNA, has been described by 3 separate models; translocation, selective release and active site hydrolysis. The translocation model is where misactivated aa-AMP is synthesized at the active site and hydrolyzed in the editing site. The selective release model describes the expulsion of the noncognate (but not cognate) aa-AMP into solution and its subsequent spontaneous hydrolysis. Misactivated aa-AMP is hydrolyzed at the active site in the active site hydrolysis model, although this mechanism has yet to be further elucidated. Post-transfer editing is the hydrolysis of the misactivated aa-AMP after it is transferred to the 3' end of the tRNA [7]. Because editing and aminoacylation are separate processes, this allows the tRNA synthetase to carry out multiple modes of editing in order to ensure adequate quality control for the cell.

Differences between Class I and Class II tRNA synthetases in editing

Class I and II enzymes differ in their editing domains [25]. Class I enzymes tend to have CPI domains, which are globular insertion domains, that are near the aminoacylation site of the enzyme [26]. LeuRS, IleRS and ValRS have homologous CPI editing domains [7]. Class II enzymes have more varying structures and the MetRS, LysRS II and SerRS are thought to edit using the aminoacylation site [7, 27]. The first class II enzyme whose distinct editing site was defined was the bacterial ThrRS, which contains an N2 domain present in bacterial and eukaryotic threonyl-tRNA synthetases but absent in most archaea, and there is no evidence that any other tRNA synthetases have N2 domains [7, 28-30]. The free-standing version of the Class II AlaRS editing domain, AlaXp, seems to have similar structure to the ThrRS N2 domain [7]. Because editing in different types of tRNA synthetases is so different between species and classes, it is difficult to make any generalizations about editing from the narrow amount of experiments that have been done.

Different cell types and compartments have different requirements for editing

Different organisms under varying conditions have diverse requirements for the fidelity of their translation and therefore different needs for the editing function of their tRNA synthetases. Although the editing function of certain tRNA synthetases is well-conserved throughout all domains of life, often an organism can remain viable after losing the editing function [27]. One study showed that *E. coli* tolerates misacylated tRNAs, demonstrating that these are not toxic to the cell despite the fact that incorrect amino acids were incorporated into proteins [31]. This study, conducted by Ruan et. al. [31], used missense suppression in which only a misacylated tRNA can correct the loss of

translation. This study showed that *E. coli* can tolerate up to 10% of mistranslated protein.

The *Mycoplasma* PheRS is naturally error prone, which results in mistranslation of proteins at a higher rate than in *E. coli* [14, 32]. *Mycoplasma* PheRS does not possess canonical editing activity and relies on kinetic proofreading, which is the ability of the reaction to have an irreversible exit step in the pathway that makes it more likely that the incorrectly activated tRNA exits the pathway; this discriminates between similar amino acids and results in a mischarged tRNA that is less likely to participate in protein synthesis [14, 33]. The editing domain in *Mycoplasma* PheRS and other related species seems to be degenerate based on substitutions of key residues in the hydrolytic active site, showing that different organisms have varying levels of needed stringency for translational fidelity [32]. Another study showed that replacement of the *E. coli* PheRS with the *Mycoplasma* PheRS causes a loss of viability in *E. coli* cells, demonstrating that while *E. coli* can tolerate some mistakes, there is a limit [14]. Substitutions in *Mycoplasma*'s nonfunctional editing domain caused a restoration of *E. coli* viability by restoring the editing activity of the *Mycoplasma* PheRS, demonstrating that editing is a specific and separate function and that *Mycoplasma* species in fact lost their separate editing function in this enzyme [14].

Eukaryotic cells have two distinct PheRS proteins, one cytoplasmic and one mitochondrial. Reynolds et al. showed that the cytoplasmic PheRS performs an editing function while the mitochondrial one does not, although the latter PheRS is very accurate. When the error rate of the mitochondrial PheRS was increased by an amino acid substitution in the active site, the cell could no longer grow on respiratory media and its

mitochondrial genome was lost. However, increasing the error rate of the cytoplasmic PheRS still supported cytoplasmic protein synthesis and cell viability. This study demonstrated that different cell types and environments require different levels of translation quality control, which explains why some tRNA synthetases do not require editing activity [25].

Removal of the editing function of *E. coli* ValRS under normal conditions only shows a slight decrease in fitness, but shows a severe defect when in the presence of elevated levels of α-aminobutyrate [34]. These data demonstrate that different levels of translational fidelity are required under different conditions, and that cellular stress plays an important role in editing.

It is thought that in some species, like *Mycoplasma* and *Candida*, mistranslation is advantageous due to upregulation of stress response proteins [14, 35, 36]. During stress response, chaperones are increased in order to help fold unfolded and misfolded proteins. When mistranslation in these species occurs, the upregulation of the stress response proteins can assist in creating properly folded and functioning proteins [7].

A curious point that comes from the studies elucidating differences between specificity of the tRNA synthetases and editing function in different situations (cell types, species and environments) is that there are different levels of stringency required in the accuracy of translation. Editing is not conserved universally in all tRNA synthetases, as cytoplasmic ProRS from archaea and some higher eukaryotes do not have post-transfer editing abilities while the bacterial ProRS does [37]. It has also been shown that mitochondrial versions of tRNA synthetase lack editing function or that their editing

ability is unnecessary [38]. It seems that different levels of stringency are required in different organisms in varying environments.

Conditions in the laboratory do not mimic how cells grow in their natural environments

Often studies showing editing-defective phenotypes do so under artificial conditions such as the addition of unnatural levels of noncognate amino acids to throw off the natural balance of amino acids in the cell. Species from all 3 domains of life have been shown to require editing ability when an excess amount of non-cognate amino acids are present [11, 27, 34, 38, 39]. It is unknown whether these amino acid imbalances are naturally encountered, although certain studies have shown that switching media or carbon source changes may alter cellular metabolites, including the levels of various amino acids in the cell [27, 40]. Often the in vivo experiments testing editing defective mutants are done when cells are in exponential growth with full nutrients supplied, while this is not how these organisms tend to grow in their natural environments. Lack of editing by tRNA synthetases has the potential to create many problems within the cell depending on what is limited in the environment and how quickly the cells are growing [7, 34]. The misuse of amino acids and tRNAs is wasteful of energy and nutrients [7, 27].

Slow growth and nutrient deprivation may be the selective pressures for editing.

Although it has been shown that editing is required in systems containing editing-defective aminoacyl-tRNA synthetases and excess noncognate amino acids [38, 41], little has been done to show that editing-defective strains are toxic in vivo under normal conditions during exponential growth [25, 27]. Because of artificial conditions in the laboratory that do not always mimic natural growth of the organisms tested, it is difficult

to understand the selective pressures that gave rise to tRNA synthetase editing in the first place. Incorrect incorporation of amino acids into a growing polypeptide chain can change its folding properties which in turn can cause the formation of toxic protein aggregates [27]. Nutrient deprivation and slow growth conditions are more similar to what most organisms experience in the natural environment. A more informative way to study editing by tRNA synthetases may be to attempt to mimic natural conditions in the laboratory.

When a cell is under slow growth conditions, it may be less able to respond to problems created by misfolding of proteins. Protein misfolding due to mistranslation can cause protein aggregation in the cell, which the cell must correct [27]. It is hypothesized that aggregation occurs more in slowly growing cells, as these cells are less able to dilute misfolding proteins [27]. This was shown when only the terminally differentiated Purkinje cells of mice were affected by an editing substitution in the AlaRS despite the fact that all of the cells had the mutation and that this mutation also affected the aminoacylation function of this enzyme [11]. It was shown by Lee et. al. that low levels of mischarged tRNAs also lead to protein aggregation in neurons [11]. One explanation for this may be that most mammalian cells have the ability to dilute misfolded proteins or aggregates before they become a problem [27].

Unfolded proteins can occur in other situations, such as addition of heat to the cellular environment. The synergistic effect of protein unfolding by heat and misfolding due to lack of quality control may have been a selective factor for tRNA synthetase editing functions. Evidence for this comes from the fact that the heat shock response is induced in *E. coli* when cells have editing defects in its ProRS, ThrS, AspRS or GluRS

[27, 31]. It has also been shown that *S. cerevisiae* and Purkinje cells upregulate expression of heat shock proteins when editing function has been compromised [11, 31, 42]. *S. cerevisiae* and *E. coli* editing mutants have been shown to grow more slowly at higher temperatures than their wild type counterparts , reflecting global changes in protein function in the IleRS editing-defective mutant [42, 43].

Diseases

Mutations in genes encoding tRNA synthetases are important in disease, and have been implicated in a variety of age-related neuropathies such as Charcot-Marie-Tooth disease [44, 45]. Lee et. al. demonstrated Purkinje cell loss and ataxia caused by an editing-defective AlaRS that shows moderately affected aminoacylation kinetics compared wild type [11]. The tissue-specific nature of a phenotype for cells expressing defective aminoacyl-tRNA synthetases in humans is consistent with the idea that certain cells can remove misfolded proteins more efficiently than other cell types, that there is a differential effectiveness in the unfolded protein response, or that the amino acid pools in these cells differ, making them more likely to misacylate. Five loci encoding tRNA synthetases with loss-of-function mutations have been implicated in varying peripheral neuropathies so far [46]. An editing-defective ValRS expressed in mouse cell culture produced a response consistent with apoptosis and was exacerbated by noncanonical amino acids that are misactivated and not cleared by properly functioning editing machinery, demonstrating that decreased accuracy in translation may be associated with disease [44]. Another study shows that a loss-of-function variant of the human HisRS is neurotoxic in *C. elegans*, although this work does not show whether the loss of this enzyme leads to protein aggregation or just mistranslation [47]. Schimmel et. al.

demonstrated that errors in $tRNA^{Ile}$ aminoacylation are mutagenic and therefore can lead to disease by showing that aging *E. coli* with editing defects display error-prone DNA repair through induction of the bacterial SOS response [48]. Overall, these studies raise more questions than they answer. A main question that must be answered in further studies is whether or not a decrease of aminoacylation function of editing-defective tRNA synthetases could in part be causing these phenotypes. The criteria under which editing is most important is an interesting topic when studying editing defects in tRNA synthetases and will help answer the question of why some cell types and compartments are affected by mistranslation and why others remain without an obvious phenotype.

Unanswered questions and the goals of this thesis

Sporulation, genetic competence and biofilm formation are all slow-growth phenotypes displayed by *B. subtilis* that can be easily observed and quantified. We believe that editing by tRNA synthetases becomes more important in situations when the cell is under stress, such as nutrient deprivation-induced slow-growth, and a phenotype will be observed under these conditions. It was demonstrated by Waas and Schimmel that a mutation of the C184 in the *E. coli* ThrRS disrupts the editing function of the enzyme [12]. Because of the sequence homology of the *E. coli* and *B. subtilis* ThrRS, this was our rationale for creating this mutation in both the *B. subtilis thrS* and *thrZ* genes [12].

REFERENCES

1. Giege, R., *The early history of tRNA recognition by aminoacyl-tRNA synthetases.* J Biosci, 2006. **31**(4): p. 477-88.
2. Ibba, M. and D. Soll, *Aminoacyl-tRNA synthesis.* Annu Rev Biochem, 2000. **69**: p. 617-50.
3. Eriani, G., et al., *Partition of tRNA synthetases into two classes based on mutually exclusive sets of sequence motifs.* Nature, 1990. **347**(6289): p. 203-6.
4. Burbaum, J.J. and P. Schimmel, *Structural relationships and the classification of aminoacyl-tRNA synthetases.* J Biol Chem, 1991. **266**(26): p. 16965-8.
5. Cusack, S., M. Hartlein, and R. Leberman, *Sequence, structural and evolutionary relationships between class 2 aminoacyl-tRNA synthetases.* Nucleic Acids Res, 1991. **19**(13): p. 3489-98.
6. Beuning, P.J., et al., *Specific atomic groups and RNA helix geometry in acceptor stem recognition by a tRNA synthetase.* Proc Natl Acad Sci U S A, 1997. **94**(19): p. 10150-4.
7. Ling, J., N. Reynolds, and M. Ibba, *Aminoacyl-tRNA synthesis and translational quality control.* Annu Rev Microbiol, 2009. **63**: p. 61-78.
8. Martinis, S.A. and M.T. Boniecki, *The balance between pre- and post-transfer editing in tRNA synthetases.* FEBS Lett. **584**(2): p. 455-9.
9. Guo, M. and P. Schimmel, *Structural analyses clarify the complex control of mistranslation by tRNA synthetases.* Curr Opin Struct Biol. **22**(1): p. 119-26.
10. Loftfield, R.B. and D. Vanderjagt, *The frequency of errors in protein biosynthesis.* Biochem J, 1972. **128**(5): p. 1353-6.
11. Lee, J.W., et al., *Editing-defective tRNA synthetase causes protein misfolding and neurodegeneration.* Nature, 2006. **443**(7107): p. 50-5.
12. Waas, W.F. and P. Schimmel, *Evidence that tRNA synthetase-directed proton transfer stops mistranslation.* Biochemistry, 2007. **46**(43): p. 12062-70.
13. Schmidt, E. and P. Schimmel, *Mutational isolation of a sieve for editing in a transfer RNA synthetase.* Science, 1994. **264**(5156): p. 265-7.
14. Yadavalli, S.S. and M. Ibba, *Selection of tRNA charging quality contorl mechanisms that increase mistranslation of the genetic code.* Nucleic Acids Res, 2012. **41**(2): p. 1104-12.
15. AN Baldwin and P. Berg, *Transfer ribonucleic acid-induced hydrolysis of valyladenylate bound to isoleucyl ribonucleic acid synthetase.* Proc. Natl. Acad. Sci. USA, 1966. **104**: p. 1907–12.
16. Fersht, A.R., *Specificity and editing mechanisms.* Enzyme structure and mechanism, 1985: p. 347-368.
17. AR, F., *Editing mechanisms in protein synthesis. Rejection of valine by the isoleucyl-tRNA synthetase.* Biochemistry, 1977. **16**: p. 1025-30.
18. Schmidt, E. and P. Schimmel, *Residues in a class I tRNA synthetase which determine selectivity of amino acid recognition in the context of tRNA.* Biochemistry, 1995. **34**: p. 11204-10.
19. Nureki, O., et al., *Enzyme structure with two catalytic sites for double-sieve selection of substrate.* Science, 1998. **280**: p. 578-82.

20. Mursinna, R.S. and S.A. Martinis, *Rational design to block amino acid editing of a tRNA synthetase.* J Am Chem Soc, 2002. **124**(25): p. 7286-7.
21. Hendrickson, T., et al., *Mutational separation of two pathways for editing by a class I tRNA synthetase.* Mol. Cell, 2002. **9**: p. 353-62.
22. Hendrickson, T.L., T.K. Nomanbhoy, and P. Schimmel, *Errors from selective disruption of the editing center in a tRNA synthetase.* Biochemistry, 2000. **39**(28): p. 8180-6.
23. Lin, L., S. Hale, and P. Schimmel, *Aminoacylation Error Correction.* Nature, 1996. **384**: p. 33-34.
24. Betha, A., A. Williams, and S. AMartinis, *Isolated CP1 domain of Escherichia coli leucyl-tRNA synthetase is dependent on flanking hinge motifs for amino acid editing activity.* Biochemistry, 2007. **46**: p. 6258-67.
25. Reynolds, N.M., et al., *Cell-specific differences in the requirements for translation quality control.* Proc Natl Acad Sci U S A. **107**(9): p. 4063-8.
26. Cusack, S., A. Yaremchuk, and M. Tukalo, *The 2 A crystal structure of leucyl-tRNA synthetase and its complex with a leucyl-adenylate analogue.* EMBO J, 2000. **19**(10): p. 2351-61.
27. Reynolds, N., B. Lazazzera, and M. Ibba, *Cellular mechanisms that control mistranslation.* Nat. Rev. Microbiol. , 2010. **8**: p. 849-856.
28. Dock-Bregeon, A., et al., *Transfer RNA-mediated editing in threonyl-tRNA synthetase. The class II solution to the double discrimination problem.* Cell, 2000. **103**(6): p. 877-84.
29. Sankaranarayanan, R., et al., *Zinc ion mediated amino acid discrimination by threonyl-tRNA synthetase.* Nat Struct Biol, 2000. **7**(6): p. 461-5.
30. Arnez, J.G., et al., *Aminoacylation at the Atomic Level in Class IIa Aminoacyl-tRNA Synthetases.* J Biomol Struct Dyn, 2000. **17 Suppl 1**: p. 23-7.
31. Ruan, B., *Quality control despite mistranslation caused by an ambiguous genetic code.* Proc. Natl. Acad. Sci. USA, 2008. **105**: p. 16502-7.
32. Li, L., et al., *Naturally occurring aminoacyl-tRNA synthetases editing-domain mutations that cause mistranslation in Mycoplasma parasites.* Proc Natl Acad Sci U S A, 2010. **108**(23): p. 9378-83.
33. Hopfield, J.J., *Kinetic proofreading: a new mechanism for reducing errors in biosynthetic processes requiring high specificity.* Proc Natl Acad Sci U S A, 1974. **71**(10): p. 4135-9.
34. Nangle, L.A., et al., *Genetic code ambiguity. Cell viability related to the severity of editing defects in mutant tRNA synthetases.* J Biol Chem, 2002. **277**(48): p. 45729-33.
35. Santos, M.A., et al., *Selective advantages created by codon ambiguity allowed for the evolution of an alternative genetic code in Candida spp.* Mol Microbiol, 1999. **31**(3): p. 937-47.
36. Bacher, J.M., et al., *Genetic code ambiguity confers a selective advantage on Acinetobacter baylyi.* J Bacteriol, 2007. **189**(17): p. 6494-6.
37. SternJohn, J., et al., *Restoring species-specific posttransfer editing activity to a synthetase with a defunct editing domain.* Proc Natl Acad Sci U S A, 2007. **104**(7): p. 2127-32.

38. Karkhanis, V.A., et al., *A viable amino acid editing activity in the leucyl-tRNA synthetase CP1-splicing domain is not required in the yeast mitochondria.* J Biol Chem, 2006. **281**(44): p. 33217-25.
39. Korencic, D., et al., *A freestanding proofreading domain is required for protein synthesis quality control in Archaea.* Proc Natl Acad Sci U S A, 2004. **101**(28): p. 10260-5.
40. Hinnebusch, A.G., *Translational regulation of GCN4 and the general amino acid control of yeast.* Annu Rev Microbiol, 2005. **59**: p. 407-50.
41. Karkhanis, V.A., A.P. Mascarenhas, and S.A. Martinis, *Amino acid toxicities of Escherichia coli that are prevented by leucyl-tRNA synthetase amino acid editing.* J Bacteriol, 2007. **189**(23): p. 8765-8.
42. Santos, M.A., V.M. Perreau, and M.F. Tuite, *Transfer RNA structural change is a key element in the reassignment of the CUG codon in Candida albicans.* EMBO J, 1996. **15**(18): p. 5060-8.
43. Bacher, J.M., V. de Crecy-Lagard, and P.R. Schimmel, *Inhibited cell growth and protein functional changes from an editing-defective tRNA synthetase.* Proc Natl Acad Sci U S A, 2005. **102**(5): p. 1697-701.
44. Nangle, L.A., C.M. Motta, and P. Schimmel, *Global effects of mistranslation from an editing defect in mammalian cells.* Chem Biol, 2006. **13**(10): p. 1091-100.
45. Zhao, Z., et al., *Alanyl-tRNA synthetase mutation in a family with dominant distal hereditary motor neuropathy.* Neurology. **78**(21): p. 1644-9.
46. Wallen, R.C. and A. Antonellis, *To charge or not to charge: mechanistic insights into neuropathy-associated tRNA synthetase mutations.* Curr Opin Genet Dev.
47. Vester, A., et al., *A loss-of-function variant in the human histidyl-tRNA synthetase (HARS) gene is neurotoxic in vivo.* Hum Mutat, 2013. **34**(1): p. 191-9.
48. Schimmel, P. and J.M. Bacher, *An editing-defective aminoacyl-tRNA synthetase is mutagenic in aging bacteria via the SOS response.* Proc Natl Acad Sci U S A, 2006. 104(6): p. 1907-1912.

Chapter 2

A codon substitution affecting editing in the threonyl-tRNA synthetase is toxic to *B. subtilis*

INTRODUCTION

Certain aminoacyl-tRNA synthetases (aaRSs) must choose between structurally similar amino acids in order to attach the correct amino acid to its corresponding tRNA, and these aaRSs have an editing site, in addition to its aminoacylation site, which hydrolyzes incorrect charged tRNAs. The aaRSs are divided into two classes, which are categorized according to differing sequence motifs [1]. The two classes differ largely in structure, having very different active sites, while maintaining functional similarity, suggesting that the two classes arose by convergent evolution [2-4]. The first class II enzyme whose editing site was defined was the bacterial ThrRS, which contains an N2 domain present in bacterial and eukaryotic ThrRSs but absent in most archaea. A similar N2 domain also contributes to editing by the AlaRS [5-9]. ThrRS attaches threonine to its cognate tRNA, but it can also occasionally charge $tRNA^{Thr}$ with serine [10]. Threonine and serine differ by only a methyl group. While it would appear critical that cells accurately translate threonine codons, the biological requirement for the editing function of ThrRS has not been tested.

Substitution of C182A in the *E. coli* ThrRS disrupts the editing function of the enzyme without drastically affecting aminoacylation activity of the enzyme [11, 12]. Comparing the sequence homology of the *E. coli* and *B. subtilis* ThrRS, we rationalized that a similar C184A substitution in the *B. subtilis* ThrRS would result in a similar disruption of the editing function. We have further hypothesized that editing by the ThrRS becomes more important in slow growth conditions, as cells may be less able to dilute misfolded proteins or dissolve aggregates and will therefore show a decrease in viability during slow growth induced by nutrient deprivation [5]. *B. subtilis* was used for

this study because it displays easily observed and quantifiable phenotypes during slow-growth induced by nutrient deprivation. These phenotypes include sporulation, genetic competence and biofilm formation. Because editing function has only been studied in bacteria during exponential growth, we wish to reproduce a growth environment similar to what is seen in nature during slow growth and nutrient deprivation.

RESULTS

Efforts to construct a *B. subtilis* strain containing the *thrS(C184A)* allele. To introduce the *thrS(C184A)* into *B. subtilis*, I first constructed a plasmid containing this allele in *Escherichia coli*. This was accomplished by cloning the entire *thrS* operon of *B. subtilis* into the pJet1.2 plasmid (Thermo Scientific) along with a chloramphenicol-resistance marker, *cat*, and an I-SceI restriction site, which is a counter-selectable marker [13]. This plasmid, pBL1037, then underwent site-directed mutagenesis to change the C184 codon of *thrS*, TGC, to an alanine codon, both the preferred alanine codon of *B. subtilis*, GCA, and separately, the non-preferred codon, GCG [14], and these individual codon substitutions were confirmed by sequencing. The two different alanine codon changes were introduced to enhance the likelihood that one of the mutant strain expressed *thrS* at a similar level to the wild-type parent strain.

The two plasmids containing the different *thrS* alleles, *thrS(C184A-GCG)* and *thrS(C184-GCA)*, were transformed into a wild-type strain of *B. subtilis* JH642. Transformants were selected based on their chloramphenicol resistance and have the plasmid integrated into the chromosome and both a wild-type *thrS* allele and a mutant *thrS* allele present on either side of the plasmid DNA. To remove the plasmid DNA and restore the *thrS* locus to its original structure, these transformants were then subjected to counter-selection with I-SceI restriction enzyme. The plasmid pBKJ223, which encodes the I-SceI restriction enzyme [13] was transformed into them. The I-SceI restriction enzyme creates a double-strand break at the I-SceI restriction site in the plasmid. This event stimulated a repair reaction involving homologous recombination between the two alleles of *thrS* flanking the plasmid. As a consequence, the pBKJ223 transformed cells

became sensitive to chloramphenicol, and DNA sequencing revealed that only one allele of *thrS* was now present. This was confirmed by analyzing sequence data, which showed one clear peak at codon 184 rather than two peaks, which would have been expected if both *thrS* alleles were present in the strain.

Based on the location of the C184A mutation in the *thrS* locus, we predicted that 30% of the chloramphenicol-sensitive transformants should have the mutant allele of *thrS*. Sixty-nine separate colonies from pBKJ223-transformed strains were sequenced. One of these sequences showed the wild type TGC (cysteine) mutated to the correct GCG (alanine) amino acid. This colony was streak purified and tested again the following day, and GGC (glycine) was instead in the mutated codon. Of the rest, 44 remained wild type (TGC), 22 had mutated into a GGC glycine codon, and1 had mutated into a GGG glycine codon. Additionally, one colony had mutated into a threonine (ACC) (Table 2-4). The inability to isolate the C184A mutation suggests that this mutation in *thrS* is lethal to the cells and that there is a selective pressure for these cells to undergo an event that leads to an alternative codon, one for either a glycine or a threonine.

Construction a *B. subtilis* strain containing the *thrS(C184G)* allele. To confirm that substitution of cysteine at position 184 in *thrS* with an alanine was toxic to the cell, but that a glycine substitution would be tolerated, we asked whether we could isolate and *thrS(C184G)* at the expected frequency for a neutral mutation. To this end, we again performed site-directed mutagenesis of pBL1037 plasmid to change the TGC (cysteine) codon at position 184 to a GGC (glycine) codon. The plasmid carrying the *thrS(C184G)* allele was transformed into *B. subtilis* cells, where it integrated into the chromosome. These transformed cells were then subjected to counter-selective pressure

with the I-SceI restriction enzyme to select for loss of the plasmid sequences and restoration of the normal *thrS* locus structure. The *thrS* locus was then sequenced from these counter-selected cells. From the 11 sequenced clones, 8 had the desired GGC mutation, 1 had retained the wild-type TGC sequence, and 1 had surprisingly mutated position 184 to the GGG glycine codon.

DISCUSSION

We have been unable isolate a strain of *B. subtilis* carrying the *thrS(C184A)* editing-defective allele. It appears that the substitution of alanine for cysteine in the putative editing site of this enzyme is toxic. Interestingly, it was very common to get a glycine in codon 184 when trying to mutate this codon to an alanine. The wild type codon was TGC (cysteine) and the glycine codon that we repeatedly found was a GGC (glycine). It may be less stressful for this wild type cysteine to mutate into a glycine than an alanine. The cysteine and glycine codons share two nucleotides, which suggests that this unexpected change could have occurred through a single base substitution or a recombination event between these two codons. Either event should have been very rare, supporting the hypothesis that the C184A substitution of *thrS* is toxic to cells. In line with our predictions that the C814G substitution of *thrS* is neutral to cells, we easily isolated strain carrying the C184G substitution when a plasmid carrying the *thrS(C184G)* allele was the starting material.

It is not clear at this time why the *thrS(C814A)* allele should be toxic when the *thrS(C184G)* allele is not. It will be necessary to assess the biochemical properties of these mutant ThrRS proteins to understand the cellular phenotypes. It is possible that despite the predictions the ThrRS(C184A) mutant protein is defective for it essential aminoacylation function. Alternatively, it may be that the ThrRS(C184A) mutant protein is specifically defective for its editing function, whereas the ThrRS(C184G) mutant protein retains wild-type function. If this latter case is true, then our data would suggest that the editing function of ThrRS is essential for *B. subtilis*. Editing functions of aaRSs

have not previously been shown to be required for normal cellular growth. Our surprising findings merit analysis to fully understand the physiological role of editing by ThrRS.

MATERIALS AND METHODS

Media and Growth Conditions:

Bacillus subtilis and *E. coli* cells were grown with shaking at either 37°C or 28°C. Both *B. subtilis* and *E. coli* cells were grown on LB medium containing ampicillin (100mg/mL), chloramphenicol (5mg/mL) or tetracycline (12.5mg/mL) as appropriate. *B. subtilis* competent cells were prepared as previously described [15].

Plasmid constructions

The pBL1038 and pBL1039 plasmids were created to introduce *thrS(C184A)* alleles, containing either the GCG or GCA codon for alanine, respectively, into the *B. subtilis* chromosome. The *thrS* locus was first PCR amplified using primers *thrS*-F and *thrS*-R (see Table 2-3 for primer sequences) and *B. subtilis* wild-type chromosomal DNA. The resulting PCR product was then cloned into pJet2.1 with the CloneJet PCR Cloning Kit, according to the manufacturers instructions (Thermo Scientific). The sequence of the *thrS* in the resulting pJet-*thrS* plasmid was confirmed using primers *thrS*-F, *thrS*-R, *thrS*-MF and *thrS*-MR for sequencing. In the second step, a fragment containing *cat*, which encodes chloramphenicol resistance, and the I-SecI restriction site, was introduced into the pJet-*thrS* plasmid. The *cat* gene [16] was amplified using primers Cat-PstI-I-SceI-F and Cat-PstI-R, which contain and PstI restriction sites at their 5' ends and the forward primer also containing the 18 bp I-SceI restriction site just to the 3' end of the PstI site. The resulting PCR product was digest with PstI and ligated to the pJet-*thrS* that had been digested with Pst1 and phosphatase treated. The resulting plasmid was named pBL1037.

The 184th amino acid in the *thrS* gene is a cysteine, which was mutated to an alanine to affect editing function of this tRNA synthetase. A substitution of this alanine

codon to glycine was also performed in order to confirm that it was easily created. In order to introduce a codon mutation (from a TGC to a GCA, GCG or GGC), a mutagenic primer was created to amplify pBL1037 with this codon substitution. Once 6.5kb linear PCR amplified plasmid was isolated, it was self-ligated with T4 DNA Ligase and transformed into *E. coli*. Sequencing confirmed the presence of the point mutation in the plasmid.

The pBKJ223 plasmid contains the I-SceI restriction enzyme, which creates a double-stranded cut at the 18 base pair I-SceI sequence [13]. This plasmid only survives in *E. coli* Top10 for a maximum of 2 passages. We have been unable to retrieve this plasmid from freezer stocks and must re-transform and prep this plasmid new for each use.

Strain construction

B. subtilis and *E. coli* strains used in this study are listed in Table 2-1. These were constructed by transformation with chromosomal DNA or plasmids using standard protocols [15]. The plasmids used in this study are listed in Table 2-2.

Once the different *thrS* alleles were constructed, the plasmids containing these different alleles, pBL1038, pBL1039, pBL1041 and pBL1042 were transformed into *B. subtilis* and chloramphenicol-resistant colonies were isolated. The subsequent strains, BAL4364, BAL4365, and the unstocked C184G and C184C (transformed with pBL1041 and pBL1042, respectively), were transformed with pBKJ223 and tetracycline–resistant colonies were selected. The strains that are isolated are patched onto both chloramphenicol and tetracycline patch plates, allowing us to choose the strains that are both confirmed to be tetracycline resistant and chloramphenicol sensitive. These cells lost

their chloramphenicol resistant and had only one copy left of the *thrS* gene. Sequencing using GeneWiz confirmed either the presence of the wild type or the mutant *thrS*.

Table 2-1. Strains used in this study

Strain Name	Relevant Genotypes	Species
BAL218 (JH642)	wild type (*trpC2, pheA1*)	*B. subtilis*
BAL4300	pBL1037 (*cat, thrS,* I-SceI)	*E. coli*
BAL4302	RecA+, pBKJ223	*E. coli*
BAL4350	pBL1038 (*cat, thrS(C184A-GCA),* I-SceI)	*E. coli*
BAL4351	pBL1039 (*cat, thrS(C184A-GCG),* I-SceI)	*E. coli*
BAL4364	*thrS::*pBL1038 (*cat, thrS(C184A-GCA),* I-SceI)	*B. subtilis*
BAL4365	*thrS::*pBL1038 (*cat, thrS(C184A-GCG),* I-SceI)	*B. subtilis*

Table 2-2. Plasmids used in this study

Plasmid Name	Relevant Features
pBL1037	pJet2.1 with *thrS*, *cat*, and I-SceI restriction site
pBKJ223	encoding I-SceI restriction enzyme
pBL1038	as pBL1037, except *thrS(C184A-GCA)*
pBL1039	as pBL1037, except *thrS(C184A-GCG)*
pBL1040	pJet-thrZ-Cat-IsceI
pBL1041	as pBL1037, except *thrS(C184G-GGC)*
pBL1042	as pBL1037, except *thrS(C184C-TGG)*

Table 2-3. Primers used in this study

Primer Name	Primer Sequence	Purpose
thrS-F	ACG AAG GAA TGA TAC CGG CTC AAA GAG AGG GAA G	PCR amplify *thrS*
thrS-R	CTC GCT CCT TAC TTC AAT GAA ATC GTC ATC TCG GGC	PCR amplify *thrS*
thrS-MF	TTC GAA AAG AAG TCA GCC GTG AAG AAG CGA AAG CC	Sequence *thrS*
thrS-MR	GTG CTG CTT GCC GTC CTC ACC GAT G	Sequence *thrS*
Cat-Pst1-IsceI-F	CTG CAG TAG GGA TAA CAG GGT AAT TAT TGG GCG CTC TTC CGC TAA GCA TG	PCR amplify Cat Cassette Containing IsceI
Cat-Pst1-R	CTG CAG GCG AGT CAG TGA GCG AGG AAG CAA GCA TG	PCR amplify Cat Cassette Containing IsceI
Cat-Seq-Top	GCG ACG GAG AGT TAG GTT ATT GGG ATA AGT TAG AGC	Sequence Cat-IsceI
Cat-Seq-Bottom	GTG GCT CTA ACT TAT CCC AAT AAC CTA ACT CTC CGT CGC	Sequence Cat-IsceI
Mut-thrS-top-1B	GAA TTC TTT GAC CTG GCA CGC GGC GTC CAT GTT CCT TC	PCR Mutagenize C184 Codon in *thrS*
Mut-thrS-top-2B	GAA TTC TTT GAC CTG GCG CGC GGC GTC CAT GTT CCT TC	PCR Mutagenize C184 Codon in *thrS*
Mut-thrS-bottomB	G CTC ATA GAT CGA AAC GGT TTC TCC TTC AGG AAT CGC	PCR Mutagenize C184 Codon in *thrS*
thrZ-F	ATC ACA ATC AAA GAA GCG GCT GGT TCG ATC AG	PCR amplify *thrZ*
thrZ-R	CTT CAG CTC CGC CTG CAC TTT TCT GCA ATA G	PCR amplify *thrZ*
Mut-thrZ-top-1C	GAA TTC GTT GAC CTG GCA CGA GGG CCG CAT CTT CC	PCR Mutagenize C184 Codon in *thrZ*
Mut-thrZ-top-2C	GAA TTC GTT GAC CTG GCG CGA GGG CCG CAT CTT CC	PCR Mutagenize C184 Codon in *thrZ*
Mut-thrZ-bottomB	CCC TTG CTG GTA CAG TGT GAT ATC CTC CCC GCG CGG AAT ATC CTT	PCR Mutagenize C184 Codon in *thrZ*
thrS-mut-gly	GAA TTC TTT GAC CTG GGC CGC GGC GTC CAT GTT CCT TC	PCR Mutagenize C184 Codon in *thrS*
thrS-mut-cys	GAA TTC TTT GAC CTG TGG CGC GGC GTC CAT GTT CCT TC	PCR Mutagenize C184 Codon in *thrS*

Table 2-4. Frequency of isolating of various codons after transformation with pBKJ223

Codon	Number	Percentage of Total Sequenced
TGC (wild type)	44	63.77%
GGC	22	31.88%
GGG	1	1.45%
ACC	1	1.45%
GCG	1	1.45%

REFERENCES

1. Eriani, G., et al., *Partition of tRNA synthetases into two classes based on mutually exclusive sets of sequence motifs.* Nature, 1990. **347**(6289): p. 203-6.
2. Ibba, M. and D. Soll, *Aminoacyl-tRNA synthesis.* Annu Rev Biochem, 2000. **69**: p. 617-50.
3. Burbaum, J.J. and P. Schimmel, *Structural relationships and the classification of aminoacyl-tRNA synthetases.* J Biol Chem, 1991. **266**(26): p. 16965-8.
4. Cusack, S., M. Hartlein, and R. Leberman, *Sequence, structural and evolutionary relationships between class 2 aminoacyl-tRNA synthetases.* Nucleic Acids Res, 1991. **19**(13): p. 3489-98.
5. Ling, J., N. Reynolds, and M. Ibba, *Aminoacyl-tRNA synthesis and translational quality control.* Annu Rev Microbiol, 2009. **63**: p. 61-78.
6. Cusack, S., A. Yaremchuk, and M. Tukalo, *The 2 A crystal structure of leucyl-tRNA synthetase and its complex with a leucyl-adenylate analogue.* EMBO J, 2000. **19**(10): p. 2351-61.
7. Dock-Bregeon, A., et al., *Transfer RNA-mediated editing in threonyl-tRNA synthetase. The class II solution to the double discrimination problem.* Cell, 2000. **103**(6): p. 877-84.
8. Sankaranarayanan, R., et al., *Zinc ion mediated amino acid discrimination by threonyl-tRNA synthetase.* Nat Struct Biol, 2000. **7**(6): p. 461-5.
9. Arnez, J.G., et al., *Aminoacylation at the Atomic Level in Class IIa Aminoacyl-tRNA Synthetases.* J Biomol Struct Dyn, 2000. **17 Suppl 1**: p. 23-7.
10. Wallen, R.C. and A. Antonellis, *To charge or not to charge: mechanistic insights into neuropathy-associated tRNA synthetase mutations.* Curr Opin Genet Dev.
11. Waas, W.F. and P. Schimmel, *Evidence that tRNA synthetase-directed proton transfer stops mistranslation.* Biochemistry, 2007. **46**(43): p. 12062-70.
12. Dock-Bregeon, A., et al., *Achieving error-free translation; the mechanism of proofreading of threonyl-tRNA synthetase at atomic resolution.* Mol Cell, 2004. **16**(3): p. 375-86.
13. Janes, B. and S. Scott, *Routine Markerless Gene Replacement in Bacillus anthracis.* Infection and Immunity, 2006. **74**(3): p. 1949-1953.
14. Kanaya, S., et al., *Studies of codon usage and tRNA genes of 18 unicellular organisms and quantification of Bacillus subtilis tRNAs: gene expression level and species-specific diversity of codon usage based on multivariate analysis.* Gene, 1999. **238**(1): p. 143-55.
15. Harwood, C.R. and S.M. Cutting, *Molecular biological methods for Bacillus*. Modern microbiological methods. 1990, Chichester ; New York: Wiley. xxxv, 581 p.
16. Stanley, N., et al., *Identification of catabolite repression as a physiological regulator of biofilm formation by Bacillus subtilis by use of DNA microarrays.* J. Bacteriol., 2003. **185**(6): p. 1951-1957.

Chapter 3

Investigating the role of editing by the minor threonyl-tRNA synthetase of *Bacillus subtilis,* encoded by *thrZ*

INTRODUCTION

In *Bacillus subtilis,* there are two independent threonyl-tRNA synthetase isoenzymes, ThrRS and ThrRZ, encoded by *thrS* and *thrZ*, respectively *[1]*. The ThrRS and ThrRZ proteins share only 51.5% identity, making them as distinct from one another as they are from *E. coli* ThrRS [1]. Either the *thrS* or *thrZ* gene alone is adequate for *B. subtilis* viability; but under normal conditions, only the *thrS* gene is expressed [3]. This has raised the question of what role ThrRZ has for *B. subtilis*.

Thus far, the ThrRZ protein is only known to be expressed under conditions of threonine starvation, which induces both the *thrS* and *thrZ* genes simultaneously [3]. The ThrRS protein normally repress *thrS* and *thrZ* expression, and conditions of threonine starvation appear to antagonize *thrS* and *thrZ* repression by ThrRS [3]. Interestingly, ThrRZ has much less of an ability to repress expression of these genes [1, 2].

ThrRZ, like ThrRS, has an editing domain whose function is predicted to be hydrolysis of mischarged $tRNA^{Thr}$. To further elucidate the role of ThrRZ in *B. subtilis*, we are investigating what role editing by ThrRZ has for cells. To this end, we are creating a *B. subtilis* strain with a point mutation in *thrZ* that results in an editing defect. This *thrZ* mutation will also be combined with a editing-defective *thrS* mutation. The strains containing the *thrZ* editing-defective mutation will be tested for defective slow-growth phenotype, including sporulation and biofilm formation, conditions under which the role of ThrRZ has not been previously tested.

RESULTS

Construction of a plasmid encoding the *B. subtilis thrZ* gene. We sought to create an allele of *thrZ* that lacks editing activity. It was demonstrated by Waas and Schimmel that a C184A substitution in the *E. coli* ThrRS disrupts the editing function of the enzyme [4]. This analogous mutation was created in the *B. subtilis thrS* gene in Chapter 2 of this work. The *B. subtilis thrS* and *thrZ* both have a cysteine as their 184th residue (Fig. 3-1), which is the residue that we will substitute with alanine in *thrZ* in order to create an editing-defective ThrRZ(C184A).

As a first step to constructing the *thrZ(C184A)* allele, we cloned *thrZ* from *B. subtilis* into an *E. coli* plasmid that contained both a selective marker, *cat*, and a counter-selective marker, I-SceI restriction site, for *B. subtilis*. This plasmid, pBL1040, contains a wild-type copy of *thrZ* and will serve as future template for site-directed mutagenesis to created the *thrZ(C184A)* allele.

DISCUSSION

Because the substitution affecting editing in the *thrS* gene seems to be toxic, it is interesting to see if the analogous mutation could be made in the *thrZ* gene. Being able to create the *thrZ* but not the *thrS* C184 mutation may show that there is something distinct about the two genes, such as the *thrS* is more ubiquitously expressed or that it is more sensitive to errors in quality control. Because *thrZ* is less expressed, it is possible that an editing mutation in this gene will not be toxic to *B. subtilis,* as was the case with *thrS*, and we may be able to see a phenotype. Another question raised here is whether the loss of editing in either ThrRS or ThrRZ could lead to compensation through increased expression of the other protein.

Upon testing the pJet-thrZ-Cat-IsceI plasmid, we found that it was incorrect. There is no evidence at this time that there was anything wrong with the primers. In the future it may be advantageous to try to reconstruct this plasmid in a different way.

MATERIALS AND METHODS

Media and Growth Conditions:

B. subtilis and *E. coli* cells were grown with shaking at either 37°C or 28°C, the latter temperature used for less growth over a longer period of time. Both *B. subtilis* and *E. coli* cells were grown in LB liquid medium or LB agar plates with ampicillin (100mg/mL), chloramphenicol (5mg/mL) or tetracycline (12.5mg/mL) as needed.

Strain Construction:

PCR amplfication of the *thrZ* gene was performed using thrZ-F and thrZ-R primers amplifying corresponding portions of *B. subtilis* wild-type chromosomal DNA (Table 3-1). Primers were designed according to the upstream and downstream regions of these genes according to Subtilist and BSubCyc. Proper PCR products were confirmed using 1% agarose gels.

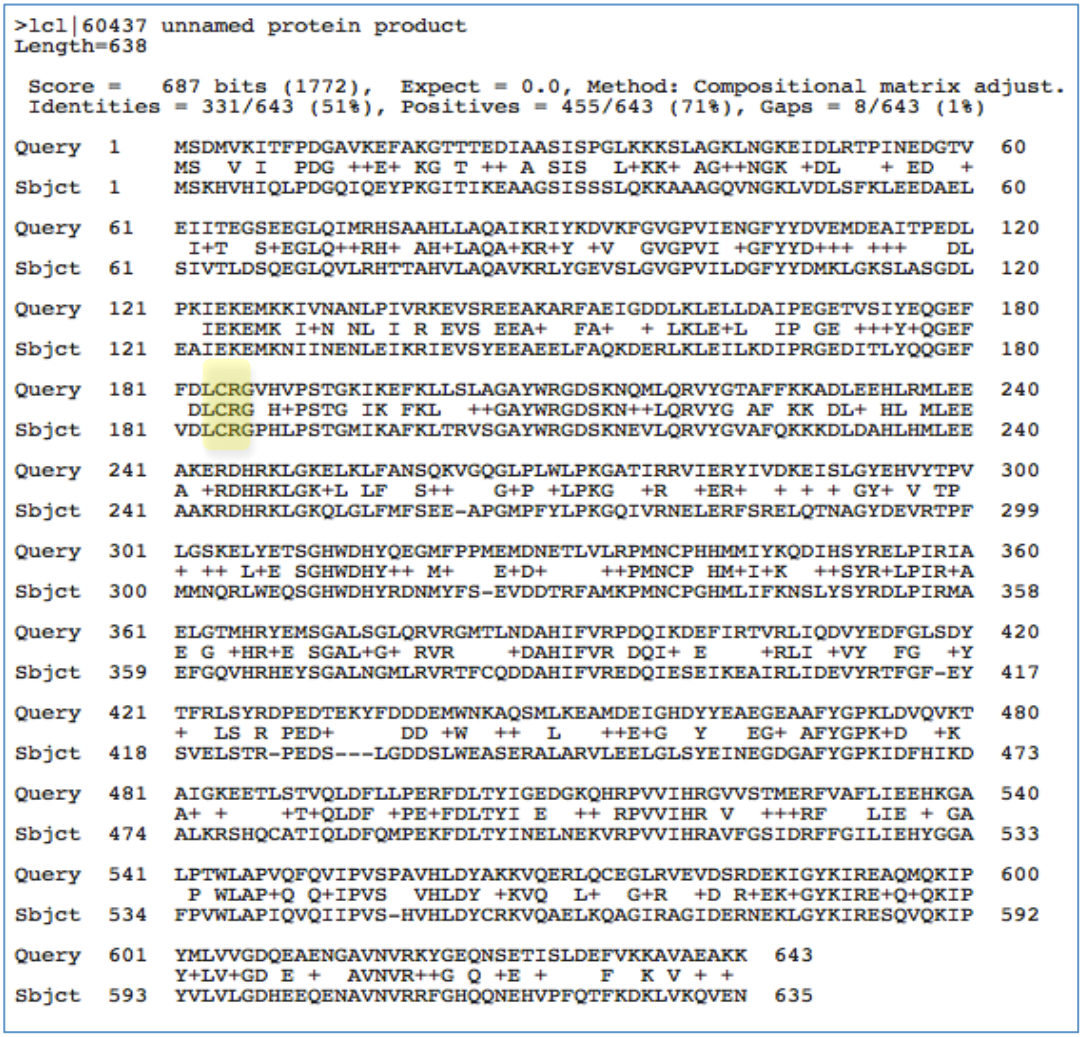
A plasmid was created that contains *thrZ*, the selectable *cat* marker, and the counter-selectable I-SceI restriction stie. This *thrZ*-containing plasmid was derived from the original pBL1037 plasmid, which contains *thrS*, *cat*, and the I-SceI restriction site and is described in Chapter 2. Two separate BglII digests were used in this process. First, the *thrZ* gene was cloned into the pJet2.1 vector (Thermo Scientific), and the subsequent pJet-thrZ vector was digested with BglII, and the 2.7kb *thrZ* fragment was gel purified. The pBL1037 plasmid was then digested with BglII, which separates *thrS* gene from the vector fragment. The vector fragment was isolated and ligated with the gel-purified *thrZ* fragment, creating pBL1040. This plasmid was confirmed to be correct by using a diagnostic BglII restriction digest.

The 184th amino acid in the *thrZ* gene is a cysteine, which was mutated to an alanine to affect editing function of this tRNA synthetase. In order to introduce a codon mutation (from a TGC to a GCA or GCG), mutagenic primers were created to amplify the pBL1040 plasmid with this codon substitution (see Table 3-1).

Table 3-1. Primers used in this study

Primer Name	Primer Sequence	Purpose
thrZ-F	ATC ACA ATC AAA GAA GCG GCT GGT TCG ATC AG	PCR amplify *thrZ*
thrZ-R	CTT CAG CTC CGC CTG CAC TTT TCT GCA ATA G	PCR amplify *thrZ*
Mut-thrZ-top-1C	GAA TTC GTT GAC CTG GCA CGA GGG CCG CAT CTT CC	PCR Mutagenize C184 Codon in *thrZ*
Mut-thrZ-top-2C	GAA TTC GTT GAC CTG GCG CGA GGG CCG CAT CTT CC	PCR Mutagenize C184 Codon in *thrZ*
Mut-thrZ-bottomB	CCC TTG CTG GTA CAG TGT GAT ATC CTC CCC GCG CGG AAT ATC CTT	PCR Mutagenize C184 Codon in *thrZ*

Figure 3-1. Amino acid residue alignment of *thrS* and *thrZ* gene products



```
>lcl|60437 unnamed protein product
Length=638

 Score =   687 bits (1772),  Expect = 0.0, Method: Compositional matrix adjust.
 Identities = 331/643 (51%), Positives = 455/643 (71%), Gaps = 8/643 (1%)

Query  1    MSDMVKITFPDGAVKEFAKGTTTEDIAASISPGLKKKSLAGKLNGKEIDLRTPINEDGTV  60
            MS  V I  PDG ++E+ KG T ++ A SIS  L+KK+ AG++NGK +DL   + ED  +
Sbjct  1    MSKHVHIQLPDGQIQEYPKGITIKEAAGSISSSLQKKAAAGQVNGKLVDLSFKLEEDAEL  60

Query  61   EIITEGSEEGLQIMRHSAAHLLAQAIKRIYKDVKFGVGPVIENGFYYDVEMDEAITPEDL  120
             I+T  S+EGLQ++RH+ AH+LAQA+KR+Y +V  GVGPVI +GFYYD+++ +++   DL
Sbjct  61   SIVTLDSQEGLQVLRHTTAHVLAQAVKRLYGEVSLGVGPVILDGFYYDMKLGKSLASGDL  120

Query  121  PKIEKEMKKIVNANLPIVRKEVSREEAKARFAEIGDDLKLELLDAIPEGETVSIYEQGEF  180
              IEKEMK I+N NL I R EVS EEA+  FA+  + LKLE+L  IP GE +++Y+QGEF
Sbjct  121  EAIEKEMKNIINENLEIKRIEVSYEEAEELFAQKDERLKLEILKDIPRGEDITLYQQGEF  180

Query  181  FDLCRGVHVPSTGKIKEFKLLSLAGAYWRGDSKNQMLQRVYGTAFFKKADLEEHLRMLEE  240
             DLCRG H+PSTG IK FKL  ++GAYWRGDSKN++LQRVYG AF KK DL+ HL MLEE
Sbjct  181  VDLCRGPHLPSTGMIKAFKLTRVSGAYWRGDSKNEVLQRVYGVAFQKKKDLDAHLHMLEE  240

Query  241  AKERDHRKLGKELKLFANSQKVGQGLPLWLPKGATIRRVIERYIVDKEISLGYEHVYTPV  300
            A +RDHRKLGK+L LF  S++   G+P +LPKG  +R  +ER+  + + + GY+ V TP
Sbjct  241  AAKRDHRKLGKQLGLFMFSEE-APGMPFYLPKGQIVRNELERFSRELQTNAGYDEVRTPF  299

Query  301  LGSKELYETSGHWDHYQEGMFPPMEMDNETLVLRPMNCPHHMMIYKQDIHSYRELPIRIA  360
            + ++ L+E SGHWDHY++ M+   E+D+    ++PMNCP HM+I+K  ++SYR+LPIR+A
Sbjct  300  MMNQRLWEQSGHWDHYRDNMYFS-EVDDTRFAMKPMNCPGHMLIFKNSLYSYRDLPIRMA  358

Query  361  ELGTMHRYEMSGALSGLQRVRGMTLNDAHIFVRPDQIKDEFIRTVRLIQDVYEDFGLSDY  420
            E G +HR+E SGAL+G+ RVR    +DAHIFVR DQI+ E    +RLI +VY  FG  +Y
Sbjct  359  EFGQVHRHEYSGALNGMLRVRTFCQDDAHIFVREDQIESEIKEAIRLIDEVYRTFGF-EY  417

Query  421  TFRLSYRDPEDTEKYFDDDEMWNKAQSMLKEAMDEIGHDYYEAEGEAAFYGPKLDVQVKT  480
            +  LS R PED+     DD +W  ++  L   ++E+G  Y   EG+ AFYGPK+D  +K
Sbjct  418  SVELSTR-PEDS---LGDDSLWEASERALARVLEELGLSYEINEGDGAFYGPKIDFHIKD  473

Query  481  AIGKEETLSTVQLDFLLPERFDLTYIGEDGKQHRPVVIHRGVVSTMERFVAFLIEEHKGA  540
            A+ +    +T+QLDF +PE+FDLTYI E  ++ RPVVIHR V  +++RF   LIE + GA
Sbjct  474  ALKRSHQCATIQLDFQMPEKFDLTYINELNEKVRPVVIHRAVFGSIDRFFGILIEHYGGA  533

Query  541  LPTWLAPVQFQVIPVSPAVHLDYAKKVQERLQCEGLRVEVDSRDEKIGYKIREAQMQKIP  600
             P WLAP+Q Q+IPVS  VHLDY +KVQ  L+  G+R  +D R+EK+GYKIRE+Q+QKIP
Sbjct  534  FPVWLAPIQVQIIPVS-HVHLDYCRKVQAELKQAGIRAGIDERNEKLGYKIRESQVQKIP  592

Query  601  YMLVVGDQEAENGAVNVRKYGEQNSETISLDEFVKKAVAEAKK  643
            Y+LV+GD E +  AVNVR++G Q +E +    F  K V + +
Sbjct  593  YVLVLGDHEEQENAVNVRRFGHQQNEHVPFQTFKDKLVKQVEN  635
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REFERENCES

1. Putzer, H., A.A. Brakhage, and M. Grunberg-Manago, *Independent Genes for Two Threonyl-tRNA Synthetases in Bacillus subtilis.* Journal of Bacteriology, 1990. **172**(8): p. 4593-4602.
2. Gendron, N., H. Putzer, and M. Grunberg-Manago, *Expression of Both Bacillus subtilis Threonyl-tRNA Synthetase Genes Is Autogenously Regulated.* Journal of Bacteriology, 1994. **176**(2): p. 486-494.
3. Putzer, H., N. Gendron, and M. Grunberg-Manago, *Co-ordinate expression of the two threonyl-tRNA synthetase genes in Bacillus subtilis: control by transcriptional antitermination involving a conserved regulatory sequence.* EMBO, 1992. **11**(8): p. 3117-3127.
4. Waas, W.F. and P. Schimmel, *Evidence that tRNA synthetase-directed proton transfer stops mistranslation.* Biochemistry, 2007. **46**(43): p. 12062-70.