A TALE OF TWO TRNAS: TRANSLATING THE SAME CODON DOES NOT IMPLY REDUNDANCY

by

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A transfer RNA (tRNA) can form codon-anticodon interactions either by standard Watson-Crick base pairing or by forming a wobble base pair between the first position of the anticodon and the third position of the codon. This means that within the same isoaccepting tRNA group, one codon may be read by two individual tRNAs each interacting in one of the two ways. This leads to the question: Why would such redundancy occur? Through the inactivation of the gene that encodes for the threonine isoaccepting tRNA with the anticodon CGU (tRNA-CGU), it was found that the tRNA-CGU, which interacts with its cognate codon through Watson-Crick base pairing, is not essential for life in *Escherichia coli*. Yet, the tRNA-CGU is present in a majority of bacteria, leading one to expect that it must have a meaningful role in the process of translation that has caused it to either be a retained or a gained molecule for such bacteria. Evidence showed that the tRNA-CGU has specific properties that allow the tRNA to contribute more at increased temperatures to the translation of its codon, while the properties of the tRNAs that "wobble" to read the codon contribute less at increased temperatures. One possible, contributing property of the tRNA-CGU is the more stable codon-anticodon interaction formed by the standard Watson-Crick base pair. Since bacteria have to live in different environments and at various temperatures, such chemical stability may be key to the survival of these bacteria and why so many bacterial genomes have a tRNA that is seemingly redundant.

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1 Introduction

1.1 Biological Background Information

The process of translation and one component of its machinery, transfer RNA (tRNA), are the topics of interest for this thesis. Translation is the molecular process that forms proteins, which are essential for most cellular functions. Proteins are molecules composed of smaller molecules, called amino acids, that link together and fold into a certain shape that dictates the protein's function. The process of protein formation begins with a cell's database, its DNA. DNA stores information concerning how a cell functions and about the proteins needed to carry this out. DNA is made from building blocks called nucleotides, of which there are four different ones. The nucleotides are linked together in a specific sequence, known as a DNA sequence. Each protein needed by the cell is encoded by a unique order of these nucleotides. However, the information stored within the DNA is not directly usable to the cell. In order for the proteins to be made and allowed to function, the information must first be transcribed into a usable form, mRNA. Once mRNA is created, it can be translated into a protein by translational machinery, including the tRNA. The overview of this process, referred to as the central dogma of biology, is depicted in Figure 1.1.

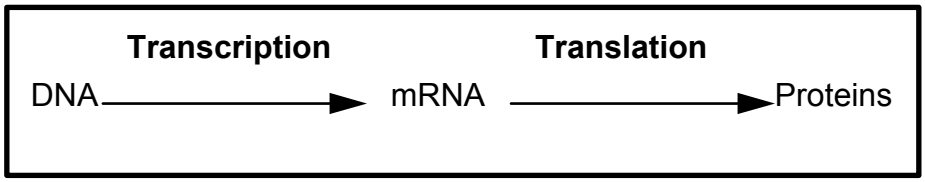

Figure 1.1: The central dogma of biology

Like DNA, mRNA is also made from the building blocks called nucleotides. In mRNA, there are four different bases: adenine (A), guanine (G), cytosine (C) and uracil (U). The nucleotides are linked together in a specific sequence that has been directly transcribed from the order of bases within the DNA. The key to mRNA being a useable form is that the codon, a unique combination of three of the four bases that gives a trinucleotide sequence, is accessible by the translation machinery. Some examples of codons are GCU and UUA. Each codon encodes one of the 20 amino acids. Going back to the prior examples, the codon GCU indicates to the translational machinery that the amino acid alanine is required. Likewise, the codon UUA encodes the amino acid leucine. All codons and the amino acids for which they encode make up what is called the genetic code. An example of some codons on an mRNA strand and the amino acids which they encode is shown in Figure 1.2. Through translation, the amino acids become linked in the order specified by the order of codons to form a protein.

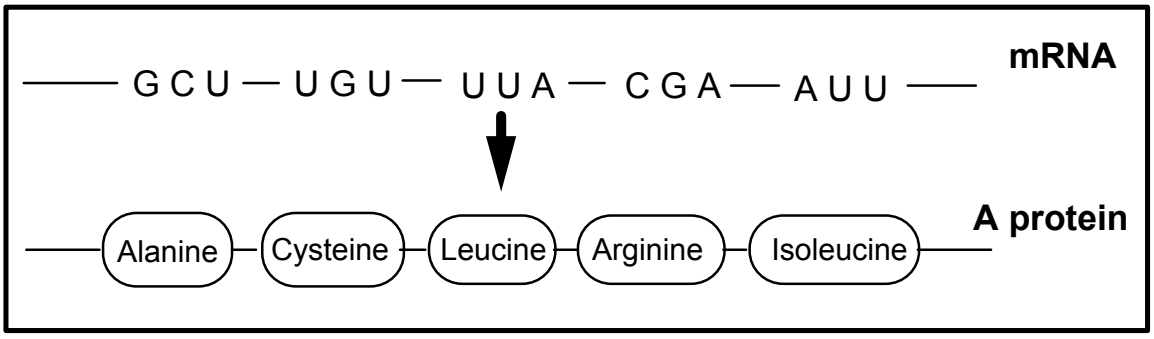

Figure 1.2: A strand of mRNA consisting of trinucleotide sequences called codons that each encodes one of the amino acids, which then are linked together the translation machinery in the order specified by the codons by to form a protein.

The job of the tRNA is to "read" the codons and bring the correct amino acids to be linked to the growing protein. tRNAs also are made of the four nucleotides linked together in a unique sequence. However, the sequence is such that the tRNA folds to give a tertiary structure. Deciphering the sequence, the structure can be converted to a secondary structure that is called a clover leaf structure, an easier way for humans to view the nucleotide interactions (Figure 1.3).

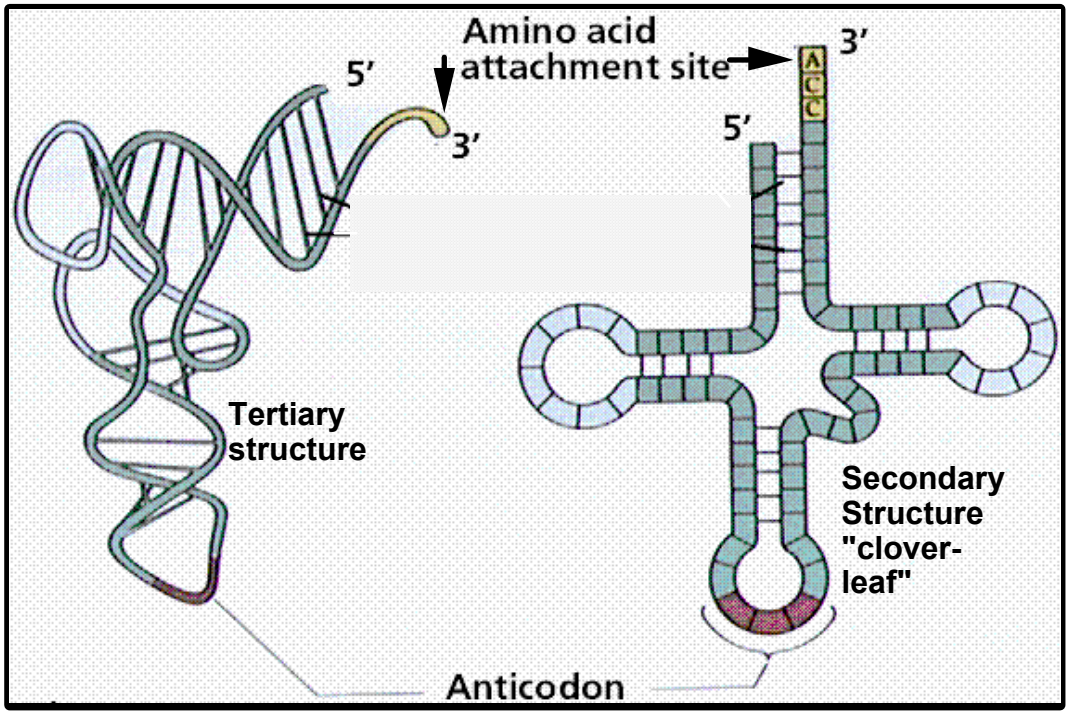

Figure 1.3: On the left is a cartoon drawing of the tertiary structure of a tRNA and on the right is the secondary structure (Farabee 2002) .

The clover leaf is the easiest way to refer to the structure of a tRNA, though in a cell, a tRNA would always be in the three dimensional structure. Every tRNA has this general structure: an anticodon, an amino acid acceptor site and the tRNA body. The body is all the other nucleotides that do not comprise the anticodon or the amino acid

acceptor site. Each tRNA has specific components that correspond to a specific amino acid. The amino acid is attached to the 3' (three prime) end of the acceptor stem. The anticodon is what reads the codons of the mRNA. This reading is actually due to a formation of chemical hydrogen bonds directly between complementary nucleotides in the codon and those in the anticodon. The common biological name for this type of bond formation is known as Watson-Crick base pairing: adenine (A) will always pair with uracil (U) and cytosine (C) will always pair with guanine (G) (Figure 1.4).

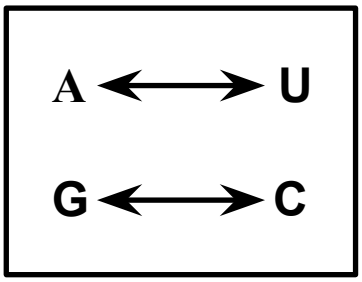

Figure 1.4: The complementary in Watson-Crick base pairing in RNA

To better address tRNA reading of mRNA, positions of the codon and anticodon must be labeled. For the anticodon CGU, C is at the 5' (five prime) end and U is at the 3' (three prime) end. In the case of the codon ACG, A is the 5' end and G is the 3' end. Both DNA and RNA strands have directionality, just like a one-way street. They only go one way, which is 5' to 3'. In order to match them together, they must go in opposite directions, like a two-way street. Refer to Figure 1.5 to see the ACG codon and CGU anticodon interaction that occurs during translation. In this case, the C of the anticodon (the first position of the anticodon) forms a base pair with the G of the codon (the third position of the codon), the G of the anticodon pairs with the C of the codon, and the U of the anticodon pairs with the A of the codon (the third position).

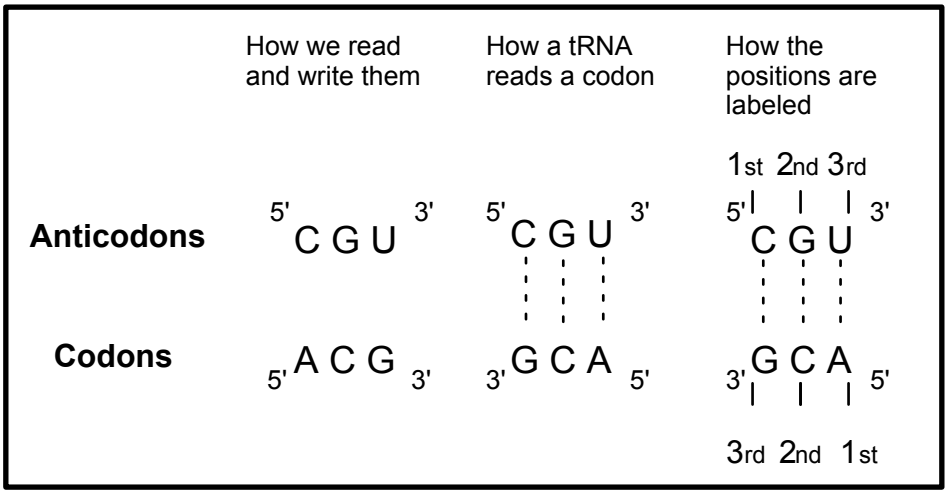

Figure 1.5

The Watson-Crick base pairing rules are easy to understand and predict with a little practice, but with respect to codon-anticodon interactions, these rules are not as strict as one would expect. With respect to the anticodon, the second and third positions always follow the traditional Watson-Crick base pairings, but the first position does not always do so. Often, a bond can form at the first position between guanine and uracil that is looser than those of traditional Watson-Crick base pairing. Such a bond is called a wobble base pair (Figure 1.6). Due to the ability of the first position to wobble, far fewer tRNAs are needed than would be required if all three positions had to be read by Watson-Crick base pairing (Crick 1966).

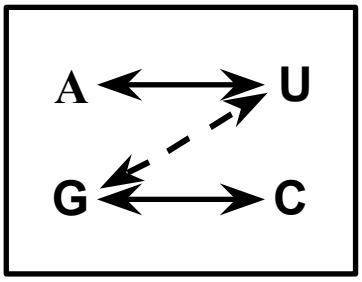

Figure 1.6: Possible base pairings--wobble base pairing (dashed arrow) and traditional Watson-Crick base pairing (solid arrows)

Once a codon has been read, whether based on traditional or wobble base pairing rules, the amino acid from the acceptor stem of the tRNA will be linked to the end of the chain of amino acids, forming a protein. This process of forming base pairs between the codon and the anticodon to give an addition of the amino acid to the protein will continue until all the codons have been read and all the amino acids for the protein have been linked together. Translation is now complete. Here it should be stated that translation is a universal process. In nearly all organisms, the same codon dictates the same amino acid. For example in every organism, the codon GCU means that an alanine is needed. However, the tRNAs vary between organisms by the specific sequence of nucleotides that comprise the rest of the tRNA other than the anticodon, as well as by which types of tRNAs are present.

1.2 Transfer RNAs, Codon-Anticodon Interactions, and Thesis Topic

Proteins are the tools of living organisms. They participate in all biological reactions and activities, and without them, life would not be. Since proteins are so important, it follows that the process to synthesize proteins from their building blocks, amino acids, is also important for life—the process called translation. The basics of translation started to be known during the 1950s. First, there was the determination of the existence of the universal genetic code, which revealed that the information for the order of amino acids to give functional proteins is encoded in the mRNA by codons. Knowing this then began the discoveries of the mechanisms for reading the genetic code. It was thought that there had to be an "adaptor" associated with each of the 20 standard

amino acids that could somehow use the template of the nucleotide encoded within mRNA to distinguish which amino acid was required to construct the desired protein. However, before the "adaptor" could be found, the specifics of the genetic code became known. F. H. C. Crick, J. S. Griffith and L. E. Orgel proposed that codons are three adjacent nucleotides whose unique order corresponds to a specific amino acid. In total, there are 64 codons, which is determined by calculating the number of trinucleotide combinations that can come from four different types of nucleotides. Crick had proposed that the interaction between the trinucleotide codon and the corresponding "adaptor" must be dictated by specific patterns of hydrogen bonds. This special type of chemical bond occurs between strands of nucleotides, in the same way that two strands of the DNA are joined together to give the double helix. P. Berg, in 1956, found that amino acids are being attached to a small piece of RNA and by further investigation found that different small pieces of these RNAs are specific to each of the standard amino acids. Shortly afterwards, M. B. Hoagland and P. Zamecnik found that the amino acids first bound to these small pieces of RNAs are eventually incorporated into a protein. These RNA molecules that are specific to each amino acid were the "adaptors" envisioned by Crick. Later the adaptors became known as transfer RNAs (tRNAs). In 1965, R. Holley sequenced the first tRNA from which several secondary structures for the tRNA were proposed, with eventually the clover leaf becoming the accepted secondary structure (Figure 1.3). Since the genetic code is comprised of a sequence of three nucleotides, these three nucleotides must interact with three complementary nucleotides at some location in the tRNA. Such a sequence was identified in the tRNAs and named appropriately the anticodon (Judson 1979). Crick (1966) proposed that no matter the

slight variations in the number of nucleotides for each tRNA in the stems or loops, the anticodon is located at a conserved position in all tRNAs. Later, the location of the anticodon was always referred to as positions 34–35–36 in the anticodon loop (Gause 1968).

By this time, the codon-anticodon interactions were thought to be standard Watson-Crick base pairing; hydrogen bonding that binds the trinucleotide anticodon to its complementary trinucleotide codon. Due to such bonding, many thought that since there were 64 codons, there would also be 64 corresponding tRNAs to recognize each codon. However, Crick (1966) began further investigations into the codon–anticodon interactions and in doing so proposed the Wobble Hypothesis. The hypothesis suggests that the nucleotides at position 35 and 36 of the anticodon form Watson-Crick base pairs with their respective nucleotides of the codon while the nucleotide at position 34 is allowed to "wobble" in its pairing. This means that between guanine and uracil there is a bond that is looser than those of traditional Watson-Crick base pairing. However, the nucleotide at position 34, even if it is a guanine or a uracil, can still form a base pair following standard Watson-Crick base pairing. This means that one tRNA anticodon can have the ability to form base pairs with two or sometimes three different codons. An example is a tRNA with the anticodon **U**CG. The U in the 34 position can form a base pair through standard Watson-Crick base pairing with an A in the third position of the codon CG**A** *and* through wobble base pairing with a G in the third position of the codon CG**G**. Through such strategies the number of tRNAs needed to successfully read all possible codons can be reduced. From the genetic code, there are a total of 61 codons that encode for the common 20 amino acids. The other three codons are responsible for

stopping translation. If tRNAs only read through standard Watson-Crick base pairing then 61 tRNAs would be required for successful translation of every codon.

However, the ability of position 34 to wobble allows for far fewer than the 61 tRNAs necessary to read each codon. In fact, the minimum number of tRNAs with wobbling abilities required for sufficient translation and synthesis of all proteins needed to maintain life is 32. Yet, this minimal amount of tRNAs is observed in very few bacteria. Rather, there are more than the minimal 32 tRNAs, because some codons are being read by multiple tRNAs, by standard Watson-Crick base pairing interactions *and* by wobble base pairing. A codon ending in G could be read two ways: by standard Watson-Crick base pairing with a tRNA having a C in position 34 or by wobble base pairing with a tRNA having a U in position 34.

One specific example is within the threonine isoacceptor system. Based on chemistry of hydrogen bonding, the codon ACG can be read by both tRNA-CGU forming standard Watson-Crick base pairing and by tRNA-UGU forming wobble base pairing (Figure 1.7).

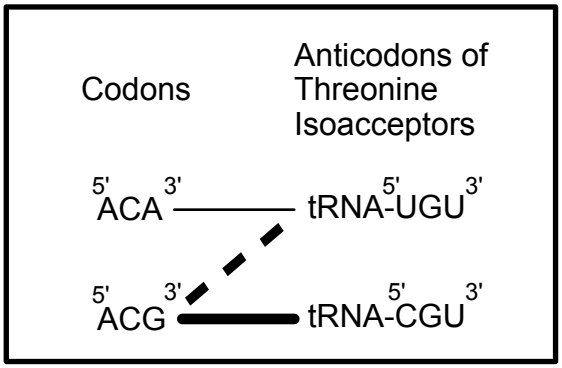

Figure 1.7: A proposed network for the translation of the codon ACG by the standard Watson-Crick base pairing with tRNA-CGU (solid line) or by wobble base pairing with tRNA-UGU (dashed line).

Interestingly, some bacteria have only the gene for the tRNA-UGU to translate the codon ACG, while others have genes for both the tRNA-UGU and the tRNA-CGU. Another way to look at this same situation is that some species have a tRNA-CGU while others do not. One could imagine that the related groups of bacteria over evolutionary time have either retained or lost the tRNA-CGU gene. This could have been due to necessity or non-necessity of the gene for more efficient translation, hence a higher fitness. However, looking at the distribution of the tRNA-CGU across related bacteria genomes, one sees heterogeneity (Figure 1.8). Within specific bacteria groups there are some species that have lost the tRNA-CGU gene while other closely related species have retained that gene. For example, the analysis revealed that the majority of bacteria in the Pasteurellales and Enterobacteriales have lost the tRNA-CGU gene, while *E. coli* and *Salmonella enterica* have retained the gene (Saks, personal communication). Overall, there is variation between and within the major bacterial lineages.

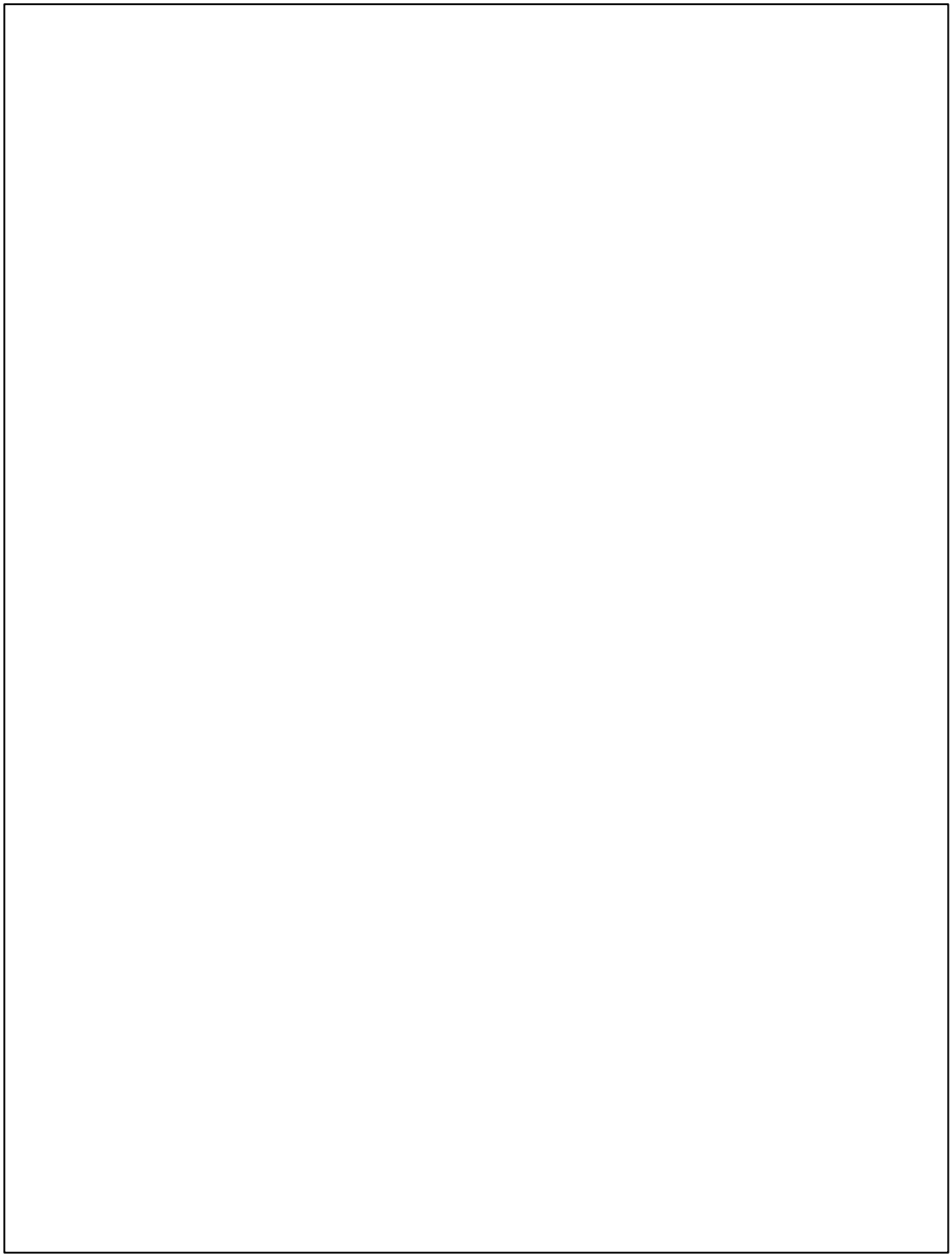

Figure 1.8: Phylogenetic analysis of the presence or absence of the tRNA-CGU.

This variation has led us to ask about the role of the tRNA-CGU. Such cases of heterogeneity are interesting, because they show that the presence or absence of the tRNA-CGU is much more complicated than just following a simple evolutionary track. Is this tRNA essential for translation in species where it is present? On the most basic level this question asks whether the organisms can survive without the tRNA-CGU, meaning that the tRNA-UGU has the ability to translate both the ACG codon as well as its own cognate codon, ACA. Perhaps, in the species that have retained the tRNA-CGU, the ability of the tRNA-UGU to wobble has ceased. Instead, due to evolutionary processes, only standard base pairing codon-anticodon interactions occur in the threonine isoacceptor system. The tRNA-UGU only reads its cognate codon, ACA, and the tRNA-CGU only reads the codon ACG (Figure 1.9). According to this idea, with the inactivation of the tRNA-CGU gene, no tRNA would read the ACG codon, and translation would cease, causing the organism to die. Hence, tRNA-CGU is essential.

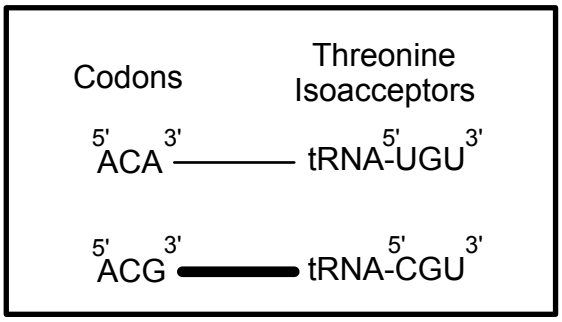

Figure 1.9: An alternative network for the translation of the codon ACG. It is translated only through standard Watson-Crick base pairing by tRNA-CGU.

However, there is a high probability that the tRNA-CGU is not essential. First, there is the ability of the tRNA-UGU to form a base pair with the codon ACG as proposed by Crick's wobble hypothesis, and upon doing so, a threonine is inserted into

the correct location of the forming protein. Secondly, there is the existence of all the bacterial species that have lost the tRNA-CGU. Of the 58 bacteria genomes analyzed for the phylogenetic tree 19 (33%) lack a tRNA-CGU gene (Saks, personal communication). The tRNA-UGU is the only possible tRNA that can read the ACG codon. If all of these species have been successful without the tRNA-CGU, the species that do have a tRNA-CGU should also be able to survive without it, which would mean that it is not essential. Though not all species are alike, in some bacteria the tRNA-CGU may be essential, while in others it may not be.

The likelihood of the tRNA-CGU not being an essential tRNA, once again leads to the question of the role and importance of the tRNA-CGU. What sets it apart from the tRNA-UGU that has allowed the tRNA-CGU to be retained in species that are closely related to other species where it has not been kept? One of the most obvious differences between the two tRNAs is the difference in the number of hydrogen bonds that comprise the base pairs between the first position of the anticodon and the third position of the codon. A difference in the number of hydrogen bonds affects the stability of the base pair. The stability of the standard base pairing, which has three hydrogen bonds between the nucleotides G and C, is greater than that of a wobble base pairing, which has only two hydrogen bonds between the nucleotides G and U. Therefore, the relative stability of the anticodon/codon interaction may have an effect when the tRNA-UGU compared to when the tRNA-CGU reads the ACG codon.

At increased temperatures, weaker, unstable hydrogen bonds have a difficult time occurring or can be broken; and therefore, stable bonds are more reliable. This may be the place at which the tRNA-CGU plays an important, functional role. Different bacteria

species can live in a variety of environments as well as ever-changing environments. One significant changing factor of different environments is temperature. When living at increased temperatures, cellular functions depending on chemical bond formations, like those between the anticodon and the codon, may differ slightly from those bacteria living at lower temperatures. The importance of stable bonds may be key for codon/anticodon base pairing within bacteria that live at higher temperatures in order to have efficient translation. Within the threonine isoacceptors and the reading of the ACG codon, this means that the tRNA-CGU, forming a standard Watson-Crick base pair, may be preferred over the tRNA-UGU, which forms a wobble base pair. The difference in the stability of chemical bonds allows us to hypothesize that the stability of the tRNA-CGU at high temperatures may be a reason that it has been retained in certain species, those that can live at high temperatures, and not in others. At higher temperatures, the tRNA-CGU is a means to efficient protein synthesis and thus increases fitness when a lone tRNA-UGU doesn't have the correct structure for efficient translation. This may be a way in which the tRNA-CGU is functionally different from the tRNA-UGU with respect to just the anticodon. Other differences may lie in the rest of the tRNA bodies.

1.3 Summary of Topic

Overall, the heterogeneity for the tRNA-CGU gene observed in related bacterial groups led us to ask what distinguishes the tRNA-CGU from the tRNA-UGU in translation. Has the tRNA-CGU evolved to be the only tRNA reading the ACG codon, making it an essential tRNA? Or does the tRNA-CGU have some functional properties that allow it to fulfill a different role than that of the tRNA-UGU during the translation of

the ACG codon? Below are hypotheses for the contributions to translation made by the tRNA-CGU.

H_{A1}: The tRNA-CGU is essential. Either, it is the only tRNA that has the ability to read the codon ACG, or it significantly contributes to the translation of the codon ACG such that it is required for survival.

H_{A2}: The tRNA-CGU has specific properties, such that in certain environmental conditions, it makes larger contributions to the translation of the ACG codon and thus fitness of the organism than does the tRNA-UGU.

H_0: There is no difference between the tRNA-CGU and the tRNA-UGU. Functionally they are the same in how effectively they translate the ACG codon. The tRNA-UGU can translate the ACG codon in every environmental condition equally well as the tRNA-CGU.

These three hypotheses are possible explanations for why some organisms have the tRNA-CGU and others do not. To investigate these topics, the tRNA-CGU gene will be inactivated in the chromosomal DNA of the model organism, *E. coli*. If *E. coli* can live without the tRNA-CGU gene, then it will be known that the tRNA-CGU is not essential for *E. coli*, that the tRNA-UGU can successfully form a wobble base pair with the ACG codon. If this is the case, then the question still remains: why would *E. coli* have a tRNA-CGU if it can get along just fine without it? This will lead to investigating if wether there are separate, functional roles between the tRNA-CGU and the tRNA-UGU.

2 Overview of methods

Here is an overview of the methods that were used to address the role of the tRNA-CGU in *E. coli* in relation to the tRNA-UGU in reading the codon ACG. *E. coli* was the chosen organism in which to test these relations, because primarily, as mentioned before, it is known to have both the tRNA-UGU and the tRNA-CGU. Its entire genome has been sequenced which facilitates manipulation of the genome. Secondarily, *E. coli* is an ideal organism with which to do research because of its extensive history within the scientific research world. Mutations are relatively easy to introduce, care for the organism is simple, and the time required to breed a new generation is less than an hour. Such a short generation time means that evolutionary effects that occur after many generations will be observed within several days. Also, the optimal temperature at which *E. coli* grows is 37˚C, but like many bacteria, it tolerates a range of temperatures (Ingraham 1987).

2.1 Inactivating the tRNA-CGU Gene

Before a gene can be inactivated, it must first be "copied." This consists of knowing the DNA sequence of the original gene, which can be found through publicly accessible databases. Then construction of an exact copy of the gene can be ordered from a company that specializes in linking nucleotides to give DNA sequences of scientific interest. Once one copy of the tRNA-CGU gene is constructed, it must be copied many times, by a process called polymerase chain reaction (PCR). PCR consists of multiple cycles of heating and cooling the specific region of DNA of the tRNA-CGU gene so that it is copied and recopied many times. Then the copies are incorporated into small circular pieces of DNA called plasmids, in this particular case, named pMAK705

(Hamilton *et al* 1989). Now, all the pMAK705s, each with the copy of the tRNA-CGU gene, are ready to go through mutagenesis. This process inserts an incorrect sequence that includes a marker into the anticodon loop of the tRNA, so that the tRNA can no longer recognize the correct codon—hence it is functionally inactive.

In the end, the entire process consists of exchanging an inactivated tRNA-CGU gene with the wildtype tRNA-CGU gene on the chromosome, the DNA naturally found within the cell. First, the plasmid carrying the inactive tRNA-CGU gene is put into, or transformed into *E. coli* cells, where it is included in normal cellular processes. Through a procedure called homologous recombination, the inactive tRNA-CGU gene is exchanged for the wildtype tRNA-CGU gene. This can occur because, despite the differences between the two genes caused by the mutagenesis, the flanking sequences are identical. During cellular replication these identical sequences of the plasmid and chromosome can bind together and exchange their non-identical DNA sequences (Figure 2.1). The inactivated gene in the chromosome is referred to as "knocked-out". After homologous recombination happens, the plasmid bears the wildtype tRNA-CGU gene, while the chromosome now has the inactive tRNA-CGU gene. The marker within the inactive tRNA-CGU gene allows the detection of the successful inactivation of the tRNA within the *E. coli*.

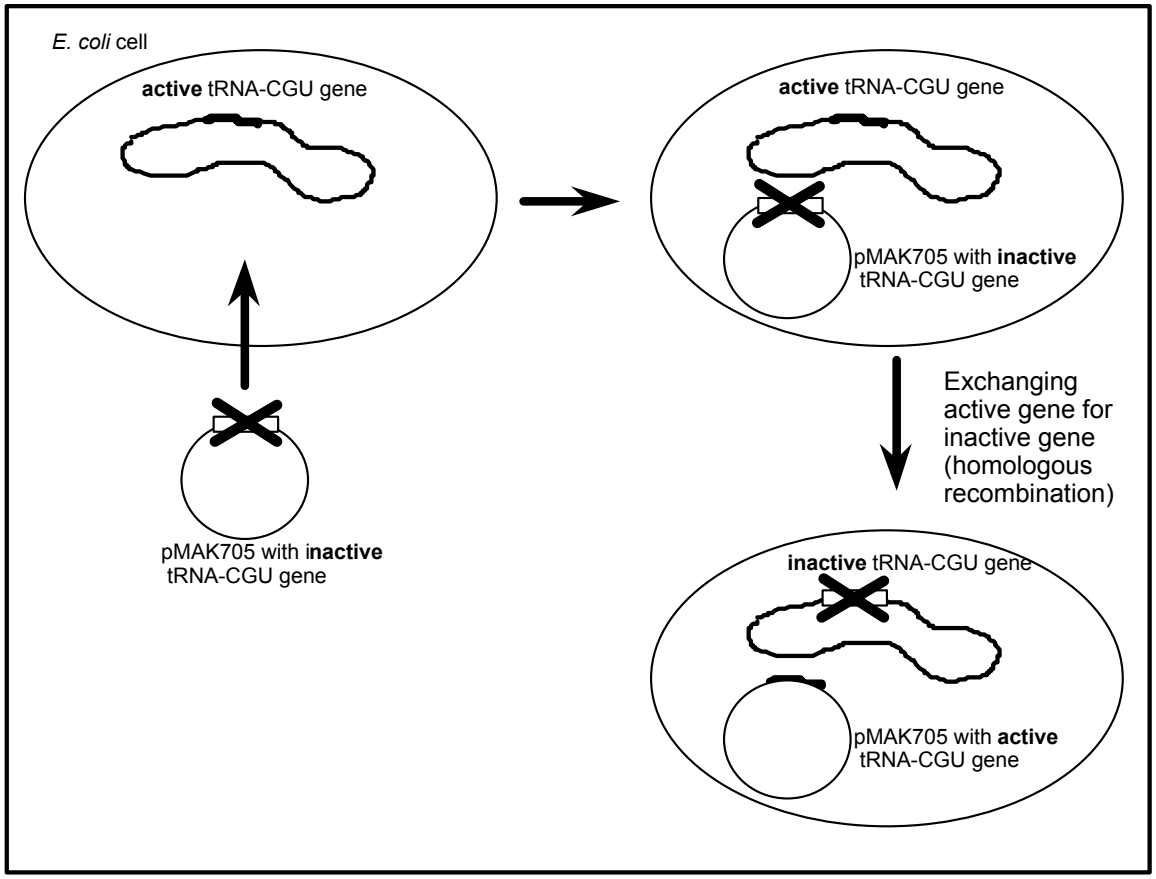

Figure 2.1: Exchanging an inactive tRNA-CGU gene for the active, wildtype tRNA-CGU gene in *E. coli*

The final step that must occur is to "chase-out" the plasmid with the wildtype tRNA-CGU gene, which can easily be done because pMAK705 has a special property of being heat sensitive during replication. It can only replicate at temperatures around 30˚C, so if the cells are grown at 43˚C, the pMAK705 cannot persist. The whole plasmid is "chased-out" of the cell; and in doing so, the wildtype tRNA-CGU gene is removed from the cell. Now, only the inactive tRNA-CGU gene is present in this particular strain of *E. coli*. This strain is named MW2. Once pMAK705 containing the wildtype tRNA-CGU is "chased-out", the cells can be checked for viability (Figure 2.2). This will determine if

E. coli can live without the tRNA-CGU and instead rely only on the tRNA-UGU to translate the codon ACG.

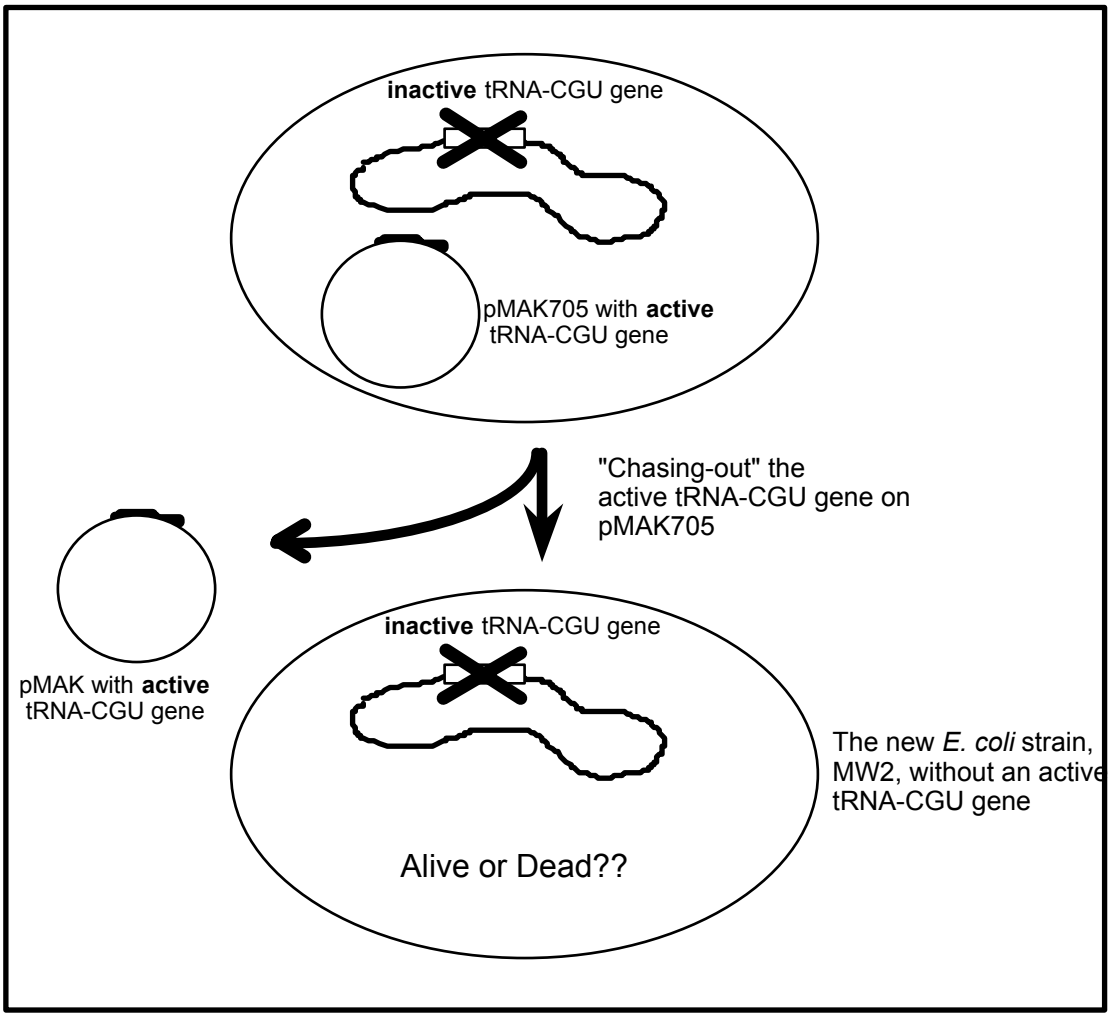

Figure 2.2: "Chasing-out" the active tRNA-CGU gene on the plasmid, pMAK705.

2.2 Measuring Translational Efficiency Under Experimental Conditions

To test the efficiency at which a certain codon is translated, depending on the tRNAs present that are thought to translate such codons, a system that reports the translational efficiency must be placed into the cells. Such a system consists of what is generally called a reporter gene; in these particular experiments, a *lacZ* reporter gene that

produces a protein that is an enzyme, ß-galactosidase. ß-galactosidase can break down complex sugar molecules into useable forms of nutrients for the cell. Also, it can break down other molecules that, instead of producing food, give a visible product, usually a color change. When the *lacZ* gene is present in a cell, on a plasmid, it is processed just like another gene. First, it is transcribed into mRNA. Then the codons within the gene are translated into the protein, ß-galactosidase. The amount of ß-galactosidase that is produced is the indicator of the translational efficiency of the cell. If the cell can translate the codons efficiently, then there will be lots of ß-galactosidase; but if the cell cannot translate them efficiently, then there will not be much ß-galactosidase.

To measure the translational efficiency in the *E. coli* cells of interest, the *lacZ* gene had to be introduced into them via a plasmid. Once in the cells, the amount of ß-galactosidase produced from the *lacZ* gene was determined by the addition of the molecule, o-nitrophenyl-ß-D-galactoside (ONPG). The ONPG is cleaved by ß-galactosidase and, upon the accumulation of cleaved ONPG, turns yellow. The intensity of the yellow can be measured spectrophotometrically. The intensity is directly proportional to the amount of ß-galactosidase present (Miller 1992). The amount of ß-galactosidase is indicative of the efficiency of the tRNAs present in the particular strain or, in other words, how well they translate the codons in the reporter gene.

To focus on the role of the tRNA-CGU and tRNA-UGU, the ACG codon must become part of the reporter system. Of course, ACG codons are present in the reporter gene and must be translated to produce a functioning ß-galactosidase. However, an amplifying effect is needed to insure a result that can be coupled with the translational efficiency of the codon ACG. For this amplifying effect, a codon cassette was created.

This segment of RNA, where the codon is repeated five times, is placed at the very beginning of the *lacZ* reporter gene. Such placement of the five codon ACG repeat means that all of them must be translated before the gene that makes ß-galactosidase can be successfully translated. Basically, the codon cassette is giving the ACG codon-reading tRNAs present in each particular strain five chances to fail at successfully creating the ß-galactosidase. If a type of tRNA is efficient, it can translate these five codons with ease and produce lots of ß-galactosidase. On the flip side, a type of tRNA may not be efficient, because perhaps there isn't enough of that type of tRNAs or they don't bind well with the codons. In such a case, the five-codon repeat will be translated with difficulty, and little ß-galactosidase will be produced.

Here it is worth noting that if a codon cannot be translated, the translational machinery cannot continue with protein synthesis. A tRNA whose anticodon can bind to the codon must finally adhere, and the encoded amino acid becomes attached to the growing protein. Only when this occurs can the translational machinery move to the next codon on the mRNA, and eventually to the *lacZ* gene. If the tRNAs present in the strain don't translate a codon well, then the translational machinery will move through the codon cassette at a slow pace; and hence, low levels of ß-galactosidase will be produced. Again, this efficiency is determined by the intensity of the solution's yellow color after the proper chemicals have been added. The amount of ß-galactosidase produced is reported in what are called ß-galactosidase units.

placZ/ACG can be used in any *E. coli* strain to test the translational efficiency of the codon ACG. If the translational efficiencies have any meaning at all about what actually occurs in a cell, then the same results should be reflected in an *in vivo*

experiment that tests the fitness of the *E. coli* strains. In other words, how well do two strains with different translational efficiencies compare when they are competing for the same nutrients.

2.3 Adding Copies of Threonine tRNAs

Either a copy of the wildtype tRNA-UGU or the wildtype tRNA-CGU is introduced into the MW2 strain cells via the plasmid *lacZ*/ACG (Figure 2.3). When the tRNA-CGU is added into MW2, it is essentially the EGM8recA$^-$ strain. Both have one copy of the tRNA-UGU gene, and both have active copies of the tRNA-CGU gene. When the tRNA-UGU is added into MW2, the created strain now has two copies of the tRNA-UGU gene and no tRNA-CGU genes. A measurement of the efficiency of ACG-reading tRNAs in this strain of *E. coli* now includes the copy of the engineered tRNA gene on p*lacZ*/ACG (either the tRNA-UGU or the tRNA-CGU) *and* the wildtype tRNA-UGU. This can be determined using the ß-galactosidase assay described above, also doing several replications and assays at various temperatures (30˚C, 37˚C and 43˚C). The results are compared to the MW2 and the wildtype strain, EMG8 recA-.

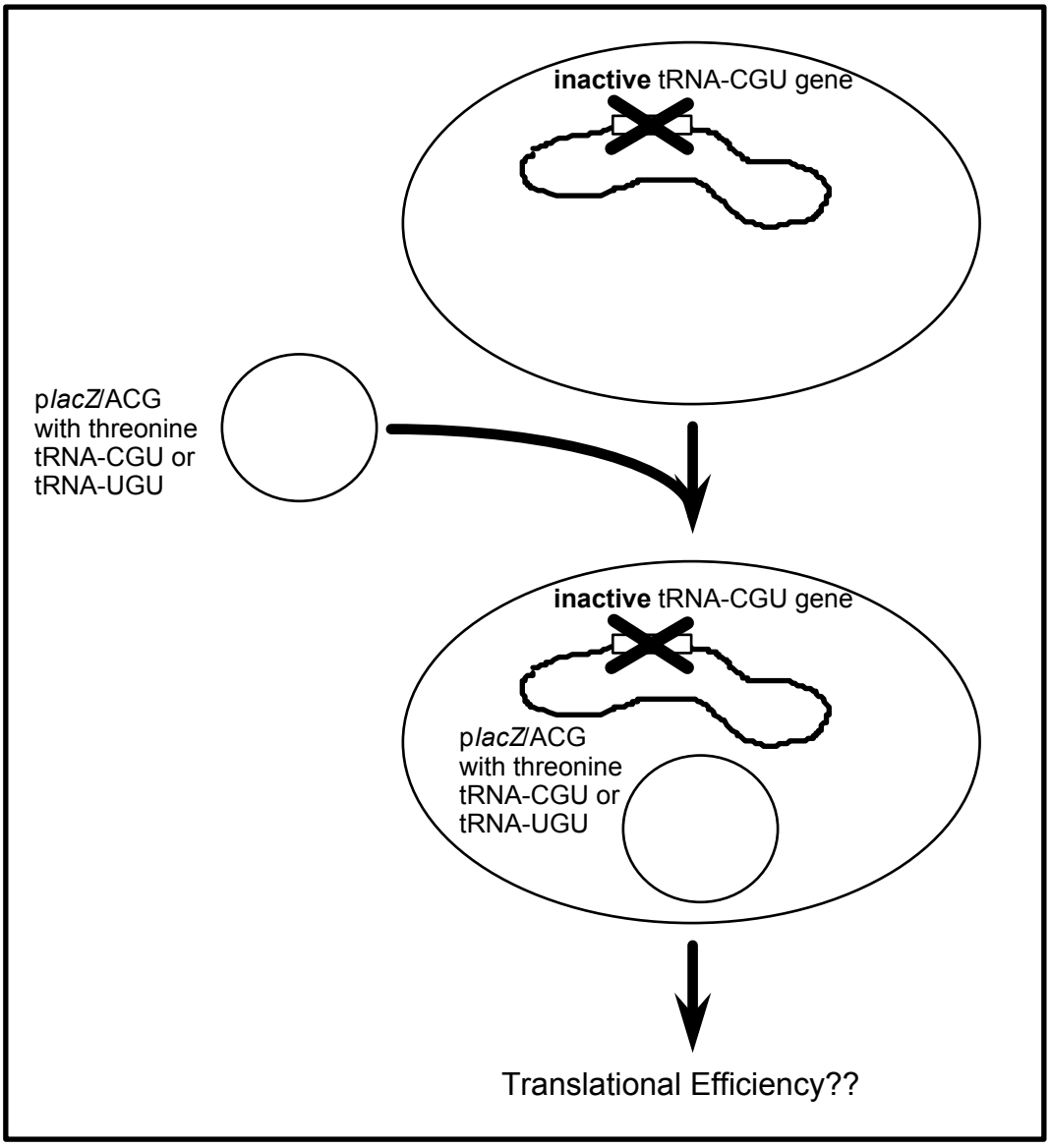

Figure 2.3: Adding either an engineered copy of the tRNA-CGU or a copy of the tRNA-UGU into MW2 via the plasmid p*lacZ*/ACG. The new strain can be tested for its efficiency at translating the codon ACG.

3 Materials and Methods

3.1 E. coli *Strains*

EMG8 was the wildtype *E. coli* strain used. MW2 was an engineered *E. coli* strain that had an inactivated tRNA-CGU gene created by the process of homologous recombination with the heat-sensitive pMAK705 that carried a mutagenesized tRNA-CGU gene. Later, with an increase in temperature to 43˚C, the pMAK705 was "chased out" carrying the endogenous tRNA-CGU gene. Both strains were made to be $recA^-$ to prevent further homologous recombinatory events. For clarification, the wildtype strain is referred to as EMG8 recA-.

3.2 Adding plac*Z/ACG and Threonine tRNAs*

Copies of the threonine tRNAs, tRNA-CGU and tRNA-UGU, were created using oligos annealed together. The annealed oligos, with a tufB promoter and a rrnc terminator, were ligated into pUC18 cut at R1 and PstII restriction sites and transformed into DH10B electro competent cells. After, successful transformations were detected through the ampecillin resistance of pUC18, PCR screening was used to identify colonies having the tRNA cloned into pUC18. Primers complementary with the flanking regions gave a fragment 327 base pairs in length when the tRNA was present. For further confirmation, the pUC18 DNA was isolated and sequenced. PCR was used to amplify the copies of each tRNA that became ligated into p*lacZ*/ACG. p*lacZ*/ACG is a derivative of pACYC179 with the *lacZ* reporter gene directly proceeded by five consecutive ACG codon repeats. The newly formed plasmids were then transformed into MW2 electro competent cells, and successful transformations were identified due to the kanamycin

resistance of p*lacZ*/ACG. Also, p*lacZ*/ACG without a threonine tRNA was transformed into both EMG8 recA- and MW2. Once again, the presence of the plasmid with the correct tRNA (or no tRNA) was confirmed by PCR, plasmid isolation, and sequencing. The successful transformation cultures were stored in glycerol at –80˚C.

3.3 ß-galactosidase Assay

The ß-galactosidase assay was the method used to determine the ability of the strains to translate the codon ACG at various temperature. For each strain and at each temperature, the assays were replicated many times. For each replicate, the same exact process, starting when the strains were removed from the –80˚C storage, was performed to insure consistent and comparable data.

The process was as follows: the desired strains to be assayed were grown from freezer stocks overnight for16-17 hours with aeration at 30˚C in 5 milliliters of 2xYT medium with kanamycin at 20µg/ml. The next day, 25 ml cultures of 2xYT medium with kanamycin at 20µg/ml and IPTG at 1mM were inoculated with an initial cellular concentration of 5.0 x 10^7 cells/ml (an optical density at 600 nm of 0.05). The cultures were grown with aeration at either 30˚C, 37˚C, or 43˚C, and cell samples were taken at several times during the log phase of cultures growth. Even when, the strains of interest were MW2+tRNA-UGU and MW2+tRNA-CGU, EMG8 recA- and MW2 were assayed as a means of control.

ß-galactosidase assays were performed according to Miller (1992). Samples of cells were taken, the optical density at 600nm was measured, and then the cells were placed on ice for 20 minutes. From each sample 0.1 ml aliquots were added to 3 replicates containing 0.9 ml of CHAPS permeabilization solution (40% of 5x Z-buffer

{0.6M of Na_2HPO_4, 0.04M of $NaH_2PO_4 \cdot H_2O$, 0.01M of KCl, and 0.001M of $MgSO_4 \cdot 7H_2O$}, 25% of 20% CHAPS in dH_2O, and 0.05 M of 14.3M ß-Mercaptoethanol). The sets of replicates were agitated at room temperature for 15 minutes in order to disrupt the membranes and then transferred to a 28˚C water bath for 5 minutes. The actual assay was initiated by adding 0.2 ml of 4mg/ml of ONPG in 1 part 5x Z-buffer and 4 parts dH_2O, at which the time was noted. Once a light yellow color appeared, 0.5 ml of 1M Na2CO3 was added to stop the reaction, and again, the time was noted. The optical density (OD) of each reaction was measured at 420nm and 550nm.

ß-galactosidase activity is calculated using the following formula:

$$\frac{1000 \times (OD_{420} - 1.75 \times OD_{550})}{t \times v \times OD_{600}} = \text{units of ß-galactosidase}$$

t is the time in minutes that the reaction takes. *v* is the volume in milliliters of the culture used in the reaction (Miller 1992). Cell density of each cell sample taken from the 25 ml culture is estimated by an absorbence of OD_{600} The calculated units of ß-galactosidase for each strain used were compared to that of other strains along the growth curve for a specific temperature of the day cultures. Statistical analyses of the data was preformed using the computer program, JMP.

3.4 Competition Experiment

The ß-galactosidase assays visualized the translational efficiency of the strains *in vitro*. The competition experiment was created as a means to see if the results about the translational efficiency of EMG8 recA- and MW2 actually reflected the fitness of the cells *in vivo*. For the competition experiment, EMG8 recA- and MW2 were grown for 16 to17

hours with aeration in 5 milliliters of 2xYT medium with 20mg/ml of kanamycin. Equal amounts (5 x 10^7 cells/ml) of each strain inoculated 25ml cultures of 2xYT medium with 20mg/ml of kanamycin (2 sets of 3 replicates). One set was incubated at 37˚C with aeration and the other set at 43˚C with aeration. Approximately 24 hours later, 0.5 ml of each culture was transferred to 25ml of fresh 2xYT medium with 20mg/ml of kanamycin, and each was returned to the appropriate temperature. Samples of cells from each culture were saved and stored in glycerol at –80˚. This procedure continued for 11 days. In order to determine the length of time that MW2 was able to persist in the culture, the Drd1 site within the inactivated tRNA-CGU gene in MW2 was amplified by a PCR reaction from each daily sample and visualized on a gel.

4 Results

4.1 Viable Strain with Inactivate tRNA-CGU

To determine whether the tRNA-CGU is essential in *E. coli*, the active, wildtype tRNA-CGU gene on the chromosome was exchanged for an inactive copy of the gene brought into the cell on a plasmid. After the exchange of genes, the plasmid with the wildtype, active tRNA-CGU gene was "chased-out" of the cell. After this occurred, the newly created strain called MW2, without an active tRNA-CGU, was shown to be viable. This means that the tRNA-CGU is not an essential tRNA for *E. coli*.

4.2 Detrimental Effects of the CGU Knockout

Knowing that *E. coli* can live without the tRNA-CGU immediately lead to the question of how detrimental is the inactivation of the tRNA-CGU gene. Perhaps without the tRNA-CGU assisting in the translation of the ACG codon, translation will slow down, because the role that the tRNA-CGU played in translation is absent. To test this idea, the ability to translate the ACG codon was determined for MW2 compared to that of the wildtype strain, EMG8 recA-. The *lacZ* reporter gene system preceded by the ACG codon cassette was used, and the amount of ß-galactosidase synthesized indicated the translational efficiency of the codon ACG for each strain. Figure 4.1 shows the results of the ß-galactosidase assays, reported in ß-galactosidase units, for EMG8 recA- and MW2. The cell cultures were grown at 37˚C. Cells were sampled during the early log growth phase (optical density of 0.25 to 0.55), log growth phase (OD of 0.56 to 0.99), and late log growth phase (OD of 1.00 to 1.75) for each strain. Comparisons of translation

efficiency through the ACG codon were made at each of these time points during *E. coli's* growth phase. From these results, we can conclude that in the strain without the tRNA-CGU, there is a consistent detrimental effect in the translation of the ACG codon, but that it is small. Therefore, in the absence of the tRNA-CGU at 37˚C, *E. coli* can translate the ACG codon, but not quite as efficiently as when there is both the tRNA-CGU and the tRNA-UGU.

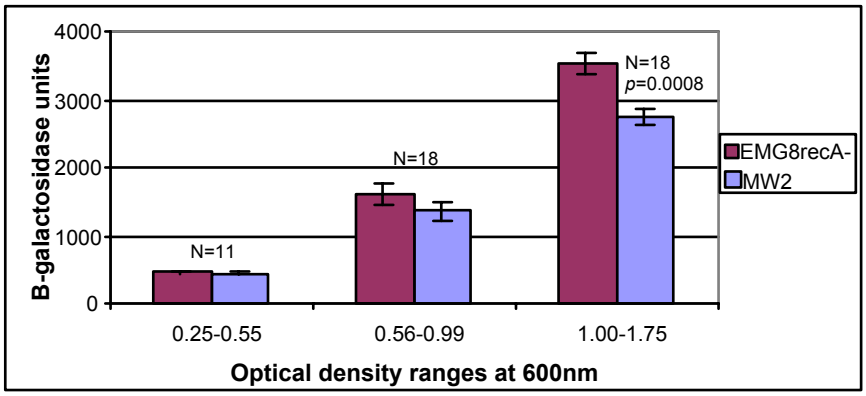

Figure 4.1: The translational efficiency, measured in ß-galactosidase units, of MW2 compared to EMG8 recA- at 37˚C for three ranges during the growth phase. Each data set is reported with plus or minus one standard error. "*N*" equals the number of days each comparison was assayed. "*p*" is a statistical probability value; $p<0.05$ means that the results are significant.

4.3 The Effect of Increased Temperature

The previous experiments was preformed at 37˚C, which is the optimal growth temperature for *E. coli*. However, outside of the laboratory, *E. coli*, like many organisms, lives in a changing environment, and one of the major changing factors of this environment is temperature. As stated earlier, more stable anticodon-codon interactions between the first position in the anticodon and the third position in the codon, those with Watson-Crick base pairing, may be required of species that live at high temperatures. *E.*

coli, being a gut bacteria, must sometimes live at temperatures higher than its optimal growth temperature, such as when the host's body temperature increases. How detrimental is the tRNA-CGU inactivation at a higher temperature of 43˚C? Looking at the other side of *E. coli's* optimal growth temperature, how detrimental is the tRNA-CGU inactivation at a lower temperature, such as 30˚C? ß-galactosidase assays were preformed on cells taken from cultures grown at 43˚C and cultures grown at 30˚C during the early log phase, mid-log phase, and late log phase of the growth cycle. Figure 4.2 shows that the detrimental effect of the tRNA-CGU inactivation on the translational efficiency of MW2 becomes even more noticeable as temperature increases. At 30˚C, the translational efficiency of the ACG codon by MW2 is not significantly different from that of EMG8 recA-. At 43˚C, MW2 has a significantly lower translational efficiency than the wildtype.

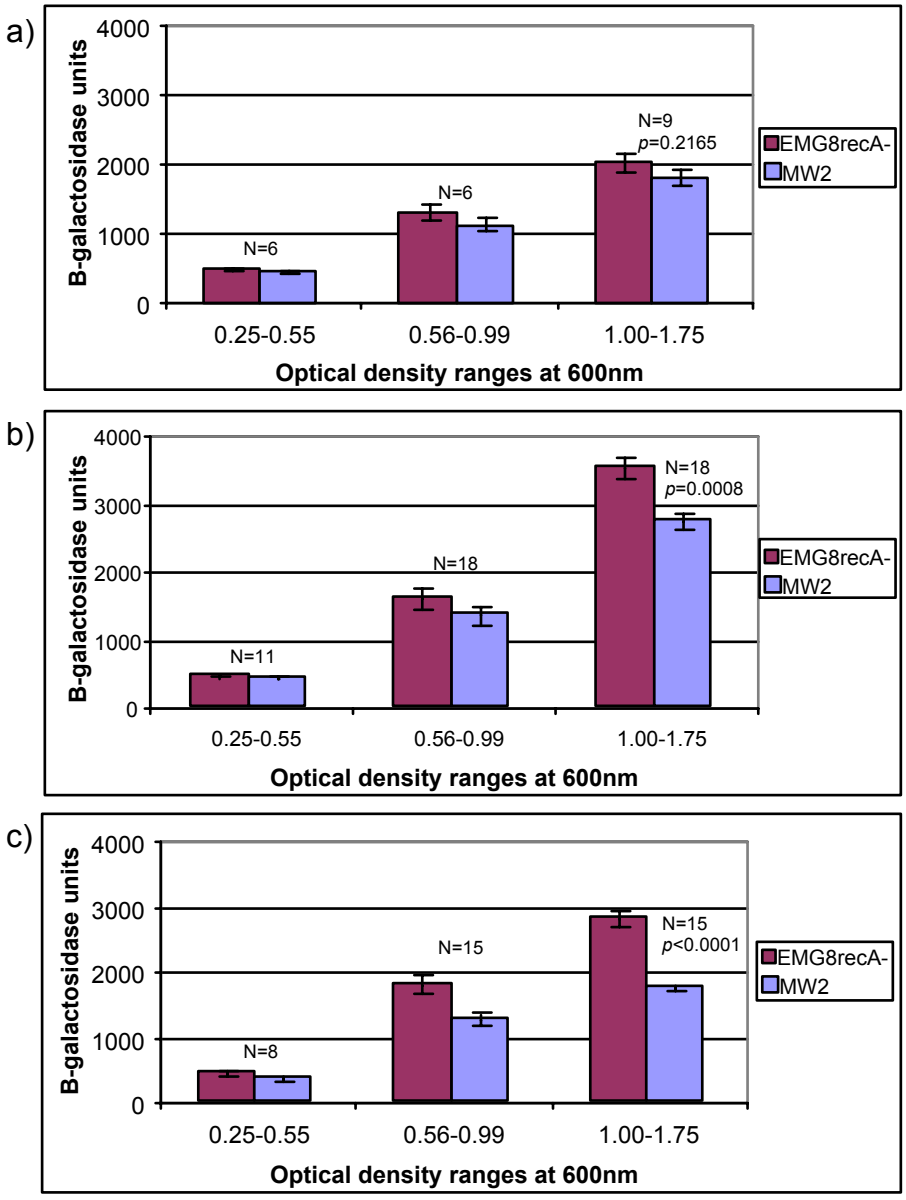

Figure 4.2: Comparing the translational efficiencies of MW2 and EMG8 recA- at different temperatures: 30˚C (a), 37˚C (b), and 43˚C (c)

The ß-galactosidase assays revealed a lower translational efficiency of the ACG codon in the absence of the tRNA-CGU, but do the results of the assay reflect what occurs *in vivo*? Is the wildtype strain of *E. coli* more fit for survival at higher temperatures, such as 43˚C, because it has a tRNA-CGU to read the ACG codon? Two *in vivo* competition experiments were performed between the wildtype strain of *E. coli* and

MW2 at 37˚C and at 43˚C. Equal amounts of each strain were used to inoculate some media and each successive day a fixed amount of cells was transferred to new medium. The daily progress of the cultures was monitored by PCR reactions that showed which strains were present in the culture. Overall, at 43˚C, but not 37˚C, the wildtype strain out-competed MW2 within three to four days (Figure 4.3). At 37˚C, the wildtype strain out-competed MW2 in ten days (data not shown). Therefore, one can conclude that at increased temperatures, it is more advantageous for *E. coli* to have a tRNA-CGU rather than only a tRNA-UGU to read the ACG codon. The results from the competition experiment about the fitness of MW2 to the wildtype strain are consistent with the ß-galactosidase assay results that the absence of the tRNA-CGU was more detrimental at increased temperatures. This also means that the translational efficiency does reflect the fitness of the organism. If translation is more efficient in the presence of the tRNA-CGU at 43˚C, then proteins can be made at optimal rates, and thus the overall fitness of the cell is better.

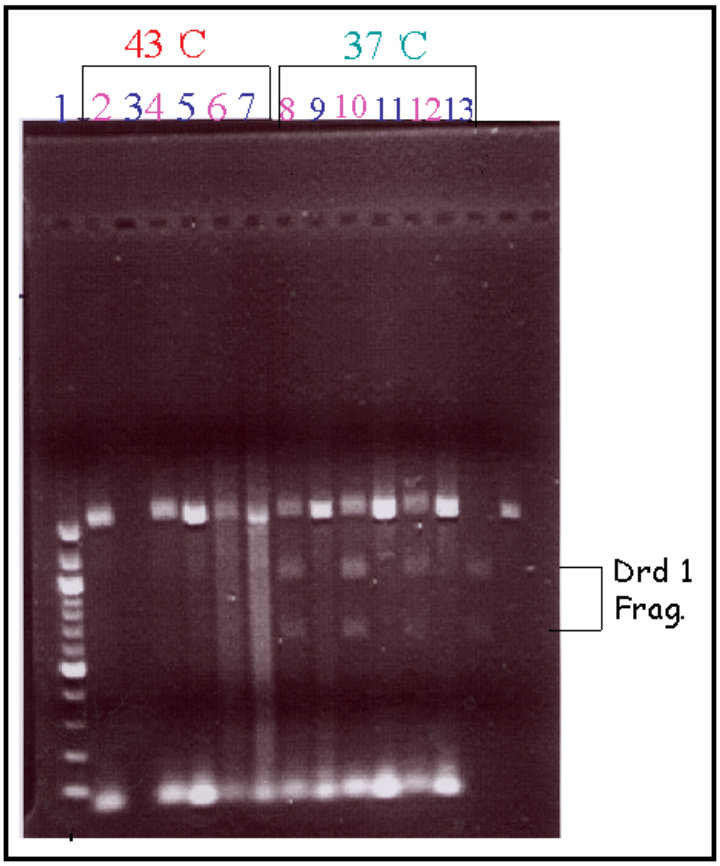

Figure 4.3: Results from the Competition Experiment at day four. Daily samples taken during the competition experiment at both 43°C and 37°C were digested with the enzyme Drd I, which cuts only the inactivated tRNA-CGU gene into two fragments. The Drd I recognizes the altered anticodon loop of the inactive tRNA-CGU gene. The normal, activated tRNA-CGU gene present in EMG8 recA- is not cut into any fragments by the enzyme Drd I. On the gel above, the lane farthest to the right shows what EMG8 recA- looks like after being digested with (there are no Drd1 fragments, just all the DNA—shown as one band). The lane second farthest to the right shows what MW2, with the inactivated tRNA-CGU, looks like after being digested (there are two Drd I fragments plus the rest of the DNA—in all three bands). For the rest of the lanes (except for lane 1 which is only a marker), all the odd numbered lanes were not digested and are shown as a control to indicate the presence of DNA. All the even numbered lanes were digested with the enzyme Drd I. For the strains grown at 37°C in competition, the inactivated tRNA-CGU gene is still present at day four, which means that MW2 has yet to be out-competed. At 43°C, no Drd I fragments are present on day four, which means that no inactivated tRNA-CGU genes are present. However, early on day three, Drd I fragments from a PCR reaction had been present on a gel (data not shown). Therefore, at 43˚C, EMG8 recA- out-competed MW2 between day three and day four.

4.4 tRNA-UGU Cannot Replace tRNA-CGU

These temperature effects suggest that the tRNA-CGU is a better translator of the ACG codon than the tRNA-UGU at 43˚C. However, another possible reason for MW2's observed decreased ability to translate the ACG codon is that there are simply fewer ACG-reading tRNAs. Perhaps, the different ways in which the two tRNAs translate the ACG codon do not have a detectable effect on the translational efficiency of each strain. If this were the case, then adding a copy of the tRNA-UGU gene to MW2 would allow its translational efficiency of the ACG codon to equal that of the wildtype. To test this, a copy of the tRNA-UGU gene, on the same plasmid as the *lacZ* gene reporter system, was added to MW2. This strain (MW2+tRNA-UGU) now has double the amount of the tRNA-UGU and no tRNA-CGU. As a control, a copy of the tRNA-CGU gene was added to MW2. This strain (MW2+tRNA-CGU) has both the tRNA-UGU and the tRNA-CGU, which resembles the wildtype strain. However, MW2+tRNA-CGU is a much better comparison to MW2+tRNA-UGU, because both have the same exact system, one gene on the chromosome and the other on the plasmid. This will produce exactly equal the number of ACG codon-reading tRNAs. The wildtype strain produces a different amount of ACG codon-reading tRNAs than the engineered strains, due to both genes being present on the chromosome.

If MW2+tRNA-UGU has the same translational ability as MW2+tRNA-CGU, then this is a strong indicator that the presence of the tRNA-CGU is just to increase the amount of ACG codon-reading tRNAs. Whether they have the CGU anticodon or the UGU anticodon does not matter. However, if MW2+tRNA-UGU's translational ability

does not equal that of MW2+tRNA-CGU, then the tRNA-CGU must have a distinct property that is necessary for the maximum translation of the ACG codon. In such a case, the tRNA-UGU is not able to compensate for the loss of the tRNA-CGU.

Again, ß-galactosidase assays were done on MW2+tRNA-UGU and MW2+tRNA-CGU, including the controls: EMG8 recA- and MW2. Cells were taken during the early log phase, the mid-log phase, and the late log phase of the growth phase of the cultures. Assays were done at 30˚C, 37˚C, and 43˚C. The results in Figure 4.4 show that at 43˚C, the strain, MW2+tRNA-UGU does not translate the ACG codon at the same efficiency as MW2+tRNA-CGU. In fact, adding the tRNA-UGU to MW2 only increased the translation of the ACG codon slightly from that of MW2. The lack of the ability of the tRNA-UGU to replace the tRNA-CGU in *E. coli* at increased temperatures is consistent with the idea that the two tRNAs have distinct roles in the translation of the codon ACG that make them functionally different in the cell.

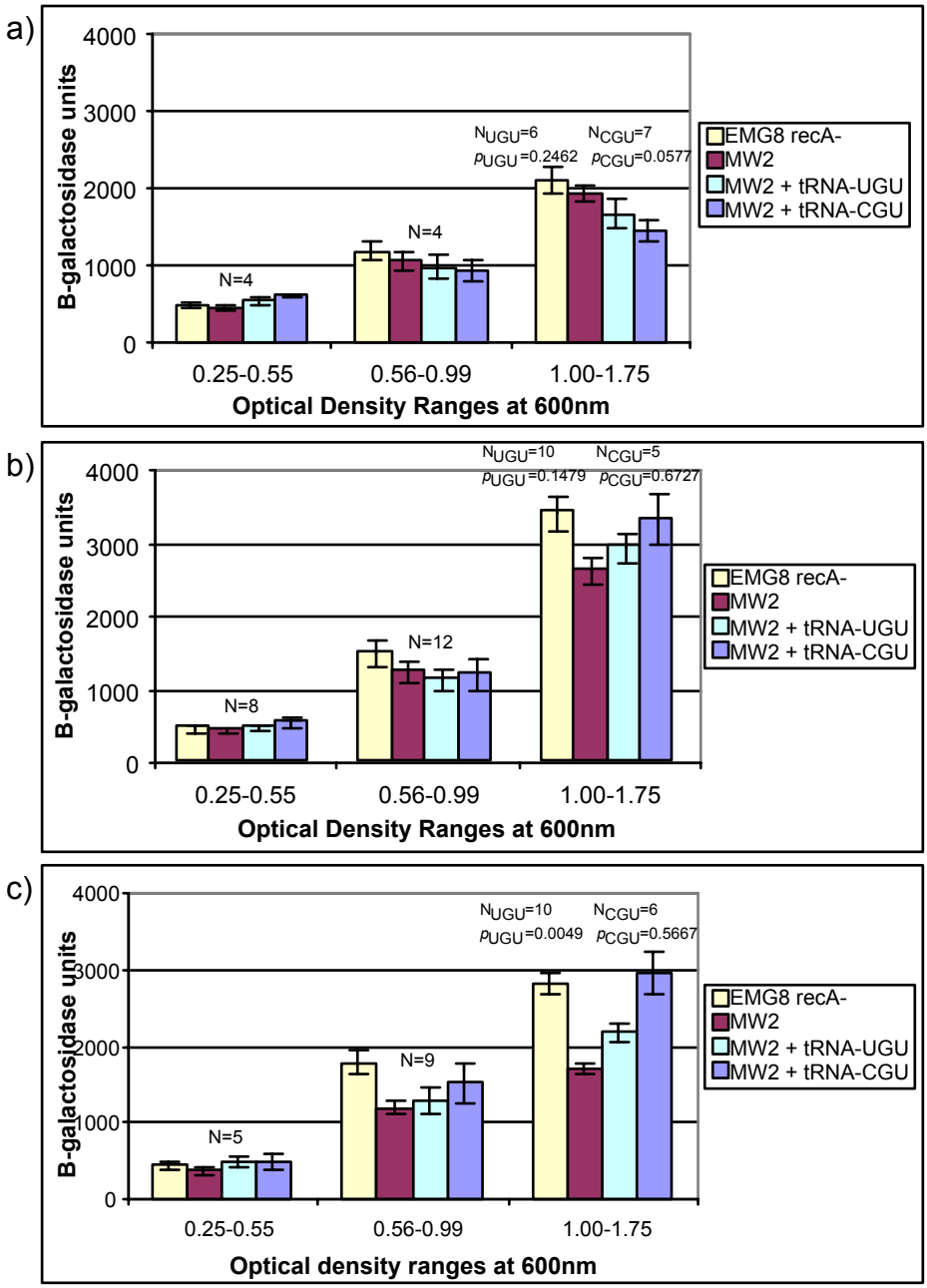

Figure 4.4: Comparing the translational efficiency of MW2+tRNA-UGU to the wildtype at various temperature: 30˚C (a), 37˚C (b), and 43˚C (c). There are two reported *p* values: p_{UGU} is for whether there is a difference between MW2+tRNA-UGU and EMG8 recA-, and p_{CGU} is for whether there is a difference between MW2+tRNA-CGU and EMG8 recA-.

5 Discussion

The heterogeneity of the presence or absence of the tRNA-CGU gene observed within closely related groups of bacteria, as seen in the phylogenetic analysis (Figure 1.9), led to the purpose of the tRNA-CGU in a bacterium. Why do some bacteria, such as *E. coli*, have the tRNA-CGU while others do not? One way to address the purpose of the tRNA-CGU is to look at whether it is essential to the bacteria species. If the tRNA-CGU were essential, then when the gene encoding the tRNA was inactivated, the cells resulting from this strain would not be viable. In such a case, the tRNA-UGU would not have the ability to form a wobble base pair with the ACG codon. Hence, there would not be any tRNA to translate the codon ACG. If the tRNA-CGU is not essential, then when the tRNA-CGU gene is made inactive, the cells are still viable. The tRNA-UGU would have the ability to form a wobble base pair with the ACG codon.

In *E.* coli, the later scenario was shown to be true. The tRNA-CGU is not essential in *E. coli*. When the tRNA-CGU gene was inactivated, the viability of the strain showed that the tRNA-UGU could translate the codon ACG through wobble base pairing, as well as translate its cognate codon, ACA, through standard Watson-Crick base pairing. Thus, one can propose that in *E. coli* Crick's wobble hypothesis is correct for the threonine system. The codon ACG can be translated through standard Watson-Crick base pairing by the tRNA-CGU and through wobble base pairing between the third position of the codon (G) and the first position of the tRNA-UGU's anticodon (U). In Figure 5.1, is an accepted figure of such a network.

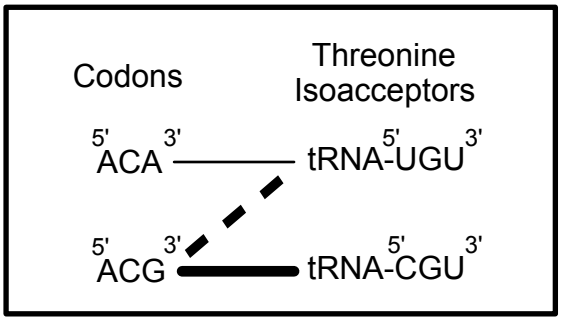

Figure 5.1: The accepted network for translating the codon ACG through both standard Watson-Crick base pairing and wobble base pairing in *E. coli*.

The accepted network above is especially interesting when considering that the tRNA-CGU is not found in all bacteria. In the bacteria lacking a tRNA-CGU, the tRNA-UGU must form a wobble base pair with the ACG codon.

Also, it is worth noting that the above network is only shown for the bacteria *E. coli*. The ability of threonine isoacceptor tRNAs to translate the codon ACG could be different in other species. The tRNA-UGU may not have the ability to translate the ACG codon, either because the ability to form wobble base pairs was never obtained or was lost. An example of when a tRNA that was thought to form wobble base pairs, but in fact could not was shown in yeast (*Saccharomyces cerevisiae*). In *S. cerevisiae* another tRNA with a C in the first position of the anticodon, the tRNA-CUG, was inactivated. Following the inactivation, the yeast was not viable. Hence, this tRNA with a C in the first position of the anticodon was essential (Weiss 1986).

However, in *E. coli*, the tRNA-CGU, also a tRNA with a C34, is present and is shown to be non-essential. This leads to the question: why would *E. coli* produce a molecule that is seemingly redundant? Does the ability of the tRNA-CGU to translate the codon ACG equal that of the tRNA-UGU? To answer these questions, ß-galactosidase

assays were used to compare the translational efficiency of the ACG codon in MW2 and EMG8 recA-. The results at *E. coli*'s optimum growth temperature, 37˚C, showed that not having an active tRNA-CGU is detrimental to the translational efficiency of the codon ACG. Hence, these results show that in the wildtype strain, the tRNA-CGU is contributing to the translation of the codon ACG. Yet, the tRNA-UGU is able to translate the codon ACG sufficiently to keep the cells alive. Once again, this brings back the question of why *E. coli* has a tRNA-CGU.

The clue to this lies within the decrease of the translational efficiency of the tRNA-UGU in MW2 as the temperature increased from 30˚C to 43˚C. To observe this, it is best to look at the optical density range of 1.00 to 1.75 for all three temperatures in Figure 4.2. At this range in the growth phase, the presence of a difference between the strains and the extent of the difference is more pronounced. At 30˚C, the translational efficiency of MW2 is not significantly different from that of wildtype, while at 43˚C the translational efficiency of MW2 is significantly different from that of wildtype. At 37˚C, the translational efficiency between the strains is also significantly different, but one can see that the difference is less pronounced than at 43˚C.

Such a decrease in the translational ability of the tRNA-UGU at increased temperatures is counterbalanced by the contribution that the tRNA-CGU makes to translation of the ACG codon as the temperature increases. At higher temperatures, the lone tRNA-UGU is unable to carry out the functional role that the tRNA-CGU fills. Such an idea is backed by the results from the *in vivo* competition experiment between the strains MW2 and EMG8 recA-, which differ by the absence or presence of the tRNA-CGU. At the higher temperature, the strain with the tRNA-CGU was able to out-compete

the strain without the tRNA-CGU in only three days, while at the lower temperature the same result took ten days. From such a difference in time, one can conclude that at higher temperatures, the presence of the tRNA-CGU is especially advantageous to the fitness of the cell. With the special functional abilities of the tRNA-CGU, the codon ACG could be translated more efficiently; and therefore, proteins could be created at an increased rate, which in the end is advantageous for cellular growth and fitness.

Though, rather than attributing the efficient translational ability of the ACG codon of the wildtype strain to the specific properties of the tRNA-CGU, perhaps the translational ability can be attributed to the simple fact that in the wildtype strain there are two ACG-codon-reading tRNAs rather than the one, as in MW2. However, consider the ß-galactosidase assays of two strains of MW2, each containing a plasmid, one with a copy of the tRNA-UGU gene and the other a copy of the tRNA-CGU gene. The results at the higher temperature showed that the particular presence of the tRNA-CGU was essential for translational efficiencies of the wildtype strain. At higher temperatures, simply having more tRNA-UGUs present in the cell did not compensate for the lack of the tRNA-CGU. However, the increase in quantity of tRNA-UGUs did improve the translational efficiency at 43˚C above that of MW2, but not within the range of the translational efficiency of the wildtype. Although at both 30˚C and 37˚C, the addition of the tRNA-UGU gene to MW2 had no significant beneficial effect.

Also, it is interesting that at the low temperature the addition of the tRNA-CGU gene does not completely compensate for the translational activity that was lost by the inactivation of the endogenous tRNA-CGU, especially since there the expression level of the tRNAs on the plasmid is greater than from the chromosome. One would expect the

translational efficiency to be higher when the tRNAs are expressed from the plasmid, simply because there would be more tRNAs in the engineered strains. Though another possible answer could be that at lower temperatures, the tRNA-CGU is actually detrimental to *E. coli*. By adding more than normal, the effect was visualized by the ß-galactosidase assays.

At this point, it has been shown that the tRNA-CGU contributes more than the tRNA-UGU to the translation of the ACG codon at increased temperatures when the tRNA-UGU cannot contribute as much. The difference between the two tRNAs means that each has distinct properties that cause such a change in their translational abilities as the temperature increases.

The decreased ability of the tRNA-UGU to translate the ACG codon as the temperature increases must lie within the specific interaction between the anticodon UGU and the codon ACG. The ability of the tRNA-UGU to translate its cognate codon, ACA, remains constant as the temperature increases (Saks, personal communication). This shows that the tRNA-UGU is not a weaker molecule at increased temperatures, but rather it has a decreased ability to interact with the codon ACG at increased temperatures.

One of the main differences between the two tRNAs is the anticodon. As mentioned several times, each forms a chemically different base pair between the first position of the anticodon and the third position of the codon. The wobble base pair, between the tRNA-UGU and the ACG codon, is a less stable bond–formed by two hydrogen bonds that cause a shift in the location of the U nucleotide that is different than what occurs in a standard Watson-Crick base pair (Figure 5.2). The standard Watson-Crick base pair, between the tRNA-CGU and the ACG codon, consists of three hydrogen

bonds; and hence, is more stable. Numbers quantifying the two different codon-anticodon interactions are unknown, but as any chemist knows, three hydrogen bonds are stronger than two.

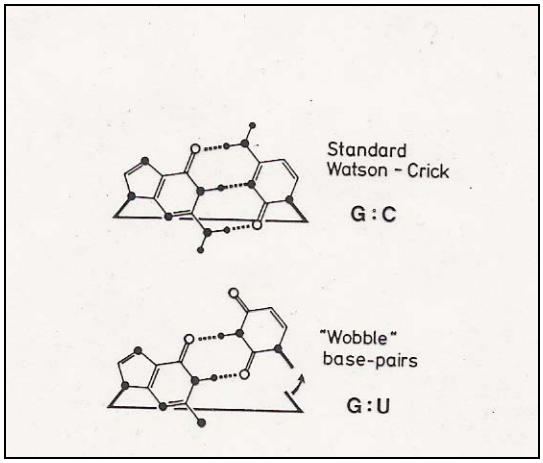

Figure 5.2: An illustration of the hydrogen bonds (dotted lines) between the nucleotides of the codon and anticodon (Saenger, 1984). The standard Watson-Crick base pair between G and C has three hydrogen bonds. The "wobble" base pair has only two hydrogen bonds. One potential hydrogen bond site on the G is left free. Also, the location of the U within a "wobble" base pair is different than the nucleotide within a standard Watson-Crick base pair (shift shown by the arrow). Such a change in location adds to instability of the wobble base pair.

As temperature increases, less stable bonds may have less of a chance of forming than stronger, more stable bonds. When referring to codon-anticodon interactions, each hydrogen bond can be assigned a certain amount of strength to hold the bases of the codon and anticodon together. At increased temperatures, an interaction with three hydrogen bonds will be stronger and more stable than an interaction of only two hydrogen bonds. This was reflected in that as the temperature increased, the ability of the tRNA-UGU to form the weaker, less stable wobble base pair at the third position of the codon was seen to decrease. When this occurs, the ability of the tRNA-CGU to form a standard Watson-Crick base pair between the first position of the anticodon and the third position of the codon becomes more necessary.

As seen at lower temperatures, the differences in the chemical stability of the codon-anticodon interaction between the two tRNAs has no effect on the ability to translate the ACG codon, when just comparing EMG8 recA- and MW2. However, as already discussed from the experiments where the tRNA-UGU and the tRNA-CGU were added to MW2, the greater amount of tRNA-CGU is detrimental to the translation of the ACG codon at 30˚C. This effect may also be related to the stability of the codon-anticodon interactions. The strong, standard Watson-Crick base pair between the anticodon CGU and the codon ACG may be so stable that the base pair does not disengage easily at a lower temperature. Translation cannot continue until the codon and anticodon disengage. Hence, when the bond is so stable, translation may be slowed down when excessive tRNA-CGU is present. In the future, it would be interesting to look at this possible phenomenon at temperatures lower than 30˚C.

Future investigations should include looking at the importance of the anticodon *and* the importance of the tRNA body for maximum translational efficiency at increased temperatures. Is maximum translational efficiency achieved by solely the correct and stable codon-anticodon interactions? Or are there other locations within the tRNA body that are important for translation and codon recognition. One way to test would be to engineer a tRNA to have a CGU anticodon but the body of the tRNA-UGU and a tRNA to have the CGU body with the UGU anticodon. Also, such body swapping experiments could be tested on the other threonine isoacceptors.

In conclusion, the tRNA-CGU was shown to have specific properties that in certain environmental conditions allow it to contribute more to the translation of the ACG codon, while the properties of the tRNA-UGU cause it to contribute less. One property

of the tRNA-CGU is the ability to form a stable Watson-Crick base pair between the first position of the anticodon and the third position of codon ACG. Such a stable bond is necessary at increased temperatures, where a wobble base pairing is shown to be less successful. Overall, the tRNA-CGU may appear to be redundant in its translating of the ACG codon, but in fact at increased temperatures the tRNA-CGU is necessary for maximum translational efficiency.

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7 Glossary

anticodon The trinucleotide sequence in each tRNA that is capable of forming base pairs with complementary codons in mRNA. When such a codon-anticodon interaction is created, the corresponding amino acid, carried by the tRNA, is linked to the growing protein.

ß-galactosidase The enzyme produced from the *lacZ* reporter gene that serves as an indicator of the rate of translation through a codon.

ß-galactosidase units A measurement of the rate of translation.

base The building blocks of DNA and RNA with distinct chemical structures: adenine (A), cytosine (C), guanine (G), thymine (T) (only in DNA), and uracil (U) (only in RNA).

base pair When two complementary bases form hydrogen bonds between them. Adenine base pairs with uracil, and guanine base pairs with cytosine.

chromosomal DNA All of an organism's information that encodes all activities and proteins.

codon A trinucleotide sequence within mRNA that encodes for a particular amino acid.

codon cassette A set of codons inserted into a reporter gene for the purpose of determining the translational efficiency of that particular codon(s).

Drd I An enzyme that cuts the inactivated tRNA-CGU at the Drd 1 site that had been inserted in place of the anticodon.

early log phase A growth stage of an *E. coli* culture before growth is limited.

EMG8 recA- The wildtype *E. coli* strain having recA- with both the active tRNA-CGU and the active tRNA-UGU present in the chromosome. It is used as a comparison for any mutated strains.

enzyme A type of protein that can cut nucleotide strands or can act in other chemical reactions.

hydrogen bond A special type of chemical bond that can occur between groups of bases (Figure 5.2).

in vitro Occurring in a cell-free environment.

in vivo Occurring in a living cell or organism.

isoacceptors A group of tRNAs that correspond to the same amino acid yet read different codons that encode the same amino acid.

late log phase A growth stage of an *E. coli* colony when growth is slightly slower than log phase. The population is reaching its maximum. At this point in culture growth, the greatest differences in translational efficiency are observed.

mid-log phase A growth stage of an *E. coli* colony when growth is rapid. The entire population is replicating approximately every 20 minutes.

MW2 The engineered *E. coli* strain having an inactive tRNA-CGU gene

mutagenesis A process of introducing a mutation into a gene; and in these experiments, inactivating the anticodon of the tRNA-CGU gene.

nucleotide The building blocks of DNA and RNA, comprised of distinct bases (adenine (A), cytosine (C), guanine (G), thymine (T) (only in DNA), and uracil (U) (only in RNA)) incorporated into a greater chemical structure that allows each component to be incorporated into a strand of DNA or RNA.

optical density (OD) A measurement of bacteria culture density which relates to the amount of cellular growth.

p*lacZ*/ACG The plasmid that carries the *lacZ* reporter gene preceded by the ACG codon cassette. It was placed in each strain to measure their translational efficiency.

plasmid A small, circular piece of DNA that can be engineered to introduce genes of interest into *E. coli* cells and is self-replicating

pMAK705 The heat sensitive plasmid used to introduce the inactivated tRNA-CGU gene into the *E. coli*. It was "chase-out" carrying the active tRNA-CGU gene (Hamilton 1989).

protein Comprised of linked amino acids, the order of which dictate the identity and function of the protein

reporter gene A gene that when translated gives an amount of a protein that upon being quantified, reports an aspect of cellular production. One example is the *lacZ* reporter gene and the production of ß-galactosidase to report the translational efficiency of the organism.

sequence A specific order of linked nucleotides.

transfer RNA (tRNA) A main component of the translational machinery. Each has a trinucleotide anticodon that forms base pairs with the nucleotide of its complementary codons within mRNA, and when doing so, donates the corresponding amino acid to the growing protein

Watson-Crick base pairing Standard hydrogen bonding between two nucleotides: adenine (A) with uracil (U) and cytosine (C) and guanine (G)

wildtype "Normal" strain that has genes commonly found in all organisms of the same species.

wobble base pairing A type of nonstandard base pairing that often occurs between the first nucleotide of the anticodon and the third nucleotide of the codon when a uracil (U) and a guanine (G) are present (Figure 5.2).

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