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Isolation of ribosomal mutants with a decreased tendency for premature release of peptidyl-tRNA

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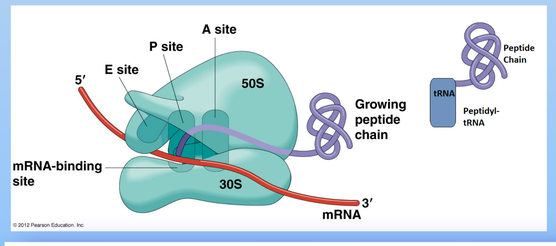
Background

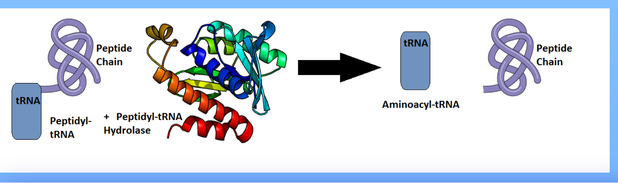
Isolating ribosomal mutations which reduce drop off has possible applications in improving protein manufacturing and identifying functions of the ribosome tunnel. Ribosomes are cellular machines that read the RNA code and translate it into proteins in a process formally known as *translation*. Beginning at the *start codon* AUG (Adenine, Uracil, Guanine) in the RNA sequence, the ribosome proceeds along the RNA matching each codon with the appropriate amino acid bound in an aminoacly-tRNA complex. The aminoacly-tRNA matches to the codon at the Aminoacly or *A-site* within the ribosome where the amino acid it carries is perfectly positioned to bond with the growing peptide chain located at the ribosome's *P-site*. The entire ribosome complex then shifts forward one codon so that the last aminoacly-tRNA on the A-site, is moved to the P-site. At this point the tRNA is bound to the growing peptide and is designated as peptidyl-tRNA. In normal translation, the process continues and the tRNA releases from the peptide then leaves through the E-site so that the next aminoacyl-tRNA can bind. Occasionally, near the beginning of translation, the entire peptidyl-tRNA is released prematurely in an event called *drop off*. **(figure 1)**

Figure 1: Ribosome structure



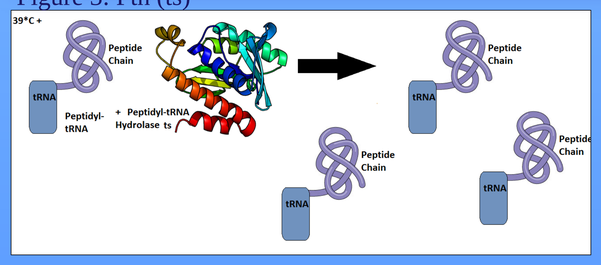
The tRNA attached to the peptide is un-usable by the cell and a large accumulation of peptidyl-tRNA decreases the amount of aminoacyl-tRNA to the point that it halts protein production resulting in cell death. Normal cells have the enzyme *peptidyl-tRNA hydrolase (*Pth)to cleave the tRNA from the peptide and return it to the pool of aminoacyl-tRNA for use in translation. **(figure 2)**

Figure 2: Peptidyl-tRNA hydrolase function (Pth)



E.coli C600 pth(ts) has a temperature sensitive peptidyl-tRNA hydrolase which does not efficiently cleave peptidyl-tRNA above thirty-nine degrees Celsius. At temperatures above 39ºC the Pth enzyme does not function and if drop off occurs the tRNA remains bound to the peptide (**figure 3**). Peptidyl-tRNA is un-usable by the cell and if enough accumulates, protein production is halted by the unavailability of amionacyl-tRNA and cell growth stops. This provides a clear method to select for cells with reduced drop off. If the Pth(ts) cells grow significantly more than normal at higher temperatures then drop off is decreased.

Figure 3: Pth (ts)



Method

Step one was to determine the exact temperature sensitivity of the C600 pth(ts) cells. This was accomplished by growing plates obtained from serial diluted cultures at 30ºC and higher until an upper limit was established **(figure 4)**. Next plasmids (circular DNA distinct from chromosomes) Pk4-16 and Pnk, containing ribosomal DNA, were randomly mutated using XL1-red competent cell. Natural mutants were also obtained. Mutated plasmids were extracted and transformed into the C600 Pth(ts) using electroporation **(Figure 6)**. The transformed cells were grown at 41ºC on ampicillin plates to select for plasmid mutations that reduced drop off. Plasmids were extracted from any colonies that grew at 41ºC, transformed into Pth(ts) cells and tested again at 41ºC to confirm the mutation was in the plasmid and not the cell's chromosome. Plasmids that resulted in colonies at 41ºC were sequenced to locate the mutation.

Figure 4: Serial dilution

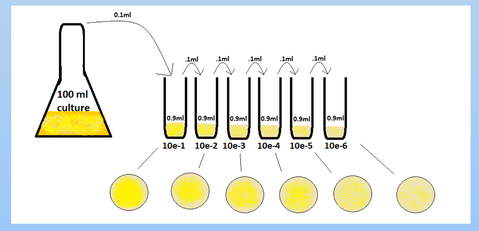


Figure 5: Plates from serial dilution

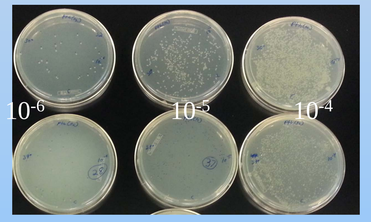


Figure 6: Transformation/ Electroporation

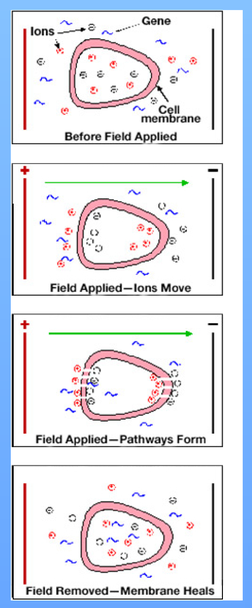
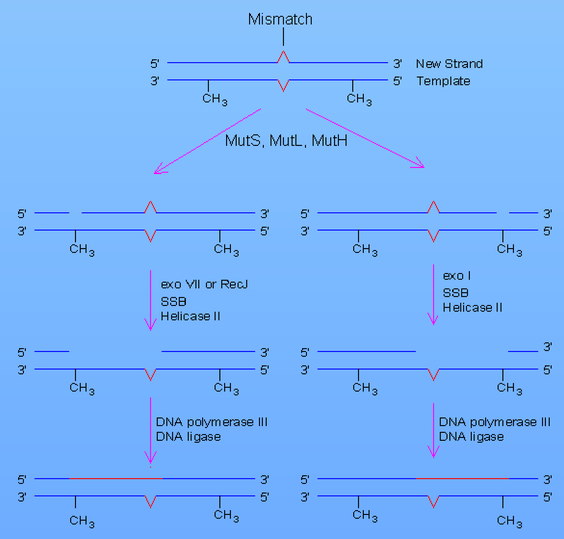


Figure 7 : Normal error repairs

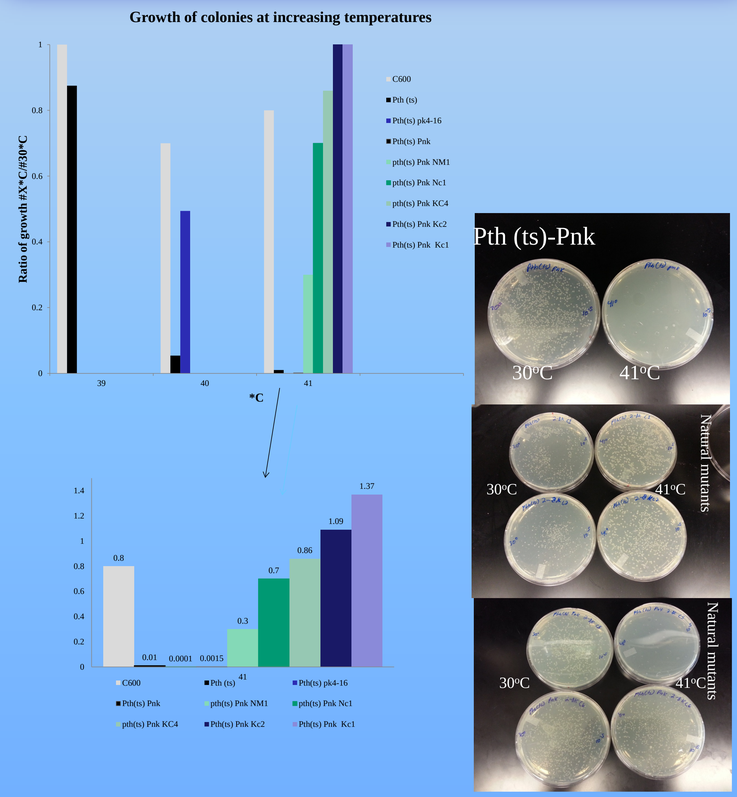


Mutations occur naturally at a very low rate when errors during DNA replication are not corrected. **(figure 7)** In Xl1-red competent cells the mechanisms for mismatch repair and DNA polymerase III exonuclease (DNA error erasing) activities are inhibited. The rate of mutation in Xl1-red cells is x5000 higher than in normal cells.

Results

The serial dilution established that C600 pth(ts) is temperature sensitive at 40ºC. When the Pk4-16 plasmid was introduced into the cell the temperature sensitivity decreased and showed 50% growth even at 40ºC. This led to the discovery that Pk4-16 contains a sequence for tRNA $^{2\,glu}$. This extra supply of aminoacyl-tRNA allows the cells to survive despite the inefficiency of the Pth(ts). At 41ºC the cells with the plasmid are again temperature sensitive.

Xl1-red mutated plasmids did not produce any plasmids capable of rescuing cells from temperature sensitivity. It is possible that the Xl1-red cells produced too many mutation in the plasmid rendering the ribosomes ineffective. Instead, natural mutants were obtained by selecting colonies that grew on plates at 41ºC. Fourteen plasmids from natural mutants were obtained and tested for decreased temperature sensitivity. Six of the transformed cells demonstrated a significant decrease in temperature sensitivity. Therefore those six plasmids should produce ribosomes with decreased tendency to prematurely abort protein synthesis. The sequence of the plasmid could provide information in determining how and why drop off occurs.



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