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DNA secondary structures and their contribution to mutagenesis in B. subtilis stationary phase cells

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It is widely known and accepted that the cause of many mutations in cells are generated during the replication process of actively dividing cells, however more recent research has shown that mutations also arise in non growing conditions, a phenomenon known stationary phase mutagenesis. Much of what is known come from studies in eukaryotic and bacterial models. It is proposed that in non-growing cells, the process of transcription plays an important role in mutagenesis. I will test the hypothesis that secondary structures formed of DNA generated transcription promote mutagenesis. The sequences transcription-generated structures are speculated to be prone to mutations by exposing regions of single stranded DNA to lesions. To test this hypothesis, I examined the *Bacillus subtilis* gene *thiF*, predicted by *in silico* analysis to be prone to mutations at particular locations during transcription. By altering the base sequence of this gene, the stability of its stem-loop structures is affected, thereby allowing us to test whether transcription of the altered sequence influences accumulation of in *thiF*. Our assay for detection of mutations is based on reversion to thiamine auxotrophy in cells under conditions of starvation. Ultimately, these experiments will increase our understanding of how mutations occur in cells of all domains of life.

DNA Secondary structures and their contribution to mutagenesis in *B. subtilis* stationary phase cells.

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Abstract

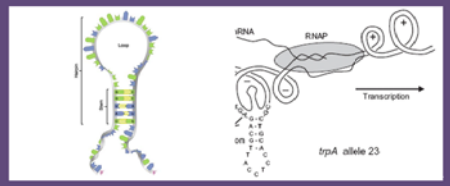
It is widely known and accepted that the cause of many mutations in cells are generated during the replication process of actively dividing cells, however more recent research has shown that mutations also arise in non growing conditions, a phenomenon known as stationary phase mutagenesis. Much of what is known come from studies in eukaryotic and bacterial models. It is proposed that in non-growing cells, the process of transcription plays an important role in mutagenesis. I will test the hypothesis that secondary structures, formed of DNA generated transcription, promote mutagenesis. The transcription-generated structures are speculated to be prone to mutations by exposing regions of single stranded DNA to lesions. To test this hypothesis, I examined the *Bacillus subtilis* gene *thiF*, predicted by *in silico* analysis to be prone to mutations at particular locations during transcription. By altering the base sequence of this gene, the stability of its stem-loop structures is affected, thereby allowing us to test whether transcription of the altered sequence influences accumulation of mutations in *thiF*. Our assay for detection of mutations is based on reversion to thiamine auxotrophy in cells under conditions of starvation. Ultimately, these experiments will increase our understanding of how mutations occur in cells of all domains of life.

Background

- In stationary phase cell division and replication are halted due to an environmental stress setting the stage for mutagenic events to occur that may enable organism to grow.
- This phenomenon has been documented since the 1950's when Francis J. Ryan's paper was published in *Genetics*. In his discussion he wrote,
  "In the meanwhile, the fact that mutations can arise in populations of bacteria whose numbers are not increasing must be accepted."
- Ryan observed a histidineless *E. coli* strain mutate to histidine prototrophy in non dividing cells in both liquid and agar media. In this early paper, Francis Ryan had to address the issue of contamination, delay in phenotype and even cells lysing to account for the mutants arising in his plates since the concept was so new.
- Current research is providing new insights on the mechanisms giving rise to these mutations.
- Research has found the importance different repair pathways and the process of transcription play in contributing to mutagenesis during stationary phase.
- In specific, secondary structures formed during the process of transcription have been found to contribute to mutations in *E.coli* and have been extended to help explain the high frequency mutability in certain cancer genes, such as p53 tumor suppressor gene.
- Stem loop structures (SLS) form as a result of transcription driven negative super-coiling by ssDNA.
- A SLS is characterized by having both, a double stranded portion that forms the stem, and a single stranded loop. It is the bases found in the single stranded portion that are speculated to be most vulnerable to mutagens.
- Different genes will form different structures with different stabilities based on their sequences. The more stable the SLS the more susceptible it is to mutagenesis.
- The mutability index (MI) of each gene can be calculated by multiplying Gibbs free energy value of the most stable secondary structure in which a base is unpaired of a particular DNA sequence by the percentage of total structures in which the base is unpaired during transcription

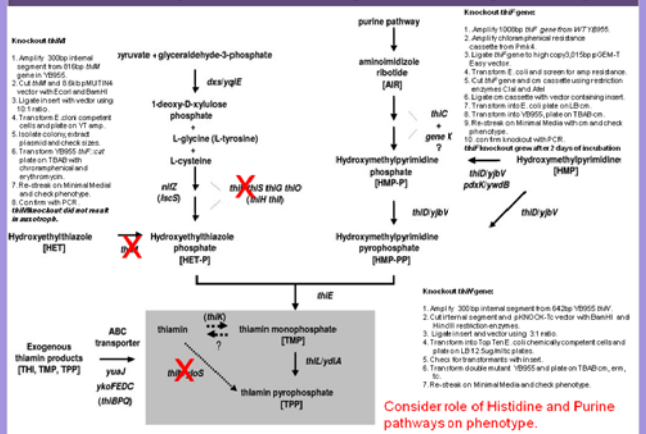
Hypothesis

The more stable the SLS the more susceptible the gene and its bases forming the structure are to mutagenesis.

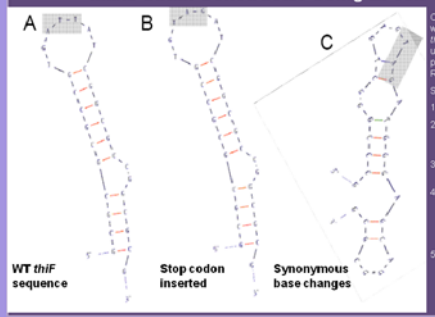


Methods

Create Auxotroph: Knockout *thiF* gene and Salvage Pathways



Make Strain Constructs: Using E. coli "RED" GENES



Once auxotrophic phenotype is obtained, we will move on to make changes within the WT *thiF* base sequence. Changes will be made using pHS vector, which includes an inducible promoter, and the E. coli Phage Recombineering system.

Steps include:

1. Ligating WT *thiF* gene to pHS vector.
2. Amplify tetracyclin cassette with *thiF* overhangs that include stop codon using PCR.
3. Use E.coli system to recombine tc cassette and stop codon into pHS vector.
4. Select vector with sequence change in *thiF* but no tc cassette by using Fusaric acid resistance. (Halving tc gene increases membrane permeability and allows acid to enter and kill the cell).
5. Once desired product is obtained pHS will be transformed into YB955 *thiF::cat thiM ::pMUTIN4 thiN::pKNOCK-Tc* into *amyE* locus where transcription will be controlled by inducible promoter.

Results

- *thiF* knockout does not yield an auxotroph for Thiamine, but a bradytroph instead.
- *thiF* knockout needs 2 days to show growth on a Minimal Medial plate.
- Supplementing Thiamine at a concentration of 1ug/ml did not completely restore WT phenotype.
- *thiM* knockout does not yield auxotroph, but a slower bradytroph then the single mutant.
- *thiM* knockout needs 4 days to show growth on Minimal Media plates.

Conclusions

At this point we are unable to reach a conclusion to answer our hypothesis on the mutability of *thiF*. We have, however, gained further knowledge on the complexity of the Vitamin B pathway in *Bacillus subtilis* and we now can proceed to obtain a Thiamine auxotroph.

Future Plans

- Knockout *thiN* gene in double mutant background to eliminate thiamine one-step salvage pathway to yield TPP auxotroph.
- Use E. coli Recombineering system to make allelic changes to WT *thiF* sequence, including stop condon and synonymous base changes.
- Once desired product is obtained pHS will be transformed into WT YB955 into *amyE* locus where transcription will be controlled by inducible promoter.
- Conduct a stationary phase assay and score mutant reversion to thiamine prototrophy.
- Conduct stationary phase assay with different levels of negative supercoiling to test effect on mutation numbers.
- Conduct stationary phase assay without transcription strand specific repair pathways such as knocking out *mfd* gene.
- Further analyze details of stem loop formation and how structure stability influences the structure and vulnerability of DNA and mRNA in its vicinity.

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References