

**The Study of Mutagenicity of AZT on a Set of lacZ<sup>r</sup> Escherichia coli**

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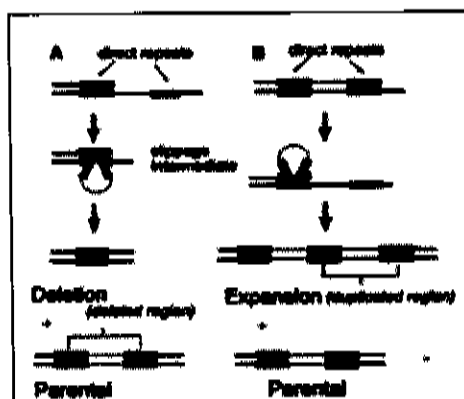
## Abstract

Azidothymidine (AZT) has been widely used since 1987 [21] to combat HIV/AIDS. AZT is thought to stop replication of viral DNA by getting incorporated into the DNA sequence and acting as a chain terminator. We constructed 15 strains of *lacZ* in wild-type and *mutS* backgrounds that contain specific point mutations, frameshift mutations or template switch mutation that can be restored to *lacZ*<sup>+</sup> by respectively correcting these mutations in only one way. Our studies investigate the effect of AZT on the reversion rates of these mutants by continuously exposing them to very doses low of AZT. For mutants carrying point mutation, it is still unclear how AZT affects their reversion due to insufficient data and technical difficulties while conducting the experiment. Mutants carrying frameshift mutation are all equally affected by AZT by about 3 to 5 fold increase. *mutS* increases spontaneous reversion rates for frameshift mutants. However, *mutS* appears to have no repair effect on the damages caused by AZT. AZT shows the strongest effects on reversion rates of mutants requiring template switching to revert to wild-type. Two types of template switching involve in this study are imperfect hairpins and sequence duplication. For mutants carrying hairpins, AZT dramatically the formation of perfect hairpins by template switch and for sequence duplicate mutant, AZT increases the rates by about 5-8 folds. *mutS* also exhibits little or no effect on the AZT damage in the sequence duplicates. However, it is unclear how and why AZT affects and favors template repeats mutants.

## Introduction I: DNA Damage

During replication, polymerases not only encounter errors in DNA sequence such as altered bases, gaps and strand breaks, but they also make mistakes by substituting or inserting and deleting the wrong bases in the newly replicated sequence. These events can cause DNA damage by producing mismatch of bases in DNA and occur more frequently when polymerases come across a sequence of repeating bases such as AAAAA [1]. In addition to producing nucleotide mismatches, polymerase can undergo simple slippage upon encountering a sequence of repeating bases, such as single 6 single nucleotide repeats, 10-12 double nucleotide repeats (GAGAGAGAGAG) or 20-25 triple nucleotide repeats (AGTAGTAGTAGTAGTAGT). Polymerase would detach itself from the newly synthesized DNA strand upon encountering error and then reattaches itself at another DNA sequence region of the template DNA downstream of the error to complete the replication. This can form a duplication or deletion of sequence in the nascent daughter strand.

In more acute cases where the sequence repeats involve much longer region (11 to 200 base pairs,) cell mediate the problem through a second method of slippage. Very large repeats go through constant deletions and expansions of base-pairs that are independent of protein RecA. For repeats that have >100 nucleotides, deletions are more prevalent. The mechanism for deletion or expansion of repeats is not recognized by the mismatch repair system and involves the formation of a very large loop on the leading strand during or shortly after replication [26].



**Figure 1. Longer repeats cause slippage of the polymerase during replication and the formation of a large loop on the leading strand to cause either the expansion of the repeated sequence or the deletion of the repeats. Deletion of the repeats is more common and is completely or partially independent of RecA [26].**

DNA polymerase I (Pol I) and DNA polymerase III (Pol III), which are both involved in normal DNA replication, have very low error rates, because they are associated with proofreading exonucleases to check for and correct the mistakes made during replication. However, during extreme conditions under general stress or exposure to mutagen, Pol I and Pol III can face difficulty carrying out their roles. These polymerases can produce errors or cease replication of damaged DNA. The cell can try to fix the problem by activating the SOS response system, which causes the activation of alternative DNA polymerases such as polymerase II and polymerase IV (Pol II and Pol IV) to continue the replication of the damaged DNA template [2]. However, Pol II and Pol IV have a higher tendency to make errors during replication because of the lack of exonucleases to proof-read the newly synthesized DNA [3]. Aside from that, scientists suggest that Pol II and Pol IV would incorporate different nucleotides to complete the replication due to the physical structure of the active sites of the enzymes. This mechanism of “guessing” increases the enzymes’ error prone tendencies, which is also intensified by their lack of proofreading. However, this proposed hypothesis of Pol II and Pol IV mechanisms have yet to be published.

Inaccuracy during replication can also result in the formation of tandem duplication or repeat which is very unstable and has a high reversion frequency. Tandem repeat involves a sequence of DNA followed by its duplicate immediately after, which can consist of a sequence of 2-6 bases or longer. The mechanism for tandem repeats formation is similar to deletion or insertion formation mechanism in DNA mutation but with distinctive properties. Formation of tandem repeats can also happen during DNA recombination [4].

DNA damage can also develop due to natural or induced physical stress to the cell. Occasionally, DNA undergoes physical stress which caused the DNA to be pulled and stretched, resulting in the breakage of the DNA backbone. More often, single stranded breaks and double stranded breaks can be the results radiation caused by x-ray or  $\gamma$ -ray exposure. When exposed to UV irradiation, DNA forms thymidine-dimers, leaving the DNA  $\alpha$ -helix distorted [5]. Replication of strands that contain thymidine-dimers results in the formation of daughter strands that contain gaps. Post-replication gap repairs will these gaps and form the correct DNA molecule [27].

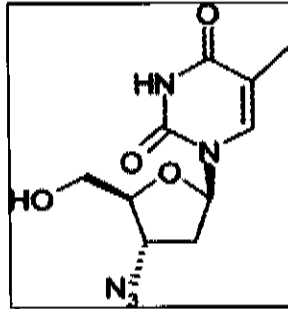
Any stress to the cell, for example chemical or physical stresses, can induce movement of mobile genetic element called transposons, which move and replicate by two mechanisms. The first mechanism, cut-and-paste mechanism, involves the transposon being cut from its donor DNA and inserted into the target DNA. This mechanism leaves the donor DNA molecule broken, which is then repaired through a number of ways resulting in deletions or rearrangements. The second mechanism is replicative transposition, where transposon is copied by DNA replication, and the end products having molecule that appear identical for both the donor DNA and the target DNA. This mechanism leaves the donor DNA remained intact. The

movements of transposon could involve the donor DNA and the target DNA residing on the same or different DNA molecule [6].

Chemical changes, or lesions, that happen to the nucleotide bases include oxidation and alkylation. For example: reactive oxygen species accumulated in the cell such as peroxide and free radicals can oxidize guanine creating 7,8-dihydro-8-oxoguanine (8-oxoG or GO), which then form lesions in the DNA [7]. Alkylation of bases or phosphates in DNA involves the addition of alkyl groups ( $\text{CH}_3$ ,  $\text{CH}_3\text{CH}_2$ , etc.) to the nucleotides [7]. Examples of alkylating agents are cisplatin, ethyl-methanesulfonate (EMS), methyl-methanesulfonate (MMS), N-methyl-N'-nitro-N-nitrosoguanidine (NTG or MNNG) [8]. Methylation is a type of alkylation and cause similar changes to the bases where an electrophilic methyl group at the 6' position of the larger ring of the adenine in the GATC sequence.

Intercalating agents and base analogs are also known to cause DNA damage. Errors in replication can occur when compounds of intercalating agents and base analogs slip between bases and substitute the bases respectively. Intercalating agents can cause deletions or insertions by slipping between two bases in the template strand of DNA by forcing polymerase to skip the inserted error or by inserting an extra nucleotide opposite the foreign compound. Examples of intercalating agents include proflavin, acridine and ethidium bromide. Base analogs on the other hand resemble the structure of a normal base and get incorporated into the DNA. However, the slight difference between analog compounds the real base result in inaccurate base-pairing and consequently replication error. An example of base analog is 5-bromouracil, a thymine analog, and causes a mispair with guanine base [9]. Another example of base analog is azidothymidine (Zidovudine, 3'-Azido 3'-deoxythymidine, AZT), which is a thymidine analog. AZT is currently

used globally as an antiretroviral drug in treating HIV/AIDS patients and healthcare and social workers who are exposed to patients affected by this virus.



**Figure 1** Structure of Azidothymidine is similar to that of deoxythymidine, where 3'-hydroxyl group (-OH) is replaced by an amino group. [10]

Due to the similarity between the chemical structure of AZT and thymidine base, AZT is activated by the enzymes that activate thymidine [10]. It cannot be linked to the 5'-phosphate group to form phosphodiester linkages of the next nucleotide downstream of it because it lacks a 3'-hydroxyl group (-OH) [11]. Azidothymidine is thought to act as a DNA chain terminator, which means that insertion or substitution of AZT into the DNA strand leads to the stall of DNA replication or can stop it altogether, and consequently lead to cell death. Research in our lab has shown that acute exposure at high dosage of AZT of 100ng/ml decreases cell growth [28]. *E. coli* stops the replication for about 25 minutes to 1 hour [12], contact. My research project will address the question of mutation caused by exposure to AZT at low dosage of 10ng/ml for prolonged period of time of two days.

### Introduction II: DNA Repair

The mismatch repair system (MMS) ensure the accuracy of DNA replication by detecting mismatches in newly replicated DNA strands and repairing them. MMS is strand specific and exclusively targets the newly synthesized and unmethylated strand that carries the wrong genetic information, thus must occur immediately after DNA synthesis [13]. The MutS protein scans the

DNA and recruits MutL protein when it encounters a mismatch. MutL protein activates MutH protein which in turn nicks the mismatch site. An exonuclease digests the newly replicated DNA single strand leaving a single stranded gap which is filled with the correct nucleotide by DNA polymerase III (Pol III) and sealed by DNA ligase. The mismatch repair system is able to repair errors that involve mismatched nucleotides and small loops [14].

The other mentioned damages in DNA can be corrected by a number of different DNA repair systems. Cells are capable of reversing the damage, excising and correcting the damage, repairing the damaged section of the strand or bypassing the damage. Two examples of direct reversal of DNA damage are removal of methyl group from O<sup>6</sup>-methylguanine and photoreactivation. Formation of thymine dimers can be corrected through the photoreaction system by the enzyme DNA photolyase [15].

For mistakes involving damaged bases, base excision repair system (BER) corrects the problem using the enzyme glycosylase which detects and removes the base and sugar from the backbone. DNA polymerase and DNA ligase then restore the affected strand using the undamaged strand as the template. This repair system would be more likely to fix the error induced by chemicals that change the structure of bases through alkylation or methylation [16]. Nucleotide excision repair (NER) comprises the recognition of distortion in the DNA double helix and the removal of a small patch or short segment of in a single strand including the lesion. A gap left on the single strand would then be fixed by the DNA polymerase through the recruitment of proteins, again using the undamaged strand as the template. In *E. coli*, four proteins are involved in this repair mechanism; proteins UvrA, UvrB, UvrC and UvrD. Damages that are usually corrected through this system involve bulky adducts on the bases or thymine dimers [17].



Homologous recombination is able to correct double-strand breaks in DNA by recovering information on the damaged sequence from its sister chromosome. In homologous recombination (HR), broken DNA strands are degraded to generate single-strand extensions, or ssDNA tails. Homologous DNA on sister chromosome is invaded by the ssDNA tails by utilizing protein RecA as catalyst. DSBs can also be fixed by non-homologous end joining (NHEJ). NHEJ is a fail-system for DSB-repair when sister chromosome is unavailable HR. The broken DNA ends are joined directly to each other with the aid of protein Ku. This repair mechanism can form deletions of bases ranging from a few to a couple thousand making it an error-causing way of repairing DSBs [18].

However, during replication polymerases occasionally encounter damages and lesions that are not yet or cannot be fixed. DNA polymerases are forced to either proceed across these lesions or stop replication. Cells can then adopt a fail-safe mechanism called translesion DNA synthesis which would carry on replication even though it is a highly error-prone measure. Translesion DNA synthesis was termed because during replication, no real repair of altered bases occurs but instead replication continues across these altered bases [19]. In *E. coli*, protein complex UmuC and UmuD' (Pol II) and DinB (Pol IV) carries out this process which incorporates nucleotides in a way that can accommodate lesions better than normal Polymerases. Translesion synthesis is associated with SOS response pathway that gets switched on when responding to DNA damage. SOS system is an alternative response to DNA damage to prevent cell death. In *E. coli*, SOS system relies on three genes, *recA*, *umuC* and *umuD*. These three genes associate themselves to Pol III which allow the replication to continue downstream of the damage [20].

### Introduction part III: AZT Background

My study focuses on the drug AZT and its effects on different LacZ' mutation in E.coli. Azidothymidine (3'-azido-2', 3'-dideoxythymidine, AZT) is a dideoxynucleoside analogue that has been proven to interfere with human immunodeficiency virus (HIV) from infecting healthy cells. AZT cannot treat cells that have already been infected nor does it cure HIV. It also has been proven to improve some certain clinical abnormalities associated with acquired immunodeficiency syndrome (AIDS) [11]. Also known as zidovudine, AZT became available as the first antiviral drug in 1987 [21] and the current standard dosage of AZT administered a day is 1500mg [22] and is cocktailed with other drugs to combat HIV/AIDS. AZT works by inhibiting the reverse transcriptase enzyme of the virus after being incorporated into the viral DNA [23], which causes the cease and incomplete replication of the virus. AZT is also administered for cancer treatment, but its success in treating cancer is limited due to adverse effects [24].

AZT is given to social workers in countries more affected by HIV/AIDS. The administration of AZT to mothers during pregnancy to reduce maternal-fetal transmission is practiced worldwide, including in the United States. However, due to its mutagenic effects on certain genes such as hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) gene and the endogenous autosomal thymidine kinase (*Tk*) gene, and alterations of cell cycle gene expression, AZT is suspected to have the potential of a carcinogen [25]. Aside from that, AZT is found to inhibit DNA polymerase gamma, a mitochondrial polymerase, and cause the decrease of mitochondrial DNA (mtDNA), with long-term side-effect that leads to myopathy [24].

Another study on AZT usage in HIV treatment uncovers that its other side effects include anemia related to severe bone marrow toxicity, neutropenia or low white blood cells count and

siderosis or derangement of iron in tissue. AZT is also found to slow down the endocytotic pathway of cells by affecting the receptors on the cell membrane [26].

Currently, the studies in our lab have shown that AZT plays an important role in template switching in *E. coli* during replication. This study hopefully will increase our understanding of how AZT affects template switching and the mechanism involved.

#### Introduction Part IV: Experiment Background

The use of *Escherichia coli* (*E. coli*) bacteria in this study is due to a number of reasons. *E. coli* has been used in genetic and molecular studies because it is easy to grow in large quantities, allowing us to perform statistical analysis of large populations. *E. coli* is also easy to genetically modify. Finally, mitochondria are more closely related to gram-negative bacteria, for example *E. coli*. This relationship will hopefully help us why and how mitochondria are so very vulnerable to AZT.

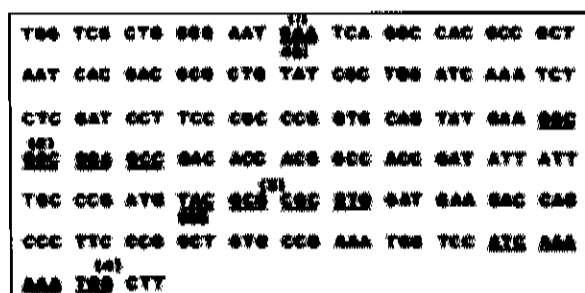


Figure 2. Glu-461 is the target site for *lacZ*. Site (1) is the codon involved in the formation of the first six *lacZ* mutants (CC101-CC106). Sites (2), (3) and (4) are the sites involved in the formation of the next five mutants (CC107-CC111) which consist of frameshift mutations [30].

Cupples and co-workers discovered that Glu-461 is important for the activity of enzyme  $\beta$ -galactosidase, which is encoded by *lacZ* gene in *E. coli* and modifications of this gene will prevent the cell from metabolizing lactose sugar, and therefore interfere with growth of *E. coli* on lactose minimal medium. Cupples constructed eleven *Lac<sup>-</sup>* *E. coli* strains that contain point mutations in the Glu-461 codon or other essential codons. To revert the strains to wild-type *Lac<sup>+</sup>*

phenotype, the sequence has to be restored back to the original Glu-461 codon, which, for each strain involves a specific mutation, such as a single base substitution, addition or deletion of one or two bases or deletion of a run of sequence.

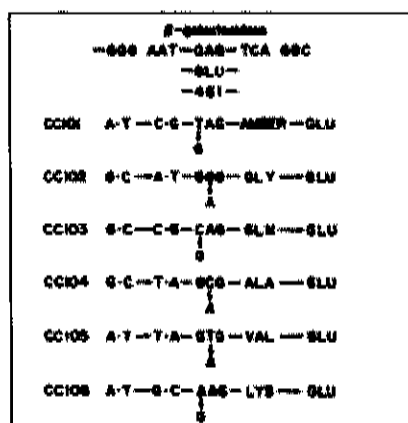


Figure 3. CC101-CC106 are mutants with base substitution in the Glu-461. The *lacZ* mutants need to restore the correct Glu-461 codon to exhibit wild-type phenotype [29].

The first six mutant strains contain point mutations of either transversion or transition at the mutation site Glu-461. CC101 has a transversion point mutation that requires the restoration of the G base from T base. CC102 is a transition point mutant and needs the substitution of G base with A base to revert to wild-type. CC103 is another transversion point mutant, requiring restoration of G base from C base. CC104 is the third transversion point mutant that needs substitution of G base from C base to become wild-type. CC105 and CC106 are both transversion mutant that need to make base substitution of T→A and A→G, respectively, to repair their *lacZ* gene. Figure 3 [29] show the site of mutation and the required restoration of the bases to revert to wild-type.

| STRAIN | (SITE)<br>SEQUENCE  | REVERSION |
|--------|---|-----------|
|        | (2)   |           |
| CC107  | GGC GGC GGA GCC WILD TYPE<br>GGC GGG GGG CC TARGET<br>GGC GGG GGG GCC REVERSION     | +1G       |
| CC108  | GGC GGC GGA GCC WILD TYPE<br>GGG GGG CGG ACC C TARGET<br>GGG GGC GGA GCC REVERSION  | -1G       |
|        | (3)   |           |
| CC109  | TAC GCG CGC GTG WILD TYPE<br>TAC GCG CGC GCG TG TARGET<br>TAC GCG CGC GTG REVERSION | -2 CG     |
|        | (4)   |           |
| CC110  | ATC AAA AAA TGG WILD TYPE<br>ATC AAA AAT GG TARGET<br>ATC AAA AAA TGG REVERSION     | +1A       |
| CC111  | ATC AAA AAA TGG WILD TYPE<br>ATC GAA GAA ATG TARGET<br>ATC AAA AAA TGG REVERSION    | -1A       |

Figure 3. CC107 and CC108 have the same site mutation (2) and require the insertion or deletion of one G. Site mutation (3) for CC109 requires the deletion of two bases of C and G. CC110 and CC111 require the insertion or deletion of A nucleotide base to restore its wild-type Glu-461 codon [30].

The next five mutant strains including CC New that we constructed all have frameshift mutation at sites away from Glu-461 that effect *lacZ* function. CC107 and CC108 both differ from wild-type gene in that they respectively contain an addition or deletion of G base in the mutation site (2). To revert back to wild-type, CC107 and CC108 need to delete or insert respectively a G base. CC109 and CC New respectively lack C and G bases and contain extra C and G bases at the mutation site (3). CC110 contains an extra A base, whereas CC111 lacks an A base at mutation site (4). Figure 3 shows the mutation sites for strains CC107-CC111 and CC New and the mutations they need to restore wild-type phenotype.

In addition the eleven strains constructed by Cupples, we constructed four other strains, all with different gene mutations. CC New has a frameshift mutation that requires the insertion of two nucleotide bases at its mutation site, which is the same mutation site as CC109. LacZ +11 is a strain with additional eleven bases after the target site for *lacZ*. The rest two mutant strains of QP3 and QP4 both contain quasi-palindromic sequence that form hair pins.

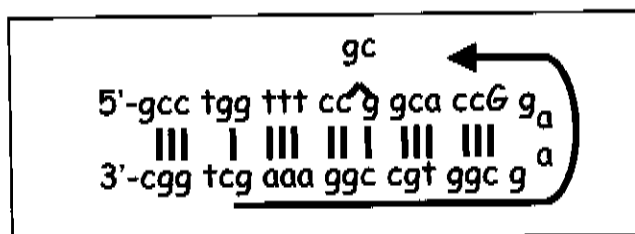


Figure 4. QP3 is a mutant with imperfect hairpin that constantly reverts to linear sequence. QP3 contains additional bases of G and C as shown in Figure 4. For the mutant to revert to wild-type, the additional bases need to be deleted from the sequence.

QP3 mutant is a *lacZ* mutant that contains quasi-palindromic sequence with additional C and G bases in the sequence that needed to be deleted for it to revert back to wild-type function. Figure 4 illustrates the position of the additional GC bases in the hairpin and their location compared to the direction of replication. During replication, cells will use the nascent leading strand of the hairpin as template after polymerase detaches from the strand. QP4 has almost similar structure as QP3 except that the additional +GC bases are located on the opposite side of the hairpin. During replication, the additional bases are usually replicated into the daughter strand through template switching, making the newly synthesized strand to contain four additional bases. QP4 acts as a control for QP3.

### Materials and Methods

**Construction of bacterial strains.** Fifteen strains of *E. coli* were constructed, each carrying a *lacZ* mutation affecting the enzyme  $\beta$ -galactosidase. We wished to study the reversion of *lacZ* in a different background than used in Cupples studies, thus we made our strains of *lacZ* in MG1655 (STL 242), which is a wild-type K-12 made F<sup>-</sup> lambda.

| Strain | Reversion Events | STL# of different mutations in mutS background |
|--------|------------------|--|
| CC101  | A.T - C.G        | 13219  |
| CC102  | G.C - A.T        | 13221  |
| CC103  | G.C - C.G        | 13223  |
| CC104  | G.C - T.A        | 13898  |
| CC105  | A.T - T.A        | 13226  |
| CC106  | A.T - G.C        | 13228  |

|         |                 |       |
|---------|-----------------|-------|
| CC107   | +1G             | 13230 |
| CC108   | -1G             | 13232 |
| CC109   | -2(-C-G-)       | 13177 |
| CC110   | +1A             | 13181 |
| CC111   | -1A             | 13186 |
| CC(New) | +2(-C-G-)       | 13233 |
| QP3     | Quasipalindrome | 13235 |
| QP4     | Quasipalindrome | 13237 |
| lacZ+11 | Duplication     | 14022 |

**Table 1: Strains constructed through recombineering carrying different mutation of *lacZ*. Second column of Table 1 shows the repair in the DNA for the strains to revert back to WT phenotype. The third column shows the STL number assigned to the new constructed strains.**

To achieve the construction of all sixteen strains, we employed the recombineering method as described by Don Court and co-workers. Through their studies, recombineering expression can be done in gram-negative bacteria through a set of plasmids that they constructed. The plasmids contain replication origin and some plasmids have temperature-sensitive replicons.

Bacteriophage  $\lambda$  genome is also included in the plasmids which consist of *red* genes (*exo*, *bet*, and *gam*).  $p_L$  operon expresses *exo*, *bet* and *gam* genes under temperature sensitive repressor C1857 and remains repressed at low temperature of 30°-34°C. Exposure to temperature of 42°C for a brief moment denatures the repressor and allows expression of Red [31].

The protocols for cloning of these various strains have been described in the Cold Spring Harbor class manual 2007 page 10 to page 12. We used the oligonucleotides obtained from Sigma-Aldrich and transformed them into *mutS* strains of either STL 13146 or STL 13149 which respectively carry plasmids pSIM5 (Cm<sup>R</sup>) or pSIM6 (Ap<sup>R</sup>) constructed by Don Court. Both these plasmids have not shown any differing abilities from each other in the past experiments [35].

The viral genes were induced by heat shock at 42°C for 30 minutes and the transformations of the oligonucleotides into the cells were done by electroporation. Transductants were selected on LB IPTG (isopropyl thiogalactoside) and X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) medium for white colonies against blue colonies. This is because

white colonies on LB IPTG X-Gal medium are an indication of defected  $\beta$ -galactosidase enzyme caused by the presence of *lacZ*. IPTG activates transcription of *lac* operon and consequently inducing transcription of  *$\beta$ -galactosidase* gene. X-gal is substrate for  $\beta$ -galactosidase and turns blue upon metabolization. Successful mutation of the *lacZ* gene produces white colonies due to inability to process X-gal. White colonies of LB IPTG X-gal medium also shows that recombination or cloning of the strains were successful. Further confirmation of correct mutation as described in Table 1 is done by sequencing the mutated region.

**Construction of P1's of the constructed strains.** P1's of each constructed strains were created to be transformed into MG1655 (STL242) WT and mutS::kan backgrounds (STL13726) [35]. P1 is a moderate bacteriophage that infects E. coli by lysogenization. Two ways viral DNA can be injected into target cells is by entering a lytic or lysogenic path depending on environmental conditions [34].

| Strain  | Reversion Events | STL # in WT background<br>MG1655 (WT) | STL # in MutS background<br>STL 13726 (mutS::kan) |
|---------|------------------|---------------------------------------|---|
| CC101   | A.T - C.G        | 13719                                 | 13773   |
| CC102   | G.C - A.T        | 13720-13721                           | 13775-13776                                       |
| CC103   | G.C - C.G        | 13814-13815                           | 13777   |
| CC104   | G.C - T.A        | 13997-14000                           | 14001-14004                                       |
| CC105   | A.T - T.A        | 13722-13723                           | 13778   |
| CC106   | A.T - G.C        | 13724-13725                           | 13724   |
| CC107   | +1G              | 13779                                 | 13663   |
| CC108   | -1G              | 14097                                 | 14095   |
| CC109   | -2(-C-G-)        | 13770                                 | 13817   |
| CC110   | +1A              | 14048                                 | 13813   |
| CC111   | -1A              | 13771                                 | 13763   |
| CC(New) | +2(-C-G-)        | 13764-13765                           | 13780   |
| QP3     | Quasipalindrome  | 13766-13767                           | 13781-13782                                       |
| QP4     | Quasipalindrome  | 14051-14054                           | 13783   |
| lacZ+11 | Duplication      | 14025-14026                           | 14027-14028                                       |

**Table 2: Nature of reversion event required to restore the Lac<sup>+</sup> phenotype along with STL number of the different strains in WT and MutS backgrounds**



**P1 Cloning into WT (MG1655/STL242) and *mutS::kan* (STL 13726).** Each of the fifteen constructed P1's was transduced into the two different backgrounds, producing a total of thirty strains. Procedures followed to achieve successful transduction are detailed in Cold Spring Harbor Manual [35].

Occasionally we deviated from the CSH protocol, for example leaving the mixtures of cells after the addition of NaCitrate at room temperature for longer than 30 minutes, sometimes up to overnight, instead of spreading the mixture on selective medium right after NaCitrate addition. This happened when we had to transduce *mutS::kan* into a strain, due cloning problems.

**Selection of transformants.** Cells were first selected for tetracycline drug resistance. Then, we confirm they are *lacZ* by showing that they produce white colonies on LB IPTG X-Gal medium. For cells with *mutS* background, further selection includes selecting colonies grown on LB kanamycin medium to confirm the *mutS::Kan* gene. Another conformation test for presence of *lacZ* in *mutS* background is to test the colonies for growth on rifampicin plates. *mutS* cells tend to survive in the presence of rifampicin at a much higher rate than WT cells. Thus, we expect to see more colony survival for strains with *mutS* background and no-growth for those with WT background. Confirmed strains are kept frozen at -70°C.

**Reversion Assay.** Frozen strains were struck out of -70°C and grown to single colonies on LB medium and LB medium with AZT (dosage: 10ng/ml) at 37°C overnight (12-18 hours). At least twelve separate single colonies were picked from each plate. Each colony is then grown in LB media or LB media with AZT 10ng/ml according to the medium they were grown on before. Cells are grown in 1.5ml of media for overnight at 37°, thus continuously exposing cells to AZT.

This is done because we are interested in studying the effect of AZT exposure over a long period of time. Moreover, the dosage we chose for this experiment is very low (10ng/ml) because we do not wish to kill the cells we wish to see if and how the exposed dosage would affect the replication of the cells to revert back to wild-type. The dosage we used for this is extremely low compared to the dosage administered to HIV/AIDS patients and this would hopefully give an insight of the effect AZT would have over long period of time [22].

1.5 mls of cells are centrifuged for 2 minutes at the speed of 13,000rpm. Supernatants are discarded and cell pellets are resuspended in 1ml of 1x56/2 salt solution. Cells are centrifuged again and washing of the cells with 1x56/2 is repeated again, for a total of two washings to completely remove AZT. It is important to use salt solution in the washing process as to prevent lysing of the cells. After the final wash, all salt solution is dispelled thoroughly by shaking the eppendorf tubes vigorously. This is to ensure that the final concentration and volume of the cells are roughly the same for each experiment. Cells are resuspended in 120ul of 1x56/2 salt solution.

**Reversion Assays.** There are two ways to perform the reversion assay according to the expected growth rate of the cells. The first method was designed for strains that have the ability to revert at a much higher rate. Strains CC107, CC108, CC109, CC111, CC New, QP3, QP4 and lacZ +11 are shown to have high rates of reversion [30]. Serial dilution of 1:30 was performed six times until 1:729,000,000 were achieved. This because we found that we need to plate the lowest concentration (1:729,000,000; 1:24,300,000; 1:810,000; and 1:27,000) on to LB medium as controls to be able to count the colonies. We need to plate out the highest concentrations (1:900, 1:30 and 1:1) onto Minimal lactose with IPTG X-gal (MinLac IX) medium to be able to

count the revertant colonies. The wide range of dilutions that we plate out will allow us to determine the rate of reversions over a wide range of possible rates.

Cells are plated onto LB medium; 20ul of each of the fourth, fifth, sixth and seventh dilution are plated onto one LB medium plate. 20ul of each of the original concentration, second and third dilution are plated onto one lactose minimal plates. LB medium plates are left for overnight growth in the 37°C and lactose minimal medium plates are left in the 37°C to grow for two nights. Lactose minimal plates cannot be left for more than two nights because the growth on the medium could arise from another mutation not related to the reversion of LacZ' to wild-type. The revertants will grow on MinLac IX medium and the high number of cells plated allows us to detect very low rates of reversion.

The second method applies to the strains that expected to have lower growth rate such as the CC101 to CC106 and CC110 also. These strains require the usage of the Failure Method. 4ul of the concentrated cells are diluted with 120ul of 1X56/2 to perform 1:30 dilution. 10ul of this diluted mixture is then diluted with 290ul of salt solution to achieve 1:900 dilution. Serial dilution is performed until the final dilution of 1:729,000,000 or the seventh dilution is achieved. 20ul of the seventh, sixth, fifth and fourth serial dilution is plated onto LB plates. To ensure the growth of on lactose minimal medium is observed, the remaining concentrated cells (about 120ul -130ul) are grown on the medium through whole-plating. LB medium plates are left in the 37°C for overnight and the lactose minimal medium plates are left for two nights.

**Calculations of Mutation Rates.** The methods we employed to plate out the cells of different strains were chosen because they were the most successful for us to analyze the cell populations

in parallel. By first starting from N=12 single colonies, we determined the number of mutants we have. By chance, we know that each population might have higher or lower number of mutants, depending on whether the initial mutation event occurred early in the growth of the culture or late. For each population we calculated the likely number of mutation events that lead to the final number of mutants,  $m$ , using the MSS method for each one. We calculated the rates of mutation by dividing the number of  $M$  with the number of cell divisions that occurred. The rates shown in this paper depicts the median rate of the N=12 rates collected [32].

For frameshift mutants (CC107-CC111 and CC New), we used the first reversion assay described above, where only 20ul of all seven 1:30 serially diluted cells were plated on the medium. We analyzed 12 cultures for each strain, with and without AZT exposure (N=12), in the first reversion assay. Reversion rates were later determined using a mathematical calculation programmed by Mohan Viswanathan which is similar to the MSS Maximum-Likelihood Method detailed in Rosche and Foster's paper [32]. Similar to the Failure Method used for CC101-CC106, the calculation designed by Viswanathan first determine the  $m$  [33].

For strains that have very low reversion rates, we used "Failure Method" to calculate the reversion rates. Instead of plating N=12, we have to plate out N=40 cultures for each strains.  $m$  is determined by the formula described in Rosche and Foster paper [Rosche].

The Failure Method takes into account the number of cultures that produce no revertants or  $p_0$  as described by Rosche and Foster in their paper. The  $p_0$  method involves the determination of  $m$  or the number of mutations per culture by dividing the number of cells that produce no mutation with the total number of cultures done in a set of experiment [32].

The equation to determine  $p_0$  is,  $p_0 = \frac{\text{Number of cultures with no mutants}}{\text{Number of cultures with } r \text{ mutants}}$

The value for  $m$  is determined using the equation  $m = \ln p_0$

Reversion rate is then determined from the value of  $m$  by dividing  $m$  by the number of colonies that grew on LB medium.

**Handling of AZT.** Through out this project, we have consistently been using the same stock of AZT 100mg/ml. This is because previous studies by Seier have shown that AZT stored at 4°C do not lose its ability for at least 6-12 months [28]. However, we store the AZT stock in 4°C. As this study is to prove the mutagenicity of the drug, it is important to handle AZT with care. Gloves should be used when working directly with AZT. Glass wares and other equipments should be cleaned thoroughly after been in contact with the drug. Furthermore, all AZT wastes, liquids and solids, should be disposed of properly.

### Results and Discussion

CC101 to CC106 are mutant strains that contain point mutation in the Glu-461 codon as shown in Figure 3 that cause them to be *lacZ*. CC101 contained the point mutation transversion that requires the nucleotide to revert from G to T to show the wild-type phenotype. CC102 contained the point mutation transition, requiring G to revert to A. CC103, CC104 and CC105 are all transversion mutated that respective need to revert from C to G, from C to A and from T to A. CC106 need to revert from A to G.

| Background | CC # | Mutation | STL # | Spontaneous Reversion Rates/ $10^8$ (mentioned in Cupples' studies) |
|------------|------|----------|-------|---|
| Wild-type  | 101  | A·T→C·G  | 13719 | 1.3   |
|            | 102  | G·C→A·T  | 13720 | 1.2   |

|     |         |       |     |
|-----|---------|-------|-----|
| 103 | G·C→C·G | 13814 | 0.4 |
| 104 | G·C→T·A | 13997 | 4   |
| 105 | A·T→T·A | 13722 | 5.4 |
| 106 | A·T→G·C | 13724 | 0.5 |

**Table 3: Transition (A↔G or C↔T) and transversion (purine ↔ pyrimidine) point mutation as shown in Table 3 above are the six strains that showed very low spontaneous reversion rates as mentioned in Cupples paper. For this experiment, we used the second method to perform the reversion assay and the Failure Method to calculate the reversion rates. The spontaneous rates shown are the rates obtained from Cupples study on the mutagen EMS [29].**

To determine the reversion rates of these six mutant strains, we first tried conducting the experiment using the second reversion assay method which is described above. In this assay, we plated only 20ul of all the seven 1:30 serial dilutions onto LB and MinLac medium. However, we observed almost no revertants for these strains. We later devised another reversion assay method. This method which is described above as the first method in the Materials and Methods section above, involves plating the maximum amount of the most concentrated cells onto whole plate of MinLac IX medium while plating the only 20ul of the most diluted cells onto LB medium. The data collected from our second trial is not enough to make any conclusion on the effect of AZT but it gives us an overview of the what to expect in future experiments.

As shown in Table 3, Cupples study discovered that the spontaneous reversion rates for CC101 to CC106 are very low. Because of this, it can be assumed that their naturally low reversion rates can be the reason we did not observe significant number of revertant colony growths. The insufficient data that we have collected could not be used to make a strong conclusion as to how AZT might affect cells carrying point mutations. It is also impossible to conclude if AZT would have a strong affect on transversion or transition mutation. Thus far, we can conclude that we will be able to determine the reversion rates for these strains using the reversion assay and rate calculation in the second experiment method described above..

Lastly, from our experiments and undocumented data, we observed that the mutS mutant background improves the number of revertant colony growths. This is somewhat expected as

mutS targets mismatch of 1-4 nucleotides. Further studies can be done by analyzing a larger number of cultures so that the Failure Method for calculating the reversion rates can be applied. Our revised reversion assay and calculation method will hopefully aid further studies on the effect of AZT on CC101 to CC106 in wild-type and mutS backgrounds.

CC107 to CC111 including CC New are all frameshift mutants that contain either additional or deletion of 1 or 2 bases in their respective mutation site as depicted in Figure 4.

| Background | CC # | Mutation | STL # | Spontaneous Reversion Rates/ $10^8$ Detailed by Cupples | 0ng/ml AZT     |       |          | 30ng/ml        |       |          |
|------------|------|----------|-------|---|----------------|-------|----------|----------------|-------|----------|
|            |      |          |       |   | Reversion Rate | Error | Low High | Reversion Rate | Error | Low High |
| Wild-type  | 107  | +1G      | 13663 | 30  | 0.48           | 0.23  | 1.9      | 1.1            |       |          |
|            |      |          |       |   | 0.95           |       |          | 1.7            |       |          |
|            | 108  | -1G      | 14095 | 54  | 0.17           | 0.1   | 0.94     | 0.53           |       |          |
|            |      |          |       |   |                | 0.3   |          |                | 1.7   |          |
|            | 109  | -2CG     | 13817 | 100   | 0.57           | 0.24  | 2.9      | 0.92           |       |          |
|            |      |          |       |   |                | 0.86  |          |                | 6.7   |          |
|            | 110  | +1A      | 13813 | 4.8   | 0.16           | 0.9   | 0.56     | 0.3            |       |          |
|            |      |          |       |   | 3.1            |       |          | 0.9            |       |          |
| 111        | -1A  | 13763    | 14    |   |                |       |          |                |       |          |
| CC New     | +GC  | 13765    | -     |   |                |       |          |                |       |          |

**Table 4. Six mutant strains of CC106 to CC111 and CC New are all frameshift mutants in wild-type background. The mutation sites for each strain are shown in Figure 3. Addition or deletion of one or two bases is required for strains to revert to wild-type. Table shows the STL number of the strain that was involved in the experiment to determine the reversion rate. Spontaneous reversion rates were obtained from Cupples paper [30]. Reversion rates of strains when not exposed to AZT and when exposed to AZT 10ng/ml are documented with low and high errors.**

The spontaneous reversion rates determined by Cupples studies for CC107-CC111 are recorded to be much higher ( $5^{-8} - 100^{-8}$ ) than the spontaneous reversion rates for CC101-CC106 ( $0.4^{-8} - 5.4^{-8}$ ). The difference in spontaneous reversion rates in the two mutant groups could be the reason for the success of determining the AZT induced reversion rates for CC107 to CC110. However, as shown in Table 4, the spontaneous reversion rates obtained from Cupples data for CC107-CC111 are higher than the spontaneous reversion rate we collected. This could be

attributed to difference in protocols or the difference in strain backgrounds (our strains are in wild-type background).

Frameshift mutants CC107, CC108, CC109, CC110, CC111 and CC New constructed in *mutS* background along with their respective STL number used in the assay are shown in Table 5. The spontaneous reversion rates we observed for these six strains are much higher than the spontaneous reversion rates we observed for frameshift mutants in *mutS* background by 1 to 2000 folds. +/- G shows the strongest effect of AZT of about 2000 fold increase but +/- A shows a much lower AZT effect. This suggests that *mutS* corrects frameshift involving G or C better than frameshifts involving A or T.

For each frameshift mutants in *mutS* background, AZT seemed to affect the reversion rates differently, with no clear pattern. As seen in Table 5, exposure of AZT to strains CC107 and CC108 increases the reversion rates by about 2 to 3 fold. Reversion rates of CC110 and CC111 after AZT exposure show lower increase of about 1 to 5 fold. CC109 shows an increase of about 5 fold after AZT exposure. The most confusing data is the +/-A because it does appear to act the similarly, which produce no clear pattern as to how AZT behaves in *mutS* background. Lack of obvious pattern could be due to instability of *mutS* which might cause it to mutate in another DNA repair gene. On the other hand, the results we are seeing could be the real depiction of AZT's effect, which means that there is a much more complex interaction between *mutS* repair system and AZT repair system.

| Background  | CC # | Mutation | STL # | 0ng/ml AZT                      |       | 30ng/ml                         |       |
|-------------|------|----------|-------|---------------------------------|-------|---------------------------------|-------|
|             |      |          |       | Reversion Rate /10 <sup>8</sup> | Error | Reversion Rate /10 <sup>8</sup> | Error |
| <i>mutS</i> | 107  | +1G      | 13779 | 1,110                           | 960   | 3,900                           | 2,100 |
|             |      |          |       |                                 | 1,500 |                                 | 6,900 |
|             | 108  | -1G      | 14097 | 200                             | 170   | 680                             | 310   |
|             |      |          |       |                                 | 370   |                                 | 1600  |
|             | 109  | -2CG     | 13770 | 19                              | 15    | 19                              | 17    |
|             |      |          |       |                                 | 21    |                                 | 21    |



|  |           |      |       |      |              |     |             |
|--|-----------|------|-------|------|--------------|-----|-------------|
|  | 110       | +1A  | 14048 | 0.19 | 0.15<br>0.24 | 1.0 | 0.67<br>2.1 |
|  | 111       | -1A  | 13771 | 11   | 7.2<br>16    | 13  | 7.4<br>19   |
|  | CC<br>New | +2CG | 13780 |      |              |     |             |

**Table 5. Frameshift mutants of CC107, CC108, CC109, CC110, CC111 and CC New in *mutS* background. The experiment to determine the reversion rates of these strains, with and without AZT exposure, is the first reversion assay method. Spontaneous reversion rates from Cupples studies are not available and incomparable with the mutant strains due to the different strain backgrounds.**

When comparing the data collected, the AZT induced reversion rates are found to be much higher than the spontaneous reversion rates by about 2 to 4 fold. This indicates that the AZT exposure increases the reversion rates for the CC107 to CC110 in wild-type background. So far, we have no data for the reversion rates of CC111 and CC New in wild-type background. This is due some problems we encountered during the execution of the experiment. Recent assay on CC New shows almost no revertant growths and could be attributed to plating errors or bad medium. We plan on fixing the problem and attempting another assay for CC111 and CC New so that we can make the comparison with CC110 and CC109 respectively.

The remaining three strains contain template mutations. Table 6 shows the reversion rates for QP3, QP4 and LacZ +11 in both wild-type and *mutS* backgrounds. We are currently in the process of collecting data for QP3 in *mutS* and QP4 in both *mutS* and WT.

| Background  | CC #     | Mutation         | STL # | 0ng/ml AZT                     |       | 30ng/ml                        |       |
|-------------|----------|------------------|-------|--------------------------------|-------|--------------------------------|-------|
|             |          |                  |       | Reversion Rate/10 <sup>8</sup> | Error | Reversion Rate/10 <sup>8</sup> | Error |
| Wild-type   | QP3      | Quasi-palindrome | 13766 | 3.6                            | 2.6   | 15                             | 1.3   |
|             |          |                  |       |                                | 6.5   |                                | 44    |
|             | QP4      | Quasi-palindrome | 14051 |                                |       |                                |       |
| Wild-type   | LacZ +11 | +11 Sequence     | 14025 | 16                             | 13    | 130                            | 90    |
|             |          |                  |       |                                | 21    |                                | 180   |
| <i>mutS</i> | QP3      | Quasi-palindrome |       |                                |       |                                |       |
|             | QP4      | Quasi-palindrome | 13783 |                                |       |                                |       |

|  |             |                 |       |    |          |    |           |
|--|-------------|-----------------|-------|----|----------|----|-----------|
|  | LacZ<br>+11 | +11<br>Sequence | 14027 | 15 | 13<br>21 | 76 | 30<br>150 |
|--|-------------|-----------------|-------|----|----------|----|-----------|

**Table 7. QP3, QP4 and LacZ +11 strains are template switching mutants that contain hairpin or sequence duplication. The reversion rates of these strains seem to increase in the exposure of AZT. Data for QP3 in *mutS* and QP4 in both backgrounds is not sufficient to calculate the reversion rates.**

QP3 in wild-type strain shows an increase of about 4 folds after exposure to AZT. For LacZ +11 in wild-type and *mutS*, we observed an increase of about 5 to 8 folds of reversion rates. From the data we collected for LacZ +11, it seemed that there is no strong effect of *mutS* to the activities of AZT.

We suspect that damages caused by AZT in frameshift mutants turn on the activity of SOS response pathways to repair these errors. The activation of SOS response pathway requires the involvement of many genes of unknown functions. Thus, one of these genes could play an integral role in the repair of AZT-induced damages. As mentioned in the introduction, when polymerase encounters an error, it would fall off the leading strand. The damage is then repaired with the help of exonuclease, most probably Exo III by removing the AZT. Replication would restart afterwards when polymerase reassociates to the DNA strand.

In template repeat mutants of QP3, QP4 and LacZ +11, AZT has a higher chance of getting incorporated in the repeated sequence. In QP3, the mutant contains two additional G and C bases in the hairpin. To revert the mutant to wild-type, the +GC has to be removed or one nucleotide has to be added near +GC bring the hairpin into frame. When AZT is induced, polymerase is more likely to randomly fall off the leading strand during replication. After the correction of the error, reattachment of polymerase to the nascent leading strand uses itself as the replication template, as illustrated in Figure 4. By using itself as the template through template switching, the additional +GC on the other side of the hairpin will not be copied in the new daughter strand, reverting the cells to wild-type. The role of AZT in causing template switching during replication is proven by Table 7. By sequencing the revertants of QP3, we saw that

majority of the revertants have perfect hairpin. Table 7 shows that only with the presence of AZT almost all the cells revert to wild-type through template switching but in absence of AZT, all three types' reversion happen.

| Types of Reversion that occur for QP3            | No. of time reversion event occurs in absence of AZT | No. of time reversion event occurs with AZT presence |
|--|--|--|
| Minus GC w/"correction" of first half of hairpin | 9  | 17   |
| Minus GC   | 3  | 3  |
| + 1 nt upstream or downstream of GC              | 5  | 0  |
| None detected                                    | 4  | 2  |
|  | 21   | 22   |

**Table 8.** The reversion events that could occur QP3 causing it to become lacZ<sup>+</sup>. Revertant colonies with and without AZT exposure were sent to be sequenced. The sequence results show that there are three possible reversion events that QP3 could undergo [date courtesy of Tracey Seier].

LacZ +11 mutant contains duplication of eleven bases. To revert to wild-type, LacZ +11 need to remove one of the +11 bases duplicate that it has. During replication, newly synthesized leading strand can stop from being replicated if the polymerase detects an error and detaches itself. Once the polymerase reattaches itself, it could use the newly synthesized lagging strand as the template to complete replication. Successful template switching occurs when only of the sequence duplicates is replicated into the daughter DNA, restoring wild-type phenotype. Table 7 shows that AZT increases the reversion rates of LacZ +11 by about 8 folds in the WT background and by about 5 folds in the *mutS* background. *mutS* background seems to have no effect on AZT's mutagenic ability, as there appears to be difference in the spontaneous reversion rates in wild-type and *mutS* background. This is no surprise as *mutS* is known to only affect the short mismatches of about 1 to 4 nucleotides long.

**Administration of AZT to AIDS/HIV patients.** Although more research works need to be done to figure out the real effect AZT have on humans, our data thus far shows that prolonged use of AZT has the potential to cause mutations and cancer. AZT is still used until now because

there has yet to be a better drug that works as well as AZT to treat AIDS/HIV patients. As we observed in our studies, very low dosage of AZT does not cause deaths of the organisms but has the ability to change the genetic structure of the organism. More studies should also be conducted to further examine the effect of AZT on mitochondrial DNA. This would hopefully help us understand the maternal transmission of mutated genes.

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**\*Notes**

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