ORIGIN OF MUTATIONS DURING GROWTH UNDER SELECTION

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Abstract

Natural selection requires heritable variation in populations to select individuals of increased fitness. Whereas natural selection is well understood, the development of variation remains controversial. Classical experiments in *Escherichia coli* demonstrated that variation arises from mutations that occur spontaneously and without the influence of selective stresses. However, those experiments made use of lethal antibiotics-resistance assays that cannot detect growth and the presumably small effects of selection. Current research systems study the role of selection using non-lethal nutrient starvation. Starvation on lactose yields a 100-fold higher mutation rate than expected. Thus, under starvation conditions, selection seems to be mutagenic and allows for sudden adaptation. In contrast, we consider that the appearance of Lac+ colonies on selective media depends on common variants with high copy numbers of the leaky *lac* gene to slowly initiate growth under selection. This work investigates the role of growth temperature and nucleases on adaptive mutation by assaying the frequency of Lac+ revertants at various temperatures and in nuclease mutants, respectively. We conclude that the temperature dependence of Lac+ reversion may be due to the temperature sensitivity of plasmid transfer functions. In addition, nucleases appear to inhibit Lac+ reversion and adaptive mutation in general.

Introduction

Evolutionary theory is based on two fundamental components: natural selection and variation. Natural selection is well-understood in principle from Darwin's demonstration of its role in the speciation of finches in the Galapagos islands. The sources of variation are also well-understood. Molecular biology has developed an understanding of how variation forms as a result of mutations during DNA replication and recombination. It is the interaction between selection and variation that still stirs debate. Two interactions have been described: 1) selection acts on pre-existing mutations, and 2) selection induces mutations.

Classical experiments in the mid-20th century demonstrated that mutations occur randomly and without the influence of selection. In 1943, Luria and Delbruck conducted a fluctuation test by selecting for T2 phage resistant mutants of *E. coli* (1). The variability in the distribution of mutants per plate demonstrated that the mutants arose in the non-selective pre-growth culture (1). Additional support for this theory was provided by Newcombe in 1949 (2). *E. Coli* cells were spread onto an agar plate containing T2 phage. This plate was then printed onto two other plates containing T2 phage. However, only one of the plates was respread. Newcombe found that the respread plate formed a lawn of T2-resistant colonies, whereas the plate that was not respread only formed a few colonies. This demonstrated that the mutants arose during the non-selective pre-growth culture and were not induced by selection.

The Cairns Assay

The understanding that mutations arose independent of selective stresses was dogmatic until the late twentieth century. There was some criticism of the classical experiments because they made use of stringent selections (resistance to bacteriophage T4), which would not allow for the presumably small effects of selection to influence mutation rates. Therefore in 1991, Cairns and Foster developed a new system which tested mutagenesis under the less stringent condition of lactose auxotrophy (3). They used Lac- cells, with

a +1 frameshift mutation in a chimeric gene made by fusing the *lacI* and *lacZ* coding sequences (Figure 1). The gene is regulated by a mutant *lacI* promoter (Iq). The +1 frameshift is in the *lacI* section of the gene, which gives a 100bp target within which a reverting -1 frameshift mutation can restore Lac+. This mutant *lac* allele is found on the conjugative F'128 plasmid in a strain with a deletion of the chromosomal *lac* gene. This allele is not completely Lac-, but retains 1-2% residual expression as compared to Lac+ cells due to ribosomal frameshifting where wild type protein can be synthesized at a low level (4). This residual activity is enough to let these cells form colonies on the lactose selection plates (4). Therefore, 10^9 scavenger cells that contain deletions of both the F'128 and chromosomal *lac* genes were used to consume all non-lactose contaminating nutrients (3).

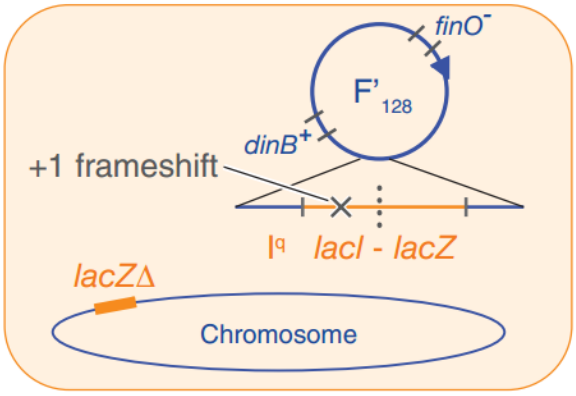

Figure 1: Genotype of the standard *E.Coli* K12 Cairns strain. A +1 frameshift mutation in the *lacI* portion of the *lacI-lacZ* gene fusion renders the cell phenotypically Lac-.
Figure citation: Roth et al., 2006.

In the assay, a culture of revertible Lac- cells was grown to stationary phase and 10^8 cells were spread on minimal medium plates supplemented by lactose. Over the course of six days of incubation, they found that approximately 100 mutant colonies formed at a nearly linear rate over time (3) (Figure 2).

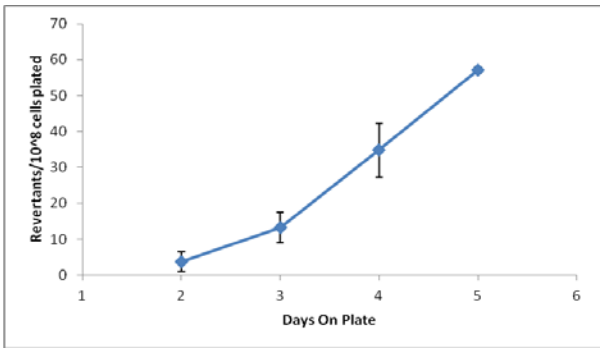

Figure 2: Reversion of Lac- cells in the Cairns assay.

Cairns and Foster noted several observations that conflict with the classical model of mutagenesis. First, the rate at which mutants arise is about 100-fold higher than would be expected. Second, the fact that mutants arise over time and not all at once suggests that a process is taking place on the selection medium that leads to the formation of the mutant. Third, mutant colonies did not fluctuate as expected (3). The assay distinguished between early revertants (Day 2) and late revertants (Day 6). Cairns and Foster performed fluctuation tests on both the early and late revertants. They found that the early revertants fluctuated with a Luria Delbruck distribution (3), which suggests that the mutations occurred in the pre-growth. The late revertants fluctuated with a Poisson distribution, suggesting that the mutants arose on the plate (under selection). Cairns and Foster concluded that these observations indicated that the stress of the lactose selection induced mutagenesis.

Several requirements must be met for this process of mutagenesis to be functional (5). 1) The *lac* mutation must be found on the F'128 and not on the chromosome, where reversion approximately occurs at a rate about 100-fold lower. 2) The *lac* allele must retain its residual activity. 3) The cells must retain their recombination machinery (RecA, RecBC). 4) The conjugative transfer genes (*tra*) must be expressed. 5) DinB, the error-prone DNA Polymerase must be located near the *lac* gene in the plasmid.

A Tale of Two Models

These main prerequisites have been interpreted into two different models: 1) stress-induced mutagenesis (SIM) and 2) the amplification model. The mechanisms for the two models are vastly different. Moreover, they differ by a crucial assumption. The SIM model states that there is no growth (cell division) by any of the tester cells, while the amplification model states that pre-existing variants of the unselected pre-growth culture can grow slowly.

Stress-Induced Mutagenesis

With the SIM model, lactose selection places a stress on the Lac- cells (7). This stress activates the stationary phase sigma factor, Rpos, which is a transcription factor that enables the expression of a set of stationary phase genes, including *dinB*. Furthermore, double strand ends (DSEs) are induced in stationary phase by the activity of the TraI endonuclease. TraI generates nicks in the plasmid, which can become DSEs after subsequent replication. DSEs are then used as substrates for repair by the recombination machinery. However, the error-prone DinB polymerase is recruited for DNA synthesis, which results in the -1 frameshift mutation needed for reversion to Lac+ (Figure 3).

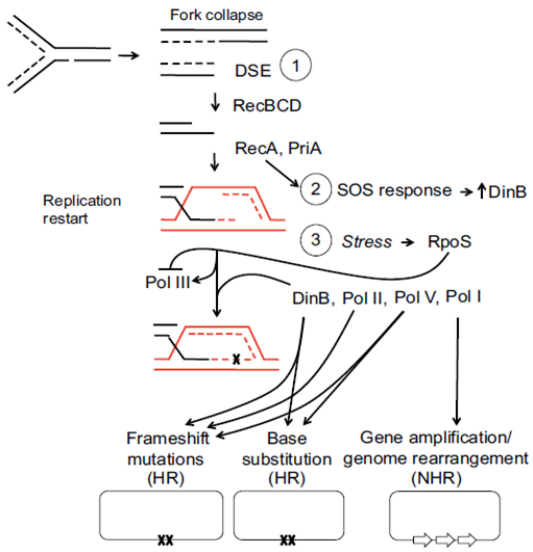

Figure 3: Model of stress-induced mutagenesis in the Cairns system.
Figure citation: Shee et al., 2012.

The Amplification Model

The gene amplification model assumes that although the population of tester cells as a whole is not growing, a small number of pre-existing variants are able to divide slowly (5). This growth is, therefore, sufficient for selection to affect the process of mutagenesis. The model proposes that high copy number variants (HCNVs) of the *lac* gene are common and allow for an improved growth fitness on the lactose selection medium. Since the *lac* allele retains 1-2% of wild type activity levels (4), a higher copy number variant will have an even greater LacZ activity level (e.g. a cell with 10 copies has about 20% of wild type activity) (5). It has been shown that duplications of the *lac* gene in Salmonella occur frequently and achieve a steady state level of 10^{-3}/cell after many generations of growth in the unselected pre-growth culture (8). Moreover, amplifications (3 or more copies) are proposed to arise at a frequency of 10^{-2} /cell for each additional copy (e.g. a cell with 3 copies of *lac* is found at 10^{-7}/cell) (8).

Before selection begins, the amplification model predicts that there is a rare population of HCNVs that are able to initiate slow growth when plated onto the selective medium (Figure 4). Under selection, these HCNVs are able to grow and produce offspring that have even more copies of *lac*. There are two possible fates for these cells. First, they can accumulate enough copies to form a colony without acquiring a revertant *lac+* allele; these are termed "unstable rich" colonies because once selection is removed, these colonies will segregate cells with few copies of the mutant *lac-* allele (6). Second, the HCNV's ability to initiate slow growth under selection can allow for the development of cells with enough copies of the mutant *lac* allele so that the normal low mutation rate can allow for the formation of the observed number of mutants (5). These colonies are termed "stable rich" because they do not segregate cells with few copies of the *lac-* allele once removed from selection (6). Essentially, this second process states that with more targets for a reversion mutation to form, the greater the apparent mutation rate. Therefore, although selection does not directly induce the formation of mutations, it

is able to drive the formation of HCNVs that have more targets within which a reversion event may take place.

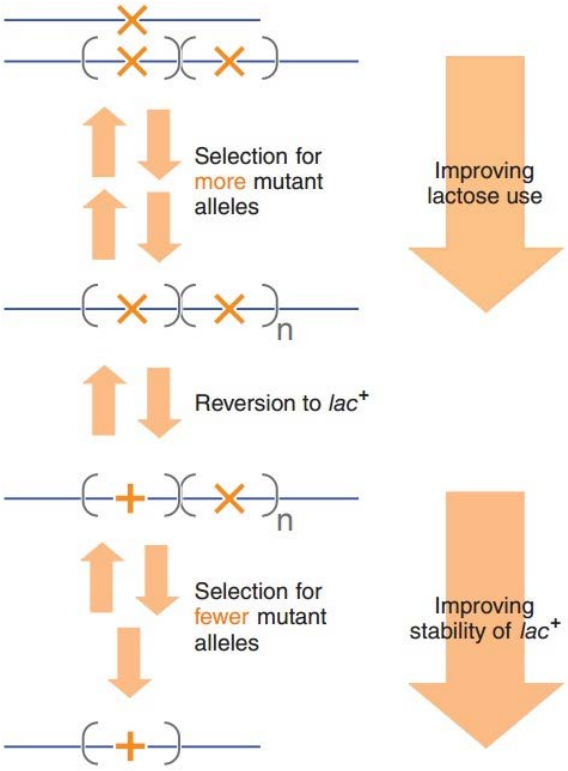

Figure 4: The gene amplification model.
Figure citation: Roth et al., 2006

The amplification model has developed an elegant mechanism to explain the observed phenomena in the lactose selection assay. Since the *lac* allele is found in the conjugative F'128 plasmid, it can acquire duplications--and amplifications thereafter--in many different ways, which we are exploring. The process of amplification is well understood by studying the unstable rich colonies. It takes place by a reversible RecA-dependent unequal crossing over event within a replication fork (Figure 5). Unstable rich colonies are unable to segregate cells with few copies of the *lac*- allele if they have a defective *recA* gene (9). Duplication formation is not fully understood and occurs through several mechanisms in the case of the F'128 plasmid. The first is by unequal crossing over (Figure 5), wherein recombination takes place between two plasmids and results in one plasmid with two copies of the *lac* allele and the other plasmid with zero copies (10). Duplications can also result from the formation of a plasmid dimer by a RecA-dependent activity (Figure 6). Furthermore, rolling circle replication with imperfect resolution of the product plasmids can result in dimers (duplications) or higher order multimers (amplifications) (10) (Figure 7).

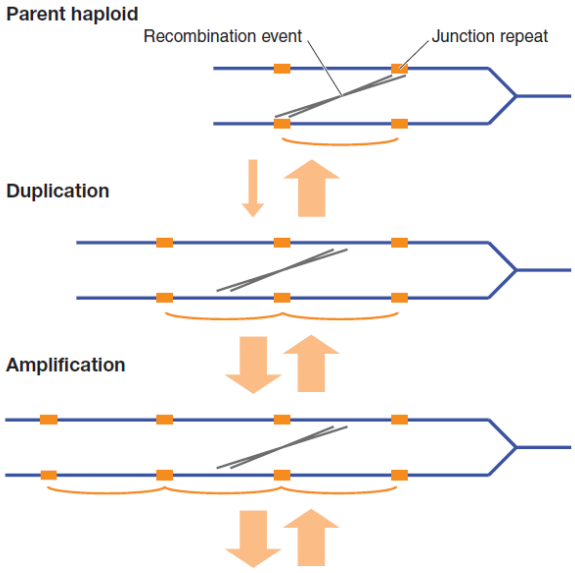

Figure 5: Formation of duplications and amplifications by unequal crossing over.
Figure citation: Roth et al., 2006

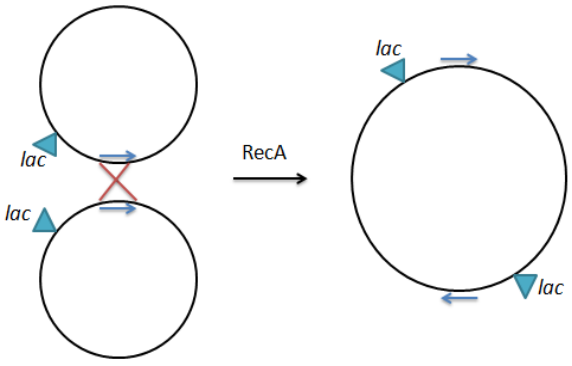

Figure 6: Duplication formation by recombination between plasmids

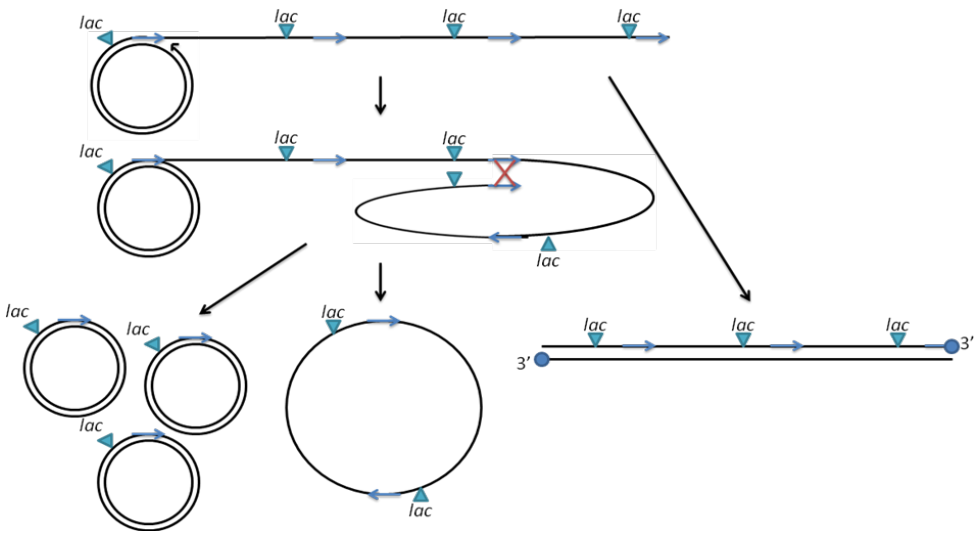

Figure 7: Formation of HCNVs by rolling circle replication.

The rolling circle mechanism may begin by the activity of the TraI endonuclease, which nicks the plasmid (11). The 5' end is spooled out and used as a template for the synthesis of a complementary strand. The 3' end is made into a replication fork that replicates the plasmid several times (12). An aberrancy in this mechanism allows for the synthesis of many copies of the F plasmid--as a linear multimer, circular multimer, or multiple F plasmids--which generates HCNVs that can initiate growth under selection (Figure 7). In this rolling circle replication mechanism, the 3' ends generated during the process can be subject to degradation by endogenous nucleases. Therefore, mutants of the nuclease activities are hypothesized to result in an improved rate of mutagenesis in the lactose assay.

SbcB In Adaptive Mutation

Two mutants of Exonuclease I, a highly processive 3'-5' ssDNA exonuclease (13), were generated. ExoI is encoded by the *sbcB* gene. The first mutant had a deletion of the *sbcB* locus. The second mutant was previously constructed by nitrosoguanidine mutagenesis and designated as *sbcB1* (14). Sequencing the region revealed that it contains a transition mutation, which replaces a guanine with an adenine at nucleotide 46 of the *sbcB* open reading frame. This results in an aspartic acid to asparagine missense mutation in amino acid 16 of the SbcB protein. This amino acid residue is involved in complexing a Fe^{2+} ion and is crucial for the nuclease activity of the enzyme (15). However, the 3' end binding activity is retained. In effect the *sbcB1* mutation generates a protein that can still bind to 3' ssDNA ends but cannot degrade them, thus essentially functioning to protect the 3' end (14). The *sbcB* deletion and the *sbcB1* allele were moved into the standard background used in the lactose assay via transduction using phage P22. These mutants were used in the lactose assay and their reversion rates were compared with that of the standard strain (Figure 8).

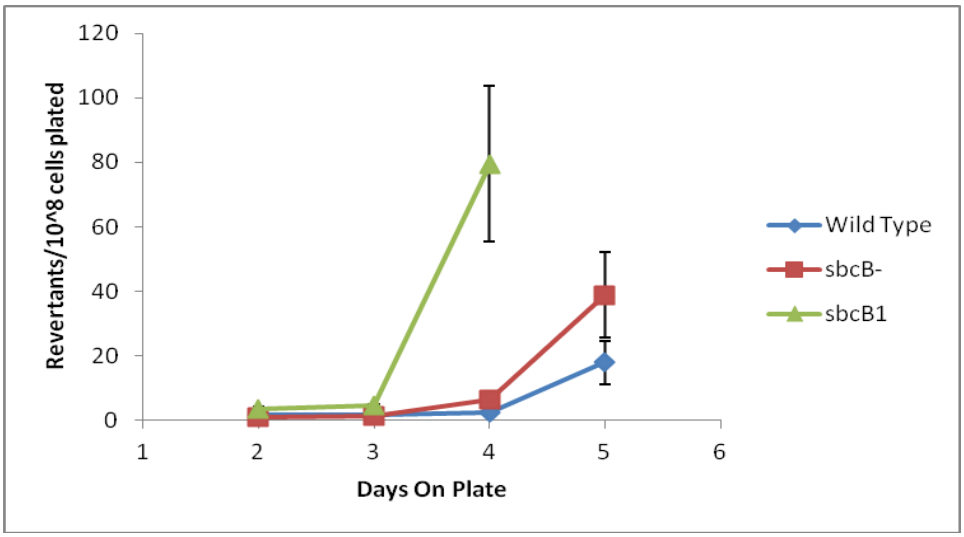

Figure 8 Effect of mutations in *sbcB* on the lactose assay.

The results of the lactose assay implicate nucleases as inhibitors of the process of adaptive mutation. The ~2-fold increase in reversion rate seen in the *sbcB* deletion strain can be attributed to the fact that in vivo this strain does not have its most processive nuclease and 3' ssDNA ends are therefore degraded less quickly. The ~16-fold increase in reversion of the *sbcB1* strain can be attributed to the fact that not only is SbcB not degrading the 3' ends, but other nucleases are unable to do so because of protection by SbcB1.

Furthermore, we hypothesized that these preserved 3' ends can now be used for generating the HCNVs described in Figure 7. We tested the hypothesis with quantitative PCR. Quantitative PCR allows for determination of the relative copy number of a locus in a population of cells. DNA was extracted from the standard LT2 strain and *sbcB1* mutant, and the copy number of two loci were compared to the standard K12 strain. *lacZ* and *traG* are sites found on opposite sites of the F'128 plasmid. Thus, we can examine whether the entire plasmid or just a portion of it is increased in copy number. The *sbcB1* mutant appears to have a 2.5-fold increase in the average copy number of the plasmid in the population of cells assayed (Figure 9). This increase suggests that *sbcB1* generates cells with increased plasmid copy number, which explains its improved efficacy in adaptive mutation and provides support for the amplification model.

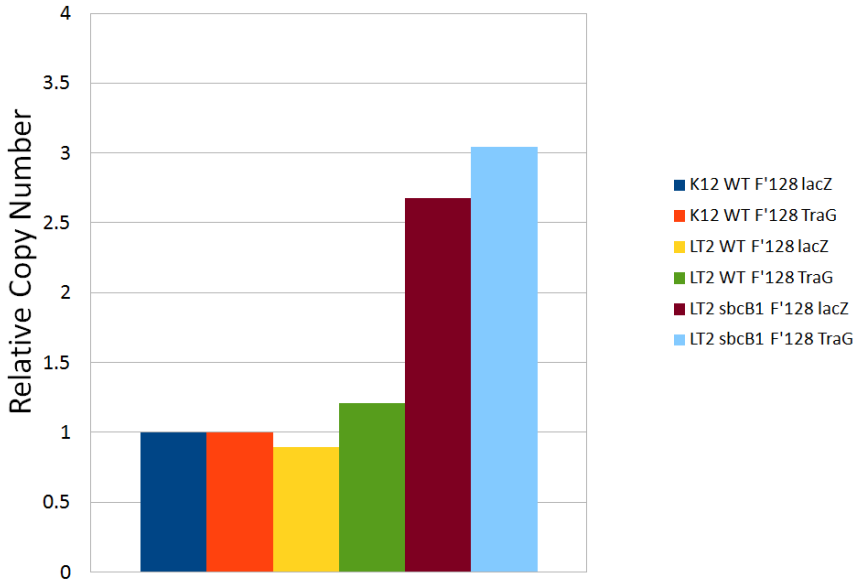

Figure 9: Quantitative PCR of the *traG* and *lacZ* loci of F'128 in the *sbcB1* mutant strain as compared to wild type.

In order to understand the exact role of SbcB in the reversion process, genetic analysis of the *sbcB* mutations with other essential components was conducted. The *sbcB* mutations were combined with mutations in *sbcCD*, *recA*, *recBC*, and *traI*. *sbcCD* encodes a nuclease that cleaves hairpin structures formed in palindromic DNA sequences, while also functioning as a 3'-5' dsDNA exonuclease (13). Therefore, in a *sbcB* mutant, it can still degrade the 3' ends. In the lactose assay, the *sbcCD* mutation increases the reversion rate by ~2-fold in strains with mutant *sbcB* genes, suggesting that SbcCD can function to degrade 3' ends in the absence of SbcB (Figure 10). However, the *sbcCD* mutation does not increase reversion rate in the strain with a wild type *sbcB* gene because SbcB is dominant to SbcCD as the main exonuclease to act on 3' ends.

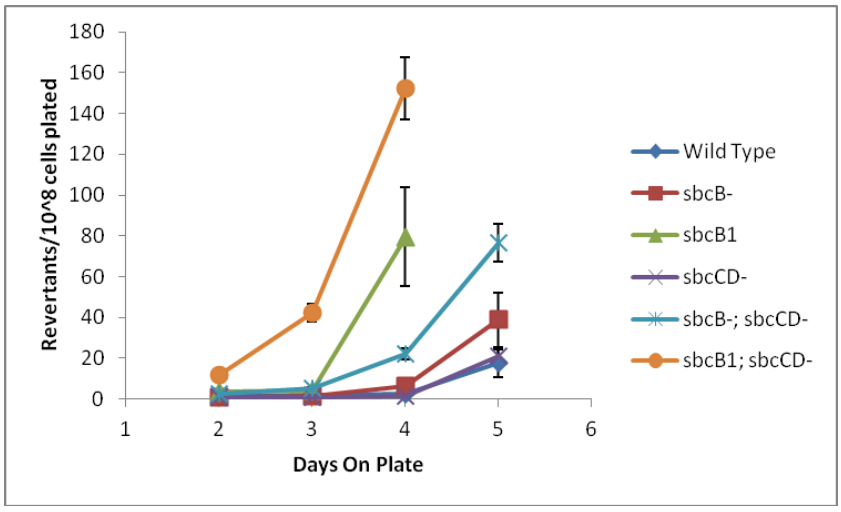

Figure 10: Role of *sbcCD* in adaptive mutation, when combined with *sbcB* mutations.

In examining the interaction of SbcB with other crucial adaptive mutation proteins, it is evident that the increase in reversion rate seen with a *sbcB1* mutation is fully suppressed by mutations in *recA* and *recB*, and partially by a mutation in *traI* (Figure 11 a, b, c). The dependence of *sbcB* on *recA* and *recB* suggests that SbcB functions in the same pathway as RecA and RecB. However, the result that *sbcB* is only partially dependent on *traI* may suggest that there may be another function that mimics that of TraI. That is, there may be another endonuclease that can generate the nick necessary. The role of such a function may be insignificant in the standard strain, but becomes pronounced with a *sbcB1* mutation because SbcB1 may prolong the lifetime of the products of this weakly active function. A complete model of the role of SbcB is shown in Figure 12.

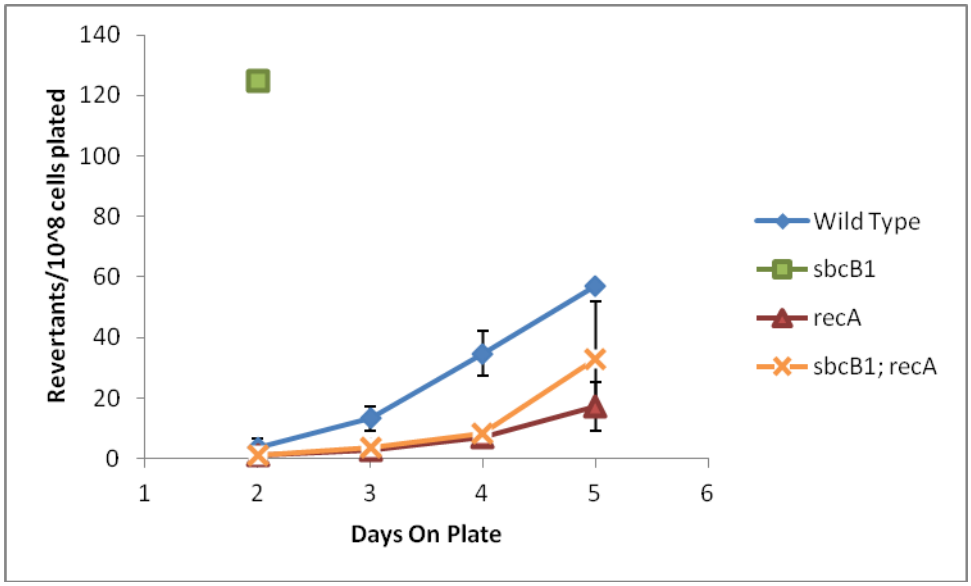

Figure 11a Role of *recA* in fully suppressing the effect of the *sbcB1* mutations in the lactose assay.

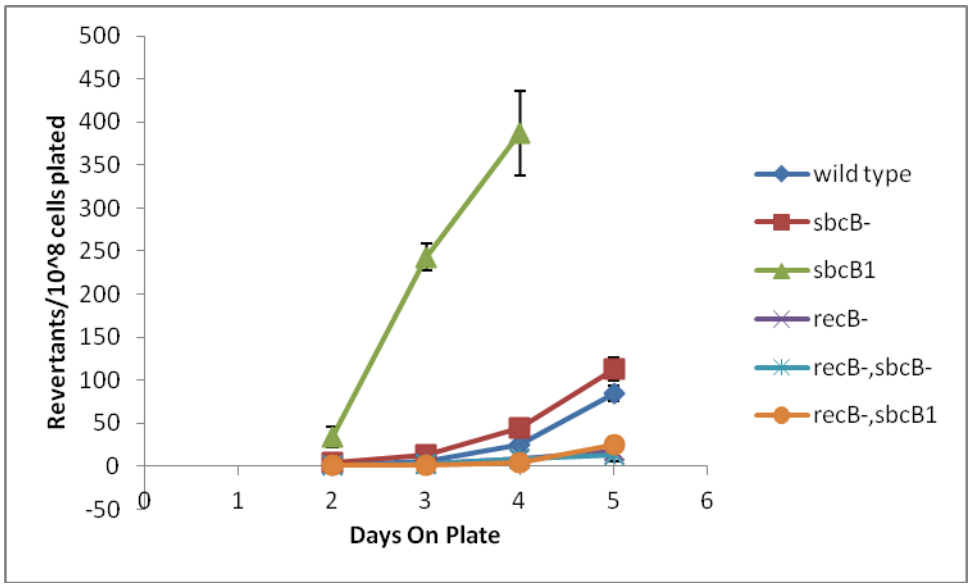

Figure 12: Role of *recB* in fully suppressing the effect of *sbcB* and *sbcB1* mutations in the lactose assay.

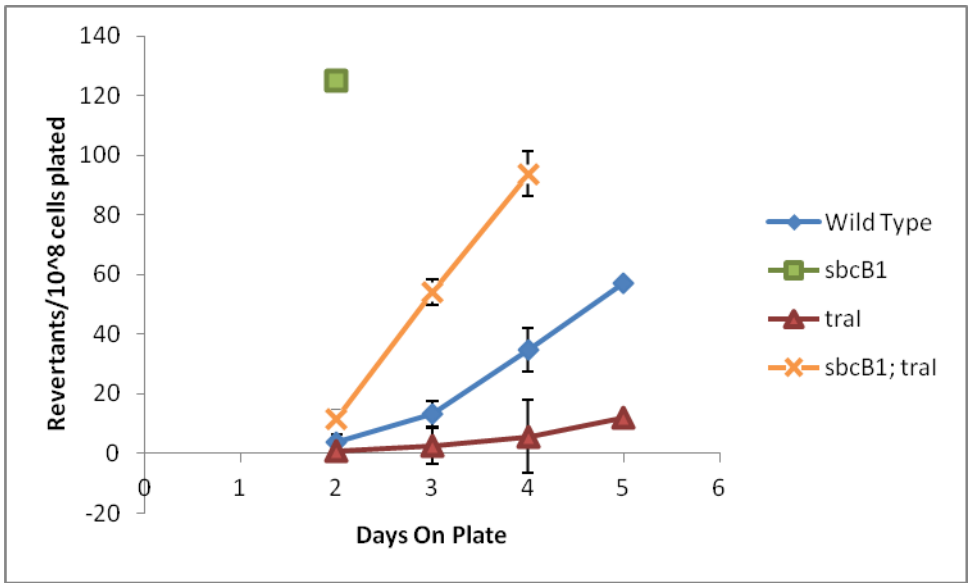

Figure 13 Role of *tral* in partially suppressing the effect of the *sbcB1* mutation in the lactose assay.

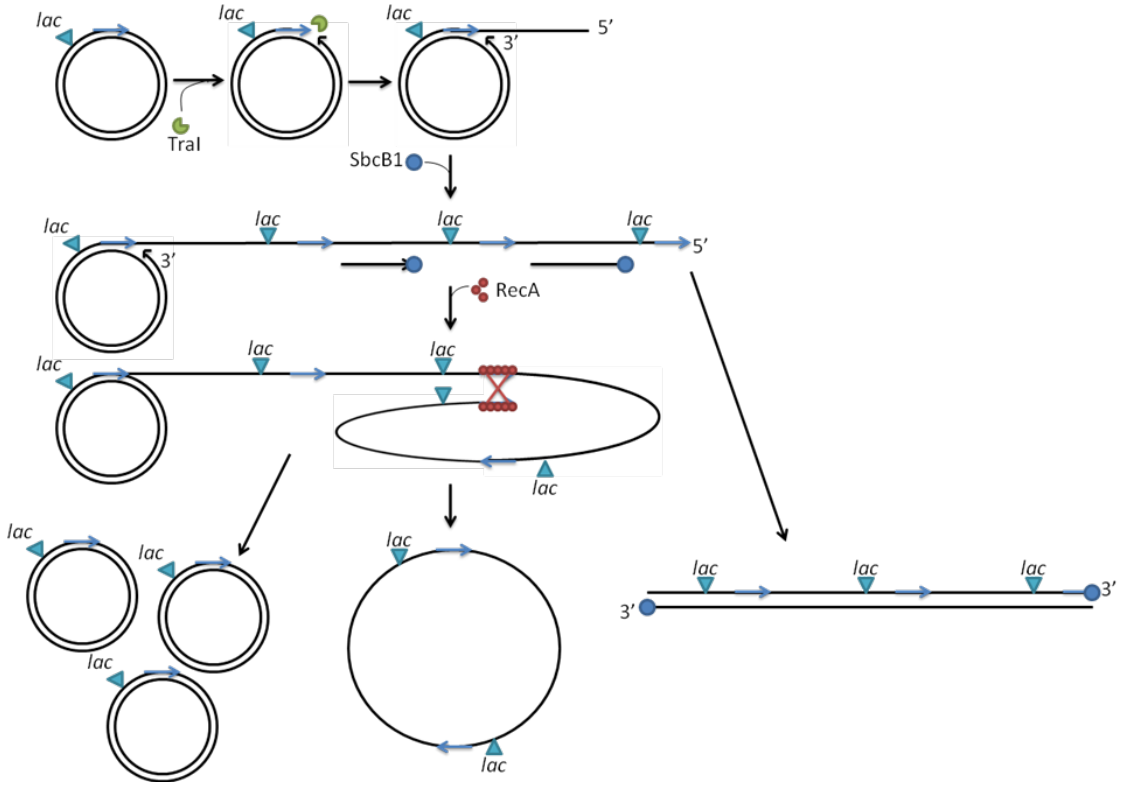

Figure 12: Model for the role of SbcB1 in adaptive mutation. Tral (green pacman) initates the process by generating a nick at a specific locus. The 5′ end is spooled out and used as a template for generating linear copies of the plasmid. The 3′ end generates a replication fork that replicates the plasmid as the 5′ end is spooled out. SbcB1 functions in protecting the 3′ ends of the linear copies from degradation by other nucleases. These copies are then able to form multiple plasmids, plasmid dimers or multimers, or linear multimers.

Temperature Sensitivity Of Adaptive Mutation

The mechanism by which adaptive mutations accumulate in the Cairns system appears to be temperature-dependent. That is, the number of revertants formed decreases approximately 10-fold at high temperature (Figure 13). A past study attributed this observation to the role of the elongation factor, NusA, in stress-induced mutagenesis (16). However, the evidence presented was not definitive, and we have developed alternative hypotheses.

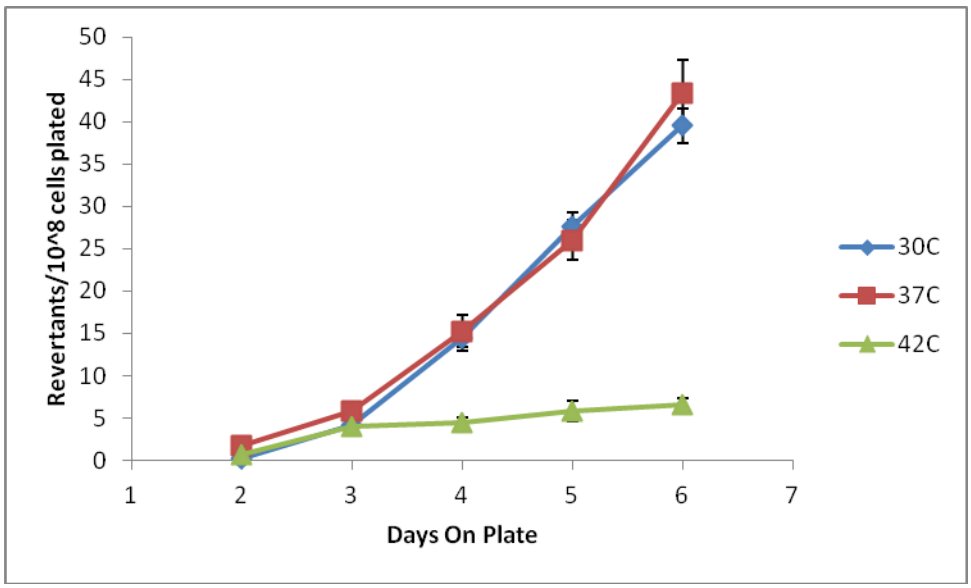

Figure 13: Temperature sensitivity of the lactose assay in *Escherichia Coli* K12 Cairns strain.

Temperature Sensitivity of the Melibiose Permease

The first hypothesis attributed the temperature sensitivity of the Cairns assay to the temperature sensitivity of the MelB permease--a transporter of melibiose, an ortholog of lactose--which is functional at 30°C but not at 42°C (17,18). MelB is also able to transport lactose into the cell (18); this activity may be able to suppress the transport defficiency in LacY--the normal lactose permease--due to the the polar effect of the +1 frameshift mutation. Ribosomal frameshifting explains the leakiness of the *lac-* allele, but it also suggests that *lacY* is likely expressed at a lower level than *lacZ* because of polarity in translating downstream genes in an operon. Therefore, insufficient lactose is transported into the cell to meet the potential of LacZ metabolism.

Furthermore, if growth under selection is essential for the formation of HCNVs, then an increase in the input of lactose into the cell may lead to improved adaptive mutagenesis.

In order to test the hypothesis, mutants of *melB* were constructed and were tested in the lactose assay where temperature was changed . A *melRAB* operon deletion was constructed by linear transformation and two *mel+* mutants (non-temperature sensitive) were positively selected on minimal melibiose plates grown at 42°C (restrictive temperature). In the lactose assay, all mutants formed few revertants at 42°C (Figure 14c). Moreover, the strains reverted normally at 30°C and 37°C, suggesting that the temperature sensitivity of the MelB permease plays no role in adaptive mutation (Figure 14a,b).

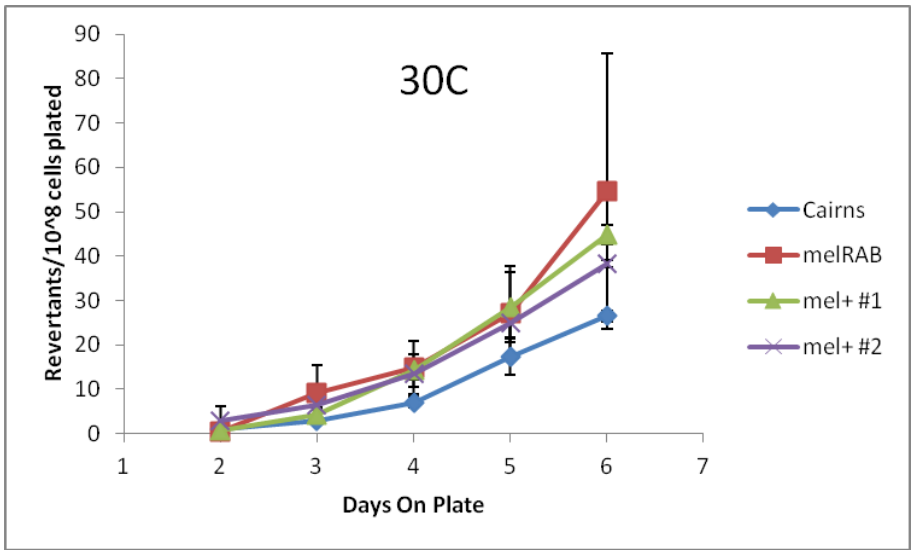

Figure 14a: Reversion of melibiose mutants at 30°C in the lactose assay. *mel* genes do not seem to play a role in the temperature sensitivity of the lactose assay.

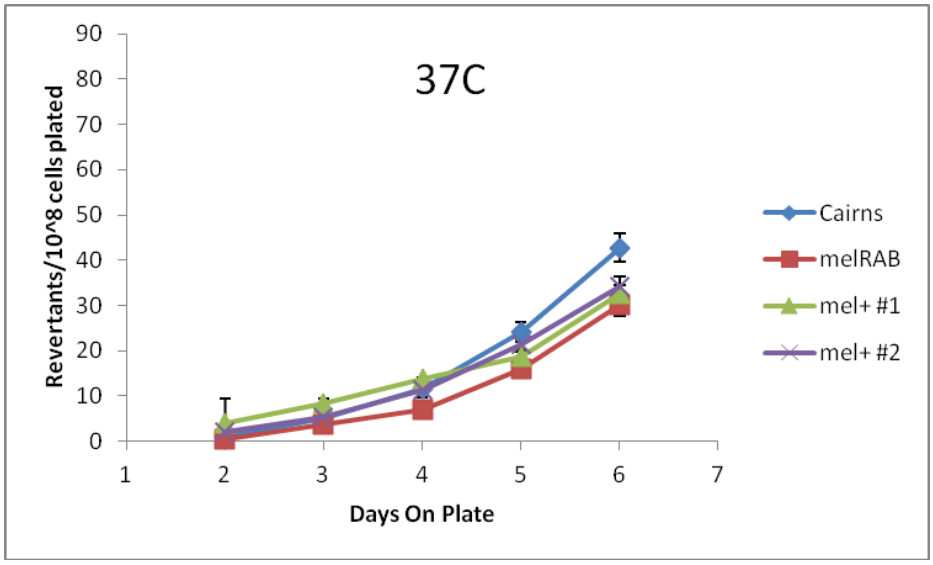

Figure 14b: Reversion of melibiose mutants at 37°C in the lactose assay. *mel* genes do not seem to play a role in the temperature sensitivity of the lactose assay.

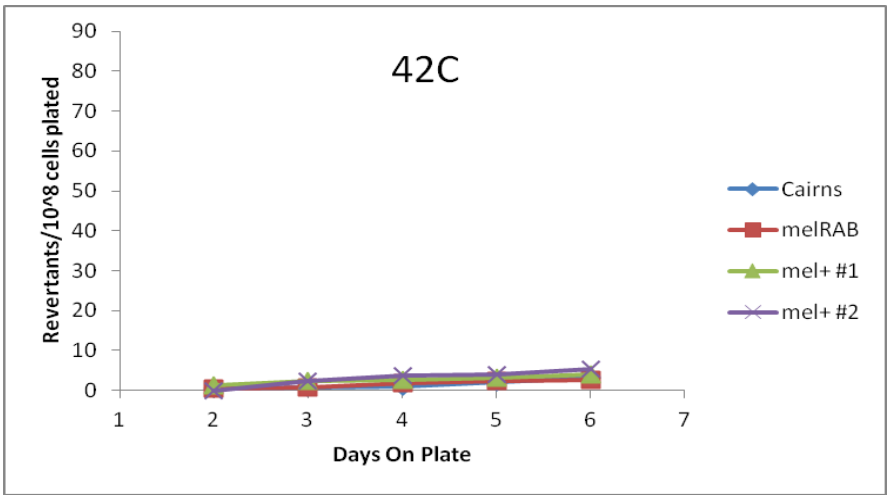

Figure 14c: Reversion of melibiose mutants at 42°C in the lactose assay. *mel* genes do not seem to play a role in the temperature sensitivity of the lactose assay.

Temperature Sensitivity of Conjugative Transfer Functions

The second hypothesis explaining the temperature sensitivity of the Cairns assay was based on observations seen in three different assay systems: standard *E. Coli* K12 strain, standard *Salmonella typhimurium* LT2 strain, and LT2 strain lacking the pSLT plasmid. The K12 strain has constitutively expressed F'128 transfer functions due to a natural mutation in *finO* (repressor of *tra*) of the F'128 plasmid (Figure 2). The standard LT2 strain (pSLT+) has repressed F'128 transfer functions due to a repressor found on the pSLT plasmid (5); thus, the pSLT- LT2 strain also has constitutive transfer functions.

Lactose reversion experiments were conducted for each of these three strains at 30°C, 37°C, and 42°C. Interestingly, the strains with constitutive transfer functions (although different species altogether) appear to produce revertants using some process favored at low temperature (Figure 15a,c). On the contrary, the *tra* repressed pSLT+ strain appears to produce adaptive mutants using a process favored at high temperature (Figure 15b).

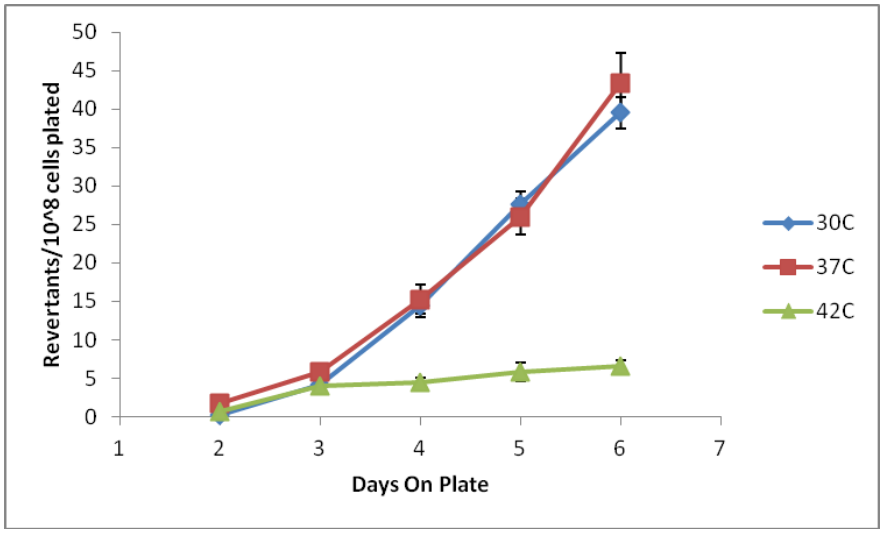

Figure 15a: *Escherchia Coli FinO-* constitutive *tra* strain.

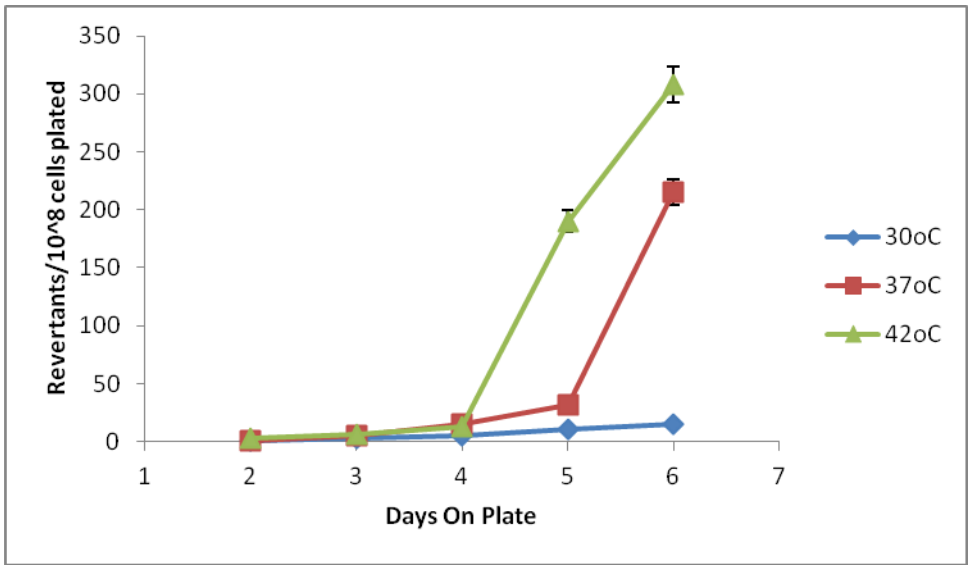

Figure 15b: Salmonella Typhimurium pSLT+ repressed tra strain.

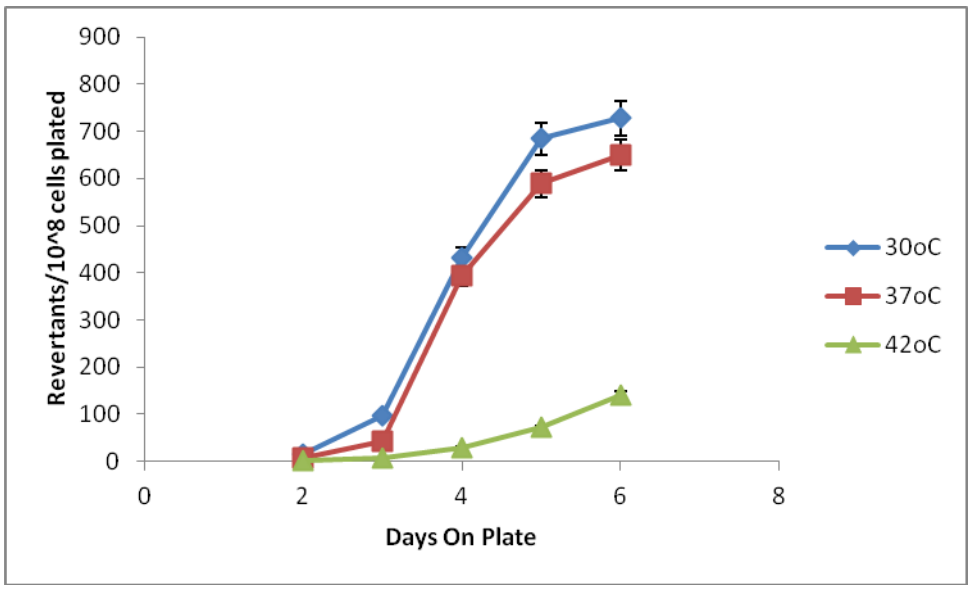

Figure 15c: *Salmonella Typhimurium pSLT – de*-repressed constitutive *tra* strain.

Thus, it appears that the temperature sensitivity of lactose reversion may be due to temperature sensitivity of some transfer function. Both models for adaptive mutation suggest that the TraI endonuclease initiates reversion by nicking the plasmid DNA (5,7). Thus, we hypothesize that TraI expression is temperature sensitive. Future work will aim to directly test this hypothesis. We will investigate whether conjugation (dependent on *tra* functions) is temperature sensitive by performing a quantitative conjugation assay at multiple temperatures. Then, we will attempt to complement the temperature sensitivity of the lactose reversion assay by placing *traI* on an expression vector.

Role of Temperature in the Pre-Growth and Plate on Adaptive Mutation.

Understanding the basis of the temperature sensitivity of the lactose assay is important. But this sensitivity to temperature can also be used as a tool to test the validity of the SIM model. The SIM model suggests that all mutants arise in response to stress on the selection medium (7). Therefore, the conditions of the pre-growth medium should not influence adaptive mutation. Furthermore, the temperature sensitivity of the reversion process provides a useful condition that can be varied between the pre-growth culture and the plate to investigate the process of adaptive mutation. This assay will demonstrate whether the adaptation is due to some process in the pre-growth or on the selective plate. To test the hypothesis, lactose reversion assays were carried out in which temperature was varied between the pre-growth and plate (Figure 16). The results demonstrate that the temperature conditions in the pre-growth medium do not affect the reversion rate nor the process of adaptive mutation. Instead, the process is affected by the selective conditions of the plates.

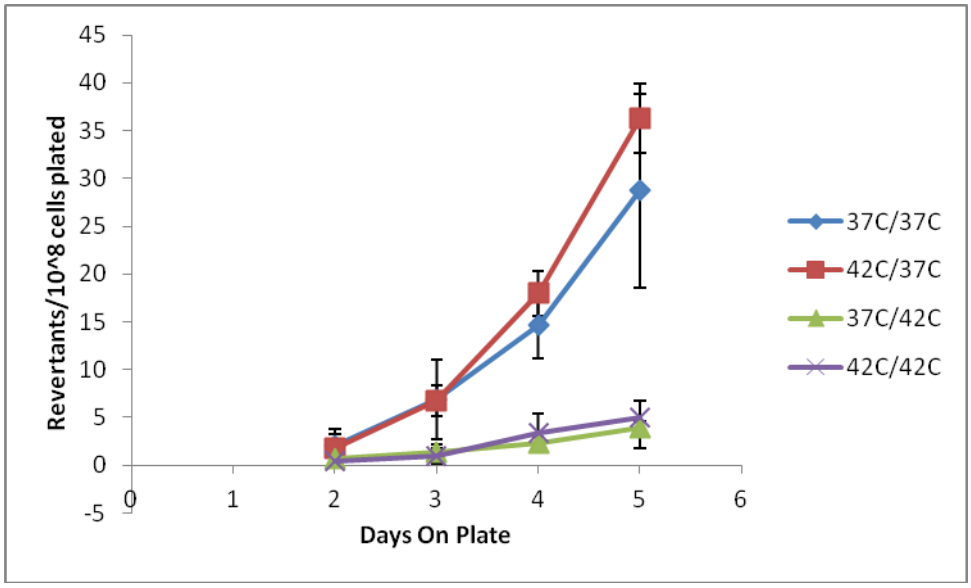

Figure 16: Role of the pre-growth or plate on the formation of revertants. The temperature of the plate appears to influence the process of adaptive mutation, with no regard to the temperature in the pre-growth.

Conclusion

Natural selection requires the continuous production of new mutants to produce a variety of individuals whose reproductive success is tested under various growth conditions. The general process of natural selection is well understood and a great deal is known about the nature of mutations that arise in the lab. However, a continuing debate has surrounded the process by which mutations arise during growth under selection. Classical experiments in *Escherichia coli* demonstrated that the large-effect mutant clones detected in the lab by strong selection are due to mutations that actually arose during previous non-selective growth. In many biological situations, however, selection is less stringent, and under these conditions, mutants appear to be more common. This has suggested the idea that growth limitation might be mutagenic (stress-induced mutagenesis). In one bacterial system used to study this question, mutant cells are unable to use lactose as a carbon source because they carry a +1 frameshift *lac* mutation on their F' plasmid. When these cells (10^8) are plated on lactose medium, they cannot grow, but give rise to Lac+ revertant

colonies that appear at a rate nearly 100-fold higher than the reversion rate during non-selective growth. This apparent increase in mutation rate has been explained in two alternative ways. (1) A stress-induced mechanism creates random mutations in non-growing cells. (2) Small-effect mutants that are weakly Lac+ form at a high rate under all growth conditions. Such cells grow slowly on lactose and acquire secondary mutations that make them fully Lac+. This adaptation can occur without mutagenesis because of the many cells within a growing colony. We have found that mutants of *sbcB* (exonuclease I) stimulate reversion by preserving 3' ssDNA ends, which we believe are essential to the formation of secondary mutations. Furthermore, we have found that the process of producing fully Lac+ cells in this system is temperature-sensitive in that 10-fold fewer revertants arise at 42°C than at 30°C. The produced mutants grow well at both temperatures regardless of where they formed. We are investigating the effect of temperature to see if it limits mutagenesis. We are testing the ability of the plasmids to replicate and the ability of the strain to generate amplifications and form frameshift mutations as a function of temperature. Furthermore, we have described a central role for nucleases in adaptive mutation. Further experiments will determine the exact role of SbcB in mutagenesis. We hope to be able to resolve this controversy regarding mutation under selection.

Materials and Methods

Strains.

All strains used for the *sbcB* experiments are derivatives of *Salmonella enteric* (serovar Typhimurium) strain LT2. Throughout, plasmid F'128 refers to a derivative carrying a triply mutant *lac* allele that includes a deletion fusing the *lacI* and *lacZ* genes, a mutation improving the *lacI* promoter (IQ), and a +1 frameshift mutation (*lacI33*).

Media and Chemicals.

Rich medium was Luria broth (LB; Difco), and minimal medium was NCE-lactose. 5-Bromo-4-chloro-3-indolyl -D-galactopyranoside (Diagnostic Chemicals, Oxford, CT) was used at 25 ug/ml in minimal medium to observe colony formation.

Construction of the melRAB Mutation by Linear Transformation.

The recipient *E. coli* K12 strain carried plasmid pKD46, which encodes recombination functions of phage lambda (*red*, *gam*, and *exo*) expressed from an arabinose-inducible promoter. The chromosomal *melRAB* gene of *S. enterica* was replaced by a Kan^R gene expressed by the *cat* gene promoter of pACYC184.

Lac Reversion Assays.

Strains were pregrown in NCE glycerol with necessary supplements, washed in NCE medium, and plated (2 x 10^8 cells) with a 10-fold excess of Lac- scavenger cells on NCE lactose medium containing 5-bromo-4-chloro-3-indolyl -D-galactopyranoside and needed amino acids. Scavengers consume carbon sources other than lactose and prevent growth of the reversion tester strain. Plates were incubated 5–7 days at 30°C, 37°C, or 42°C, and revertants (Lac+) were counted daily. Plotted numbers are the mean number (with standard deviation) of revertants per 10^8 cells plated.

Acknowledgements

I would like to thank Distinguished Professor John Roth for his mentorship and for enabling me to pursue this project.

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