# DIRECTED MUTATION:
# ARE MUTATIONS ALWAYS SPONTANEOUS AND RANDOM?

by

Tricia Ann Len

Submitted in Partial Fulfillment of the Requirement

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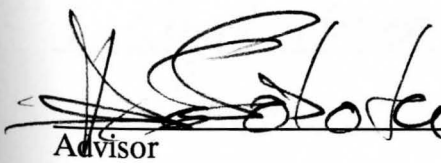

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# ABSTRACT

Directed Mutation: Are Mutations Always Spontaneous and Random?

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Master of Science

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The term directed mutation describes those mutations occurring as a specific response to stress. *Escherichia coli* strain K12, lac-(i-, z-) was used in this study. Overnight cultures were plated on MacConkey's basal media or MacConkey's basal media plus lactose. Revertants to lac+ which occur within two days of plating are considered to be due to phenotypic lag. Revertants produced after two days, which can be observed as red papillae on larger white colonies, are considered to be the result of mutations occurring after plating. The data shows an increase in the number of lac+ revertants growing in the presence of lactose. It has been suggested that this observed increase is due to transcriptional bias. This hypothesis suggests that during transcription, the DNA of the lac operon becomes single-stranded and more vunerable to mutation. In the strain we are using there is a mutation in the *lac I* gene that allows constitutive transcription of the operon and thus the DNA in this region is continually in a single-stranded state. However, with this strain, we find an increase in revertants only in the presence of lactose suggesting that another mechanism may be operating in this operon.

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# CHAPTER I

## INTRODUCTION

Salvatore Luria and Max Delbruck (1943) published a manuscript on mutation theory which is currently considered to be the birth of bacterial genetics. Evaluation of their experimental evidence led them to the conclusion that mutations are of a random, spontaneous origin and furthermore to reject the thought that mutations might be caused by some outside pressure or physiological change. To support their conclusions they used a strain of phage sensitive bacteria which was found to mutate to resistant cells. Exposure to the phage particles produced no overall effect on the mutation rate. They suggested that mutation in bacteria to a resistant phenotype occurred in culture spontaneously before the cells were exposed to the phage particles. At the time this manuscript was published, little was known regarding the molecular mechanisms since the structure of DNA had not yet been determined. Although Luria and Delbruck limited their conclusions to their experimental system, their work has been considered as the basis of mutational theory and remained primarily unchallenged for nearly half a century. This was due to the neo-Darwinian belief that mutations were spontaneous, random and independent of natural selection. This mindset was held firmly by most biologists. Evelyn Fox Miller (1992) chose two quotes in a 1992 paper that typified this mindset. The first was from Francis Crick

(1957):

> once 'information' has passed into the protein *it cannot get out again*. . . . In more detail, the transfer of information from nucleic acid to nucleic acid, or from nucleic acid to protein may be possible, but transfer from protein to protein, or protein to nucleic acid is impossible.

The second observation came from Jacques Monod just a few years later which stated molecular biology has proven

> beyond any doubt but in a totally new way the complete independence of the genetic information from events occurring outside or even inside the cell--to prove by the very structure of the genetic code and the way it was transcribed that no information from outside, of any kind, can ever penetrate the inheritable genetic message (Judson,1979).

Luria and Delbruck's (1943) results were based on statistical inferences, and have become known as the Luria-Delbruck distribution. This distribution indicated that the utants, when present, occurred as a result of rare spontaneous mutations in culture before selection. This was based on the observation that the number of mutants varied from culture to culture, some of which occurred early in culture yielding a large number of mutants, while other mutations occurred later with a smaller distribution of mutants. In addition, they examined the possibility that mutations might arise in direct response to the presence of the phage, but they found no evidence to support this assumption. This distribution is known as a Poisson distribution where the mean equals the number of bacteria times the probability that a cell will become resistant. From this statistical data and their experimental results, they concluded that mutations conferring resistance to the bacteria arose independently of the action of the virus. Thus, random and spontaneous mutations may occur in a culture prior to exposure to the virus and subsequently render those cells and their progeny resistant. The presence of the virus is not necessary nor in

any way affects this process.

For the most part, it seemed the issue had been settled. In the words of Joshua Lederberg, "many, perhaps most, readers of the 1943 article did not understand its abstruse mathematical argument and would respond either with uncritical acceptance or uncritical rejection" (Lederberg,1989). It appeared to put to rest any lingering theories of Jean Baptiste de Lamarck dealing with the "inheritance of acquired characteristics" (Jordanova,1984). It was not until nearly half a century later that anyone truly challenged this theory.

In the mid 1980's, John Cairns, working in the Department of Cancer Biology at the Harvard School of Public Health, was interested in determining the cause of mutations resulting in cancer. After some initial work, he felt that some mutations may result from the influence of environmental factors.

In their control investigations it appeared that Luria and Delbruck had tested the effects of physiological changes on an organism. However when they searched for mutants caused by exposure to the virus, they may have been killing off those that had mutated due to the presence of the virus. Currently we have a better understanding of this mechanism. The mechanism by which a phage can kill a bacteria cell involves the binding of the phage to the surface of the cell through the recognition of a surface receptor. The phage DNA is then injected, ultimately killing the cell. For bacteria to become resistant to a virus, its genotype may be altered in order to prevent production of surface receptors for the virus. Although a cell may have mutated genotypically from sensitive to resistant, it may still be killed off because cell surface receptors may remain due to phenotypic lag thus allowing the

phage to gain entrance to the cell and terminate its existence. The cell will exhibit phenotypic resistance only after several generations when the surface receptors for the phage are sparse, but in Luria and Delbrucks work these cells were killed off before this could be shown to have happened (Goodman,1992). Luria and Delbruck were unaware of the consequence of phenotypic lag and therefore unknowingly may have misinterpreted their data.

With the advantage of this insight, Cairns re-examined the experiments of Luria and Delbruck in an attempt to determine the true role of an organism's environment on its survival. This was accomplished by, as Cairns put it, giving the organism something to "worry" about rather than killing it off before it has a chance to do anything about the situation. In order to achieve this, Cairns and his colleagues needed to stress the bacteria without killing them thus providing sufficient time to respond to their situation and successfully adapt. To do this , they chose a strain of *Escherichia coli* with an amber mutation in the *lac Z* gene producing a *lac-* phenotype, that is, the strain was unable to use lactose as a energy source. The bacteria were plated on a medium containing lactose as the sole carbon source for energy. It was found that more revertants accumulated in the presence than in the absence of this pressure. Using this same strain, Ryan found that the distribution of mutants was more compressed than would be expected for spontaneous mutations as described by Luria and Delbruck (Ryan,1952). Ryan (1955) distinguished between mutations occurring in exponential growth and those occurring in stationary phase. From this data he concluded that "mutations can take place in non-dividing bacteria," another oversight of Luria and Delbruck. In 1949, Lea and Coulson published tables for

**the Luria-Delbruck distribution. According to these calculations, the Poisson distribution is expected to be less than that of Luria-Delbruck. Cairns determined the distribution found in Ryan's original paper to be a combination of the Poisson and Luria-Delbruck distributions. This suggested that in addition to rare, spontaneous mutations in a growing culture, other mutations can occur in response to pressure. They also tested for the accumulation of a second neutral mutation. They noted that the pressure did not cause an accumulation of valine resistant mutants in the *E.coli* strain and therefore the pressure probably did not cause a general overall increase in mutation events (Cairns et al.,1988).**

**In addition to these experiments, Cairns and his colleagues also investigated other systems to support his theory of "directed" mutation. It had been demonstrated that a Mu segment separating two genes, *ara C* and *lac Z* (for the arabinose and lactose operons , respectfully), can be excised enabling the bacteria to grow on lactose in the presence of arabinose. This produced a *Lac(Ara)+* phenotype (Shapiro,1984). Using this system Cairns determined that *Lac(Ara)+* cells did not accumulate in the presence of only one of the two required nutrients in lag phase. While in stationary phase, in the presence of arabinose, the *Lac(Ara)+* cells began to accumulate after lactose was added. Again it was demonstrated that accumulation of mutants does not occur in the absence of pressure. Additionally Cairns and his colleagues demonstrated directed mutation under more natural means of selective pressure, where several changes in base sequence were required to obtain a primary mutation. This mutation was found to occur more frequently under selective pressure. These results lend more support to his theory, for:**

**it is difficult to imagine how bacteria are able to solve complex problems like these--and do so without, at the same time, accumulating a large number of neutral and deleterious mutations--unless they have access to some reversible process of trial and error (Cairns et al., 1988).**

**Cairns did not set out to disprove the neo-Darwinian theories, his intention was to, as he put it "show how insecure is our belief in the spontaneity (randomness) of most situations" (Cairns et al.,1988).**

**Lamarck, although he did not originate the theory, was the first to include inheritance of acquired characteristics in his evolutionary theory (Burkhardt,1977). He was highly criticized for his beliefs but most seem to believe that this was due more to his unorthodox attitude and methods for that time period, rather than actual scientific findings. Many think that Lamarck has long since been discredited, but Cairns and his colleagues appear to have initiated what has been termed a neo-Lamarckian revolution. This is not to say that Darwin was wrong in his theories but instead maybe a new chapter must be added to the story.**

**One of Cairns' most published supporters was Barry Hall at the University of Rochester. In a article published simultaneously with Cairns', Hall pointed out that "we have ignored the possibility that mutation rates might be highly variable and subject, like other cellular processes, to environmental modulation" (Hall,1988). In this paper he worked with a strain of *E.coli* which required two mutations in the ß-glucosidase operon in order to utilize salicin. This was accomplished by excision of an insertion sequence. He reported that excision was not advantageous to survival of the organism nor was the first of the two mutations to occur on selective media. The second mutation, however, would provide the organism with a means to increase its survival. He did state that:**

> this has the appearance of anticipatory evolution, a phenomenon that would itself be strongly selected, but for which it is very difficult to imagine a mechanism. Despite appearances, it is not necessary to invoke anticipatory evolution to explain these results.

But, he continued to emphasize that it was important " to examine the notion implied by these results, that mutation, like other biological processes, is subject to regulation by environmental factors" (Hall,1988).

Hall cited Cairns' work and suggested that this might explain the observations of Ryan, in which he demonstrated that mutation rates of growing cells were different in "suboptimal" conditions. Hall (1988) also sought to explain Shapiro's (1984) studies, the rates of Mu excision on rich vs. selective media, in the same way.

Hall's (1989) initial attempt to study this phenomena came when he reported that a reversion of a mutation for methionine biosynthesis occurred more frequently under selective conditions than under nonselective conditions in growing cells. He also found that methionine starvation, the selective mechanism he employed, did not cause a general increase in other nonadvantageous mutations. He suggested that these observations supported the work of Cairns. He notes that the classical experiments did not address the possibility of an organism having some means of directing advantageous mutations under selective conditions. The other types of mutation that exist are those which are spontaneous and random which produce a means of selection whose purpose is only to increase or decrease the "livelihood" of a mutation in subsequent generations. Hall also noted that the mutation rates as calculated by Luria and Delbruck apply only to growing cultures, and not those occurring under selective conditions. It was therefore concluded that enough evidence

has been presented to "warrant thorough and systematic investigation."

Another set of experiments supporting the theory of directed mutation was conducted by Hall (1989). In this paper, Hall indicated that mutations occur more frequently when they are advantageous. For these experiments he chose a strain of *E.coli* that contained a mutation in a gene requiring tryptophan in the medium for growth. He found that point mutations in the tryptophan operon occurred more frequently when the cells were starved for tryptophan than with tryptophan present; the previous being more advantageous to the cells survival. He also noted that the rate of reversion to tryptophan synthesis was not found to increase when the cells were starved for cysteine. This indicated that the increased rate of mutation in the tryptophan operon was specific to that advantageous mutation (Hall,1990). In his conclusion he pointed out that Luria and Delbruck considered bacteria only in exponential growth rather than under more natural conditions which normally occur in the environment. Testing the mutation rate of cells under starvation or stress conditions is more representative of the natural state. This challenge to the central dogma of molecular biology caused quite a controversy. However, Cairns and his supporters were not attempting to replace spontaneous, random mutations as a mechanism to explain evolution but to recognize the possibility that other mechanisms may be operating (Goodman,1992). The most compelling criticism came from Mittler and Lenski. They challenged Cairns' results on Mu excision. They repeated Shapiro's experiments but suggested another interpretation (Mittler and Lenski,1990a). With a slight variation in technique, they found that lactose and arabinose did not act to selectively induce mutations but that in general these starving cells increased Mu excision. Their interpretation

supported the view that physiological stress was the cause of Mu excision.

In a manuscript appearing in *Nature*, Cairns (1990) stated that: "we could not find fusion in liquid, stationary phase cultures until arabinose and lactose were added" and went on to give suggestions for the discrepancy such as differences in *E.coli* at different times of the year which may be due to changes in water supply. In a reply appearing with Cairns' rebuttal, Mittler and Lenski reaffirmed their results and pointed out that, in addition to other support, they included essential information in their paper which Cairns' lacked. This information included:

> (1) explicit laboratory methods; (2) experimental designs with appropriate controls; (3) the level of replication performed for each experiment; (4) the computational method used to estimate mutation rates from directly observable data; and (5) inferential statistics to guide the rejection of acceptance of competing hypotheses (Mittler and Lenski, 1990a).

In another publication, Mittler and Lenski (1990b) took issue with some of the conclusions of Hall. Their criticism involved the circumstances by which excision of a sequence can be accomplished (the first mutation in Hall's double mutants). They reported that this mutation can occur without selective pressure for the second mutation, which would be advantageous to survival. This contradicted the conclusions of Hall (1988). They failed to address the suggestion that there was no advantage for the first mutation occurring more frequently under selection except to lay the ground work for the second mutation. This, in turn, would increase survival of the cell. They also reported a mechanism for their findings which Symonds (1989) first suggested and Lenski et al. (1989) supported. This mechanism called for the formation of mutagenic intermediates which would allow selection to play a role.

Along with the criticisms came other possible explanations, which attempted to make these mutations consistent with neo-Darwinianism. Boe (1992) and Stahl (1988) separately supported a mismatch repair system explanation for Cairns' observations. Stahl suggested that in starving cells which are in stationary phase the mismatch repair system (which functions in stationary phase) is slowed down due to the decrease in the available energy supply. If an error occurred that is not immediately corrected, it may prove to be beneficial to the cell and subsequently allow replication and thus the mutation would then become part of the genome. If an error was present that did not increase the cells viability under its present circumstances, the nonadvantageous mutation would eventually be repaired while the cell remained in stationary phase (Goodman,1992). Boe (1992) suggested that translational errors were the result of amino acid starvation which lead to an increased mutation rate and subsequently selectable mutants. He did, however, leave open the possibility for other explanations.

Stewart and his colleagues re-examined the work of Luria and Delbruck and suggested that deviations from the Luria-Delbruck distribution should not be held as evidence for directed mutation (Stewart et al.,1989). The reasons for this belief varied but the "key conclusion. . . is that almost any biological complication, such as mutants growing faster or slower than the parental strain, shifts the distribution toward the Poisson" (Goodman,1992).

Davis (1989) investigated a "non-Lamarckian" mechanism for Cairns' results. He suggested that single-stranded regions of the genome are more vulnerable to mutation. Substrates, which induce the single-stranded state, provide a "bias" for a mutation. He

calls this transcriptional bias. He noted that spontaneous mutations in stationary phase are the most important types of mutations in evolution since they most closely mimic the natural state. His conclusion is based on a suggestion of Hall that "physiological regulatory feedback loops could modulate the probabilities of mutations at specific loci when the cell is under stress" (Hall,1988; Davis,1989). Davis suggested a mechanism in which transcription is the indirect cause of mutation since it requires that DNA be single-stranded. According to this mechanism, single-stranded DNA is subject to more damage by mutagens than double-stranded DNA and therefore mutations occur more frequently in the area of the DNA. Thus there is the potential for advantageous mutations to accumulate maximizing the survival of a cell. He cited several studies which support vulnerability of single-stranded DNA.

Davis also examined the role of excision of transposons in directed mutation (1989). He cited two possible alternatives. The first states that during starvation, the repressor for the transposase is depleted an excision occurs rapidly. He also cites recent evidence that supports his transcriptional bias theory in cases of excision in which a copy choice mechanism was also involved. This mechanism requires terminal repeats of single-stranded regions on the transposable element on which transcriptional bias occurs leading to rapid excision. In his conclusion, Davis stated that clarity is needed in addressing this phenomena of directed mutation since, "we are dealing with environmentally induced, but not necessarily directed, mutations" (Davis, 1989).

This was the first publication which suggested a transcriptional explanation. Cairns et al. excluded transcription as a solution (Cairns et al.,1988). They used lactose and

**isopropyl ß-D-thiogalactoside (IPTG), both inducers of the lac operon, on *lac*- cells. They found that lactose, which also acts as a substrate for the *lac Z* gene, caused accumulation of revertants while IPTG, which is not a substrate for the gene but induces transcription, showed no apparent increase. Davis suggested that induction requires, in addition to an inducer, building blocks and energy. Lactose provides the low level of energy required for transcription and IPTG did not. He cited an experiment (Shapiro,1984) in which it was found that the addition of limiting concentrations of glucose with IPTG yielded an increase in the number of mutations.**

**The purpose of this study to examine the effect of lactose on a strain of *E.coli* which contains two mutations in the lac operon. The first mutation is in the *lac I* gene which is the repressor of the lac operon and which causes constitutive synthesis of the structural genes. The second mutation is found in the *lac Z* gene which is a structural gene coding for ß-galactosidase. An attempt will be made to determine if directed mutation is involved in the reversal of the mutation in the *lac Z* gene when this organism is cultured to stationary phase in the presence of lactose. Also, it will be determined if the addition of lactose to the medium has any effect on the reversal of the *lac I* gene in the same operon. This will be accomplished by comparing by direct count the number of mutations occurring under pressure in the presence of selection and absence of selection. A *lac*- strain of *E.coli* will be used in which revertants to the positive genotype can be identified by changes in the physical appearance of the colony. It will be determined if lactose applies selective pressure to starving cells to produce more revertants than starving cells without the lactose where the new genotype would not necessarily be advantageous to survival.**

# CHAPTER II

## MATERIALS AND METHODS

### Bacterial Strain

**The strain of bacteria used in this study is *Escherichia coli* K12 *lac-(i-,z-)F-*, which was purchased from American Type Culture Collection (Clowes et al.,1968).**

### Media

**MacConkey's agar base (17g Bacto Peptone, 3.0g Protease Peptone, Difco, 1.5g Bacto Bile Salts No. 3, 5.0g Sodium Chloride, 13.5g Bacto Agar, 0.03g Bacto Neutral Red, 0.001g Bacto Crystal Violet, deionized water to one liter) was purchased from Difco and prepared by adding 40 grams per liter of water. This was then autoclaved for 15 minutes and poured into petri plates (100x15mm) to a volume of about 20 milliliters. Liquid cultures were prepared in Trypticase Soy Broth (TSB) (17g Pancreatic Digest of Casein, 3.0g Papaic Digest of Soybean Meal, 5.0g Sodium Chloride, 2.5g DiPotassium Phosphate, 2.5g Dextrose, deionized water to one liter) to a concentration of 30 grams per liter.**

**Nutrient Agar was prepared by adding Agar to TSB. This medium was used to maintain cells in stationary phase and for cell counts.**

### Lactose

**When $\alpha$-D-lactose ($C_{12}H_{22}O_{11.H2}O$, MW 360.31, Eastman) was used it was added to the**

**agar to a final concentration of 1%.**

**Other reagents**

**X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) was prepared to a concentration of 2% in N,N-Dimethylformamide ($HCON(CH_3)_2$). Both were purchased from Sigma. This reagent was diluted 1:1 with sterile water in order to permit the solution to spread more evenly on the agar plates. This was accomplished by transferring 100 microliters to the plate with a micropipetor and spreading with a sterilized glass rod. Since X-gal is not very soluble in water the mixture was briefly vortexed before the sample was added to the plate. Plates were then allowed to dry until all the liquid was absorbed by the agar.**

**Growth of Cultures**

**Initially *E.coli* cultures were obtained by inoculating 20 milliliters of TSB and incubating at 37° Celsius for 18-24 hours with shaking. Stationary phase cells were maintained on MacConkey's base agar, MacConkey's base agar plus lactose or MacConkey's base agar plus X-gal at 37° Celsius.**

**Plating Cells**

**Liquid cultures were inoculated and allowed to grow overnight. These cultures were diluted before plating by a 1:100 serial dilution. To each of four microcentrifuge tubes one milliliter of sterile water was added. Ten microliters of bacteria was taken from the overnight culture and transferred to the first tube to start the serial dilution. Ten**

**microliters was used throughout the dilution series and that volume was also added to the appropriate agar plate and spread on the plate with a sterilized glass rod. Plates were then transferred to the incubator. The final dilution plated was $10^{-10}$.**

**Revertant Counts**

**Revertants to the *lac*+ genotype are determined in a similar way on each of the two types of plates. On the MacConkey's base agar plus lactose plates revertants are seen as red papillae on larger white colonies (Ryan,1952). Revertants on MacConkey's base agar plus X-gal appear as blue papillae. Revertants were counted and recorded for 14 consecutive days.**

**Testing for Double Mutants**

**The possibility of double mutants occurring under selective pressure was examined by plating purified revertant cells onto MacConkey's base agar plus X-gal. Double revertants would be seen as white colonies whereas single mutants in the ß-galactosidase gene only would appear blue. This distinction is due to the fact that X-gal is not an inducer of the lac operon, so if the repressor gene has reverted to *lac I*+, the ß-galactosidase gene would not be transcribed due to the absence of an inducer and X-gal will not be split, even though it is a substrate for the *lac Z* gene. However, a reverse mutation in only the *lac Z* gene would result in constitutive synthesis of this enzyme, splitting of X-gal and the formation of a blue colony.**

Cell Viability

To determine the number of viable cells in a culture, the method commonly employed is based on a serial dilution of the culture and plating aliquots onto agar plates. After the colonies begin to grow, they can be counted and the number of colonies equals the number of live bacteria per milliliter in the original culture times the dilution factor (CFU/ml). In this study the above technique was utilized with a slight variation to suit the conditions. To determine the number of viable cells per colony after plating on lactose, one colony grown on MacConkey's base plus lactose was cut out of the agar using sterile technique and placed in one milliliter of sterile water. This was vortexed until a smooth suspension was obtained. Ten microliters of the sample was serial diluted into one milliliter of sterile water. To determine the final dilution plated, it was necessary to determine the volume of the colony cut out of the original plate. This was accomplished by dropping small aliquots of water onto a piece of paraffin until the size of the original piece of agar cut from the plate was simulated. This volume (500 microliters) was assumed to be the volume of the first aliquot in the serial dilution. A final dilution of $5x10^5$ colony forming units per milliliter was plated on both MacConkey's base plus lactose and nutrient agar to determined cell viability.

# CHAPTER III

# RESULTS

## Revertants

Overnight cultures were diluted to $10^{-10}$ and were plated on MacConkey's base agar plus or minus 1% lactose and allowed to grow in the incubator at 37° Celsius. Colonies reached maximum size after two days of plating. Revertants were counted daily and results tabulated for a period of 14 days. Revertants appearing within 48 hours of plating are considered to be spontaneous mutants (Cairns et al.,1988). In the presence of lactose a total of 55 colonies on four plates yielded a total of 174 red papillae in 14 days (Table 1). Of these, no spontaneous mutants appeared since their were no papillae visible within the first two days. The number of papillae varied within the colonies but the average number of revertants (i.e. mutation events in the lac operon) per colony was 3.2 during starvation in the presence of lactose.

The revertants in the absence of lactose are tabulated in Table 2. X-gal was used on these plates as an indicator for revertants on MacConkey's base agar. A total of 56 colonies yielded 31 blue papillae. The average number of revertants here is 0.55 per colony. Figure 1 shows a graph summarizing the results of both these experiments.

A second set of experiments were performed. This data is summarized in Tables 3 and 4. The number of revertants on a total of 44 colonies grown on MacConkey's plus lactose after 14 days was 169, for an average of 3.84 revertants per colony. A total of 199 colonies grew on MacConkey's plus X-gal, with 134 papillae, with an average of 0.67 revertants per colony. It can be observed that the trends in both experiments were the same.

**Table 1.**

**Daily Total Revertants per Plate on MacConkey's plus Lactose**

| DAY | PLATE 1 | PLATE 2 | PLATE 3 | PLATE 4 | TOTALS |
|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 1 | 0 | 1 |
| 4 | 0 | 0 | 2 | 1 | 3 |
| 5 | 0 | 0 | 2 | 2 | 4 |
| 6 | 0 | 0 | 2 | 3 | 5 |
| 7 | 0 | 0 | 4 | 14 | 18 |
| 8 | 0 | 0 | 5 | 30 | 35 |
| 9 | 1 | 0 | 7 | 42 | 50 |
| 10 | 2 | 1 | 18 | 57 | 78 |
| 11 | 6 | 6 | 24 | 60 | 96 |
| 12 | 13 | 10 | 34 | 64 | 121 |
| 13 | 22 | 15 | 40 | 64 | 161 |
| 14 | 35 | 22 | 55 | 64 | 174 |
| Colonies per plate | 8 | 21 | 17 | 9 | 55 |

**Table 2.**

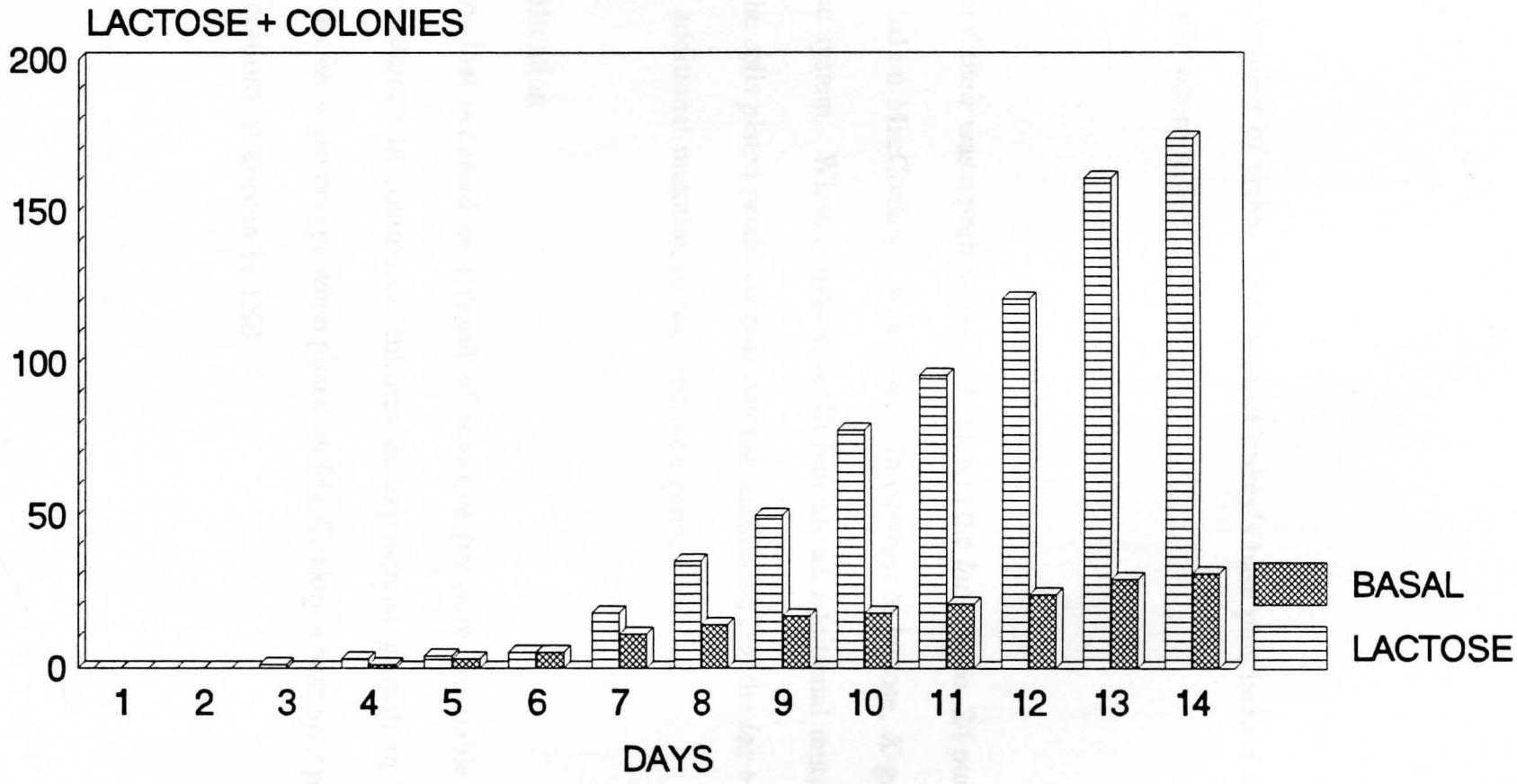
**Daily Total Revertants per Plate on MacConkey' plus X-gal**

| DAY | PLATE 1 | PLATE 2 | TOTALS |
|---|---|---|---|
| 1 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 |
| 4 | 0 | 1 | 1 |
| 5 | 0 | 3 | 3 |
| 6 | 0 | 5 | 5 |
| 7 | 3 | 8 | 11 |
| 8 | 5 | 9 | 14 |
| 9 | 7 | 10 | 17 |
| 10 | 7 | 11 | 18 |
| 11 | 10 | 11 | 21 |
| 12 | 11 | 13 | 24 |
| 13 | 14 | 15 | 29 |
| 14 | 16 | 15 | 31 |
| Colonies per plate | 18 | 38 | 56 |

Figure 1.

Total number of revertant papillae per day for 55 colonies on MacConkey's base agar plus lactose and 56 colonies with X-gal.

# LACTOSE REVERTANTS ON LACTOSE AND BASAL MEDIUM




KeyChart 2000

REVERTAN

Cell Viability

The average number of viable cells on MacConkey's base plus lactose was $1.9x10^7$ versus nutrient agar where it was $2.3x10^8$.

Reversions

To determine if their was a preferential reversion in the *lac Z* gene, 24 purified *lac*+ isolates were plated on MacConkey's plus X-gal. In contrast to lactose, X-gal is not an inducer of the lac system. White colonies would indicate an additional mutation in the *lac I* gene. All the cells plated produced blue colonies indicating that the *lac*+ revertants did not show an additional mutation in the repressor gene.

Stability of the Mutation

The mutations that occurred as a result of selective pressure are stable. Purified revertants were sustained in continuous cultures of exponential growth in TSB. All progeny retained the *lac*+ phenotype when plated on MacConkey's base agar plus lactose after several generations of growth in TSB.

Table 3.

Trial 2. Daily Total Revertants per Plate on MacConkey's plus Lactose

| DAY | PLATE 1 | PLATE 2 | PLATE 3 | PLATE 4 | PLATE 5 | PLATE 6 | TOTALS |
|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 0 | 0 | 1 | 1 | 0 | 1 | 3 |
| 5 | 0 | 1 | 3 | 3 | 5 | 3 | 15 |
| 6 | 1 | 1 | 5 | 5 | 7 | 3 | 22 |
| 7 | | | | 6 | 10 | 4 | |
| 8 | 1 | 2 | 9 | 7 | 13 | 6 | 38 |
| 9 | 2 | 4 | 10 | 11 | 20 | 6 | 53 |
| 10 | 3 | 4 | 15 | 17 | 22 | 7 | 68 |
| 11 | 3 | 7 | 15 | | | | |
| 12 | 4 | 7 | 16 | 38 | 40 | 10 | 115 |
| 13 | 4 | 12 | 18 | 48 | 55 | 11 | 148 |
| 14 | 5 | 15 | 22 | 57 | 58 | 12 | 169 |
| Colonies per plate | 16 | 14 | 4 | 4 | 2 | 4 | 44 |

Table 4.

Trial 2. Daily Total Revertants on MacConkey' plus X-gal

| DAY | PLATE 1 | PLATE 2 | PLATE 3 | PLATE 4 | PLATE 5 | PLATE 6 | TOTALS |
|---|---|---|---|---|---|---|---|
| 1 | 1 | 3 | 0 | 0 | 0 | 0 | 4 |
| 2 | 1 | 3 | 0 | 0 | 0 | 0 | 4 |
| 3 | 1 | 3 | 0 | 0 | 0 | 0 | 4 |
| 4 | 1 | 3 | 0 | 0 | 0 | 1 | 5 |
| 5 | 2 | 4 | 1 | 2 | 0 | 1 | 10 |
| 6 | 2 | 11 | 5 | 7 | 0 | 2 | 27 |
| 7 | 6 | 15 | 9 | 8 | 0 | 2 | 40 |
| 8 | 7 | 20 | 11 | 9 | 1 | 5 | 53 |
| 9 | 8 | 23 | 14 | 11 | 4 | 7 | 67 |
| 10 | 12 | 23 | 15 | 11 | 4 | 9 | 74 |
| 11 | 16 | 23 | 16 | 13 | 7 | 14 | 89 |
| 12 | 22 | 23 | 22 | 13 | 9 | 16 | 105 |
| 13 | 22 | 29 | 25 | 15 | 9 | 20 | 120 |
| 14 | 27 | 33 | 30 | 15 | 9 | 20 | 134 |
| Colonies per plate | 21 | 97 | 50 | 27 | 1 | 3 | 199 |

# CHAPTER IV

## DISCUSSION

The theory of directed mutation as proposed by John Cairns attempts to explain the observation that the appearance and frequency of some mutations appears to be influenced by the environment (Cairns et al.,1988). Most of the investigations, including the present study, that have contributed to this theory have been based on manipulation of the lac operon. The lac operon consists of one regulatory gene and three structural genes (Figure 2). The regulatory gene is the repressor, *lac I,* and is followed be two other sequences: the promoter, *lac P*, and the operator, *lac O*. They occur in this sequence on the DNA and are followed by the structural genes *lac Z, lac Y* and *lac A*, respectively. The *lac Z* gene codes for the enzyme ß-galactosidase, which cleaves lactose to yield glucose and galactose. The *lac Y* gene codes for a permease which is necessary to transport lactose into the cell. The third structural gene, *lac A,* codes for transacetylase, whose function is not well understood. *Lac I* synthesizes a repressor which binds specifically to the operator due to a unique sequence. When this repressor is bound to the operator, the operon is in a repressed state. Lactose enters the cell initially in small amounts due to basal amounts of permease activity. Once in the cell, it acts as an inducer binding to and changing the conformation of the repressor so it is unable to bind to the operator. The operon is said to be derepressed when this happens and the cell is now able to transcribe the structural genes. A CAP protein plays a role in positive control of the operon since its binding is required for transcription of the structural genes in the absence of other

carbon sources.

The strain of *E.coli* used in this study bears mutations in the *lac I* and *lac Z* genes. A mutation in the *lac I* gene causes constitutive synthesis of the structural genes since the repressor is non-functional. Even though its synthesis is constitutive, the *lac Z* gene is not functional due to a mutation that renders the enzyme nonfunctional. This strain was chosen to examine the process of directed mutation in a system in which there is an uncontrolled or constitutive synthesis of the lac enzymes and thus the DNA in this section of the genome in continually being transcribed.

To observe directed mutation, the bacteria must be placed in a stressed environment where it is difficult for the organism to survive. In this study, the strain of bacteria was chosen because it could not normally use lactose as a carbon source. The bacteria were stressed by plating on MacConkey's base agar plus lactose. White *lac-* colonies begin to appear after one day and reach maximum size after two days. Continued growth on this nutritionally deprived medium is due to peptone carbon sources in the media, the nutrients still in the cell from its original growing culture and by utilization of carbon sources from cells which did not survive the initial plating. Once the peptone carbon sources are deplete the colony stops growing. However any *lac+* revertants in the colony form papillae which grow out as red microcolonies on the surface of the white main colony. As mentioned earlier, the strain of *E.coli* used in this study bears two mutations in the lac operon, one of which (Z) is required to back mutate in order for the bacteria to utilize lactose as a carbon source. This back mutation would confer an

advantage to survival under the stress conditions when lactose is present for the cell to utilize. In an otherwise nutritionally stressed medium the lactose acts to increase the number of revertants to the *lac*+ phenotype as opposed to those under the same stress without lactose present.

Directed mutation has been subjected to intense investigation and there have been several additional proposals to explain the observed experimental results (Boe,1992; Davis,1989; Lederberg,1989; Lenski et al.,1989; Mellon and Hanawalt,1989; Mittler and Lenski,1990; Stewart et al.,1989). One of the proposals is of particular interest in the present study. This is the theory of transcription bias proposed by Davis (Davis,1989). This model suggests that single-stranded DNA is more vulnerable to mutation than double-stranded DNA. In this case, a substrate, such as lactose, would induce unwinding of the DNA into single-stranded form in preparation for transcription. The single-stranded section of DNA would be vulnerable to damage by enzymes or other mutagens in the cell. Also, because the cells are starving, replication errors due to metabolic changes are more likely to occur, increasing the chances of an advantageous mutation occurring. Cells with limited available nutrients also take longer to transcribe proteins due to decreased availability of building blocks and, therefore, their DNA remains single-stranded for a greater period of time. Thus the observed increase in *lac*+ revertants in the presence of lactose would be the direct result of the unwinding of the DNA in the *lac Z* region.

Cairns (Cairns and Foster,1991) has provided some experimental evidence that

suggests that the transcriptional bias model may be incorrect. Lactose and IPTG (isopropyl ß-D-thiogalactoside) were used in this study. As mentioned earlier, IPTG is an inducer of the lac operon but not a substrate. Both IPTG and lactose would cause constitutive synthesis of the structural genes. Cairns found that accumulation of *lac+* mutants occurred only in the presence of lactose. Davis suggested this was due to lactose providing a low level of energy to the cell which IPTG did not supply. This would then favor transcription in the cells which are in the presence of lactose and not IPTG. This would also account for the accumulation of mutants.

The results of the present study are not in agreement with the transcriptional bias model. A mutation in the *lac I* gene of the strain used in this study results in constitutive synthesis of *lac Z* as well as the other structural genes. Since it was found that *lac I* double revertants do not exist, even under stress, the structural genes are always being transcribed. This means the structural genes are in a perpetual single-stranded state, with or without substrate present. If Davis' model were true, the number of revertants on the control plates (without lactose) would be the same or nearly the same as those in the presence of lactose. It is clearly seen from the data presented here, that is not the case. Instead, it is the presence of lactose in the media that seems to be determining the fate of the cells. In addition, Davis' theory that lactose is providing a low level of energy to the cell favoring transcription in these cells appears to be incorrect also since X-gal is also a ß-galactoside just as lactose, and yet an increase in revertants occurs only in the presence of lactose.

This data had several implications. First, the only revertant mutation that occurred was advantageous for the cells survival. Second, a general increase in non-advantageous mutations did not appear to occur since no *lac I+* double revertants were found. Finally, the phenotypic alteration which occurred to increase viability under specific stress conditions is retained even after the stress conditions are removed and therefore becomes a permanent part of the genome.

In reviewing the data it can be observed that as early as day 6, a difference in the number of revertants is evident. By day fourteen, the colonies in the presence of lactose show nearly six times as many revertants. Lactose confers a selective advantage to survival of the cells in their stressed environment and therefore its presence by some yet unknown mechanism "directed" the mutation of the cells to a viable genotype. The most astonishing part of this study was the dramatic accumulation in revertants compared to that of previous studies. One possible explanation for this may be the amount of lactose entering the cell. Due to the *lac I* mutation, constitutive synthesis of the lactose permease takes place. This in turn increases the amount of lactose present in the cell very early. This increased concentration of lactose in the cell may act enhance the process of reversion. Results from other investigations demonstrate that strains bearing a mutation only in the *lac Z* gene do not show such a dramatic increase in revertants (Cairns and Foster,1991). If the *lac I* gene is functional, only a small amount of lactose enters into the cell due to basal activity of the permease.

It is important to note here that spontaneous mutants arising in culture before plating

were also considered. Any revertants appearing within two days of plating, when colonies reach maximum size, would be considered spontaneous mutants arising in culture before plating (Cairns et al., 1988). In this study, no spontaneous mutations occurred, probably due to their rarity and the small number of cells plated.

Recall the strain of bacteria used in this study contained two mutations in the lac operon, one in the *lac I* gene and the other in the *lac Z* gene. A reversion in the mutation in the *lac I* gene is not required to confer a selective advantage to survival of the cell in the presence of lactose. A reversion mutation in the *lac Z* gene is sufficient to confer an advantageous genotype on the cell. A second reversion in the *lac I* gene would be neutral. Whether the *lac I* gene has mutated or not, synthesis of ß-galactosidase will occur and permit utilization of lactose as a carbon source. Thus while a large increase in *lac*+ revertants was observed in the presence of lactose, no detectible increase in *lac I* double revertants was observed either with or without lactose in the medium.

This is important for two reasons. First, it appears that overall increase in mutation due to stress is ruled out since no other detectable *lac I* double revertants were found. Second, the fact that the gene tested here is within the lac operon further suggests that genes in close proximity to those with high reversion rates are not effected. Cairns et al. (1988) found a similar increase in revertants of the lac operon and no overall increase in mutations when using the lac operon and valine resistance as a neutral mutation. Other studies have found similar accumulation of mutants with amino acid requirements and frameshift mutations (Hall,1989; Cairns and Foster,1991).

Although the studies presented may not provide absolute proof for the theory of directed mutation, they do provide undeniable support for it. The theory is not an attempt to challenge the "central dogma" of molecular biology but instead suggest another possible mechanism by which advantageous mutations occur. Proponents of the theory seem to consider the function of directed mutation only as being another mechanism by which mutations can occur. Most studies on mutations have previously been done on cells growing under optimal conditions. In nature, on the other hand, starving, stationary phase cells seem to be the norm. Directed mutations therefore seem more likely to be the mechanism by which bacteria mutate in nature.

In summary, it has been demonstrated that 1) the presence of lactose in a stress medium significantly increases the number of revertants in a *lac-* strain, 2) this increase in *lac+* revertants appears to be specific since no comparable increase in *lac I-* revertants was observed and 3) the increase in *lac+* revertants is much higher than that for other *lac-* strains reported in the literature a may reflect the increased level of lactose in the cell which is the result of continued synthesis of the permease in this *lac I-* strain.

# LAC OPERON

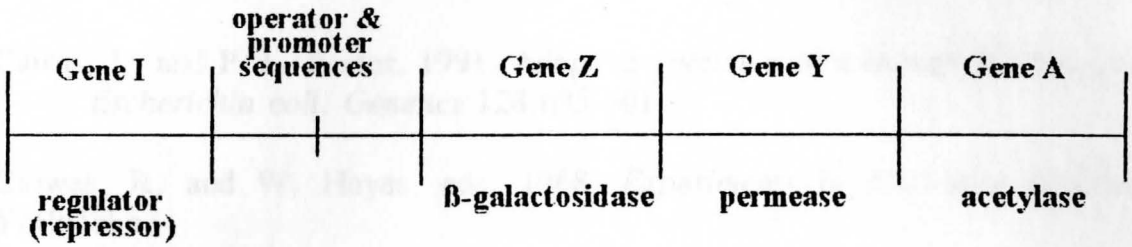



**Figure 2.**

Figure 2.

The Lactose Operon

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