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Neg354
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Isolation and Characterization of a Lac- Mutant strain of *Escherichia coli*

When exposed to ultraviolet light, bacteria absorb the energy, which damages DNA. Bacteria have mechanisms for UV repair that involve reversal, removal of the damage, or even DNA mutation. *Escherichia coli* in particular possesses an SOS response to UV exposure, which can result in mutant strains. The purpose of this series of experiments observe the effects of mutagenesis on bacteria by isolating a mutant strain of *E. coli* that was not able to ferment lactose. After isolation of the mutant strain, we determined the generation time of the exponentially growing culture and induced the *lac* operon by adding IPTG, a chemical that mimics lactose, in order to test the mutant strain for the presence of the enzyme β-galactosidase.

After exposure to UV light, *E. coli* utilizes repair methods in reaction to the damage done to the DNA. Two of these mechanisms include a reversal of the thymine-thymine dimers caused by the UV damage by light-activated photolyase and a method encoded by the *uvr* genes that cuts the damaged DNA strand out and fills in the gap using DNA polymerase. The repair mechanism we focused on is unlike the other two in that it results in a high number of mutants. As part of the SOS response, this method introduces a new DNA polymerase called Pol V that replaces DNA polymerase III, the DNA polymerase active in normal chromosome replication. Pol V is responsible for mutant strains because it often adds the wrong base and creates mutations. In order to eliminate the activity of other two repair mechanisms for this experiment, we used the *E. coli* K-12 strain *W3110*, which does not contain the gene for the photylase, and the *uvr* gene system is not present. Using this strain of *E. coli* increased the chances for mutant strains due to the lack of repair mechanisms other than the SOS response. We were specifically looking for mutant strains that involved an error in the *lac* genes, which are responsible for fermenting lactose in normal *E. coli.* In order to form mutant strains of *E. coli*, we exposed the bacteria to high-intensity, short-wave, germicidal UV with a wavelength of 254 nm. Since UV light damages DNA, it can also kill many cells in the process. It was important to find a balance in exposure time and intensity so that we could successfully obtain mutated strains without

killing all of the cells. To do this, we worked in groups of two people with each group using a different irradiation time of up to twenty-four seconds. We also performed serial dilutions in order to decrease the concentration of cells in a culture and make it possible to count the cultures after spreading them on a plate. My partner and I irradiated our sample under UV light for our assigned time of four seconds, and then diluted the irradiated sample by factors of 10^-3, 10^-4 and 10^-5. We then plated 100 µl of our three different diluted samples onto MacConkey medium with each dilution on two plates.

The purpose of plating the irradiated *E. coli* on MAC plates was to test for lactose fermentation because MacConkey medium contains lactose, and normal *E. coli* would ferment lactose on the medium and form red colonies. Those mutated cells that do not ferment lactose would show up as white or pale pink. After approximately 24 hours, I checked the plated *E. coli* and found what appeared to be one whitish/clear colony on the plate containing the 10^-3 dilution. *Figure 1* shows the expected mutated colony that I then restreaked on a new MAC plate next to a normal lac+ colony to confirm the mutation. *Figure 2* shows the restreaked sample, which reveals whitish colonies and confirms that the colony was a mutated strain of *E. coli* that was unable to ferment lactose.

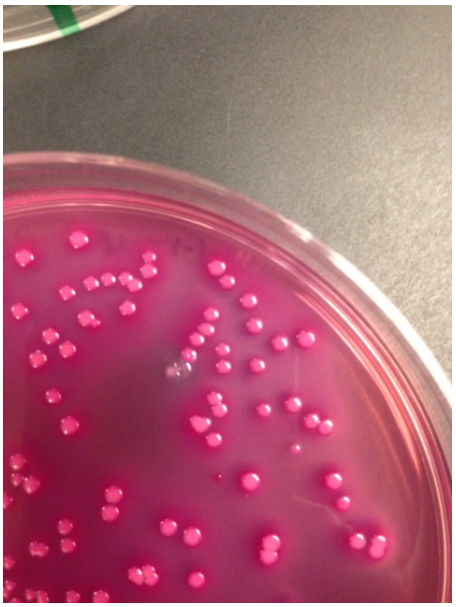


Figure 1: Identification of suspected mutant strain of E. coli on MAC medium

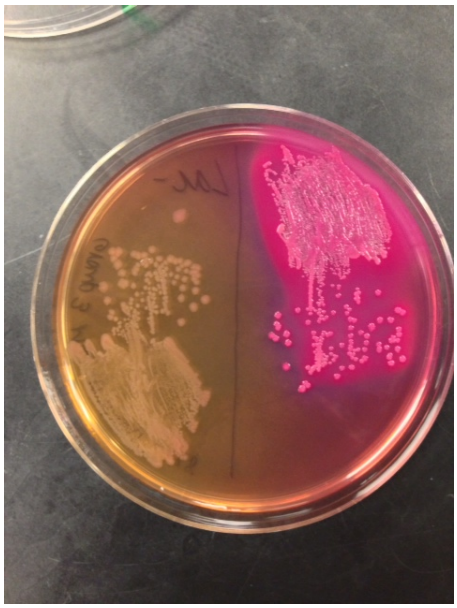


Figure 2: Isolation of suspected mutant E. coli strain and lac+ E. coli strain on MAC medium

The SOS response initiated by UV exposure causes the cell division of *E. coli* to be reduced; however, the cells continue to grow without this division. We observed samples of irradiated cells taken by each group at different exposure times under phase contrast microscopy. Upon observation, phase contrast microscopy of the samples revealed that those cells that had undergone longer UV exposure appeared longer than those of shorter or no exposure. *Figure 3*, which was obtained from Group 5, shows a phase contrast image of the irradiated *E. coli* cells that were exposed for eight seconds. In contrast to normal *E. coli* cells, these appear longer with some cells clumped together. The longer length of the irradiated cells can be explained by the growth but lack of division of cells due to UV damage. For samples that were exposed for 24 seconds, there were significantly less cells apparent in the images, which can be explained by long exposure of UV light causing enough damage to kill most cells.

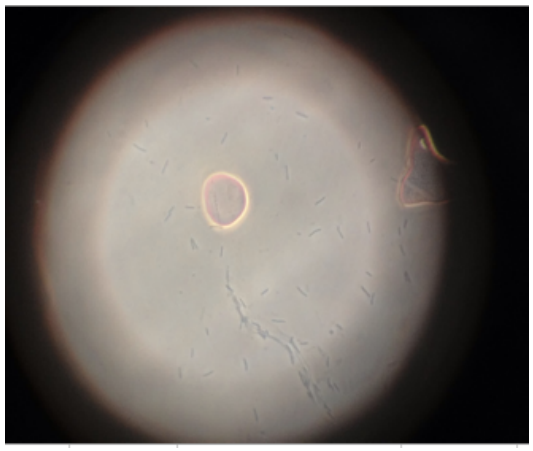


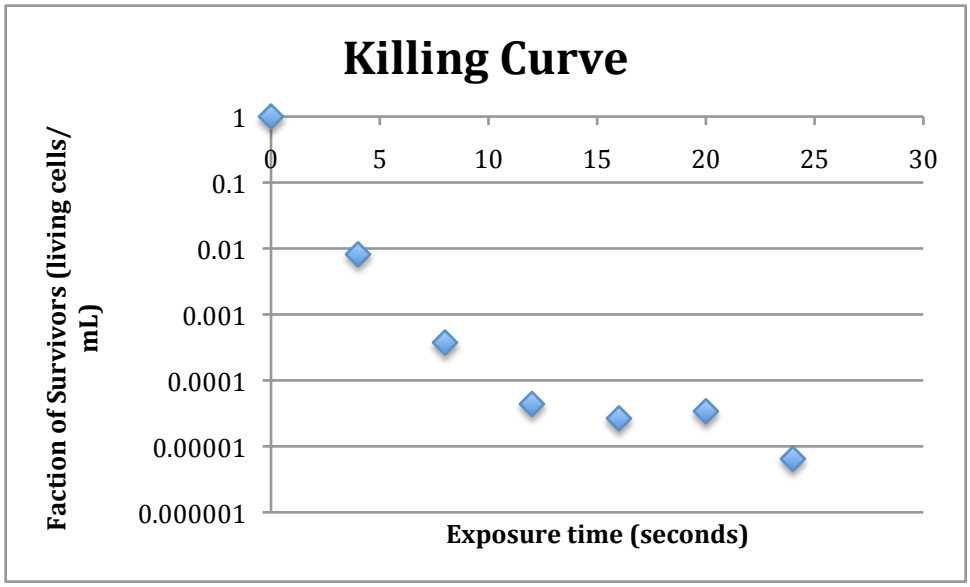
Figure 3 (taken from Group 5): Phase contrast image of irradiated E. coli cells after exposed to 8 seconds of UV radiation

After observing the irradiated *E. coli* cells under phase contrast and analyzing whether or not mutant strains grew on MacConkey medium, I counted the cultures grown on each of the six MAC plates with the three different dilutions. I recorded the values that were higher to ensure greater accuracy and posted them to a Google document with the rest of the class data. *Table 1* shows the class data of the number of cultures for each sample of different exposure times and dilutions. Using the class data from *Table 1*, I constructed a killing curve for the strain, which measures the fraction of survivors based on the exposure time. The purpose of forming a killing curve was to be able to compare the effects of exposure time to UV radiation to the number of *E.*

coli cells killed due to UV damage. It was important to take the dilutions into account by multiplying the cultures counted by the dilution factor used to get the true number of survivors. I then found the fraction of survivors at each exposure time by dividing the number of survivors by the number of cultures in the sample that was not exposed to UV radiation. Using Excel, I graphed the fraction of survivors as a function of exposure time on a logarithmic scale in order to obtain a linear graph. *Graph 1* shows the results, which indicate a negative correlation between number of survivors and exposure time. These results were expected because as UV exposure increases, we would predict that the damage would kill more cells.

Time (sec)	Group Number	Name	No dilution	10^-1	10^-2	10^-3	10^-4	10^-5
0	1	Samantha Salcedo				TNTC	TNTC	262
0	1	Jon Mercado				TNTC	TNTC	258
0	2	Anndee Shaunessy				TNTC	FC	235
0	2	Kellie Kilcline				TNTC	FC	180
4	3	Nancy Gannon				236	42	3
4	3	Karina Ruano				204	31	6
4	4	Karlee Bispo				196	39	2
4	4	Lindsey Bank				399	43	3
8	5	Melissa Soto			132	18	3	
8	5	Karen Duong			63	13	0	
8	-							
8	-							
12	7	Dyan		110	77	24		
12	7	Meredith		129	143	53		
12	8	Eli Arbov		103	22	35		
12	8	Tony Tran		202	36	36		
16	9	Roanne Pueyo	>618	67	31			
16	9	Alice Nguyen	>724	71	32			
16	-							
16	-							
20	11	Peter Tran	TNTC	58	8			
20	11	Samuel Gomez	TNTC	82	26			
20	12	Justin Stapp	TNTC	151	23			
20	12	Kunnel	TNTC	95	15			
24	13	Micah Asadi	175	21	9			
24	13	Kumar Sudhakaran	161	29	7			
24	14	Roxanne E.	278	24	9			
24	14	Vinh Nguyen	521	24	8			

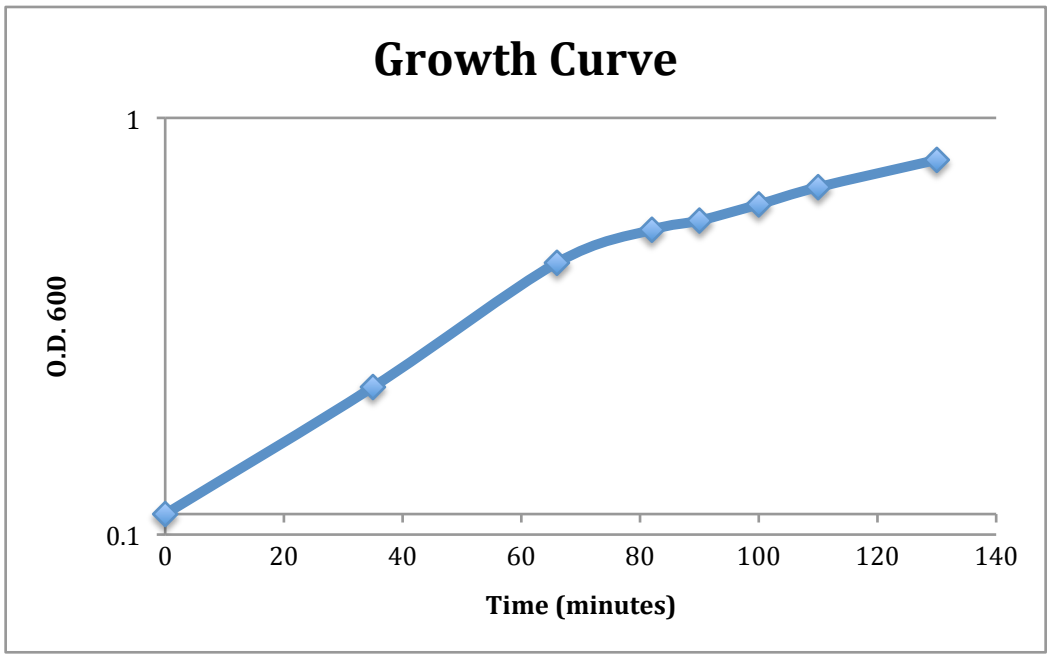
Table 1: Class data of counted cultures of irradiated E. coli samples under various exposure times and dilution values; FC = confluent. TNTC = too many to count



Graph 1: Fraction of survivors of irradiated E. coli as a function of exposure time to UV light on a logarithmic scale

The next part of the experiment involved calculating the generation time of the potential Lac- mutant *E. coli* strain. In our lab class, 9 Lac- mutants were recorded and the one chosen was taken from group 11, Sam and Peter, whose potential mutant strain came from a sample that was subjected to 20 seconds of UV exposure. To avoid confusion, we named their mutant the Sam-Peter mutant. When bacteria have access to adequate nutrients, optimum temperature, and other necessary resources for growth, they are thought to be capable of balanced growth. In balanced growth, each cell in the culture has the same average composition and all major components of the cell are increasing at the same rate. The cells are said to be in exponential phase in which they are growing exponentially, and the generation time is constant. The generation time is the time between cell divisions during exponential phase, in which the cell is undergoing balanced growth and is growing under a specific set of conditions. Knowing and comparing the generation time helps in determining if the cell is in an optimal physiological state. Optical density (OD) is a way of measuring generation time because it is proportional to the number of cells per milliliter in a culture. Calculating the time it takes for the OD to double will give the generation time, which can be compared to a culture with a known OD. For *E. coli,* we knew that 0.8 OD600 = 5 x 10^8 cells/mL, so we measured the OD values of our cultures at 600 nanometers. One problem

with measuring optical density of a culture is that the process doesn't show whether the cells are alive or dead because it only measures the scattering of light. A typical growth curve of a culture consists of a stationary phase in which resources are depleted and cells stop growing. In order to obtain accurate results, we made sure our OD600 measurements didn't exceed much past 0.6, which is a value in the middle of the exponential phase when all cells are alive and growing. Each pair of students was divided into one of three different groups and assigned to measure the optical density of either the Lac+ *E. coli* strain, the Sam-Peter mutant, or a known Lac- mutant *E. coli* strain. The Lac- mutant strain was a negative control, which had already been isolated as a known Lac- and unable to induce β-galactosidase. My group was assigned to test the Lac+ *E. coli* strain, which was a positive control. We inoculated 2 mL of our sample into 20 mL broth and incubated it in the shaker water bath at 37 degrees Celsius. After dilution, we measured the OD600 of the sample at 30-minute increments using the spectrophotometer. It was important to blank the sterile medium before each measurement to ensure accuracy. We plotted the OD600 at each time point on a logarithmic scale, and the results are shown in *Graph 2*. From the graph, I was able to calculate generation time as 32 minutes for the Lac+ *E. coli* positive control. This is the time it took for the optical density to double, which is proportionate of the time it took for the cells per milliliter to double.



Graph 2: Growth curve of W3110 uvr phr (Lac+) E. coli sample with optical density at 600 nanometers as a function of time after dilution

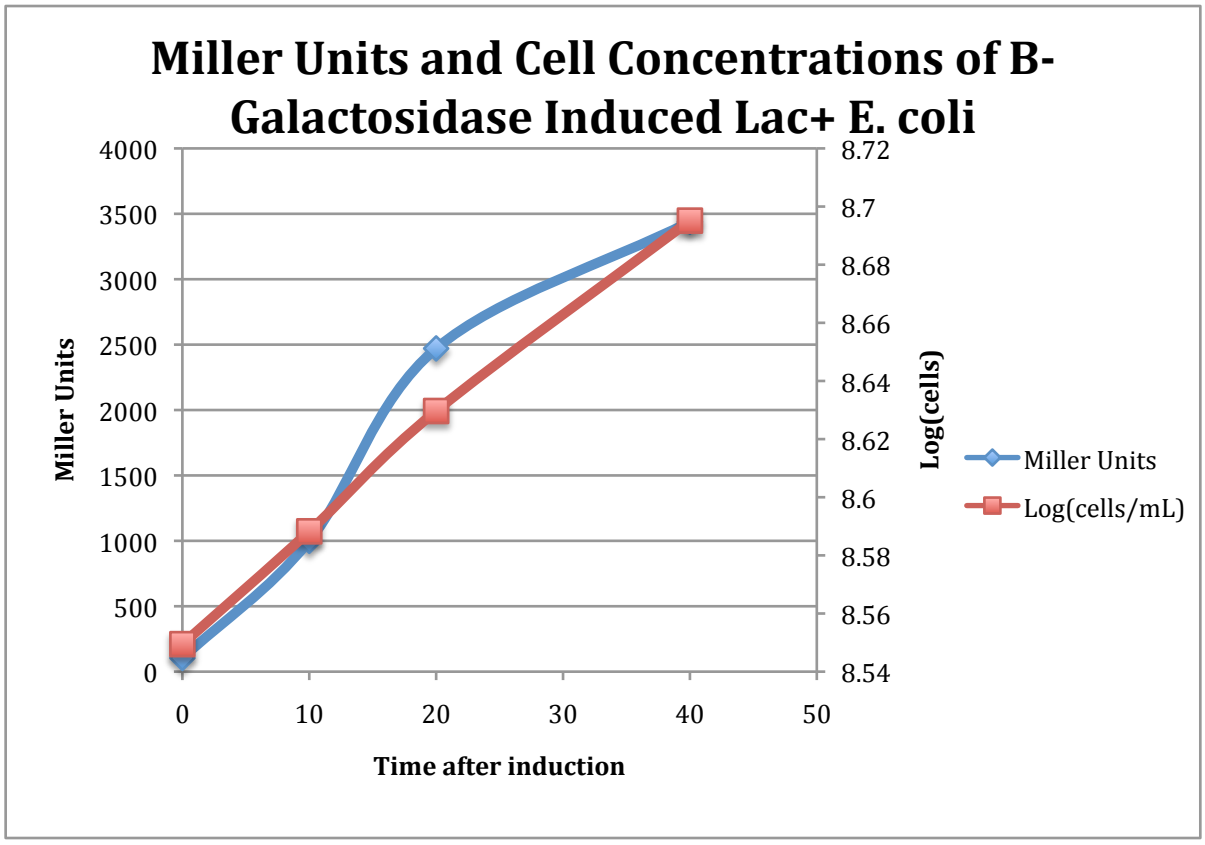
Another way to analyze the suspected mutant *E. coli* strain was to induce the *lac* operon and later assay for the enzyme β-galactosidase. The lacZ gene in the lac operon of E. coli encodes β-galactosidase, and this enzyme is responsible for breaking down lactose into glucose and galactose. One way the β-galactosidase is induced is by the addition of lactose, which enters the cell, binds to the lac repressor, and allows for the enzyme to be transcribed. It can also be induced by the compound IPTG, which mimics lactose and binds to the repressor, inactivating it, but is not affected by β-galactosidase. Since β-galactosidase breaks down lactose once it is induced, it was more beneficial to use IPTG since the concentration wouldn't be affected. During the experiment in which we determined the generation time by measuring optical density, we also induced β-galactosidase with IPTG. We waited until the OD600 reached a value of 0.6 because this value was known to be in the middle of exponential phase of the cells, during balanced growth. At that point, which was at 90 minutes in our case, we added IPTG to the sample of Lac+ *E. coli* to produce a final concentration of 0.5 mM in 22 mL of the culture. We then added 1 ml of the culture to a microfuge tube and spun it in the microfuge rotor for two minutes in order to break open the cells and release the DNA, allowing the IPTG to induce the lac operon. We repeated this sampling at time intervals of 10, 20, and 40 minutes.

After adding the IPTG to the samples of cells and incubating them in the freezer for one week, we prepared to analyze the samples for the presence of β-galactosidase. Since my group was testing the control Lac+ normal *E. coli* sample, we expected the IPTG to complex with the lac repressor, preventing it from blocking transcription of the *lacZ* gene, and causing the amount of β-galactosidase produced to increase. For the Sam-Peter mutant that was Lac- on the MAC medium, we would expect different results with a wide range of possibilities. A mutation to the *lacZ* gene, the *lac* operon, or the *lac* operon promoter would cause an absence of β-galactosidase production. The *lacI* gene that encodes for the lac repressor could also have a mutation, preventing it from binding to inducers and even affecting normal cells by binding to the normal cell *lac* operon even in the presence of inducers. This would cause cells to show Lac- properties even if normal cells were present. Another possibility involves the *lacY* gene, which encodes a permease needed for uptake of lactose into the cell. If *lacY* is mutated, lactose is unable to enter the cell and the sample appears as Lac- on MAC medium. In this case, the IPTG is still able to enter the cell and induce the *lac* operon, causing production of β-galactosidase. β-galactosidase

can be measured by a colorimetric assay because cleavage of ONPG by β-galactosidase produces a yellow product that can be measured with a spectrophotometer. This procedure involved adding 1 drop of chloroform and 1 drop of 1% SDS to the resuspended cells we prepared the week before. We then made mixtures of 0.02 ml and 0.05 ml of each sample of permeabilized cells with buffer. To those mixtures, we added 200 microliters of ONPG and recorded the time of addition. We noticed that for the mixture containing 0.05 ml of permeabilized cells that had been taken from the sample after 40 minutes of the IPTG being added, the color immediately began turning yellow. Since our sample was the Lac+ normal *E. coli*, we would expect the reaction to happen quickly since β-galactosidase should have been produced and cause cleavage of the ONPG. We then took OD readings at 550 and 420 nanometers of our samples that contained 0.02 ml of permeabilized cells. Since the results we obtained were adequate, we did not have to measure the OD readings of the samples that contained 0.05 ml of permeabilized cells. The purpose of making the samples with 0.05 ml of permeabilized cells, however, was for reliability to ensure that our OD420 readings were between 0.1 and 1.0. We then calculated the Miller units of our values, which represent the amount of β-galactosidase produced per cell. *Graph 3* shows our results of analysis of the Lac+ W3110 *uvr phr* positive control sample, with Miller units and Log(cells/ml) as a function of time in minutes after induction of β-galactosidase. As was expected for the Lac+ *E. coli* strain, Miller units increased as time after induction increased, indicating that β-galactosidase production increased due to IPTG being introduced and caused hydrolysis of ONPG, which produced a yellow color and changed the optical density. The concentration of cells also increased, due to exponential growth of the cells under balanced growth conditions.

When comparing my results of the positive control Lac+ sample to those of the Sam-Peter mutant and the Lac- strain, I found some expected differences. The Miller unit values were significantly lower in both of the other samples, indicating that not as much β-galactosidase was produced. The results between groups using the same sample were variable, which could be explained by error or mistakes in procedure. The generation times for the Lac- and mutant samples were slightly shorter than for the Lac+ sample. This observation was surprising since I would expect mutated DNA to take longer to replicate and form new cells. I noticed that for the Sam-Peter mutant data, Miller units didn't steadily increase or decrease, but instead seemed to fluctuate. This could be explained by a mutation in the *lacY* gene previously mentioned, which

could account for a small amount of β-galactosidase being produced even though the mutant is Lac- on MacConkey medium. The low Miller unit values could also be explained by a mutation in the *lacZ* gene coding for β-galactosidase or to the *lac* operon itself. Since we had already assessed that the Sam-Peter mutant *E. coli* strain was Lac- on MacConkey medium, and this data further supports that the mutant sample produced a much lower amount of β-galactosidase, it is evident that the Sam-Peter mutant contains some type of mutation in the lac operon or the genes involved in lactose fermentation.



Graph 3: Miller Units and concentration of cells/mL of Lac+ E. coli as a function of time after induction of B-galactosidase

Overall, mutagenesis and characterization of W3110 *uvr phr E. coli* was a successful experiment that allowed for careful observation of the effects of UV damage and mutation on bacteria. From this series of experiments it can be concluded that UV exposure has an effect on the lactose fermentation system of *E. coli*, including the *lac* operon, *lac* genes, and enzymes such as β-galactosidase. While I was unable to analyze the exact type of mutation that was caused by

the UV exposure, it would be very interesting to experiment further to gather more specific data and conclusions on the mutations.