

# Controlling Fluorescence in E. coli

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## 1 Introduction

In a simulated environment, we can design artificial chemistries that allow a bacteria to move along a chemical gradient. The objective in this project was to try to apply these ideas to a real chemistry in a real cell. Capturing movement of real cells is extremely difficult due to the size of bacteria and the speed of movement. My goal was to control the output of some cellular protein by controlling the cell's environment. Specifically, it would be useful if we could precisely control the production of some protein and determine a quantitative relationship between the concentration of a chemical in the environment with the output of a protein.

For this project, the output proteins used were fluorescent proteins, chosen for the ease of measuring them. The standard E. coli bacteria was used as the target organism. To regulate the output of the proteins, a well-studied regulatory system in E. coli, the lac operon, was used.

### 1.1 lac operon

In E. coli, the *lac* genes codes for a set of proteins that help the bacteria metabolize lactose [2]. The genes for these proteins are controlled by a regulatory region that control the transcription of the genes. The genes and the regulatory regions are all a part of the lac operon and is shown in Figure 1.

*lacZ*, *lacY*, and *lacA* code for proteins needed by the bacteria to metabolize lactose. The region of the lac operon that we care most about is the regulatory region. The regulatory region consists of three parts: *lacI*, *lacP*, and *lacO*. *lacI* codes for an inhibitory protein that binds to the operator region, *lacO*. *lacP* is the promoter region and is where the RNA polymerase binds to begin transcription.

In the absence of lactose, the *lacI* protein binds to the operator region, preventing the transcription of the lac proteins. With lactose present in the environment, the lactose binds to the *lacI* inhibitor, effectively disabling the

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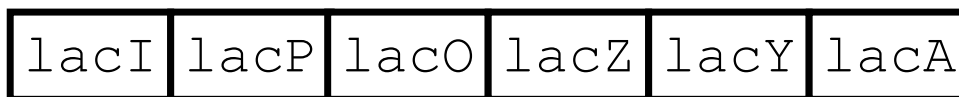


Figure 1: Various parts of the lac operon.



Figure 2: Parts of the lac operon combined with gene for fluorescent protein.

inhibitor, and allowing the expression of the lac proteins. Thus, *E. coli* expresses the proteins to metabolize lactose only when lactose is present in the environment. Lactose induces the production of the lac(Z,Y,A) proteins.

However, lactose is not the only chemical that can control the expression of the lac proteins. Through a different mechanism that is not as well understood, the presence of glucose inhibits the expression of the lac proteins, known as catabolite repression. The cell prefers to use glucose over other sugars when glucose is available, because glucose is faster and easier to metabolize. Thus, glucose represses production of the lac proteins.

## 2 Methods

### 2.1 Fluorescence Proteins

The ECFP gene (Enhanced Cyan Fluorescent Protein) codes for a protein that makes a cell fluoresce when viewed under ultraviolet light. We combine the promoter and operator region of the lac operon with ECFP as shown in Figure 2. This construct is inserted into a plasmid and transformed into *E. coli*. The cells can then be grown on various mediums and checked for fluorescence. We expect the expression of the ECFP fluorescent protein to be high in the presence of lactose and low in the presence of glucose. The same construct is also made with the EYFP gene (Enhanced Yellow Fluorescent Protein) and inserted into a separate set of bacteria.

### 2.2 Gradient Plates

Various plates were created that contained a chemical gradient across the plate. These were done as follows. Plates were placed at an angle and a solution containing a chemical A was poured in. Due to the angle, one side of the plate will contain a higher concentration of chemical A than the other side. After the solution hardens, we level out the plate and pour on another solution with chemical B. Thus, we see in Figure 3 that there exists a gradient of the chemicals

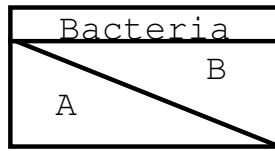


Figure 3: A gradient plate with varying concentration of chemicals A and B.

across the plate. Once these plates are made, some amount of bacteria is mixed with LB Top Agar and poured on top of the plates. The agar solidifies and fixes the bacteria to the top of the plates.

### 2.3 Chemicals

Various chemicals and mixes were used in the experiments. Here are descriptions of some of the more important ones.

**LB** The standard rich medium used to grow cells.

**M9** A minimal medium that cells can barely live on.

**Amp** Ampicillin is a standard antibiotic used to select for cells that contain an inserted plasmid with the fluorescent protein.

**Glucose** Primary sugar and energy source for *E. coli*. We expect glucose to inhibit the production of the fluorescent proteins.

**Lactose** A sugar source. Lactose induces the production of the lac proteins and we expect it to also induce the production of the fluorescent proteins.

**Sucrose** Another sugar source that should have no effect on fluorescent protein production.

**IPTG** isopropyl-beta-D-thiogalactoside is similar enough in chemical structure to lactose that it can bind to the lacI inhibitor and induce production of the lac proteins. We thus expect that IPTG and lactose would have similar effects on the fluorescence proteins. IPTG, however, cannot be used as a sugar source.

**Agar** A substance that solidifies at room temperature. Used to make plates.

### 2.4 Measuring Fluorescence

The fluorescence of cells on the gradient plates were initially looked at under UV light. It was expected that the fluorescence would be obvious to the naked eye and that a gradient could be seen across the plate.

To get more quantitative results, a fluorescence reader was also used for cells grown in liquid medium. The fluorescence reader has filters for excitation

and emission and these filters were set to roughly correspond to what we would expect from the ECFP protein or the EYFP protein. To measure ECFP, the excitation filter was set to 420 and the emission filter was set to 485. To measure EYFP, the excitation filter was set to either 485 or 508 and the emission filter was set to 560. The fluorescence reader was set to a sensitivity of 100. As the growth medium is fluorescent by itself, when using the fluorescence reader, it was necessary to centrifuge the solution and remove the medium to obtain reasonable values.

## 3 Results

### 3.1 Gradient Plates

The first experiments involved gradient plates with glucose and phenol red on one side and IPTG on the other side. Phenol red was a dye added to track the glucose and also changes color when bacteria grow on it. A non-fluorescent colony of cells and two ECFP colonies were grown on these plates. Under UV light, an obvious difference in fluorescence could be seen between the ECFP and non-fluorescent colonies, but it was not so obvious whether the fluorescence varied along the gradient.

Part of the problem came from the dye turning yellow and also perhaps the fluorescence of the LB. The next experiment involved using the minimal M9 medium instead of LB and eliminating the dye. M9 medium was expected to be less fluorescent than LB. Solutions of M9+Amp+Glucose and M9+Amp+Lactose were made. Gradient plates were made using these two solutions and both the non-fluorescent and fluorescent bacteria were grown. Initially, the results showed the non-fluorescent plates to be uniformly blue. The fluorescent plates were blue on both the glucose and lactose ends of the plate, but in the middle, it was greenish. After a day, the high glucose end became very greenish and the lactose end remained bluish. Thus, the results were exactly opposite of what was expected. The highest fluorescence ended up being on the glucose end of the plate, not the lactose end.

To explain the above, some experiments to control for various factors were made. Plates were made that were all glucose or all lactose in addition to trying more gradient plates. There turned out to be a small amount of fluorescence visible on the all glucose plates. On the gradient plates, the same results as before was seen, with the most fluorescent area being in the middle of the gradient and migrating towards the glucose end over time. Surprisingly, no fluorescence was seen on the all lactose plates.

To test whether the cells on the all lactose plate were even growing or could become fluorescent, a colony from the plate was picked and grown on a gradient plate. When grown on the gradient plate, fluorescence was obvious. Thus, there was something about the all lactose plate that was not allowing the cells to become fluorescent.

It appeared that these cells may have required glucose to grow and may not

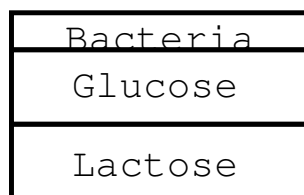


Figure 4: A plate with a constant amount of glucose and lactose.

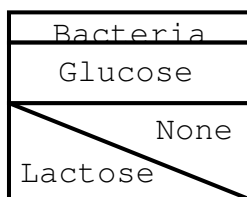


Figure 5: A plate with a constant amount of glucose and a gradient of lactose.

be able to grow too well on all lactose. Plates serving as controls were made containing an even amount of glucose and lactose as seen in Figure 4. Furthermore, a solution of M9+Amp was created that did not have either glucose or lactose. A gradient plate was created as seen in Figure 5. There exists a gradient of lactose across the plate but the amount of glucose is constant across the plate. On this plate, we expect that all cells will be able to grow as happily as all other cells on the plate, due to the even spread of glucose. The results showed that there was more fluorescence on the plate with an even distribution of both glucose and lactose than on a plate with only glucose, showing that lactose is having an inducer effect. However, on the plates in Figure 5, more fluorescence was seen on the side with the least lactose.

At this point, it was discovered that the strain of *E. coli* being used, DH5 $\alpha$ , is missing the lac operon. In particular, it did not contain the lacI gene. Without lacI, there would be no repressor protein, and so the amount of fluorescent protein produced would not depend on the amount of lactose. However, glucose should still have had the expected effect. In addition, without the lac operon, the bacteria would not have been able to grow on lactose.

We found another strain of *E. coli*, XL1-Blue, and transformed it with both the lacP-lacO-ECFP and lacP-lacO-EYFP constructs. XL1-Blue contains the lacI<sup>Q</sup> gene, where the Q indicates a strong promoter attached to lacI, and so the expected regulation of the fluorescent protein is expected.

To control for the fact that glucose and lactose are sugars and may be used by the cell in other ways, sucrose was used instead of glucose and IPTG was used instead of lactose. One solution contained M9+Amp+sucrose and another solution contained M9+Amp+sucrose+IPTG. Gradient plates with these two

solutions were made in addition to control plates that consisted of only one of these solutions. Both the original DH5 $\alpha$  and XL1-Blue cells were grown on these plates. Fluorescence was obvious on all the plates containing DH5 $\alpha$  cells, but there was no difference in fluorescence across the DH5 $\alpha$  plates. This is expected, because DH5 $\alpha$  does not have lacI and so IPTG should not be an inducer. However, no fluorescence was seen from any of the XL1-Blue cells. The all sucrose and sucrose+IPTG plates were grown again with the XL1-EYFP and XL1-ECFP cells. The result of this attempt showed that XL1-EYFP was slightly more fluorescent on sucrose+IPTG than sucrose alone. However, the opposite was seen for the XL1-ECFP, so the results are inconclusive.

Later, this experiment was repeated adding a plate with glucose also. Both the XL1-Blue and DH5 $\alpha$  cells were again used. The glucose plates were distinctly more bluish than all other plates for all types of cells. In addition, there was visible fluorescence from the DH5 $\alpha$  cells on both the sucrose and sucrose+IPTG plates. For the XL1-Blue cells, there was not much visible difference between sucrose and sucrose+IPTG plates. This is essentially the same result from before, but shows that glucose does have an inhibitory effect in both the DH5 $\alpha$  and XL1-Blue cells.

### 3.2 Serial Dilution with IPTG

Next, the XL1-Blue cells were grown overnight in LB+Glucose. The idea was to grow the cells in glucose to inhibit fluorescence. Then, the cells were used for a serial dilution as follows. Five tubes were filled with 6 ml of LB+Amp. A 32 mM IPTG solution was created. 3 ml of the IPTG solution was put into tube 1 and mixed together. Then 3 ml from tube 1 was taken out and put into tube 2 and mixed together. This was repeated down the tubes and for the last tube, 3 ml was taken out and removed. Then, 0.5 ml of bacteria was added to each tube. At the end, each tube was expected to have about a third the concentration of IPTG as the one before it. The cells were allowed to grow for 4 hours. A sample was taken from each tube and centrifuged. The medium was removed and resuspended in a buffer. The fluorescence was then measured by the fluorescence reader. Note that the units of fluorescence are arbitrary. As can be seen in Figures 6 and 7, the relationship between IPTG and fluorescence is not perfect and jumps around. From these data, it is not clear that there exists a definitive relationship between the concentration of IPTG and amount of fluorescence.

### 3.3 Glucose and IPTG

In the next experiment, XL1-Blue cells were grown in the following four liquid mediums:

1. 10 ml LB+Amp
2. 10 ml LB+Amp + 200  $\mu$ l 20% glucose

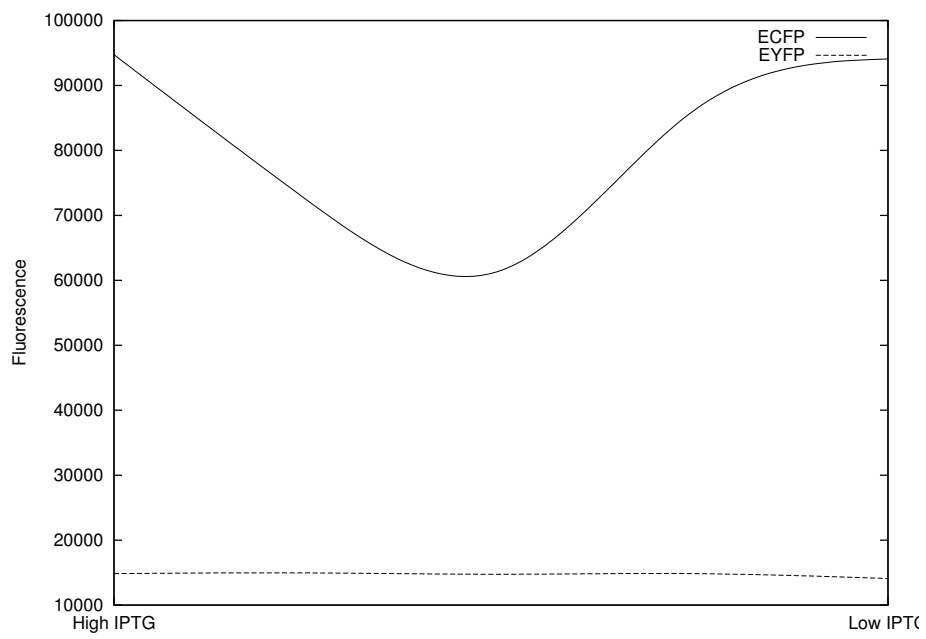


Figure 6: Fluorescence in the ECFP range (420/485) for cells grown with various concentrations of IPTG.

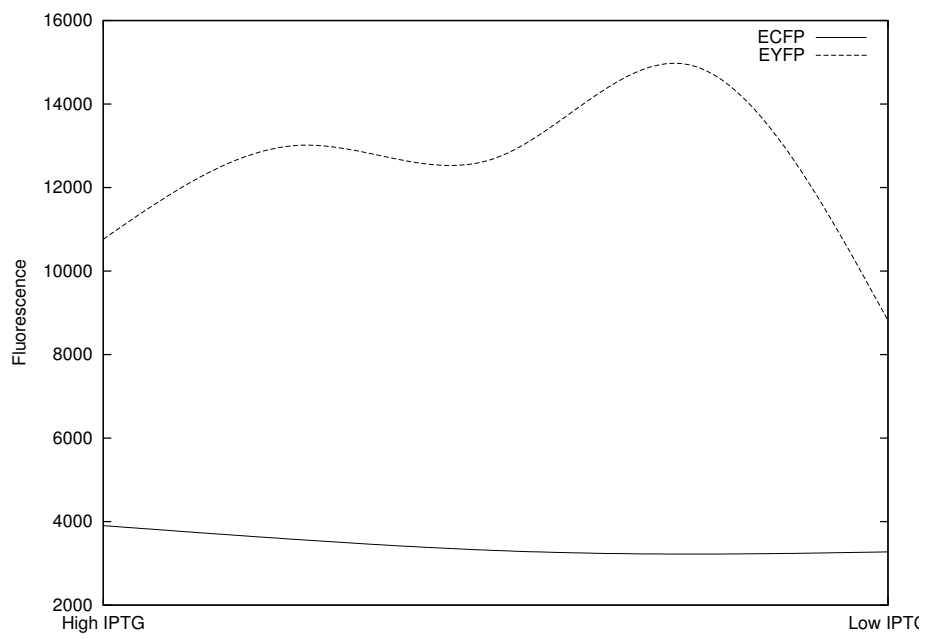


Figure 7: Fluorescence in the EYFP range (485/560) for cells grown with various concentrations of IPTG.

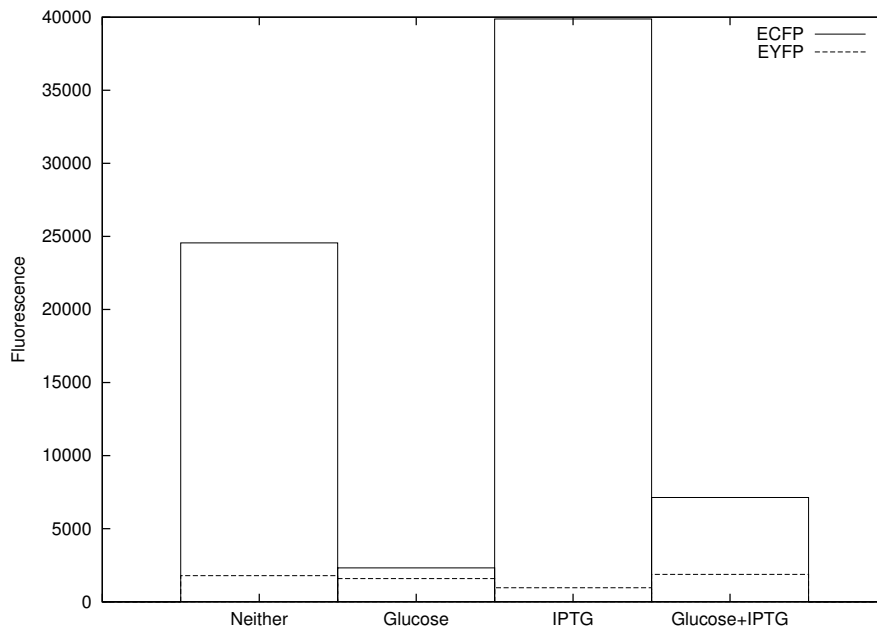


Figure 8: Fluorescence in the ECFP range (420/485) for cells grown in medium with various combinations of glucose and IPTG.

3. 10 ml LB+Amp + 200  $\mu$ l 1M IPTG
4. 10 ml LB+Amp + 200  $\mu$ l 20% glucose + 200  $\mu$ l 1M IPTG

To measure the concentration of ECFP protein, the excitation filter was set to 420 and the emission filter was set to 485. For EYFP, the excitation filter was set to 508 and the emission filter was set to 560. These different filters for EYFP appeared to work better than the filters used previously.

In Figures 8 and 9, we see data that match well with the expected results. An obvious separation can be seen between the ECFP and EYFP cells. The first bar shows the baseline fluorescence of cells grown in only the medium. When the cells are grown in the presence of glucose, the amount of fluorescence drops to the background fluorescence level compared to the baseline. With IPTG, the amount of fluorescence is increased. With both glucose and IPTG added, the inhibitory effect of glucose appears to be slightly stronger than the inducing effect of IPTG, leading to a slightly decreased level of fluorescence.

### 3.4 Lactose

The next experiment involved varying the amount of lactose. In tubes with 10 ml LB+Amp, various amounts from 0 to 1 ml of 20% lactose were added.

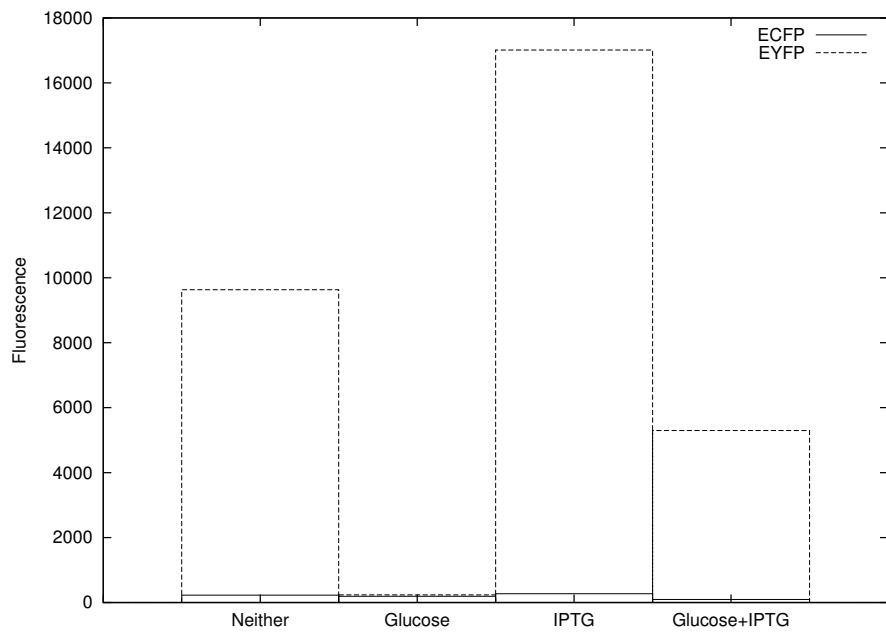


Figure 9: Fluorescence in the EYFP range (508/560) for cells grown in medium with various combinations of glucose and IPTG.

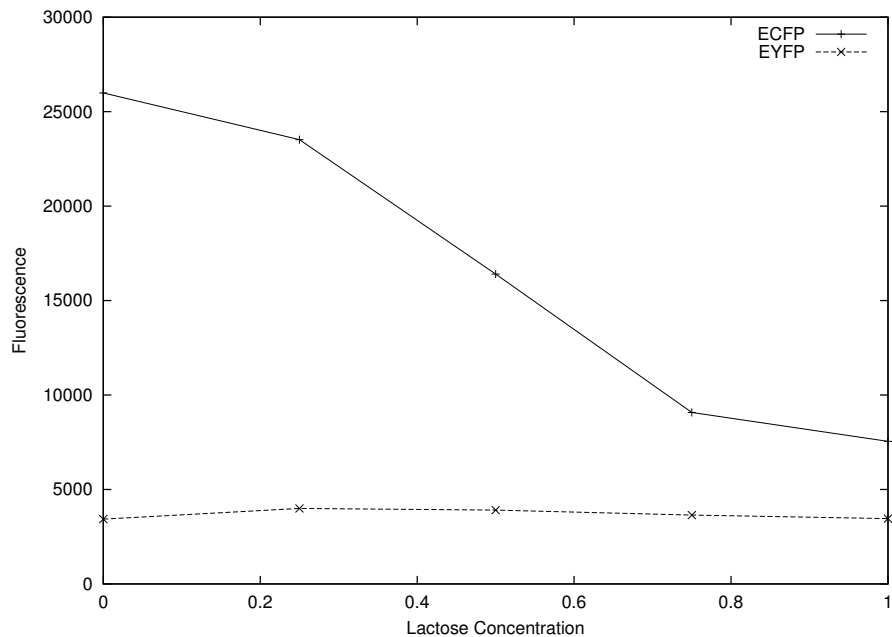


Figure 10: Fluorescence in the ECFP range (420/485) for various concentrations of lactose.

The cells were grown and the next day, the fluorescence was measure using the reader. The same settings for the fluorescence reader as in the last experiment were used. In Figures 10 and 11, we see a mostly monotonic relationship but it is in the opposite direction from what is expected! Higher levels of lactose led to lower levels of fluorescence.

## 4 Discussion

The first results with cells grown on glucose and lactose gradient plates could be possibly explained by the particular strain of *E. coli* used, the DH5 $\alpha$  cells. Being unable to grow on lactose, the plate can be treated as just a glucose gradient. Initially, the cells showed the most fluorescence on the side with least glucose as expected. We suppose that the cells on the end with more glucose grow faster and denser due to having more nutrients. Once the glucose is all used up, it stops acting as an inhibitor and we see the most fluorescence on the end of the plate that had the most glucose.

However, even after switching to the XL1-Blue strain, no interesting results were seen on the gradient plates. The results in Figures 8 and 9 show that IPTG should be an inducer for the XL1-Blue cells. But no fluorescence gradient was seen on the gradient plates with sucrose and IPTG. One possibility is

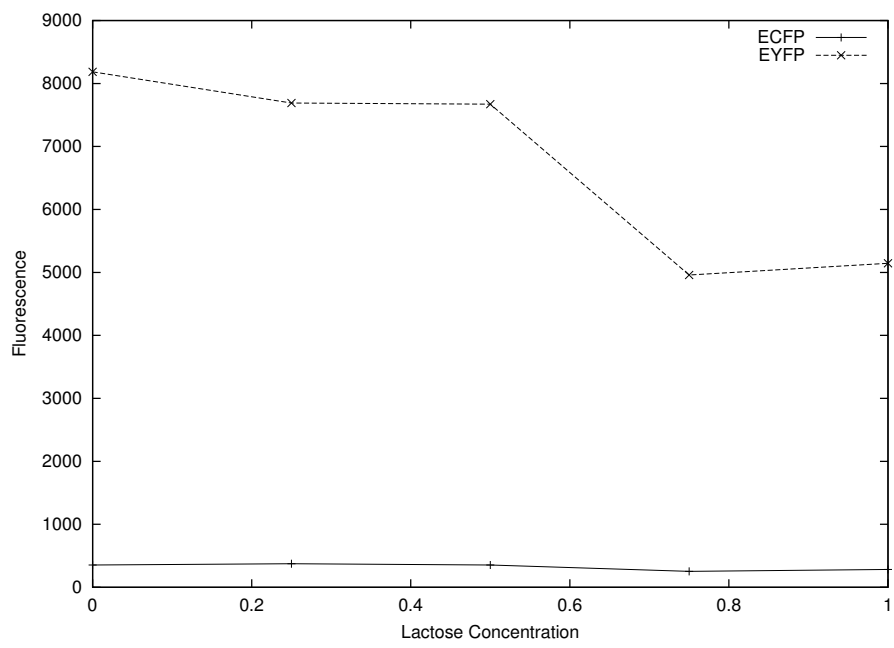


Figure 11: Fluorescence in the EYFP range (508/560) for various concentrations of lactose.

the concentration of IPTG used. The amount of IPTG in the sucrose+IPTG solution may not have been enough to induce production of the protein. The actual concentration of IPTG put in the solution was not recorded at the time. One problem with the gradient plates is that both the LB and M9 medium are fluorescent, and when comparing plates with the naked eye, this fluorescence may have obscured any effect that was present.

For the serial dilution experiment with IPTG, it is not clear if any conclusions can be drawn. It is certain that IPTG has an effect on the cells, but the relationship is not consistent. However, when the same samples used in this experiment were tested again for fluorescence many days later, the numbers turned out to match the predicted direction much better, with cells grown in higher levels of IPTG having higher fluorescence. This was not a controlled experiment, so the data is not reported. It is unknown what effect the passage of time had on the fluorescent proteins, and it is almost certain that the cells were dead at the time.

The data from the last experiment with lactose went completely against expectations. Several possibilities exist to explain the data. It is possibly due to experimental errors if the tubes were somehow labelled incorrectly, but this is unlikely. Another possibility is that it may be due to the strain of *E. coli* (again). Although the experiment was done with the XL1-Blue strain that contains the *lacI* gene, it may not have contained *lacY*. *lacY* codes for lactose permease, a protein that allows lactose to enter the cell, and lactose permease is required for the cells to grow on lactose. Lactose and IPTG may not have the same effects on the production of the fluorescent protein if lactose could not make it into the cell. However, even this does not explain the shape of the curve. The complete answer is probably much more complex and may never be completely known.

## 4.1 Things Learned

In the process of obtaining the above results and trying to understand the results, many things were learned about biological systems and techniques. It took some time to become comfortable with biological techniques, but after a while, the more interesting thing to focus on was understanding the biological system. Understanding a biological system from a theoretical perspective is easy compared with engineering a system. The *lac* operon may be one of the most well understood regulatory systems, but that does not make designing a system based around it easy.

The failures are in many cases as important, or even more important, than the successes. If the experiment worked as expected from the beginning, it would have been over almost immediately. In fact, the initial attempts did not involve using fluorescence proteins but instead the violacein protein from *Chromobacterium violaceum* [1]. The violacein protein makes bacteria visibly purple, and it was originally thought that this would be easier to see than fluorescence. However, after four unsuccessful attempts at cloning it into *E. coli*, the fluorescent proteins were used instead. The data presented above might have been drastically different if the cloning of the violacein gene had been successful.

## 4.2 Limitations

In any experiment, there are limitations of various types. One of the limitations in this experiment that was unexpected had to do with the strain of *E. coli*. The particular strains used very likely had a large effect on the results. A different strain of *E. coli* than the two used in this experiment may have had better results. One obvious difference observed while working with the strains in this experiment was that the XL1-Blue cells seemed to die much faster than DH5 $\alpha$  cells. It is not known whether this had any unintended effect on the results.

Another limitation was that the density of cells was not well controlled. Ideally, we would want to know the amount of fluorescence from an individual cell. When we have many cells, we are not certain whether the fluorescence is due to the sum over a large number of cells or if there are only a few cells with high fluorescence. In the experiments above, we assume that the cells grow equally well in all conditions and so there are roughly the same number of cells in all conditions. This is almost certainly not the case, as for example, we expect cells to grow better with glucose.

In the experiments of the type described here, there are many possibilities for experimental errors. Each experiment required many human actions and humans can easily make errors, especially when dealing with systems that are not visible to the naked eye. It would not have been too difficult to contaminate a sample with a single cell from somewhere else that could have nullified all results that followed. In addition, biological systems are complex, not particularly reliable, and are difficult to debug. Experiments need to be repeated before a higher confidence can be placed in the results. However, cells take a relatively long time to grow and with only limited time, only so many experiments could be performed.

## 4.3 Conclusion

In theory, it should have been relatively straightforward to control the expression of one protein using the lac operon. But this project has shown that, especially for biological systems, practice and theory are only weakly related. The results of Figures 8 and 9 show that it is possible, both in practice and theory, to control the output of a specific protein by controlling the chemicals in the cellular environment. In addition, the specific protein should not matter. Theoretically, the gene for any protein could have been put into the place of the fluorescent protein and the same effect would be seen, if the protein could be measured in some way.

What was not shown is the capability for more fine-grained control over fluorescence. None of the gradient plates showed any interesting gradients and neither did various experiments done in liquid medium. It would be much more useful if we could determine the exact concentration of an environmental chemical needed to produce a desired level of fluorescence. It would also be useful to characterize how the fluorescence output changes over time.

The unpredictability and complexity of even simple bacteria is one of the greatest strengths of working directly with biology. It is unlikely that the unpredictability of biological systems could have been modeled by any computer simulation even with liberal use of a random number generator. Thus, although the results in this project may not be completely explainable, the results, or the lack of results, are undoubtedly real.

## References

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