Frequencies of UV-Mediated Mutagenesis in Amino Acid Biosynthetic Pathways in *Escherichia Coli*

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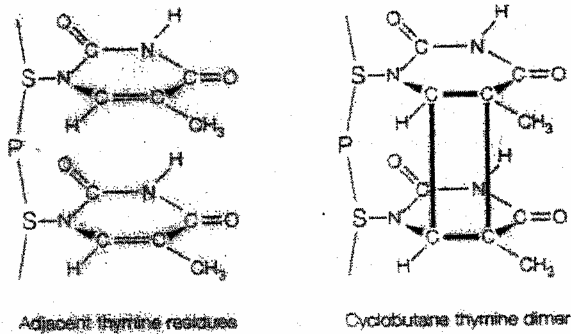
Abstract:

Using an ultraviolet transilluminator of the sort commonly used for viewing gels, I exposed diluted cultures of the BL21 strain of *E. Coli* to ultraviolet light, killing many of them and mutating others. The surviving cells were then cultured on media deficient in one of each of the four following amino acids: alanine, aspartic acid, asparagine, and methionine, in an attempt to determine the relative frequencies of UV-mediated mutagenesis in the genes that control the biosynthetic pathways for these amino acids; an exposed colony that died on such media could be assumed to have a destructive mutation in the genes that encode its amino acid synthesizing enzymes. By comparing the numbers of mutants found on each type of media, ratios could be determined to compare the likelihood of mutation in one biosynthetic pathway versus that of another. However, for reasons that remain unknown, the experiment failed to produce any colonies with confirmed mutations in their amino acid biosynthetic pathways. Even when the mutated cells were cultured on media lacking all 20 amino acids, they all survived.

Introduction:

Ultraviolet light is commonly used for sterilization in many different settings because of its germicidal properties. UV rays damage cells by introducing mutations into their DNA, destroying the cell's capacity to carry out its essential functions. Specifically, UV light changes monomers in adjacent pyrimidines to cyclobutane pyrimidine dimers (Ritchie, p 3). This process is diagrammed below:



Source: Ritchie, p 3

When a strand of DNA with such a dimer is used as a template for replication, the cell's DNA replication machinery cannot recognize the dimer, and inserts an adenine by default (Rao, p 2). Thus, since adenine is the complement to thymine, thymine-thymine dimers do not cause mutations. Cytosine-thymine dimers, on the other hand, create a point-mutated strand of DNA.

Because of the selective nature of the mechanism of UV-mediated mutagenesis, it is possible that some genes, specifically those with more adjacent thymine-cytosine pairs, are more likely to suffer mutations from UV light than others. To test this hypothesis, I decided to look at a specific class of genes that would be easy to test for mutations: those genes which encode proteins involved in amino acid synthesis.

Amino acids are among the most important of a bacterium's resources, forming the building blocks for all the proteins necessary for life. The production of amino acids is catalyzed in multiple steps by various enzymes, most of which have been observed and cataloged. Amino acid synthesis is important enough that many cells have multiple biosynthetic pathways for the same acid, allowing synthesis to continue even if one of the relevant enzymes or genes becomes damaged. The purpose of this experiment was to determine which, if any, of the proteins that catalyze the amino acid biosynthetic pathways was more likely to suffer mutations after exposure to ultraviolet light.

In order to achieve this goal, the experiment first had to be focused and narrowed in scope. Testing all of the proteins involved in synthesizing all 20 of the amino acids would have been a gargantuan task, so I decided instead to focus on only the final catalytic step in each of the pathways. Several possibilities were then thrown out: glutamate and glutamine, because each can be synthesized from the other; threonine and tryptophan, because their immediate precursors are highly charged phosphates that would be difficult to pass through a cell's membrane from its environment; and tyrosine, because of the multitude of pathways leading to its synthesis. From the remaining candidates, I narrowed the field down to four: alanine, aspartic acid, asparagine, and methionine. These were chosen because their immediate chemical precursors (pyruvate, oxaloacetate, aspartic acid, and homocysteine, respectively) are already present in normal cells as intermediates of respiration and other common cellular processes. The proteins I would test for mutations would be: methyl transferase, which converts homocysteine to methionine; asparagine synthetase, which synthesizes asparagine from aspartic acid; alanine transferase, which makes alanine from pyruvate, and aspartate transaminase, which converts oxaloacetate to aspartic acid.

Next, some method had to be devised to identify those cells with mutations in the enzymes under study. I considered sequencing the mutant colonies, but decided that this method would be too time-consuming and unreliable. I settled on a procedure in which "minimal " media, containing only the essentials for bacterial life, were created. To this media I added amino acid in five different combinations, producing plates containing all amino acids but one. I then spread BL21 cultures onto complete LB media and exposed them to UV light for several seconds. After incubation, I expected to see a clear kill curve; as the time of exposure increased, I expected the number of surviving colonies to decrease steadily. Those cells which had survived

the exposure, but presumably had mutations that were not life-threatening on LB, would appear as colonies. The next step required the use of what is known as a replica plating device: a plastic cylindrical object with a diameter slightly less than that of a petri dish, to which a sterile velveteen square can be attached. When a plate containing bacteria is pressed down onto the device, cells are left on the square in the same positions as on the original plate. Pressing fresh plates onto the device creates exact replicas of the original, “master” plate.

Using this tool, I made copies of the UV-exposed plate on each of the four amino acid deficient plates, plus a control plate with all amino acids. Since healthy *E. Coli* bacteria can synthesize all 20 of the amino acids, and the immediate chemical precursors of the missing amino acids were present in the media, any cells which did not have mutations in the protein which catalyzes the final synthetic step of a particular pathway would be able to survive on a plate lacking that amino acid. For instance, assume that a cell has a mutation in the gene that encodes the protein that catalyzes the first step of the sequence for asparagine. This cell will survive on the -asn plate (and, obviously, all the other plates), since it can still synthesize asparagine from its precursor, aspartic acid, which is present in the -asn plate. On the other hand, if the cell has a mutation in asparagine synthetase, it will not survive on the -asn plate, since it cannot synthesize asparagine from aspartic acid and thus has no way to obtain asparagine. If I observed a particular colony that appeared on the original and control plates, and all but one of the incomplete plates (for example, on -ala, -asp, -asn, but not on -met), then I could assume that this colony had a mutation in the final step of the pathway for the relevant amino acid (in this case, methionine).

To ensure that the colonies were real mutants and not simply errors in the replication procedure, I made test plates of each of the four amino acid deficient media. Any colony thought

to have a mutation, for example, in its alanine pathway, would be streaked onto media lacking alanine; if it grew, then it would be declared a false alarm, but if it did not grow, it could be considered a confirmed mutant.

In order to make sure that the experiment's procedure was not flawed, and to screen for a wider variety of mutations, I also made some plates from M9 minimal media, which lacked all of the amino acids, and carried out the same basic procedure of exposure and replica plating described above. In theory, a cell with mutations in any of the proteins that synthesize amino acids or their essential intermediates would not survive on M9 with no amino acids (referred to as "M9 -" from here on). This test would eliminate some possible sources of error in the main experiment; it would show whether or not any mutations in genes related to amino acid synthesis were occurring, and help to determine whether the other M9 media were providing an accurate screen. In addition, any colonies that showed up on the LB master plate but not on M9- could be tested on the other M9 plates to determine which amino acid the mutant colony could not produce. This step provided, in effect, a backup measure to ensure that the main experiment was working properly; if I observed a large number of mutants through this method but not through the first, I could reasonably assume that I had made an error in preparing the single-acid-deficient media; if I observed few or no mutants through this method, I could conclude that either mutations in genes related to amino acid synthesis are very unlikely, or there was some other, larger flaw in the setup of the experiment.

By comparing the number of colonies with mutations in the four different pathways, I could establish which genes, if any, were more likely to mutate than the others. If I found that one pathway was significantly more likely to suffer mutations than another, I would have what I was looking for: what is known as a "mutation hot spot."

Methods:

Part I: Prepare M9 base media.

1. To 750 ml distilled water, add the following, to a total volume of 1 l:

   -200 ml 5x M9 salts:

   To make M9 salts, add the following to 1 liter distilled water:

   64 g $Na_2HPO_4 \cdot 7H_2O$

   15 g KH_2PO_4

   2.5 g NaCl

   5.0 g NH_4Cl

   - 2 ml 1 M $MgSO_4$
   - 20 ml 20% glucose
   - 0.1 ml 1 M $CaCl_2$
   - 0.1 ml 0.5% thiamine solution
   - 20 ml amino acid 50x stock:

   To make 50x amino acid stock, add the following to 20 ml distilled water:

   40 mg each of all amino acids EXCEPT ala, asn, asp, met.

2. Add bacto-agar: 15g / liter of media.

3. Prepare a total volume of 150 ml of the same ingredients in the same concentrations, but omit the amino acids and thiamine solution. Label this media M9-.

4. Autoclave both types of media on liquid cycle for 30 minutes.

5. Pour liquid media into sterile plates, label carefully, refrigerate.

Part II: Mix and add amino acid combinations.

1. Label 5 small, screw-top glass containers: -ala, -asp, -asn, -met, +.

2. To each jar, add the following:

-ala: 5 mg each of asn, asp, met powder, 1 ml sterile deionized water.

-asp: 5 mg each of asn, ala, met powder, 1 ml sterile deionized water.

-asn: 5 mg each of ala, asp, met powder, 1 ml sterile deionized water.

-met: 5 mg each of asn, asp, ala powder, 1 ml sterile deionized water.

+: 5 mg each of asn, asp, met, ala powder, 1 ml sterile deionized water.

3. Sterilize by autoclaving for 15 minutes on liquid cycle.

4. Using a micropipettor and a sterile glass spreader, add 250ml of each solution to each of 4 M9 plates (regular M9, not M9-), for a total of 20 plates, four of each type. Be sure to label carefully.

5. Refrigerate.

Part III: Culture BL21 cells, dilute.

1. Using a sterile inoculating loop, transfer a visible clump of cells from frozen stock of BL21 competent cells to a tube containing 10 ml LB broth.

2. Incubate 24 hours at 37°C.

3. Dilute to 1/10000 concentration by adding 1 μl BL21 culture to 10 ml LB broth.

Part IV: Expose to ultraviolet light.

1. With a sterile-tipped micropipettor, transfer 50 μl of dilution onto each of 6 LB plates.

2. Sterilize a glass spreader by dipping it in 95% ethanol and igniting the ethanol with a bunsen burner.

3. Use the spreader to spread the diluted cells across the surface of the plate. Be careful not to push all the cells to the edges of the plate, because colonies near the circumference

are often missed by the replica plating device.

4. Turn the plates upside down, and label them with a time of exposure: 0, 3, 4, 5, 6, and 7 seconds.

5. Place the 0 second plate directly into the incubator.

6. Remove the lid from the plate labeled "3 seconds," and place it upside-down into the UV transilluminator. Make sure that the plate is horizontal and aligned with the center of the UV lamp.

7. Close the cover of the UV transilluminator, and turn it on for 3 seconds. Remove the plate, and replace the lid.

8. Repeat steps 6 & 7 for each of the plates, changing only the length of exposure.

9. Incubate all plates at 37°C for 18 - 24 hours. Longer incubation times produce larger colonies, which are more difficult to replicate cleanly.

Part IV. Replica Plate.

1. Look at the plates that were just incubated, and check to see that the number of surviving colonies decreases with increasing time of exposure. The colonies on the control plate should be uncountable, and the longest exposure should have few or no colonies.

2. If a kill curve is observed, choose a plate with a reasonable number of colonies. Around 100 or so colonies is ideal, but less is acceptable.

3. Mark each colony with a permanent marker on the bottom of the plate, and record the total number. Also in permanent marker, draw a line on the circumference of this plate as a reference marker.

4. Take one plate from each of the five types of media that contain amino acids, as well

as an LB control plate. Mark each of these with a line on the circumference.

5. Place a sterile velveteen square onto the replica plating tool, and secure it with the locking ring. Make a mark on the locking ring as well.

6. Press the UV-exposed LB plate firmly down onto the replica plating tool, matching up the two reference lines, and remove.

7. Press each of the 6 replica plates onto the device, once again lining up the reference markers.

8. Incubate for 24 hours.

9. Repeat steps 3-9, using a new master plate, on M9- media. Once the replica process has been observed to work on LB, there is no need to repeat the use of an LB control plate.

Step V. Compare replicas and original, test mutants.

1. With each replica plate, remove its cover and that of the master plate. Hold the plates face down, with the reference points aligned and the master plate on the bottom.

2. If a dot on the bottom of the master plate is visible in a location where there is no corresponding colony on the replica plate, mark the dot, and check if a colony appears in that position on the + plate.

3. If no colony exists, cross out the dot, and do not consider it further.

4. If a colony does appear on the + plate, check to see if there are any other plates where the colony does not appear, and if so, write them down.

5. Using a sterile instrument, pick the colony from the + or master plate, and scrape it onto a marked region of the same kind of plates that the colony does not appear on. (example: colony A shows up on -ala, -asp, and +. To test, scrape A onto -met and -asn

plates.)

5. Repeat as necessary, until all possible mutants have been found and transferred to the appropriate test plates.

6. Incubate for 24 hours, and observe growth or non-growth of each colony.

7. Repeat this part with the M9- replica and LB master plates.

Results:

Kill Curves:

This section of the results shows the data I collected after exposing E. Coli cells to UV light for varying lengths of time. Each table shows the strain used, the concentration, and the number of colonies visible after incubation for each length of exposure. All of the tables show a clear downward trend; the longer the exposure, the less likely the exposed cells were to survive. The time courses vary somewhat because as the term progressed, I gained a better understanding of what an appropriate time of exposure was. The numbers of surviving colonies vary, even within the same length of exposure, because the cells were not always evenly distributed in the dilutions. Thus, sometimes two plates exposed for the same length of time had different numbers of survivors, because they had different numbers of cells to begin with.

Trial 1, BL21 1/10000:

Time of Exposure (sec.)	Surviving Colonies
0	uncountable
5	54
10	4
15	0
30	0

Trial 2, BL211/10000:

Time of Exposure (sec.)	Surviving Colonies
0	uncountable
7	106

Trial 3, DH5α 1/10000:

Time of Exposure (sec.)	Surviving Colonies
0	uncountable
2	83
3	27
4	3
5	0

Trial 4, BL21 1/10000:

Time of Exposure (sec.)	Surviving Colonies
0	uncountable
6	106
7	47

Trial 5, BL21 1/10000:

Time of Exposure (sec.)	Surviving Colonies
0	uncountable

6	70
7	39

Trial 6, BL21 1/10000:

Time of Exposure (sec.)	Surviving Colonies
0	uncountable
3	130
4	127
5	38
6	13
7	4

Trial 7, BL21 1/1000:

Time of Exposure (sec.)	Surviving Colonies
0	uncountable
3	63
4	25

Replica Plate Results:

This section shows the results of the replica plating procedure. The control experiment was performed to ensure that the procedure worked, and that healthy cells could survive on the different M9 media. All other tables shows the dilution and strain used, as well as the kill curve from which the master plate came and the number of colonies visible on each type of replica

media. Those colonies that did not appear on certain media were given a number, and tested on the appropriate test plates. The results of these tests are listed in the “mutant status” column.

Control trial: BL21 undiluted, 0 second exposure

Media	# colonies
LB (replica)	lawn
-asp	lawn
-asn	lawn
-ala	lawn
-met	lawn
+	lawn

Trial 1: BL21 1/10000, 7 sec, from kill curve 2.

Media	# of colonies	Mutant status
LB (master)	106	none
-asp	105	#5 confirmed non-mutant
-ala	104	#1, 3 confirmed non-mutant
-met	102	#2, 3, 5, 6 confirmed non-mutant
-asn	103	#2, 3, 4 confirmed non-mutant
+	105	#2 confirmed non-mutant

Trial 2: BL21 1/10000, 6 sec, from kill curve 4.

Media	# of colonies	Mutant status

LB (master)	106	none
-asp	106	none
-ala	106	none
-met	106	none
-asn	106	none
+	106	none

Trial 3, DH5α 1/10000, 2 sec, from kill curve 3.

Media	# of colonies	Mutant status
LB (master)	106	none
-asp	106	none
-ala	106	none
-met	106	none
-asn	106	none
+	106	none

Trial 4, BL21 1/10000, 6 sec, from kill curve 5.

Media	# of colonies	Mutant status
LB (master)	70	none
M9-	70	none

Trial 5, BL21 1/10000, 5 sec, from kill curve 6.

Media	# of colonies	Mutant status
LB (master)	38	none

M9-	38	none

Trial 6, BL21 1/10000, 4 sec, from kill curve 6.

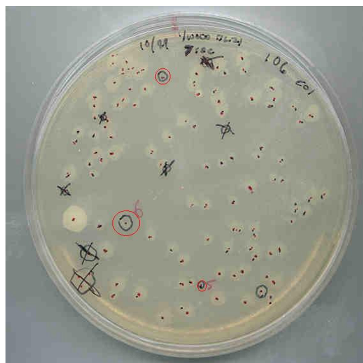
Media	# of colonies	Mutant status
LB (master)	127	none
M9-	126	#7, confirmed non-mutant

Trial 7, BL21 1/10000, 3 sec, from kill curve 7.

Media	# of colonies	Mutant status
LB (master)	63	none
M9-	63	none

Pictures:

This section contains photographs of an exposed LB plate and a replica M9 –met plate. I have circled three colonies that appear on LB but not on –met . I thought at first that these colonies must have mutations in the methyl transferase gene. However, when they were transferred onto –met test plates, they all survived, confirming that they were not, in fact, truly mutants.



Above: the LB master plate

Above: the –met replica plate

Conclusion:

Clearly, as my results demonstrate, I was unable to draw the kind of conclusion I had hoped to when I first conceived this experiment. With no confirmed mutants at all, I was unable to determine any ratios between the mutation frequencies in the four enzymes I studied. Of the hundreds of bacteria that survived the initial irradiation, not one proved to have a mutation in any of these enzymes.

This lack of mutants might be due simply to the small number of colonies and enzymes I tested. However, this explanation fails to account for the total absence of mutants on the M9-plates. In these trials I vastly increased the number of proteins under survey, and it seems unlikely that not a single irradiated bacteria would have a mutation in any of its genes related to amino acid synthesis.

The explanation may be that mutations in these genes are not likely to be replicated without correction; genes involved in amino acid synthesis are fairly essential to a bacterial cell, so it is possible that DNA repair mechanisms may be more concentrated on them than on genes less important to survival. It is also possible that many of the genes involved in amino acid biosynthesis have other functions without which a bacterium cannot survive, even on complete media. Indeed, recent research into the *E. Coli* genome has found that the genes asd and dapE, both of which encode the enzymes involved in lysine synthesis, are essential for the survival of the bacterium. Once again, however, the M9- experiments suggest that this is not the answer, since although some genes may be essential, certainly not all or even most amino acid biosynthesis genes are so critical that *E. Coli* cannot survive without them under any conditions.

Fortunately, a simpler answer that explains more of the findings presents itself. Research, of which I was not aware at the time I devised the experiment, has found that exposure to visible

light activates the DNA repair mechanisms of many bacteria. “The cyclobutane photodimer [the kind of DNA damage caused by UV light] can be repaired by a photolyase that has been found in bacteria and lower eukaryotes… The enzyme binds to the photodimer and splits it, in the presence of certain wavelengths of visible light, to generate the original bases.” (Griffiths et al., p 511)

To understand how this might undermine the findings of my experiment, recall how mutation by ultraviolet light occurs. Mutations do not happen immediately; instead, they happen when damaged DNA is used as a template for replication. If a cell’s built-in repair mechanisms can correct the damage before any replication occurs, the mutation will not be expressed or incorporated permanently into the cell’s genome. What seems likely to have happened in my experiment is that, while I was irradiating the plates, I set some of them down on my lab bench, since I could only expose one at a time. By doing so, I exposed the mutated cells to visible light, inadvertently allowing their photolyase repair mechanisms to activate, wiping out any photodimer damage that might have been present immediately following the irradiation. When I grew the cells on the various M9 plates, it appeared that none of them had suffered any mutations to the genes in question.

A simple way to fix this would be to simply cover the culture tubes and plates in tin foil to prevent the cells from being exposed to visible light before or immediately following irradiation. The kind of repair described above could only take place in the first generation after exposure, since by the second generation the damaged DNA would have already been used as a template and incorporated as a permanent mutation. Thus, the remainder of the experiment could be repeated without modification.

Although this experiment did not produce the kind of data I had hoped for, due to a lack

of knowledge about some pre-existing research, I do not feel that it was a failure. I learned quite a bit about the mechanisms underlying UV-mediated mutagenesis, and became significantly more skilled at carrying out the laboratory procedures involved. I am confident that if I were to repeat the experiment with only slight modifications, as described above, the project would succeed in determining the mutation frequencies I had originally hoped to find.

References:

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