AN ANALYSIS OF THE EXPRESSION OF MUTATIONS IN

Escherichia coli (E.coli)

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An Analysis of The Expression of Mutations in Escherichia coli (E.coli)

RESEARCH PROBLEM

Do the mutations in the Lactose (Lac) Operon of the E.coli genotypes DH1, DH5 alpha, AMA 1004, and XL-1-Blue produce the predicted phenotypic expressions of the Lac Operon?

PURPOSE

The purpose of this research was to determine if the mutations in the **lac operon** of DH1, DH5 alpha, AMA 1004, and XL-1-Blue produce the expected **phenotypic expressions** of the Lac Operon. Selected phenotypic expressions of the lac operon were tested at the temperature for optimum E.coli growth (37°C) and at 25°C. The phenotypic expression of each strain was tested in the laboratory by measuring the levels of proteins the Lac operon codes for in each strain. The protein selected for examination was **Beta-Galactosidase,** an enzyme coded for by the **lac Z gene,** located on the lac operon.

The initial objective of this research endeavor was to test various research protocols for protein evaluations and make any necessary modifications. Protocols were then developed which gave consistent results.

BACKGROUND

Researchers at East Carolina University and North Carolina State University developed the mutant bacteria strains to be utilized in this research. Table 1 gives the

genome (List of Genes) for each strain.

Table 1

GENOMES OF BACTERIA USED

DH1

F^-, end A1, hsd R17 (rk^-,mk^+), sup E44,

thi -1, λ^- rec A1, gyr A96, rel A1

DH5α

F^-, ϕ 80 d lac Z Δ M15 Δ(lac Z YA^- arg F) U196

deo R, rec A1, end A1, hsd R17 (rk^-, mk^+)

sup E44, λ^-, thi-1, gyr A96, rel A1

XL-1-BLue

rec A1, end A1, syr A96, thi-1,

hsd R17, sup E44, rel A1, lac,

[F' pro AB, lac^9 ZΔM15, Tn 10 (tet^r)]

/ puc 19

AMA-1004

Δ (lac 1 POZ) C29, leu B6, trp C9830,

hsd R, gal U, gal K rec A56, Str A^r

An **operon** is a group of linked genes, including the associated **promoter,**

operator, and any regulatory genes present.(Darnel, Lodish, & Baltimore 1990) A **promoter** is a part of the operon which makes the operon accessible to the polymerase enzyme responsible for transcription. The **operator** is the location where the polymerase attaches to the operon to begin transcription. **Regulatory** (controller) genes encode proteins which can attach to the operator preventing transcription(Bains 1987).

When transcription is allowed to continue, the genes of the operon produce specific **messenger RNAs** which are translated into specific proteins(Hartl 1991). This study involved a qualitative and quantitative analysis of the results of genetic engineering in the lac operon of E.coli(DH1, DH5 alpha, AMA 1004, and XL-1-Blue).

Lactose is a sugar which is not normally metabolized if glucose is present. This occurs because the lac operon is normally inhibited by the presence of glucose and will function only when induced by lactose or an analog of lactose. **Induce** means to initiate or cause to take place(Darnell, Lodish, & Baltimore 1990). When the operon is induced, it will operate because the operator is released to accept the polymerase complex even if glucose is present.

Analogs are complex molecules which function in a similar manner to another molecule. The analog for lactose that was utilized in this research was **Isopropyl Beta-D-Thiogalactopyranoside (IPTG)**(Cesareni & Murray 1987). Its function was to induce the lac operon into producing the enzymes which it codes for.

Of the four strains of E.coli used in this research, one strain (**DH1**) was the wild type. This strain has not been changed in the lac operon of the bacterium. The other three strains have had changes made in their lac operons. Gene fusion is one type of alteration

which can be used to achieve desired changes in phenotypic expression, but due to the nature of these mutants they are not as easily used. They require additives in the media. When the lac operon is fused with another operon which is very accessible, the result can be a lac operon which does not respond to negative regulation. Only by growing these mutants on media with thiophenyl-ethyl-Beta-D-galactoside (TPEG), can the lac operon be turned off. None of the mutants in this research were mutated in this manner, so the need for the special media was eliminated(Beckwith 1991). The mutants used in this research were created by removing sections of the lac operon.

DH1 is a wild type with no alteration in the lac operon(Streips 1991). It was predicted that this type would produce higher levels of Beta-Galactosidase when induced by lactose or an analog such as IPTG.

DH5-alpha is a mutant strain which has had the amino-terminal end of the **lac Z gene** (which codes for the protein enzyme Beta-Galactosidase) removed. This organism must have a complimentary part called **pUC-18** in order to utilize lactose. pUC-18 is a plasmid containing the missing portion of the Z gene. A plasmid is a loop of DNA which can be taken up by cells. This part was not provided in the media, so the bacteria should not have been induced by the IPTG(Suzuki, Griffiths, Miller, & Lewontin 1989).

AMA 1004 is a mutant strain in which the genes coding for **lactase** and **permease** (two other proteins coded for by the lac operon) have been removed leaving only the gene for β-Galactosidase. The production of β-Galactosidase should not be affected by this type of mutation. It was predicted that this strain would be induced by IPTG (Jewell 1994).

XL-1-Blue is a mutant strain in which the regulatory gene has been deleted, thus rendering the operon fully functional at all times, regardless of inducement with IPTG (Jewell 1994). A regulatory gene produces a proteins which binds to the operator of an operon and renders it unable to transcribe. Normal levels of β-Galactosidase should have been produced with or without inducement with IPTG. This strain also has a gene for resistance to the antibiotic ampicillin incorporated into it. The **pUC plasmid** was used to incorporate the **pUC 19** gene into this organism. The antibiotic ampicillin can now be used to select for this strain(Streips 1991). This can be done by exposing the bacterium successively to heat and then to cold in order to open pores in its cell wall. Electric shock can also be used to open pores in the bacterium. This process is known as electroporation. The plasmid is capable of multiplying itself and doesn't require incorporation into the genome of the host. Based on these modifications in genotype it was predicted that the test organisms would respond to inducement in the following ways:

(1) **DH1** should produce higher levels of Beta-Galactosidase when induced by IPTG as compared to DH1 not induced by IPTG.

(2) **DH5α** should not respond to inducement by IPTG. Levels of Beta-Galactosidase should be the same with or without IPTG.

(3) **AMA 1004** would be expected to produce amounts of β-Galactosidase similar to those produced by DH1. Total protein production by this strain when induced should be less than DH1 due to the two missing genes coding for lactase and permease. Without inducement with IPTG, protein levels should be comparable to those of DH1. With inducement there should be less proteins produced by AMA 1004.

(4) **XL-1-Blue** should produce consistent amounts of β-Galactosidase with or without inducement with IPTG because there is no regulator gene to stop the operon from functioning. There is no regulatory gene present which produces the protein that attaches to the operator to prevent transcription.

Growing the strains of E.coli at a lower temperature was hypothesized to produce an overall lower growth rate as well as lower protein production rate. The amount of proteins produced with respect to density of growth; however, was hypothesized to be comparable to that of the bacteria grown in optimal conditions of temperature.

Several laboratory techniques were utilized and perfected throughout the course of this research(Table 2).

TABLE 2

SKILLS MASTERED DURING THIS RESEARCH

Aseptic technique	Media preparation
Spectrophotometer use	Antibody probing
SDS-PAGE gel preparation	Culture maintenance
Western Blot preparation	Beta-Galactosidase Assay
BCA protein assay	

JUSTIFICATION

This research project has provided expertise in utilizing the tools and techniques listed previously, which will be needed for further study and future employment. Aseptic technique will be crucial for reproducible results. The ability to make and maintain media and cultures will be necessary. In addition, techniques involving the use of electrophoretic gels and Western Blots are required for use in most research labs.

This research also provided information to the scientific community concerning the functions of operons, and the ways in which genetic manipulation can either hamper or enhance the expression of undesirable or desirable traits. This research has the potential to benefit Pembroke State University as well. The protocols perfected during the course of this research will assist the laboratories at Pembroke State University to achieve better experimental results and assist in the training of future students.

EXPERIMENTAL DESIGN

I. PROTOCOL DEVELOPMENT AND MODIFICATION

The protocols for several experiments were attempted and subsequently modified. The following is a representation of the numbers of protocols attempted and the modifications which eventually yielded successful results.

For growth of the bacteria used in the study, two protocols were tried. The first, involving 20 mls of media in 50- ml tubes was used 12 times. The growth obtained required 24 hours. The second protocol involved the use of 15- ml tubes containing only 10 mls of media. This protocol originally appeared to produce quicker growth, however, 6 repetitions of this protocol yielded no quicker growth. Finally, the protocol involving the 50- ml tubes was adopted due to the greater number of sub-samples which could be obtained from each tube.

The protocol for cell preparation for protein synthesis was used for 14 repetitions before it was discovered that a step was missing which yielded unacceptable results. The step missing was a wash step requiring the use of 250 µl of Z Buffer to wash any remaining media from the cells. This extra wash step was inserted in the protocol and four more repetitions completed. Results were more consistent with the revised protocol.

The protocols for Bicinchoninic acid protein assays (BCA) and β-Galactosidase (β-Gal) assays were not modified for this research. The original protocols developed by Dr. Barker Bridgers were used throughout the research for the testing of the E.coli strains. The BCA protocol was utilized 18 times, and the protocol for β-Gal assays was utilized 9 times.

A total of 10 Sodium Dodecyl Sulfate-Polyacrylamide gels were poured and used in this research. Four gels were poured and run to perfect the polymerization process and determine the proper voltages and running speeds for separation of proteins. Two gels were used for coomassie blue staining to determine the location of β-Galactosidase on the gel. A high molecular weight marker containing β-Galactosidase was used to determine the exact location of the protein in the samples. The final four gels were used for Western Blots to transfer the proteins present in the gels nitrocellulose membranes for probing with a monoclonal antibody for β-Gal.

The probing protocol used in this research was developed specifically for this research project. The times of exposure to blocking solutions, antibody solutions, and staining solutions were changed to optimize results. Each of two protocols were used twice. The experiments conducted utilized the protocols as modified in the preliminary portion of the research.

II. FINAL PROTOCOLS UTILIZED

GROWTH OF TEST BACTERIA POPULATIONS

Cultures of 4 strains of Escherichia coli(E.coli) (**DH1, DH5 alpha, AMA 1004, and XL-1-Blue**) were established from streak plates into liquid broth media. The medias used were LB broth, LB broth with the antibiotic ampicillin, and 2YT broth (See Table3).

BROTH MEDIA RECIPES

TABLE 3

LB BROTH		2YT BROTH	
Tryptone	10g	Tryptone	16g
Yeast extract	5g	Yeast extract	10g
NaCl	10g	NaCl	5g
H_2O	1L	H_2O	1L

For solid media, add 15 grams agar per liter of media.

For media with ampicillin, add 100 microliters ampicillin per 100 mls of media after it has cooled to below 60°C.

Ampicillin is prepared at a concentration of 50 mg/ml.

The working area under the laminar flow hood was sterilized using 70% ethyl alcohol in a spray bottle. All materials used were also sterilized by placement in a beaker of 70% ethyl alcohol and subsequent flaming using a bunsen burner.

Four clear 50 ml polypropylene conical tubes were prepared each containing 20 mls of the appropriate media. The strains, DH1 and DH5 alpha use LB broth. AMA 1004 required 2YT broth, while XL-1-Blue uses LB broth with ampicillin.

The streak plates were placed top up in the laminar flow hood. An innoculating loop was flamed to redness and air cooled under the hood for 10 seconds. The streak plate was opened by partially lifting the top of the plate off of the bottom half. An individual colony was gently removed from each plate, and used to innoculate the conical tubes of broth media. As each

colony was removed from the streak plate, the plate was closed and the appropriate tube was selected. With the little finger of the transfer hand, the cap was removed. The innoculating loop, with the colony, was lowered into the liquid media in the tube. With a vigorous up and down motion, the colony was dislodged into the liquid media. The cap was replaced and the loop flamed.

This step was repeated with all plates and tubes until colonies of all four types were transferred from the streak plates into broth tubes.

These cultures were incubated overnight in a shaking waterbath at 37°C, insuring that the water level in the waterbath never dropped below the level of the media in the tubes. The caps were kept loose (approximately one turn) to insure adequate oxygen for the bacteria.

These cultures were used as source cultures throughout the remainder of the project. They were used as a source of bacteria to introduce into fresh media when new cultures were needed.

II. OPTICAL DENSITY TESTS FOR GROWTH

1. One ml of each of the source cultures was placed into tubes containing 20 ml of appropriate media as outlined in Table3. The tubes used were 50- ml polypropylene conical tubes. This procedure was done aseptically using the laminar flow hood. Two tubes for each culture were prepared.

2. Twenty microliters of IPTG (Isopropylthio-Beta-D-Galactosidase) were added to one of the tubes of each culture.. This substance acted as a lactose analog. (**See appendix A**) The tube containing IPTG was labeled "+IPTG", and the other tube without IPTG was labeled "-IPTG".

3. The tubes were placed in a shaking waterbath at 37° C overnight as outlined in experiment I. The water level in the waterbath was maintained above the media level in the tubes. The caps were monitored regularly to ensure they remained loose while the tubes were constantly shaking.

4. The cultures were harvested in 24 hours, and the density of cells in the media was measured for each culture using a spectrophotometer. Readings were recorded for each culture using a spectrophotometer set at wavelength of 600 nm. Blanks of LB and 2YT media were used to zero the spectrophotometer.

5. When DH1 reached an optical density of 0.8-1.2, the cultures were ready to use. (DH1 was a wild type bacteria with no genetically engineered changes in its DNA. It was used as a control to compare with the other strains. An optical density of 1.0 for DH1 is required as a standard of measurement to quantify differences in growth of the other cultures.) DH1 -IPTG was used as the control because it was in the natural state without

inducement of the lac operon.

6. The IPTG was used to induce the lac operon of the bacteria and cause formation of catabolic enzymes like Beta-Galactosidase, lactase, and permease. One of these protein enzymes, Beta-Galactosidase, was the object of this research. The calculation needed for the conversion of the readings is:

TEST READING / 1ML = 1 / X

EXAMPLE: DH1=1.0 AND DH5α=0.66

X=1.5ML

7. The entire protocol II was repeated incubating the cultures at 25°C.

PREPARATION FOR PROTEIN ASSAYS

Introduction

1. All cultures from experiment II were placed on ice immediately after reading the optical densities to determine if they were in the acceptable range. This prevented any additional cell multiplication.

2. Using the quantities calculated from protocol II.-6, the equivalent of 1 ml of 1.0 optical density units of culture were put into a 1.5 ml microfuge tube (A 1.5 ml tube can only hold 1.0 ml of working sample. Only 1 ml of sample can be centrifuged at the time.). This was done for all eight tubes. The tubes were centrifuged at 14,000 rpm in an Eppendorf Centrifuge 5415 for 10 seconds. This was done by centrifuging 1 ml of each culture, pouring off the supernatant, and adding the appropriate amount of culture required from calculations in protocol II. The tubes were centrifuged again and the supernatant poured off. If only 1 ml was required from the calculation in protocol II, then one centrifugation wasall that was needed. Example: If DH5 alpha had a calculation of 1.5 from protocol II, the second centrifugation only required 0.5 ml added to the tube containing the pellet from the first centrifugation. This 0.5 mls of culture would have been centrifuged and the supernatant poured off.

3. The pellets were resuspended in 250 microliters of pure **Z buffer (See Appendix A)**. The cells were resuspended by pipetting the solution (with cells) up and down with a micropipetter.

4. The tube was centrifuged at 14,000 rpm for 10 seconds and the supernatant poured off. This step washed the cells and removed any remaining growth media.

5. The pellet was resuspended in 0.5 ml of Z Buffer using the micropipetter.

6. 50 microliters of **chloroform** were added to the solution and the cap closed securely (Beta-Galactosidase is soluble in chloroform and will separate out of the solution into the chloroform.). The cell suspensions were vortexed for 5 seconds, then centrifuged at 14,000 rpm for 30 seconds. The microfuge tubes were removed carefully from the centrifuge to avoid disturbing the layers which developed during centrifugation.

7. The aqueous layer on top of the interface of the two phases was removed carefully without disturbing the chloroform phase below or the interface. Denatured proteins were present at the interface. The aqueous layer was transferred to a new 1.5 ml microfuge tube previously labeled **BCA** and placed on ice immediately.

8. 100 microliters of the remaining lower phase were transferred from each tube to a new 1.5 ml microfuge tube previously labeled **β-Gal**. Care was taken not to include any of the denatured proteins located at the interface. The new tubes were placed on ice awaiting further analysis.

BCA ASSAY

WORKING REAGENT PREPARATION:

The working reagent was prepared by mixing 50 parts of Reagent A (Provided in Pierce BCA protein assay kit) with 1 part of Reagent B. The reagent mixture was green in color.

REAGENT A

1000 mls of base reagent containing sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.2N NaOH.

REAGENT B

25 mls of 4 % Copper Sulfate solution.

PROCEDURE:

1. A set of protein standards of known concentrations were prepared by diluting the stock BSA solution (bovine serum albumin at 2 mg/ml) as in Table 4.

RECIPE FOR MIXING BCA STANDARDS

TABLE 4

Volume of Stock Albumin	Volume of Water	Concentration
0.10 ml	0.90 ml	0.2 mg/ml
0.20 ml	0.80 ml	0.4 mg/ml
0.30 ml	0.70 ml	0.6 mg/ml
0.40 m	0.60 ml	0.8 mg/ml
0.50 ml	0.50 ml	1.0 mg/ml

2. 0.1 ml of each standard was pipetted into an appropriately labeled test tube. 0.1 ml of each test sample from the 1.5 ml microfuge tubes labeled "BCA" **(Protocol II)** was also pipetted into an appropriately labeled test tube. A **blank** was prepared using 0.1 ml water. A blank is a sample of no optical density, or a known optical density used to zero the machine.

3. 2.2 ml Working Reagent(The mixture of Reagents A and B) were added to each tube and the tubes were vortexed for 5 seconds.

4. All tubes were incubated in a waterbath at 37° C for 30 minutes. After incubation, the tubes were cooled to room temperature.

5. The blank was used to zero the spectrophotometer at 562 nm. The standards and test samples were then read and the results recorded.

6. A standard curve was prepared by plotting the known standards of protein concentrations on a graph. The concentrations of the test samples were found by comparing them to the standard curve.**(Table 6 in results and discussion)**

BETA-GALACTOSIDASE ASSAY

1. The total contents of each sample tube labeled "β-Gal"were transferred to a 20- ml glass tube with the appropriate sample label. 1.5 mls of Z buffer were added to the samples. 5.4 µl of β-Mercaptoethanol were added to the tubes. The β-mercaptoethanol is designed to break the disulfide bonds between polypeptides resulting in smaller protein subunits. This frees-up the β-Galactosidase for binding with a substrate. A substrate is a substance which provides a binding surface for an enzyme.

2. 400 microliters of o-nitrophenyl-β-D-galactopyranoside (ONPG) were added to each glass tube. ONPG is a substrate for the protein enzyme β-Galactosidase. The tubes were then incubated in a shaking waterbath at 37° C for one hour. When a distinct yellow color appeared in at least 25 % of the tubes, the reaction was stopped by the addition of 0.5 ml 1M Na_2CO_3 to each tube with a micropipetter. The tubes were placed on ice.

3. The optical density of each sample was recorded at 420 nm, and the results discussed in the results and discussion section.

PREPARATION FOR SDS-PAGE GEL

1. The equivalent of 1 ml of culture with 1.0 optical density (Calculated in protocol II) was transferred to a 1.5 ml microfuge tube. One tube was prepared for each of the 8 samples(**DH1+/-, DH5-alpha+/-, AMA 1004+/-, and XL-1-Blue+/-).**

2. The tubes were centrifuged at 14,000 rpm for 10 seconds and the supernatant discarded.

3. The cell pellets were resuspended in 40 microliters of double distilled water and 40 microliters of 2X sample buffer.(**See Appendix A)** A 1000 µl micropipetter with a standard pipet tip was used to resuspend the pellets in the 2X buffer.

4. Using a 1000- ml beaker of water on a hot plate, the tubes were heated to 100° C for 5 minutes. They were stored at 4.0° C until needed for loading the gel.

VI. ELECTROPHORETIC SEPARATION OF PROTEINS

SDS PAGE

SODIUM

DODECYL SULFATE-POLYACRYLAMIDE GEL

ELECTROPHORESIS OF PROTEINS

INTRODUCTION

The SDS-PAGE system is used to separate the proteins in an extracted cell solution so that they can be stained and observed, or removed from the gel and identified. This process is a laboratory technique often used to separate and identify macromolecules according to their purity and molecular weight. The rate of movement in the gel depends on the molecular weight of the molecule. Due to the nature of the gels, the molecules are forced to squeeze through tiny openings and pores in the gel. Smaller molecules will generally travel further than larger molecules.

In this experiment, the gel was sandwiched between two glass plates. The gel was suspended in buffer solutions and an electrical current passed through it. The charge at the bottom of the gel was opposite that of the proteins being forced through the gel. Due to the fact that the proteins appeared invisible in the gel, a tracking dye was added to the samples which would travel through the gel at a faster rate than the samples. The proteins had moved through the gel when the tracking dye reached the end of the gel.

The gel was then placed in an organic dye (coomassie blue) to reveal the bands formed by the proteins. More gels were prepared, and were used to transfer the proteins

to a nitrocellulose membrane to be probed with an antibody for β-Galactosidase. These gels were not stained with the organic dye because staining with the dye damages the proteins and will not allow for their proper transfer to a membrane for probing.

PREPARATION OF SEPARATING GEL

1. The gel unit was assembled in the casting mold with 1.5mm spacers.

2. In a 125-ml side arm vacuum flask, 30 mls of separating gel solution were mixed according to Table 5a.

Table 5a

RUNNING GEL RECIPE

30% T, 2.7% C (1)	10 ml
Buffer (2)	7.5 ml
10% SDS (3)	0.3 ml
H_2O	12.05 ml

3. The flask was stoppered and a vacuum applied for approximately 15 minutes, while stirring at low speed.

4. 15 microliters of TEMED and 225 microliters of ammonium persulfate were added(See Appendix A). The flask was swirled gently to mix and avoid generation of any bubbles.

5. The separating gel solution was taken up into a glass syringe and transferred to the sandwich slowly, but not so slowly as to let it gel in the syringe.

6. The gel was water-layered using a syringe fitted with a needle. About 1 ml of water was used to place a thin, even layer over the entire surface of the gel.

7. The gel was approximately 4 cm from the top of the sandwich plate, and polymerized in 30 - 45 minutes.

PREPARATION OF THE STACKING GEL

1. In a 50 ml side-arm flask, 15 mls of stacking gel were mixed according to Table 5b.

STACKING GEL RECIPE

TABLE 5b

30% T, 2.7% C (1)	2.0 ml
Buffer (2)	3.75 ml
10% SDS (3)	0.15 ml
H_2O	9.15 ml

2. A stirring bar was added and the solution was deaerated in the same manner as the separating gel.

3. To the solution were added 7.5 microliters of TEMED and 75 microliters of ammonium persulfate.**(See Appendix A)** The solution was swirled gently to mix.

4. The water layer was poured off the separating gel, and the stacking gel was immediately transferred onto the separating gel with a glass syringe. A gel comb was inserted into the gel carefully to avoid trapping any bubbles. Oxygen in the bubbles will inhibit polymerization of the gel solution. The gel was then covered with Saran Wrap and stored in the refrigerator until gel could be run.

LOADING AND RUNNING GELS

1. The comb was carefully pulled straight out of the gel to ensure integrity of the wells. Any walls which were crooked were straightened with a syringe tip. Any trapped air bubbles were removed with a syringe tip.

2. Each well was filled with tank buffer.**(Table 7)** Using a 20 µl pipetteman, 15 microliters of 1%Bromphenol Blue were added to the samples stored for **SDS-Page gels.** These were underlayered into each well. The bromphenol blue acted as a tracking dye.

TANK BUFFER

TABLE 7

Tris	12 grams
Glycine	57.6 grams
SDS	40 ml 10% solution
H_2O	4.0 liters

3. The upper buffer chamber was put into place. The lower cams were removed from the sandwich which was cammed to the bottom of the upper buffer chamber. The upper buffer chamber was put in place on the heat exchanger in the lower buffer chamber.

4. The lower buffer chamber was filled with tank buffer until the sandwich was immersed in buffer. Any trapped bubbles were coaxed away with a pipet.

5. A magnetic stirring bar was added to the lower chamber and the chamber was placed on a magnetic stirrer. By circulating the lower buffer, the temperature of the buffer was kept uniform throughout. This is important because uneven heating distorts the

banding pattern of the gel.

6. The upper buffer chamber was filled with tank buffer. The buffer was not allowed to pour directly into the top of the sandwich. This would wash out the samples.

7. The lid was placed on the unit and was connected to the power supply. The cathode was connected to the upper buffer chamber. The power supply was set for constant current.

8. The power supply was turned on and adjusted to 30 milli-amps per gel. Example: If two gels are used, set to 60 ma. The voltage should start at about 70-80 volts, but may increase during the run.

9. When the dye reached the bottom of the gel, the power supply was turned off and disconnected. This process took about 3 hours.

STAINING AND DESTAINING GELS

1. The sandwich was disassembled and the gels put into Coomassie Blue stain.

2. Gloves were worn to ensure there were no fingerprints on the gels.

3. The gels were gently shaken for 3-4 hours on an automatic rocker.

4. The gels were removed and put into Destaining Solution 1 and shaken for one to two hours.(**Table 6**)

DESTAINING SOLUTIONS UTILIZED

TABLE 6

DESTAINING SOLUTION 1

Methanol	500 mls
Acetic Acid	100 mls
H_2O	1.0 liters

DESTAINING SOLUTION 2

Acetic Acid	700 mls
Methanol	500 mls
H_2O	10.0 liters

5. The gels were transferred to Destaining Solution 2 and shaken for one to two hours.(Table 6)

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NOTE:

The coomassie blue stain was used to see the bands in the first gels ran. For gels

which were going to be transferred to a membrane for probing with antibodies, the coomassie blue stain was not used. This would have rendered the proteins un-retrievable.

WESTERN BLOT

VII. TRANSFER FROM GEL TO MEMBRANE

1. This step required the use of Transfer Buffer(**See Appendix A**), two pieces of filter paper, and one piece of nitrocellulose membrane.

2. Immediately after the SDS gel had finished running, the top glass plate was removed from the sandwich and the stacking gel discarded.

3. A tray was filled with about one inch of transfer buffer. The gel/glass plate was soaked in the buffer for 10-15 minutes to facilitate removal of glass plate.

4. The transfer tank was filled with transfer buffer and a spin bar added.

5. A large tupperware tray was filled with enough transfer buffer to cover the transfer cassette used in the procedure(About 1 inch).

6. The taller half of the casette was placed into the buffer in the tupperware tray. A prewet sponge, followed by a piece of wet filter paper, was placed on top of the cassette half. The pre-wet nitrocellulose membrane was placed on the stack next. All air bubbles were removed from the stack by rolling them out with a large test tube. The gel was then added to the assembly.

7. The gel was removed from the glass plate by placing a dry filter paper on top of the gel after the gel/glass plate was lifted out of the transfer buffer and drained. The gel adhered to the dry filter paper and the glass plate could then be removed.

8. The gel was placed on top of the membrane on the cassette and the dry filter paper allowed to become thoroughly soaked.

9. The second pre-wet filter paper was added to the stack. The other pre-wet sponge

was placed on top of the filter paper and all bubbles removed.

10. The other half of the cassette was placed on top of the sponge and the apparatus hooked together.

11. The cassette was quickly lifted out of the buffer and transferred to one of the center slots in the buffer chamber. The cassette was oriented with the nitrocellulose membrane on the anode side of the cassette. The cassette was tapped to dislodge any bubbles that were trapped by the cassette.

12. The power lid was placed on the unit and the two banana plugs were engaged on the electrode panels.

13. The power supply was turned on and run at 0.5 amps for two hours. The amperage was checked every twenty minutes because of the tendency to increase with time.

14. After two hours, the power was turned off and the cassette removed and opened. The filter paper above the gel was removed carefully.

15. A black pen was used to trace the outline of the gel onto the membrane. This aided in orientation of bands.

PROBING FOR β-GALACTOSIDASE

1. Each membrane to be probed was initially blocked to prevent binding of unwanted proteins. Blocking was achieved by shaking the membrane in a blocking solution for one hour. The blocking solution was prepared by dissolving 3.0 grams of nonfat powdered milk in 100 mls of Tween Tris Buffered Saline (TTBS). The blocking solution was heated to 50oC to ensure the milk was in solution.

2. After one hour the blocking solution was poured off and the membrane rinsed with 50 mls of TTBS for five minutes.

3. The TTBS was poured off and the primary antibody added. This was a new monoclonal antibody developed by Sigma. The antibody was mixed in blocking solution at a 1:1000 ratio. 50 µl of Anti-β-Galactosidase antibody were added to 50 mls of blocking solution and the mixture poured onto the membrane. This was shaken for one hour to allow the antibody to bind .

4. After one hour the primary antibody was poured off and the membrane rinsed twice in TTBS for five minutes, then two quick rinses with TTBS.

5. The secondary antibody was prepared by adding 50 µl of Mouse IgG labeled with peroxidase compliment to 50 mls of blocking solution.

6. This mixture was added to the membrane and shaken for one hour to allow binding.

7. After one hour the secondary antibody was poured off and, again the membrane was rinsed twice in TTBS for five minutes and in two quick rinses with TTBS.

8. Fast DAB (3,3-Diaminobenzidine)stain was prepared by dissolving a DAB tablet and Urea tablet in 15 mls of ddH_2O. (The Fast DAB labels the peroxidase with a brown color). This was accomplished by utilizing a vortex.

9. The Fast DAB was added to the membrane and allowed to stain overnight.

10. Any bands of β-Galactosidase on the membrane could then be seen as dark brown.

RESULTS AND DISCUSSION

The bacteria grown at 25° C showed no difference in the amount of proteins present or in growth rate when compared to bacteria grown at 37°C. The hypothesis that there would be differences in the sets of bacteria grown at two different temperatures was dismissed.

The first strain of E. coli examined, DH1, (wild type) produced an average total protein concentration of 0.08 mg/ml when not induced. When induced with IPTG, DH1 produced an average total protein concentration of 0.11 mg/ml. The difference in protein amounts was consistent with each replication, and at a 95% comfort level, the lac operon of DH1 produced significantly more protein when induced with the lactose analog IPTG.

The β-Gal Assay results also correlated well with the genotype of this strain. At a 1:5 dilution with distilled water, DH1 produced 0.085 β-Gal when not induced. When induced DH1 produced 0.935. When not induced, the lac operon of DH1 did not stimulate the production of proteins. When induced, the lac operon of DH1 stimulated the production of a substantial amount of β-Gal.

DH5α , when not induced, produced an average protein concentration of 0.11 mg/ml on the BCA assay. When induced, this strain produced an average of 0.18 mg/ml. It is 90 percent probable that this represented a similar ratio in protein production to that of DH1.

Since the gene in the lac operon which codes for β-Gal has been deleted in this strain, a lack of β-Gal correlates well with its genotype. When not induced the absorbance of DH5α was 0.01 for the β-Gal assay. When induced, the absorbance was 0.009. These values were statistically equivalent and represented an absence of β-Gal production at a 95 % comfort level.

XL-1-Blue, when not induced, produced an average protein concentration of 0.185 mg/ml in the BCA assay. When induced, the average concentration of protein was 0.26 mg/ml. There was

a 90% probability that these values represented similar protein production. This production should have been a full complement of proteins from the lac operon regardless of inducement. This was supported by the data.

The results of the β-Gal assay were less conclusive with XL-1-Blue. When not induced, the absorbance was 0.024 at a 1:5 dilution. When induced, the absorbance was 0.95. This represented a likely inducement of the lac operon by the IPTG. This should not have been the case, as XL-1 Blue had no regulatory gene in the lac operon, and should have produced the full complement of proteins. Statistically, the lac operon of XL-1 Blue behaved identically to that of DH1. There could not have been contamination of the cultures with DH1; however, because XL-1 Blue was grown in media containing ampicillin. DH1 was not ampicillin resistant.

AMA 1004, when not induced, produced a total protein concentration of 0.215 mg/ml. When induced, this strain produced an average total protein of 0.266 mg/ml. These results correlated well with its genotype. There should have been only slightly more protein produced when induced with IPTG, as was the case.

The amounts of β-Galactosidase produced by AMA 1004 were negligible. The amounts resembled those of DH5α which produced none.

DH1 produced results which were consistent with its genotype. DH5α produced slightly higher amounts of β-Galactosidase than DH1, but in a ratio consistent with its genotype. XL-1-Blue produced total protein amounts similar to DH1, but showed a response to the IPTG, which did not correlate with the genotype. AMA 1004 produced slightly higher protein levels than DH1, but the lack of β-Gal present did not correlate with its genotype. Table 6 lists the data obtained from the BCA assays.

The data obtained from the BCA assays correlated well with the genotypes of the bacteria. The β-Gal assays yielded data which did not correlate well in the case of XL-1-Blue amd AMA 1004.

BCA ASSAY RESULTS

TABLE 6

NOT INDUCED

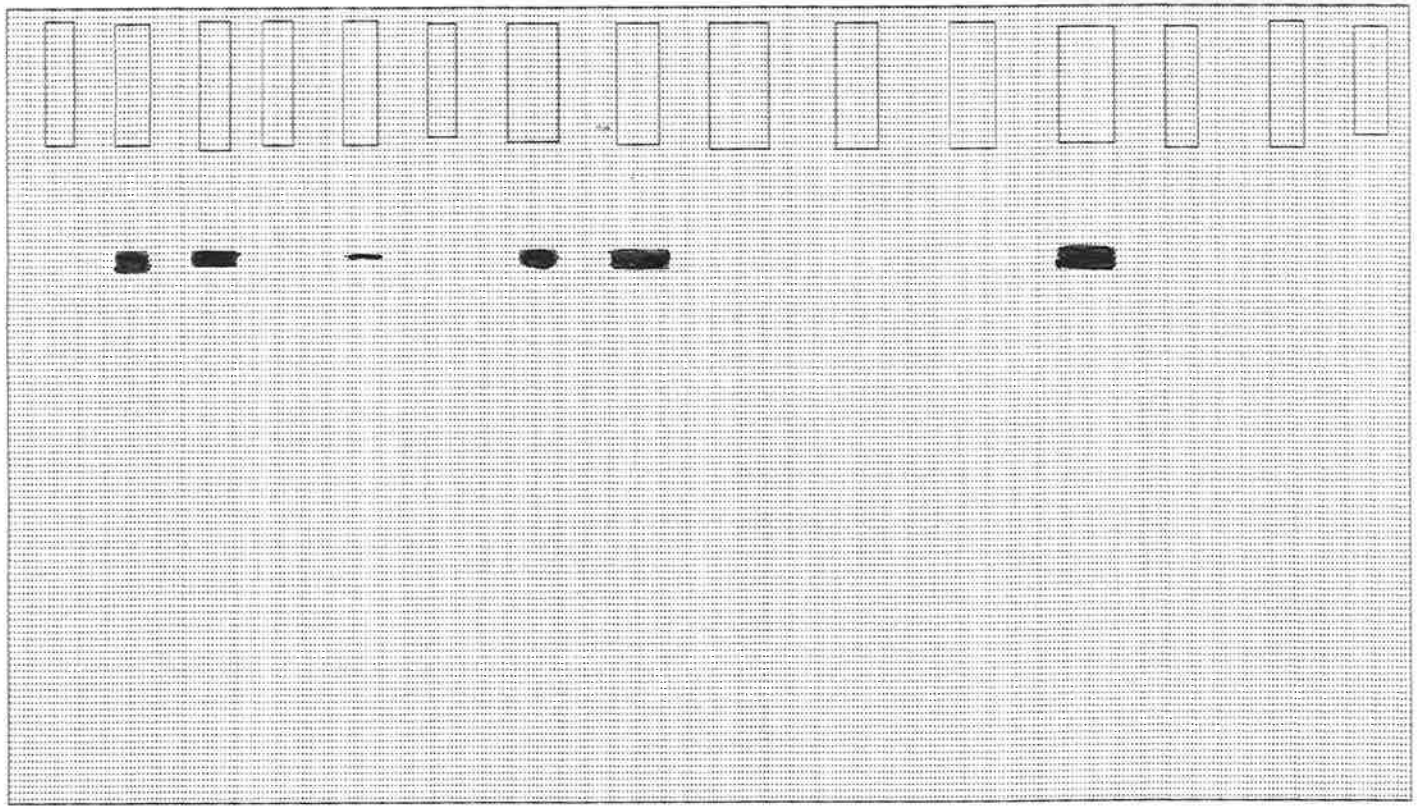
E.coli TYPE	REP 1	REP2	REP3	REP4	AVERAGE	MASS
DH1	0.134	0.201	0.134	0.130	0.150	0.08 mg/ml
DH5α	0.133	0.188	0.147	0.238	0.177	0.11 mg/ml
XL-1-BLUE	0.197	0.228	0.232	0.285	0.236	0.185 mg/ml
AMA 1004	0.193	0.314	0.375	0.159	0.260	0.215 mg/ml

INDUCED

DH1	0.187	0.193	0.172	0.152	0.176	0.11 mg/ml
DH5α	0.225	0.351	0.131	0.190	0.224	0.18 mg/ml
XL-1-BLUE	0.247	0.318	0.308	0.253	0.282	0.26 mg/ml
AMA 1004	0.212	0.301	0.238	0.311	0.266	0.22 mg/ml

The membranes which were probed for β-Galactosidase yielded varied results. One of the four membranes probed, showed β-Galactosidase in the high molecular weight markers and two of the lanes. A map is provided on the following page.

Membrane Banding



APPENDIX A

1. **Z Buffer** .06 M Na^2HPO^4

   .04 M $Na\ H^2\ PO^4$

   .01 M KCl

   .001 M $MgSO^4$

2. **TEMED**

   Ammonium persulfate Agents for polymerization of SDS Gel.

Transfer Buffer (0.192 M glycine, 0.025 M tris, pH 8.3, 20% v/v methanol)

Tris	12 grams
Glycine	57.6 grams
methanol	800 ml
water	to 4 liters(deionized, distilled water)

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