Lac Operon Expression in Steady State Cells of Escherichia coli

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LAC OPERON EXPRESSION IN STEADY STATE CELLS

OF Escherichia coli

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Presented to
The Faculty of the Department of Biology
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirement for the Degree of
Master of Arts

by
SungAe Cho
1985
APPROVAL SHEET

This thesis is submitted in partial fulfillment of the requirement for the degree of

Master of Arts

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DEDICATION

To my parents for their endless encouragement and support.
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>viii</td>
</tr>
<tr>
<td>SECTION 1. THE EXPRESSION OF LacZ AND LacA</td>
<td>2</td>
</tr>
<tr>
<td>IN STEADY STATE CELLS OF E. COLI</td>
<td></td>
</tr>
<tr>
<td>SECTION 2. TEMPERATURE EFFECTS ON LAC-OPERON EXPRESSION</td>
<td>21</td>
</tr>
<tr>
<td>SECTION 3. IPTG EFFECTS ON LAC-OPERON EXPRESSION</td>
<td>33</td>
</tr>
<tr>
<td>SECTION 4. THE EFFECT OF GENETIC NEIGHBORHOOD ON LAC-OPERON EXPRESSION</td>
<td>45</td>
</tr>
<tr>
<td>APPENDIX 1. COLONY FORMING UNIT ENUMERATION</td>
<td>57</td>
</tr>
<tr>
<td>APPENDIX 2. &quot;Z-BUFFER&quot; INGREDIENTS</td>
<td>58</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>59</td>
</tr>
<tr>
<td>BIOGRAPHICAL SKETCH</td>
<td>65</td>
</tr>
</tbody>
</table>
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## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title of Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td>IPTG Concentration Effects on LacZ Expression</td>
<td>39</td>
</tr>
<tr>
<td>4-1</td>
<td>The Chromosomal Markers of F' and F- Strains of <em>Escherichia coli</em></td>
<td>48</td>
</tr>
<tr>
<td>4-2</td>
<td>Beta-Galactosidase Concentrations in Various Strains of Steady State <em>E. coli</em></td>
<td>52</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title of Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>The Expression of $\text{lacZ (BG)}$ and $\text{lacA (TA)}$ per cell over the Growth Curve</td>
<td>14</td>
</tr>
<tr>
<td>1-2</td>
<td>The Expression of $\text{lacZ (BG)}$ and $\text{lacA (TA)}$ per milliliter over the Growth Curve</td>
<td>16</td>
</tr>
<tr>
<td>2-1</td>
<td>The Expression of $\text{lacZ (BG)}$ per cell at Different Temperatures</td>
<td>24</td>
</tr>
<tr>
<td>2-2</td>
<td>The Expression of $\text{lacA (TA)}$ per milliliter at different Temperatures</td>
<td>26</td>
</tr>
<tr>
<td>3-1</td>
<td>The Expression of $\text{lacZ (BG)}$ per cell versus IPTG Concentration</td>
<td>37</td>
</tr>
<tr>
<td>3-2</td>
<td>The Expression of $\text{lacA (TA)}$ per milliliter versus IPTG Concentration</td>
<td>40</td>
</tr>
<tr>
<td>4-1</td>
<td>The Expression of $\text{lacZ (BG)}$ in different Genetic Environments</td>
<td>50</td>
</tr>
</tbody>
</table>
Abstract

Within the range of steady state growth of *Escherichia coli*, the activities of beta-galactosidase (BG) and thiogalactoside transacetylase (TA) were measured and the influence of temperature, inducer and linked genetic loci were assessed. Under the normal conditions of lac expression in the prototypic W3110 (cultured at 37°C with 0.6 mM IPTG), BG activity remained constant while TA was apparently not induced in steady state cells. Pertaining to the effect of temperature, BG concentration in cells grown at 20.5°C was half that of cells grown at 37°C. BG was maximally induced overall IPTG (isopropyl-beta-D-thiogalactoside) concentration range of 0.01-0.1 mM and decreased above this range. TA was not induced in cells under any condition. BG activity was unchanged regardless of the genetic composition and location of lac-operon in several F', an Hfr and the prototypic strain. Therefore, it seems likely that the expression of lac-operon is unaffected by genetic location in steady state cells.
LAC-OPERON EXPRESSION IN STEADY STATE CELLS

OF Escherichia coli
SECTION 1
THE EXPRESSION OF LacZ AND LacA IN STEADY-STATE CELLS

INTRODUCTION

The operon is defined as a polycistronic unit which is controlled coordinately by a single regulator sequence of DNA. The evolutionary tendency for gene expression in the form of an operon unit has been explained to be very advantageous for specificity and coordination of physiological and biochemical mechanisms in both eucaryotic and procaryotic cells (Jacob and Monod, 1961a).

The control mechanisms of operons are mainly classified into two categories: repression and induction. For example, the biosynthetic tryptophan operon is repressed its product, tryptophan, while the lactose operon is induced by its substrate, lactose (Jacob and Monod, 1961a). In spite of the differences, either is very worthy of study to understand how effectors regulate gene expression.

The lac-operon is composed of control elements and structural cistrons. The control elements consist of a structural gene (lacI) for the production of repressor
protein which follows \textit{lacI} promoter sequence, another promoter (\textit{lacP}) for the binding of the RNA polymerase and cAMP-CRP (cAMP receptor protein) complex, and an operator gene (\textit{lacO}) to which the repressor protein binds actively. The structural cistrons consist of \textit{lacZ}, coding for beta-galactosidase (BG); \textit{lacY}, coding for lactose permease; and \textit{lacA}, coding for thiogalactoside transacetylase (TA).

In the absence of lactose, expression of the structural genes is almost completely abolished by the binding of repressor to the DNA of the operator. When the operator DNA was mixed with radioactively-labeled repressor protein, it was found that the mixture sedimented as a single band on a glycerol gradient. When inducer was added to this mixture, the mixture dissociated. Therefore, it is clear that the repressor inhibits the promoter activity, however, it has not been demonstrated that the RNA polymerase is prevented from attaching to the promoter region and then transcribing the structural elements (Gilbert and Muller-Hill, 1970). In the presence of lactose, the sugar molecule binds to the repressor and weakens its ability to bind \textit{lacO}. The repressor then can not bind to the operator, or the
repressor is removed from the operator. The result is that the translation of structural genes increases 3,000 to 10,000 times over that of the uninduced state (Jacob and Monod, 1961b).

In addition to lactose or its analogs, the CRP-cAMP complex is necessary for initiation of lac-operon transcription. Cyclic-AMP is made from cellular ATP by the action of adenylate cyclase. Cya- mutant which can not produce normal adenylate cyclase or Crp- mutant which can not make normal cAMP receptor protein was found to be unable to synthesize BG (Perlman and Pastan, 1969; Emmer et al, 1970). This complex seems to destabilize the DNA of operator or directly interact with RNA polymerase for its binding to the operator. The conversion of cellular ATP into cAMP is strongly substrate-inhibited. Thus if glucose is supplied to the cells, the resultant ATP inhibits cAMP formation (Makman and Sutherland, 1965). This phenomenon is known as catabolite repression. Such repression by glucose is found also in the synthesis of enzymes which break down other carbohydrates (e.g.: galactose and maltose) (Magasanik, 1962; Benoit de Crombrugghe and Ira Pastan, 1978).
Let us now consider the properties of the protein products of structural genes in the lac-operon.

The \textit{lacZ} locus for the production of BG occupies 0.7 map units or 3,500 bp in the total DNA of \textit{E.coli} (Jacob and Monod, 1961b). BG, which can normally account for 5\% of the total cellular protein, is a tetrameric structure of identical polypeptide chains. The molecular weight of the tetramer is estimated to be from 490,000 to 540,000 daltons (Zabin and Fowler, 1970). The protein subunit seems to be assembled into tetramers very rapidly even at very low monomer concentrations, therefore, BG has a high association constant (Adamson \textit{et al}, 1970). BG has two types of enzymatic activity: transferase (transgalactosylase) and hydrolase. The absorbed lactose is isomerized to allolactose (\(\beta\)-D-galactosyl-\(\alpha\)-1,6-D-glucoside) which is the true inducer neutralizing the repressor. Ultimately BG cleaves lactose into glucose and galactose by its hydrolase activity (Muller-Hill \textit{et al}, 1964; Burstein \textit{et al}, 1965; Jobe and Bourgeois, 1972). The BG activity can be measured easily by the ONPG (o-nitrophenyl-\(\beta\)-D-galactoside) procedure which produces yellow o-nitrophenol when hydrolyzed by BG (Pardee \textit{et al}, 1959).
The lacA cistron, for TA, occupies an as yet unknown number of map units. The concentration of TA in total cellular protein is about 0.3% (Zabin, 1963). TA has a dimeric structure totalling 60,000 daltons. There are about 3 to 5 times more BG subunits synthesized than of TA subunits (Zabin and Fowler, 1978). The dissociation constant of TA is regarded quite high (Micheles and Zipser, 1969). Enzymatically, TA transfer the acetyl group from acetyl coenzyme A to the 6-OH of a thiogalactoside acceptor such as IPTG (isopropyl-β-D-thiogalactoside) (Zabin and Fowler, 1970). A lacA- mutant does not show any difference in lactose metabolism relative to normal lacA+ strains (Fox et al, 1966; Wilson and Kashket, 1969). However, it has been found that in lacA- strains, the growth rate was inhibited in the culture containing non-metabolizable lactose structural analogs (e.g.: IPTG or TMG; methyl-β-D-thiogalactoside) and that acetyl thiogalactosides were left inside the cell. The acetyl thiogalactoside was pumped out in A+ strains. In lacA- strains the growth rate was decreased when both lactose and its analog were present. In lacA+ strains growth rate remained unaffected. Recently, it has been accepted that TA is essential for the detoxification by acetylation of non-metabolizable analogs in the late

LacY for lactose permease is located between lacZ and lacA and is of undetermined length. The permease is localized in the membrane for the transport of galactosides from outside to the inside of the cell by specific binding. Many inducers for lac-operon are good substrates for the permease system. Nevertheless, chemically pure permease has not been isolated and the transport mechanism of lactose is not yet fully understood (Kennedy, 1970). In the ML strain, permease is estimated about 0.35% of the total protein and its molecular weight is 30,000 daltons (Zabin and Fowler, 1978). In my experimentation the expression of LacY has not been studied.

Overall, this project has its objective in measurement of lac-operon expression as lacZ and lacA expression under steady state growth range of E.coli W3110. Steady state growth is defined as that in which the statistically average cell remains unchanged over time, regardless to the parameter measured. There are two theoretical phases in the bacterial growth curve where
this occurs: one is the static steady state of dormancy, while the other is the dynamic steady state of exponential growth. I am here concerned with the latter only as "steady state cells". As the population density increases to high levels, nutrient is depleted, oxygen utilization exceeds the diffusion rate and metabolic waste products accumulate to toxic levels. Therefore, the maintenance of physiological state has always been an important theory behind the quantification of cellular activities. Constancy of cell size and biochemical composition (RNA, DNA and ribosomes, for example) has been emphasized as major criteria for steady state growth (Schaecter et al, 1958).

While classical dynamic steady state growth has been defined in the range of $1 \times 10^8$ to $1 \times 10^9$ cells/ml (turbidity between 0.2 to 0.6), it was recently reported that *E. coli* maintains steady state growth only at cell concentrations of less than $1 \times 10^7$/ml (Cho et al, 1984). In this thesis, I termed the classical log phase as post steady state to distinguish it from the truly steady state. Steady state growth can theoretically be continued infinitely in appropriate constant dilution devices such as turbidostats. Over the usual turbidity
range of 0.1 to 0.6, batch grown cells were found not to be in steady state with regard to both BG/cell or mean cell diameter. However, at much lower cell concentrations steady state conditions did prevail and were maintained up to approximately $10^7$ CFU/ml (Cho et al., 1984).

In this experiment, concentrations of BG and TA were measured in steady state cells of *E. coli* grown in extreme dilution in batch broth cultures. In further sections, the effects of temperature, inducer concentration and genetic environment were studied in steady state growth range. Temperature is the important environmental factor which can affect cellular metabolism rate and IPTG is the most effective inducer for lac-operon. Therefore, the activities of *lacZ* and *lacA* were measured under these conditions. Also the lac operon expression on different genetic backgrounds were quantified. Because the quantification of gene expression is a precise measurement of gene efficiency, this data shows gene efficiency depending on the genetic locations.
MATERIALS AND METHODS

1. Bacterial Strain: The prototypic *E. coli* strain W3110 (*E. coli* K12 F-thi-) was used.

2. Media: Overnight cultures contained 0.8% tryptone, 0.7% lactose, and 0.5% NaCl ("L-broth"). The overnight cells were inoculated into fresh broth containing 0.6 mM IPTG instead of lactose.

3. Bacterial Growth: 150 ml of fresh thermally equilibrated L-broth was inoculated with 0.05 ml of overnight culture containing about 1x10^6 cells. This was swirled vigorously for aeration. At regular intervals, one milliliter samples for the BG assay and colony counting and five milliliter samples for the TA assay were taken until the culture reached late log-phase (about 1x10^6 cells/ml).

4. BG was assayed according to Miller (1969): Each sample was mixed with assay medium (mixture of 1 ml Z-buffer, 3 drops of chloroform and 3 drops of 0.1% sodium dodecyl sulfate) and 0.5 ml of ONPG (o-nitrophenyl -D-galactoside) solution (0.64 mg/200 ml Z-buffer) was
added into mixture. The reaction was run at 23°C. When the mixture turned a yellow color, one milliliter of 1M sodium carbonate was added into the mixture to stop the reaction by changing pH. The cell debris was centrifuged down at 8,000 rpm for 10 minutes. The optical density of ONP concentration was measured spectrophotometrically at 420nm. One unit of BG is defined as the amount which hydrolyzes one millimicro-mole of ONPG per minute per milliliter. BG units per cell were calculated thusly:

\[
BG \ \mu \text{UNITS} = \frac{\text{ABS}(420\text{nm}) \times 100 \times 2.46 \times 10^6}{0.5 \times t \times n}
\]

where:
- \(t\) = reaction time in minutes
- \(n\) = CFU/ml
- 2.46 = correction to adjust 37°C data to standard 25°C temperature
5. TA was assayed according to Alpers et al (1969): Five ml samples were centrifuged down with 5 ml of uninduced heat-killed (60°C) carrier cells at 12,000 rpm for 15 minutes. The pellet was suspended in 1 ml of 0.05 M Tris and 0.01 M EDTA at pH 7.9. The cells were lysed by alternate freezing plus thawing. The disrupted cells were heated at 70°C for 5 minutes to inactivate heat-labile BG and other enzymes and the debris was centrifuged down at 13,000 rpm for 40 minutes. 50 µl of suspension was reacted with 50 µl of assay reagent (5mg/ml acetyl coenzyme A plus 250mg/ml IPTG in 0.05M Tris, 0.01M EDTA, pH 7.9) and incubated at 25°C for one hour. The reaction was stopped by the addition of 3 ml of DTNB (5,5'-dithiobis-2-nitrobenzoic acid, 25 mg/100 ml in 0.05M Tris, pH 7.9) Quantification of the color reaction was made spectrophotometrically at 412 nm. Even though the relation of TA activity to the number of molecules has not been determined, TA units per cell were calculated according to the following equation:

\[
\text{TA \, \mu\text{UNITS}} = \frac{\text{ABS}(412\text{nm})}{t \times n} \times 2.379 \times 10^6
\]

where: 
- \( t \) = reaction time in hours
- \( n \) = CFU/ml
- 2.379 = correction for standard temperature.
Results

In the steady state growth range with inducer present, BG showed invariant steady state expression, but TA was not perceptibly induced and the dilution of pre-existing TA molecules was continued until stationary phase (cell concentrations of $1 \times 10^9$ cells/ml) (Figure 1-1). When the sensitivity of an analytical method is low, such as with TA, precision can be amplified if production is measured on a per volume basis as the output of many cells combined. In Figure 1-2, the amount of TA per ml of culture began to increase at cell concentrations of about $1 \times 10^9$ cells/ml. Therefore, TA activity seems to be elevated in the very late stage of population growth. On the other hand, the amount of BG per ml of culture increased at cell concentrations of $1 \times 10^7$ cells/ml.
The Expression of LacZ (BG) and LacA (TA) per Cell over the Growth Curve. Upon inoculation of the medium containing 0.03 mM IPTG the enzyme levels per cell decrease at a rate corresponding to their being diluted among the cells in the expanding population. After approximately 90-100 minutes, BG production equals the dilution rate, and the cells are in the steady state with respect to this enzyme. TA levels meanwhile continue to decrease indicating that there is no production during this steady state period (see Fig. 1-2 for further evidence). Upon the population's reaching $1 \times 10^5$ CFU/ml, hyper induction of the lac-operon commences and both BG and TA are produced at highly elevated levels, which become those associated with the later dormant cells and thus become the starting values for an inoculation of a new growth curve.
Figure 1-2: The Expression of LacZ (BG) and LacA (TA) per milliliter over the Growth Curve. During the earlier portion of the population's growth curve corresponding to the range of the steady state production of BG in Fig. 1-1, BG level is shown to be increasing thus indicating a net synthesis of the enzyme among the population of cells. Since TA does not increase in this range, there is no significant synthesis of it during steady state.
Discussion

The stoichiometry of the coordinated induction of structural genes within lac-operon has been widely accepted since it was first posited by Jacob and Monod (1961a,b). However, in this experiment, the expression of \textit{lacZ} (BG) and \textit{lacA} (TA) did not show coordinate induction in steady state cells. While the concentration of BG exhibited a steady state level, the pre-existing TA was still being diluted and the amount of TA per cell seemed to be too low for measurement in steady state growth range (Figure 1-1).

Generally, the BG subunits in a cell is much higher than the TA subunits (molecular weight of 12 to 20:1, molecular ratio of 3 to 5:1) and the suggested reasons for this are the much longer code probably due to highly repetitive sequences, the higher initiation rate of translation by ribosomes and the slower message decay rate of \textit{lacZ} than those of \textit{lacA} (Zabin and Fowler, 1978). However, the main reason for noncoordinate induction in steady state cells can be better explained as different assembly properties of two genes' products. The noncoordinate induction of \textit{lacZ} and \textit{lacA} was already
found by Zipser et al. at low levels of induction. They found that the ratio of active BG to active TA molecules was not proportional at low IPTG concentrations, but was proportional only at high IPTG concentrations. They suggested that a threshold number of TA monomeric units is needed for the formation of active dimeric TA molecules (Michels and Zipser, 1969). Accordingly, the extremely low production of active TA molecules in the steady state growth range is due to high dissociation of the monomers. Meanwhile, BG is highly associated, therefore it forms active tetramers even at low monomer concentrations as well as at low levels of induction (Michels and Zipser, 1969; Adamson et al., 1970). The noncoordinate induction of lacZ and lacA in steady state growth range is illustrated in Figures 1-1 and 1-2. The synthesis of TA began only after the cell concentration exceeded 1x10^8 cells/ml, while BG maintained a measurable steady state level at cell concentrations up to 1x10^7 cells/ml, after which it rapidly increased.

The main role of TA is not yet understood. The hypothesis that it helps in the transport of lactose is found wanting, because it was proved that lactose is metabolized normally in lacA- strains. However, the de-
toxification of non-metabolizable analogs by acetylation is now generally accepted as its main role (Andrews and Lin, 1976; Zabin and Fowler, 1984). My results also support this hypothesis because lacA is actively turned on at a later growth stage. It is becoming increasingly clear that TA is synthesized only in the late portions of the culture's growth. The detoxification and the removal of deleterious by-products by acetylation is an important physiological mechanism for cells in late culture.
SECTION 2
TEMPERATURE EFFECTS ON LAC-OPERON EXPRESSION

INTRODUCTION

Since Schaecter et al (1958) found that cell mass, size and composition of Salmonella typhimurium are constant at both high (37C) and low (25C) temperatures, many other experiments have shown that cell size, ribosome and protein concentration of the cell are maintained at constant levels at different temperatures. However, there are several reports which demonstrate that the synthesis of macromolecules is affected by temperature change and that cell size is also slightly changed at the extremes of normal temperature range (under 22C and above 42C) (Trueba et al, 1982). Such temperature effects are likely to influence the synthesis of ribosomes, specific proteins, enzymes or directly on the existing molecules like fatty acids (Herendeen, 1979; Garwin, 1980; Bortolussi et al, 1983).

To understand temperature effects on lac-operon expression, the concentrations of BG and TA were measured in steady state cultures grown at 20.5C and 37C. BG activity was approximately twice the level at the high
temperature as that present at the low temperature. TA activity was not detected at both temperatures. The decrease of BG level at low temperature is thought to be related to the decrease of ribosomal components (Golosow, et al, 1985).

Materials and Methods

Bacterial cultures were incubated at 37C and 20.5C in L-broth. After one hour for acclimation, samples were taken periodically and assayed for measurement of BG activity at 23C and TA activity at 25C. While BG was measured on a per cell basis, TA was measured on a per milliliter of culture basis since when a product is synthesized at very low rates per cell, the accumulated production of all cells amplifies the sensitivity of the analysis.
Results

The amount of BG per cell was twice as high in cells grown at 37C as in cells at 20.5C (Figure 2-1). TA was not perceptibly produced at either temperature (Figure 2-2). The growth rate of the cells (doubling time) was 6-times slower when the culture was grown at 20.5C instead of 37C.
Figure 2-1: The Expression of LacZ (BG) per Cell at different Temperatures. Over the most commonly used laboratory cultivation temperatures, the steady state production level of BG is not significantly affected. However for temperatures below 23°C, the level assumes a value of approximately 60% that of the higher temperature.
Figure 2-2: The Expression of LacA (TA) per milliliter at different Temperatures. Temperature is seen to have no releasing effect allowing steady state production of the enzyme.
Discussion

In the previous section, it was already shown that TA was not induced in steady state cells. Here we see that TA was neither produced in cells grown at high nor at low temperatures. This result thus supports the general idea that TA is not induced during steady state growth phase. On the other hand, the level of BG decreased at low temperature.

In addition to media components, temperature is one of the most important environmental factors in cellular metabolism. Temperature can change physical properties (e.g. cell size and specific gravity) (Trueba et al, 1982; Golosow et al, 1985) or metabolic products of organisms (e.g. K1 antigen and fatty acids) (Bortolussi et al, 1983; Garwin et al, 1980). Although the nutrients in a culture affect cell size very significantly (Begg and Donachie, 1978), temperature has not been regarded as an important factor of size. However, Trueba et al (1982) reported that cells grown at 22C became smaller and shorter than those at 37C. They suggested that DNA replication was initiated earlier at 22C than at 37C, thus the constriction and cell division occurs
earlier and the cell size was reduced. Therefore, they proposed that cell morphology could be changed at low temperatures. Generally, it is widely accepted that DNA replication is initiated when cell size per chromosome reaches a certain value (Donachie, 1968). Pierucci (1972) found the pattern of DNA replication is not affected by temperature change. However, at present, it has not yet been determined how the DNA replication mechanism is influenced outside the normal temperature range and whether it is related to cell size. Bortolussi et al (1983) found the Kl-antigen of E.coli was not produced at a growth temperature of 22°C, although it was synthesized copiously at both 37°C and 30°C. Yamamori et al (1980) showed some proteins including a product of the mop (morphogenesis of phages) gene were induced by temperature shift-up and they demonstrated that regulation of expression occurred at the transcriptional level. Garwin et al (1980) reported that fatty acid synthesis was affected by temperature change. Highly relevant to my study, Nishi et al (1963) found the induction of lacA and lacZ is affected differently by temperature change. They suggested that the temperature effects on lac-operon indicated that the translation of the cistrons was regulated separately, and not coordinately. However, the
regulation mechanism of the lac-operon on the translational level has not been studied.

Although the mechanism and target of temperature effect on cellular metabolism is not yet understood completely, my result can be simply explained by the experiment of Golosow et al. (1985), who found that the specific gravity which expressed the concentration of ribosomal contents per cell decreased by a half when E. coli was cultured below 25°C. On the assumption that the rate of protein synthesis per ribosome is constant according to Schaecter et al. (1958), it can be reasoned that the more ribosomes there are, the more proteins are synthesized. Therefore, it can be concluded that the BG production could be diminished by the decrease of ribosomes at the low temperature. The initial rate of galactosides has been found to be higher when the temperature increased. However, the constant level of absorption of galactosides (e.g. lactose, ONPG and TMG) has been found higher at 14°C than at 37°C (Kepers and Cohen, 1962). Therefore, the accumulation rate of galactosides which is controlled by permease does not appear to affect BG expression.
Heredeen et al (1979) defined the normal temperature range for as being 23 through 37°C. They explained all cellular metabolisms were controlled together proportionally to the whole growth rate within this temperature range. A similar result was reported by Zarisky (1982). The relative number of ribosomes per cell was found to be constant over the temperature range of 25 through 37°C. Therefore, temperature effects on bacterial metabolism seem to be under coordinated regulation within the normal temperature range. However, Herendeen's group (1979) found the growth rate decreased rapidly and the concentration of major proteins changed variably outside this range. They did not identify all proteins, but they found the levels of ribosomal proteins and other translational and transcriptional proteins decreased at both extremes of this range.

In conclusion, lacZ expression was decreased by lowering the temperature to 20.5°C. This temperature was outside generally accepted normal range from the point of view of growth rate or cell size. It also seems clear that the temperature effect is related to the cellular ribosomal content. Temperature can modulate enzyme
activities and change the level of enzyme synthesis and ribosomal components or possibly the initiation time of DNA replication. If the mechanism of response and adaptation of bacteria to external temperature change is discovered, it will be very beneficial industrially and clinically.
INTRODUCTION

IPTG (isopropyl-β-D-thiogalactoside) is the gratuitous inducer whose activity is not coupled with chemical modification (Gilbert and Muller-Hill, 1966). The lac-operon is induced rapidly such that approximately within 3 minutes after addition of 0.35 mM IPTG gene-product first begins to accumulate. Also, this active induction ceases immediately upon removal or dilution of IPTG (Boezi and Cowie, 1961).

IPTG causes the repressor to either dissociate from the operator or prevents the free repressor from binding to the operator (Barkeley & Bourgeois, 1978). The repressor has a high affinity for the operator ($K_a = 1 \times 10^{13}$ M$^{-1}$), but IPTG directly decreases the affinity of the repressor for the operator about 1000-fold under standard conditions (Barkeley et al, 1975). The repressor is composed of four identical subunits (38,000 daltons) and each subunit has two independent binding sites: one for operator and the other for inducer. Therefore, the repressor is an allosteric protein having two conformation-
al forms. Within each subunit, the two binding sites are separated. However, they do interact with each other and mutually induce a conformational change in the repressor protein (Barkley and Bourgeois, 1978).

Now concerning the induction mechanism of IPTG, there are two models for conformational transition of repressor caused by the binding of inducer: the non-cooperative and the cooperative models. The cooperative model holds that the inducer binding to only one subunit of the repressor causes a conformational change affecting all the other subunits such that the repressor separates from the operator. The non-cooperative model, on the other hand, suggests that the conformational change should occur independently in each subunit leaving the others unaffected. The non-cooperative model is supported with more confidence. Nevertheless, the mechanism is far from being completely elucidated. The degree of destabilization of the operator-repressor complex is found to be dependent on the magnesium ion concentration, but not on the rate of IPTG absorption, temperature, pH or ionic strength (Barkley et al, 1975).
In my experiments, the optimal concentrations of IPTG for induction of \textit{lacZ} and \textit{lacA} were determined. In the range of 0.01 mM through 0.1 mM IPTG, the \textit{lacZ} induction was maximal, although TA activity was not observed over the whole range of IPTG concentrations (0.01 to 5.0 mM), so long as the cells were in the range of steady state growth. This concentration value for maximal BG activity in steady state cells is different from the concentrations (0.3 to 1.0 mM) for \textit{lac}-operon expression that have been widely used over the past three decades.

\textbf{MATERIALS & METHODS}

Bacterial cultures were grown at five different IPTG concentrations (0.01, 0.1, 0.5 and 1.0 mM) for one hour in L-broth. After this period of acclimation, BG concentration per cell and TA concentration per millimeter were measured regularly in the steady state growth phase, according to the same method as in first section of this thesis.
RESULTS

The BG activity was maximal in the range of 0.01 to 0.1 mM IPTG and decreased as IPTG concentration increased over the IPTG range tested. When the BG activity was plotted versus the logarithm of IPTG concentration, there was a linear decrease in expressed enzyme (Figure 3-1 and Table 3-1).

TA activity was not detected over the IPTG range tested: the TA level per milliliter of culture increased only in the late growth stages when the cell count had exceed $1 \times 10^8/\text{ml}$, as discussed in the first section (Figure 3-2).

All cultures grew at the same rate of growth ($g=23$ minutes), regardless of the concentration of IPTG.
TABLE 3-1: IPTG CONCENTRATION EFFECTS ON LacZ EXPRESSION

The effect of IPTG concentration on the steady state level of induction of beta-galactosidase (BG) and on the growth rate of Escherichia coli W3110.

<table>
<thead>
<tr>
<th>IPTG (mM)</th>
<th>BG (µUnits)</th>
<th>Generation Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>1.09</td>
<td>23</td>
</tr>
<tr>
<td>0.1</td>
<td>0.965</td>
<td>23</td>
</tr>
<tr>
<td>0.5</td>
<td>0.684</td>
<td>23</td>
</tr>
<tr>
<td>1.0</td>
<td>0.577</td>
<td>23</td>
</tr>
<tr>
<td>5.0</td>
<td>0.285</td>
<td>23</td>
</tr>
</tbody>
</table>
Figure 3-1: The Expression of LacZ (BG) per Cell Versus IPTG Concentration. In steady state cells, the optimal concentration of IPTG is in the range of 0.03-0.1 mM. Below that range, no enzyme production is supported, and above that range the production decreases proportional to log [IPTG]. For post-steady state cells, IPTG concentration is seen to have less proportional effects at the higher concentrations. Generally the threshold supporting any enzyme production remains the same (0.03 mM), although cells that are closely approaching dormancy seem to be induced even at extremely dilute concentrations of IPTG.
Figure 3-2: The Expression of LacA (TA) per Milliliter Versus IPTG Concentration. The production of TA does not seem to be unlocked at any reasonable concentration of inducer.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>IPTG Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>.</td>
<td>0.01 mM</td>
</tr>
<tr>
<td>x</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>o</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>●</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>■</td>
<td>5.0 mM</td>
</tr>
</tbody>
</table>
DISCUSSION

While the amount of BG decreased as the concentration of IPTG increased, the generation times of five bacterial cultures grown in the presence of different IPTG concentrations were all the same: 23 minutes. This is in good agreement with the idea that IPTG does not influence the growth rate, metabolic yield and biosynthetic activity except for the lac-operon products themselves, even though lactose does.

Since the IPTG concentration for half the maximal differential rate of the operator-repressor complex has been determined as $2 \times 10^{-4} \text{ M}$ in a post-steady state culture in which the turbidity exceeded 0.2 and it has been reported that BG induction was saturated maximally above at this concentration, higher concentration of IPTG than $2 \times 10^{-4} \text{ M}$ has been generally used for lac-operon induction (Barkley and Bourgeois, 1978). However, my data demonstrated that the expression of lacZ is rather inhibited in steady state cultures containing excessive concentrations of IPTG, that is more than 0.1 mM. The non-metabolizable lactose analogs such as IPTG were found to be toxic to lacA- strains (Andrews and Lin, 1976).
Since TA is not active in steady state cells, it seems likely that the excessive IPTG is deleterious to the lac-operon, although the mechanism for inhibition of IPTG has not yet been elucidated. Referring to the experiment, which was expanded upon by my colleagues, the maximal induction of BG occurred at 0.1 mM IPTG, not only in steady state cells but also in the post-steady state cells. However, the BG concentration in post-steady state cells was four-fold higher than that in steady state cells. Because cAMP is suggested to be but little produced in steady state cells, while it is produced in very high amounts in post-steady state and especially in stationary phase cells due to decreasing energy sources, it is reasoned that if cAMP is sufficient for induction, the lac-operon might be turned on actively and continuously before inducer inhibits lac-operon expression. Therefore it is thought that the more cAMP in a cell, the more BG is induced, while the amount of IPTG should be used for optimal concentration (about 0.1 mM) for maximal induction (Cho et al, 1985).

In conclusion, the IPTG concentration for maximal induction of lacZ is in the neighborhood of 0.1 mM and concentrations over that value induction decreased. TA
activity was not detected at all over a wide range of IPTG levels, even though the IPTG concentration range for maximal TA induction is known to be 0.7 to 1.25 M (Alpers et al, 1964). The mechanism of lacA induction in late stages was previously explained in the first section of this thesis.
SECTION 4

THE EFFECT OF THE GENETIC NEIGHBORHOOD ON THE EXPRESSION OF LAC-OPERON

INTRODUCTION

The F-factor is a relatively large plasmid with 94.5 kilobases in length and 63 megadaltons in molecular weight (Davidson et al., 1975). Its copy number is maintained stringently to one or two per cell (Prichard et al., 1975). The host chromosome has a fair number of sites for the integration of F, resulting in an Hfr (Beckwith et al., 1966). The incorporated F may either remain in the host chromosome or it may excise to again become a plasmid. When the inserted F releases from the host chromosome, it can excise chromosomal DNA. If so, the plasmid is called F-prime (e.g. F'-lac) and the linked genes can be transferred together in the form of either Hfr or F-prime (F') (Hann and Stouthamer, 1963). The gene exchange through conjugation of F has been thought as one of the major evolutionary mechanisms of bacteria (Jones, 1983).
By the formation of merozygotes through F'-lac, the "cis/trans" effect of control elements on structural gene expression was found and the cytoplasmic production of repressor was fully understood (called "PaJaMo" experiment) (Pardee et al, 1959; Beckwith, 1970). Since its discovery, F'-lac has been used for the elucidation of plasmid structure and replication mechanisms.

In this experiment, the level of BG per cell was measured in steady state cultures of a wild-type strain (W3110), an Hfr transposition mutant (PB3004) where lac was situated near its usual site, and in a set of several related F'-lac strains. It should be noticed that BG production has been heretofore thought to be higher in F'-lac strains than when the lac is located on the chromosome. On the contrary, my data indicate that there is little or no obvious difference if steady state cells of Hfr, F'-lac and normal strains are assayed on a per cell basis.
Materials and Methods

1. Bacterial strains: Wild type (W3110), Hfr (PB3004) plus several F' strains were used. X478 (F-) was used for verification of F' strains. The chromosomal markers of F' and F-strains are listed in table 4-1.

2. Verification of F'-strains: To verify that lac is located on the F and that the plasmid is not integrated in the host chromosome, each F' was tested by cross-streaking with X478 (F-). One streak of the F' was literally crossed with a streak of X478. When the latter streak crossed the former, some of the former bacteria were picked up, mated with, and were the recipients of the plasmid. Since the foregoing would also hold for Hfr's, a second level of screening was taken. Mixed cultures of X478 paired with each of the F' strains were grown for several generations. In these conditions, only rapid transfer of plasmids is able to take place with concomitant mutual transfer of both the sex factor and lac rapidly throughout the population. These mated cells were then washed with sterile saline to eliminate nutrients and then grown in a liquid medium containing a medium that prevented the growth of either the donor
TABLE 4-1: The CHROMOSOMAL MARKERS OF F' AND F- STRAINS of *E. coli*

<table>
<thead>
<tr>
<th>E. coli strains</th>
<th>Chromosomal Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGSC-5218</td>
<td>F'-proAB, lac</td>
</tr>
<tr>
<td>CGSC-5886</td>
<td>F'-proAB, lac, tsx, purE</td>
</tr>
<tr>
<td>CGSC-6450</td>
<td>F'-lac, lip</td>
</tr>
<tr>
<td>PB3004(Hfr)</td>
<td>(proC-lac) lac+(10min)</td>
</tr>
<tr>
<td></td>
<td>[a lac-transposition produced by Paul Brody]</td>
</tr>
<tr>
<td>X478(F-)</td>
<td>lac-, proC-, purE-, str , trp-, lys-, metE-, leu-</td>
</tr>
</tbody>
</table>

=============================================
F'-strains or the recipient F- strains. The resultant cell suspension was plated on Lactose-McConkey plates.

3. Measurement of steady state \textit{lacZ} expression: Bacterial cultures of F', Hfr and wild type strains were incubated with vigorous aeration at 37°C. Samples were taken at regular intervals from each culture about one hour after inoculation and were assayed for the estimation of BG activity, as stated in the first section of this thesis.

\textbf{Results}

By two concurrent and independent methods, it was verified that each strain of F' possessed a \textit{lac} situated on the plasmid and not of the somatic chromosome.

BG was produced at approximately the same level in all F', Hfr and wild type strains tested. Only one F' strain (CGSC 5218) gave an expression which was somewhat less than the level of the others. In this strain the F'-\textit{lac} has the opposite orientation in its neighborhood as compared to the other F'-\textit{lac}'s (Figure 4-1 and Table 4-2).
**TABLE 4-2**

**Beta-Galactosidase Concentrations in Various Strains of Steady State *E. coli***

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli Strain</strong></td>
<td><strong>Run Number</strong></td>
<td><strong>Generation time (min.)</strong></td>
<td><strong>Sample size</strong></td>
<td><strong>μUnits of Enz avg (s.e.)</strong></td>
<td><strong>Average of Runs</strong></td>
</tr>
<tr>
<td><strong>W3110 (prototype)</strong></td>
<td>1</td>
<td>19.33</td>
<td>3</td>
<td>1.382 (0.015)</td>
<td>1.227</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>21.33</td>
<td>8</td>
<td>1.071 (0.130)</td>
<td></td>
</tr>
<tr>
<td><strong>PB3004 transposed lac</strong></td>
<td>1</td>
<td>22.67</td>
<td>7</td>
<td>0.931 (0.229)</td>
<td>0.843</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>23.67</td>
<td>10</td>
<td>0.755 (0.125)</td>
<td></td>
</tr>
<tr>
<td><strong>C5218 (F' lac)</strong></td>
<td>1</td>
<td>23.00</td>
<td>14</td>
<td>0.396 (0.064)</td>
<td>0.520</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20.25</td>
<td>7</td>
<td>0.644 (0.106)</td>
<td></td>
</tr>
<tr>
<td><strong>C5886 (F' lac)</strong></td>
<td>1</td>
<td>36.67</td>
<td>12</td>
<td>0.973 (0.154)</td>
<td>1.119</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>37.50</td>
<td>15</td>
<td>1.265 (0.216)</td>
<td></td>
</tr>
<tr>
<td><strong>C6450 (F' lac)</strong></td>
<td>1</td>
<td>23.60</td>
<td>9</td>
<td>1.203 (0.205)</td>
<td>1.062</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>28.70</td>
<td>7</td>
<td>0.920 (0.114)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4-1: The Expression of LacZ (BG) in Different Genetic Environments. Whether the operon is placed normally or transposed to a nearby location on the somatic chromosome, or is placed on the F-plasmid, there is little significant affect on the production level of BG in steady state broth-grown cells.
Discussion

There have been several reports in which the expression of lacZ on Hfr or F-plasmid was measured. Beckwith et al (1966) found BG was produced in about same amount in several different Hfr-strains. However, and very importantly, they did not use steady state cells. In contrast, Masters and Vermeulen (1985) recently showed that the amount of BG per gene dosage was rather constant in steady state cells of Hfr strains with lac's widely transposed around the chromosome. However the closer the F'-lac was transposed to oriC, the origin of chromosomal replication, the more the BG per cell was produced. Therefore, it is reasoned that the inconsistent results of Beckwith's group and that of Masters and Vermeulen were caused by the different physiological conditions of the bacteria used. Because the integrated F' replicates as a part of the host chromosome (Beckwith et al, 1966) and DNA replication nearly stops in the late phases of growth, the gene dosage is thought to be almost constant regardless of chromosome position at this phase, which was used by Beckwith's group. Hence, the BG level per cell in this stage seems to be relatively constant, independently of the transposed locations. On the
contrary, DNA replicates actively in steady state cells, so gene dosage is different depending on the distance from chromosomal origin and BG is produced proportionally to the gene dosage per cell (Masters and Vermeulen, 1985). O'Farrell et al (1978) also showed that the production of BG is proportional to the copy number of pBG120, although the induced BG was different in structure and enzyme activity from normally induced one.

Since there are a large number of lac-transposition mutants available, we chose PB3004 because the lac-operons of both W3110 (Bachmann, 1984) and PB3004 are located within two minutes of each other, there is little expected difference in the gene dosages in these two strains.

BG activity in cells carrying F'-lac has been measured to be about twice as much as that on normal chromosome (Revel, 1965). Independently it was also concluded that the copy number of F is about two. However, these measurements were not made in steady state cells. Again these former results do not agree with my result. Since it seems that the copy number of F increases in older cells (Andresdottir and Masters,
1978), it might be suggested that the copy number is one in steady state cell and increases to two later in the growth cycle. Again this leads to the concentration of BG as being approximately the same in the F', the wild strain and the Hfr (Beckwith et al, 1966).

The control mechanism of plasmid replication has not yet been understood completely. However, it is clear that F controls its own replication, since the replication time of F is not coupled with that of the host chromosome (Steinberg and Helmstetter, 1981). Moreover, the product of the dnaA gene, which is necessary for initiation of new cycles of chromosomal replication, is not required for the replication of F, even though host RNA and protein synthesis is essential for new replicative cycles. Therefore, the present evidence suggests that the replication is regulated by F itself and its copy number is adjusted physiologically by the host chromosome (Nordstrom, 1983). The pattern of F replication is not affected by DNA homologies on the F'-lac and the host chromosome (Steinberg and Helmstetter, 1981).
Comparing the concentration of BG with regard to the length of F'-lac, there was not a significant difference among two F'-strains, although the lengths differed by more than two-fold. CGSC-5218 produced somewhat less BG than in the other strains. This might be due to orientation (neighborhood) and not to plasmid size. However, this cannot be assumed to be definitely so because of the variability of the data. In late exponential cells, it has been shown by Beckwith et al (1966) that Hfr's with opposite orientation produced the same amount of BG as those with different orientation. However, there has not been any explanation about the gene efficiency depending on the orientation of F'.

In the study of genetic environmental effects on lac expression, there does not appear to be any difference in lac expression due to genetic neighborhood except in one F'stRAIN, or by differences in length of F-plasmids of equal orientation. Also, the difference of BG level within all the F'strains was less than two-fold, and not as high as one would expect if there were high copy numbers. Unfortunately, the strains studied in this experiment were not all in the same somatic chromosomal background. These and similar quantitative studies should be of great advantage to the understanding of control mechanism of plasmid and chromosome replication.
APPENDIX I

Colony Forming Unit Assay Procedure

One milliliter samples were periodically removed from the growing cultures. These samples were then serially diluted in what can be called a pi-fold dilution series: each subsequent tube is diluted by the 0.5 power of 10. This is accomplished by transferring 1.0 ml through a series of tubes each containing 2.16 ml of sterile saline. Ten microliter samples of each of these serial dilution tubes were spotted in a line upon a petri plate and then streaked out in a lane. After overnight incubation, the colonies were enumerated in the countable lanes. From these counts, the number of CFU/ml of original culture could be ascertained.
APPENDIX II

"Z-buffer" Composition

0.06 M $\text{Na}_2\text{HPO}_4$
0.04 M $\text{NaH}_2\text{PO}_4$
0.01 M KCl
0.001 M MgSO$_4$
0.05 M beta-mercaptoethanol

Adjust to pH 7.0.
REFERENCES


SungAe Cho was born in Seoul, Korea, on June 21, 1959. She graduated from Chung-Shin Girl's High School. Upon obtaining the Bachelor of Science degree in Biology from Sogang University, Seoul in February, 1982, she joined the graduate program at the College of William and Mary in August, 1982, and received the Master of Arts degree in August, 1985.