

## Impact of carbon dioxide gas and carbonate ions on the growth of *Escherichia coli* K-12 B23 and on the induced $\beta$ -galactosidase activity: exploring models for global regulation

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**Global regulation of cell metabolism and *lac* operon expression in response to CO<sub>2</sub> in *Escherichia coli* K-12 B23 was investigated. Two experimental systems were applied to test the effects of CO<sub>2</sub>. One of the systems involved adding CO<sub>2</sub> as bicarbonate (ion-phase system) to three separate test cultures; the concentrations of sodium bicarbonate tested were 0.0 M, 39.4 mM, and 61.5 mM Na<sub>2</sub>CHO<sub>3</sub>. Carbon dioxide was added as a gas in the second system, known as the gas-phase system. The types of gases used in this system were air, CO<sub>2</sub> mixture (5% CO<sub>2</sub>, 10.4% H<sub>2</sub>, and 84.5% N<sub>2</sub>) and pure N<sub>2</sub> at 850 cc/min, 110 cc/min and 120 cc/min respectively. Our results indicate the bicarbonate had limited effects on cell growth and enzyme activity in  $\beta$ -galactosidase ( $\beta$ -gal). But we found that anaerobic conditions in our gas system hindered cell growth greatly but significantly increased the levels of  $\beta$ -galactosidase in the gas-phase experiment. The results were tied into three models of global regulation: catabolite regulation, stringent response and redox regulation. From this experiment we concluded that anaerobic conditions could have affected intracellular levels of adenosine 3',5'-cyclic monophosphate (cAMP), cyclic AMP receptor protein (CRP), and/or guanosine 3',5'-bispyrophosphate (ppGpp); which in turn regulated downstream activities such as growth rate and expression of the *lac* operon.**

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Cell physiology and metabolism is dependent on its environment. The ability of a cell to sense and respond to its surroundings, such as fluctuating levels of nutrient or oxygen, determines how well it and its genetic information will survive. This phenomenon of cellular response to changes in a cell's environment is termed global control or global regulation [16]. This area provides many avenues of exploration, most notably gene regulation and intracellular signalling. Investigation into this area also provides many practical benefits such as the control of bacteria growth, virulence and the long-term persistence of pathogenic bacteria [5].

The organism of choice for this experiment is *Escherichia coli* because it is one of the better-characterized organisms. The determining environmental factors of this investigation are carbon dioxide (CO<sub>2</sub>) and presence or lack of oxygen (O<sub>2</sub>). To observe cellular response to these conditions, cell growth and the induction of the *lac* operon was monitored, specifically the production of  $\beta$ -galactosidase (coded for by the *lacZ* gene).

Induction of the *lac* operon involves two different steps. One involves removing the negative control, the *lac* repressor (coded by the *lacI* gene) which is an allosteric molecule with binding sites for the *lac* operon (*lacO*) and for the inducer, thio-galactosidaseactosides such as isopropyl-thio-galactosidaseactoside (IPTG). Once an inducer has bound the repressor molecule, it will dissociate from the operator allowing RNA polymerase to bind the *lac* promoter (*lacP*). However, transcription of the *lac* gene requires a second step, a positive regulatory control system has to be satisfied. To promote transcription a complex of adenosine 3',5'-cyclic monophosphate (cAMP) and cyclic AMP receptor protein (CRP) is required. This cAMP-CRP complex binds the CRP binding site on *lacP* and causes the DNA strand to unwind, allowing RNA polymerase binding and transcription [for more information about the *lac* system see references 1, 21, 23].

Both cyclic AMP and a second nucleotide, guanosine 3',5'-bispyrophosphate (guanosine tetraphosphate, ppGpp) are key signal nucleotides that regulate metabolism under starvation or stressed conditions [35]. In addition to promoting *lac* transcription, cAMP along with CRP controls many other operons similar to *lac* such as *gal* (genes for galactosidaseactose metabolism) and *fru* (genes for fructose metabolism). Furthermore, cAMP's role as a second messenger and as an alarmone [15] is indicative of how general its use is in global control. The cAMP-CRP system is part of a larger, global control system that is responsible for co-ordinating carbon source utilization. This control system is the amalgamation of a century's amount of work. Starting with the ideas of diauxic growth and the "glucose effect" [15, 34]. Followed by the development of catabolite repression and inducer exclusion by Magasanik and Neidhardt [17, 18]. And completed with the discoveries of cyclic AMP, CRP (also know as

catabolite activator protein, CAP), and the phosphoenolpyruvate (PEP) dependent carbohydrate: phosphotransferase system (PTS) [13, 15, 26, 32, 34, 36].

Guanosine tetraphosphate (ppGpp) is similar to cAMP in many ways; both are nucleotides that act as alarmones, they are triggered in response to nutritional deprivation or to environmental stress, and both modulate the synthesis of many molecules. Guanosine tetraphosphate is an integral part of the stringent system (regulon) [2] or stringent response. The accumulation of ppGpp in response to environmental stress leads to global changes in cellular metabolism such as the downregulation of nucleic acid and protein synthesis, and the upregulation of amino acid synthesis and protein degradation [5]. Therefore, studying the levels of  $\beta$ -galactosidase production provides insights into global regulation.

A third system of global regulation, redox regulation, controls cell physiology while growing in aerobic or anaerobic environments. In aerobic conditions, transcription factors OxyR and SoxR are activated to induce oxygen defence proteins in response to elevated levels of oxygen and oxygen-reactive species. When there is a shift in oxygen levels, cells respond through histidine sensor kinases such as ArcB and RegB, and DNA-binding proteins such as FNR. These proteins regulate many global processes such as respiration, photosynthesis, carbon fixation, and nitrogen fixation [3]. Thus, the "redox control system" provides another model to explore global regulation.

It is shown in many previous experiments (see review [7]) that carbon dioxide inhibits cell growth in *Escherichia coli* and other microorganisms. From this same reasoning that cell growth is inhibited, we expected  $\beta$ -galactosidase induction to be affected as well. The results from this investigation are considered in the context of the previous three described models of global regulation.

## MATERIALS AND METHODS

**Chemicals and Equipment.** All chemicals, gases, and enzymes used were standard reagent grade chemicals. All equipment is standard, readily available; otherwise, they're noted.

**Strains and Culture Conditions.** In both the gas-phase and ion-phase experiments, the wild type strain B23 of *Escherichia coli* K-12 (provided by Dr. Ramey, UBC) was used. Before each experiment, overnight cultures of *E. coli* B23 were set-up in M9 minimal media supplemented with 0.2% glycerol (M9-0.2G) by inoculating 19.98 ml of M9-0.2G media with 0.02 ml of refrigerated *E. coli* B23 inoculum. Overnight cultures were then incubated in a shaking 37°C water bath at power level 4 (Metabolyte by New Brunswick Scientific Company). Turbidity of the overnight cultures were greater than 1.5 OD<sub>460nm</sub>.

**Ion-phase Culture Sampling and Induction.** For the ion-phase experiment, the procedure was adapted from lab number nine of the 1999-2000 MICB321 Laboratory manual [29]. Using the overnight culture, fresh M9-0.2G was inoculated to make up a culture with a final volume of 160 ml in a 500 ml Erlenmeyer flask; the optical density should be approximately 0.1 OD<sub>460nm</sub>. This culture was placed in a 37°C shaking water bath at power level 5, and grown until approximately 0.42 OD<sub>460nm</sub>. At that point, the culture was induced to produce  $\beta$ -galactosidase by adding IPTG to the culture to make up a final concentration of 10mM IPTG. Sampling consisted of removing 3 ml of culture and recording an OD<sub>460nm</sub> reading. Cell growth in the sample was then arrested by vortexing with toluene for 30 seconds; sample was saved for later ONPG assay of  $\beta$ -galactosidase. Sampling was done right after induction, at 2 minutes and at 4 minutes after induction with IPTG. At 6 minutes after induction, the culture was split into three separate 250 ml Erlenmeyer flasks; each flask containing a final volume of 50 ml of induced culture but with different final concentrations of sodium bicarbonate. The final concentrations of sodium bicarbonate in the flasks were 0.0 M Na<sub>2</sub>HCO<sub>3</sub> for the control, 39.4 mM Na<sub>2</sub>HCO<sub>3</sub> (equivalent of 0.64 atmospheric pressure of CO<sub>2</sub> [22]) for the first test condition and 61.5 mM Na<sub>2</sub>HCO<sub>3</sub> (equivalent of 1 ATM of CO<sub>2</sub>) for the last test condition. Sampling of the control condition occurred at 9, 13, 17, 21, 25, 30, 35, 40, 46, 53, 61 and 70 minutes after initial induction with IPTG. For the first test condition (0.64 ATM of CO<sub>2</sub>), sampling was taken one minute after the control was sampled and for the second test condition (1.0 ATM of CO<sub>2</sub>), sampling was taken two minutes after the control was sampled.

**Gas-phase Culture Sampling and Induction.** A sub-culture of 350 ml with 0.1 OD<sub>460nm</sub> was made from inoculating fresh M9-0.2G with the overnight culture. This was placed in a 37°C shaking water bath at power level 5 and grown to 0.4 OD<sub>460nm</sub>. Methylene blue was added to the sub-culture, final concentration of 0.0002% [20], as an anaerobic indicator and as a reducing agent to facilitate the anaerobic process; when the culture is under anaerobic conditions it will appear cloudy white, otherwise it will be a deep aqua blue indicating aerobic conditions. The sub-culture was then split into three separate glass jars with screw-top lids (made by UBC Microbiology Workshop) by filling each jar with 100 ml of culture; these lids have two circular openings allowing the insertion of a redox probe (ORP Redox Combination Electrode, Broadley James Corp.) and air stones. These jars were then placed into a 37°C non-shaking water bath. The redox probes were attached to pH-volt meters which were in turn hooked up to the MPLI data acquisition software (Vernier Software). The redox probes were calibrated using quinehydrone solutions at various pH values (4.0, 7.0, 10.0). The calibrated probes were inserted into each test tube to measure the redox potential of the cultures. An air diffuser (#A-983, Hagen) hooked up to a regulator on a gas cylinder containing compressed air was inserted into the first jar; this was our control condition. The same setup was used to provide a CO<sub>2</sub> mixture comprising of 85.6% N<sub>2</sub>, 10.4% H<sub>2</sub>, 5% CO<sub>2</sub> to the second jar, and N<sub>2</sub> gas to the third jar. Carbon dioxide mixture was bubbled into the second jar at a flow rate of 110 cc per minute and N<sub>2</sub> was bubbled into the third jar at a rate of 110 cc per minute. The first jar was allowed to sit in the water bath. These cultures were allowed to go anaerobic (redox potential reading between -180mV and -200mV) for 20 minutes. After 20 minutes, air was supplied to the first jar at a flow rate of 850 cc per minute. IPTG (10 mM final concentration) was immediately added to the first jar and sampling was performed. One minute afterwards, the second jar was induced with IPTG (10mM) and sampled. The last jar was induced with IPTG (10mM) 2 minutes after the first jar was induced and sampled. Additional samples were removed at 5, 10, 15, 20, 25, 30, 35, 40, 50 and 60 minutes after induction for each jar respectively.

**Preparation of Samples for O-nitrophenol-galactosidase assay (ONPG) assay.** Before the actual ONPG assay for  $\beta$ -galactosidase activity, 0.4 ml of the toluene-permeabilized cells was removed from each sample and placed into its own respective tube. To each of these tubes, 1.2 ml of 20 mM Tris (pH 8.0) was added. However, the gas-phase experiment required extra steps. The toluene permeabilized samples collected at 0, 5, 10, and 15 minutes after induction with IPTG were placed into microcentrifuge tubes and microfuged at 8000 RPM (Brinkman) for 5 minutes. The supernatant was removed and set aside. The pellet was then resuspended with 1 ml of 20 mM pH 8.0 Tris buffer. After resuspending the

cells, 0.4 ml was transferred into a separate test tube and 1.2 ml of 20 mM Tris (pH 8.0) was added. These extra steps served to concentrate (3 fold) the small amounts of  $\beta$ -galactosidase in these earlier samples for the ONPG assay; thus, improving the precision of the reading.

**Assay of  $\beta$ -galactosidase by O-nitrophenol-galactosidaseactoside.** The newly prepared samples were then placed in a 25°C water bath for 15 minutes to warm up. When the samples have warmed up, 0.2 ml of 5 mM ONPG was added to each sample to initiate the assay. The time required for the reaction to produce sufficient yellow colour (approximately 0.2 OD<sub>420nm</sub>) was noted for each individual sample. The reaction was stopped by adding 2 ml of 0.6 M sodium carbonate to the samples. Absorbance of the enzyme reaction was read at 420 nm on a spectrophotometer.

## RESULTS

**Ion-phase System.** During the course of the ion-phase experiment, samples from the three cultures with varying concentrations of carbonate ions (0.0, 0.64, and 1.0 ATM CO<sub>2</sub>) were taken to monitor the growth rate of *Escherichia coli* B23 by observing the changes in optical density (Fig. 1). This set of data suggests that there are no appreciable differences in growth rates between the cultures growing under various carbonate levels within the first forty minutes after induction. After forty minutes, the data points begin to diverge from one another, maybe suggesting some sort of delayed response to the carbonate ions. The generation time ( $g$ ) and growth rate constant ( $\mu$ ) of these cultures were calculated as follows, where  $t$  represents the amount of time for the initial cell number ( $N_0$ ) to grow to the final cell number ( $N$ ):

$$g = t / (\ln N - \ln N_0) \quad (\text{Eq. 1})$$

$$\text{growth rate constant } (\mu) = \ln 2 / g \quad (\text{Eq. 2})$$

**Table 1. Generation time and growth rate constants of E. coli B23 growing in M9-0.2G supplemented with various concentrations of Na<sub>2</sub>CHO<sub>3</sub>**

| [Na <sub>2</sub> HCO <sub>3</sub> ] mM | generation time (g) in hours | Growth rate constant ( $\mu$ ) |
|--|------------------------------|--------------------------------|
| 0.0                                    | 1.17                         | 0.59                           |
| 39.4                                   | 1.23                         | 0.56                           |
| 61.5                                   | 1.22                         | 0.56                           |

Table one shows how the generation time is essentially the same amongst the three conditions. Thus, our results suggest that carbonate ions have little, if any, noticeable influence on cell growth.

Enzyme activity of  $\beta$ -galactosidase was determined with the following equation:

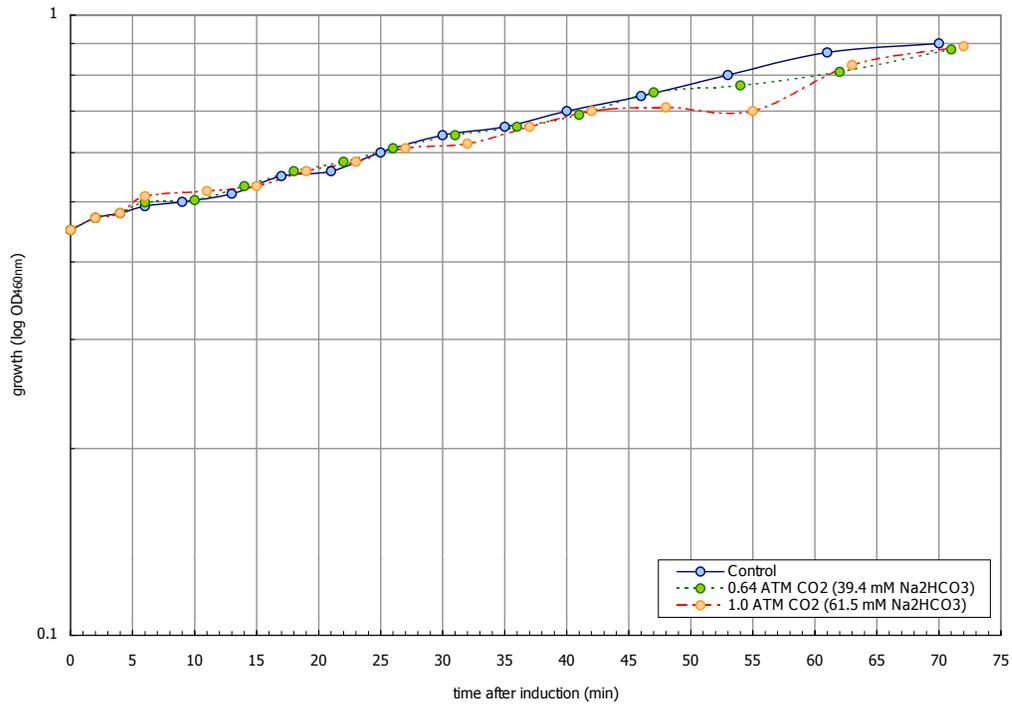
$$\text{enzyme activity / ml of enzyme} = A \times (1 / t) \times (10^6 / 15,000) \times (Nv / Ev) \quad (\text{Eq. 3})$$

Where  $A$  is the absorbance reading of assay reaction at 420 nm,  $t$  is the elapsed time for assay reaction,  $Nv$  is the total assay volume (ml) of the reaction, and  $Ev$  is the volume of enzyme (ONPG) in the assay. The value 15,000 is the molar extinction coefficient for O-nitrophenol in the spectrophotometer tube and  $10^6$  is a correction to change the molar part of the extinction coefficient from one mole per litre to nanomole per millilitre.

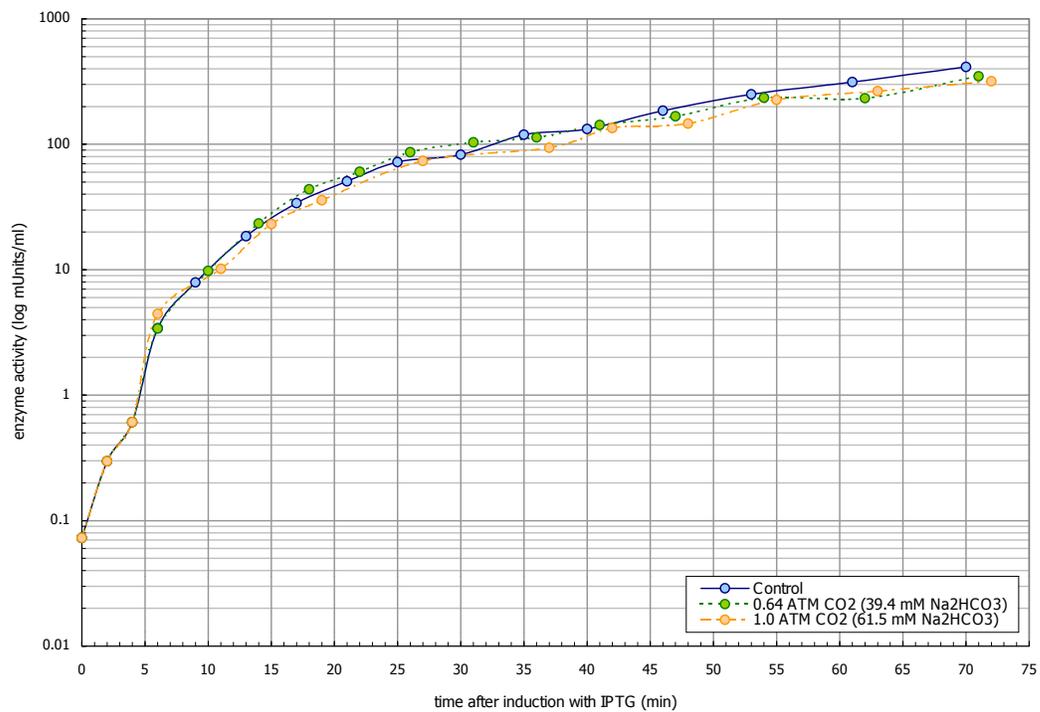
$\beta$ -galactosidase activity in permeabilized cells shows a similar trend to that of cell growth (Fig. 2). This trend indicates that increased levels of carbon ions do not greatly affect the induced  $\beta$ -galactosidase activity because all three test-conditions behaved similarly. Figure 2 also demonstrates sharp increase in enzyme activity in the first 20 to 30 minutes after that the trend begin to plateau off; it seems like  $\beta$ -galactosidase activity and production has reached a maximum level. The absorbance of the first few points (enzyme assays for time 0, 5, 10 and 15 minutes) tended to be hard to detect due to the very small amounts of  $\beta$ -galactosidase activity. To improve the sensitivity of the assay of these early points, the samples were microfuged and resuspended in Tris buffer (see methods for detail).

To explore the results for the induction of  $\beta$ -galactosidase, the activity was looked at a per cell basis (Fig. 3) by taking the total enzyme activity in the sample and dividing it by the optical density. This ratio looks at the induction of  $\beta$ -galactosidase in each cell and how it changes over time. Enzyme activity under all three conditions was similar up until the first 40 minutes but after 40 minutes, the points begin to deviate from one another and the trends become unclear. The trends seem to diverge for several reasons. This observed effect could be due to complications by variations in the values recorded but the general trend is reproducible. Or the observed effect is trying to imply that

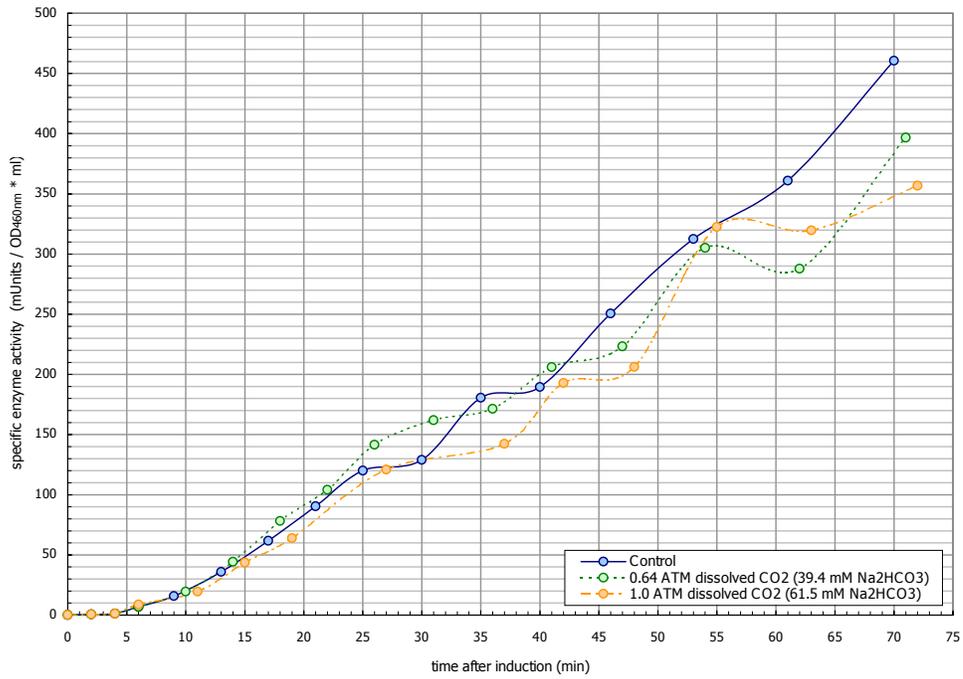
**Figure 1. Effect of CO<sub>2</sub> (carbonate ions) on *E. coli* B23 growth in M9-0.2G**



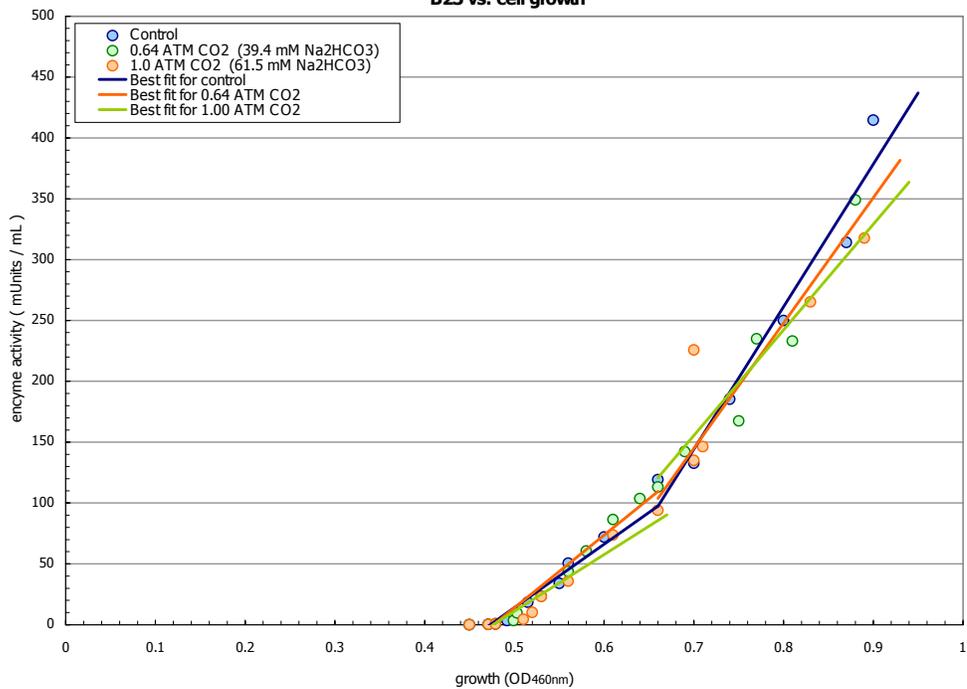
**Figure 2. Effect of CO<sub>2</sub> (carbonate ions) on activity of B-galactosidase from *E. coli* B23 growing in M9-0.2G**



**Figure 3. Effect of CO<sub>2</sub> (carobnate ions) on specific activity of B-galactosidase *E.coli* B23 in M9-0.2G**



**Figure 4. Correlation between effect of CO<sub>2</sub> on enzyme activity of B-galactosidase from *E. coli* B23 vs. cell growth**



there is a difference in response to carbonate levels but only after a period of lag because the levels of  $\beta$ -galactosidase activity is higher in the non-carbonated culture than the levels observed in the carbonated cultures. Plotting  $\beta$ -galactosidase activity as a function of optical density (Fig. 4) provides support for the latter assessment. This plot illustrates that within a test condition there appears to be two stages of response as described by what appears to be a concave curve, suggesting the induction is actually improved as the cell cultures get denser. Either case, it is clear that  $\beta$ -galactosidase activity per cell continues to increase over time in all conditions.

**Gas-phase System.** Growth of *E.coli* in the three different test conditions (850 cc/min air, 120 cc/min CO<sub>2</sub> mixture, and 110 cc/min N<sub>2</sub>) was monitored in the same manner as the ion-phase experiment. The optical density was recorded at 460 nm and plotted against time (Fig. 5); methylene blue was tested to see if it affected the OD<sub>460nm</sub> reading, but it did not affect the reading greatly (about  $\pm 0.05$  OD units). The generation time and growth rate constants were then calculated using equations 1 and 2.

**Table 2. Generation time, specific growth rate constants, and redox levels of *E. coli* B23 growing in M9-0.2G incubated with various gases**

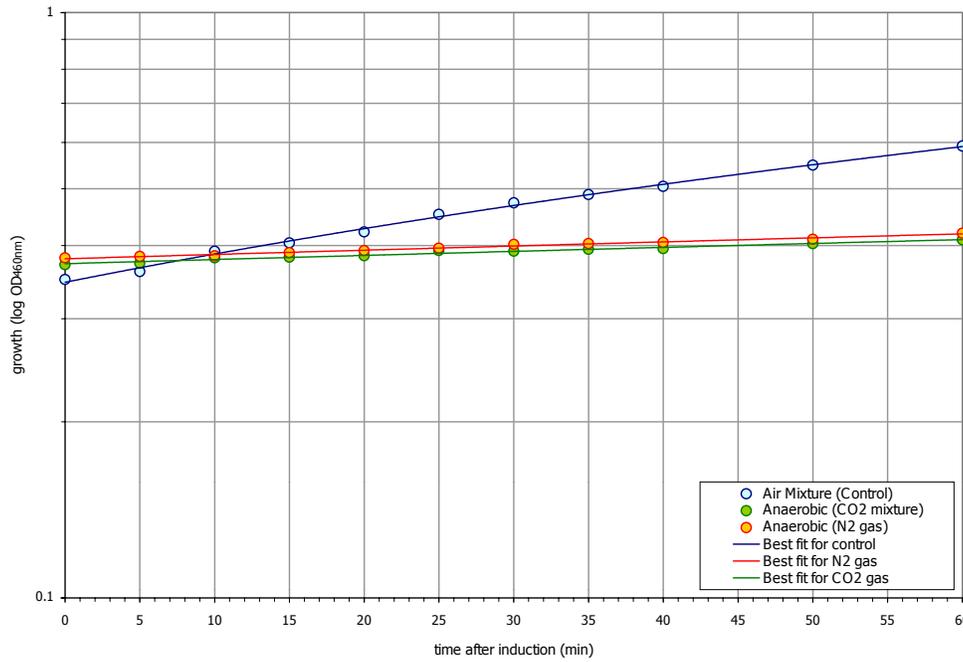
| test condition                  | generation time (g) in hours | Growth rate constant ( $\mu$ ) | Redox potential (mV) |
|---------------------------------|------------------------------|--------------------------------|----------------------|
| 850 cc/min air                  | 1.33                         | 0.52                           | +300                 |
| 110 cc/min CO <sub>2</sub> mix. | 7.17                         | 0.09                           | -500                 |
| 120 cc/min N <sub>2</sub>       | 7.15                         | 0.09                           | -190                 |

The trends between the control with air and the test conditions are significantly different as indicated by the differences in slopes of the growth plot (Fig. 5) and by the differences in generation time (Table 2). Significant growth was observed in the control jar but growth was slow in the other two test conditions. Redox potentials of the three conditions was also recorded (Table 2). The most reduced culture was the one bubbled with CO<sub>2</sub> mixture and the control was the most oxidized.

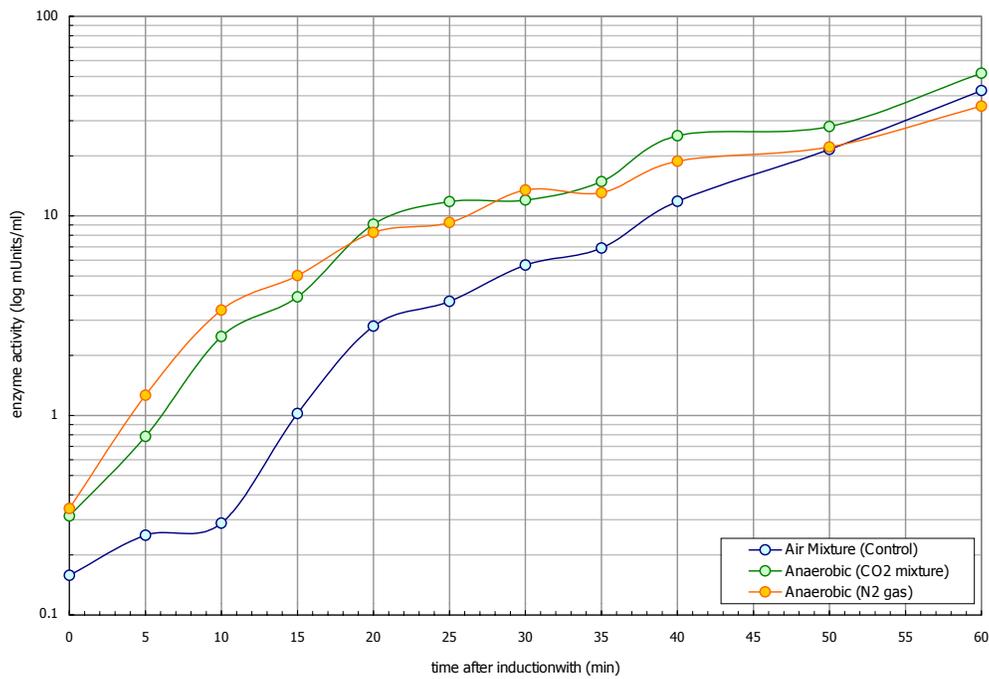
$\beta$ -galactosidase activity was monitored against time after induction with IPTG as well (Fig. 6). Surprisingly, the results differed from the ion-phase system generating carbonate because the observed levels of activity were significantly different between the anaerobic cultures and the control culture. Despite the differences in redox potential, the behaviour of the two different anaerobic conditions were fairly similar and levels of  $\beta$ -galactosidase activity observed in these conditions was significantly higher than  $\beta$ -galactosidase activity observed in the aerobic condition. The observed trends were enhanced when specific  $\beta$ -galactosidase activity was plotted against time (Fig. 7). Anaerobic conditions induced greater  $\beta$ -galactosidase activity than the aerobic condition, however, this plot reveals that the specific activity of  $\beta$ -galactosidase is the greatest in the culture bubbled with CO<sub>2</sub> gas mixture.

The plot of  $\beta$ -galactosidase activity against optical density (Fig. 8) continues to show that there is a marked difference in the production of  $\beta$ -galactosidase activity due to the varying conditions. The two anaerobic conditions display a lot of similarities, the slope of the two anaerobic trends are almost vertical; meaning that the rate of  $\beta$ -galactosidase activity production is much greater than the growth rate. However, for the aerobic condition, the trend is more exponential than linear. Production of  $\beta$ -galactosidase activity increases slowly under aerobic conditions but at optical densities over 0.5 OD<sub>460nm</sub>, the production of  $\beta$ -galactosidase activity begins to increase rapidly.

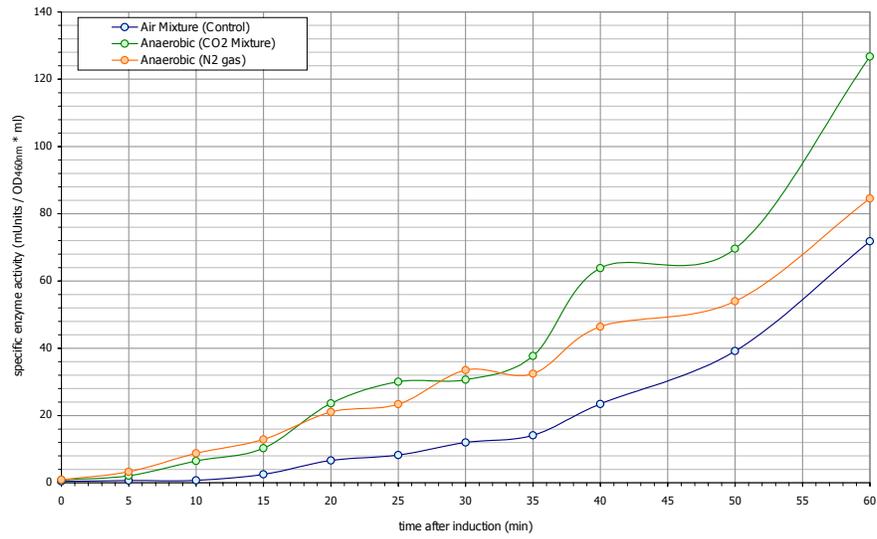
**Figure 5. Effect of Aerobic and Anaerobic conditions on *E. Coli* B23 growth in M9-0.2G using the gas-phase system**



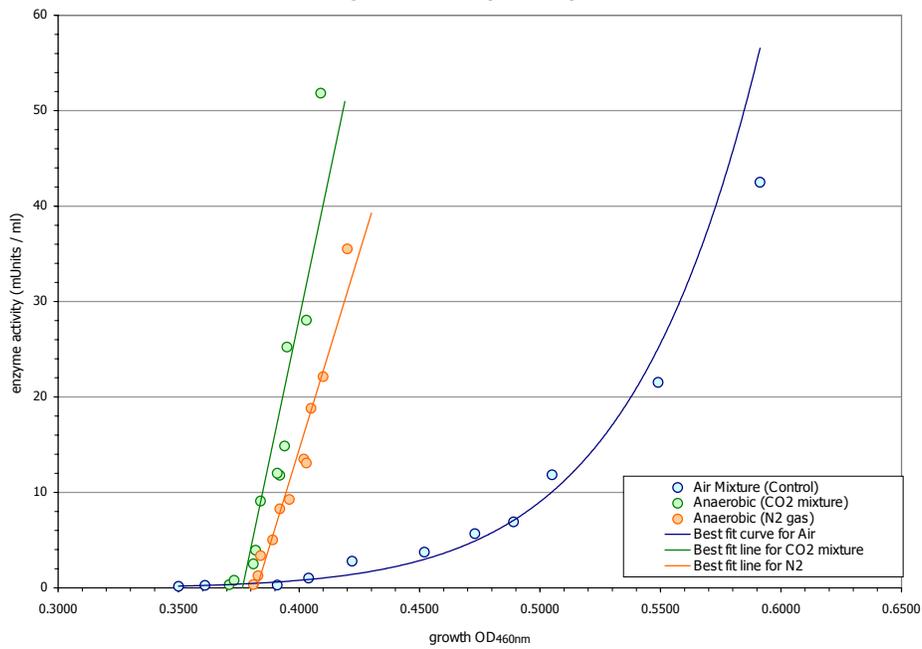
**Figure 6. Effect of Aerobic and Anaerobic conditions on enzyme activity of B-galactosidase from *E. coli* B23 growing in M9-0.2G using the gas system**



**Figure 7. Effect of Aerobic and Anaerobic conditions on B-galactosidase specific activity of *E. coli* B23 in M9-0.2G media using the gas system**

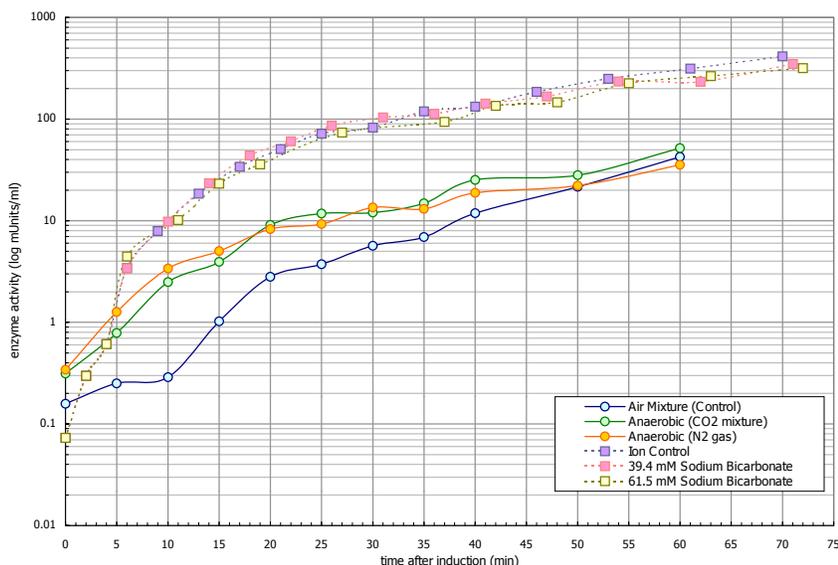


**Figure 8. Correlation of effect of Aerobic and Anaerobic conditions on *E. coli* B23 growth and B-galactosidase enzyme activity**

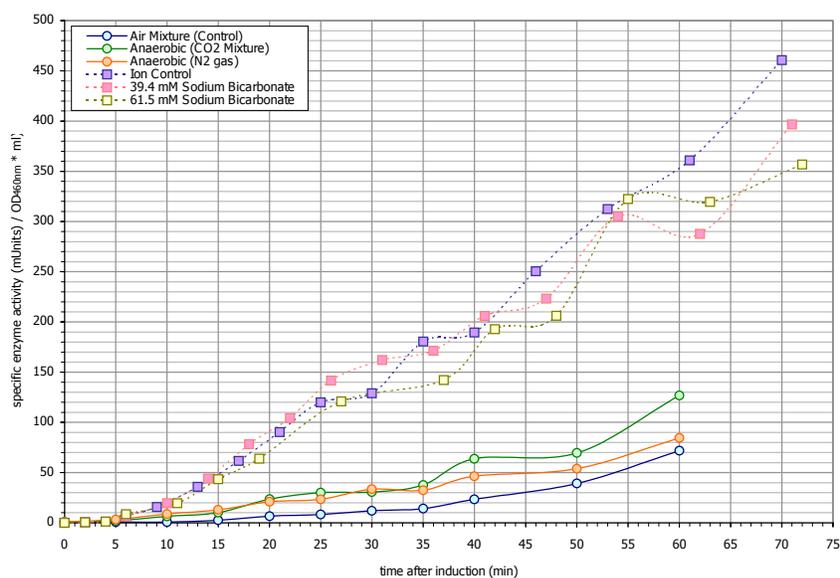


**Comparing ion-phase results and gas-phase results.** When the plots of  $\beta$ -galactosidase induction over time for the ion-phase system (Fig. 2) and gas-phase system (Fig. 6) were combined (Fig. 9), there is a striking difference in the levels of  $\beta$ -galactosidase activity between those produced by cells in the ion system compared to those in the gas system. There's approximately 50 to a 100 times greater enzymatic activity for  $\beta$ -galactosidase produced by cells grown in the ion system than those produced by cells in the gas system. Furthermore, the production of  $\beta$ -galactosidase within the first 15 minutes appears to increase greatly in the ion system compared to the gas system. After 15 minutes, the trends of both systems appear to be increasing at about the same rate because the slopes of the trend appear to be parallel. The combined specific activity plot for level of  $\beta$ -galactosidase activity per optical density per millilitre against time (Fig. 10) provides the same relationship between the two systems except on a non-logarithmic plot.

**Figure 9. Effect of Aerobic and Anaerobic conditions on enzyme activity of B-galactosidase from *E. coli* B23 growing in M9-0.2G using the gas system vs. ion system**



**Figure 10. Effect of Aerobic and Anaerobic conditions on B-galactosidase specific activity of *E. coli* B23 in M9-0.2G media using the gas system vs. ion system**



## DISCUSSION

**Ion-phase System.** At the beginning of this experiment, we expected the carbonate ion system to inhibit the growth of *Escherichia coli* and the induction of  $\beta$ -galactosidase because it is well documented that carbon dioxide inhibits growth in *E. coli* [7, 21]. However, neither expectation was satisfied. In a separate experiment, non-neutralized (pH 9.0) carbonate solutions were used and the results in that experiment indicated that cell growth was inhibited under basic, carbonated conditions. Thus, it seems that carbonate ions alone do not physiologically cause the cells to respond differently. The reason as to why we're not seeing any physiological changes is unclear. It could be possible that the carbonate ions are unable to enter the cell and affect intracellular processes because they're unable to interact with cytoplasmic proteins and nucleic acids. After consultation with Dr. Ramey, this explanation seemed unlikely because  $\text{CO}_2$  is known to enter plant cells and autotrophic cells. Thus, the likely scenario is that we may have underestimated the amount of carbonate ions to add to the cultures. Mori [22] et al. reported that inhibition of *E. coli* growth was observed in fed-batch cultures with 64%  $\text{CO}_2$  in the exhaust gas; this  $\text{CO}_2$  was produced as a result of metabolic waste as no  $\text{CO}_2$  was supplied to their fed-batch cultures. We assumed that 64% of exhaust gas equals 0.64 ATM of  $\text{CO}_2$ . Since 1 ATM  $\text{CO}_2$  equals 24.6 mM dissolved  $\text{CO}_2$  [7] and the ratio of dissolved  $\text{CO}_2$  to  $\text{HCO}_3^-$  is 2.5 [22], we calculated the concentration of carbonate ions to be 39.4 mM  $\text{Na}_2\text{HCO}_3$ . Therefore, it could be that our assumption of the pressure of exhaust  $\text{CO}_2$  in Mori's experiment is faulty leading to an underestimate of the concentration of carbonate to use.

**Gas-Phase System.** The gas-phase system provided more interesting results than the ion system because there's a clear distinction in cellular response to aerobic (850 cc/min air) and anaerobic conditions (110 cc/min  $\text{CO}_2$  mixture and 120 cc/min  $\text{N}_2$ ). Since cellular response to both anaerobic gas conditions were fairly similar and that the ion-system showed no appreciable difference in response to carbonate ions, carbon dioxide does not appear to be key in eliciting the differences observed in enzyme activity and cell growth. Rather it is the absence (anaerobic) or presence of oxygen (aerobic) that is causing the cells to respond in such fashion. This is because the only difference between the two anaerobic conditions is that the  $\text{CO}_2$  mixture contained  $\text{H}_2$ ,  $\text{CO}_2$ , and  $\text{N}_2$  gases, whereas the other condition was all  $\text{N}_2$  gas. Thus, it's possible to state that  $\text{CO}_2$  appears to have very little effect on  $\beta$ -galactosidase activity or cell growth in *E. coli* B23 because the responses to both types of gases were very similar. Here we'll try to fit the results in a broader context known as global control or global regulation.

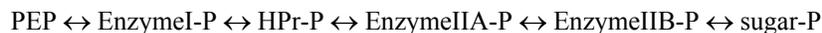
**Redox control and observed *lac* response.** SoxR and SoxS are proteins that control cell response to increased levels of oxidative stress such as intracellular redox values and levels of superoxides [10]. Both proteins contain an iron sulphur centre therefore any changes in redox potential will cause the iron to have different levels of oxidation or reduction [3]. When SoxR is activated, it'll upregulate the production of anti-superoxide proteins such as superoxide dismutase (SOD) and catalase [10]. Since the redox potentials between the various experimental conditions were quite different (+200 mV, -190 mV, and -500 mV), it is certainly possible that SoxR/S were activated by the redox potential. Thus, in the aerobic culture, activated SoxR, SoxS and OxyR (see below) were causing the cells to devote most of its protein synthesizing machinery to making superoxide dismutases and catalases in the early stages of the experiment. Since the "aerobic culture" was sitting in the water bath prior to the start of the experiment, the cells were accustomed to an anaerobic environment. Suddenly introducing these cells to an aerobic environment by bubbling large quantities of air requires the cells to make quick changes, such as increasing the production of SOD and catalase to combat the increased levels of oxygenic species. As a result, most of the protein synthesizing machinery was occupied with production of SOD and catalase. Therefore, this could account for the lag (the first 25 minutes) in  $\beta$ -galactosidase production and the subsequent return to normal levels of  $\beta$ -galactosidase production (after 30 minutes) because the cell was busy making enzymes in response to oxidative stress and only later, when enough SOD and catalases were produced, did  $\beta$ -galactosidase production return to normal levels.

Another key regulator of global control responsible for responding to anaerobiosis is FNR, known as the anaerobic transcriptional regulator because it controls over 120 genes in *E. coli* [3, 8]. It is suggested that FNR responds to redox potentials through a mechanism similar to that in SoxR/S system by using an iron containing centre. In anaerobic conditions, FNR induces proteins such as fumarase, isocitrate dehydrogenase, and other proteins involved with anaerobic respiration. Meanwhile, FNR represses other proteins such as cytochrome oxidase and those involved with aerobic respiration. FNR sequence analysis revealed that its amino acid sequence and conformation is very similar to the catabolite activator protein (CAP, also known as CRP) [3]. ArcB and ArcA are global regulators of gene expression much like FNR; in fact, they share identical regulation of several genes. ArcB and ArcA achieve control by repressing expression of enzymes involved with aerobic respiration when exposed to

anaerobic conditions [3]. There are still many other redox regulatory proteins that are part of the global control system such as FixL (regulates nitrogen fixation), and OxyR (responds to H<sub>2</sub>O<sub>2</sub> levels and responds in a similar fashion as SoxR/S) [3].

With the anaerobic test condition, the high levels of  $\beta$ -galactosidase activity can be explained in part by FNR regulation. Since most of the proteins involved with aerobic respiration are turned off, this may free up polymerases and ribosomes for the production of  $\beta$ -galactosidase if the levels of production for anaerobic respiratory proteins is less than that of aerobic respiratory proteins. The same can be said for ArcB and ArcA regulation because they too repress the production of proteins involved in aerobic respiration. Also, there is large homology in amino acid sequence and in binding site recognition between FNR and cyclic AMP receptor protein (CRP or CAP) [8]. Since CRP and cAMP is involved with activation of transcription at the *lac* locus, there may be a possibility that FNR can activate or influence *lac* transcription; for example, the colicin gene E1 (*cea*) is activated by FNR and is also dependent on catabolite repression [8]. Or FNR may affect the levels of cAMP and ppGpp through FNR regulated genes involved with respiration, and subsequently affect *lac* expression.

**Carbon Catabolite Regulation and *lac* expression.** The catabolite regulation responds to nutrient levels in its surrounds. When abundant glucose, a preferred carbon and energy source, is available the cell will respond accordingly by transferring glucose into cytoplasm with the aid of the PTS (see [27] for review). The phosphoryl transfer chain is basically the transfer of a phosphate group through a series of proteins (phosphorylation cascade):



The key protein of interest is the glucose-specific EnzymeIIA (EIIA<sup>Glc</sup>) because EIIA<sup>Glc</sup>-P stimulates adenylate cyclase and adenylate cyclase controls the level of cAMP and hence, the level of cAMP-CRP complexes because activated adenylate cyclase synthesizes cAMP from ATP. Thus, if the nutrient levels are rich in rapidly metabolized carbohydrates, PTS will be used and the amount of EIIA<sup>Glc</sup>-P will be low because the phosphate group is constantly transferred to glucose. As a result, less adenylate cyclase will be active and the levels of cAMP and cAMP-CRP complex will go down. Furthermore, non-phosphorylated EIIA<sup>Glc</sup> will bind other permeases through allosteric interactions and inactivate these permeases. As a result, this will prevent the formation of intracellular inducers because the permeases are unable to transport them into the cytoplasm (known as inducer exclusion). Thus, catabolite repression is achieved by lowered cAMP levels and permeases inactivation by EIIA<sup>Glc</sup> [6, 15, 19, 21, 30, 31, 34].

Magasanik and Niedhardt noted that expression of *lac* is influenced strongly by sources of carbon and energy available in growth media [19]. Also, they noted that the levels of  $\beta$ -galactosidase production increases when the cells are grown in media that supports slow growth rates [19]. Further studies have shown that anaerobic conditions do favour the production of  $\beta$ -galactosidase through a process called depression of catabolic repression [14]. These findings agree with the results obtained from the gas system. Growth rates were very slow in the anaerobic test conditions of the gas system. Since the growth rates were low, we should expect high levels of  $\beta$ -galactosidase production from these anaerobic conditions compared to the aerobic condition. This is no different from what we observed with our gas system, the levels of  $\beta$ -galactosidase are noticeably higher in the anaerobically grown cultures.

Also, Hogema et al. found that if the *lac* operon is induced by IPTG, the induction of  $\beta$ -galactosidase is only dependent of the levels of cAMP and CRP and not on the phosphorylated state of EIIA<sup>Glc</sup> [11]. Therefore, inducer exclusion is not a factor when IPTG is used because IPTG do not require transportation by lactose permeases. If the increase in  $\beta$ -galactosidase production is due to anaerobic conditions and not to inducer exclusion, because we used IPTG as our inducer and glucose was absent in our growth media, the anaerobic condition is still somehow affecting the levels of cAMP and CRP. This was observed in experiments done by Lee and Dobrogosz as well [14]. In their experiments, they noticed distinct changes of cAMP and  $\beta$ -galactosidase levels in response to aerobic and anaerobic conditions; anaerobic conditions led to higher cAMP and  $\beta$ -galactosidase levels [14]. Thus, anaerobiosis may be affecting the production of cAMP from ATP by several mechanisms such as activating adenylate cyclase either directly or through another phosphorylating protein similar to EIIA<sup>Glc</sup>. Cyclic AMP receptor protein is also a requirement for initiation of transcription of the *lac* gene. Therefore anaerobiosis may have caused the cell to upregulate the production of CRP. The regulatory signal to increase the production of CRP and cAMP may be regulated by the redox control system because it is able to sense changes in redox potential and adjust cellular processes accordingly. Therefore, this area requires further investigation because it is known that cAMP and CRP levels change in accordance to anaerobic or aerobic conditions but how this is accomplished is still a mystery.

**Stringent response and *lac* expression.** Stringent response is a regulatory system used by bacteria to control the production of various molecules as a function of environmental stresses such as nutritional or energy starvation; specifically amino acid starvation. When cells are starved for nutrients they'll respond by increasing the levels of the alarmone guanosine 3',5'-bispyrophosphate (ppGpp) [2]. Accumulation of ppGpp occurs when cellular levels of aminoacylated tRNA to uncharged tRNA are unbalanced [9]. Guanosine tetraphosphate is produced when an uncharged tRNA enters the A site of ribosomes, causing the translation process to stall. When this stalling occurs, ribosome bound RelA (ppGpp synthetase I) synthesizes ppGpp from GTP and ATP [2, 5, 35]. Levels of ppGpp are also controlled by another protein called SpoT, which is basically a pyrophosphohydrolase [35]. During starvation, RelA is actively synthesizing ppGpp while the degrading activity of SpoT is inhibited [2]. The promoters for tRNA and rRNA synthesis are under regulation by ppGpp; ppGpp represses the production of tRNA and rRNA by interfering with the binding of RNA polymerase to the promoters. In addition, ppGpp is also known to inhibit cell wall synthesis [35] and DNA replication by slowing the rate of initiation of replication at *oriC* and by negative regulation of *dnaA* [4]. Thus ppGpp is able to control the growth rate of bacteria by limiting synthesis of stable RNA, cell wall, and by slowing down DNA replication.

This model of cellular response to environmental stress fits well with our results for the gas-system. The lack of cell growth due to anaerobiosis, which can be considered a form of environmental stress even though the cell is a facultative anaerobe, indicates that these regulatory steps in stringent response may be activated by ppGpp. Since cells are unable to produce as much ATP energy under aerobic conditions than anaerobic ones, they may see the situation as being stressed; this could be amplified by the fact that they are growing in minimal media. Therefore, since the sources of energy and carbon are low and the cells are unable to efficiently use the sparse sources, the ratios of uncharged and aminoacylated tRNA becomes unbalanced. This caused the cells to produce many molecules of ppGpp, resulting in the drop in growth rate.

Johansson et al. noted that the promoter of *crp* (gene coding for CRP) is regulated through negative stringent control [12]. If stringent response is able to affect the transcription of CRP and thus control the levels of cytoplasmic CRP, the stringent regulatory system is able to indirectly affect the levels of  $\beta$ -galactosidase by controlling the complex (cAMP-CRP) required for transcription of *lac*.

Beside from negatively regulation gene expression and cell growth, studies have shown that ppGpp can act as a positive regulator and activate catabolic and amino acid biosynthetic operons [5, 35]. Primakoff and Artz have done in vitro studies on positive control of *lacZ* expression by ppGpp and cAMP [28]. They observed that ppGpp and cAMP were able to increase the expression *lacZ* by 19-fold. Consequently, our results for anaerobiosis have two characteristics of stringent response. Firstly, cell growth was presumably limited by the anaerobic condition. Secondly, the amount of  $\beta$ -galactosidase produced from cells grown in the aerobic condition was also much higher than the ones produced by cells growing in aerobic conditions. Thus, there's a strong possibility that anaerobiosis is affecting the levels of ppGpp as the cell tries to adapt to deprivation of oxygen.

Our findings of increased  $\beta$ -galactosidase activity in anaerobically grown cells compared to aerobically grown cells agrees with the general consensus that cells in media supporting slow growth rate produce more catabolic enzymes. The affect could arise if anaerobiosis is somehow affecting the production of cAMP and CRP, as these are key in the initiation of *lacZ* expression. If this explanation is true then by adding cAMP to cell grown aerobically the levels of  $\beta$ -galactosidase should increase. Or we can construct or obtain a *crp* mutant in which CRP (CAP) is constitutively expressed at high levels and see how it responds in our aerobic gas-system, with and without cAMP added.

The stringent response model can provide a model to explain the data obtained from the gas-system. Since levels of ppGpp is the hallmark of stringent response, future experiments can assay the amounts of ppGpp [24] in cells growing aerobically and anaerobically to determine whether there is a difference and if the difference in levels are actually related to a certain response. Other possible methods of testing the effects of ppGpp under aerobic and anaerobic condition, would be the addition of ppGpp to cells growing aerobically or the removal of ppGpp from cells (such as constructing or obtaining a mutant that is RelA<sup>-</sup>) growing anaerobically.

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