Journal of Experimental Microbiology and Immunology (JEMI) Vol. 3:35-40

Effect of changing osmotic stress on *lac* operon expression in *Escherichia coli* K-12 wild-type strain B23

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Previous studies have focused primarily on the effects of changing hyperosmotic stress on the expression of particular sigma factors, and stress regulons in *Escherichia coli.* Prior experiments have shown that stress regulons play a prominent role in transcriptional regulation of various cellular osmoprotectants, and other genes involved in the osmotic stress response. This experiment was conducted in order to test whether the expression of the *lac* operon in *E. coli* is under the direct influence of hyperosmotic stress. *E. coli* B23 cells were exposed to various environmental concentrations of sodium chloride and the activity of the *lac* operon was monitored by assaying β-galactosidase production. Two separate trials were conducted in order to investigate the effect of different stages of cell growth on the expression of the *lac* operon in a hyperosmotic environment. The results indicated that there was an exponential decrease in β-galactosidase production in with increasing osmolarity in stationary phase cells, implying that the *lac* operon is repressed under such conditions. The cells in exponential phase of growth were also affected by varying external osmolarity; however, the pattern of β-galactosidase production differed remarkably from that observed by stationary cells, which was likely a result of prior exposure of the cells to high temperatures. In essence, the *lac* operon is amongst many genes affected by osmotic stress; however, additional factors, including the stage of growth and environmental stresses, such as increased temperature, may influence transcription of the *lac* operon.

The osmolarity of the environment is one of the physical parameters that affect the proliferative capacity of microorganisms. Bacteria have evolved various mechanisms to cope with osmotic stress, one of which is use of compatible solutes. Compatible solutes are synthesized in response to changes in turgor pressure, which function to increase the osmolarity in the cytoplasm. *Escherichia coli* copes by varying the amount of cytoplasmic potassium and solutes such as glutamate and putrescine (8).

Hyperosmotic shock results in a decrease in cytoplasmic water activities (3). This can have a variety of detrimental effects on the cell such as impairing the normal functioning of proteins and other macromolecules (3). Sudden plasmolysis can also inhibit a variety of physiological processes, including metabolism, growth, respiration, enzyme synthesis and nutrient uptake (9). Several enzymes of the *mal* regulon are strongly repressed at increased osmolarity (2). Similarly, the *bet* regulon of *E. coli,* which allows the synthesis of the osmoprotectant glycine betaine from choline, displays upregulated expression, 10-fold, in response to hyperosmotic stress (5).

Sigma factors assist in directing RNA polymerase to specific regions of DNA to transcribe a subset of stress genes (1). Hyperosmotic shock results in the induction of a variety of sigma factors, which control expression of a large number of other genes (1,6). For example, σ^S acts primarily as a positive regulator and exerts transcriptional control of over 50 genes, one of which is LacZ of the *lac* operon (7). Thus it is expected that transcription of the *lac* operon is also altered in the face of hyperosmotic stress.

The aim of this present work was to examine the impact of changing osmotic stress on the expression of the *lac* operon in the form of β-galactosidase production in *Escherichia coli* K-12 wild-type strain B23.

MATERIALS AND METHODS

Growth of Cells: *E. coli* K-12 wild type strain B23 was incubated overnight in a 37°C shaking water bath. In the morning, the turbidity at 660 nm (OD_{660}) of the culture was measured, and sufficient culture was transferred to a 2L Erlenmeyer flask containing 400ml of Luria broth (4), to give an initial OD_{660} of 0.15. This culture was incubated in a water bath at 37°C with shaking for 4 h. After 4 h, 25 ml of the culture was transferred to each of twelve 250 ml Erlenmeyer flasks. In Trial 2, the overnight culture was incubated on a platform shaker at 37°C for 2 h in a 1L Erlenmeyer flask, at which time 25 ml of the culture was transferred to each of twelve 125 ml Erlenmeyer flasks. In both trials, sufficient IPTG was added to each of these flasks to give a final concentration of 0.1 mM. Sodium chloride (NaCl) was added to the flasks with final concentrations ranging from 0 to 1 M. These flasks were incubated in a 37°C shaking water bath for 1 h, at which time the turbidity at OD_{660} was taken and 1 ml of the culture was removed for plating purposes. Cell suspensions were diluted and then spread plated on Luria agar plates and incubated at 37°C for 48 h. The cells in the remaining 24 ml sample were concentrated and then broken open using the bead-bashing method.

After incubation with NaCl and IPTG in Trial 2, 3 ml of the culture was removed for plating and OD measurements. The cells that were spread plated were incubated at 37°C for 24 h. The cells in the remaining 22 ml sample were concentrated and then broken open using the bead-bashing method. It is important to note that there was some salt inherent in the media, so the control (no NaCl) flask actually had 5 g of NaCl per liter, which is equivalent to 0.086 M. Each subsequent flask had an additional 0.086 M NaCl on top of the value stated. The values stated in this experiment (0, 0.2, 0.4, 0.6, 0.8 and 1.0 M) are the values added to the flask on top of the NaCl inherent in the media.

Bead Bashing Method: Prior to bead bashing, all samples were centrifuged for 10 min at 14,000 rpm using a Beckman J2-21 centrifuge. Samples 9-12 (0.8 and 1.0 M NaCl samples) were centrifuged for an additional 10 min at 18,000 rpm. Prior to bead bashing in Trial 2, all samples were centrifuged for 10 min at 14,000 rpm, as in Trial 1, however, samples 9-12 (0.8 and 1.0 M NaCl samples) were centrifuged for another ten min at 17,000 rpm. The supernatant was removed and the pellets were resuspended with 1 ml of 10 mM Tris buffer (pH 8). These resuspended samples were transferred to screw-capped microcentrifuge tubes approximately half full of small glass beads. The samples were bead bashed for 1 min at 50,000 rpm using a Biospec Mini Bead Beater at room temperature. After the cells were broken open, the amount of protein present was determined using the Bradford assay.

Bradford Assay: Samples were diluted to appropriate dilutions then assayed as previously described (6). A standard curve was prepared using chicken egg white albumin standard. The protein concentration of each sample was determined using this standard curve.

β-galactosidase Assay was used to determine the amount of enzyme activity present in each sample. 1.2 ml of 10 mM Tris (pH 8) and 0.2 ml of 5 mM ONPG were added to a test tube and warmed in a 45°C water bath for 2 min. A total sample volume of 0.1 ml was added, and the test tube incubated in the 45°C water bath until a bright yellow color developed. At this time, 2 ml of 0.6 M $NaCO_3$ was added to stop the reaction, and the A_{420} of the sample was taken using a Spectronic 20. If the reading was too high, the assay was performed again, after dilution of the sample with 10 mM Tris (pH 8.8) buffer, again for a final sample volume of 0.1 ml.

SDS-PAGE gel: A 10% SDS gel was run in this experiment to confirm the presence and size of β-galactosidase in each sample, as well as the proportion of β-galactosidase in each sample relative to the amount of protein. The samples were standardized to contain 1.5 µg of protein per lane, and adjusted to 24 µl using dH_2O and 4X sample buffer. The gel was run at 191 V for 90 min, at which time it was stained using the silver-staining method, as previously described (6). Gel images were captured using a HP Scanjet IICX/T. In Trial 2 the 10% SDS-PAGE gel was run at 200 V for 3 h due to the occurrence of buffer spillage upon movement of the gel, which led to the gel ceasing to run until the buffer spillage was noticed.

RESULTS

Two notably different trends were observed between cells in stationary phase (Trial 1) and exponential phase (Trial 2), in regards to the enzyme activity of β-galactosidase per *E. coli* B23 cell when grown in various hyperosmotic growth environments. As indicated in Figure 1, *E. coli* exposed to high salt concentration stationary phase of growth exhibited an overall decrease in β-galactosidase production as the osmolarity of the media increased. Enzyme activity per cell appeared to follow an exponential decay as the environmental NaCl concentration increased from 0 to 1 M NaCl, with maximum enzyme activity per cell occurring in an environment without NaCl.

The enzyme activity per cell in exponential phase cells showed a markedly different trend (Figure 1). The enzyme activity per cell displayed a relatively stable pattern up to 0.8 M NaCl, at which point there was a noticeable decrease. There was a problem with exponential phase cell data, since the cells were exposed to temperatures up to 50°C. This heating compromised our results for comparison purposes to the data obtained using stationary phase cells, and the results of both experiments will be treated separately.

Table 1. Concentration of stationary phase *E. coli* B23 cells incubated at 37°C, and exponential phase *E. coli* B23 cells exposed to high incubation temperatures. Both cultures were incubated in the presence of various concentrations of NaCl and 0.1mM IPTG for 1 h, at which point the OD_{660} was taken to obtain an estimate of cell concentration.

NaCl Concentration (M)	Cell Concentration (x10^8 cells/ml)	
	Stationary Phase Cells	Exponential Phase Cells
0.0	9.8	1.8
0.2	9.8	2.0
0.4	9.0	2.0
0.6	8.4	2.0
0.8	8.8	2.1
1.0	9.6	2.1

The cells remained viable as osmolarity was increased in both exponential and stationary phase cells, as plate counts for all samples resulted in cell growth (data not shown). The concentration of both stationary and exponential phase cells was relatively constant with increasing osmolarity, although the stationary phase cell concentration was approximately five times higher than exponential phase cell concentration (see Table 1). This was consistent with expectations, as the stationary phase cells had reached maximal cell density, while the exponential phase cells had not. In addition, heating the exponential phase cells may also account for this large difference in concentration.

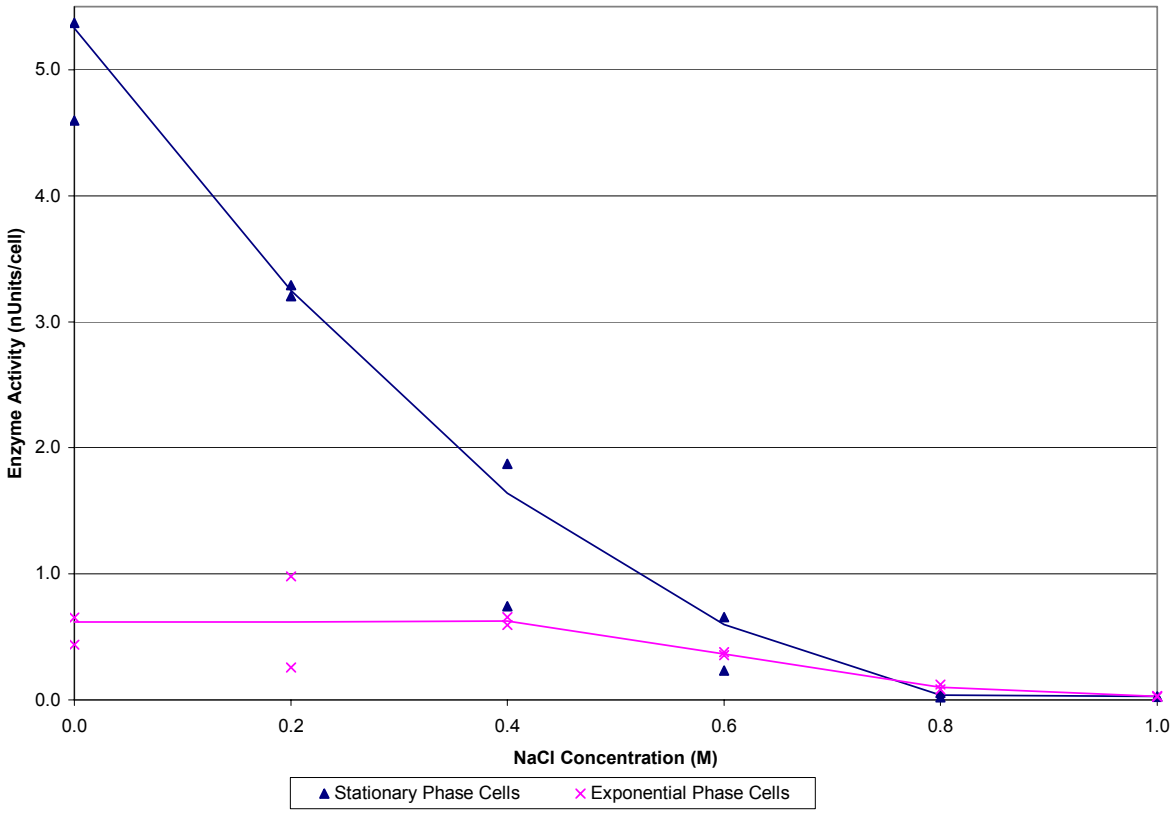

Figure 1. Enzyme activity per *E. coli* B23 cell, incubated in various NaCl concentrations. Cells in stationary phase (▲), and exponential phase (x) were incubated with NaCl and 0.1mM IPTG.

The SDS-PAGE gel containing stationary phase cell samples, Figure 2, showed a β-galactosidase band in lanes 1 through 8, which contained samples ranging from 0 to 0.6 M NaCl. This β-galactosidase band was not detected in lanes 9 through 12, which contained the 0.8 and 1.0 M NaCl samples. At NaCl concentrations of 0.8 and 1.0 M, the enzyme activity was very low, shown in Figure 1, and was likely present in quantities too small to be detected on the gel. These results concur with the graph of β-galactosidase activity per cell in Figure 1. This gel also showed lanes pushed out to the sides, and slanted bands, which could have been be due to interactions of the salt with the gel.

An SDS-PAGE gel was obtained for exponential phase overheated cells, however, it yielded very poor results due to overstaining (data not shown). The β-galactosidase band was identified in both gels using a provided figure showing the banding pattern of the protein standards used (figure not shown).

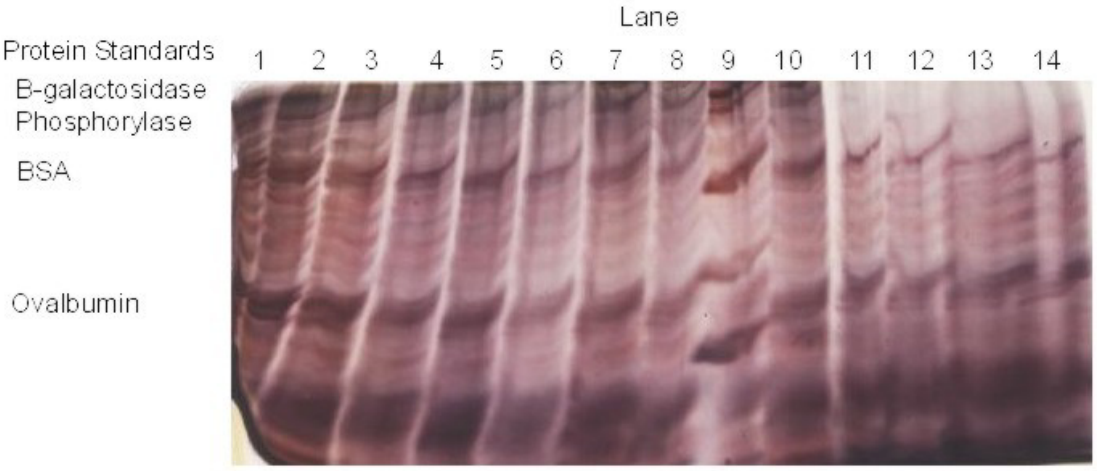

Figure 2. Silver-stained SDS-PAGE gel for Trial 1, stationary phase cells. Lane 1 = protein standard ladder; Lanes 2 and 3 = samples without NaCl; Lanes 4 and 5 = 0.2 M NaCl samples; Lanes 6 and 7 = 0.4 M NaCl samples; Lane 8 = 0.6 M NaCl samples; Lane 9 = protein standard ladder; Lane 10 = 0.6 M NaCl samples; Lanes 11 and 12 = 0.8 M NaCl samples; Lanes 13 and 14 = 1.0 M NaCl samples.

DISCUSSION

Results indicate that stationary phase *E. coli* cells (Trial 1) exhibit a decrease in β-galactosidase production as osmolarity increases, while cell concentration remains the relatively constant. Thus, *lac* operon expression is reduced as NaCl concentration increases, indicating that expression of some genes, such as the *lac* operon, are very repressed at environmental salt concentrations of 1.0M. The question remains, however, if these trends are due to the osmotic stress imposed on the cells, or if it is actually the ions affecting cellular processes, including *lac* operon expression. Further experimentation could further investigate this question, using an alternate osmotic compound. This experiment has shown a decrease in *lac* operon expression under increasing osmolarity. This effect may also have associated implications in a variety of other osmodependent systems. Osmoregulation of the *lac* operon could be a result of osmodependent alterations in a vast number of factors involved in lactose utilization in *E. coli* B23. For example the *lac* repressor, which is transcribed elsewhere in the genome, may be subject to osmoregulation by osmosensitive sigma factors.

The exponential phase *E. coli* cells were exposed to relatively high temperatures, which limits our ability to compare results between trials. This heating may account for the very low enzyme activity per cell at low NaCl concentrations. Higher enzyme activity per cell is expected from exponential phase cells, however, results show a much higher activity in stationary phase cells, up to 0.8 M NaCl. Interestingly, above 0.8 M NaCl, stationary phase cells exhibit higher enzyme activity than exponential phase cells. It appears that exposure to elevated temperatures may somehow protect the cells from the stresses associated with high osmolarity. It is possible that this exposure to high temperature may stimulate a general stress response, thereby allowing these heated cells to continue cellular process under high osmolarity conditions more effectively than the unheated stationary phase cells. Further experiments could be conducted using heated cells under high osmolarity conditions to determine the effect of prior stress on cellular processes over time.

One major problem encountered in the experiment was that the cells were not washed following centrifugation of the cultures, prior to bead bashing. This led to protein concentration anomalies in the samples, due to remaining broth components, which contained significant amounts of non-cellular protein. Unfortunately, this left us unable to make any comparisons or draw any conclusions from the Bradford assay results. This evidence of broth-related protein presence is visible in both gels (see Figures 2 and 3). Several unidentified bands are present in both gels, and are consistently present in every lane. These bands are likely due to media-related protein carryover. It would be advisable for future experiments of this nature to wash the cells prior to preparing samples.

Our experiment clearly demonstrated that the stationary phase cells exhibited lower *lac* operon expression with increasing osmolarity while cell concentration remained relatively constant.

FUTURE EXPERIMENTS

It would be interesting to determine how *lac* operon expression in *E. coli* is altered by osmotic stress during different phases of growth. The present experiment attempted to test this difference, however due to exposure to high temperature, results between trials were not comparable. To reduce this ambiguity, the difference in β-galactosidase expression in *E. coli* should be tested when they are in the lag phase, log phase and stationary phase of growth.

Another important experiment is to determine if the salt effect is due to osmotic stress. This could be determined by monitoring synthesis of various osmoprotectants such as glycine betaine in *E. coli* to determine if this significantly enhances their ability to withstand osmotic stress. Preliminary tests could be done using exogenous betaine on growth conditions that promote betaine formation to see if further tests are worthwhile.

The results of our experiment leave us with a major pending question: How or why does β-galactosidase expression decrease when subjected to hyperosmotic stress? It could be simply that *E. coli* diverts its metabolism to the synthesis of osmoprotectants to restore cell vigor, while consequently decreasing *lac* operon expression. Incubating *E. coli* in a hypertonic solution could determine the mechanism responsible for the decrease in enzyme activity. Samples taken at various time intervals could be analyzed for suspected elements thought to indirectly or directly regulate *lac* operon expression in the face of osmotic stress. Manipulation using *lac* operon plasmid constructs, while mechanically varying the concentration of the suspected osmoregulator, would aid in this process.

ACKNOWLEDGEMENTS

We would like to thank Dr. Ramey, UBC, for supplying us with *E. coli* K-12, strain B23. We would also like to thank Dr. Ramey, Karen Smith and Andre Comeau for their assistance preparing and executing the lab, and also the media room people for providing us with materials and assistance.

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APPENDIX I

Sample Calculations for Trial 1, Sample 1.

Enzyme Activity (mUnits/ml) = A/t * 10^6/15,000 * Nv/Ev

Where:

1 mUnit =	1 mole of product formed per minute
A =	absorbance measured at 420nm
T =	time in minutes
15,000 =	molar extinction coefficient for ONPG
10^6 =	conversion factor to convert mol/L to nmol/ml
Nv =	total assay volume
Ev =	volume of enzyme sample used

Enzyme Activity (mUnits/ml) = (0.15/3.97min)*(10^6/15,000)*(3.5/0.002) = 4411.8 mUnits/ml

Cell Concentration (from OD reading): 1 OD_{660} = $8x10^8$ cells/ml

1.20 OD_{660} * ($8x10^8$ cells/ml)/(1 OD_{660}) = $9.6x10^8$ cells/ml

Enzyme Activity per cell = (Enzyme activity/ml)/(# cfu/ml)

Note: In this case, #cfu/ml is averaged from OD and Plate counts.

Enzyme activity/cell = (4411.8 mUnits/ml)/[($9.6x10^8$ cells/ml+$3.2x10^8$ cfu/ml)/2]

Enzyme activity/cell = $6.9x10^{-6}$ mUnits/cell = 6.9 nUnits/cell

NOTE: For Trial 2, values for Enzyme Activity should be multiplied by 24/22, to account for differences in the volume used in each Trial.