

The Effect of Ionic (NaCl) and Non-ionic (Sucrose) Osmotic Stress on the Expression of β -galactosidase in Wild Type *E.coli* BW25993 and in the Isogenic *BW25993ΔlacI* Mutant

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A sudden increase in the osmolarity of the environment is highly detrimental to the growth and survival of *Escherichia coli*, thus it must promptly sense changes in the external osmolarity and instigate appropriate adaptation responses. To determine how the cell is affected by ionic and non-ionic hyperosmotic stress, *lac* operon expression was monitored indirectly by measuring the activity of β -galactosidase. The results indicated that the Lac repressor was affected under hyperosmotic conditions. In particular, there was higher β -galactosidase activity under higher osmolarity due to the Lac repressor. Results also revealed that transcription of the *lac* operon was affected differently under NaCl (ionic) and sucrose (non-ionic) osmotic stress. A 3 fold higher β -galactosidase activity in NaCl osmotically induced cells in contrast to sucrose treatments at 0.6 Osm and 1.2 Osm was observed. The results for NaCl induced osmotic stress correlated with our hypothesis, however the effects on β -galactosidase activity under sucrose induced osmotic stress were contrary to our hypothesis, as the expected up-regulation of the enzyme activity was not observed.

Escherichia coli's ubiquity in soil, water and normal intestinal flora of mammals (32), where it frequently encounters varying osmotic conditions, reflects its extreme adaptability. Osmolarity is a significant physiological challenge for cells, as semipermeable cytoplasmic membranes only allow water and a limited number of solutes to cross (27). Consequently, to minimize the loss of cytoplasmic water under hyperosmotic stress conditions, *E.coli* has a number of mechanisms to increase its internal pressure. These include increasing the uptake of potassium ions (K^+) (21, 27, 28) and osmoprotectants (17), biosynthesis of compatible solutes glutamate and trehalose (4, 5, 9), and greater control of ion flux across the cytoplasmic membrane (30).

Traditional methods of examining cell response, using two-dimensional electrophoresis gels (2, 13) together with current techniques of analysing global transcription patterns (6, 32, 33) suggest that increase in osmolarity has a global effect on gene expression (27). A number of genes involved in cross-protecting the cell against stress conditions are upregulated by master stress regulator RpoS (σ^S) in a time-dependent manner. Only a few genes, mainly involved in housekeeping processes, are recognized to be down-regulated upon osmotic stress (32). Physiological adaptations are often associated with changes in metabolic activities. Therefore, one way to determine how the cell is

affected by a hyperosmotic environment would be to monitor the activity of β -galactosidase. The lactose (*lac*) operon of *E. coli* is an example of a system where gene expression (β -galactosidase) is affected by global regulation. For instance, cyclic AMP (cAMP) receptor protein (CRP) is a global regulator, involved in the repression as well as activation of a vast number of *E. coli*'s genes, plays a part in activation of the β -galactosidase expression (24).

The *lac* operon is expressed when two conditions are met. First, negative control through the Lac repressor binding to the *lac* operon (*lacO*) needs to be removed. The structural gene for this repressor (*lacI* gene) is separate from the *lac* operon, and therefore the repressor is synthesized constitutively in the cell at a low rate. Lac repressor control can be removed by adding an inducer, such as isopropyl-thio-galactosidase (IPTG) to the system, where it binds to the repressor molecule and dissociates it from the operator, allowing RNA polymerase to bind to the *lac* promoter (*lacP*). Second, a complex of cAMP and CRP is required for maximum transcription from the *lac* operon (23). The intracellular level of cAMP possibly plays a key role in osmoregulation and is critical for bacterial adaptability to changing osmotic conditions involving both ionic and non-ionic solutes (14). Previous studies suggest that increased level of cAMP results in hyperexpression of *lac* operon during

elevated osmotic stress (24).

Overall, we expected transcription of the *lac* operon to be affected differently under ionic and non-ionic osmotic stress. The general effect that was expected from the increase of cAMP is the hyperexpression of the *lac* operon leading to an increase of β -galactosidase levels under both NaCl and sucrose treatments. In isotonic concentrations of NaCl and sucrose, higher β -galactosidase production would be expected in cells under NaCl treatment than those treated with sucrose, since some of ATP is likely to be consumed in the process of compensating for the depleted cytosolic K⁺. Moreover, higher β -galactosidase expression in *BW25993Δlac I* mutant would be expected since the Lac repressor can no longer play a role in regulation of lac operon.

To understand whether the type of osmotic shock could influence the cell differently, β -galactosidase activity was studied in wild-type *E.coli* *BW25993* and its isogenic *lacI* mutant subjected to hyperosmotic stress imposed by metabolically inert ionic (NaCl) and non-ionic (sucrose) solutes. Wild-type cells were grown in concert with isogenic mutant to attribute changes in β -galactosidase activity to the effects on the Lac repressor.

MATERIALS AND METHODS

Bacterial culture. *E. coli* strains *BW25993* and *BW25993ΔlacI* were obtained from the University of British Columbia MICB 421 culture collection.

Growth conditions. Strains were inoculated separately into 25 ml LB - Lennox (1% tryptone, 0.5% yeast extract, and 0.5% sodium chloride (NaCl) medium in 125 ml Erlenmeyer flasks and incubated overnight at 37 °C in shaking water bath. To generate log phase cultures, overnight cultures were diluted and grown for 2hrs in 160 ml LB -Lennox media with isopropyl β -D-1-thiogalactopyranoside (IPTG) at 0.1 mM at 37 °C and verified by turbidity measurements at OD 460nm. Samples were diluted in PBS buffer (29) and plated on LB-Lennox agar plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar) and incubated overnight in 37 °C incubator for initial cell viability before osmotic induction.

Induction of osmotic stress. Log phase cultures were centrifuged at 1000 x g (21,000 rpm) for 10 minutes in the J2-21 Beckman centrifuge. Osmotic shock was induced when the cell pellets were re-suspended in 50 ml pre-warmed 42°C LB- Lennox media containing 0 M NaCl (control), 0.3 M NaCl, or 0.6 M NaCl in 125 ml Erlenmeyer flask. Each 50 ml re-suspension was then further divided into two 125 ml Erlenmeyer flasks; therefore duplicates of each varying concentration of NaCl were performed. The cells were then incubated at 42°C in shaking water bath for 2 hours. If osmotic stress were to be induced by sucrose instead of NaCl, pre-warmed 42°C LB-Lennox media with 0 M (control), 0.6 M or 1.2 M sucrose would be added instead to the cell pellets and the following procedure would be the same. In order for the same osmotic pressure to be asserted by NaCl and sucrose, sucrose was added in twice the concentration, as osmolarity is measured in moles of solute particles. For instance, NaCl can dissociate in solution into Na⁺ and Cl⁻ ions. Thus, for every 1 mole of NaCl in solution there are 2 osmoles (Osm) of solute particle since both ions affect the osmotic pressure of solution. Sucrose does not dissociate and 1 M sucrose is a 1 Osm sucrose solution (31). After incubation, the cells were diluted with PBS and plated on LB-Lennox agar plates, to determine cell viability post-

stress induction. The plates were then incubated at 37 °C for overnight.

β -galactosidase Assay. The cells were permeabilized by toluene assay as described by Miller (23). β -galactosidase assay was used to measure the amount of active β -galactosidase present in the cells as described by Miller (22).

Bradford Assay. Lysates were analyzed for total protein concentration by the Bradford assay as described by Bradford (3). Bradford reagent was purchased from Bio-Rad Laboratories, and chicken egg albumin (Sigma-Aldrich) was used as a protein standard.

RESULTS

Effect of NaCl treatment on β -galactosidase production. Enzyme activity was normalized to the number of viable cells where cellular machinery required for enzyme production was active at the time of NaCl or sucrose treatment. It was assumed that enzyme activity is proportional to the amount of enzyme present. Figure 1 shows both wild-type and mutant cells showed significantly higher β -galactosidase activity under nonzero concentrations of NaCl. Wild-type cells treated with NaCl showed a 0.6 fold enzyme activity increase in 0.6 Osm NaCl and a 3.6 fold increase in 1.2 Osm from 0.0 Osm. Mutant cells treated with NaCl increased β -galactosidase production by 1.2 fold and 3.8 fold at 0.6 Osm and 1.2 Osm, respectively from 0.0 Osm. Figure 1 also shows

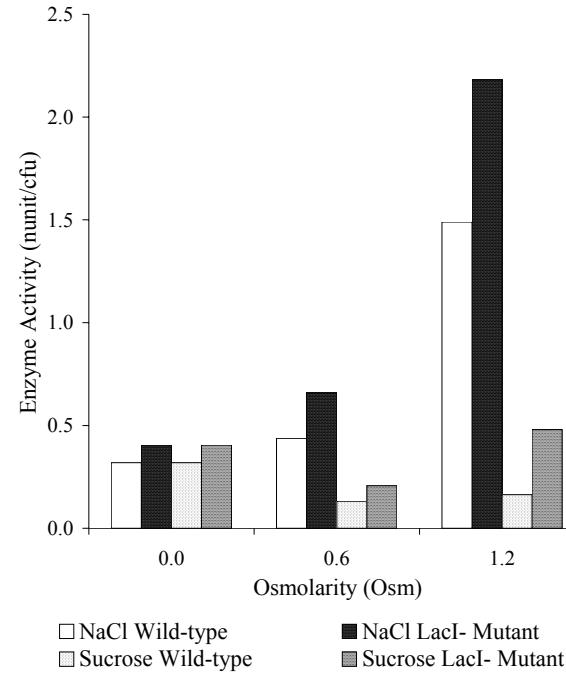


FIG. 1. Effect of the osmotic pressure on the β -galactosidase activity, expressed when NaCl and sucrose were used as osmoreagents in wildtype *E.coli* *BW25993* and isogenic *BW25993ΔlacI* mutant. Values were normalized to the viable cell counts. Two trials were performed per treatment and data shown was representative of one of two trials. Control data is represented at zero osmolarity.

that there was 2.5 fold higher β -galactosidase activity at 1.2 Osm NaCl than under 0.6 Osm NaCl osmotic conditions in both wild-type and mutant. When comparing β -galactosidase activity between wild-type and mutant strains, Figure 1 shows that the mutant strain produced more enzyme at each NaCl osmolarity treatment - by 1.3 fold difference in both 0.6 Osm and 1.2 Osm and 1.2 fold difference at 0.0 Osm. It is clearly evident at higher osmolarity that treatment with NaCl resulted in higher enzyme activity as compared to treatment with sucrose. Wild-type cells under at 0.6 Osm NaCl treatment is shown to produce 2.2 fold more enzyme than wild-type cells under sucrose at the same osmolarity while mutant type cells at 0.6 Osm NaCl and sucrose showed 2.3 fold more β -galactosidase in NaCl than in sucrose treatment. Wild-type cells under NaCl treatment produce 8.1 fold more enzyme than wild-type cells under sucrose treatment at the same 1.2 Osm while mutant cells treated with NaCl produced 3.5 fold more enzyme than those treated with sucrose.

Effect of sucrose treatment on β -galactosidase production. The trend of β -galactosidase activity under sucrose treatment was not as clear as demonstrated in NaCl treatment. Figure 1 shows wild-type cells treated with sucrose had 0.6 fold and 0.5 fold (at 0.6 Osm and 1.2 Osm, respectively) lower β -galactosidase activity from 0.0 Osm control. In general, cells treated with sucrose also tended to show increase in β -galactosidase activity between 0.6 Osm and 1.2 Osm; however it is seen more clearly in mutant than in wild-type. Enzyme activity from 0.6 Osm to 1.2 Osm sucrose treatment in wild-type only showed a 1.2 fold increase while the *LacI* mutant showed a 1.3 fold increase. When compared between wild-type and mutant, enzyme activity also showed an increase at each osmolarity sucrose treatment, which parallels the observation for NaCl treatment. Figure 1 shows mutant cells produced 1.4 fold and 1.7 fold more enzyme than wild-type cells at 0.6 Osm and 1.2 Osm respectively.

DISCUSSION

Without the lac repressor, *lac* operon would be constitutively expressed through the action of CRP (24), which correlates with our results where there was a higher background β -galactosidase levels in the *E.coli* *BW25993ΔlacI* in contrast to wild-type, assuming that measured enzyme activity is proportional to the amount that was produced. The β -galactosidase activity was consistent between wild-type and *lacI* mutant in all trials, where under different osmolarity concentrations (0.6 Osm and 1.2 Osm) and different solutes (ionic or non-ionic), wild-type *E.coli* *BW25993* consistently showed lower β -galactosidase activity than *lacI* mutant. However, levels of β -galactosidase activity were not consistent. For instance, 1.2 Osm sucrose osmotic stress

resulted in average about 3 fold higher β -galactosidase activity in *lacI* mutant than wild-type, while 0.6 Osm sucrose osmotic stress resulted in less than 0.5 fold on average. This is also evident in NaCl osmotic treated cells, where at 1.2 Osm NaCl osmotic stress, there was about 1.5 fold higher β -galactosidase activity in *lacI* mutant than wild-type, while 0.6 Osm NaCl resulted in about 0.7 fold higher activity. The results presented seem to suggest that under higher osmolarity, induced by sucrose or NaCl, there was higher β -galactosidase activity. This could be attributed to increased stability of lac repressor-DNA complex with increasing external osmolarity (10).

The overall increase in β -galactosidase due to osmotic stress caused by NaCl might be due to global regulatory protein, CRP which positively regulated the expression of β -galactosidase (24). It has been shown that the level of CRP is influenced by external osmolarity, specifically by NaCl. Furthermore, cAMP is a key component in the osmoregulation of *cpr* expression (1). Previous studies found that there was a significant cAMP level increase, as well as *cpr* expression, at 0.4 Osm NaCl in rich medium, indicating that osmolarity directly affected the production of cAMP (1). This correlates with the results in Figure 1, since NaCl induced osmotic stress resulted in the higher overall β -galactosidase activity under both 0.6 Osm and 1.2 Osm treatments in comparison to control, perhaps in response to CRP-cAMP regulatory complex. The signalling molecule, cAMP in *E. coli* is mostly known for its role in catabolite repression (24). However, in this study and also in Balsalobre *et al.*, (1) cAMP can also be considered as a component in regulatory network involved in osmoregulation. Sensory signal, cAMP is produced by adenylate cyclase from ATP and activated by phosphorylated IIA^{Glc} enzyme (20). Normally, in catabolite repression, glucose would reduce phosphorylated IIA^{Glc} enzyme levels, therefore decreasing adenylate cyclase activity and cAMP levels (20). The main issue lies in how osmolarity due to NaCl might activate adenylate cyclase to increase cAMP. There are reasons to believe that osmolarity does not affect the phosphorylation of IIA^{Glc} enzyme in the PTS system like glucose does since, it has been found that the level of phosphorylated IIA^{Glc} enzyme are not affected by the osmolarity in the presence of glucose. Also, osmoregulation of CRP was previously observed when either glucose or glycerol was used as a carbon source (1). This drives the speculation that perhaps osmolarity affects adenylate cyclase through influencing *cya* gene expression or interacting with the enzyme directly. For instance, *cya* gene transcription could be similar to *E.coli proU* gene expression, where under high osmolarity growth conditions, the region of DNA would enter a reversible underwound DNA conformation (8). Alternatively, adenylate cyclase may

act similar to the osmosensor KdpD protein of *E.coli*, which functions under hyperosmotic stress (21). Overall there was an increase in β -galactosidase activity due to osmotic stress induced by NaCl, however, higher β -galactosidase activity was observed in 1.2 Osm NaCl then at 0.6 Osm NaCl osmotic conditions. In particular, there was about 2.5 fold higher β -galactosidase activity, on average between wild-type and *lacI* mutant, produced at 1.2 Osm NaCl then at 0.6 Osm NaCl osmotic conditions. This was expected since different levels of osmolarity possibly result in different adaptive processes, which mainly aims at restoration of turgor inside the cell.

It has been demonstrated that at low osmolarity of about 0.32 Osm, most cytoplasmic K⁺ serves to balance change on macromolecular anion in a bounded state. At higher osmolarity (above 0.8 Osm) cytoplasmic K⁺ increases (in particular the level of the free K⁺ increases), mainly due to a decrease in putrescine levels, and glutamate takes on the role of major cytoplasmic anion (19). In permeabilized toluene-treated *E.coli* cells, free K⁺ has been shown to stimulate adenylate cyclase in an additive manner through an effect on the PTS, and is associated with an increase in V_{max} and Km for ATP (16). Since higher K⁺ levels are associated with higher osmolarity, adenylate cyclase activity in cells perhaps increased and subsequently higher β -galactosidase activity was measured under higher osmolarity of 1.2 Osm NaCl then 0.6 Osm NaCl.

Overall no increase in β -galactosidase activity under the osmotic stress induced by sucrose was observed in comparison to control levels. Furthermore, results indicate lower β -galactosidase activity at higher osmolarities, which was unexpected. A possible explanation comes from a previous study suggesting that degradative enzymes are released from exponentially growing *E. coli* by osmotic shock from sucrose at 0.5M concentration (25). Cyclic phosphodiesterases, which degrade cAMP, are among the number of degradative enzymes released. This would explain the decrease in β -galactosidase levels at 0.6 Osm and 1.2 Osm sucrose osmotic induction from the 0.0 Osm control. These cyclic phosphodiesterases would also contribute to the low levels of β -galactosidase activity only seen in sucrose treatments and partially account for the huge difference in β -galactosidase activity between NaCl and sucrose treatments at non-zero osmolarities. Since sucrose is a disaccharide like lactose the effect might also arise if sucrose has a direct effect on the enzyme activity or induction.

In Figure 1, higher beta galactosidase activity at both nonzero concentrations of NaCl than sucrose was observed and the difference was more pronounced in 1.2 Osm. The main response of bacteria due to hyperosmotic stress, regardless of the type of solutes

used to induce stress, is elevated uptake of inorganic ions, primarily through increasing K⁺ influx (30). Bacteria favour this over biosynthesis of compatible solutes, process that is highly energy and time costing (30). Depolarization of membrane rendering K⁺ uptake thermodynamically unfavourable is only observed in NaCl but not in sucrose treatment. Yet there is no difference in intracellular K⁺ concentration at 0.6 Osm or isotonic concentrations of NaCl and sucrose (30). This suggests that NaCl treated cells would necessitate more energy expenditure than sucrose treated cells for the uptake of K⁺ in bringing intracellular osmolarity up to the equilibrium level. At higher concentrations of NaCl, a net K⁺ efflux increases in magnitude, whereas no significant change in amount net K⁺ uptake is observed in most non-ionic solutes including sucrose thus the gap between energy expenditure to K⁺ uptake in NaCl-treated and sucrose-treated cells grows larger at higher isotonic concentration of NaCl and sucrose. It is known that intracellular accumulation of cAMP is associated with membrane depolarization (15). Therefore, the presence of membrane depolarization in NaCl but its absence in sucrose treatment suggests that difference in beta galactosidase activity between NaCl and sucrose treatments may result either by direct regulation of adenylate cyclase and cAMP level by depolarization or by varied level of inhibition of adenylate cylase by different ATP expenditure in the cells (7).

In this study we expected that both ionic and non-ionic osmotic stress would result in the hyperexpression of the *lac* operon, thus leading to an increase of β -galactosidase levels and that osmotic stress induced by an ionic solute would have a greater affect on β -galactosidase activity than a non-ionic solute. Our results indicated that the anticipated affects of NaCl (ionic) induced osmotic stress on the β -galactosidase activity correlated with our hypothesis. However, the affects on β -galactosidase activity in sucrose induced osmotic stress were found to be contrary to our hypothesis, where treatments did not result in the enhanced β -galactosidase activity. Overall, there was 3 folds higher β -galactosidase activity in NaCl osmotically induced cells in contrast to sucrose treatments of 0.6 Osm and 1.2 Osm. These results suggest that the nature of the stress has the potential to cause different effects on the enzyme activity.

FUTURE EXPERIMENTS

In this experiment, there was no definitive evidence that difference in enzyme activity was due to different amount of enzyme produced but not a direct change in enzyme activity. Thus, western blot could be accompanied with the experiment to compare actual amount of enzyme with observed enzyme activity. Also,

using enzyme activity normalized to total protein concentration along with the one normalized to cell viability data would prove to be useful. In this study, measured enzyme activity was normalized to cell viability and expressed in nUnits/cfu. Plate counts were used to estimate cell viability and this poses a limitation, as in determining the number of colony forming units (cfu) cell size is not a factor. However, total protein levels per cell vary greatly depending on the size of the cells studied (18). Although total protein analysis has a number of limitations, it would eliminate the inherent bias imposed by cell size in cell viability data.

A single cell sample was only taken two hours after induction of osmotic stress, after which the cells would have presumably been done adapting to the osmotic solutes (31). This raises the question of the pattern of increase or decrease of β -galactosidase during the two-hour adaptation period. To investigate this, a modified version of this experiment, by sampling cells at regular time intervals to measure the change in β -galactosidase production as a function of time at the different osmotic stress levels.

In this study we indirectly demonstrated that both ionic and non-ionic osmotic stress had an effect on β -galactosidase production, which we hypothesized was due to the action of cAMP. It would be useful to further investigate the link between *lac* operon expression and cAMP regulation by directly measuring intracellular cAMP levels inside *E.coli* using cAMP assay as described by Notley-McRobb *et al.* (26). We can also look at β -galactosidase production in the absence of cAMP-CRP complexe by using *crp*⁻ mutant cells to investigate possible effects of varied cAMP levels at different osmotic stresses.

It would also be helpful to know if sucrose was directly affecting the enzyme activity.

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