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Differential Effects of Sucrose or NaCl Osmotic Shock on β-galactosidase Activity in *Escherichia coli* BW25993 is Dependent on Normalization Method by Total Viable Count or Total Protein Content

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The purpose of this study was to determine if the osmoregulatory response in *Escherichia coli* BW25993 varied depending on the solute used to cause osmotic stress, as measured by β-galactosidase enzyme activity. β-galactosidase enzyme activity was measured in *E. coli* BW25993 after osmotic shock with NaCl or sucrose for 2 hours. β-galactosidase activity was normalized against number of colony forming units or to total protein content, to demonstrate comparable, standardized activities. The normalization of the results eliminated the issue of variable culture concentration and viability. By offering several normalization methods, this study has provided a more comprehensive assessment of the complexity of osmoregulation. With either normalization method, β-galactosidase activity showed no significant difference compared to the Luria-Bertani Lennox medium control after a 0.3 M NaCl stress condition, but β-galactosidase activity increased at 0.6 M NaCl. When normalized against total protein, β-galactosidase activity decreased with increasing sucrose concentration (0, 0.6 and 1.2 M stress conditions). In contrast, β-galactosidase activity normalized against colony forming units decreased at 0.6 M sucrose, but increased at 1.2 M sucrose, relative to the control. To investigate the cause of differing results between normalizations, a sucrose dose-response curve was performed and it was determined that both cell viability and culture turbidity decreased with increasing sucrose concentrations. The discrepancy between the two different β-galactosidase activity normalization methods at 1.2 M sucrose is therefore likely a mathematical result of low cell viability, but somewhat higher protein content, due to the presence of dead cells. In the sucrose dose-response, the turbidity decreased faster than the viable cell count, which may be explained by higher bacterial aggregations at higher sucrose concentrations. Therefore, the β-galactosidase activity normalized against total protein or viable cell counts differs when *E. coli* BW25993 is osmotically shocked with NaCl or sucrose. This suggests that osmoregulation is dependent on the source of the osmotic stress, and is not restricted to a single physiological response.

Bacteria have evolved several mechanisms, including changes in cellular composition and metabolism, to respond to the changing osmolarities of their environment (1). *Escherichia coli* have three structures that help them survive changing osmolarities: the inner cytoplasmic membrane, a peptidoglycan layer, and an outer membrane (2). These layers form a barrier between the cytosol and the environment (2), and maintain a concentration gradient of solutes existing inside and outside of the cell. This gradient is affected differently depending on the solute itself. For example, some molecules may simply diffuse across the barrier (e.g. those that are non-polar and low in molecular weight) whereas diffusion of others may be limited (2). For those solutes that cannot diffuse, but are needed by the cell, there are often transport proteins that facilitate transport across the membrane. For example, outer membrane porins allow the transport of some mono-, di- and tri-saccharides (3).

A cell is in a hyperosmotic state and experiences water efflux when solute concentration is higher in the environment (4). If the solute is diffusible across the cell membranes, it will diffuse into the cell until equilibrium is reached (2). If the solute is unable to diffuse, the concentration will equilibrate via osmosis (i.e. the diffusion of water across the membrane), where the water moves into the environment (4). Consequently the cell may experience plasmolysis, where the cytoplasmic membrane shrinks away from the bacterial cell wall (5, 6). Generally cells can recover from plasmolysis, but extended periods in this state may cause permanent damage or cell death (4, 5). This recovery is, in part, due to osmoregulation.

Osmoregulation, or the regulation of a cell's internal osmotic pressure in response to changing environmental osmolarities (1), can be observed by varying the concentration of solutes in the media of a cell suspension (4). Previous studies have used solutes, such as sodium chloride and sucrose (7) in an effort to study the effects of osmoregulation (7). One of the genes affected by osmoregulation (as part of the global response), is *lac*Z.

β-galactosidase is an enzyme encoded by the *lac*Z gene that participates in lactose catabolism in the

absence of glucose (1). β-galactosidase expression has been shown to respond to osmotic stress, making it a model gene for studying osmoregulation (8, 9). Its response has been linked to the levels of cyclic AMP receptor protein (CRP) levels (9). As the CRP-cAMP (cyclic AMP) complex is involved in positive regulation, *lac*Z expression is also subject to osmoregulation (1). One study found that both NaCl and sucrose osmoregulate CRP levels, with higher osmolarity resulting in lower CRP expression (9). It was reported that higher osmolarity, created by NaCl, decreased CRP levels but increased β-galactosidase activity, which was attributed to an increase in cAMP levels (9). When comparing the levels of β-galactosidase activity resulting from osmotic shock by NaCl and sucrose, they found that increasing osmolarities of either solute decreased *crp* transcription in *E. coli* (9). Cells subjected to osmotic stress show varying responses in CRP and therefore β-galactosidase production, dependent in part on the solute responsible for the stress (9). β-galactosidase was therefore used in this experiment to study the effects of osmoregulation induced with different solutes.

To ensure that the activity of β-galactosidase measured in this study was not misrepresented due to variable cell viability or protein concentration, it was normalized against both total protein and viable cell count. By normalizing the results, the values of enzyme activity can be compared amongst samples, regardless of differing protein concentrations or viability at each concentration of solute.

The purpose of this experiment was to test whether *E. coli* BW25993 responds differently to osmotic stress induced by equal osmolalities of NaCl or sucrose as measured by β-galactosidase activity. The results of Kimata *et al.* and Balsalaobre *et al.* suggest that β-galactosidase expression would be solute-independent (8, 9). However, Cheung *et al.* suggested that cells grown in the presence of sucrose showed lower levels of β-galactosidase activity compared to cells grown in the presence of NaCl (7). Furthermore, Shabala *et al.* suggested that *E. coli* may respond differently to ionic and non-ionic environmental stresses (10), supporting the use of NaCl and sucrose as opposing solutes in this study. Should the expression of β-galactosidase be shown to be solute-dependent, it would suggest that osmoregulation is not a single response mechanism, but varies depending on the source of stress. Should the expression of β-galactosidase be shown to be solute-independent, it would suggest that osmoregulation is a single response, produced regardless of the source of stress. This information would assess the complexity of the osmoregulatory response in *E. coli* BW25993. Our aim was to re-examine the effect of osmotic stress induced by sucrose or NaCl.

MATERIALS AND METHODS

Bacterial Culture. *E. coli* BW25993 (*lac*Z$^+$) was obtained from the culture collection of the Department of Microbiology and Immunology at the University of British Columbia.

Osmotic Shock Solution Preparation. Luria-Bertani Lennox (LB-Lennox) medium components (1% tryptone, 0.5% yeast extract, 0.5% NaCl) and additional NaCl or sucrose were dissolved in distilled water. Osmotic shock solutions for the NaCl and sucrose comparison (NSC) included 0 M sucrose (control), 0.6 M sucrose, 1.2 M sucrose, 0.3 M NaCl, and 0.6 M NaCl in LB-Lennox broth. Note that these are the final concentrations of NaCl, and account for the initial 0.5% NaCl already in the media. The sucrose range comparison (SRC) included 0 M (control), 0.2 M, 0.4 M, 0.6 M, 0.8 M, 1.0 M, 1.2 M, and 1.4 M sucrose LB-Lennox.

Culture Conditions. All liquid cultures were incubated at 37°C on a platform shaking at 175 rpm. Cultures were prepared by a 1/40 dilution of an overnight culture grown in LB-Lennox medium. Cultures were induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and incubated for 2 hours until an OD_{460} of at least 0.5 was reached. Then the culture was diluted to 0.5 OD_{460} prior to osmotic shock treatment.

Induction of Osmotic Shock. The pre-shock culture was aliquoted into 5 (NSC) or 9 (SRC) tubes then pelleted at 1000 x g for 10 minutes in the J2-21 Beckman centrifuge, (NSC) or 3300 x g for 1 minute in the Eppendorf microcentrifuge (SRC). To induce osmotic shock, pellets were resuspended in 30 ml (NSC) or 5 ml (SRC) of the appropriate shock solutions then incubated for 2 hours. After incubation, OD_{460} was read after blanking each sample with their respective shock solution.

Viability Assay. Samples of the 0.5 OD_{460} pre-shock culture and post-shock cultures were plated. Samples were diluted in phosphate-buffered saline (PBS) (0.8% NaCl, 0.03% KCl, 0.155% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.4) then plated in duplicate on LB-Lennox agar plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl and 1.5% agar) and incubated at 37°C for 18-24 hours.

β-galactosidase Assay. Cells were pelleted at 3300 x g for 2 minutes in an Eppendorf microcentrifuge, washed with Z buffer (60 mM Na_2HPO_4•$7H_2O$, 40 mM NaH_2PO_4•H_2O, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0), re-pelleted, then resuspended in Z buffer. The wash step was performed in order to prevent medium components from potentially interfering with β-galactosidase activity. Next, cells were permeabilized with toluene and β-galactosidase activity was measured using the β-galactosidase assay, described by Miller (11). Absorbance was measured at both 420 nm and 550 nm, then the enzyme activity was calculated as follows:

$$\text{Enzyme Activity}\left(\frac{\text{mUnits}}{\text{ml of sample}}\right) = \frac{\text{OD}_{420} - 1.75 * \text{OD}_{550}}{\text{reaction time (minutes)}} * \frac{10^6}{11{,}500} * \frac{\text{N}_\text{V}}{\text{E}_\text{V}}$$

where N_V = total assay volume, E_V = volume of sample used

Bradford Assay. For whole cell lysis and trichloroacetic acid (TCA) precipitation, 12 mL of each treatment sample was added to 12 mL of chilled 10% TCA for approximately 15 minutes then centrifuged at 9000 x g at 4°C for 10 minutes in the J2-21 Beckman Centrifuge with the JA-20 rotor. The protein pellet was washed with 10 ml 5% TCA, re-pelleted at the same speed, and resuspended in 1.4 ml 0.1 M NaOH. Samples were diluted as necessary (often by 1/200), and analysed as described by Bradford (12). The Bradford reagent was purchased from Bioshock and the protein standard used was bovine serum albumin (BSA) from Sigma.

Microscopy. 5 ul cell samples of each post-treatment culture were stained with crystal violet dye for thirty seconds, rinsed with water and then visualized at a 1000X magnification with a Zeiss Axiostar plus microscope and AxioCam ICm1 camera adapter. Cell sizes were determined using KLONK Image Measurement, where the cell lengths of five randomly chosen cells were averaged for each treatment and the differences were determined using a percent difference calculation.

RESULTS

β-galactosidase activity increases with increasing NaCl concentration. To assess the response of *E. coli* BW25993 to osmotic stress, cells were incubated in LB-Lennox with increasing concentrations of NaCl for 2 hours, then β-galactosidase activity was measured. After osmotic shock with 0.6 M NaCl, cells responded with a 2.5 to 3-fold increase in β-galactosidase activity relative to the control (LB-Lennox alone) (Fig. 1A and B). Cells exposed to 0.3 M NaCl did not differ significantly from the control in terms of β-galactosidase activity (Fig. 1A and B). The same trend was observed when β-galactosidase activity was normalized against colony-forming units (CFU) or total protein content (Fig. 1A and B). This data shows that *E. coli* BW25993 responded to an increased NaCl concentration with increased β-galactosidase activity.

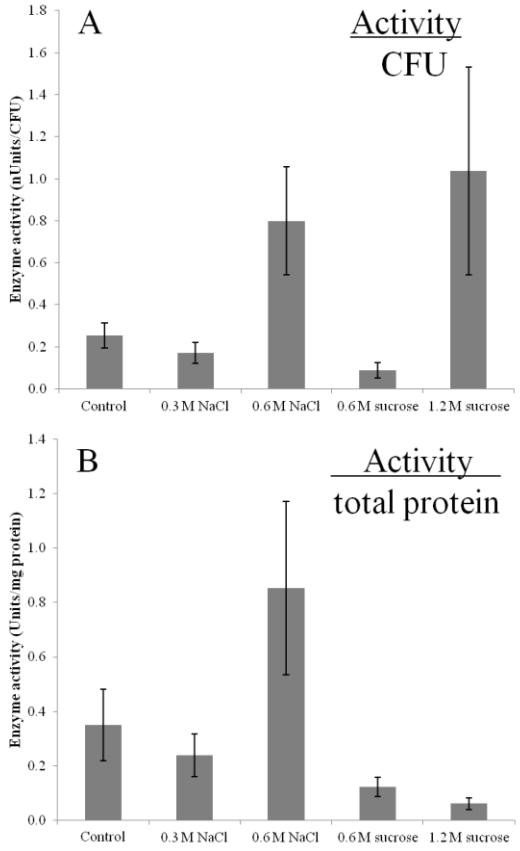

FIG 1 β-galactosidase response of *E. coli* BW25993 to osmotic stress by NaCl or sucrose, normalized against CFU (A) or total protein (B). Results shown are the average of three trials. The control was grown in LB-Lennox medium alone.

Apparent β-galactosidase activity is dependent on the normalization method used and depends on the range of sucrose concentrations tested. Next, osmotic stress induced by sucrose was assessed in the same manner as with NaCl. Following osmotic shock with 0.6 M sucrose, β-galactosidase activity decreased 3-fold relative to the control regardless of the normalization method used (Fig. 1A and B). In contrast, the β-galactosidase activity in cells treated with 1.2 M sucrose was dependent on the normalization used. When normalized against CFU, β-galactosidase activity of the 1.2 M sucrose-treated cells was comparable to that of 0.6 M NaCl treatment condition (Fig. 1A). However, when normalized against total protein, the β-galactosidase activity of the 1.2 M sucrose-treated cells showed a marked decrease (Fig. 1B).

It is worth noting that the β-galactosidase activity of cells treated with 1.2 M sucrose was low and relative to the other samples, only a faint yellow colour was observed after up to an hour. Furthermore, the CFU/ml for 1.2 M sucrose-treated cells was about 50 times lower than that of the other treatments while the total protein was only 2-fold lower. These results show that β-galactosidase activity changes with different sucrose concentrations and the method of normalization can significantly change the reported β-galactosidase activity for the same treatment conditions.

Cell viability decreases with increasing sucrose concentration. To investigate the effect of sucrose on viability, cells were subjected to osmotic stress with increasing concentrations of sucrose. The number of CFU/ml decreased slowly as the sucrose concentration increased from 0.2 to 0.8 M, and then more rapidly as the sucrose concentration increased beyond 0.8 M (Fig. 2A). As a test, one sucrose solution was prepared as described by Cheung *et al.* (7). An increase in volume due to the mass of sucrose added was noted. This gave a final concentration of 0.9 M, although a concentration of 1.2 M was intended. This solution is labelled as 0.9 M sucrose in Fig. 2. The viability of our 0.9 M sucrose treatment was 3.6-fold higher than that of the 1.2 M treatment prepared by standard practices (Fig. 2A). The viable cell count of 0.9 M to 1.4 M sucrose-treated cultures were lower than the culture viability prior to osmotic shock (Fig. 2A). Therefore, sucrose has a negative effect on cell viability at concentrations greater than 0.8 M.

Optical density is a poor predictor of cell viability in sucrose-containing media. Interestingly, as sucrose concentration increased, the culture turbidity began to decrease before the cell viability as determined by spectroscopy and viable cell counts, respectively (compare Fig. 2A and B). Culture turbidity began to decrease at 0.2 M sucrose, and plateaued around 1.2 M and 1.4 M sucrose (Fig. 2B). The discrepancy between turbidity and viability is more apparent when comparing 0.2 M to 0.6 M sucrose: the turbidity dropped 3.5-fold, whereas the viability dropped only 1.2-fold (Fig. 2A and B). The turbidity of 0.8 M to 1.4 M sucrose-treated cultures was less than the culture turbidity prior to osmotic stress (Fig. 2B).

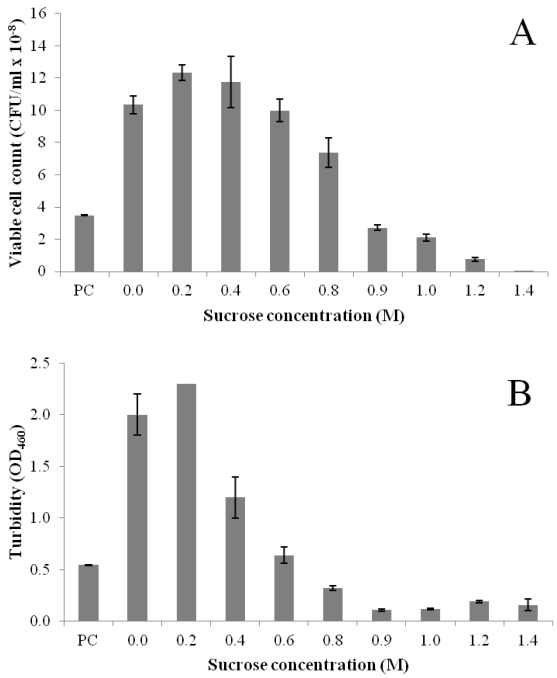

FIG 2 Effect of increasing sucrose concentrations on CFU/ml (A) and turbidity (B) in *E. coli* BW25993. OD_{460} was read at the same time as dilutions were made for plating. Results shown are the average of two trials. The preculture (PC) represents CFU/ml (A) or turbidity (B) of the culture immediately prior to osmotic stress with sucrose.

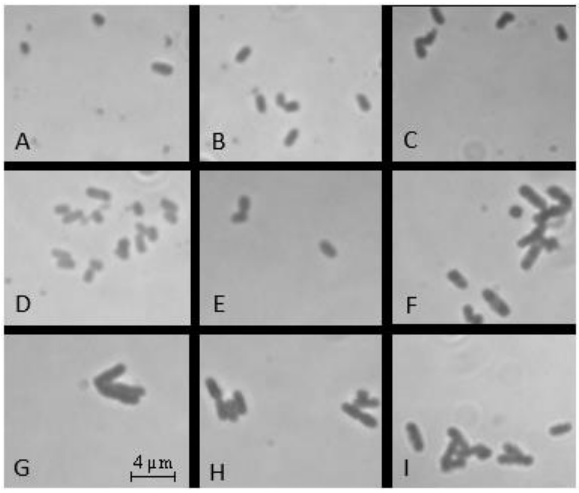

FIG 3 Crystal violet stain of *E. coli* BW25993 stressed with increasing sucrose concentrations. A = 0 M, B = 0.2 M, C = 0.4 M, D = 0.6 M, E = 0.8 M, F = 1.0 M, G = 1.2 M, H = 1.4 M, I = 0.9 M.

The media also became increasingly clearer with increasing sucrose concentration, supporting the measured decrease in turbidity. No visible clumping was observed with the unaided eye. Collectively, these data highlight a discrepancy between culture turbidity and viable cell count in sucrose-treated cultures

Cells form aggregates with increasing sucrose concentrations. In Fig. 3, observation using a light microscope showed an increase in cell aggregation at concentrations of sucrose greater than 0.6 M. Cells increased 40% in length from the 0 M sucrose to the 1.4 M sucrose condition. Cells were seen to be dividing more frequently in the lower sucrose concentrations, demonstrated by the small cell sizes, as well as what appeared to be cells undergoing division. Some evidence of lysis was seen in the higher sucrose concentrations (data not shown). No plasmolysis (e.g. invagination or shrinkage, particularly near cell poles) was evident, but this could not be ruled out due to insufficient magnification. Collectively these data indicate a change in cell morphology and aggregation depending on the sucrose concentration.

DISCUSSION

Bacteria often experience harsh conditions as a result of environmental exposure, and therefore must possess mechanisms to cope with various stresses. cAMP, required for the induction of the *lac* operon, is involved in the response to osmotic stress in *E. coli* (9). Therefore, this study used β-galactosidase activity to characterize the response to osmotic stress induced by two different solutes, NaCl and sucrose.

In order to study the osmoregulation of β-galactosidase using different solutes, a model system first had to be established. This was done by building on the methods used in a previous study (7). After revisiting and analyzing the methods used in a past study (7), several modifications were introduced. Using this modified protocol, two different solutes and two different normalization methods were tested.

While reviewing the previous study (7), it was noted that the osmotic shock solutions were prepared in an unconventional way which may have resulted in inaccurate concentrations of sucrose. The solutions were prepared by adding sucrose to the final desired volume of LB-Lennox. The late addition of sucrose increased the volume, thereby decreasing the actual concentration of sucrose. Therefore, the first modification with respect to Cheung *et al.* (7) was to prepare the osmotic shock solutions by standard practices. Secondly, a wash step was added prior to both the Bradford and β-galactosidase assays to decrease the chance of the shock solution components interfering with either assay.

The results of this study were consistent with Cheung *et al.* (7). Their results also showed an increase in β-galactosidase activity at 0.6 M NaCl. However, in contrast to our results, β-galactosidase activity at 1.2 M sucrose normalized against CFU was decreased relative to the control. This could be explained by the different methods of preparing the shock solution. Since the sucrose concentration as prepared by Cheung *et al.* (7) was effectively 0.9 M, not 1.2 M, the viable cell count for this treatment could have been about 3 times higher than in this study. This would translate to a lower reported value for β-galactosidase activity per CFU.

When comparing the β-galactosidase activity normalized against total protein between shock conditions (NaCl or sucrose), a difference in β-galactosidase activity is observed. This finding is supported by Shabala *et al*., who observed that 40% of the genes in *E. coli* that are upregulated during osmotic stress share no similarity in their responses to NaCl and sucrose treatment (10). This suggests that *E. coli* may respond differently to ionic versus non-ionic environmental stresses (10).

Two methods of normalization (CFU and total protein) were used to account for the possibility that cell death might skew the results. The differences in β-galactosidase activity of sucrose-treated cells, depending on the normalization, can be attributed to a decrease in viable cell count of *E. coli* at the higher sucrose concentrations (1.2 M). Studies have shown that the doubling rate of *E. coli* suffers a 3-fold increase at 1.0 M sucrose (13). This would dramatically reduce the number of cells present. Due to the low numbers of CFU, the ratio of β-galactosidase activity to CFU becomes high.

When considering NaCl and comparing the two different normalization methods, the β-galactosidase activity trend of post-shock cells was similar in both cases. According to a previous study, NaCl concentrations higher than 7% are lethal (14). The concentration of 0.6 M translates to 3.5%, which is only half of that threshold. The survival of the cells allows for both normalization methods to be meaningful, as the activity of β-galactosidase is not artificially inflated due to low cell numbers.

The β-galactosidase activity value for 1.2 M sucrose normalized against CFU was thought to be due to cell death caused by sucrose. This was explored by determining cell viability of *E. coli* in sucrose. A decreasing trend in both viability and turbidity was observed as concentrations of sucrose in media increased. The decrease in turbidity compared to cell viability can be addressed by at least two possible explanations. First, sucrose can interfere with turbidity readings. A study by Shlafer and Shepard noted that sucrose interfered with absorbance readings even when included in the blank (15). If the turbidity readings were affected by sucrose in some way, the graph may not accurately reflect what is actually occurring with the bacteria. Second, Daneo-Moore *et al*. studied the differences in growth rate when expressed using turbidity versus the accumulation of DNA, RNA, and protein (16). They found that sucrose-containing cultures exhibited a linear growth curve when measured by turbidity, instead of the expected exponential curve (16); however, when the growth was monitored by the accumulation of DNA, RNA and protein, the growth rate was exponential (16). This difference was attributed to the formation of cellular aggregates in sucrose-containing medium (16). Even though their study was done on *Streptococcus mutans*, it shows that growth of cells in sucrose could be underestimated when measured by turbidity.

Interestingly, cellular aggregation in sucrose-containing media was also observed in our study. This aggregation may explain the more rapid decrease of turbidity as compared to viable cell count in the sucrose-dose response assay. The aggregation seen by the cells may be a survival mechanism, and has been shown to increase survival in certain situations (17). By aggregating, the surface area of the cell that is exposed to the osmotic stress would be decreased, which may contribute to survival.

To determine whether or not plasmolysis occurred following exposure to sucrose, samples were visualized microscopically. It was observed that the cells appeared structurally similar, although there was an increase in length that was seen at increasing concentrations of sucrose, as well as an increase in aggregation. Other studies reported plasmolysis of cells treated with sucrose, but no change in length relative to the control (18). The increase in length observed in our results may, however, be an artifact of the fixing or the staining of the cells. This is because a crystal violet stain was performed, whereas Alemohammad and Knowles (18) used electron microscopy. It should also be noted that this result may reflect the stage in the cellular division cycle, when more small, recently divided cells are present.

In conclusion, β-galactosidase activity measured after osmotic stress is dependent both on the solute and on the normalization method used. NaCl caused an increase in β-galactosidase activity, regardless of the normalization method. Sucrose caused a slight decrease in β-galactosidase activity when normalized against total protein; however, an increase in sucrose caused an increase in β-galactosidase activity when normalized against viable cell count. The increase in β-galactosidase per CFU at high sucrose concentrations was thought to be a mathematical result of cell death. Cell death at high sucrose concentrations was confirmed by comparing cell viability after shock at increasing sucrose concentrations. Cell death begins to occur after 0.8 M sucrose or around 0.9 M sucrose using turbidity or viable cell count, respectively. These results provide further support for the conclusion that the differences in β-galactosidase activity are a product of both the solute and the normalization method used.

FUTURE DIRECTIONS

To explore the cause of the discrepancy between turbidity and viability in the sucrose dose-response experiment, it would be useful to measure the culture turbidity immediately after the cells were resuspended in their shock solutions, and at several time points during the 2 hour

shock. This would help to describe the kinetics of the observed turbidity drop. If the turbidity drop is due to sucrose directly interfering with turbidity readings, one might expect the turbidity to drop instantaneously. If the turbidity drop is due to a change in cellular morphology, death, or aggregation, one might observe a more gradual change in turbidity. The findings would help to elucidate the cause of the turbidity-viability discrepancy observed in this study. If sucrose interferes with turbidity readings, it would produce unreliable results.

It would also be informative to perform a growth curve of a 1.2 M sucrose culture, sampling CFU/ml at several time points to determine whether the low viability was a result of an initial adjustment to high osmolarity or if the cells were gradually dying over time. The significance of this study would be to better understand behaviour of cells in sucrose media, and to take into consideration the different growth curves of cells in different sucrose concentrations.

In this study, cells were diluted in PBS prior to plating. It is important to know whether the sudden change in osmolarity from shock solution to PBS could affect cell viability. To test this, dilution from a shock solution into an isotonic or a hypotonic saline solution would be compared, with plate counts as a readout.

Once the model system for measuring β-galactosidase activity in response to osmotic stress has been well-established, future studies can move forward to test solutes other than NaCl and sucrose. This would provide a broader perspective on the response to osmotic stress. Does β-galactosidase activity depend on the charge of the solute, and does it vary with other solute properties?

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