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Effect of Sequential Treatment with High Temperature and Hyperosmolarity or Low pH on RpoS Expression and Cell Viability in *Escherichia coli* B23

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RpoS, a sigma subunit of RNA polymerase in *Escherichia coli*, is seen as the master regulator of the general stress response which is triggered by stress signals such as high temperature, hyperosmolarity, and low acidic pH, allowing the bacteria to up-regulate the expression of specific proteins that confer resistance against the particular stress environments. The objective of this study was to investigate if cross-protection exists when *Escherichia coli* B23 was first subjected to high temperature shock followed by hyperosmolarity shock or low acidic pH shock. Various techniques, including OD measurement of cultures at 660nm, cell viability assay, Bradford assay, and Western immunoblotting with monoclonal RpoS-specific antibody were used to assess protection and the expression levels of RpoS in sequential exposure to different stress signals. Cell viability assays showed that when cells were grown at 37 °C throughout the experiment, cell viability did not vary much whether shocked with high salts or not and the data results suggested no significant cross-protection was observed in dual-treated cells with high temperature (at 42 °C) followed by sequential hyperosmolarity. The assay also illustrated that when cells were exposed to low pH media without initial incubation at higher temperature (42 °C) condition, cell viability decreased dramatically, which suggested a possible cross-protection effect in cells treated with high temperature followed by sequential low acidic pH. These results suggested that heat shock at 42 °C can confer protectivity against low pH but not high osmolarity.

Gram-negative enteric bacteria, such as *Escherichia coli*, are characterized by their ability to quickly adjust to different physiological conditions in order to survive under various stress environments. Indeed, various strains of *E. coli* have developed mechanisms to survive in many different stress conditions through RpoS and RpoS-dependent genes which have been shown to confer resistance against oxidative stress, near-UV irradiation, starvation, heat shock, hyper-osmolarity, acidic pH, and ethanol (4, 9).

RpoS, or σ^S, is a 38 kDa sigma subunit of RNA polymerase in *E. coli* that has been reported to control a regulon consisting of up to 70 or more genes expressed in response to starvation, many stress signals, and during transition to stationary phase (3). In the general stress response of *E. coli* triggered by many different stress signals, RpoS acts as the master stress regulator that activates several downstream effector proteins to render the cell broadly resistant to damage. Often, this conferred resistance is accompanied by a reduction or cessation of cell growth which then provides the cells with the ability to survive the current stress as well as the stresses not yet encountered (cross-protection) (2, 3, 11). It has been found that RpoS-activated gene products result in modification of cell envelope composition and overall morphology to contribute to resistance mechanisms. Another area of effect is metabolism, consistent with RpoS being important under conditions where the switch from metabolic mechanisms that promote maximal growth to growth maintenance is essential. σ^S also controls genes that mediate programmed cell death or apoptosis in stationary phase, which may support the survival of a bacterial population as a whole under certain growth conditions by sacrificing a fraction of the population. Elimination of some bacterial cells by RpoS activated genes provides nutrients for the remaining surviving cells, benefiting the population as a whole (4).

The active RpoS activity directs RNA polymerases to alternative promoters in order to activate expression of relevant gene products. This suggests that triggering RpoS expression by only a single stress condition may lead to elevated expression of many gene products that may elicit increased protection against many other stresses, assuming that the same subsets of gene products are involved (4, 5). Indeed, it has been

suggested that the elevated levels of RpoS due to high temperature stress would result in elevated general stress response proteins, making the bacteria more tolerant and able to adapt more quickly to other stresses such as hyperosmolarity or acidic pH (8, 10). Previous studies have also shown that even before the characterization of RpoS, it was noted that *E. coli* at stationary-phase showed increased resistance to exposure to high osmolarity or high temperature growth conditions (6).

In this paper, we investigated whether the sequential heat stress (growth at 42°C) and high osmolarity (0.8 M NaCl) or heat stress and low pH (pH 2) conditions on *E. coli* B23 would induce high RpoS level in *E. coli* B23 to confer protectivity. We hypothesized that high temperature stress at 42°C would cause elevated levels of RpoS in *E. coli B23* which, in turn, would provide cross-protection against high osmolarity (0.8M NaCl) and acidic pH (pH2) growth conditions.

MATERIALS AND METHODS

Bacterial culture. *E. coli* K-12 strain B23 was provided from the MICB421 culture collection (Department of Microbiology & Immunology, University of British Columbia).

Growth conditions and induction of stress for assessing the time course of RpoS development. Cells from the *E. coli* K-12 B23 culture were grown overnight, at 37°C, in LB media (0.5% yeast extract, 1% tryptone, 0.5% NaCl, 0.2% glucose). A sample of this overnight culture was taken, as the stationary phase sample, for further analysis. The overnight culture was diluted at a ratio of 1/5 to give an OD_{660} reading of 0.3. This culture was allowed to grow up to an OD_{660} of 0.5. At this point, t = 0 min, samples were taken for further analysis. The remaining solution was divided as 35 mL parts into 6 different flasks and incubated for 5, 10, 20, 30, 45 and 60 minutes in a 42°C waterbath with gentle shaking. Samples were taken for further analysis, at the end of all incubations.

Growth conditions and induction of stress for testing the effect of stress on other stressors. Cells from the *E. coli* K-12 B23 culture were grown overnight, at 37°C, in LB media. A sample of the overnight culture was taken, as the stationary phase sample, for further analysis. The overnight culture was diluted at a ratio of 1/5 to give an OD_{660} reading of 0.3. This culture was allowed to grow up to an OD_{660} of 0.5. At this point, the culture was divided into two flasks, and the each flask was incubated at either 37°C or 42°C for 45 minutes. Three 35 mL samples from 37°C incubation and three 35 mL samples from 42°C incubation were taken and centrifuged in a JA-20 rotor at 700 x g, for 5 minutes at 17°C. Pelleted bacterial cells were re-suspended in 70 mL of low pH LB media (pH=2), control LB media or high salt LB media (0.8M NaCl). The six samples were incubated at 37°C, in their respective media, for 30 minutes. Samples for further analysis were taken at 0, 15 and 30 minutes past the incubation.

Viability Readings. At each time point listed above, 1mL culture samples were taken for viability assay. Samples were first diluted to 10^{-5}, 10^{-6}, and 10^{-7} and plated on LB agar plates as a single replicate. The plates were incubated overnight at 37°C, and after 24 hours, the number of isolated colonies was counted in order to compute a viability trend for each type of growth condition.

Cell lysis. 25 mL samples were taken at indicated time points of the experiments. 25 mL samples were also taken from the overnight cultures (representing the stationary phase samples). These samples were centrifuged in a JA-20 rotor in a J2-21 centrifuge, at a speed of 7500 x g at 17°C for 5 minutes. The supernatant resulting from the centrifugation was removed and the pellet was washed in 10 mL 25 mM Tris buffer. The washed pellet was re-suspended in 1 mL of 25 mM Tris buffer. The re-suspended pellet was sonicated with Microson Ultrasonic Cell Disruptor at a setting of 8 with a small probe. The samples were sonicated for 10 seconds, followed by 30 seconds of cooling. This cycle of sonication was repeated 3 times on each sample. The samples were centrifuged in a 5414 centrifuge at 14000 x g for 1 min at 4°C. They were stored at -20°C for further use.

Bradford Assay. The lysed cell samples were tested for total protein concentration using methods described by Bradford (1). The Bradford reagent was made by mixing 20.6 mL of Bio-Rad Bradford Reagent with 50.4 mL of distilled water. The Sigma-Aldrich chicken egg albumin powder was used to a make a 250 µg/mL sample of the protein in distilled water. The protein solution was then used for Bradford standard measurements.

SDS-PAGE. The cell lysates were normalized to have 200 µg of protein within a 100 µL sample. The normalization was based on the total protein concentration values predicted by the Bradford assay, and the dilutions were made in 25 mM Tris buffer. The 100 µL diluted samples were mixed with 100 µL of 2X Sample buffer (50mM Tris pH 6.8, 100mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) to give a total concentration of protein at about 1ug/uL. The samples were run in running buffer (25mM Tris base at a pH of 6.8, 192mM glycine, 1% SDS) on two 12% SDS-polyacrylamide gel. The samples were run at 180 V in a Bio-Rad PAGE apparatus.

Comassie Blue staining. One of the SDS page gels was stained with Comassie blue stain to test for equal protein loading in all of the lanes. The gel was first washed with a fixing solution (50% ethanol, 40% dH2O and 10% acetic acid) for 30 minutes, at room temperature. The gel was then stained with the staining solution (comassie blue, 40% methanol, 10% acetic acid and 50% dH2O) for 30 minutes at room temperature. The gel was destained with the fixing solution for 30 minutes at room temperature. The gel was finally destained with 10% acetic acid, overnight.

Western blotting and immuno-detection. The second gel from the SDS-PAGE was used for a western transfer. A Hybond-P nitrocellulose membrane was cut to size and pre-soaked in methanol for 10 seconds. The membrane was then soaked in transfer buffer (25mM Tris base pH 6.8, 192 mM glycine, 20% methanol) for 10 minutes. The membrane and the gel were set up for western transfer and the transfer was run at 100 V for 1 hour, with ice to cool the environment. The membrane was then washed with TBST buffer (50mM Tris, 150 mM NaCl, 0.2% KCl, 0.5% Tween 20, pH 7.5) 3 times. The washed membrane was blocked with blocking reagent (6% Casein, 1% polyvinylpyrolidone, 10 mM 0.5M EDTA in 1X PBS) for 15 minutes. Following another 3 washes with TBST, the membrane was incubated overnight with 1/1000 solution, in blocking reagent, of primary goat anti-RpoS antibody (Neoclone Biotechnology, cat #W0009). After 3 additional 5 minute washes with TBST, the membrane was incubated with a 1/3000 dilution, in blocking reagent, of alkaline-phosphatase linked goat anti mouse IgG (Chemicon International, cat. # AP124A) for 1 hour. The membrane was given another three 5 minute TBST washes, and another two 5 minute washes with substrate buffer. Finally, the membrane was treated with 10 mL of BCIP/NCT substrate (Sigman-Aldrich cat. #B3804), and a picture was taken upon color formation. The membrane was then washed with distilled water and stored at -20°C away from light.

RESULTS

RpoS (σ^{38}) protein level increased when *E. coli* was heat shocked at 42°C for 60 minutes. The samples of cell lysates with equal protein concentrations were run on a SDS-PAGE gel to study the expression level of RpoS. It is previously known that RpoS expression level starts to increase as early as after 10 minutes of growth at 42°C media (7). High

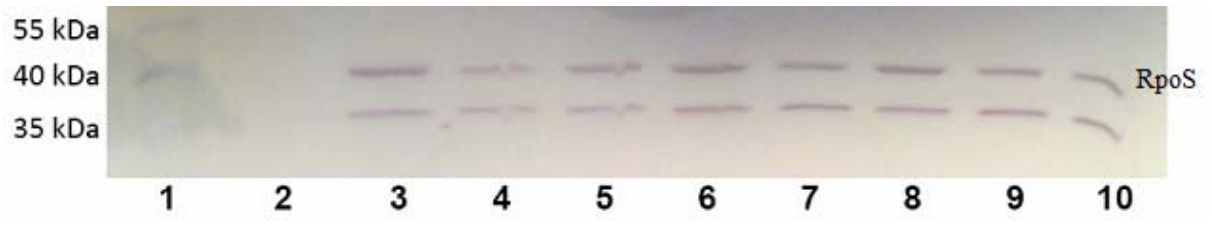

FIG. 1. Preliminary experiment visualization of RpoS levels by western immunoblotting of B23 *E. coli* cell lysates treated at 42 ºC over 60 minutes resolved with 12% SDS-PAGE. 1) PageRule™ Prestained Protein Ladder, 2) blank, 3) stationary phase control, 4) 0 mins, 5) 5 mins, 6) 10 mins, 7) 20 mins, 8) 30 mins, 9) 45 mins, 10) 60 mins.

temperature is known to increase the half-life of RpoS by slowing down the proteolysis of the protein (4), hence resulting in increased presence of RpoS in the cells. The Coomassie dye G-250 stained SDS-PAGE gel picture showed uniform intensities of bands in the gel, suggesting that there was fairly equal loading of cell lysates in all samples. Figure 1 shows the expression of RpoS at 38 kDa. The band intensities suggest the induction of RpoS expression at 42°C. While RpoS was expressed to a lesser degree at the start of incubation at 42°C growth media (Lane 4), the amount of RpoS expressed in bacterial cells increased as the time of incubation at 42°C increased to 30 minutes (Lane 8). From 30 minutes incubation, the amount of RpoS in bacteria seems to stay fairly constant. Since the expressed amount of RpoS is sufficiently high at 45 minute (Lane 9), we decided to use this time point as the end time point of 'heat incubation' step for subsequent experiments.

Effect of heat and NaCl exposure, individually and sequentially, on *E. coli* B23 viability. Figure 2 suggests that cells grown at high concentration of NaCl tend to have lower viability when their viability was tested right after transferring to the high salt media. When grown at 37°C throughout the experiment, cell viability did not vary much whether shocked with high salt or not. Looking at the viability for cells grown at 42°C prior to exposure to high salt media, their initial viability is lower than those initially grown at 37°C but at 30 minutes, the viability seems to be recovered, although can't be concluded due to size of the standard deviation.

Effect of heat and low pH exposure, individually and sequentially, on *E. coli* B23 viability. Figure 2 shows that when cells were exposed to pH2 media incubated at 37°C, cell viability decreased over time. At 30 minutes, *E. coli* grown at pH 2 after initial growth at 37°C has a significantly lower viability compared to the control at 37°C at 30 minutes as illustrated in Fig. 2. Cells grown at 42°C and then in pH 2 media at the 30 minutes interval had higher cell viability at than pH2 at 37°C at 30 minutes. It is observed that cell viability of pH2 at 42°C grown cells decreased initially at 15 minutes and recovered back at 30 minutes. OD measurements at 660 nm were taken at the time 0, 15 and 30 minutes (data not shown), and throughout all samples, OD measurements indicating cell density showed increase over time in all samples at about the same rate.

RpoS (σ^{38}) protein level in *E. coli* B23 lysate when grown subsequently after heat shock at 42°C for 45 minutes and high osmolarity (0.8M NaCl) or low pH (pH 2). Through the Coomassie blue stained gel, equal loading of all lanes was confirmed, thus Western immunoblot visualization of RpoS levels can be compared between samples directly, without the use of an internal control. The lysate collected from stationary phase cells (Lane 2) and control sample (Lane 3) collected after 30 minutes of growth at 37 °C showed

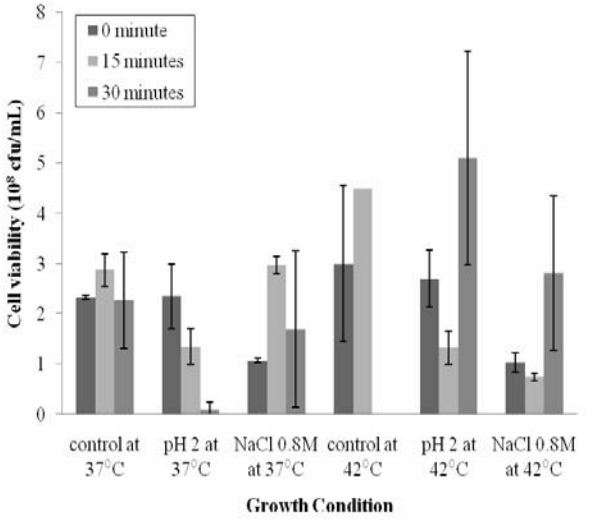

FIG. 2. Effect of initial heat shock on cell viability following stress by low pH or hyperosmolarity Cell viability was tested by plating the cells at three different dilutions after indicated time periods of growth at indicated conditions and counting the colonies after 24 hours. Cell viability is expressed in colony forming units (cfu) per mL of inoculum. Uncertainty is demonstrated by standard deviation of cell counts obtained by two different dilutions.

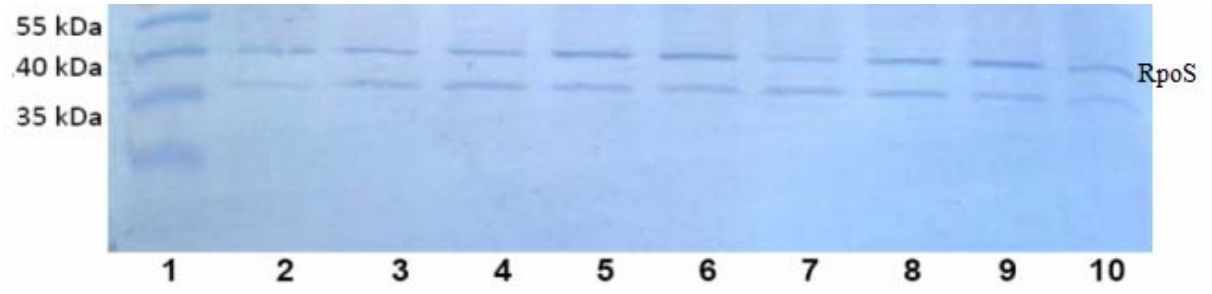

FIG. 3. Visualization of RpoS levels by Western immunoblotting of B23 *E. coli* cell lysates treated to high osmolarity or low pH after initial heat treatment at 42 °C or control at 37 °C resolved with 12% SDS-PAGE. 1) PageRule™ Prestained Protein ladder, 2) Stationary phase control, 3) 37 °C control^{+}, 4) 42 °C control^{+}, 5) 37 °C 0.8M NaCl, 6) 42 °C 0.8M NaCl, 7) 37 °C initial*, 8) 42 °C, initial*, 9) 37 °C pH 2, 10) 42 °C pH 2.

similar RpoS levels. Stationary phase cells did not have dramatically elevated expression of RpoS (Lane 2) compared to other samples. This is unusual, because stationary phase growth usually happens in starvation conditions (4). Under starvation induced stress a greater amount of RpoS production must be seen (4). Increased RpoS level in *E. coli* B23 after 45 minutes of 42°C heat shock was observed in Fig. 3 (Lane 8) compared to no heat shock (Lane 7). This shows that prior to the second subsequent stress of high osmolarity or low pH, cells that were heat shocked at 42 °C had higher RpoS level compared to the no heat shock control sample. Compared to samples that received no sequential shock (Lanes 3 and 4), RpoS level is visually much higher in samples treated to high osmolarity (Lanes 5 and 6) and also higher in low pH samples (Lanes 9 and 10), but to a lesser extent compared to the high osmolarity samples.

DISCUSSION

Previously, Hung *et al* (5) attempted to observe whether heat shock conferred production to high osmolarity in *E. coli* B23 strain, based on their previous knowledge that RpoS (σ^{38}) is expressed at an elevated quantity during high-stress situations such as high temperature and that presence of this transcriptional activator will turn on expression of various gene products that play essential roles for homeostasis. Their results showed that cross-protection was not observed when the cells were first grown at 45°C media and then transferred to high salt environment of 0.8M NaCl. However, their result was inconclusive since the Western blotting result showed no significant difference in expression of RpoS between *E. coli* grown at 37°C and 45°C. This indicated that their method of incubation at 45°C may not have been suitable to increase the expression of RpoS in the cells. Moreover, it was evident that their data needed more replicates to improve validity. Hence, our aim was to conduct a similar experiment to see if high temperature growth of *E. coli* could provide cross-protection when the cells were sequentially subjected to a high osmolarity environment. In addition, we attempted to study the presence of cross-protection when cells were exposed to a low pH environment because some strains of *E. coli* such as the food-borne pathogen *E. coli* O157:H7 are known to survive the low-pH environment of the stomach after food products undergo canning (2).

In the principle experiment, a cell viability assay was conducted after *E. coli* B23 was transferred to and grew in the second stress media for time periods of 0, 15 and 30 minutes in an attempt to generate death curve for comparison. Except for the 37°C treated pH 2 sample, cell viability fluctuated, especially in the high osmolarity samples, making generation of a death curve not possible (Fig. 2). Due to the inconsistent colony counts from different dilutions, large error bars were generated making our cell viability data inconclusive for the osmolarity experiments at 30 minutes. This was also the case with control samples where no significant differences in cell viability between different time points and between non-heat shocked and heat shocked cells (Fig. 2). One of the main problems was the fact that only one plate was plated per dilution, making determination of the actual cell viability difficult and unreliable. Nonetheless, some analyses could be done with the viability data. At 15 minutes, non-heat shocked cells had an increased number of cell colonies compared to 0 minutes, whereas heat shocked cells had roughly the same number of colonies at 0 and 15 minutes. This seems to show a trend that is consistent with Hung *et al*'s results (5) where heat shock and subsequent high osmolarity treated cells had a lower viability compared to no heat shock and just osmolarity treated cells. On the other hand, both heat shocked and non-heat shocked cells had an initial decrease of roughly 2 fold in cell viability at 15 minutes when grown in LB at pH 2 (Fig. 2). However, at 30 minutes,

cell viability of non heat shocked cells drastically decreased while cell viability of previously heat treated cells increased to a level that is equal or above the cell viability of cells at 0 minute. This trend suggested that the heat shocked cells could better adapt and resume their growth in low pH condition, whereas the non-heat shocked cells did not have this capability. Confirmation of this possibility could be achieved by evaluating cell viability in later time points to see whether non-heat shocked cells can recover at all. Repeating the experiment with heat shock and subsequent low pH stress would be essential as well.

Protein lysate was collected from all samples and RpoS level was detected by western immunoblotting using a RpoS specific antibody. Lane 7 and 8 (Fig. 3) confirms that before the cells were subjected to the subsequent stresses: control, low pH or high osmolarity, heat shocked cells treated to 42 ^{o}C for 45 minutes has sufficiently elevated RpoS expression. This increased RpoS level in the heat shocked cells was not observed by Hung *et al* (5). In their experiment, cells were grown at 37 ^{o}C for additional 15 minutes after heat shock which may have resulted in the RpoS proteolysis system to return to normal. Similar results were demonstrated in our heat shocked control sample (Fig. 3 Lane 4), which was left in normal LB media for 30 minutes at 37^{o}C following 45 minutes of 42^{o}C heat shock. In fact, the level of RpoS here is very similar to the not heat shocked sample (Lane 3). This suggests that prolonged incubation at 37^{o}C may have contributed to Hung *et al*' result. Furthermore, by increasing our heat shock treatment duration to 45 minutes, the heat shocked cells had a higher RpoS level (Fig. 1). In comparison, the high osmolarity stressed cells (Fig. 3 Lanes 5 and 6) had slightly higher RpoS levels compared to our controls (lane 3 and 4). This increase is in agreement with Hung *et al*'s result. The same trend was observed in the low pH treated cells (Lanes 9 and 10) which is in agreement with literature that low pH stress induces increased RpoS levels (11). The stationary phase sample (Fig. 3 Lane 2) have a similar RpoS level compared to control (lane 3 and 4) which is in contrast to our results from the preliminary experiment (Fig. 1). This inconsistency may be due to the fact that the same stationary phase protein lysate were used for both Western experiments, hence it was not processed at the same time as the rest of the sample and that the lysate underwent more freeze-thaw cycles which may have resulted in the degradation of RpoS. Furthermore, when control cells were being collected for protein analysis, the cells had been growing at 37 ^{o}C for 75 minutes from OD_{660} of 0.5, thus cells may have begun to enter stationary phase and have an increased RpoS level. An extra band is consistently seen in Western blots (Fig. 1 and 3) with a weight of roughly 35 kDa. This is consistent with the immunoblotting results from Hung *et al*, who used the same RpoS specific antibody from Neoclone. This may be a degraded form of RpoS or other similar forms of RNA polymerase sigma subunits. Aside from the consistently seen extra band, fainter bands were also visible above the RpoS band in Fig. 3. These faint bands may be a result of non specific binding, caused by insufficient blocking period (15 minutes) which was shorter than 1 hour as practiced in conventional protocols.

Cell viability comparison of the samples placed under cross protective treatment seemed to show some interesting patterns. The samples induced with 0.8M NaCl stress at 42°C showed increased viability after 30 minutes, rather than at the start of the stress. The same samples, when compared with the control at 42°C, should show higher viability after the sequential treatment because of the presence of expected increased RpoS levels. However, we were not able to verify this due to a lack of viability data from our 30 minutes 42°C control treatment. Although we were not able to support this point, we were able to conclude that cell viability does not go up if the salt treatment is given without the heat shock. This would be true because there would be no RpoS present in non-heat shocked cells to further the growth of cells in the culture (3, 4). This conclusion is inferred from the comparison of samples, which underwent 0.8M NaCl treatment after growth at 37°C, with the control samples at 37°C. At 30 minutes, we see a greater loss of viability in the sample population induced with the 0.8M NaCl treatment. The difference in the two viabilities is not statistically significant. It would need a repetition of the experiment with greater number of replicates in the viability assay to confirm the significance of our predictions.

During many parts of this experiment we have been able to directly proportionate the change in viability to the change in RpoS levels. This has led to many questions regarding cellular pathways regulating RpoS levels, and the cellular pathways regulated by the RpoS transcription factor. We predict that each cross protective stress induces its own unique set of genes in order to combat stress. Some members of these sets are common to all stresses, and others are common to a specific stress. The members of this stress response set that are common to both temperature and pH would cause cross protection in a sample which is placed under extreme pH conditions after heat shock. We also predict that the greater the number of common stress fighting genes between the two treatments being treated for cross protection, the higher the cell survival rate of the sample that underwent cross protection. These predictions and some ideas to test them are discussed in further in the future experiments.

Our experiments concluded that heat shock treatment capable of inducing elevated RpoS levels appeared to provide protection against the detrimental effects

caused by low pH; however, we could not assess cross protection of heat shock to hyperosmolarity conditions as the salt concentrations used did not appear to be sufficient to induce cell death in non-heated shocked cells.

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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