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The Effect of Sucrose-induced Osmotic Stress on the Intracellular Level of cAMP in *Escherichia coli* using Lac Operon as an Indicator

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The effect of sucrose-induced osmotic stress on *Escherichia coli* cells is mediated by the regulation of cAMP synthesis. Previous studies concluded that hyperosmotic stress induced by ionic solutes will lead to an increase in the cAMP levels in the cell as a response to changes in osmolarity; however, such conclusions could not be made for osmotic stress induced by non-ionic solutes such as sucrose. To determine whether the global regulator cAMP receptor protein (CRP) plays a role in adaptation under hyperosmotic stress induced by sucrose, *E. coli* cells were grown under varying sucrose concentrations and the growth rate over a three hour period was correlated with the β-galactosidase activity of these cultures. In the absence of sucrose, *crp* mutants and wild-type strain have similar growth rates. However, *crp* mutants show increased growth rate when subjected to growth in increased amount of sucrose, while the growth rate for the wild-type is not affected by the increasing sucrose concentration. β-galactosidase assays were performed on the wild-type, *crp* mutant and *crp/lacI* double mutant as an indirect assessment of intracellular cAMP levels, but the results were inconclusive due to sucrose-induced turbidity which interfered with absorbance measurements.

Escherichia coli have the ability to grow in environments ranging from dilute solutions scarce of essential nutrients to media containing molar concentrations of ionic or non-ionic solutes (10). Growth in a wide range of osmolarities poses significant physiological challenges for cells and requires *E. coli* to manipulate a number of cytoplasmic solution variables such as the flux of cytoplasmic water and of charged and uncharged solutes (12). It was suggested that increase in osmolarity has a global effect on gene expression (4). One important player in the regulation of stress related responses is the master stress regulator, RNA polymerase sigma factor (RpoS), which is responsible for up-regulating a number of genes involved in cross-protecting the cell against stress conditions such as hyperosmolarity (13). The lactose (*lac*) operon of *E. coli* is another system controlled by global regulation, since expression of the β-galactosidase gene (*lacZ*) is up-regulated by the binding of cAMP to the cAMP receptor protein (CRP), a global regulator involved in the repression as well as activation of a large number of *E. coli* genes (9). Therefore, it was assumed that β-galactosidase activity could be correlated to intracellular cAMP levels, to assess whether cAMP and the cAMP-CRP complex play a role in the adaptation to hyperosmotic stress.

Transcription of the gene encoding β-galactosidase, the enzyme involved in hydrolysis of lactose, is negatively regulated by the Lac repressor (encoded by *lacI*) and positively regulated by the cAMP-CRP complex. Constitutive expression of β-galactosidase requires the removal of the LacI repressor from the lac operator (*lacO*) via specific gene knockout mechanisms. In the absence of LacI, RNA polymerase is able to bind to the Lac promoter (*lacP*), which would allow a basal amount of β-galactosidase to be produced. However, maximal expression of the β-galactosidase gene is controlled by the binding of the cAMP-CRP complex to the Lac promoter (6). β-galactosidase activity is measured through the standard assay which involves measuring the formation of the yellow chromophore o-nitrophenol (ONP) via spectrophotometry. ONP is the hydrolytic product that is formed when β-galactosidase acts on the colourless substrate o-nitrophenyl-β-D-galactoside (ONPG) (5).

Previous studies confirmed that NaCl-induced osmotic stress resulted in an elevated level of β-galactosidase activity, while sucrose-induced osmotic stress did not (4). Therefore, to investigate further into the correlation between sucrose-induced osmotic stress and the levels of β-galactosidase activity, it is important to use an *E. coli crp* mutant and a *E. coli crp/lacI* double mutant as controls. The *crp* mutant will be unable to form the cAMP-CRP complex and will lead to lower β-galactosidase activity compared to the wild-type. On the other hand, the double mutant is expected to have higher levels of β-galactosidase compared to the single mutant due to the deletion of the Lac repressor, leading to constitutive expression of the β-galactosidase gene. Since a *crp/lacI* double knockout mutant was not available in any stock center, it was necessary to create

this double mutant via site-specific mutagenesis using the Lambda-Red Recombinase (λ-Red) system.

Cheung *et al.* measured β-galactosidase activity of cell cultures subjected to varying concentrations of solutes only at the end of a two-hour incubation period, whereas notable fluctuations of β-galactosidase could have occurred during earlier stages (4). Therefore, it was decided that cell density and β-galactosidase activity should be measured every thirty minutes for three hours so the trend for β-galactosidase activity changes could be observed more clearly. Furthermore, a growth curve could be plotted and used to calculate the growth rate of cell cultures and correlate this to changes in β-galactosidase activity.

Since the cAMP-CRP complex regulates a number of genes expressed during hyperosmolarity, an increase in β-galactosidase should be observed in the wild-type when subjected to increasing concentrations of sucrose, but not in the *crp* mutants. It is also expected that increasing osmolarity will lead to lower growth rates in all three strains. A correlation between cell growth and β-galactosidase activity in all controls and experimental conditions was also expected.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strains MG1665/pKD46, DH5α/pACYC177 were obtained from the University of British Columbia's MICB 421 collection and *E. coli* strains CA8000 (wild-type) and CA8445-1 (*crp* mutant) were obtained from the Coli Genetic Stock Center at Yale University (Table 1). *E. coli* strains DH5α/pACYC177, CA8000, CA8445-1, and LCH01 (*crp/lacI* double mutant) were grown at 37°C in Luria-Bertani (LB) medium (1.0 % w/v tryptone, 0.5 % w/v yeast extract, 0.5 % w/v NaCl) where the DH5α/pACYC177 strain was supplemented with 50 μg/ml kanamycin (Sigma, St. Louis, MO) and the LCH01 strain was supplemented with 100 μg/ml ampicillin (Sigma, St. Louis, MO). *E. coli* strains MG1655/pKD46 and CA8445-1/pDK46 were grown at 30°C in LB medium supplemented with 100 μg/ml ampicillin. All the strains were shaken at 200 RPM during incubation.

Construction of a *crp/lacI* double knockout (LCH01) using the Lambda-Red Recombinase system. Plasmids pKD46 and pACYC177 were isolated from *E. coli* strains MG1665/pKD46 and DH5α/pACYC177 using the Fermentas GeneJETTM plasmid purification kit. Complete characteristics of each of the plasmids has previously been described by Woo (14). Amplification of the kanamycin resistance gene from pACYC177 plasmid and confirmation of upstream and downstream junctions in recombinants were performed with the following reagents: 1X PCR buffer, 0.2 mM dNTP, 1mM MgCl, 0.4 μM forward primer (3), 0.4 μM reverse primer (3), 1.25 U Pfx polymerase (Invitrogen, Burlington, Ontario, Canada), and distilled H_2O to a final volume of 25 μl. The PCR reaction conditions used to amplify *kanR* were: an initial denaturation of 5 minutes at 95°C; 35 cycles of 45 seconds at 94°C, 45 seconds at 52°C, and 90 seconds at 68°C; followed by a final extension of 10 minutes at 68°C. The PCR reaction conditions used to amplify the junctions were: an initial denaturation of 10 minutes at 95°C, followed by the addition of pfx polymerase; 34 cycles of 45 seconds at 94°C, 30 seconds at 60°C, and 60 seconds at 68°C; followed by a final extension of 10 minutes at 68°C. The PCR amplified *kanR* from pACYC177 plasmid was purified using the Fermentas GeneJETTM PCR purification kit. In preparing electrocompetent cells of CA8445-1 strains and CA8445-1/pKD46 strains, overnight cultures were made based on the growth conditions specified above. Preparation of electrocompetent cells, transformation of cells, and confirmation of *lacI* knockout cells using PCR were carried out as described by Beamish *et al.* (3).

Transformation confirmation tests. To confirm successful transformation of the CA8445-1 strain with the pKD46 plasmid, potentially transformed colonies were streaked on to plates with or without 100 μg/ml ampicillin and incubated at 30°C or 42°C overnight. Another confirmation test was done by running the purified plasmids isolated from potentially transformed colonies on a 1.5 % w/v agarose gel at 120 V at room temperature. A 1 kb GeneRulerTM linear DNA ladder was used for band size estimation.

Sucrose treatment. Overnight cultures of CA8000, CA8445-1, and LCH01 strains were diluted to 0.3 OD_{460nm}, incubated at 37°C, and shaken at 200 RPM until the cells reached 0.5 OD_{460nm}. At this point, equal volumes of culture and sucrose solution (0 M, 0.5 M or 1 M sucrose in distilled water) were mixed together. The flasks were vortexed immediately and samples were taken from each flask for subsequent experiments and put on ice. The flasks were then re-incubated at 37°C and shaken at 200 RPM with additional aliquots taken every 30 min for 3 hrs. Growth was simultaneously measured at 460 nm with a Beckman UV/VIS spectrophotometer for both the CA8000 and CA8445-1 strains sampled after sucrose treatment.

β-galactosidase assay. β-galactosidase assays were adapted from Miller (8), with minor modifications. Briefly, a drop of toluene was added to each sample, incubated at 37°C and shaken at 200 RPM for 30 min with the caps open to allow evaporation of toluene. To test tubes containing equal volumes of 25 mM Tris (pH 10) and sample, ONPG was added to a final concentration of 0.7 mg/ml. Tubes were incubated for 1 hr at room temperature, and the reaction was terminated by the addition of 1 M Na_2CO_3 solution (to a final

TABLE 1. *E. coli* strains used

***E. coli* Strains and Plasmids**	**Strain characteristics**	**Reference**	**Source**
MG1655/pKD46	*&lambda⁻; rph-1;* pKD46*	CGSC# 7669	MICB 421 collection
DH5α/pACYC177	Δ(*argF-lac)169*; φ80d*lacZ58*(M15); *glnV44*(AS); *&lambda⁻*; *rfbC1*; *gyrA96*(NalR); *recA1; endA1; spoT1; thi-1; hsdR17;* pACYC177	Beamish *et al.* (3)	MICB 421 collection
CA8000	*&lambda⁻; e14-; relA1; spoT1; thi-1*	CGSC# 6026	Yale University Coli Genome Collection
CA8445-1	*&lambda⁻; e14-; relA1; rpsL136*(strR); *Δcrp-45; spoT1; thi-1*	CGSC# 7043	Yale University Coli Genome Collection

*pKD46 marker/mutation: *repA101*(ts); *araBp-gam-bet-exo*; *oriR101*; *bla*(ApR)

concentration of 0.3 M). β-galactosidase activity was measured spectrophotometrically by the absorbance at 420 nm. β-galactosidase activity was calculated using the following equation:

$$\frac{\text{Enzyme activity}}{\text{ml of enzyme}}\ (\text{mUnit/ml}) = A \times \frac{1}{t} \times \frac{10^3}{\varepsilon} \times Nv \times \frac{1}{Ev}$$

where 1 milliunit equals to 1 nmol of product formed per time, A depicts absorbance of solution at 420 nm, t is the reaction time in min, ε is the extinction coefficient for product (ONP at alkaline pH is 4.5 $mM^{-1}cm^{-1}$(11)), Nv is total assay volume (ml) for absorbance reading, and Ev is enzyme sample volume (ml). β-galactosidase activity inside the cells was normalized to the cell density at each time point.

RESULTS

Successful cloning of the *crp*/*lacI* mutant using the λ-red recombinase system. The *E. coli crp* mutant was successfully transformed with the pKD46 plasmid which contains the recombinase gene necessary for recombination; this was confirmed by two different methods. The gel electrophoresis of purified plasmids from potentially transformed cells showed bands of two different sizes (Fig. 1). pKD46 is known to be 6.3 kb (16), however, the two bands on the gel did not have an apparent size of 6.3 kb due to the ladder being linear rather than circular since only linear ladders were available in the lab. A circular ladder would have given correct size information pertaining to circular plasmids such as pKD46 while a linear ladder would only provide correct size for linear molecules. Therefore, another confirmation method was conducted to test the existence of the heat-sensitive, ampicillin-resistant pKD46 plasmid by growing the potentially transformed colonies at 30°C or 42°C, with or without ampicillin. At 42°C, truly-transformed colonies grew only sparsely on plates with ampicillin compared to the plates without ampicillin (Fig. 2). Positive confirmation was observed in two out of the nine colonies tested, and the successfully transformed colonies were labeled colony 1 and colony 3.

These two colonies were then transformed with a *kanR* cassette to knockout the *lacI* gene in the CA8445-1/pKD46 strain, ultimately creating a *crp*/*lacI* double mutant. Figure 3 presents the confirmation results of the deletion of *lacI* by amplifying the upstream and downstream junctions of *kanR*. Both colony 1 and 3 showed the presence of *kanR* at a correct site in the genome (Fig. 3). Since colony 1 exhibited a clearer band for the amplified upstream junction of *kanR* compared to colony 3, subsequent experiments used colony 1 as the *crp*/*lacI* double mutant (Fig. 3). Colony 1 was named as LCH01.

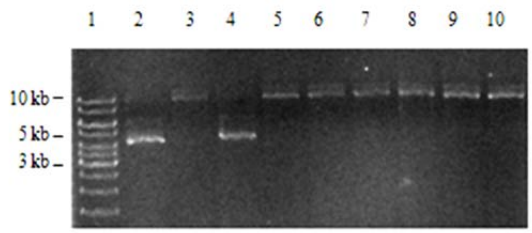

FIG. 1. Visual confirmation of the presence of pKD46 plasmid transformed into CA8445-1 strain. Gel electrophoresis of isolated plasmids of nine selected prospective CA8445-1/pKD46 colonies (lanes 2 to 10). Colony 1 and 3 (lane 2 and 4) show pKD46. The ladder in lane 1 is linear rather than circular.

FIG. 2. Confirmation of temperature-sensitive pKD46 plasmid transformed into CA8445-1 strain. Properly transformed colonies 1 and 3 exhibit scarce growth compared to non-transformed colonies 2 and 4 on a LB plate supplemented with ampicillin grown at 42 °C.

Effect of sucrose on growth of *E. coli* in the presence and absence of CRP. Prior to assessing the effect of sucrose on β-galactosidase activity, growth was monitored in the presence of sucrose concentrations of 0 M, 0.25 M, 0.5 M, 1 M and 1.5 M. This was done to normalize the β-galactosidase activity by the cell density. However, it was observed that the wild-type, the *crp* mutant and the *crp/lacI* double mutant cells all died upon the addition of sucrose at concentrations of 1 M and 1.5 M. Therefore, subsequent assays were carried out using only three sucrose concentrations: 0 M, 0.25 M and 0.5 M. The growth curves of the three bacterial strains at three different sucrose concentrations were determined (Fig. 4). Growth rate was calculated based on the cell densities at 120 min and 180 min after sucrose treatment. It was expected for the growth rate to be similar between the three strains and to decrease with increasing sucrose concentration. High osmotic pressure on any cells was expected to cause decreased growth rate, as the cells would focus on producing osmolytes to prevent water loss and subsequently lead to a lag in growth (10). However, increasing the sucrose concentration had negligible effect on the growth rate

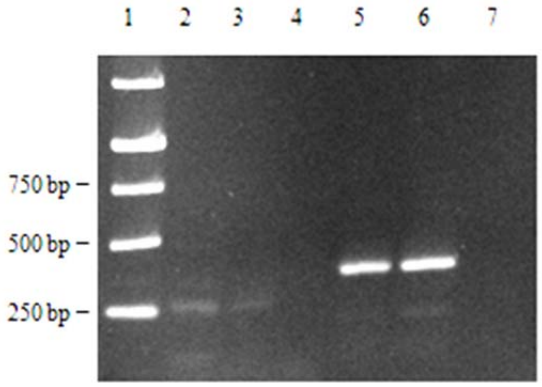

FIG. 3. PCR amplification of kanR junctions of CA8445-1/pKD46/kanR colonies 1 and 3 from Fig. 1. Gel electrophoresis of PCR products. The amplified upstream junction of *kanR* is shown in lane 2 and 3 and the amplified downstream junction of *kanR* is shown in lane 5 and 6 for CA8445-1/pKD46/kanR colonies 1 and 3, respectively. Lane 4 and 7 are negative controls containing no DNA.

of wild-type cells while in the two strains of *crp* mutants, the growth rate increased with increasing sucrose concentration (Fig. 5); in the *crp* mutant, increasing the sucrose concentration from 0 M to 0.5 M tripled the growth rate, while in the *crp/lacI* mutant, the growth rate was doubled (Fig. 5). As *crp* mutants and the wild-type strain exhibited very different growth trends (Fig. 4), it can be speculated that CRP may be playing a role in regulating the growth of *E. coli* during osmotic stress.

Effect of sucrose on β-galactosidase activity. In using β-galactosidase activity as an indicator for the level of intracellular cAMP, increased levels of cAMP inside wild-type cells represented by increased levels of β-galactosidase activity was expected in increasing amounts of extracellular sucrose. As seen in Figure 6A, wild-type cells exhibited higher levels of β-galactosidase activity when exposed to higher sucrose concentrations; this was observed throughout each sampling point during the three hours of sucrose exposure. The *crp* mutant and *crp/lacI* double mutant were expected to show lower β-galactosidase activity compared to the wild-type due to *crp* deletion. This was observed in Figure 6, where the wild-type cells was monitored to have at least 50 % higher levels of enzyme activity at every corresponding time point and sucrose concentration than both mutant strains. It was also expected for the crp/*lacI* double mutant to have slightly higher β-galactosidase activity compared to the *crp* mutant due to *lacI* deletion. This trend could not be observed from Figure 6B and 6C. For all three strains tested, 0 M treated samples appeared clear, while cells grown under 0.25 M and 0.5 M sucrose concentrations appeared turbid. For the wild-type, cells grown in the

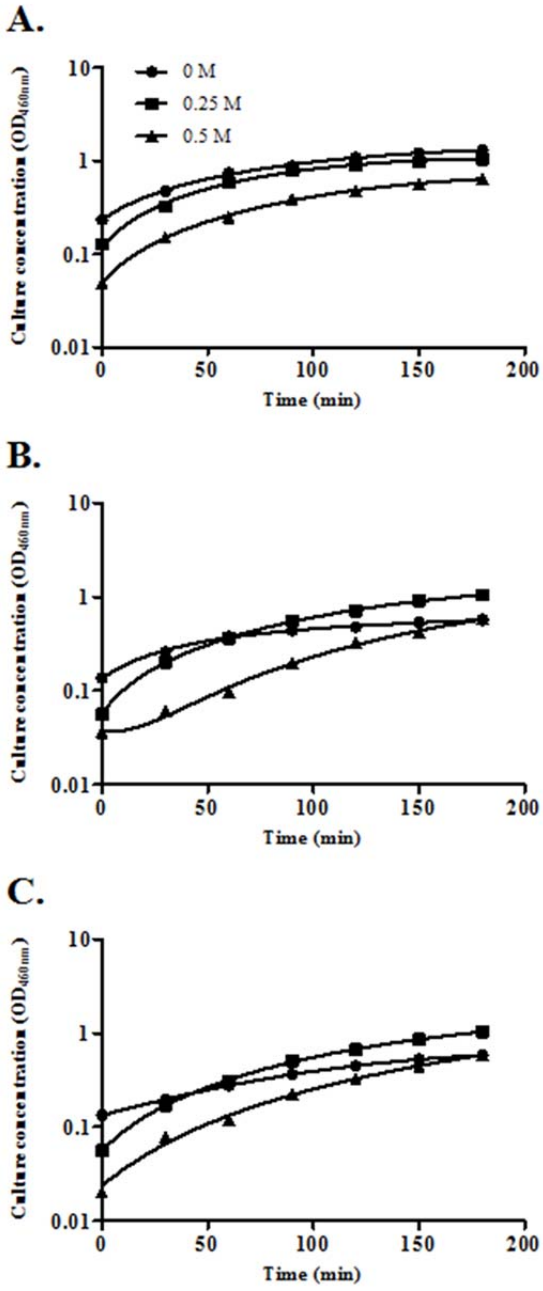

FIG. 4. Effect of varying levels of sucrose on the growth of the wild-type strain (A), *crp* strain (B), or *crp/lacI* strain (C). Data shown is representative from one of two independent experiments.

absence of sucrose produced a fairly intense yellow color indicating the formation of the ONP product, while in the presence of sucrose, no observable yellow color was produced. *crp* mutants treated under 0 M, 0.25 M and 0.5 M sucrose concentrations did not appear yellow towards the naked eye. Therefore, β-galactosidase activity values measured on the

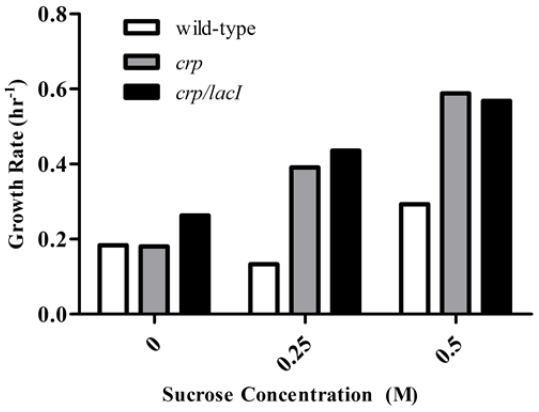

FIG. 5. Effect of varying levels of sucrose on the calculated growth rate of the wild-type strain, *crp* strain, and *crp/lacI* strain. The growth rate of each sample is calculated based on the growth curve in Fig. 4.

spectrophotometer did not correspond well with the actual yellow colour formation in the β-galactosidase assay; in terms of colour intensity observed with the naked eye, increasing sucrose concentration actually decreased β-galactosidase activity and consequently would indicate a decreased level of intracellular cAMP. Hence, it was suggested that after toluene treatment the cellular debris should be centrifuged down at a gentle speed, so that the spectrophotometer is measuring intensity of yellow due to the formation of ONP rather than being influenced by the turbidity of the cultures. This should give a more accurate reading of β-galactosidase enzyme activity.

DISCUSSION

Biological membranes are permeable to water but relatively impermeable to most ionic and non-ionic solutes. Living cells are affected by changes in the total solute concentration of the environment. For instance, increase in external osmolarity will cause loss of water from the cell, whereas a decrease in external osmolarity will cause uptake of water (7). To reduce fluctuations in the cellular water content due to osmotic stress, *E. coli* is able to activate the synthesis of compatible solutes though the expression of compatible solute synthesis genes *otsA* and *otsB*, both regulated by the global regulator *rpoS* sigma factor, which is responsible for gene expression under stress (10) .

Like the *rpoS* gene, the lactose (*lac*) operon of *E. coli* is also controlled by global regulation, where expression of the β-galactosidase gene (*lacZ*) is up-regulated by the cAMP-CRP complex. Intracellular cAMP is therefore expected to increase when cells encounter hyperosmolarity, and is assumed to correlate positively with the level of β-galactosidase produced.

Figure 6 shows generally higher β-galactosidase activity for the wild-type strain in comparison to the *crp* mutant and *crp/lacI* double mutant, but the correlation between the β-galactosidase activity and the sucrose concentrations was inconclusive. For all three strains, β-galactosidase enzyme activity was generally the highest for the cells subjected to 0.5 M sucrose and decreased with lower sucrose concentrations. Some variations were observed for the *crp* mutant and *crp/lacI* double mutant graphs in Figure 6, but a general trend was still observed. However, observations made by eye during the experiment noted that cells from all three strains grown in 0 M sucrose solution presented a clear and transparent solution, while the cells subjected to 0.25 M and 0.5 M sucrose treatments were of high turbidity despite toluene treatment and did not appear yellow. It was also noted that wild-type cells subjected to 0 M sucrose treatment presented an intensely yellow solution due to the formation of the yellow chromophore ONP, compared to wild-type cells treated with 0.25 M and 0.5 M sucrose, which appears to be a translucent white colour. During spectrophotometry, samples with high cell turbidity but no yellow color

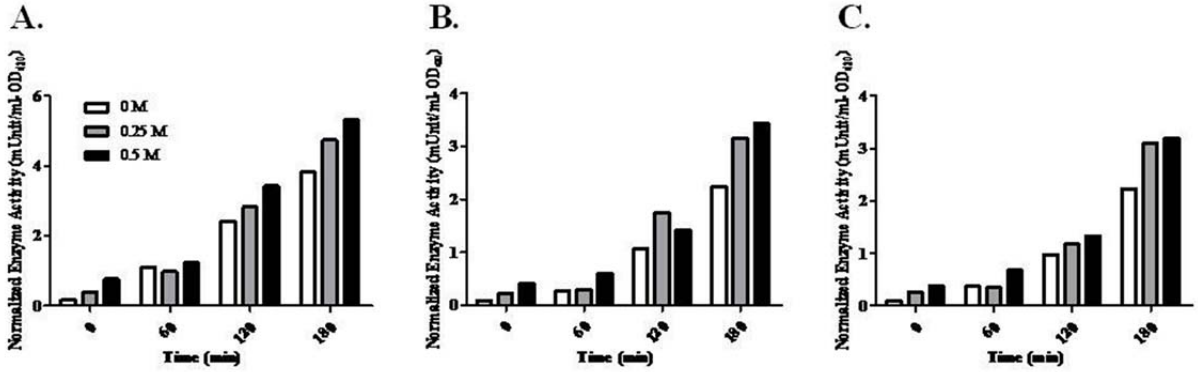

FIG. 6. Effect of varying levels of sucrose on the β-galactosidase activity in wild-type (A), *crp* (B), and *crp/lacI* (C) strains. The enzyme activity values are normalized to bacterial cell density. Data shown is representative from one of two independent experiments.

gave higher absorbance readings than clear solutions with intense yellow colour. This suggested that the absorbance readings at 420 nm were more indicative of turbidity of the samples rather than the level of β-galactosidase as reflected in the degree of yellowness. This is supported by the fact that the absorbance readings at 420 nm were consistently higher for samples with higher turbidity. According to Miller, toluene added during the β-galactosidase assay would permeabilize the cells leading to disruption of cellular compartments and cell lysis (8). It was hypothesized that the extracellular sucrose might have roles in balancing the flux of water through the cellular membrane to maintain the integrity of the membrane barrier, leading to increased turbidity of the sample. This was not taken into consideration and could be the explanation of the inconclusive β-galactosidase enzyme assay results.

In comparison to wild-type, both the *crp* mutant and the *crp/lacI* double mutant were expected to demonstrate similar growth rates when subjected to 0 M sucrose, since CRP is not needed to counter changes in the absence of sucrose induced osmotic stress. This is mostly consistent with the results presented in Figure 5, as the growth rates for wild-type and *crp* mutant are similar, but both are slightly lower than the growth rate of the *crp/lacI* double mutant. The growth rates for *crp* mutant and *crp/lacI* double mutant at 0.25 M and 0.5 M sucrose were expected to be lower than the wild-type due to the absence of CRP protein which has essential regulatory roles in hyperosmolarity. This is inconsistent with the observations that the growth rate for the wild-type is significantly lower than both the *crp* mutant and the *crp/lacI* double mutant. Finally, it was hypothesized that increasing osmotic stress will lead to lower growth rates of all three strains. However, it was observed that for the wild-type, osmotic stress does not seem to affect growth rate since cells treated with 0 M, 0.25 M and 0.5 M sucrose have similar levels of growth rates. Surprisingly, both the *crp* mutant and the *crp/lacI* double mutant show significant increase in growth rate as the sucrose concentration increases, which is clearly different from the behaviour of the wild type cell and contrary to the hypothesis.

There are two potential explanations for the increase in growth rate of *crp* mutants during sucrose induced osmotic stress. The first is that the active CRP or cAMP-CRP complex has the ability to either limit growth during osmotic stress by repressing transcription of genes required for growth, or by inducing the transcription of stress-related genes that would normally limit growth during osmotic stress. However, some evidence suggest that the *rpoS* gene encoding the sigma factor for the RNA polymerase that recognize certain promoters under stress conditions is inversely correlated to growth rate and negatively regulated by cAMP-CRP complex. This would suggest that in *crp* mutants, there will be a higher level of *rpoS* expression, leading to lower growth rates. This is completely opposite to what was observed in Figure 5. The second explanation is that if CRP is actually regulating the growth in osmotic stress, the absence of CRP might lead to changes in the membrane composition so that the energetics are more efficient under a stressed environment. Evidence for this has yet to be found but could be an interesting area of research.

Prior to the β-galactosidase assay, the λ-Red Recombinase system was used to disrupt the *lacI* gene in the *crp* mutant to obtain a *crp/lacI* double mutant. This method had been successfully utilized by Beamish *et al.* to disrupt the *lacI* gene in other *E. coli* strains (3). Our study replicated the methods of Beamish *et al.* (3). The cloning of the *crp/lacI* double mutant was successful with a positive confirmation test (Fig. 3). An additional phenotypic confirmation though the results of the β-galactosidase assay would have been preferred, but due to difficulties encountered in the β-galactosidase assay, that confirmation was not done.

FUTURE DIRECTIONS

To further explore the explanations for increasing growth rates in crp mutants subjected to sucrose induced osmotic stress, it would be interesting to test membrane proteins expressed in the presence and absence of *crp* to identify genes that are expressed in one condition but not another, then assess whether those proteins are involved in energy metabolism or transport systems. To address the question as to whether sucrose is critical at causing the high growth rate observed in *crp* mutants, other osmotic stress inducers can be tested to see if they would cause the similar effect.

As a clear correlation between osmotic stress and intracellular cAMP levels could not be established using β-galactosidase activity as an indicator, levels of intracellular cAMP might not be directly linked to β-galactosidase activity, and other methods for measuring cAMP such as ELISA should be sought after.

For the experiment measuring growth rate, a few adjustments can be suggested. The sucrose solution can be made with LB rather than distilled water so that the concentration of the nutrients of LB in the cell culture is not changed when adding the sucrose solution. More sucrose concentrations can be explored as well, since our study only measured the effect at 0.25 M and 0.5 M.

For further optimization of the cloning procedure to maximize chances of obtaining a successful clone, it is recommended to minimize salt in the solution after using PCR clean up kits thereby minimizing arcing during electroporation.

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