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Characterization of *Escherichia coli* Alkaline Phosphatase Response to Osmotic Stress Conditions in Limited and Rich Media

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The *pho* regulon in *Escherichia coli* responds to osmotic stress. Previous studies indicated that in nutrient limited medium, expression of *phoA*, one gene of the *pho* regulon, increased as the osmolarity increased. By contrast, in nutrient complex medium, *phoA* expression was not directly proportional to osmolarity. In this experiment, the effect of supplementing nutrient-limited medium with either glutamate or proline was characterized to test the hypothesis that osmoprotectants in nutrient-complex medium may avert induction of *phoA* expression. Compared to nutrient-limited conditions, lower alkaline phosphatase specific activity was detected under nutrient rich condition suggesting the presence of osmoprotectants in the nutrient-rich medium. Our results suggest that, in addition to intended osmotic stress, starvation was unexpectedly induced. Consequently, conclusive results were difficult to deduce.

Most bacterial cell cytoplasms, including that of *Escherichia coli*, are much more concentrated in particles than the surrounding cell environment. Due to osmosis, water tends to flow into the cell, exerting a pressure directed outward against the cell wall, termed turgor pressure. To maintain internal turgor pressure, necessary for cell integrity, bacteria are able to adjust their internal osmolarity in response to changes in external osmolarity. For example, cells placed in media of high osmolarity increase their intracellular concentration of specific solutes called osmolytes. Organic osmolytes, termed osmoprotectants, frequently accumulate in cells through importation from the surrounding environment, rather than intracellular synthesis. These imported osmoprotectants aid osmotic homeostasis.

An example of an operon present in *E. coli* that responds to osmotic stress is the *pho* regulon (10). *Pho* regulon genes are involved in the phosphate starvation response and are expressed when *E. coli* is in phosphate-limiting conditions. One gene of the *pho* regulon, *phoA*, encodes alkaline phosphatase (AP). When expressed, AP is translocated via a signal peptide to the periplasm, where it scavenges and recycles phosphate. In the presence of phosphate, the regulatory protein, PhoR, inhibits *pho*A expression.

Villarejo *et al.*, (12) have previously studied *phoA* expression under various osmotic shock conditions. To eliminate the necessity for phosphate-free medium, Villarejo *et al*. conducted experiments with *E. coli phoR* mutants, in which *phoA* expression is constitutive and independent of growth medium phosphate concentration. To measure *phoA* expression, Villarejo *et al*. used a *lac* fusion to the *phoA* promoter and assayed β-galactosidase activity. They observed that in a nutrient limited medium, *phoA* expression was directly proportional to medium osmolarity. However, in a nutrient complex medium, *phoA* expression did not increase with increasing medium osmolarity.

We hypothesize that osmoprotectants, present in the nutrient complex medium and absent from the nutrient limited medium, allowed *E. coli* to avoid a change in internal osmotic pressure significant enough to induce *phoA* expression. Peptone and tryptone present in the nutrient complex medium act as a complete source of essential amino acids. Further, proline is a well-characterised osmoprotectant, commonly applied to both prokaryotic and eukaryotic experiments (1, 4, 5, 6, 8). Glutamate is an identified compatible solute (1), however its osmoprotective ability has not been well characterized.

In this experiment, the osmoprotective capacity of glutamate was evaluated by monitoring AP specific activity in osmotically stressed *E. coli* cultures grown in nutrient limited medium. Proline was used as a positive control. The results obtained were difficult to interpret. However, attenuation of AP specific activity in osmotically stressed cultures grown in nutrient complex medium, as compared to that of nutrient limited medium, was due to numerous nutrient complex medium components rather than a single osmoprotectant.

MATERIALS AND METHODS

Media and reagents. Luria-Bertani (LB) medium contained 1% tryptone (w/v), 0.5% yeast extract (w/v), 0.5% NaCl (w/v), 0.2% glucose, and was adjusted to pH 7 with NaOH. Nutrient limited (M63) medium contained 1.3% KH_2PO_4 (w/v), 0.5% $FeSO_4 \cdot 7H_2O$ (w/v), 0.4% glucose (w/v), 0.2% (NH4)2SO4 (w/v), 0.02% MgSO4 7H2O (w/v), 0.001% thiamine (w/v) and was adjusted to pH 7 with KOH. Nutrient Broth (NB) was prepared following standardized protocol (Difco).

Bacterial strains and growth conditions. *E. coli* phoR mutant strain C29 (2) was used in osmotic shock response experiments. *E. coli* B23 with wild type *phoR,* used as a negative control for *phoA* expression, was grown in NB medium. Cultures were grown aerobically in a shaking water-bath (Metabolide Water Bath Shaker, New Brunswick Scientific Co.) at 37°C.

Osmotic stress conditions. *E. coli* strain C29 subcultures, in either M63 medium or NB medium, of ~ 0.6 optical density (OD_{600}) were subjected to osmotic stress conditions of either 200 mM NaCl, 400 mM NaCl, 800 mM NaCl, or no NaCl (negative control). Cultures were aerobically shaken in a water-bath at 37°C under osmotic stress conditions for 65 min, 105 min and 155 min. Then, cultures were harvested and the alkaline phosphatase specific activities of periplasmic fractions were assayed.

Osmoprotective conditions. Osmotic stress was induced and osmoprotectants were added simultaneously to *E. coli* strain C29 subcultures, in M63 medium, of ~ 0.6 OD_{600}. Each subculture was subjected to osmotic stress conditions of 200 mM NaCl, 300 mM NaCl, 400 mM NaCl, or no NaCl (negative control). Simultaneously, osmoprotectants, either 0.1% (w/v) proline or 0.1% (w/v) glutamic acid were added to a subculture. Under experimental conditions, cultures were aerobically shaken in a water-bath at 37°C for 105 min. Then, cultures were harvested and the alkaline phosphatase specific activities of periplasmic fractions were assayed.

Periplasmic fraction isolation. To isolate periplasmic fractions, cells were first harvested by centrifugation (International Equipment Company Centra-4B Centrifuge) at 8000 x g for 5 min at room temperature. To allow assay of alkaline phosphatase released by leaky or lysed cells, one ml of medium was sampled. The cell pellet was washed with ice-cold pH 7.4 phosphate buffered saline (PBS) and centrifuged at 8000 x g for 5 min at room temperature. Then, the supernatant was discarded and the pellet was resuspended in 1 ml Tris – (20% w/v) sucrose solution (Tris 50 mM and pH 6.8). The pellet was mixed with
40 µL EDTA-lysozyme solution (0.25 M EDTA, 25% w/v chicken egg white lysozyme) and allowed to react for 10 min at room temperature. The reaction was then centrifuged at 8000 x g for 5 min at room temperature. The supernatant (periplasmic fraction), was transferred to a fresh microcentrifuge tube, and immediately assayed for alkaline phosphatase activity.

Assay alkaline phosphatase activity. The assay was performed as described in the Microbiology 421 laboratory manual (7).

Assay periplasmic fraction total protein. 0.5 ml periplasmic fraction sample was added to 1 ml 1XBradford dye reagent (7), vortexed briefly and incubated for 10 min at room temperature. Then, absorbance was assayed at 595 nm on a spectrophotometer.

Qualify changes in alkaline phosphatase expression in response to osmotic stress. Equal amounts of protein samples were electrophoresed through 15% SDS-PAGE gel using constant voltage of 120 V for 90 minutes. The gel was stained using Coomassie Blue dye (9) and destained with distilled water. The gel photograph was taken using Hewlett Packard ScanJet IIcx/T and Adobe Photoshop 6.0. The contrast of the gel picture was adjusted in Photoshop.

RESULTS

The experiment documented in Figure 1 was carried out to confirm the results reported by Villarejo *et al*., specifically the increase in *E. coli* C29 alkaline phosphatase specific activity in response to osmotic stress, when grown in nutrient limited medium. Data is presented here in terms of specific activity in order to normalize for differences in total protein concentration. Graphs of enzyme activity exhibited similar trends and are not shown. Our results demonstrated a different relationship from the one observed by Villarejo *et al*. The difference in AP specific activity between M63 and NB media was pronounced at 105 min post-osmotic shock induction, as NaCl concentration increased from 0 mM to 400 mM (Fig. 1). Over this range of NaCl molarities, the observed AP specific activity in M63 medium was higher than that in NB medium, demonstrating induction of either AP expression or activity in nutrient limited conditions. However, at 800 mM NaCl concentration AP specific activity declined in M63 culture to a similar level in NB cultures.

Figures 2 and 3 present time courses of AP specific activity of cells grown in M63 and NB media, respectively. For osmotically stressed cells grown in M63, AP specific activity did not vary significantly over time within treatments (Fig. 2). Moreover, comparing treatments of varying NaCl concentration, similar levels of AP specific activity were observed in osmotically stressed cultures over the entire time course and in the 0 mM NaCl treatment at earlier time points. Interestingly, in the absence of osmotic stress AP specific activity increased over time, as demonstrated in the 0 mM NaCl culture from 105 to 155 min time points.

The AP specific activity trends were approximately reversed among cultures grown in NB medium (Fig. 3). The activity in the 0 mM NaCl culture did not change significantly over time, whereas 200 mM and 800 mM NaCl cultures increased in AP specific activity from 105 to 155 min post-osmotic stress induction, parallel to the trend observed for 0 mM NaCl M63 culture (Fig. 2). Thus, osmotic stress in the nutrient rich medium induced AP specific activity over time. The AP specific activity levels in all NB cultures were similar at earlier time points, parallel to the results in M63 cultures, indicating that more than 105 min were required for evident AP specific activity induction. Results for the 400 mM NaCl NB culture at 155 min post-osmotic stress induction are not available because the sample was accidentally discarded. Therefore, whether the AP specific activity in the 400 mM NaCl NB culture would have held constant or increased is unknown.

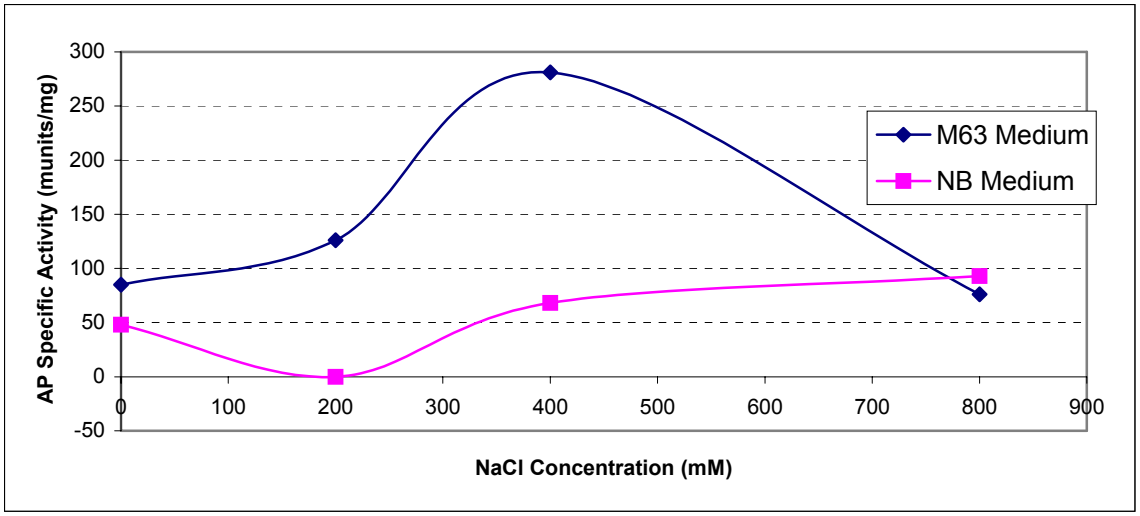

FIG. 1 Alkaline phosphatase specific activity at 105 min post-osmotic shock induction in periplasmic fractions of *Escherichia coli* C29 grown in cultures of increasing NaCl molarities.

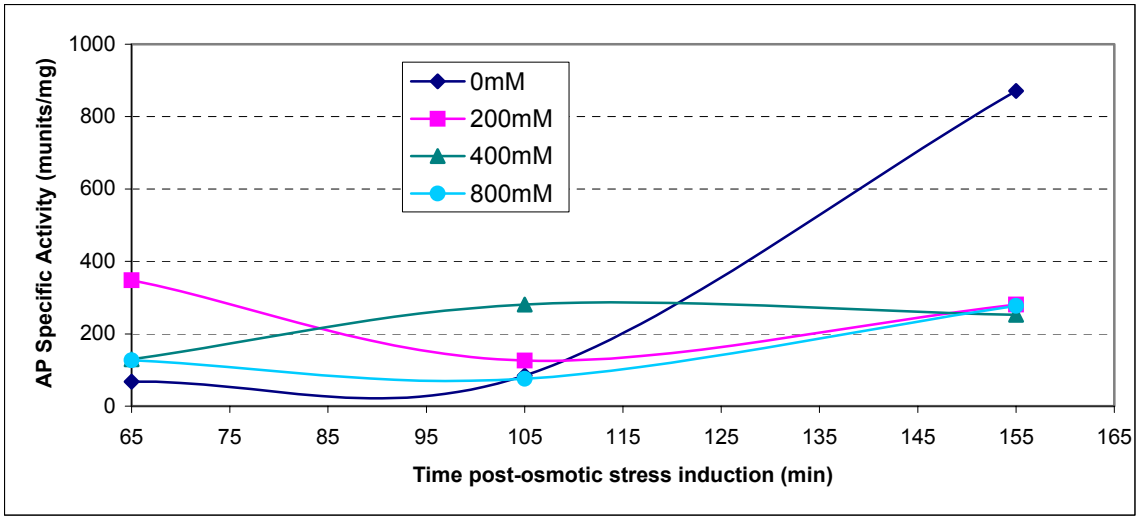

FIG. 2 Time course of alkaline phosphatase specific activity in periplasmic fractions of *Escherichia coli* C29 grown in **M63** cultures of specified NaCl molarities.

Figure 4 shows the effects of proline and glutamate on AP specific activity in *E. coli* C29 grown in M63 medium of increasing osmolarity. NaCl concentrations of 200 mM to 400 mM were tested in this assay, since higher salt concentrations appeared to deter cell growth in M63, as was apparent from Figure 1. Addition of amino acids to M63 grown *E. coli* cultures stimulated AP specific activity, as the basal level of AP specific activity (in 0 mM NaCl cultures) was 10- to 15- fold higher in proline- and glutamate-containing cultures, than in non-supplemented M63 cultures. The increase in AP specific activity observed for cells grown in M63 medium of 200 mM to 400 mM NaCl (Fig. 1) is negligible when compared to the increase in AP specific activity in M63 cultures supplemented with proline and glutamate (Fig. 4). The specific activity in proline and glutamate supplemented cultures continually climbs with increasing salt concentrations. Overall, the specific activity levels in the osmoprotectant-supplemented cultures are similar.

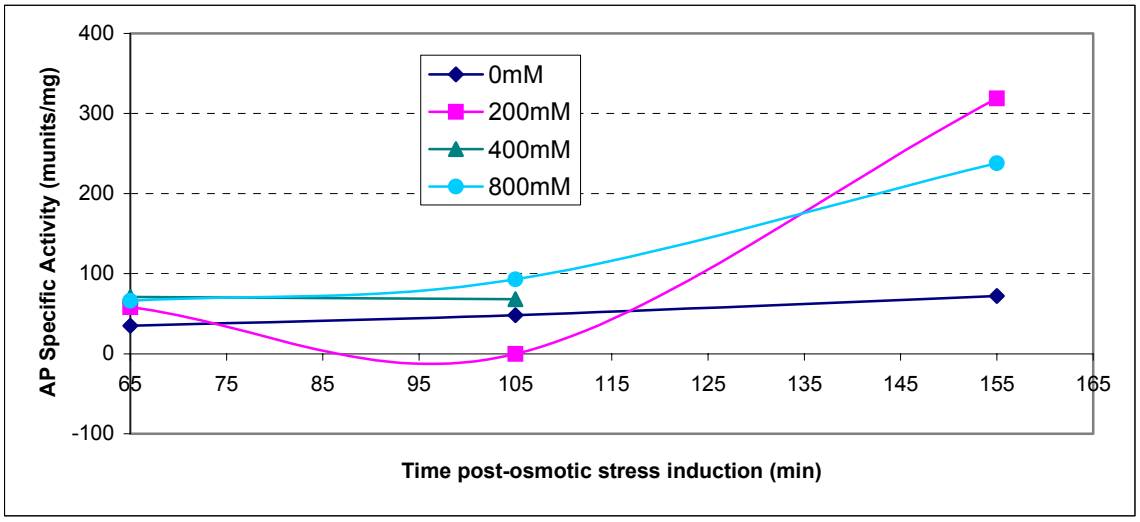

FIG. 3 Time course of alkaline phosphatase specific activity in periplasmic fractions of *Escherichia coli* C29 grown in **NB** cultures of specified NaCl molarities.

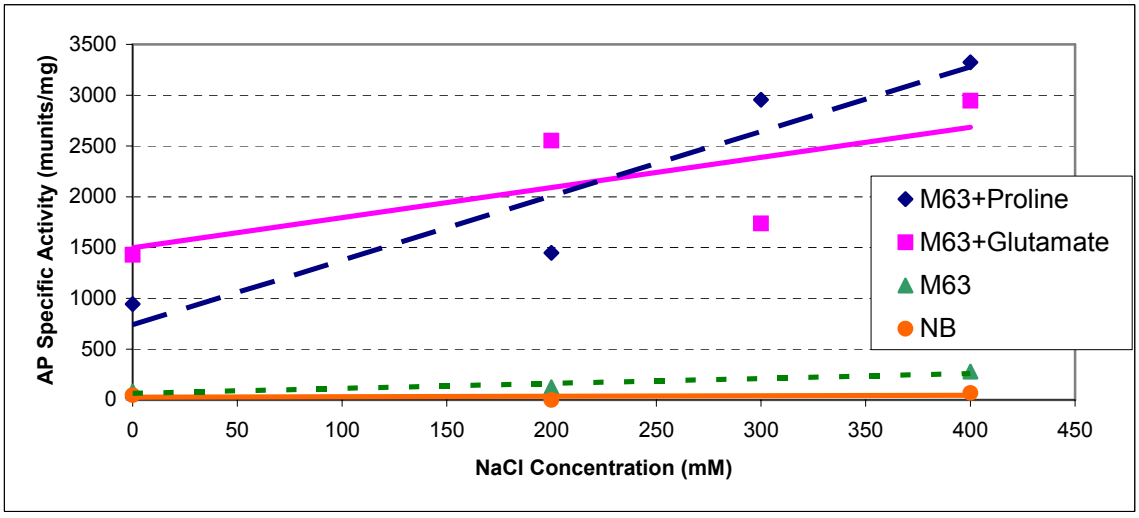

FIG. 4 Alkaline phosphatase specific activity at 105min post-osmotic shock induction in periplasmic fractions of *Escherichia coli* C29 grown in cultures of increasing NaCl molarities with and without putative osmoprotectants.

Figure 5 shows the total periplasmic protein profile of M63 samples featured in Figure 4. The sizes of molecular weights used are also shown. In its native conformation AP is a homodimer, and protein bands presumed to be dissociated ~50kDa AP subunits are highlighted in the boxed area. The bands at the box center are likely AP, because they electrophoresed slightly above the 48 kDa marker and are nearly absent from the negative control sample (wild type *E. coli* grown in the presence of phosphate). Results from Bradford assays were used to load equal amounts of total protein in each well. However, the SDS-PAGE gel indicates that lanes differed in total amounts of protein loaded, as evident from varying overall lane intensities. Thus, although the greater intensity of presumptive AP bands in lanes 5-12 indicates higher levels of AP expression in the corresponding treatments, accurate comparisons between lanes cannot be inferred. Refer to Discussion for further analysis.

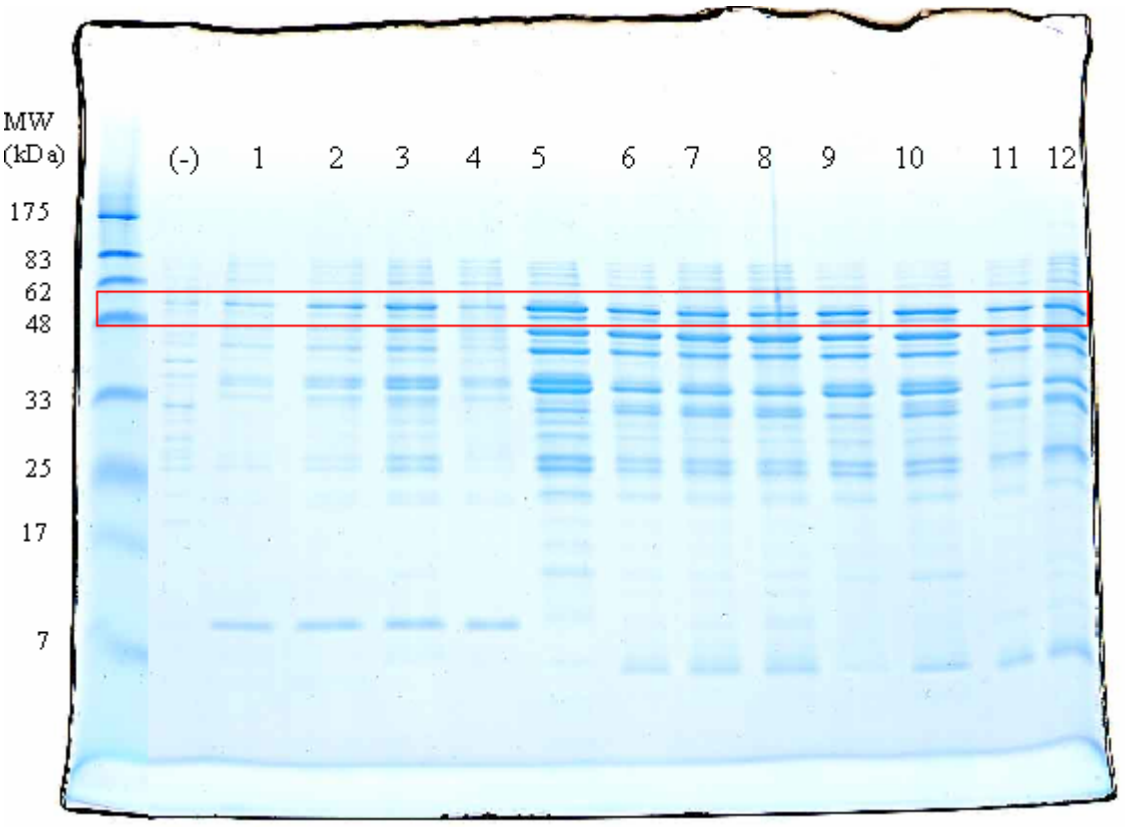

Figure Legend:

MW – size of protein markers
(-) negative control
1. 0 mM NaCl
2. 200 mM NaCl
3. 400 mM NaCl
4. 800 mM NaCl
5. 0mM NaCl + proline
6. 200 mM NaCl + proline
7. 300 mM NaCl + proline
8. 400 mM NaCl + proline
9. 0mM NaCl + glutamate
10. 200 mM NaCl + glutamate
11. 300 mM NaCl + glutamate
12. 400 mM NaCl + glutamate

FIG. 5 Coomassie staining of SDS-PAGE gel containing periplasmic fractions of *Escherichia coli* C29 grown under specified experimental conditions. All samples are from cultures grown in M63 medium under specified conditions and harvested at 105 min post-osmotic stress induction.

DISCUSSION

In contrast with the results of Villarejo *et al*, our results suggest that AP specific activity in rich medium was unstable as NaCl molarity increased. Also in contrast, in limited medium constant increase of AP specific activity with increasing osmolarity was not observed (Fig. 1). This discrepancy between our results and those reported by Villarejo *et al* may be due to a number of differences between experimental set-ups. In our experiments, increasing sodium chloride concentrations were used to induce osmotic stress, whereas Villarejo *et al* used glycerol at concentrations that were not apparent from their paper. Perhaps the discrepancy between protocols may account for the differences observed. For example, high salt concentrations inhibit many enzymes (3, 11) and this may have affected the relative cell growth rates and consequently influenced AP expression and AP activity. This is demonstrated by the sudden decrease in AP activity at the 800 mM NaCl treatment in Figure 1 for M63 grown cultures. Furthermore, AP specific activity was measured in the periplasmic fraction of *E. coli* C29 cells only, whereas cell samples of the Villarejo *et al* group were not fractionated. The presence of a *phoA::lacZ* fusion in the chromosome of the *E. coli* C29 strain used by Villarejo *et al*, and the absence of the fusion from the strain we applied may also contribute to differing data.

The 0 mM NaCl treatment acted as a negative control to expose possible environmental stresses other than osmotic stress. An increase in AP specific activity would indicate the presence of other environmental stresses (10).

In the case of the 0 mM M63 culture, AP specific activity increased over time (Fig. 2). Contrary to expectations, M63 cultures of higher NaCl molarities (200 mM, 400 mM or 800 mM) did not exhibit an increase in AP specific activity at 155 min. Likely, the 0 mM NaCl culture underwent a normal rate of cell division, and may have reached starvation state (10) at the 155 min time point. However, in higher NaCl concentration treatments, cells were osmotically stressed and therefore the rate of cell division may have been impeded and nutrients not depleted. Therefore, osmotically stressed cells avoided starvation stress.

Results of the AP specific activity time course of NB cultures differed from those of M63 cultures (Fig. 3). In NB cultures, ascent of AP specific activity over time in the 0 mM NaCl culture was not apparent, whereas, at higher NaCl concentrations (200 mM and 800 mM), AP specific activity gradually increased. Differing specific activity trends between NB and M63 treatments may be due to higher nutrient concentrations in NB that are able to sustain cell growth at the 155 min time point. In addition, a relatively constant AP specific activity in the 0 mM NaCl NB culture, suggests that NB cultures were not nutrient-starved. Because cells grown in NB were not starved, osmotic stress would have been responsible for any observed changes in AP specific activity. At the earlier time points, an osmoprotectant in NB may have allowed cells to evade AP expression induction. However, at later time points, the ratio of presumptive osmoprotectant to cells decreased as cells grew and divided. Therefore, at 155 min, conditions in which osmoprotectant was relatively dilute among cells and less effective, may have induced the observed increase in AP specific activity.

Our subsequent experiment aimed to test the effects of the previously described osmoprotectants proline and glutamate on AP specific activity in osmotically stressed cultures of *E. coli* C29. We chose to assay the extent of osmoprotection at 105 min post-osmotic stress induction, as it showed a linear increase in AP specific activity among M63 cultures, at NaCl concentrations of 200 mM to 400 mM (Fig. 1) that resembled the trend observed by Villarejo *et al* (12). In this experiment, NaCl concentrations of 200 mM, 300 mM and 400 mM acted as osmotic stress treatments, because AP specific activity displayed a linear increase over these concentrations. The additional reason for using 300 mM NaCl in place of 800 mM NaCl was the apparent growth-inhibiting effect of this salt concentration in M63 medium, as discussed above (Fig. 1).

Comparison of AP specific activity in M63 cultures without osmoprotectants, and M63 cultures containing either proline or glutamate demonstrate an unexpected increase in AP specific activity in response to presence of amino acids in the medium (Fig. 4). The basal level of AP specific activity (in 0 mM NaCl cultures) was 10- to 15- fold higher in proline and glutamate containing cultures, than in non-supplemented cultures. Moreover, neither proline nor glutamate reduced AP specific activity among osmotically stressed cells (200 mM, 300 mM and 400 mM NaCl). Both proline and glutamate have been previously described as osmoprotectants, and other researchers have demonstrated that the addition of proline to osmotically stressed cultures, at concentrations as low as 0.2 mM provided significant osmoprotection (5, 12). Therefore, the increase in AP specific activity in NaCl supplemented M63 cultures is unlikely due to osmotic stress, but instead due to another factor, such as nutrient limitation. Further, we cannot deduce that the increase in AP specific activity was due to added amino acids alone, as AP specific activity immediately upon adding either proline or glutamine was not measured as an internal control to assess background AP specific activity. This highlights the potential for experimental set-up optimization.

Apparently, experimental conditions induced at least two different stresses. Osmotic stress was introduced with intent and data evidently supports the hypothesis that one or more components of NB medium is an osmoprotectant and thus, decreases AP specific activity (Fig. 3). Inadvertently, starvation stress was also induced. Addition of either proline or glutamate did not seem to alleviate starvation, as deduced from AP specific activity.

Qualitative assessment of the amount of AP in isolated periplasmic fractions was attempted through SDS-PAGE (Fig. 5). By adjusting the overall lane intensities to account for differences in total protein concentrations, it appears that all lanes contain similar levels of presumptive AP. Therefore, osmotic stress may affect AP specific activity through catalytic modifications rather than influencing enzyme expression. This hypothesis would explain why an increase in AP specific activity (Fig. 4) was not reflected by an increase in protein levels (Fig. 5). However, our proposal is visually inferred, and a more accurate intensity adjustment could be performed digitally.

The assumption that the Bradford assay did not accurately determine protein concentration could alter calculated AP specific activities. Unlike AP specific activities, total protein concentrations are not required to calculate AP activities. Therefore, AP activity calculations were done on all samples (data not shown) and similar patterns of AP activity changes were observed.

With no error bars, assessment of the significance of the observed changes is difficult to deduce. We decided to deem the changes significant when the observed values differ by a factor of four or more. Similarly, as none of the experiments were repeated, the reproducibility of the results has not been established.

AP specific activity is regulated in a very complex manner and it is hard to discern between osmotically induced regulation and regulation induced by other factors. However, our results clearly demonstrated that under test

conditions, osmoprotectants stimulated AP specific activity, while cultures grown in minimal M63 medium tended to have higher AP specific activity than cultures grown in rich media NB. This indicates nutrient limitation may also increase AP specific activity.

FUTURE EXPERIMENTS

The inability to distinguish between osmotic stress and starvation stress made it difficult to propose a single explanation for increased AP activity in nutrient limited media. This was in part due to a strong reliance on the results reported by Villarejo *et al.,* which led us to overlook potential pitfalls. To account for nutrient limitation, a follow-up experiment, utilizing a continuous culture to incur nutrient homeostasis, and eliminate the starvation variable, is advised.

In addition, the presumptive AP band in the gel was not positively identified. It would be beneficial to confirm the presence of the AP band in the gel to aid in interpreting results. Also, quantification of the intensity of the bands would make expression levels of AP proteins more comparable. This would help to distinguish between the effects of osmotic stress on AP expression versus activity.

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