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Periplasmic Alkaline Phosphatase Activity and Abundance in *Escherichia coli* B23 and C29 during Exponential and Stationary Phase

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Alkaline phosphatase (AP), encoded by *pho*A, is a periplasmic, homodimeric protein that is responsible for the hydrolysis of phosphate molecules from non-transportable organophosphates. Osmotic shock was employed to specifically isolate periplasmic proteins from *E. coli* C29 constitutive AP strain during exponential and stationary phase growth. Assays were performed for AP activity and abundance. Our findings confirm previously observed results that levels of AP specific activity and protein abundance increase in stationary phase compared to exponential phase growth. In addition, we examined AP trends in wild type *E. coli* B23 to determine whether the observed increase in AP activity and protein levels in *E. coli* C29 was the result of modified AP regulation at the transcriptional level. The rapid increase in AP specific activity at the onset of stationary phase in *E .coli* B23 suggests an induction event that may be due to *pho*A up regulation mediated by phosphate starvation. σ^s-dependent global down-regulation of protein synthesis in stationary phase may contribute to higher AP specific activity due to a proportional increase of AP relative to the decreasing amount of cellular proteins.

Alkaline phosphatase (AP) is a homodimeric, nonspecific phosphomonoesterase that is produced by *Escherichia coli* in phosphate-deficient environments (2). The function of AP is to scavenge inorganic phosphate for the cells' survival by deconstructing phosphorylated compounds in the bacteria's surroundings (4). The 50 kDa AP monomeric subunit is first produced in the cytoplasm and is transported to the periplasm due to a specific signal sequence at the N terminal which is then cleaved off during the secretion process (9). Once outside the cytosol, the AP subunits naturally form a dimer through disulphide bond interaction. Dimerization cannot be done inside the cell as the reducing environment of the cytosol prevents the formation of the disulfide bonds, which serves as a safeguard mechanism against self-damage (8).

The *pho*A gene which encodes AP is normally regulated and is not constitutively expressed due to the destructive nature of the AP protein (2). The *pho*A gene is part of the Pho regulon and its expression is controlled by PhoBR, a two-component regulation system that involves PhoR, a phosphate-sensory protein, and PhoB, a transcriptional activator (2). Under low phosphate conditions in the environment, PhoR will first auto-phosphorylate and then transfer the phosphate group to phosphorylate PhoB. This chain of events will trigger PhoB's activation role for AP gene expression and other phosphate-regulated genes in the Pho regulon (2). The phosphorylated activation of PhoB can also be completed by the phosphorylated form of CreC kinase (18). However, the interaction between PhoB and CreC activates AP expression at a slightly lower level compared to the interaction between PhoB and PhoR (19). Furthermore, CreC responds to carbon sources and can only activate PhoB in the absence of PhoR (19).

A previous investigation had shown an increase in AP specific activity and protein level during stationary phase growth compared to exponential phase in *E. coli* C29 (5). This particular strain constitutively expresses AP due to a combination of a mutation in the *cre*C gene that causes the CreC protein to be permanently phosphorylated and a null mutation in the *pho*R gene (18). The previous investigation had proposed that the apparent increase in AP specific activity in stationary phase may be influenced by incomplete lysis of stationary phase cells leading to an increased proportion of periplasmic proteins, such as AP, and thus an underestimation of total cellular protein.

This study attempted to verify previous results by employing osmotic shock to selectively release periplasmic proteins from *E. coli* C29 in stationary and exponential phase (11). To evaluate the effectiveness of the technique, AP activity was assessed in periplasmic, cytoplasmic, and culture media fractions. In addition, beta-galactosidase activity was monitored in periplasmic fractions to demonstrate that the cellular proteins were not being released during osmotic shock. When repeating the experiment of Chou et al. (5) using the periplasm-specific lysis technique, results showed an increase in AP specific activity and relative protein levels in stationary phase.

In parallel to the validation study, wild type *E. coli* B23 with normal regulation of AP expression was also

studied because there were concerns as to whether the dysregulation of AP expression in *E. coli* C29 was influencing the levels of AP during stationary phase. Cells enter the stationary phase of growth when the available nutrients, such as phosphate, become exhausted. Thus, it was anticipated that AP expression would not be induced in wild type *E. coli* B23 until the onset of stationary phase. Results from this study have validated the anticipation by demonstrating a sharp increase in AP expression during stationary phase.

MATERIALS AND METHODS

Cultivation and sampling of *E. coli* strains C29 and B23. *E. coli* strains C29 and B23 were obtained from the MICB 421 culture collection at the University of British Columbia, Department of Microbiology and Immunology. *E. coli* C29 (Hfr PO 12.20' CW *tonA22, phoR19* (constitutive) *ompF627* ($T2^R$) *fadL701* ($T2^R$) *relA1 pit-10 spoT1 rrnB-2 mcrB1 creC510 Shu A22*) is a mutant strain that constitutively produces AP due to a combination of a loss of function mutation of an inhibitory AP expression regulator and a mutation that causes permanent activation of an AP expression activator. *E. coli* B23 does not have any mutations so wild type expression levels of AP are expected. To start the growth curve, overnight cultures were inoculated into fresh Luria-Bertani broth (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.4) with a starting turbidity of 0.30-0.35 OD_{660}. Both cultures were stimulated with IPTG (280 µg/ml) and grown aerobically at 37°C on a shaking incubator at 150 rpm. Optical density readings were measured at 660 nm (Spectronic 20D+, Milton Ray) every 0.5 h from 0 to 6 h. Exponential and stationary phase time points were estimated from the growth curve generated by the optical density readings. Accordingly, 30 ml samples were removed from each culture at 0.5, 1.5, and 2 h for exponential phase time points, and at 3, 5, and 6 h for stationary phase samples. The 30 ml samples were centrifuged at 11700 x *g* for 5 min at room temperature. The culture media was removed and stored at -20°C for further analysis. The cell pellet was resuspended in a total volume of 1 ml chilled Tris-HCl (10 mM, pH 8.0) and microfuged at 11700 x *g* for 5 min at room temperature. The Tris-HCl was removed and the cell pellet was resuspended in 0.7 ml Tris-HCl and stored at -20°C for further analysis. In addition, plate counts were performed at the six sampling time points to confirm culture viability during stationary phase.

Osmotic shock. Periplasmic proteins were isolated by osmotic shock as previously described (11), with slight modifications. Culture samples were thawed on ice and microfuged at 11700 x *g* for 5 min at 4°C. The supernatant was removed and the cell pellet was resuspended in 1 ml of 20% w/v sucrose-0.03M Tris-HCl (pH 8.0). Then 0.25 ml of 5 mM disodium EDTA (pH 8.0) was added to each sample and mixed on a rotating shaker (Orbit Shaker, Lab-Line) at 180 rpm for 10 min at room temperature. Afterwards, cells were pelleted at 13000 x *g* for 10 min at 4°C. The supernatant was removed and the pellet was resuspended in 1.25 ml of ice-cold distilled water and mixed on a rotating shaker at 180 rpm for 10 min in an ice bath. After the 10 min incubation in the ice bath, the cells were then pelleted again at 13000 x *g* for 10 min at 4°C. The supernatant containing the periplasmic fraction was removed and stored in a microfuge tube at -20°C until protein assays could be completed. The pellet containing the cytoplasmic contents was stored on ice for further processing.

Bead bashing. Cytoplasmic proteins were isolated using the bead bashing method as previously described (13). The supernatant containing the isolated cytoplasmic proteins was collected and stored at -20°C until protein assays could be completed.

Determination of AP and β-galactosidase enzyme activity. Both enzyme assays were performed on the culture media samples, the periplasmic samples, and the cytoplasmic samples. AP assays were performed as previously described (12). Detection of β-galactosidase was included as a negative control for the osmotic shock method and this cytoplasmic enzyme was assayed as previously described (13). Specific activity of both AP and β-galactosidase was calculated by dividing sample enzyme activity by the respective sample protein concentration as determined by the Bradford assay as previously described (13).

SDS-PAGE and Western blot analysis. The maximum constant amount of protein at all time points for each strain was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% acrylamide gel as previously described (4.5 µg/lane for strain B23 versus 15.6 µg/lane for strain C29) (13). The gel was run at 200 V for 1 h and then at 10 V for 10 min. Protein samples and PageRuler Prestained Ladder (Fermentas Cat. # SMO671) were transferred at 100 milliamps for 60 min to a Hybond-P membrane (Amersham Biosciences Cat. # RPN1416F) in a BioRad Transfer Blot Cell (Serial #19586) with transfer buffer (25 mM Tris, 0.2 M glycine, 20% v/v methanol). Following transfer, the membrane was blocked overnight in 5% (w/v) skim milk powder in TBST (0.1% Tween 20 in TBS (50 mM Tris pH 7.5, 150 mM NaCl)) at 4°C. After a brief wash in TBST, the membrane was probed overnight with a 1:10000 dilution of rabbit polyclonal anti-*E. coli* alkaline phosphatase IgG (Chemicon Cat. # AB1204) at 4°C on a rotating shaker (Orbit Shaker, Lab-Line) at 180 rpm. The membrane was then washed twice with TBST for 5 min and incubated with a 1:10000 dilution of horseradish peroxidase-linked goat anti-rabbit IgG (Chemicon Cat. # AP132P) for 1 h and 45 min at room temperature with rotational shaking at 180 rpm. Both primary and secondary antibody dilutions were performed in TBST. The membrane was then washed 3 times for 5 min in TBST followed by chemiluminescent detection using the ECL Western Blotting Detection Reagent (Amersham Biosciences Cat. # RPN2106) according to the manufacturer's protocol. The membrane was exposed to Bioflex Scientific Imaging Film (Clonex Corporation) for 1, 2, 5, and 30 min. Densitometry of protein bands was performed using the Spot Densitometry program provided with the Alpha Imager Gel-doc (Alpha Innotech).

RESULTS

***E. coli* B23 and *E. coli* C29 growth curves.** Late exponential growth of the wild type strain *E. coli* B23 and the AP constitutively active strain *E. coli* C29 was observed between 0 and 3 h post-inoculation (Fig.1). The onset of stationary phase was observed at approximately 3 h post-inoculation, with *E. coli* C29 and *E. coli* B23 growth stabilizing at 1.0 OD_{660} and 1.1 OD_{660} respectively (Fig. 1). Stationary phase persisted until the termination of the experiment, as turbidity values remained relatively constant between 3 and 6 h post-inoculation.

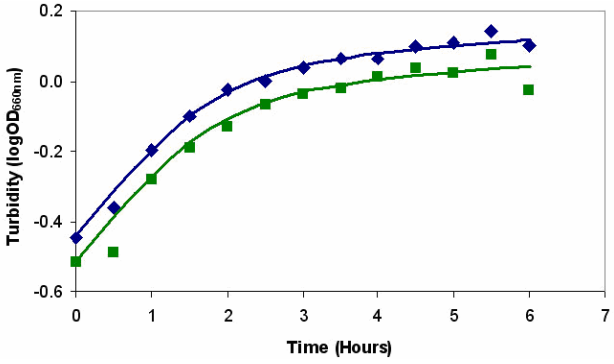

FIG. 1. Growth of *E. coli* B23 (♦) and C29 (■) in late exponential (0 to 3 h) and stationary phase (3 to 6 h). Cells were cultured at 37°C in Luria Bertani Broth with mild aeration. The optical density at 660nm was monitored every 0.5 h over a 6 h period.

Alkaline phosphatase specific activity. *E. coli* B23 exhibited a sudden increase in AP specific activity in the periplasmic fraction at 3 h post-inoculation. This increase in activity resulted in an 8-9 fold increase in AP activity at 6 h post-inoculation with a specific activity of approximately 77 munits/mg as compared to 9 munits/mg at 3 h post-inoculation (Fig. 2A). Since AP is located in the periplasm, cytoplasmic fractions exhibited near non-detectable levels of AP specific activity throughout the experiment (Fig. 2A).

E. coli C29 exhibited much higher overall levels of AP specific activity than wild type, but retained the same overall patterns. AP specific activity in periplasmic fractions increased with time, ultimately displaying higher specific activity in stationary phase cultures compared to their exponential phase counterparts (Fig. 2B). Minimal periplasmic AP specific activity (30 munits/mg) was observed in exponentially growing cells at 0.5 h post-inoculation. AP activity gradually increased until it reached a maximum at approximately 1100 munits/mg of AP activity in stationary cells at 5 h post-inoculation. Unlike the observations for *E. coli* B23 samples, this increase was gradual and was initiated earlier at 0.5 h post-inoculation instead of
3 h post-inoculation. This increase in AP activity was not observed in cytoplasmic fractions, which retained their basal AP activity levels far lower than that observed in periplasmic fractions (Fig. 2B). Throughout all sampling time points, the culture media of *E. coli* C29 exhibited extremely low levels of extracellular AP activity that were below the threshold of detection (data not shown).

Alkaline phosphatase relative protein levels. Western blot analysis of AP in exponential and stationary phase cells was performed to investigate whether there was a correlation between AP specific activity and AP protein levels. AP levels in the cytoplasmic (data not shown) and periplasmic fractions (Fig. 3) of *E. coli* B23 wild type were too low for western blot detection. The *E. coli* C29 constitutive strain exhibited intense bands corresponding to ~50 kDa AP in all periplasmic samples, with the 0.5 h post-inoculation as the only exception (Fig. 3). Differences in band intensity were not observable by eye, and therefore relative AP abundance was evaluated via spot densitometry. Densitometric analysis revealed an increase in AP abundance in the periplasmic fraction of *E. coli* C29 through time (Fig. 3). For both strains, culture media was also subjected to western blot analysis, but the corresponding AP levels at all sampling time points were too low for detection (data not shown).

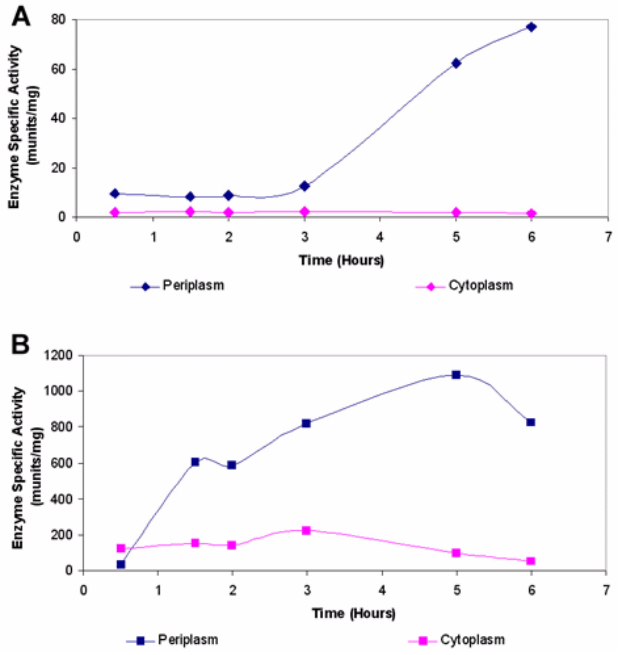

FIG. 2. Increase in alkaline phosphatase specific activity in stationary phase of *E. coli* strains. Specific activity of alkaline phosphatase was determined in late exponential (0 to 3 h) and stationary phase (3 to 6 h) of periplasmic and cytoplasmic fractions derived from *A) E. coli* B23 (♦) and *B) E. coli* C29 (■). Samples were taken at 0.5, 1.5, 2, 3, 5, and 6 h.

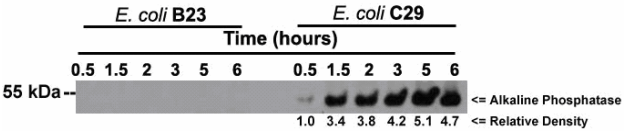

FIG. 3. Increase in relative alkaline phosphatase levels of *E. coli* C29 during stationary phase. Western blot analysis of the relative levels of alkaline phosphatase derived from the periplasm of *E. coli* B23 and *E. coli* C29 in late exponential (0 to 3 h) and stationary phase (3 to 6 h). Band densities were determined relative to the lowest integrated density value of the 0.5 h sample using Alpha Imager Gel-doc.

DISCUSSION

Previous experiments (5) revealed that there was an increase in AP activity and protein abundance in cell lysates of the constitutively active *E. coli* C29 strain during stationary phase. However, there were concerns as to whether the bead bashing method that was employed failed to effectively release AP from stationary phase cells due to cellular modifications (5). Stationary phase cells are reported to be more resistant to mechanical lysis due to a highly structured, cyclopropyl fatty acid rich inner membrane (3, 7). Thus, incomplete lysis of gram negative bacteria would lead to an increased proportion of periplasmic proteins,

when compared to total protein amounts, such as AP in cell lysates, and subsequently result in an overestimation of AP specific activity. In our study, we attempted to validate the results of Chou et al. (5) and further investigate AP activity in stationary phase *E. coli* cells by employing osmotic shock for the isolation of AP. The use of osmotic shock was found to be selective for periplasmic proteins of *E. coli* and was equally effective for both exponential and stationary phase cells (11). To evaluate the effectiveness of osmotic shock in releasing periplasmic specific AP, cytoplasmic fractions were also retained and assayed for AP activity. Comparatively lower AP specific activity observed in cytoplasmic fractions than periplasmic fractions in both B23 and C29 strains indicate specificity in lysis and fractionation techniques (Fig. 2A, 2B).

In our study, we observed that the periplasmic fractions of the constitutively active *E. coli* C29 strain had higher AP specific activity and relative AP protein levels in stationary phase compared to growth in exponential phase (Fig. 2B, Fig. 3). Overall, these results agree with the findings reported by Chou et al. (5), despite the different lysis methods that were used in the isolation of AP. By employing a lysis method that specifically isolates periplasmic proteins, our findings demonstrate that the higher AP specific activity observed in stationary phase *E. coli* C29 cells was not the result of incomplete cell lysis. Instead, the increase of AP abundance and subsequent activity observed in strain C29 is more likely the result of accumulation of AP in the periplasm, as the rate of constitutive AP synthesis may exceed the rate of cell division. Cell division occurs less frequently during stationary phase (1). According to this model, one would expect a gradual increase of AP activity and protein abundance over time, which agrees with our findings (Fig. 2B, Fig. 3). Our results show an increase in both AP specific activity and relative AP protein levels, with the exception of the 0.5 h time point where we believe low AP activity and protein levels were the result of a technical error. Resuspension of the cell pellet in the osmotic shock solution and ice-cold water at that time point was difficult; therefore, AP may not have been successfully extracted for the periplasmic fraction, resulting in an underestimation of AP activity. The data from the first time point in the mutant strain was expected to be higher than that of the wild type, which differs from the results shown in Fig. 2. Therefore, the data from that time point is suspicious and should be repeated to validate the observations.

To further this study, we investigated the possibility that the observed increase in AP activity and abundance in *E. coli* C29 was the consequence of AP dysregulation at the transcriptional level. By using the wild type *E. coli* B23 strain, we were able to examine AP under the control of its natural regulatory components. At the onset of stationary phase, periplasmic fractions isolated from the *E. coli* B23 strain exhibited a sudden and continual increase in AP specific activity (Fig. 2A). However, the corresponding amount of AP protein in the periplasmic fractions of wild type cells was undetected by western blot analysis, in contrast to the overwhelming amount found in constitutive cells (Fig. 3). This discrepancy is most likely due to the low amount of total protein loaded for wild type samples in comparison to constitutive samples. Nonetheless, the abrupt increase in AP activity from the assay results suggests an induction event where the cell switches its regulation patterns.

This apparent increase in AP specific activity and protein abundance in cells during stationary phase may be a physiological response to starvation stress known as the global stringent response. Characteristics of stationary phase cultures, which are regulated by the product of the *rpo*S gene σ^s, include reduced metabolic activity, cessation of cell division, and resistance to environmental stresses though adjustments in the cell wall (6, 15, 17). In addition, starvation for inorganic phosphate (P_i) in stationary phase would trigger the induction of genes pertaining to the Pho regulon, including AP, in an attempt to release P_i from phosphorylated compounds (16). Thus, if cell division is reduced in stationary phase as a stress response, but AP is still being made due to P_i starvation, the relative levels of AP would be higher than that of total cellular protein.

This proposed model then presents two opposing mechanisms working at stationary phase. While σ^s-induced stationary phase gene expression attempts to shut down the majority of protein synthesis in preparation for survival during the stringent response, the Pho regulon is induced due to external factors of depleted P_i (16). In addition, σ^s is targeted by ppGpp, an alarmone which promotes transcription from σ^s-dependent promoters (6). Levels of ppGpp are increased during nutrient starvation and the activity of σ^s is positively regulated. However, there are conflicting reports as to whether ppGpp synthesis is induced in P_i-starved cells (6, 10). Taschner et al. (16) have reported that σ^s negatively regulates the expression of *pho*A by out competing σ^D, which is required for transcription of genes in the Pho regulon. This finding appears to contradict our results in which AP specific activity and protein levels are increased in stationary phase. Although we did not directly investigate AP at the transcriptional level, it would be of interest to investigate the levels of *pho*A transcript with the activity and abundance of its functional enzyme product in exponential and stationary phase.

In conclusion, we demonstrated that the levels of AP activity and protein in *E. coli* C29 were elevated in stationary phase compared to exponential phase, thus confirming that the previous results of Chou et al. was not due to an artifact of incomplete stationary lysis (5). We further demonstrated that the increase in AP activity and abundance is not the result of incomplete cell lysis, as we assayed both the periplasmic and cytoplasmic fractions, which were obtained using specific isolation techniques. Instead, we hypothesize that the observed increase in AP specific activity and protein level is due to accumulation of AP in the periplasm. Accumulation of AP occurs as the rate of constitutive protein synthesis eventually exceeds the rate of cell division, thus resulting in a gradual increase in AP over time. In contrast, our results show an abrupt rather than gradual increase in AP activity in *E. coli* B23 during stationary phase. This sudden increase suggests that an induction event has occurred, with two response mechanisms at play. We speculate that decreased cell division due to a stress response, in conjunction with increased AP production mediated by P_i starvation, are responsible for the observed increase in AP specific activity in *E. coli* B23.

The significance of this study regarding AP expression and functional activity is useful for optimizing conditions for AP isolation, which is relevant in industrial production of AP for various biotechnological applications. For example, AP is used in conjunction with antibody-mediated targeting in chemotherapeutic treatments (14). The anti-tumor prodrug etoposide-phosphate requires the cleavage of a phosphate group by AP to convert it into its active form, etoposide, at the desired target location (14). Our study demonstrated that higher AP expression and activity is induced during stationary phase in *E. coli*. Therefore, stationary phase *E. coli* might be the preferred system for optimizing AP production.

FUTURE EXPERIMENTS

The western blot showing the relative levels of alkaline phosphatase derived from the periplasm should be repeated in order to validate whether there is an increase in protein abundance in wild type *E. coli* B23 during stationary phase, as seen in the constitutive strain. To improve the results, the proteins from the wild type strain should be concentrated before undergoing SDS-PAGE gel electrophoresis, which can be done using ultrafiltration.

A major speculation in our study is that the activation of AP production in *E. coli* B23 is due to the exhaustion of available phosphate in the bacteria's surroundings during stationary phase. To provide evidence of this correlation, the growth and sampling experiment for *E. coli* B23 could be repeated in the presence of radioactive-labeled phosphate in a controlled medium where the phosphate content is known. The labeled phosphate would allow monitoring of phosphate reduction from cellular activities. If the conjecture from this study is correct, the increase of AP expression during stationary phase would occur in parallel to the depletion of phosphate in the cells' surroundings.

Previous investigations reported that the presence of σ^s, a stationary phase gene regulator, may suppress *pho*A transcription (16); however, increased levels of AP activity and protein abundance were observed in our study. A search for a σ^s promoter in upstream sequence of the *pho*A gene will confirm whether *pho*A transcription is indeed controlled by σ^s. If the promoter was located for *pho*A, deletion of this region should remove the regulating effects of σ^s and increase *pho*A transcription. To further elucidate whether *pho*A transcription is suppressed during stationary phase, Northern blot analysis could be conducted. RNA could be isolated from exponential and stationary phase cells and hybridization would be done using DNA probes specific for the *pho*A gene. A quantitative RT-PCR experiment measuring the expression of *pho*A mRNA could also be employed. By assessing the abundance and function of AP through protein and enzyme assays in parallel, we could decipher whether the stringent response during stationary phase has differential effects on AP at the transcriptional and translational level.

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