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Escherichia coli C29 Alkaline Phosphatase Enzyme Activity and Protein Level in Exponential and Stationary Phases

JACK CHOU, JENNIFER MORSE, STEPHANIE OMEIS, AND ERIK S. VENOS
Department of Microbiology and Immunology,UBC

Alkaline phosphatase is a homodimeric periplasmic protein that catalyzes the hydrolysis of phosphate molecules from non-transportable organophosphates. The *Escherichia coli* (*E. coli*) C29 strain constitutively produces alkaline phosphatase; however, it has been previously reported that alkaline phosphatase enzyme activity ceases during stationary phase. The aim of this study was to confirm these previous results by investigating the trends of alkaline phosphatase specific activity and protein levels throughout exponential and stationary phases. *E. coli* C29 cells were assayed for alkaline phosphatase activity and protein levels throughout 120 hours of culture. Contrary to the previous observation, the alkaline phosphatase activity in *E. coli* C29 did not decrease upon entry into stationary phase but, rather, increased following exponential growth. Moreover, the increase in alkaline phosphatase activity in stationary phase did not correspond to an equivalent increase in the amount of alkaline phosphatase protein. Further investigation into this phenomenon would assist in the elucidation of these trends in alkaline phosphatase activity observed over time in *E. coli* C29.

Alkaline phosphatase (AP) is a homodimeric periplasmic protein comprised of 50kDa subunits and is encoded by the *phoA* gene of *E. coli* (5, 6). As its name indicates, AP functions optimally at an alkaline pH and catalyses the hydrolysis of a phosphate molecule (P_i) from non-transportable organophosphates (11, 17). Normally, transcription of the *phoA* gene is upregulated under conditions of limiting environmental P_i while small amounts of transcription occur under conditions of excess environmental P_i. P_i is an essential constituent of nucleic acids and phospholipids; thus, the enhanced production of AP during P_i limitation allows the *E. coli* to scavenge P_i from the environment and continue to divide.

P_i-dependent *phoA* transcription is regulated by a two-component signal transduction system, PhoBR, which consists of PhoR as the kinase/phosphatase and PhoB as the response regulator (17). Phosphorylated PhoB binds to a specific sequence in the promoter of the *pho* regulon and activates transcription of several genes, including *phoA* (17). Upstream of the PhoBR system, the Pst transporter subunit PstS serves as the sensor of extracellular P_i levels and negatively regulates PhoR activation under high P_i conditions through the action of PhoU (17). In addition, PhoB activation can be regulated independently of environmental P_i levels. CreC is a kinase involved in the poorly-defined CreBC two-component regulatory system, which is thought to be modulated by the type of available cellular carbon source and has also been reported to phosphorylate PhoB and activate the *pho regulon* (2, 17).

E. coli strains that possess a PhoR19 null mutation and CreC510 mutation, which results in the constitutive phosphorylation of CreC, have been reported to produce AP at an intermediate level between the extremes of strong AP production in limiting P_i conditions and weak AP production in excess Pi conditions (16). A molecular basis for these observations has been proposed where the PhoR19 mutation would prevent activation of PhoB by PhoR in response to P_i-limiting conditions (17). The CreC510 mutation would result in constitutively activated CreC and thus constitutively activated of PhoB regardless of the growth phase and independent of environmental P_i levels (17). Interestingly, the *E. coli* C29 strain, which contains PhoR19 and CreC510 (17), has been observed to deviate from this behavior. When growing in modified Luria broth with glycerol as the carbon source, the initial AP activity observed in log phase of growth disappeared after 72 hours of growth, a time when the culture would be in stationary phase (W. D. Ramey, personal communication). This study attempted to test a model for this phenomenon whereby the levels of AP protein remained the same but the AP specific activity decreased during stationary phase due to the inhibitory stationary phase conditions.

MATERIALS AND METHODS

***Escherichia coli* C29 Cultivation.** *E. coli* C29 E. coli C29 (tonA22, PhoR19, ompF62, relA1, pit-10, spoT1, T2r, Hfr(PO2A)) was obtained from a laboratory stock from the University of British Columbia Department of Microbiology. The cells were cultured aerobically in modified Luria broth (10 g/l tryptone, 5 g/l yeast extract, 5 g/L NaCl, 0.4% (w/v) glycerol, pH 7.4). Optical density of the culture was measured at 660nm (Spectronic 20D+, Milton Ray) every 0.5 hours from 0-5 hours and at 24, 48, 72, and 120 hours. Additionally, at these times 15 ml samples were removed from the culture, pelleted by centrifugation at 11700 x *g* for 5 minutes, and

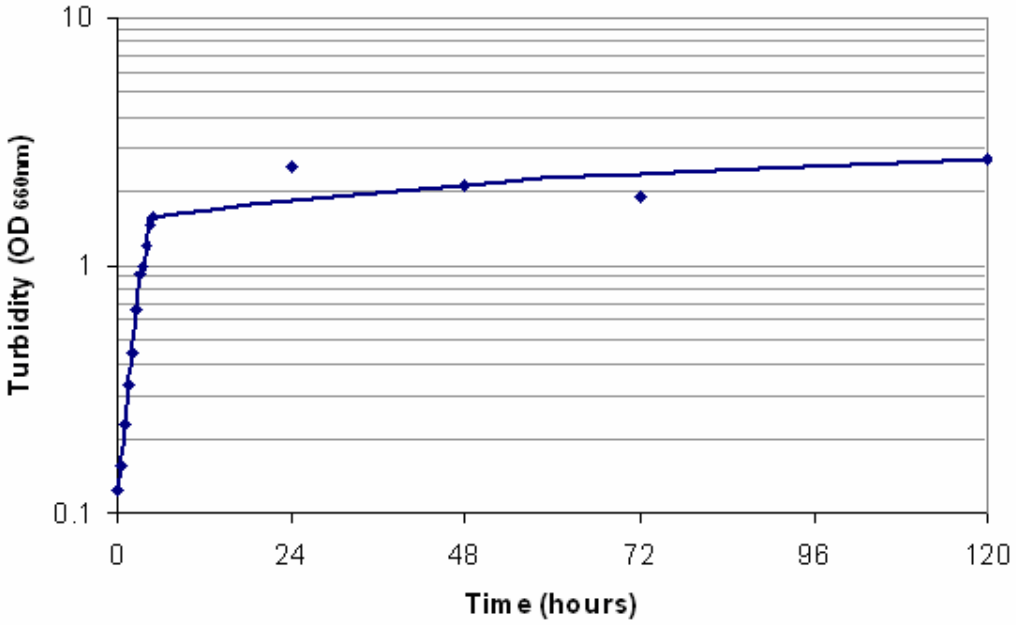

FIG. 1 Growth curve of *E. coli* C29 culture over a 120 h period. Cells were cultured in modified Luria broth with 0.4% glycerol at 37°C with mild aeration. The optical density at 660nm was monitored from time 0 to 120 hours.

resuspended in Tris (10 mM, pH 8) and stored at -20°C until lysis and enzyme assay, a period of about one week.

Preparation of Cell Lysate. Cells were broken by adding an equivalent volume of 0.1 mm diameter glass beads (Cat.# 11079105, Biospect) to the thawed cell pellets resuspended in Tris buffer (10mM, pH 8). The suspension of beads and cells were then mechanically disrupted using the FastPrep FP120 (Thermo Electron Corp.) at speed setting 6.5 for 45 seconds. After bead bashing, the cell lysate was cooled on ice for 10 minutes followed by centrifugation at 13800 x *g* for 2 minutes. Supernatants were then harvested for subsequent experimental procedures.

AP Enzyme Activity Assay. Alkaline phosphatase (AP) assays were performed as previously described (14). Specific activity of AP was determined using sample enzyme activity normalized by the sample protein concentration as determined by Bradford assay (4).

SDS-PAGE and Western Blot Analysis. Proteins in cell lysates from equivalent total protein, as determined by the Bradford assay (4), were separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE), as described previously (10). Silver staining of SDS-PAGE gels was performed using the Bio-Rad Silver Stain Plus Kit (Cat.# 161-0449, Bio-Rad) as per the manufacturer's instructions. Proteins and PageRuler Prestained Ladder (Cat.# SM0671, Fermentas) were electrophoretically transferred at () V for 90 min onto a pure nitrocellulose membrane (Cat# 1703956, Bio-Rad) in transfer buffer (2.5 M Tris Base, 0.2M glycine and 20% (v/v) methanol). After transfer, the membrane was blocked with 5% (w/v) skim milk powder in 50mM Tris and 150 mM NaCl at pH 7.4 overnight at 4°C with shaking. The blot was subsequently probed with rabbit polyclonal anti-*E. coli* alkaline phosphatase IgG (Cat.# AB1204, Chemicon) at a dilution of 1 in 10000 in TBS-T (50mM Tris, 150 mM NaCl and 0.05% (v/v) polyoxyethylene sorbitan monolaurate/Tween 20, pH 7.4) for 3h at room temperature with shaking. Following washing 3 times for 10 min in TBS-T, the blot was then incubated with horse-radish peroxidase-linked goat anti-rabbit IgG (Cat.# AP132P, Chemicon) at a dilution of 1 in 1:10000 in TBS-T for 45 min at room temperature with shaking. The blot was washed 3 times for 10 min in TBS-T followed by chemiluminescent detection using the Enhanced Chemiluminescent Kit (Cat.# RPN2108, Amersham) according to the manufacturer's instructions. Light emitted from the blot was captured using auto radiographic X-ray film (BioMax ML, Kodak) after an exposure time of 1-10 seconds, and was quantified using an AlphaImager accompanied by AlphaEase FC Image Analysis Software (Alpha Innotech).

Statistical Analysis. Group means were compared using a two-sample, two-tailed, t-test. P values were considered significant at a value of 0.05.

RESULTS

***E. coli* C29 Growth Curve.** Cells were observed in exponential phase during the first 5 hours of growth. The turbidity reached a plateau by 24 hours and remained relatively constant between 24 and 120 hours (Fig. 1) at which time the culture was considered to be in stationary phase.

Alkaline Phosphatase Specific Activity. At specific time points, culture samples were pelleted, lysed, and assayed for AP activity. The data demonstrated that AP specific activity was higher in stationary phase culture compared to that of exponential phase (Fig. 2A). As the culture entered stationary phase, AP specific activity continued to increase, though at a lower rate compared to that observed in exponential phase. A comparison of the means of exponential phase and stationary phase AP specific activity demonstrated an increase of 3.7 fold ($p<0.001$) in the stationary phase samples (Fig. 2B).

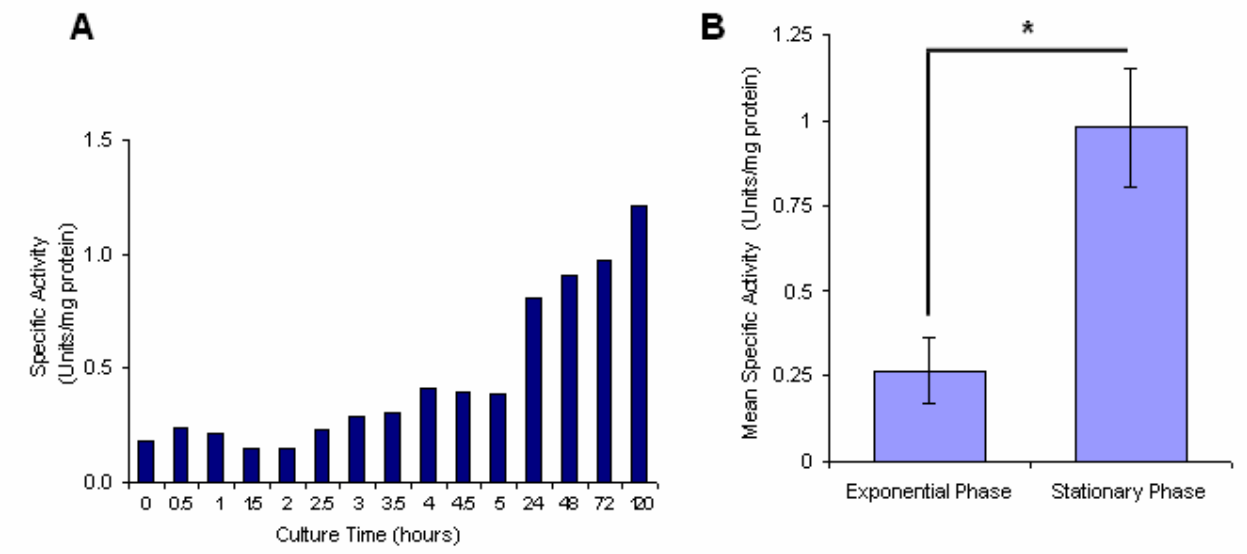

FIG. 2 Alkaline phosphatase specific activity in *E. coli* C29 cells over a 120 h period. *A)* Time course assay of AP specific activity in total cell lysates of *E. coli* C29. Culture conditions were as described in the legend to Fig. 1. *B*) Mean values of exponential phase (0 to 5h) (n = 11) and stationary phase (24 to 120h) (n = 4) AP specific activity ± standard deviation. *p<0.001.

Alkaline Phosphatase Protein Levels. To investigate whether the increase in AP specific activity correlated to increased levels of AP protein, the cell lysates were analyzed by Western blot and quantified by densitometry (Fig. 3A). Equal loading of total protein for each sample was controlled for by means of silver staining (Fig. 3C). From the immunoblot, a strong band at approximately 45 to 50kDa was present in all of the samples, putatively representing AP. By visual inspection, band intensity of AP protein remained relatively constant during exponential phase and increased at the beginning of stationary phase, remaining constant throughout (Fig. 3A). Densitometry analysis confirmed the visual trends observed. A comparison of the means of exponential phase and stationary phase relative AP levels demonstrated an increase of 0.6 fold ($p<0.001$) in the stationary phase samples (Fig. 3B).

Slightly above 45kDa in the silver stained SDS-PAGE gel a band is present in each of the samples which may correspond to AP (Fig. 3A). Additionally, though the gel showed approximately equal amounts of protein between lanes, the protein composition differed between the exponential and stationary phase samples. For example, below 31kDa a single band appears in the exponential phase samples but not in the stationary phase samples, and a doublet band appears in the stationary phase samples but not in the exponential phase samples.

Relative Ratios of Alkaline Phosphatase Specific Activity to Alkaline Phosphatase Protein Level. To determine whether the observed increase in AP specific activity correlated to the observed increase in AP protein the ratio of relative specific activity to relative AP protein was compared for each culture sample (Fig. 4A). A comparison of the means of exponential phase and stationary phase ratios demonstrated an increase of 2.1 fold ($p<0.001$) in the stationary phase samples (Fig. 4B).

DISCUSSION

Upon analysis of AP specific activity of the *E. coli* C29 culture over a 120 h period, the mean AP specific activity was found to be 3.7 fold higher in stationary phase compared to that of exponential phase (Fig. 2A, 2B). The relative levels of AP protein were also elevated in stationary phase (Fig. 3A, 3B); however, the ratio of specific activity to AP protein levels was not constant between samples (Fig. 4A, 4B). The mean ratio of stationary phase culture was 2.1 fold greater than that in exponential phase (Fig. 4B). This infers that AP specific activity in stationary cells increased to a greater extent than the AP protein levels.

The observed increase in both AP specific activity and AP protein levels may be the influenced by differences in the effectiveness of cell lysis between exponential and stationary phase cells. It has been reported that the stationary phase cells may be more resistant to mechanical lysis as the membranes are in a more highly ordered state due to membrane modifications such as increased amounts of cyclopropyl fatty acids (7, 9). As AP is a periplasmic protein, ineffective membrane lysis of the stationary phase cells may result in an underestimation of total

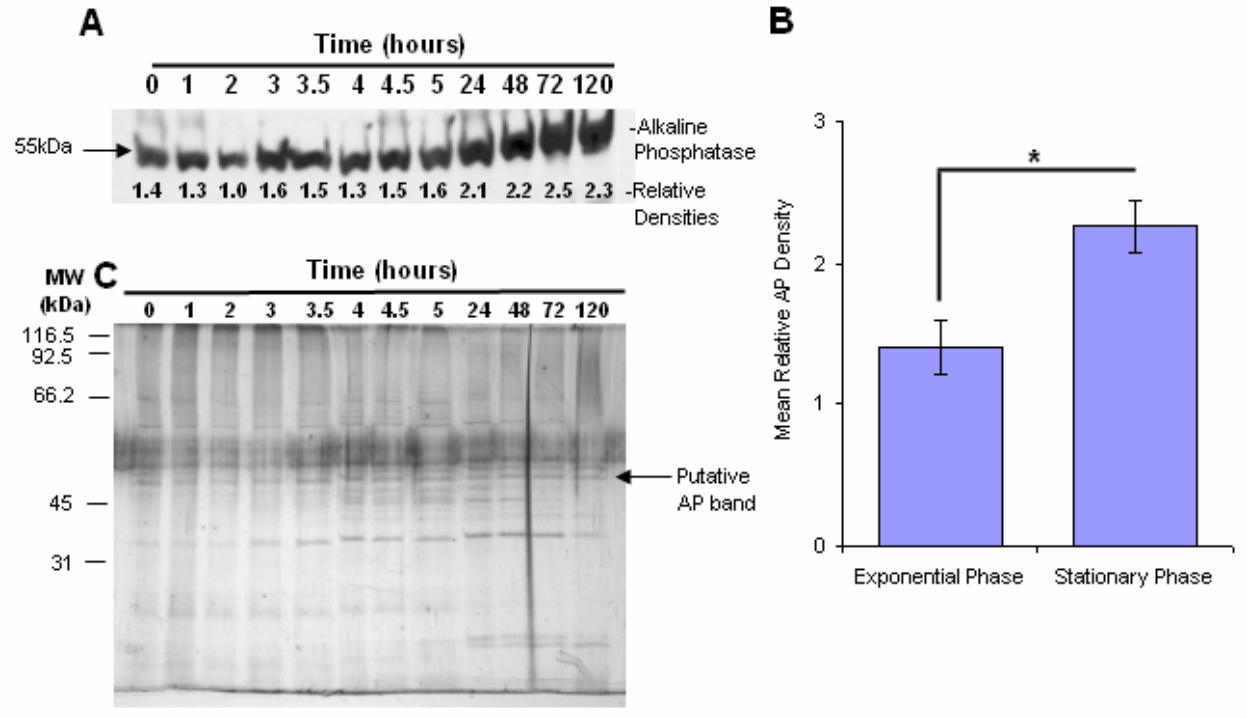

FIG. 3 Relative levels of alkaline phosphatase protein in *E. coli* C29 cells over a 120 h period. Culture conditions were as described in the legend to Fig. 1. *A)* Equal amounts of whole cell lysates (30µg/lane) were subjected to 10% SDS-PAGE, and then Western blot was performed using a specific antibody against *E. coli* alkaline phosphatase followed by a secondary antibody of anti-rabbit-HRP. Relative densities determined using Alpha Imager Gel-doc. Densities were determined relative to the lowest integrated density value (IDV). *B*) Mean values of exponential phase (0 to 5h) (n = 8) and stationary phase (24 to 120h) (n = 4) AP protein relative densities ± standard deviation. *p<0.001. *C)* Equal amounts of whole cell lysates (300ng/lane) were subjected to 10% SDS-PAGE followed by silver staining (10.5 minutes) the gel to visualize relative levels of total protein.

cell proteins and thus an overestimation of AP specific activity. Likewise, ineffective cell lysis would alter the relative distribution of AP protein in the prepared cell lysates resulting in misleading trend of increased AP protein levels in stationary phase samples (Fig. 3A).

An alternate explanation for the increase in AP specific activity and AP protein levels may be accumulation of the enzyme within stationary phase cells. As cells enter stationary phase, overall protein synthesis rates decrease (12). Since the synthesis of AP in *E. coli* C29 is constitutive (17), cells in stationary phase would have higher levels of AP proteins relative to the total cellular protein. Furthermore, as cell division rates decline during stationary phase, over time AP may continue to accumulate. This would again lead to an overestimation of both AP specific activity and AP protein levels in stationary phase samples. Evidence supporting this was provided by the differential banding patterns present between the exponential and stationary phase samples, suggesting that the samples' overall protein composition differed (Fig. 3C).

An unexpected result was the increase in ratio of relative AP specific activity to relative AP protein in stationary phase (Fig. 4A & 4B). This implies that the AP protein may have undergone modification between exponential and stationary phases, altering its enzyme activity or its ability to be detected by the primary antibody. Covalent modifications of enzymes that are common to bacteria include acetylation, phosphorylation, methylation, and disulfide bond formation (3, 8, 15, 18). The addition or removal of such modifications in stationary phase cells may have decreased the K_m of AP resulting in a higher level of activity compared to exponential phase cells. For example, *E. coli* AP requires the formation of disulfide bonds within subunits in the periplasm to become active (8). If in stationary phase, more disulfide bond formation occurred, presumably an increase in enzyme activity would be observed. In addition, if the exponential cells had a greater ratio of inactive subunits that were detected by the primary antibody, this would lead to a lower ratio of relative activity to relative density. Furthermore, other uncharacterized modifications may have altered the affinity of the primary antibody to AP in the stationary phase form, thus reducing the relative density of AP (Fig. 3A). In this study, it was demonstrated that the AP specific activity in *E. coli* C29 did not decrease in stationary

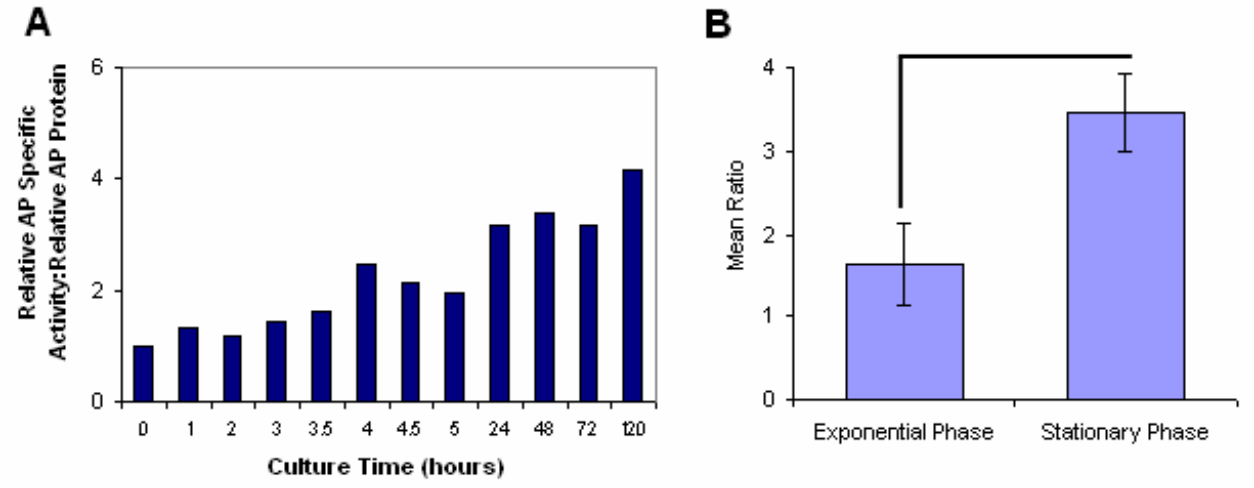

FIG. 4 Ratios of the relative AP specific activity to relative levels of AP protein in *E. coli* C29 cells. Specific activity and amount of AP protein were calculated relative to the 0 h sample. *A)* Relative ratios of AP specific activity to relative protein densities over a 120 h period. *B*) Mean values of exponential phase (0 to 5h) (n = 8) and stationary phase (24 to 120h) (n = 4) ratios of the AP specific activity to AP protein densities ± standard deviation. *p<0.001.

phase but rather increased as compared to exponential phase. This result may be due to the lysis method employed or the accumulation of AP protein in stationary phase cells. Furthermore, the increase in activity in stationary phase did not correspond to an equivalent increase in the amount of AP protein. Covalent modification may have altered the activity or detection of AP. Examining these variables in further detail would assist in the elucidation of these trends in AP activity observed over time in *E. coli* C29.

FUTURE EXPERIMENTS

In this study, we observed that the specific activity of AP was 3.6 fold higher in stationary phase compared to exponential phase ($p < 0.001$). We thought that some of the difference in AP specific activity might be explained by the lysis method employed. The bead bashing method we used releases cytoplasmic and periplasmic proteins. During stationary phase, *E. coli* cells have been reported to be more resistant to lysis compared to exponential phase cells because of cellular modifications to the membrane (7, 9). Therefore, periplasmic proteins such as AP would comprise a larger proportion of the total cell protein in stationary phase samples and would cause these samples to have a higher AP specific activity. However, it may be possible to improve the comparison of AP specific activity between exponential and stationary phase cells by using an osmotic shock method for protein release that isolates only periplasmic proteins and including a specific cytoplasmic marker such as β-galactosidase as a control. This method could be compared to bead bashing method to evaluate our hypothesis.

Osmotic shock is a well-characterized method for releasing periplasmic proteins (1, 13). Specifically, cells would be pelleted by centrifugation at room temperature, resuspended in a hypertonic solution of sucrose and EDTA, and incubated at room temperature for 10 min. Having been osmotically shocked, the cells would then be pelleted by centrifugation at 4 °C. The pellet would be resuspended with a volume of cold water equal to volume of hypertonic solution added earlier and incubated on ice for 10 min. The periplasmic proteins would then be released into the supernatant and could be isolated from the cells by centrifugation. A disadvantage of this method may be that concentration of protein in the supernatant may be low if the volume of water used is high or protein levels are low. It may be necessary, then, to optimize this process for the volume of hypertonic solution and water used or to concentrate the protein by filtration.

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