

# The relative release of alkaline phosphatase and beta-galactosidase of *E. coli* C29, in exponential and stationary phases, through different lysis methods

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**Alkaline phosphatase (AP) in *Escherichia coli* C29 is constitutively expressed, unlike wild type *E. coli* in which AP expression has to be induced. However, it has been observed that AP activities drop upon entry into stationary phase. In contrast, another study demonstrated an increase in AP levels in stationary phase. It is speculated that ineffective bead bashing lysis of stationary cells resulted in an underestimation of total protein and thus overestimation of AP specific activity. The aim of this study was to confirm this hypothesis by investigating the lysis efficiency of bead-bashing and chemical whole cell lysis on the release of AP and  $\beta$ -galactosidase ( $\beta$ -gal), a cytoplasmic protein. AP levels were also investigated throughout exponential and stationary phase. The AP/ $\beta$ -gal ratio confirmed the incomplete lysis of the bead bashing method, and that AP levels decreased upon transition into stationary phase, but a subsequent substantial increase in the ratio needs further investigation.**

Alkaline phosphatase (AP) is a 100kDa homodimeric enzyme localized to the periplasm of *Escherichia coli* (2). In wild type *E. coli* K-12, AP is produced only when the levels of inorganic phosphate are limiting and it functions by harvesting inorganic phosphates ( $P_i$ ) from the environment (14). AP is encoded by the *phoA* gene and is transcriptionally regulated by 2 two-component signal transduction systems: PhoBR and CreBC (8,14). The PhoBR signal transduction system induces the expression of AP when environmental levels of inorganic phosphate are low through the PhoR sensor and the PhoB response regulator (16). Phosphorylated PhoB recognizes and binds to the promoter region of the *pho* operon, upregulating several genes including *phoA* (16). The CreBC signal transduction system consists of the CreC sensor and the presumed CreB global regulator (8, 10). CreC can phosphorylate PhoB resulting in the activation of the *pho* operon (8). Thus, the CreBC signal transduction system upregulates AP production independent of  $P_i$  availability.

*E. coli* C29 contains a *phoR19* null mutation and a *creC510* mutation. The *phoR19* mutation prevents activation of the *phoA* gene in response to  $P_i$ -limiting conditions, while the *creC510* mutation results in a constitutive expression of the *phoA* gene (16). Thus, it is expected that in *E. coli* C29, the production of AP will occur constitutively due to the *creC510* mutation.

However, in a previous investigation, when grown in modified Luria broth with glycerol, the activity of AP in *E. coli* C29 fell to zero after 72 hours of growth, where the cells were in stationary phase (W.D. Ramey, personal communication). Subsequent investigation of this phenomenon produced the opposite results; the levels of AP protein and AP activity increased as cells entered stationary phase and when assays were done at 72 hours of culture growth (4). The authors hypothesized that the increase in AP specificity activity and protein levels observed in the stationary phase was due to ineffective cell lysis of stationary phase cells using the bead bashing method (4). The incomplete lysis of inner membrane resulted in underestimation of total proteins, thus an overestimation of AP specific activities in the stationary phase.

As *E. coli* cells enter stationary phase, cell composition changes in response to environmental stresses, such as lower nutrients and pH changes (13). The cell membrane in stationary phase has a higher ratio of cyclopropane fatty acids to unsaturated membrane fatty acid resulting in a cellular membrane with an increased resistance to acid shock (3, 13). The composition of the cell wall also gradually changes during stationary phase including the increase in the number of cross-links between murein subunits (11). These structural changes to the peptidoglycan increase the mechanical integrity of the cell wall (11). When

such modification is extended to 90% of the murein sacculus, bacterial cells can be resistant even to the disruptive effects of freeze-thawing (14). In general, these major structural changes to the membrane and murein sacculus allow stationary-phase *E. coli* cells to be more resilient to the damaging effects of heat, oxidative shock and osmotic shock (13).

This study attempted to show that the increase in AP specific activity and protein level was due to inefficient lysis of stationary phase *E. coli* by the bead-bashing method (4). As a control for complete cellular lysis, chemical lysis in the presence of high SDS concentrations combined with boiling conditions was used. Cell lysis method efficiencies were assessed using the ratio of AP to  $\beta$ -gal levels.  $\beta$ -gal is a 464kDa homo-tetramer cytoplasmic enzyme encoded by *lacZ*. In this experiment, it is used as a cytoplasmic protein marker to evaluate the efficiency of lysis methods. The AP/ $\beta$ -gal ratio provided more information than an AP/total protein ratio, as the total protein content in a cell may change between exponential and log phase. Induction of *lacZ* at a constant concentration of isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) during the growth curve produced a consistent level of  $\beta$ -gal to use as normalization for AP protein level for comparison between different samples in exponential and stationary phase. Comparison of the AP/ $\beta$ -gal ratio between cells lysed with bead bashing and chemical lysis revealed that bead bashing resulted in incomplete lysis. This plays a role in the differential release of cytoplasmic proteins from exponential phase and stationary phase cells, an important consideration when comparing proteins from exponential and stationary phase cells.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *E. coli* C29 (fhuA22, fadh701, tonA22, phoR19, ompF627, relA1, pit-10, spoT1, rrnB-2, mcrB1, creC510, T2r, Hfr (PO2A)) was obtained from MICB421 culture collection at the University of British Columbia Department of Microbiology and Immunology. In the generation of the growth curve, cultures of *E. coli* C29 were grown in modified Luria Broth media (10g/L tryptone, 5g/L yeast extract, 5g/L NaCl, 0.4% (w/v) glycerol, pH 7.4) at 37°C. When  $\beta$ -galactosidase production was to be induced, a final concentration of 0.10 mM IPTG was used.

**Growth curve and sampling.** Optical density measurements of culture were taken at 660 nm (Spectronic 20D+, Milton Ray). An overnight culture of *E. coli* C29 was used to inoculate a culture of modified LB media such that the initial OD<sub>660</sub> was 0.01. The OD<sub>660</sub> as measured every 0.5 hours for the first 5 hours. Fifteen milliliters of samples was removed at time points 3.0 and 3.5, and 6.5 and 7.5, which represent cells in exponential and stationary phase, respectively. Samples were pelleted by centrifugation at 8,000 x g for 10 min. Cell pellets were stored at 4°C until cell lysis the next day.

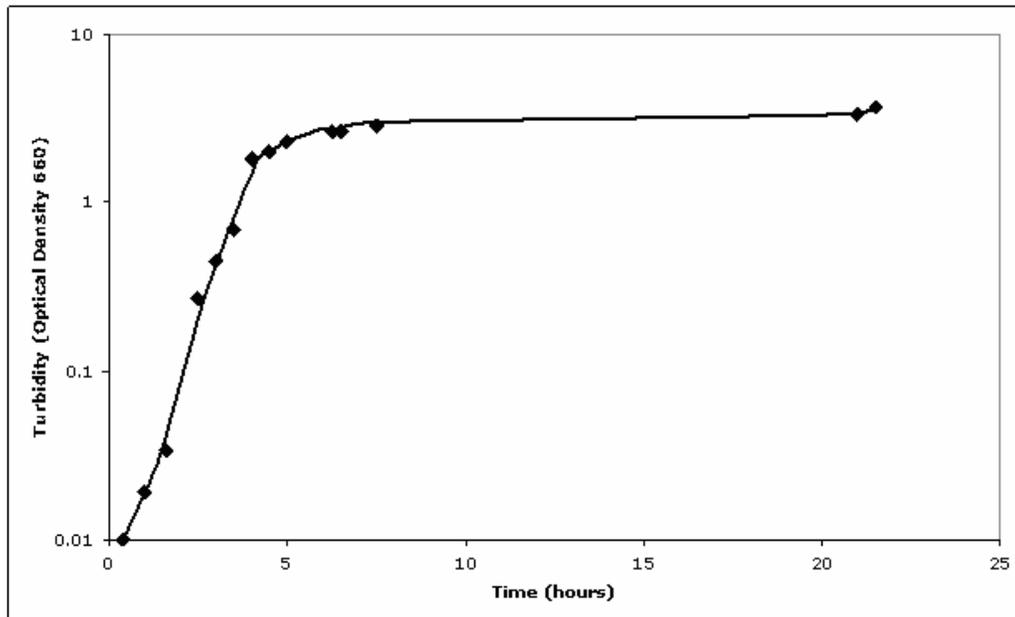
**Whole cell lysis (WCL).** Pelleted samples were resuspended in 500  $\mu$ L of Tris:HCl (20 mM, pH 7.4) then mixed with an equal volume of a 2X disruption buffer (0.1 M Tris-HCl (pH 6.5), 4% (w/v) SDS, 10% (v/v) 2- $\beta$ -mercaptoethanol). Samples were mixed and immediately boiled at 100°C water bath for 5 minutes. Samples were cooled on ice and stored at -20°C until immunoblot analysis.

**Bead bashing.** Pelleted samples were resuspended in 1 mL of Tris:HCl (20 mM, pH 7.5) then transferred to a 2.0 mL microfuge tube, which was half filled with 0.10 mm diameter glass beads (Cat.#11079105, Biospect.). Mechanical disruption of the cells by the beads was done using FastPrep FP120 (Thermo Electron Corp.) at speed 6 for 40 seconds. The broken samples were then clarified by two successive 5 minute centrifugations at 14,000 xg then mixed with 100  $\mu$ L of a 300  $\mu$ g/mL DNase/RNase solution and stored on ice for 30 minutes. Supernatants were then stored at -20°C until immunoblot analysis.

**Osmotic shock.** Cells were pelleted and resuspended in 1 mL of Tris:HCl (33 $\mu$ M, pH 7.3) and 1 mL osmotic shock solution (1.5 mM EDTA, 40% sucrose, 9.9 mM Tris-HCl, pH 7.5). Samples were incubated at 25°C for 15 minutes followed by centrifugation at 5,000 x g for 10 minutes. After removal of the supernatant, 1 mL ice-cold 1 mM MgCl<sub>2</sub> was added to the pellet, the mixture was incubated on ice for 10 minutes, the samples were centrifuged at 6,000 x g for 10 minutes. Supernatants were stored at -20°C until immunoblot analysis.

**BCA protein assay.** Three bovine serum albumin (BSA) standard curves were prepared by diluting the appropriate stock concentration with the lysis buffers used above (2x disruption buffer, 20mM Tris:HCl (pH 7.5) or osmotic shock solution). The BCA protein assay protocol was followed as previously described in (7). Standard curves were only produced for lysates from beading bashing and osmotic shock treatments.

**SDS-PAGE and Western Immunoblot Analysis.** Twenty-five microliters of lysate from bead bashing, whole cell lysis and osmotic shock were resolved on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Eighteen and three quarter microliters cell lysate and 6.25  $\mu$ L 6X sample buffer



**FIG.1** Growth of *E. coli* strain C29 grown in modified LB at 37°C shaken at 250 rpm. Optical density was read at 660 nm on a Spec20D+.

were mixed together and loaded for each lane of the SDS PAGE (12). Ten microliters of PageRuler Prestained Ladder (Cat.#161-0449, Bio-Rad) was loaded. A total of three SDS PAGEs were loaded for immunoblot assays. Proteins were electrophoretically transferred to a PVDF membrane (Cat.#PVM020C810CS, Pall) at 100 V for 60 minutes in cold transfer buffer (125 mM Tris:HCl, 0.2 M glycine, 20% (v/v) methanol). The transfer was followed by blocking the membrane in 5% (w/v) skim milk powder in Tris blocking solution (TBS) (250 mM Tris:HCl, 750 mM NaCl, pH 7.5) for 30 min. All blot incubations were done at room temperature with shaking.

The blots were incubated with either rabbit polyclonal anti-alkaline phosphatase IgG (Cat.# AB1204, Chemicon) at a dilution of 1:10000 TBS-Tween (TBS-T)(TBS with 0.25% (v/v) Tween-20) or rabbit polyclonal anti- $\beta$ -galactosidase IgG (Cat.#R901-25, Invitrogen) at a dilution of 1:5000 TBS-T, or mixture of the two antibodies at their respective dilutions, for 1 hr. The blots were then washed three times at 5 min, 10 min and 15 min respectively in TBS-T followed by incubation with horse-radish peroxidase conjugated goat anti-rabbit IgG (Cat.#AP132P, Chemicon) at a dilution of 1:10000 in TBS-T for 1 hr. The blots were washed three times at 5 min, 10 min and 15 min respectively in TBS-T. Chemiluminescent detection of the blot was accomplished with the Enhanced Chemiluminescent Kit (Cat.#RPN2108, Amersham) according to instructions provided by the manufacturer. Autoradiographic X-ray films (BioMax ML, Kodak) were exposed for 1 hour to the light emitted by the blot.

Quantification of band densities was accomplished using AlphaEase FC Image Analysis Software following the provided instructions (Alpha Innotech). Selection of the band of interest was done manually with a manual removal of background intensity by selection of an area near the band of interest.

## RESULTS

***E. coli* C29 Growth Curve.** Within the first five hours, exponential growth was observed (FIG.1). A plateau in turbidity readings was obtained by approximately 7 hours and little change in culture turbidity was observed subsequently between 7 and 24 hours.

**Cellular lysis.** At each time point, samples of culture were pelleted and subjected to lysis methods that preferentially released periplasmic proteins and cytoplasmic proteins. For the preferential release of periplasmic proteins, an osmotic shock protocol was used. For the release of cytoplasmic proteins, WCL (through chemical treatment), and bead bashing protocols were used. Only protein concentrations from bead bashing and osmotic shock lysates were available. The total protein concentration from cells treated with chemical lysis was unavailable due to the high concentration of SDS, which interfered with the BCA protocol. Bead bashing lysates had a greater average amount of released protein per cell in exponential phase than that in stationary phase (Table 1). However, the opposite was seen for osmotic shock lysates; released protein per cell was higher in stationary phase than in exponential phase. In bead bashing, the amount of protein lysed per cell dropped by 2.5-fold upon entering stationary phase yet the total number of cells lysed actually increased by 3.9-fold. Similarly, the amount of protein lysed per cell dropped by 5.0-fold from sample

taken at 6.5 hr to 7.5 hr while the total number of cells lysed increased from 1.1-fold. Following osmotic shock, the concentration of released protein was at undetectable levels.

**Table 1:** The effect of different breakage methods on the average amount of released protein/cell.

| Breakage Method | Time (hr) | Protein in 25 $\mu$ L ( $\mu$ g) | Cells in 25 $\mu$ L ( $\times 10^7$ cells) | Amount of protein per cell ( $\times 10^{-5}$ ng/cell) | Average protein per cell ( $\times 10^{-5}$ ng/cell) |
|-----------------|-----------|----------------------------------|--|--|--|
| Bead Bashing    | 3.0       | 2.11                             | 3.05                                       | 6.09   | 17.21  |
|                 | 3.5       | 10.64                            | 4.67                                       | 28.33  |  |
|                 | 6.5       | 15.94                            | 18.06                                      | 11.24  | 6.75   |
|                 | 7.5       | 4.04                             | 19.60                                      | 2.26   |  |
| Osmotic Shock   | 3.0       | 0                                | 3.05                                       | 0  | 0  |
|                 | 3.5       | 0                                | 4.67                                       | 0  |  |
|                 | 6.5       | 12.8                             | 18.06                                      | 7.12   | 7.16   |
|                 | 7.5       | 14.1                             | 19.60                                      | 7.20   |  |

**Western Immunoblot Analysis.** Western immunoblot produced the expected bands corresponding to AP and  $\beta$ -gal respectively, at 50 kDa and 116 kDa (FIG.2). There were several other unexpected protein bands in both the  $\beta$ -gal and AP western immunoblots (FIG.2). There was also a certain degree of non-specific binding as seen with the smear in both AP and  $\beta$ -gal immunoblots (FIG.2).

**Levels of Alkaline Phosphatase and  $\beta$ -galactosidase expression.** AP expression was constitutive, whereas the expression of  $\beta$ -gal was induced via IPTG (16). There was 3 to 4.5-fold increase in AP/  $\beta$ -gal ratio in samples released by chemical lysis compared to bead bashing (Table 2). In lysates from bead-bashing, the amount of  $\beta$ -gal that constitutes the total loaded protein showed a general increasing trend, with a 2.6-fold increase in  $\beta$ -gal expression when transiting to stationary phase. In lysates from bead bashing, the AP to total protein ratio showed a continuous decrease over time, with a 1.6-fold decrease in value upon entering the stationary phase. However, an increase in the AP to total protein ratio was observed in the later stationary phase time point. The

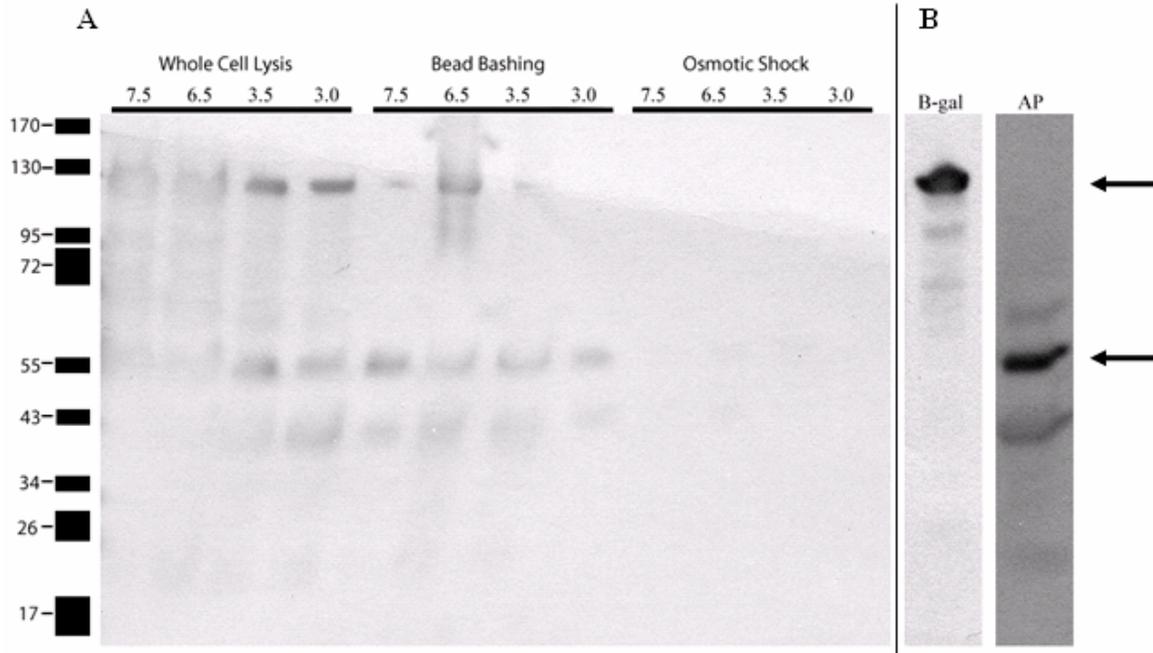
lack of total protein data for lysates from WCL prevented calculations of AP or  $\beta$ -gal to total protein ratio. AP and  $\beta$ -gal intensities for osmotic shock samples were below the lower detection limits.

## DISCUSSION

Analysis of protein release per cell for stationary and log phase cells lysed by bead bashing provides evidence that stationary phase *E. coli* C29 are less susceptible to bead bashing lysis (Table 2). Cells treated with the bead bashing lysis showed that the amount of protein released per cell decreased 61% in cells from stationary phase as compared to exponential phase (Table 1). This decrease can be explained by changes to the inner membrane and murein sacculus when *E. coli* enters the stationary phase. In the stationary phase, it is known that *E. coli* alters its cellular membrane composition and the increased number of cross-links in the murein sacculus increases the mechanical integrity (11) and increases general resistance to a variety of harsh environmental conditions (3, 11, 15). Therefore, we expect that stationary phase cells will be more resistant to mechanical lysis, such as bead bashing.

As the protein concentration from cells treated with WCL was not able to be determined, we were unable to compare lysates produced via bead bashing and via WCL. The WCL was meant to act as a control as we believe it is able to completely lyse cells, resulting in the release of the majority of periplasmic and cytoplasmic proteins. Without data on the WCL lysates protein concentration, we cannot conclude whether the observed differences between bead bashing lysates from exponential and stationary phases were due to alterations in membrane integrity or due to changes in levels protein expression. Cell protein levels may decrease in stationary phase through induction of the stringent response, which induces proteolysis and decreases protein synthesis by 50% in response to amino acid starvation (9).

To further investigate the efficiency of cell lysis at different growth stages, the amount of AP and  $\beta$ -gal present in the lysates were assayed by western blot. AP is a 100 kDa homodimeric protein which was dissociates into two 50 kDa subunits upon denaturation by SDS-PAGE (2).  $\beta$ -gal is a 464 kDa tetrameric protein thus, consisting of four 116 kDa subunits. The amount of  $\beta$ -gal present was chosen to represent the release of cytoplasmic proteins.  $\beta$ -gal is naturally



**FIG. 2** Western immunoblot of lysates resolved on NEXT Gel SDS-PAGE with 1:5000 anti-AP rabbit primary antibody and/or 1:5000 anti- $\beta$ -gal rabbit primary antibody with 1:10,000 anti-rabbit goat secondary antibody. **A.** Immunoblot of samples from 3.0 hr, 3.5 hr, 6.5 hr and 7.5 hr broken by chemical lysis, bead bashing and osmotic shocking incubated concurrently with both primary antibodies. **B.** Immunoblot of lysates from 3.0 hr treated by chemical lysis then incubated with either anti- $\beta$ -gal rabbit antibody or anti-rabbit AP goat antibody. The arrows denote the  $\beta$ -gal and AP protein bands used to determine densitometry data.

induced in the environment by the presence of lactose or artificially by the presence of IPTG. Although IPTG-induced  $\beta$ -gal expression is constitutive, these levels may change depending on growth stage and environment through regulation by CAP:cAMP or other global regulatory systems (6). The intensity values reported in Table 2 reflect the given band as a percentage of the total density in all the AP or in all the  $\beta$ -gal bands. The ratio of AP/ $\beta$ -gal intensity or the normalized AP and  $\beta$ -gal intensity can be used for comparison between the samples. Simple visual inspection of the band intensity trends is not accurate given the different amount of total protein loaded. The AP/ $\beta$ -gal ratio indicates the ratio of periplasmic and cytoplasmic proteins released. This is under the assumption that during lysis, there is no preferential release of specific proteins from the periplasm or cytoplasm and  $\beta$ -gal levels remain relatively constant. The 4% SDS and 100°C used in the WCL was expected to release the maximal amount of cellular protein, if not all of the cellular protein.

The AP/ $\beta$ -gal ratios for bead bashing lysates were on average 420% higher than WCL lysates (Table 2). Since the periplasm is surrounded by one membrane, as opposed to two, it is believed to be more susceptible to lysis than the cytoplasm. For this reason it is believed that close to all of the AP present in the periplasm was released by treatment with bead bashing and WCL. It is important to note that during bead bashing, some membrane segments may have reformed trapping small amounts of periplasmic and cytoplasmic proteins (W.D. Ramey, personal communication). As the amount of AP release is expected to stay constant between different lysis methods, the increase in AP/ $\beta$ -gal ratio, suggests that the observed increased ratios may be due to a decrease in the amount of  $\beta$ -gal present in the lysate. These observations suggest that bead bashing may indeed result in less than complete inner membrane lysis. This lysis efficiency may be affected further by the growth stage of cells.

| Lysis Method              | Time (hr) | Intensity of AP band (% of all density) | Intensity of $\beta$ -gal band (% of all density) | Ratio of AP to $\beta$ -gal intensity | Total Protein loaded ( $\mu$ g) | Normalized AP band Intensity (Intensity/Total Protein $\mu$ g) | Normalized $\beta$ -gal band Intensity (Intensity/Total Protein $\mu$ g) |
|---------------------------|-----------|---|---|---------------------------------------|---------------------------------|--|--|
| Whole cell chemical lysis | 3.0       | 13.6                                    | 24.9  | 0.6                                   | -                               | -  | -  |
|                           | 3.5       | 14.1                                    | 21.8  | 0.6                                   | -                               | -  | -  |
|                           | 6.5       | 2.1                                     | 11.5  | 0.2                                   | -                               | -  | -  |
|                           | 7.5       | 10.6                                    | 8.8   | 1.2                                   | -                               | -  | -  |
| Bead bashing              | 3.0       | 11.1                                    | -   | -                                     | 1.1                             | 5.26   | -  |
|                           | 3.5       | 14.1                                    | 5.3   | 2.7                                   | 7.9                             | 1.33   | 0.50   |
|                           | 6.5       | 13.2                                    | 23.6  | 0.6                                   | 12.2                            | 0.828  | 1.31   |
|                           | 7.5       | 20.8                                    | 4.1   | 5.1                                   | 2.6                             | 5.15   | 1.01   |
| Osmotic Shock             | 3.0       | -                                       | -   | -                                     | 0*                              | -  | -  |
|                           | 3.5       | -                                       | -   | -                                     | 0*                              | -  | -  |
|                           | 6.5       | -                                       | -   | -                                     | 12.8                            | -  | -  |
|                           | 7.5       | -                                       | -   | -                                     | 14.1                            | -  | -  |

\* protein levels were below the lower detection of the BCA assay and assumed to be 0  $\mu$ g  
 - no data available

**Table 2.** Proportion of AP to  $\beta$ -gal released by whole cell chemical lysis, bead bashing lysis and osmotic shock lysis. Band intensities were calculated using densitometry with the AlphaEase FC Image Analysis Software (Alpha Innotech).

The ratios of AP/ $\beta$ -gal for bead bashed samples were expected to increase during the transition from exponential to stationary phase according to the previously reported data (4). The observed results, however, showed a 3-fold decrease between the 3.5 hr and 6.5 hr phases followed by a 7-fold increase in the 7.5 hr sample (Table 2). These same trends are observed in the WCL samples as well. This may suggest that the fluctuations in AP/ $\beta$ -gal ratios between different time points may be the result of changes in protein expression and not lysis method. Furthermore, the availability of only limited samples does not allow

us to determine if these trends are merely random fluctuations.

The 3-fold decrease in AP/ $\beta$ -gal ratios from 3.5 hr to 6.5 hr observed in both the bead bashing and WCL samples may be due to growth phase dependant changes in the regulatory control of  $\beta$ -gal synthesis. As cells approach the stationary phase, carbon catabolite repression is eliminated. The “hunger” state in stationary phase is signaled by an increased intracellular concentration of cAMP (6). This leads to increase levels of cAMP:CAP complexes which bind to, and activate, promoters of lacZ (6). Although  $\beta$ -gal was

artificially induced using IPTG, the combination of cAMP:CAP together with IPTG, may lead to increased expression beyond the “constitutive” levels in stationary phase (6). This increase in  $\beta$ -gal levels due to cAMP:CAP action may result in the lower AP/ $\beta$ -gal ratio observed in the 6.5 hr time point (Table 2). Unfortunately this cannot be supported using  $\beta$ -gal expression levels as each lane of the western blot contained differing amounts of protein and the densitometry data could not be normalized. Similarly the 7-fold increase in AP/ $\beta$ -gal ratios from the 6.5 hr to 7.5 hr stages could be due to increase in AP levels due to modifications in gene transcription or translation beyond the constitutive levels (Table 2). This is supported by the protein release data which shows that total protein released per cell by bead bashing decreased by 80% from 6.5 hr to 7.5 hr (Table 1). This decrease in total protein is too drastic to be explained merely by  $\beta$ -gal expression but may suggest a more widespread transcriptional control. Alternatively, it is possible that cells from the 6.5 hr sample did not fully complete the transition to stationary phase. For this reason, cell regulation and protein expression changes may have further occurred between the 6.5 hr and 7.5 hr samples therefore causing decrease a decrease in  $\beta$ -gal or increase in AP expression.

A set of samples lysed by osmotic shock was included on the western blot in an attempt to provide support for differential lysis. These were also included to help control for changes to AP expression through the experiment. These samples, however, were not detected on the membranes treated with each of the three antibody treatments mentioned earlier. This is likely due to the fact that small amounts of periplasmic proteins were released by this method, which were further diluted according to the lysis procedure (1:1 osmotic shock solution to cell lysate).

Although our experimental procedure has focused on comparison of AP and  $\beta$ -gal levels in cell lysates, we have been unable to control for changes in AP expression. Although AP expression in *E. coli* C29 is considered to be constitutive, the levels of expression may still vary depending on growth stage or environment (4). Our lack of control arose from limitations in the BCA assay for WCL samples and poor resolution of the osmotic shock sample immunoblot. For these reasons, it is not possible to conclusively determine if changes in observed AP/ $\beta$ -gal ratios are due to differences in lysis method or changes in AP expression.

During analysis of the immunoblot results, the reliability of the data comes into questions, especially during comparison of triplicate membranes blotted with

either anti-AP only, anti- $\beta$ -gal only anti-AP and anti- $\beta$ -gal antibodies (FIG.2). Although each of the three immunoblots were loaded in the exact same manner, the banding patterns between them differed in intensity (FIG.2). It is believed that the discrepancy in band intensity may be due to interference. Interference may be due to competitive interactions of the two primary antibodies used to stain the membrane used to calculate AP/ $\beta$ -gal ratios (N.R. Mawji, personal communication). The time between the addition of the chemiluminescence detection reagent and exposure of membranes may also contribute to the different banding intensities of the separate blots as the detection intensity decreases rapidly with time.

Inspection of the banding patterns on these gels also reveals the presence of unexpected bands (FIG.2). In the immunoblot with the anti- $\beta$ -gal rabbit primary antibody, two bands are found in the 6.5 hr and 7.5 hr WCL sample lanes (FIG.2). Since  $\beta$ -gal is a homo-tetramer and the sample preparation is expected to fully separate these subunits, it is unlikely these bands are due to incompletely separated monomers. These bands may be due to partial protein degradation due to sample handling. This banding pattern may also be due to non-specific binding of the antibody (FIG.2). Due to time constraints in the procedure, the blocking step was only performed for 30 minutes, which may have contributed to this non-specific binding. There is also one band, which presumably stains with the anti-AP rabbit primary antibody at approximately 43kDa and appears in all bead bashing sample and 3.0 hr and 3.5 hr WCL samples (FIG.2). The origin of these bands may also be due to non-specific binding of the primary or secondary antibody.

This data provides conflicting evidence regarding the differential lysis of the *E. coli* C29 inner membrane in exponential and stationary phase. Analysis of protein release by bead bashing suggests that the inner membrane may be more susceptible to lysis during exponential than stationary phase. Similarity between AP/ $\beta$ -gal ratios in WCL and bead bashing samples, however, suggest that any differences in protein release may be due to expression levels instead of lysis efficiency. Further experimentation is required to elucidate the true impact of *E. coli* C29 growth stages on inner membrane lysis susceptibility and how this may impact the determination of AP specific activity.

## FUTURE EXPERIMENTS

In this study, one of the main issues was the lack of complete data. Total protein present in whole cell lysis samples cannot be assayed because the whole cell lysis

buffer is not compatible with the BCA assay. The absence of total protein assay made it impossible to normalize beta-gal and AP levels detected. Comparison of total protein between whole cell lysis and bead bashing samples would have been useful in understanding the efficiency of the lysis method since chemical lysis is expected to completely lyse the cells.

Secondly, the use of  $\beta$ -gal levels as an indicator, for lysis efficiency is questionable.  $\beta$ -gal, encoded by the *lacZ*, is under the negative control of LacI and the positive control of CAP:cAMP complex. For this reason fluctuations in  $\beta$ -gal levels may occur which impact the calculated AP/ $\beta$ -gal ratios. The possible fluctuations in AP expression should also be controlled for by inclusion of another periplasmic protein. This would enable us to determine if AP expression varies and allow us to attempt to normalize any variability to lysis efficiency.

This experiment should be repeated with these issues in mind. The first issue can be overcome by utilizing another total protein assay, such as the Bradford protein assay, which was originally not chosen as the SDS in the chemical lysis buffer was incompatible with the chemical lysis buffer. The BCA assay protocol was believed to be more suitable, as it is able to detect protein concentration in the presence of SDS. However, the BCA assay is not compatible with the reducing agents present in the chemical lysis solution. The Bradford assay is more superior because it is compatible with reducing agents and the SDS incompatibility issue can generally be overcome by sample dilutions. The second issue can be addressed by choosing another cytoplasmic protein as a reference protein. This choice must meet several requirements. First, transcription of this gene must be constant throughout the growth phases, especially during the transition from exponential to stationary phase. It should be noted that finding such a protein may require intense literature research, even after which, such a protein may not be found. As an alternative, using a protein with a known expression rate in the exponential and stationary phase may be suitable as well. Secondly, protein levels must be assayable with available reagents. Further research is needed to determine a more ideal reference cytoplasmic protein as well as a reference for periplasmic proteins. Such modifications would enable more conclusive interpretation of results.

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