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The Effects of Extraneous Autoinducer-2 Addition to a Young *Escherichia coli* B23 Culture

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The advantages of living in a regulated bacterial system include greater access to nutrient sources, enhanced protection and establishment of environmental niches. Cell growth and regulation within bacterial systems is partially mediated by diffusible autoinducers. When populations reach high cell densities in closed systems, the increase in concentration of autoinducer results in an overall change in cellular activity. Autoinducer 2 (AI-2), an acyl homoserine lactone, is an example of a signaling molecule that functions in both interspecies and intraspecies communication. *Vibrio harveyi* was the first bacteria in which AI-2 activity was observed, as the genes impacted by AI-2 regulate bioluminescence. The luminescence emitted by *V. harveyi* provides a system often exploited for bioassays in order to assess autoinducer activity. AI-2 was used to induce a low cell density culture of *Escherichia coli* B23, previously shown to be capable of induction, to behave in a similar fashion to a high-density cell population. It was found that AI-2 did not affect growth rate but it did enhance the extent of growth. Analysis of protein abundance in young cultures upon exposure to AI-2 was performed in order to compare protein profiles between young and old cultures. Comparison of young cultures treated with AI-2 revealed points of similarity with old cultures that were not present in young cultures grown in the absence of AI-2.

Quorum sensing is a cell-to-cell signaling system that allows for the synchronization of bacterial activities by initiating regulated gene expression (7). This mechanism of communication has evolved as a way of improving an organism's access to nutrients, environmental niches and providing protection by enhancing defense capabilities (7). Quorum sensing is mediated by detection of low molecular weight chemical signaling molecules produced and secreted into the external environment (8). These signaling molecules are called autoinducers (AI). Most autoinducers, such as AI-1, are species specific; however AI-2 an acyl homoserine lactone and its synthase *LuxS* are widely distributed in over 50 gram negative species of bacteria (20). Genetic and biochemical evidence has suggested that AI-1 mediates intraspecies communication while AI-2 mediates interspecies communication (2,8). Once a bacterial population reaches optimal autoinducer concentration, termed 'quorum', a signal transduction cascade is triggered resulting in the induction or repression of target genes. *Vibrio harveyi* was the first organism in which AI-2 was identified (6). In this case, the target genes are responsible for bioluminescence and thus *V. harveyi* is often used to quantify AI-2 as it has an easily measurable reaction.

When a population reaches appropriate cell density and therefore optimum autoinducer concentration, induction or repression of specific genes occurs in all cells in the population. In a rich media, AI-2 is produced maximally in mid-exponential phase (1) and is degraded when population density reaches stationary phase (15). In this experiment, the effects of AI-2 addition on a low cell density (young) culture of *Escherichia coli* B23 were observed. A better understanding of the effect of AI-2 on *E. coli* B23 was gained by monitoring of the culture's growth curve, growth rate, AI-2 production over time and comparison of protein profiles with the use of two-dimensional gel electrophoresis.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. The three strains used in this experiment were: *E. coli* B23 (wild type), *V. harveyi* BB152 (AI-1$^-$, AI-2$^+$) and *V. harveyi* BB170 (sensor 1$^-$, sensor 2$^+$) (supplied by Dr. W. Ramey, UBC). *V. harveyi* BB152 can produce AI-2 while *V. harveyi* BB170 can only sense AI-2, not produce it. *E. coli* B23 was grown in 0.2% Glycerol Luria Bertani (LB) media (10g tryptone, 5g yeast extract, 5g sodium chloride, 2g glycerol, 1L H_20, pH 7) in a shaking water bath (140rpm) at 37^{o}C. Glycerol was used as a carbon source based on a previous study (4). *V. harveyi* BB152 and *V. harveyi* BB170 were grown for 16 hours in Marine Media (MM) (3) (10g tryptone, 5g yeast extract, 20g sodium chloride, 1L H_20, pH 7) in a shaking water bath (130rpm) at 30^{o}C.

Bacterial Growth Curve. The turbidity (OD_{660}) of the *E. coli* B23 culture was determined at a wavelength of 660nm (Spectronic20D+). Overnight cultures were added to LB broth to a final concentration of 0.01 at OD_{660}. N-hexanoyl-DL-homoserine lactone (AI-2) (Sigma Aldrich, cat. # 09926) was resuspended in methanol to a final concentration of 100mM. AI-2 was added to a final concentration of 5nmol/ml (13) in one culture, while the other culture (control) was not manipulated (no methanol added). The turbidity of both samples was measured at 660nm every 30 minutes for 330 minutes.

Bioluminescence assay. Cell-free samples were prepared by sampling cultures at 30 minute intervals during *E. coli* B23 growth, followed by centrifugation at 13,200 rpm for 7 minutes. *E. coli* supernatant (SN) was filtered through 0.45µm sterile membrane filters (Millipore TYPE HA). Filtered SN was stored at -20ºC. Cell-free SN was also prepared from *V. harveyi* BB152 grown overnight in MM as a positive control. The luminescence assay was performed as previously described (5,16). Briefly, cell-free supernatants from *E. coli* B23 and *V. harveyi* BB152 were each added to 1:5000 dilutions of a *V. harveyi* BB170 overnight culture and incubated for 3 hours in a 30ºC shaking water bath (130rpm). Luminescence was measured for each sample with a delay time of 20 seconds and a read time of 120 seconds (TD-20/20 Luminometer, Turner Biosystems).

Protein Isolation. Cell harvest for the control (untreated culture) and AI-2 cultures was performed after 150 minutes and 300 minutes of growth. Proteins were isolated as previously described (14). All steps were performed at 4ºC. One hundred fifty millilitres of 150 minute culture and 75 ml of 300 minute culture were harvested by centrifuging in the Beckman J2-21 at 10,000rpm for 10 minutes. SN was decanted and pellets frozen in acetone/dry ice bath, followed by storage at -80ºC. Pellets were washed three times with 25 ml of 0.8% sterile saline and centrifuged at 10,000rpm for 10 minutes. SN was decanted and pellets were resuspended in 3 ml of Tris-EDTA, pH 8.0. One-millilitre samples were aliquoted into pre-chilled microfuge tubes and centrifuged at 13,200 rpm for 5 minutes (Brinkman Centrifuge 5415). Protein was released by the addition of 500µL lysis buffer (0.1% CHAPS, 30mM Tris pH 7.5), and 1X protease inhibitors (Complete Mini, cat. # 1836153, Boehringer Mannheim) then incubated on ice for 20 minutes. Cell membranes were disrupted using bead bashing (FastPrep FP-120 BIO 101) utilizing 0.1mm glass beads (Biospec Products, cat. # 11079101). Bead bashing was repeated three times at speed 6 for 15 seconds with 3 minutes on ice to cool samples between each round. Samples were then centrifuged at 14,000rpm for 10 minutes, transferring SN to fresh microfuge tube. Multiple centrifugations were performed to ensure all glass beads were removed from the samples.

Protein Quantification. Bradford assays were performed using 100mg/ml chicken egg albumin (Sigma-Aldrich, cat. # A5503) in Tris pH 8 as a standard for determining the concentration of the protein preparation samples. The standards and samples were incubated with Coomassie Brilliant Blue (Bradford reagent, BioRad, cat. # 500-0201) and absorbance was read at a wavelength of 595nm (Spectronic20D+).

2-Dimensional (2D) gel electrophoresis. 2D gel electrophoresis was performed as previously described (10,11,14). Briefly, isoelectric focusing (IEF) of proteins was done using 24 cm linear immobilized pH gradient (IPG) strips of pH 3-7 (Amersham Biosciences, cat. # 17-6002-43). Ninety micrograms of protein was suspended in rehydration buffer (10M urea, 2M thiourea, 30mM dithiothreitol (DTT), 3% CHAPS, 2% Pharmalyte pH 3-10). The protein samples were sonicated at temperatures that did not exceed 25ºC for 10 minutes and then were applied to the IPG strips. IEF was carried out under mineral oil for 23 hours using ETTAN IPGphor (Amersham Biosciences) starting at a low voltage (20V), allowing for the diffusion of proteins into the gel matrix of the strips, and then increasing the voltage to a maximum of 8000V. The strips were then equilibrated in buffer (50mM Tris-Cl pH6.8, 30% glycerol, 6M urea, 4% SDS, 3.5mg/mL DTT, 0.045g/mL iodoacetamide) to remove substances that would interfere with running the samples in the acrylamide gel. The IPG strips were placed on top of 12% acrylamide gels, all air pockets were removed and 1% agarose was added as a sealant. Electrophoresis was performed using the ETTAN DALTtwelve System (Amersham Biosciences). Broad-range molecular mass markers (Invitrogen, cat. # 10747-012) were run on either side of the gel. Tank and buffer temperatures were equalized to 25ºC and gels were run at 2.5 W/gel for 40 minutes, then at 17W/gel until dye front reached 1cm from the bottom of the gel. The gels were fixed with 40% methanol and 10% acetic acid and then incubated in Sypro Ruby Protein Stain (Sigma, cat. # S4942) overnight, followed by a wash with 10% methanol and 6% acetic acid to remove any unbound stain. Gels were imaged using Variable Mode Imager Typhoon 9400 (excitation 488 nm, emission 610 nm; Amersham Biosciences, cat. # 63005588). Manipulation and analysis of the gels was performed using Progenesis Workstation software (Nonlinear Dynamics, Durham, NC). The 300AI protein sample gel was arbitrarily selected as the template. Each gel was aligned to the template using the warping function of the editing software then compared to all other gels in terms of protein spots. Progenesis is capable of comparing two gels and listing all the spots that are found in one reference gel but not found in the comparison gel. A normalized volume threshold of 0.02 was set to remove spots less than this as most were assumed to represent background detection. All other spots were closely examined to confirm that the spot was absent in the comparison gel. Due to time constraints each spot could not be perfectly matched during the warping step. However, determining spot similarities and differences by eye allowed for the assessment of whether spots of interest did in fact have a match.

RESULTS

Growth Curve. The addition of AI-2 at time zero to a freshly inoculated *E. coli* B23 (experimental sample) resulted in a similar growth rate as the control sample (no AI-2 added) (Fig. 1). However, the final culture density of 2.3 OD_{660} in the treated culture is almost 35% higher than the maximum culture density of 1.7 OD_{660} observed in the control. Both cultures were in lag phase until a point between 120 minutes and 150 minutes.

Luminescence Assay. When *V. harveyi* BB170 sensor was treated with SN from *V. harveyi* BB152, greater amounts of luminescence were detected than when the sensor was treated with media alone. This positive control was used to confirm that the BB170 strain had the capacity to detect and react to autoinducer. *V. harveyi* BB170 was also observed to respond to the pure AI-2 added to the experimental culture. Since *V. harveyi* was able to respond to the purchased AI-2, it was expected that the *E. coli* B23 would also respond, as AI-2 is known to function in interspecies signaling (8). SN from *E. coli* B23 produced luminescence indicating that the strain does produce its own AI-2. The control culture and experimental culture were observed to have similar levels of luminescence until approximately 150 minutes of growth (Fig. 2). After this point, the amount of luminescence in the experimental culture increased at a higher rate than observed in the control culture. Between 330 minutes and 360 minutes the amount of luminescence was observed to decrease in both cultures. These differences might be real but subsequent tests of the assay showed that the luminescence was unstable and diminished as the assay proceeded (Fig. 3).

2-D Gel Electrophoresis. Four gels from the protein preparations were run in duplicate: a 150 minute control (150), 150 minute AI-2 (150AI), 300 minute control (300) and 300 minute AI-2 (300AI). The quality of the gels was poor thus the gel with the best quality from each duplicate was analyzed (A1-A4).

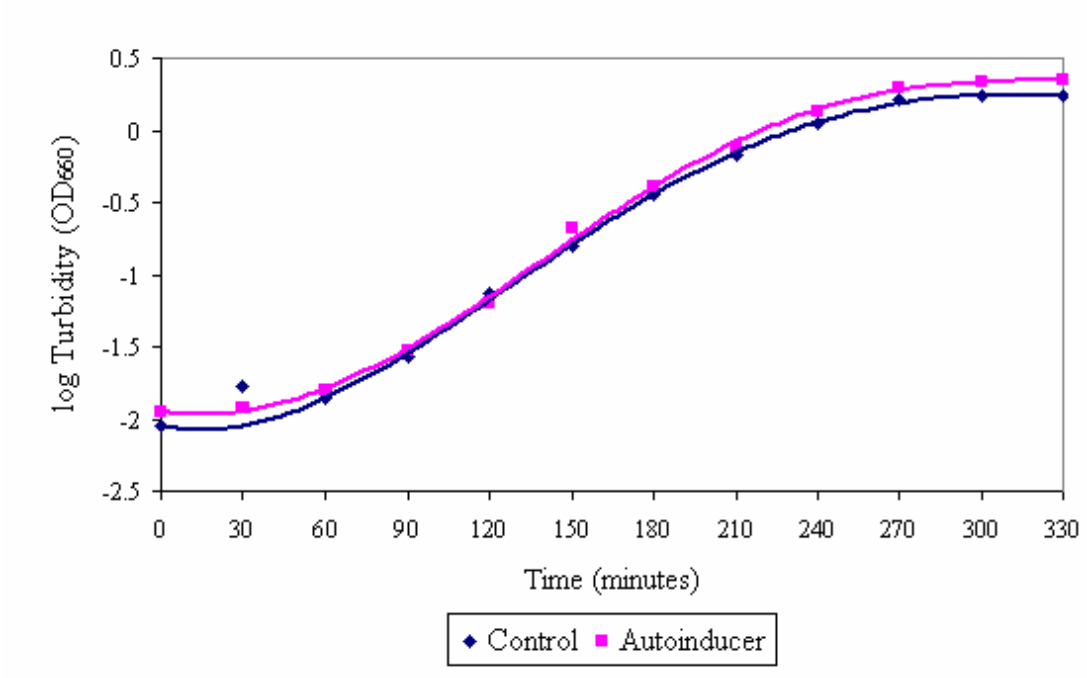

FIG. 1 Growth rate of *E. coli* B23 grown in LB media supplemented with 0.2% glycerol in the presence and absence of AI-2. The AI-2 was supplied in 7.5 µl of methanol to a final concentration of 5nmol/ml at time zero.

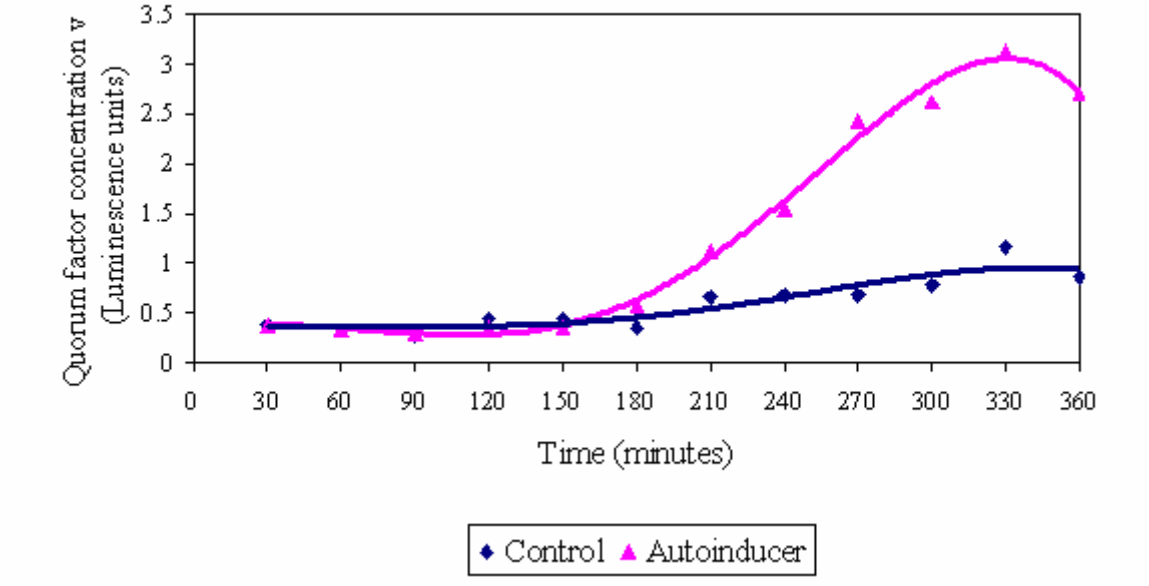

FIG. 2 Luminescence of supernatants harvested from *E. coli* B23 in the presence and absence of additional AI-2 read after 3 hours of incubation with *V. harveyi* BB170. A final concentration of 5nmol/ml of AI-2 was added at time zero.

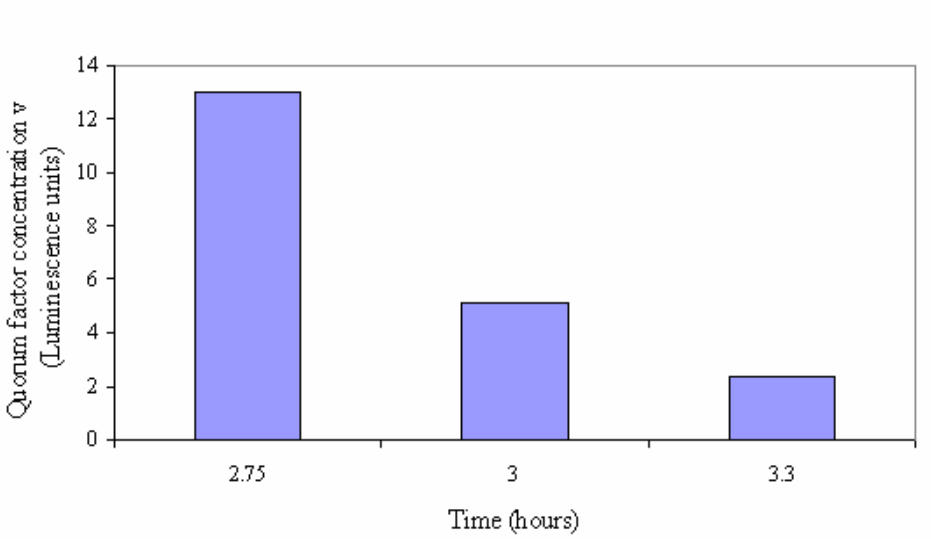

FIG. 3 Variation in the luminescence reading of one sample read at different times after the addition to the *V. harveyi* detection assay.

Some proteins resolved as multiple spots causing difficulty in analysis. However, due to the fact that all gels had similar abnormalities, comparison between samples was still possible.

Only spots that were not present in one or more gels were examined. There were no spots found in the 150 gel that were not present in the 150AI gel however, the 150AI gel had 12 spots that were not present in the 150 gel (table I). The differences between the 300 and the 300AI gels were minimal representing only 3 different spots. The 150AI gel resembled the 300 and 300AI gels more so than the 150 gel resembled the 300 and 300AI gels. In some cases, the same protein spots were absent from multiple gels. A single protein of interest was found to be in all gels except 150 (Fig. 4). This protein was found to exist in varying amounts in the other three gels (Fig. 5).

TABLE 1. Additional unique protein spots found when each gel is compared to each of the other three gels. The identification numbers refer to the time the sample was taken after the start of the culture. Samples labeled AI were treated with AI-2 at the initiation of the culture.

Comparison Gel	Reference Gel			
	150	150AI	300	300AI
150		0	4	7
150AI	12		1	3
300	26	11		1
300AI	21	7	2	

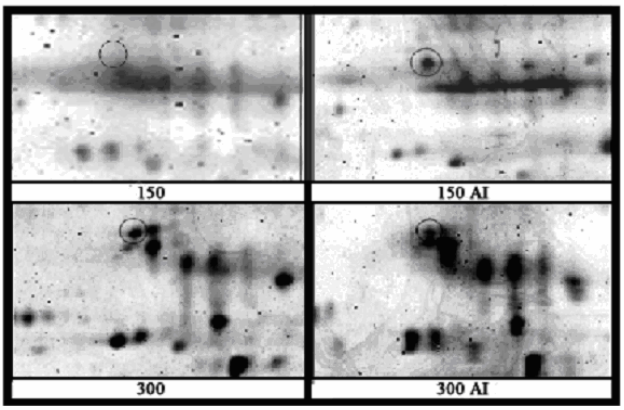

FIG. 4 Montage of an area containing a protein of interest (circled) that is specific to 150AI, 300 and 300AI and not present on the 150 gel. The identification numbers refer to the time (in minutes) after the start of the culture at time zero. Samples labeled AI were treated with AI-2 to a final concentration of 5nmol/ml at the initiation of the culture.

DISCUSSION

The results suggest that the addition of AI-2 at the beginning of a freshly inoculated culture of *E. coli* B23 may cause premature aging. This phenomenon was initially observed during analysis of the growth curve. The growth rates were observed to be similar in both cultures (Fig. 1) however the cell productivity differed. The growth curve suggests that the experimental culture may have left lag phase earlier than the control culture because it had higher turbidity readings than the control culture at each time point after 120 minutes. The AI-2 concentration is usually highest during late log and the entry into stationary phase (20). There are

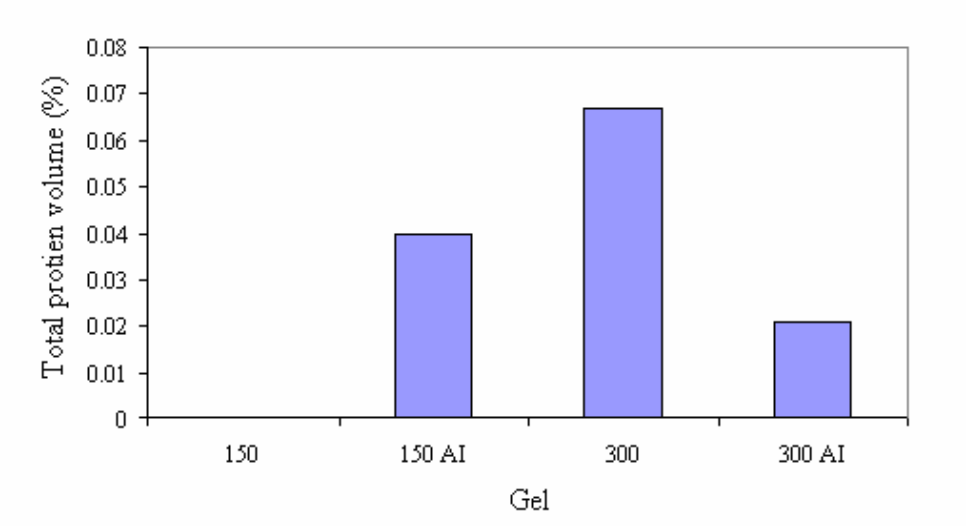

FIG. 5. Comparison of the volume of protein spots present in the individual gels that were not present on the control at 150 minutes. The volume of the unique spots was compared to the total volume of protein within that gel to get a normalized volume. The identification numbers refer to the time the sample was taken after the start of the culture. Samples labeled AI were treated with AI-2 at the initiation of the culture.

several factors that induce increased production of AI-2 including exponential growth, preferred carbon sources, low pH, and high osmolarity (1,17). Many of these AI-2 inducing factors are present during the log phase of growth in the rich LB media used. This suggests that the added AI-2 at the beginning of the culture may have provided a signal indicating that the culture was in log phase when in reality it was not.

Leaving lag phase early does not provide an explanation as to why the final OD_{660} where stationary phase is entered was higher in the experimental culture than the control culture. The control culture stabilized at approximately 1.7 OD_{660} between 270 and 300 minutes (Fig. 2). Coincidently, the experimental culture was also seen to enter stationary phase during this time period though at a slower rate (Fig. 1). The experimental culture was still growing while the control culture had stabilized. If AI-2 had not effected entry into stationary phase, it would be expected that the experimental culture and control culture would enter stationary phase at a similar cell density. It has been suggested that there is a stationary signal that leads to the breakdown of AI-2 (15). A correlation between stationary phase supernatant and a decrease in AI-2 concentration has been demonstrated though the mechanisms have not yet been elucidated (15). Since AI-2 was added at the initiation of the experiment, greater amounts of AI-2 were expected to be present during all phases of growth until after the entry into stationary phase (Fig. 3). However, during the first 120 minutes the two conditions appear to be essentially the same (Fig. 2). This data suggests that a threshold concentration of AI-2 is required for accurate luminescence readings using *V. harveyi*. This is potentially due to *E. coli* having different AI-2 concentration requirements for reaching quorum than *V. harveyi.* The tested level of diluted AI-2 in the SN samples during the first 120 minutes of either sample might not have been sufficient to stimulate light production in *V. harveyi*. The eventual greater accumulation of AI-2 in the treated culture compared to the control culture may account for the slow entry of the experimental culture into stationary phase. The cells receive stationary phase signals because of other factors (e.g. nutrient depletion and indole signal) (19) but also a conflicting high AI-2 concentration, suggesting to the culture that it is still in log phase.

The variability in the *V. harveyi* luminescence assay has been previously noted (9). A 5.5-fold decrease was seen over a 40 minute time period surrounding the 3 hour sampling point for one test sample (Fig. 3). This data suggests that approximately 0.25 luminescence units were lost per minute during this time period. Since many of the luminescence values were less than one, small changes between incubation times of the samples may have caused large variation in the luminescence observed. The degree of variability of luminescence experienced during the assay demonstrates the need to incubate each sample identically in order to obtain results with greater reproducibility and comparability. The instability might have caused some of the fluctuations in this study (Fig. 2) and should be investigated in more detail in future studies. It would be expected to have a higher basal level of AI-2 in the treated culture since it included both the normal AI-2 from the *E. coli* B23

plus the added AI-2. The absence of enhanced basal level may suggest that the added AI-2 was degraded or that it inhibited autogenous production. However, even with the apparent turnover, the added AI-2 appears to enhance subsequent production of AI-2 as the cells enter late log phase.

The protein gels obtained were not representative of reference gels found in the literature (A1-A4) (18). Some proteins were observed as three discrete spots on all the gels. This suggests that the isoelectric point (pI) of the proteins was altered and not their molecular weight, as it was observed that the proteins migrated in the same horizontal plane. There are many reasons why this may have occurred. The most likely cause is chemical modification of the proteins during the protein preparation for the 2-D gels. This event may have occurred due to carbamylation from urea or protein oxidation from dithiothreitol, both found in the rehydration buffer (M. A. Patrauchan, personal communication). The Amersham Biosciences web page (http://www1.amershambiosciences.com) indicates that the minor horizontal streaking may have resulted from impurities in the agarose overlay or equilibration solution. Horizontal streaking was also potentially caused by incomplete solublization of proteins due to their poor solubility in the rehydration buffer. Some problems may have occurred because the protocol used was optimized for *Rhodococcus*, a gram positive bacteria (10,11,14).

All the spots on the 150 gel also appeared to be on the 150AI gel, while the 150AI gel had additional spots when compared to the 150 gel (table I). This is quite significant as it implies that the addition of autoinducer resulted in an increased abundance of 12 proteins not seen in a mid-log culture without additional autoinducer. However, this did not result in changes in any proteins normally found in a culture without the addition of AI-2 (table I). It is well known that protein expression changes in stationary phase (12). It is expected that 300 and 300AI gels would be quite similar to each other but would express a novel set of proteins compared to mid-log cultures. This was indeed found to be the case. More than 20 new spots were observed in the 300 and 300AI gels that were not present in the 150 gel. While there were differences between the stationary phase gels and the 150AI gel, they were not as numerous as those seen in the 150 gel. This suggests that the 150AI sample shares a greater number of proteins with the 300 and 300AI gels than the 150 gel. An example of this is shown in figure 4 describing a single protein present in all gels except on the 150 gel. This data suggests that the addition of AI-2 causes the 150AI culture to behave in a similar manner to a culture in late log and early stationary phase in terms of protein expression. There is also a change in this single protein's abundance between the samples (Fig. 5). This suggests that there are factors other than AI-2 also regulating the expression of this protein. It is possible that the abundance of other proteins is also altered between gels. This would have to be examined more thoroughly in future experiments.

While this study was not able to conclude that AI-2 can prematurely age a young *E. coli* B23 culture, it did show promising results that the addition of AI-2 does alter various aspects of a young culture. With further study, a more exact role of AI-2 could be determined.

FUTURE EXPERIMENTS

These experiments suggested that excess autoinducer is capable of altering protein expression in a young culture, resulting in similar protein expression to an old culture. It would be of interest to identify the novel proteins expressed in the young culture upon induction. Potential identification of proteins could be performed through the comparison of samples to a 2-D gel database or with the use of mass spectrometry. In order to analyze the samples using mass spectrometry, new samples would have to be attained and the gels run again. Before experiments were performed again, it would be crucial to determine the cause of the problems observed during the 2-D gel analysis. However, before these types of experiments are performed it would be necessary to sort out the fundamental problems with the AI-2 assay to know the actual response in the treated culture.

It was observed that the culture with AI-2 added had higher productivity and a higher final turbidity than the control culture. By examining cell productivity and final turbidity of the cultures with varying concentrations of AI, its role in the latter may be elucidated. When these studies are done, it would be important to use samples that are less diluted in order to determine the levels of AI-2 in the first 180 minutes of the culture and confirm that the test and control had different levels.

During the luminescence assay, the importance of keeping the incubation period identical for all samples was observed, as luminescence is extremely unstable when time is altered. Knowledge of the optimum time period for sample readings would be beneficial for reproducibility of the assay. To gain understanding of the specific requirements of this assay, a time course experiment should be done. Preparing samples in replicate and reading sequentially would be essential in determining if or when the luminescence activity is stable.

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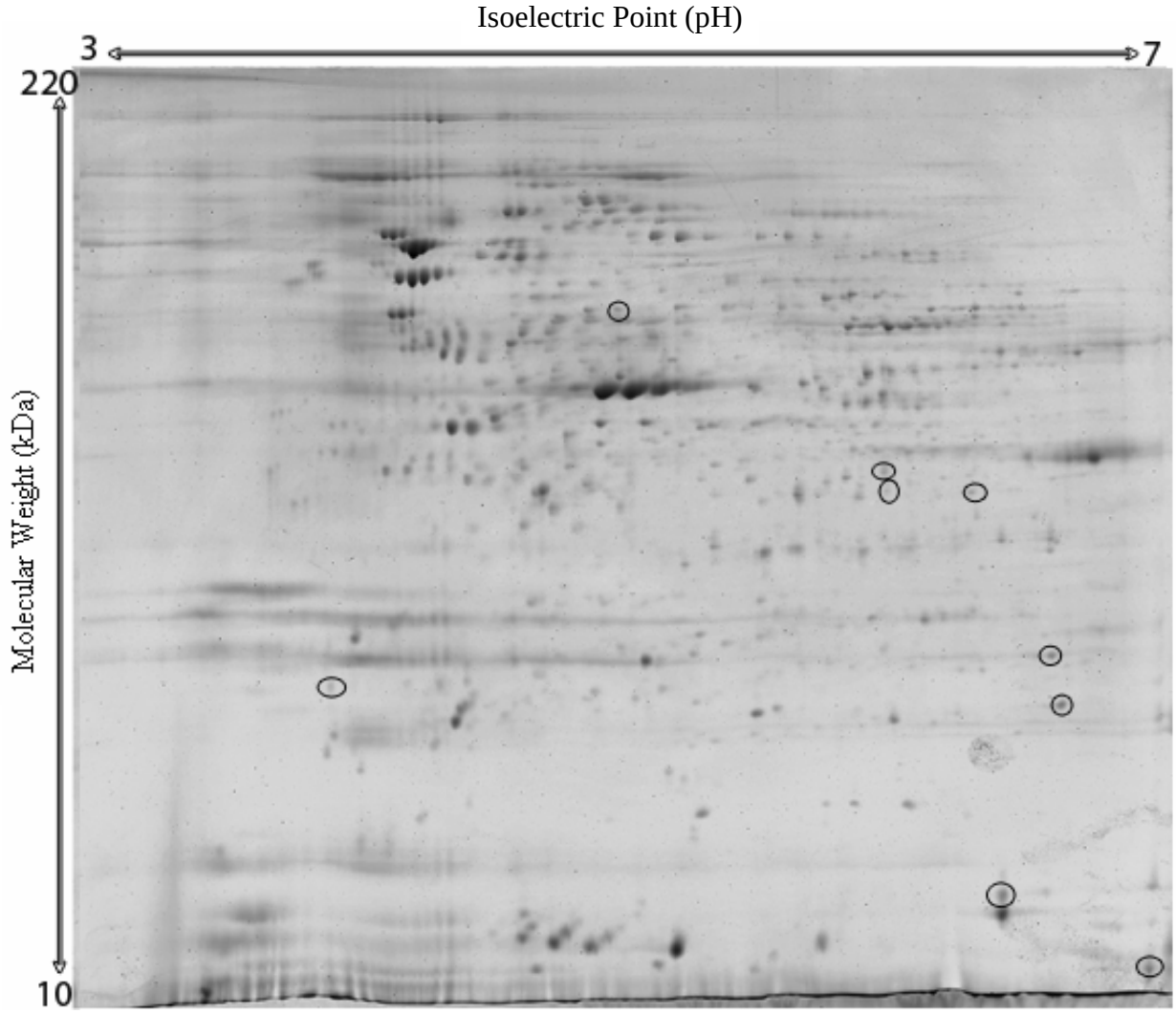
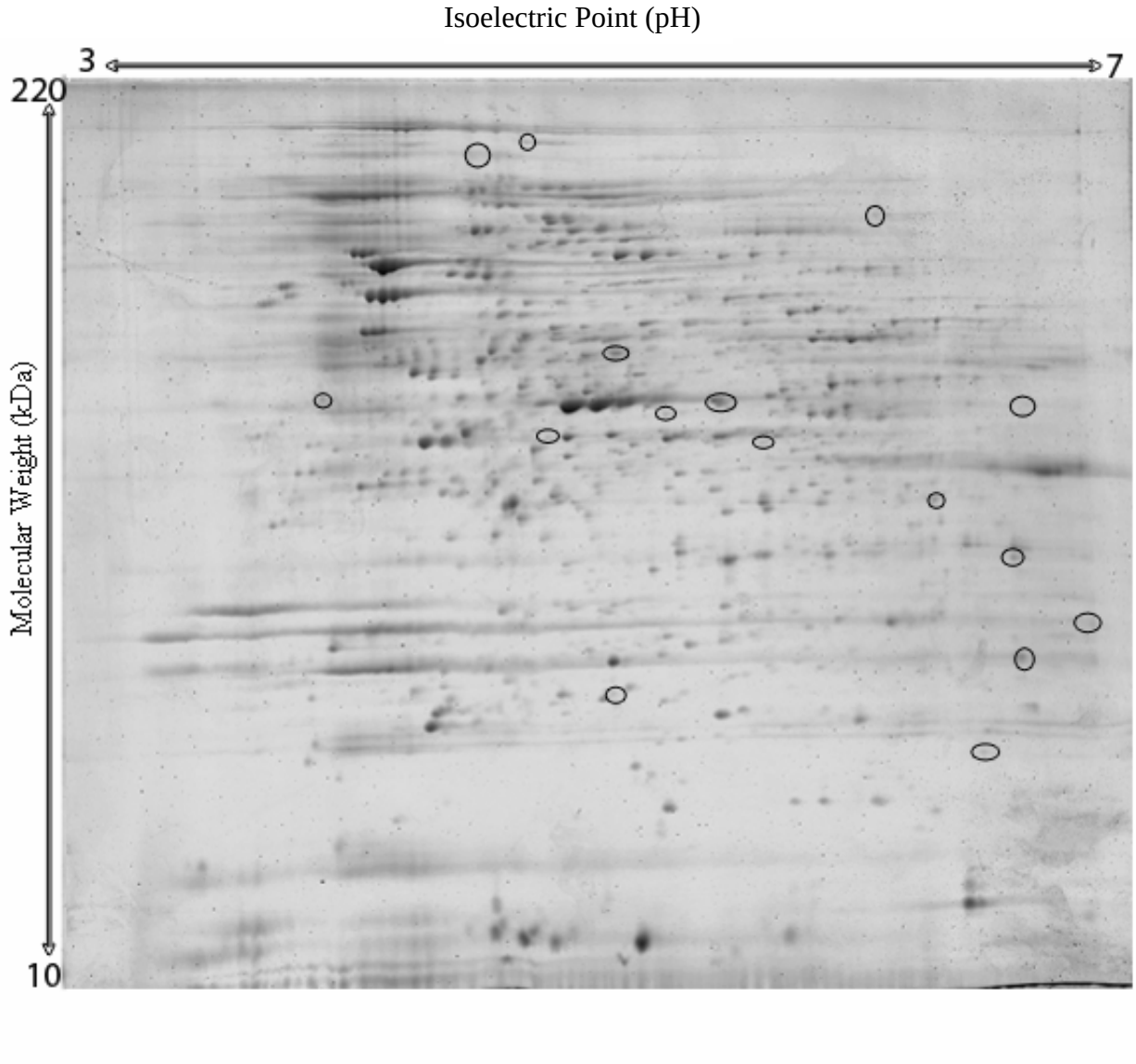
APPENDIX

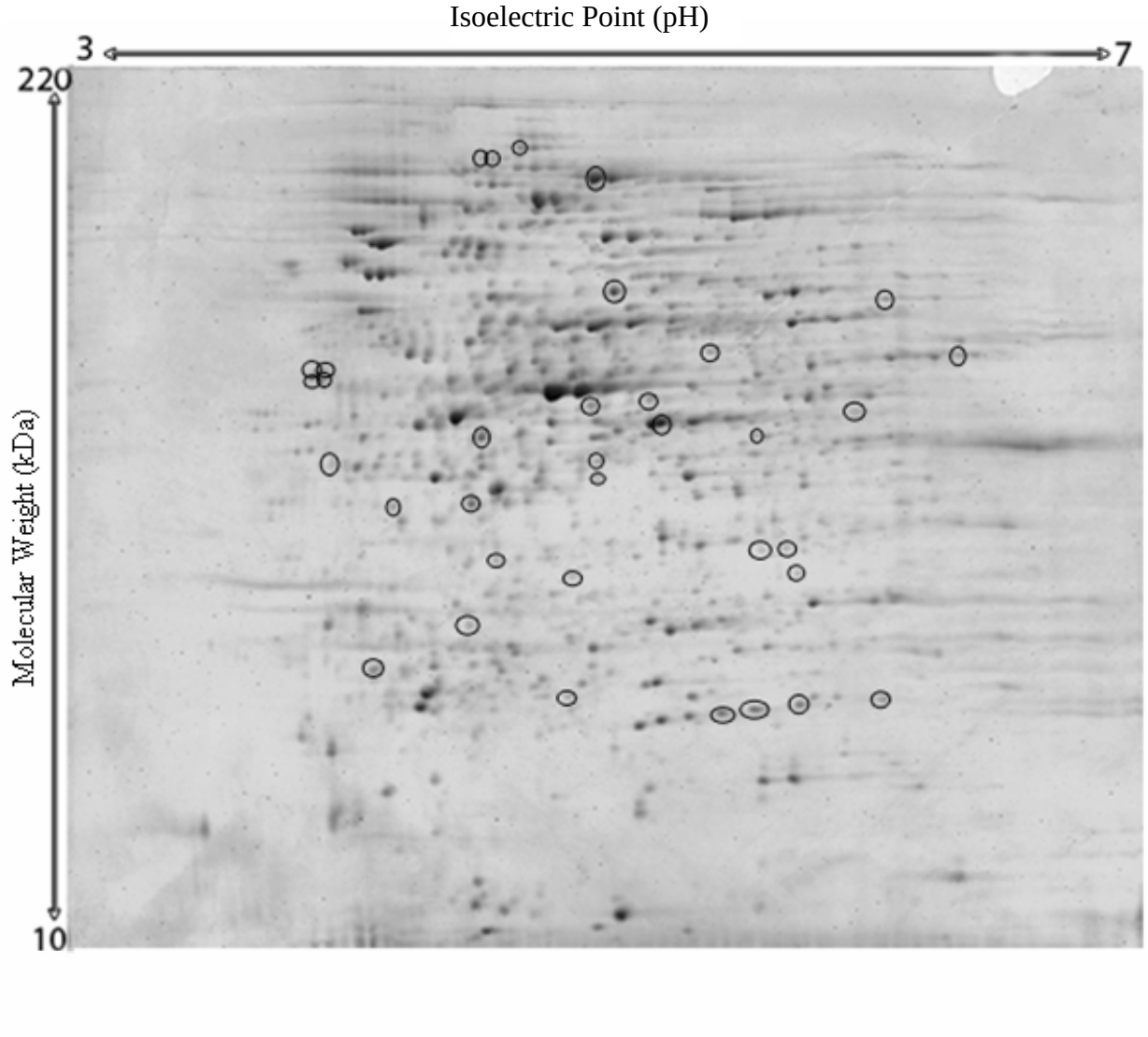
A1. Two-dimensional gel electrophoresis on the total protein from *E. coli* B23 after 150 minutes of growth without exposure to AI-2. The circles represent protein spots found in this gel but not one or more other gels.



A2. Two-dimensional gel electrophoresis on the total protein from *E. coli* B23 after 150 minutes of growth with addition of AI-2 at the start of the culture. The circles represent protein spots found in this gel but not one or more other gels.



A3. Two-dimensional gel electrophoresis on the total protein from *E. coli* B23 after 300 minutes of growth without exposure to AI-2. The circles represent protein spots found in this gel but not one or more other gels.



A4. Two-dimensional gel electrophoresis on the total protein from *E. coli* B23 after 300 minutes of growth with addition of AI-2 at the start of the culture. The circles represent protein spots found in this gel but not one or more other gels.