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The Environmental Conditions That Influence the Induction of the Autoinducer-2 Quorum Sensing System In *Escherichia coli* AB1157 and the Effects of This System on Growth

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***Escherichia coli* strain AB1157 is known to employ the autoinducer-2 (AI-2) quorum sensing system in certain environmental conditions. This system is hypothesized to allow the bacterium to alter its growth rate in response to environmental stimuli such as the nature of the carbon sources available and the pH of the environment. Maximum AI-2 production was expected in acidic, glucose-supplemented media. This maximal level of AI-2 was expected to reduce the growth rate of the test cultures relative to a control situation. The small AI-2 molecule can be quantitatively detected through the use of a *Vibrio harveyi* reporter strain; this bacterium luminesces at a threshold stimulatory concentration of the autoinducer. The trend observed in the data indicates that maximal autoinducer production may occur when AB1157 is grown in media supplemented with glucose. However, no direct correlation between maximal levels of autoinducer production and alterations in growth pattern were observed. Our results do not conclusively show a relationship between AI-2 production and growth nor can a comparison be made on the effects of glucose versus glycerol metabolism on AI-2 production.**

Quorum sensing involves bacterial production, release and response to hormone-like molecules called autoinducers (13). The ability to modify behaviour in response to these molecules is useful to bacteria in many ways (3, 8). Bacteria often need to respond promptly to sudden environmental changes to facilitate survival. Such responses involve defending against other microorganisms in order to compete for the same nutrients or adapting to changes in availability of nutrients. In a similar manner, it is crucial that pathogenic bacteria coordinate their virulence and evade the immune response of the host therefore establishing a successful infection within the host (8).

As would be expected due to the wide variation in bacterial types, there is also variation in the quorum sensing mechanisms employed. Gram-negative species typically produce acyl-homoserine lactones as autoinducers whereas gram-positive species produce uncharacterized peptides as autoinducers (8,10). Recent studies suggest that more than one type of autoinducer is produced by some gram negative bacteria. A bacterium that has been found to possess this second type of quorum sensing system is *Salmonella typhimurium*. In the *Salmonella* system, an organic molecule is produced for signalling purposes (12). The locus of the gene responsible for this novel quorum sensing mechanism was mapped and the gene was called lux_{St}. (13)

Due to the discovery of this second system in *Salmonella*, a well-known enteric pathogen, libraries of other enteric pathogens were screened looking for homologous quorum sensing systems. One such system was found in *Escherichia coli* and subsequently named lux_{Ec}. The *lux* gene was apparently induced in conditions where a good carbon source and low pH were present (13). Use of genomic arrays to try and determine the function of this system in *E. coli* have shown that when AI-2 is produced, the genes involved in cell division are down regulated and those involved in suppression of cell division are up regulated (13).

However, no conclusive direct correlation between the production of AI-2 and growth has been found. Some previous attempts at studying this phenomenon were marred by a lack of understanding of the conditions that induce AI-2 production (6). This study aimed to show that both a preferred carbon source and low pH are necessary to induce the maximal activity of the *E. coli* AI-2 quorum sensing system. This was attempted through the use of different growth inducing conditions combined with an assay using *Vibrio harveyi* as a reporter strain (11). This assay is based on the interaction of AI-2 with the *LuxP* receptor protein, resulting in a signal cascade that leads to the production of light (2). We also attempted to correlate AI-2 production to changes in bacterial growth patterns through comparison of the growth curves of *E. coli* AB1157 (quorum sensing) and a non-quorum sensing strain, *E. coli* DH5α, in the various test conditions.

Due to problems with strain growth and time limitations, the spectrum of conditions tested was not as great as originally intended. The effect of carbon source variation, but not pH manipulation, on the production of AI-2 by AB1157 was monitored. The hypothesis for this protocol was that a preferred carbon source, glucose, would result in greater amounts of AI-2 being produced and therefore slower growth relative to control conditions.

MATERIALS AND METHODS

Media. The Luria Bertani (LB) broth, agar plates and M9 minimal media were prepared as described (9). 20% sterile stock solutions of glucose and glycerol were prepared and used at final concentrations of 0.25%. Marine Broth 2216 (MB) (Difco) was used for the autoinducer assay.

Bacterial Strains and Culture Conditions. Initially, *Escherichia coli* AB1157 (Dr. Ramey, UBC) was grown overnight in LB broth at 37ºC with aeration. The cultures were diluted 1:40 in M9 minimal media supplemented with 0.25% glucose or glycerol at pH 6.0 or 7.0.

For the second trial, the overnight culture of AB1157 was diluted in LB broth and LB broth supplemented with 0.25% glucose or glycerol. Cultures were grown for 8 hours. A wild type *Vibrio harveyi* strain was grown overnight in MB at 30 ºC with aeration.

Growth. Culture turbidity was monitored at 600 nm using a Spectronic 20+ spectrophotometer (Spectronic Instruments). Viable cell counts were set up by serial dilution of samples in 0.85% sterile saline and plating onto LB agar. The pH was monitored using an Accumet® 900 pH meter (Fisher Scientific).

Autoinducer Assay. Cell-free extracts were prepared as described (11); samples were spun for 20 min, filtered through a 0.2 μm Millipore filter and frozen at –20ºC. *V. harveyi* was grown up overnight in MB. The autoinducer assay was set up in a 96-well white microtitre plate as described (11) with MB instead of AB broth (4). Luminescence was measured in a Wallac Victor 1420 Multilabel Counter (Perkin-Elmer Life Sciences) set in luminescence mode with readings recorded every half hour for three hours. The samples were incubated at 30˚C with rapid shaking

RESULTS

There was a markedly long lag phase (~ 5 h) of growth in the cultures in the first trial. These AB1157 cultures had a final OD_{600} of 0.2 after 8.5 h of growth (results not shown). This was attributed to the transfer from rich media to supplemented minimal media. Attempts to rectify this by growing the cultures overnight in supplemented M9 minimal media before transfer into the same media were not successful for AB1157 due to the lack of essential amino acids and vitamins required for its growth. Due to time constraints, the protocol was modified to compare the effects of glucose and glycerol on AI-2 production and AB1157 was grown in LB broth with and without the required carbon supplements.

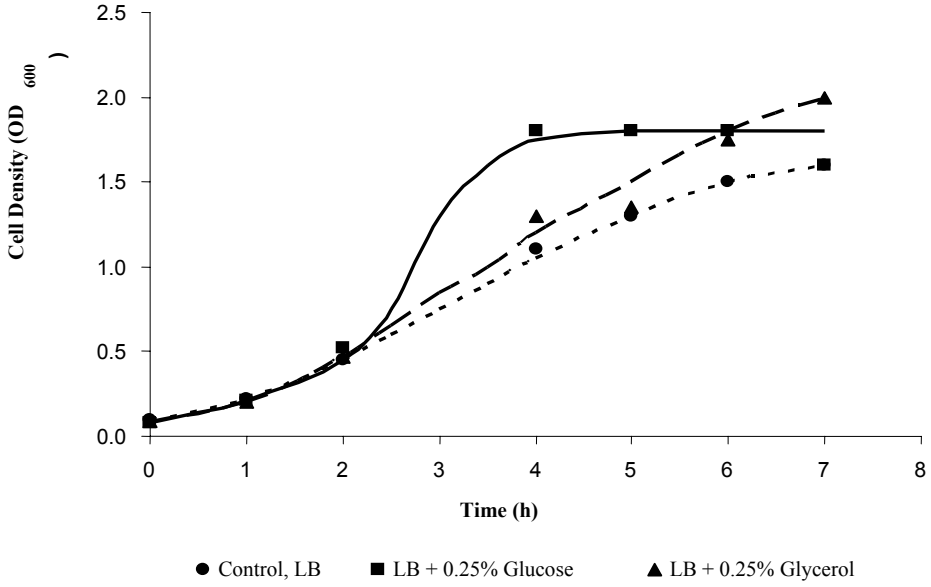

Fig. 1: A comparison of cell density measurements for AB1157 in different growth conditions as determined by OD_{600}.

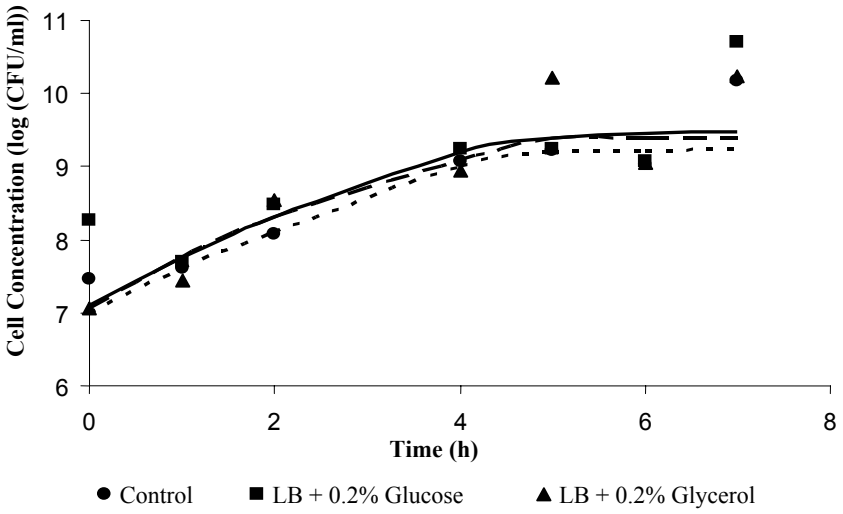

Fig. 2: Plate count measurements of AB1157 cell concentrations (cfu/ml) in glucose, glycerol or non-supplemented LB. - - - control, ─ glucose, ─ ─ glycerol

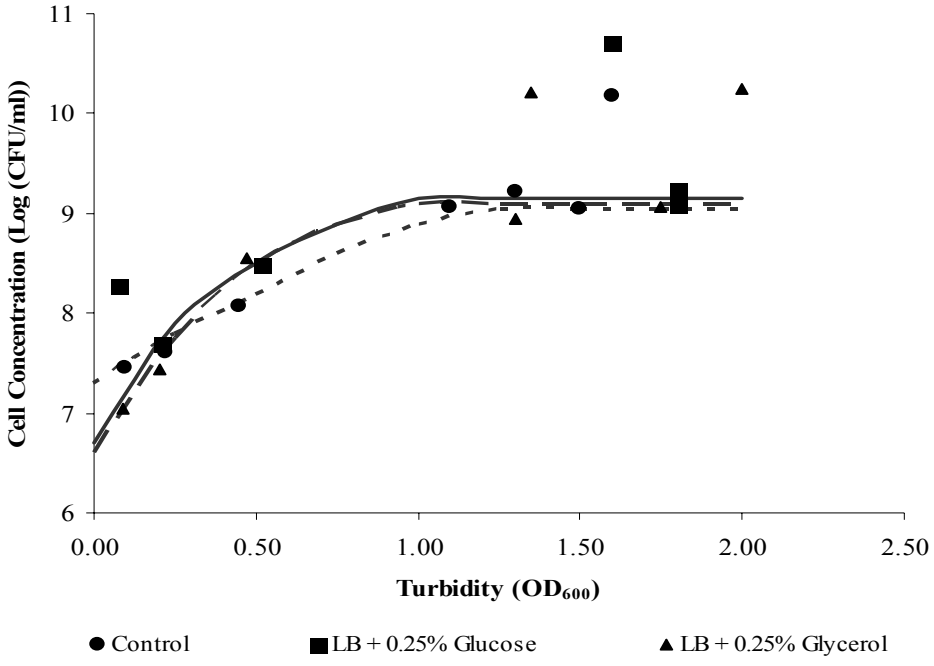

Fig. 3: Standard growth curves for AB1157 grown in glucose, glycerol and non-supplemented culture conditions. - - - control, ─ glucose, ─ ─ glycerol

From the standard curves (Fig. 3), we were able to determine that 1.0 OD_{600} is approximately 10^9 cells/ml. All the cultures levelled off at approximately the same cell concentration. As shown in Figure 1, the turbidity of the three

cultures increased at different rates. The glucose treated culture entered stationary phase sooner (4 h) than the control or the glycerol treated cultures which seemed to be in the late exponential phase of growth.

The trends for changes in cell concentration were similar for all cultures despite the differences in the change of turbidity. There was a similar increase in cell number in the glucose and glycerol treated cultures (Fig. 2). Cell division in the control may have been slower, however it reached the same plateau as the other cultures by 5 h. The curve in the lines suggests that the growth rates of all the cultures were not constant.

Although pH was not manipulated in the second trial, results from monitoring it showed some interesting observations (Fig. 4). The pH of the cultures differed despite similarities in growth. The pH of the glucose-supplemented culture dropped rapidly from an initial level of 6.25 to a low of 4.5 in 4 h; the pH of the glycerol supplemented culture also dropped to 5.5 pH units. However, the control culture had a rise in pH from the initial 6.25 to 6.9.

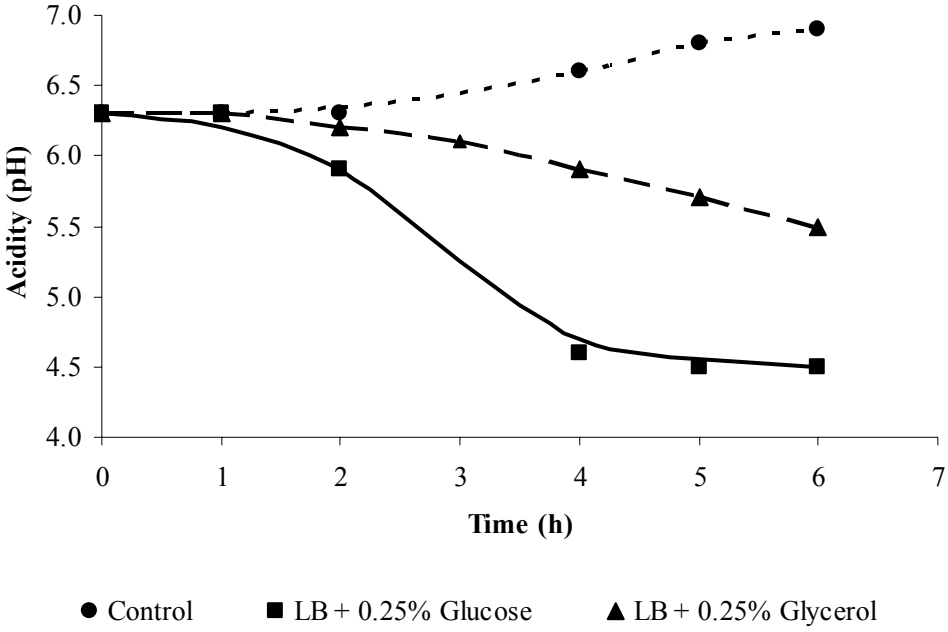

Fig. 4: Change in pH of LB medium as a result of AB1157 growth.
- -control, ─ glucose, ─ ─ glycerol

The luminescence readings for AI-2 activity were not conclusive (Fig. 5). We expected autoinducer production to be proportional to light production in the *V. harveyi* reporter strain. Levels of light intensity were low to begin with (< 200 relative light units (rlu)) and decreased within the first hour of incubation. The luminescence value obtained for the positive control (*V. harveyi* in MB, no supernatant) was subtracted from all the other values to account for endogenous luminescence (Table II).

A pattern can be observed in the luminescence levels which are correlated to the % of autoinducer present in the samples. A moderate level of intensity is present which then peaks at 6 h for the glucose-supplemented culture and at 3 h for the control culture. The intensity of luminescence over time in the glycerol-supplemented culture is relatively constant (Figures 5 and 6).

DISCUSSION

The hypothesis of the experiment focused upon observable differences in the growth curves of AB1157 grown in different experimental conditions. These differences were then correlated to autoinducer production. The investigation of this hypothesis relies upon the construction of an accurate and reliable growth curve. Initial attempts to grow AB1157 in glucose or glycerol-supplemented M9 minimal media failed. This strain is an

auxotroph that requires additional amino acid and vitamin supplementation to allow growth. pH manipulation also presented a problem due to the high buffering capacity of the media. The changes to the experimental protocol eliminated pH manipulation. Instead, comparisons between glycerol and glucose metabolism on AI-2 production were addressed.

Table 2. Normalized % luminescence values for each of the AB1157 cultures.

	Glucose				Glycerol				Control			
Sample	Average	Error	Normalized	Error	Average	Error	Normalized	Error	Average	Error	Normalized	Error
0 Hour	15	2.3	30	15.7	30	2.3	60	8.8	27.5	2.3	55	9.4
1 Hour	22.5	2.3	45	11.1	15	2.3	30	15.6	20	2.3	40	12.2
2 Hour	27.5	2.3	55	9.4	12.5	2.3	25	15.8	47.5	2.3	95	6.6
4 Hour	22.5	2.3	45	11.1	17.5	2.3	35	13.7	12.5	2.3	25	15.8
5 Hour	15	2.3	30	15.6	27.5	2.3	55	9.4	17.5	2.3	35	13.7
6 Hour	50	2.3	100	6.4	25	2.3	50	10.2	22.5	2.3	45	11.1
7 Hour	15	2.3	30	11.1	17.5	2.3	35	13.7	32.5	2.3	65	8.3

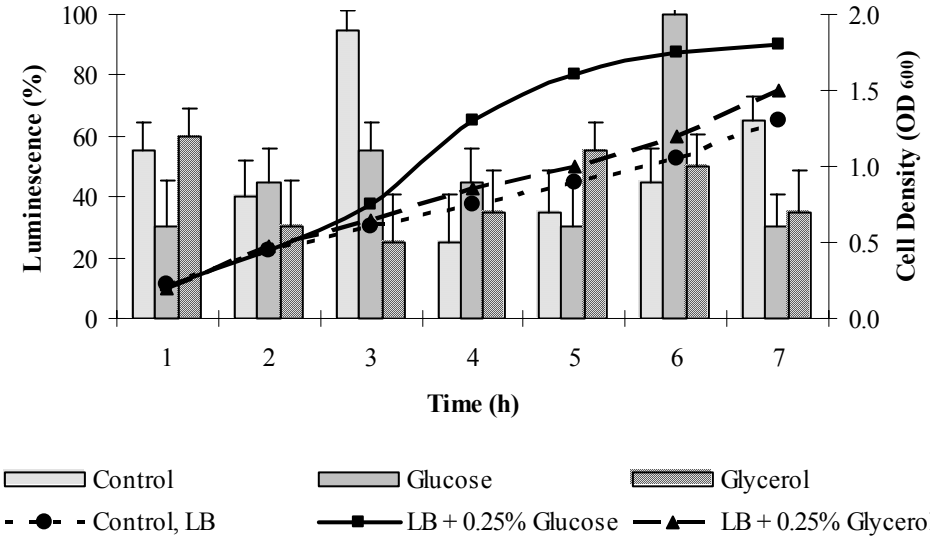

Fig. 5: Comparison of AI-2 production measured as luminescence (bars) to AB1157 cell density (lines).

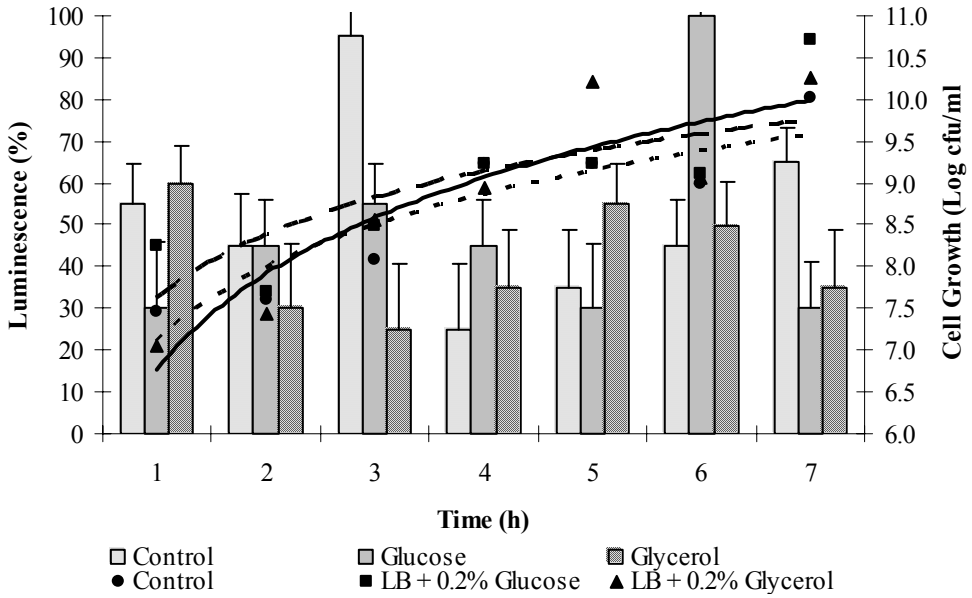

Fig. 6: Comparison of AI-2 production measured as luminescence (bars) with cell concentration (lines).
- - - control, ─ glucose, ─ ─ glycerol

Figure 2 shows relatively similar growth curves for all three conditions. This suggests that either AI-2 was not produced, or else it did not accumulate to stimulatory levels required to alter the growth rate within the time allocated. It also may not have had an effect on the cells since there was little difference between the control and the other culture conditions. These data also suggest that growth was not constant and may have been due to the uncertainty in the measurements. The results in Fig. 1 indicate that the turbidity of the glucose-treated culture rose faster than the other conditions with stationary phase attained sooner. However, the cell division rate in Fig. 2 did not follow this trend suggesting that cell division may be 'suppressed' compared to the turbidity. This suppression may the expected effect that is induced by AI-2.

It is difficult to compare the results as the viable counts are subject to error and may not be accurate. Due to the variability of the data, it is difficult to ascertain the trend in the results. In addition, small changes in cell concentration may be masked. All the cultures seem to have similar growth curves when comparing cell concentrations but significantly different curves when comparing cell density. A source of error in cell counting was due to the clumping of cells making it difficult to distinguish between colonies. In addition, many cells were growing along the edges, therefore the cell concentrations as determined by plate counts are possible under-representations of the actual number of cells present during the various time points. These two flaws make the accuracy of the growth curves questionable. Ideally several replicates would have been done to allow more valid comparisons of the differences.

Another factor that is thought to influence AI-2 productions is pH (11). Although pH was not factored in the hypothesis of the second trial, monitoring showed that there was the expected drop in the pH of the glucose-supplemented culture due to the production of acidic by-products of metabolism. The control, however, showed a rise in pH to neutral indicating production of alkaline by products as a result of amino acid metabolism. It is possible that a drop in pH could induce autoinducer production as a signal that the environment is becoming unfavourable and therefore the growth rate would be slowed to prepare cells for stationary phase (5). In addition, AI-2 has been observed to be acid stable and labile in alkaline conditions (11). We speculate that secretion into an acidic environment would be required to maintain its structural integrity.

The original assay for the production of autoinducer involved the use of *Vibrio harveyi* reporter strains: BB170 which is capable of sensing AI-2 but mutated in its AI-1 sensor system and BB152 which is capable of producing AI-2 and not AI-1 (11). Unfortunately we were unable to obtain the desired strains within the time allocated for the experiments and substituted with a wild type strain. It is possible that endogenous luminescence stimulated by both

AI-1 and AI-2 in the reporter strain affected the assay results. This would be evident in samples with AI-2 concentrations below the threshold required to stimulate significant light production above that naturally produced by the reporter strain. Assay conditions also required the use of AB media for the growth of the reporter strain (4). However, the wild type strain used did not grow to the desired cell density in an overnight culture. The protocol was therefore modified to use Marine Broth in which there was a better growth response. This medium is designed to mimic mineral compositions in seawater (Difco Laboratory Manual online, http://www.bd.com/industrial/difco/manual.asp) whereas AB media is similar to a minimal media with the required carbon and amino acid supplementation.

It was predicted that autoinducer activity would be highest at mid-exponential phase resulting in a slowing of growth; however, results were not as expected. There were significant differences in cell turbidity, however similar growth was observed in all culture conditions when comparing cell concentrations. The glucose and glycerol treated cultures seemed to have similar rates of cell division despite the changes in turbidity and cell division may have been slower in the control (Fig.2) suggesting that perhaps AI-2 may have a suppressive effect on cell division as expected. AI 2 is thought to affect the down-regulation of genes involved in cell division therefore maximal production is expected to be correlated to rapid logarithmic growth (10) with a resulting effect on growth rate. AI 2 production is also thought to be regulated in response to changing cell densities (1, 11). Production was therefore expected to be minimal in the early stages of growth, increase rapidly toward late exponential phase then decrease in stationary phase. It is possible that AI-2 is produced as a result of increasing cell density or in response to decreasing nutrient availability. Studies suggest that bacteria may have evolved a means to monitor crowding as competition for nutrients and enable the cells to begin the transition into stationary phase sooner. This stage of growth enables the cells to adapt to stressful environmental conditions such as the absence of nutrients and low pH (5).

In this experiment, AI-2 production did not correlate to the expected mid exponential growth phase. Peak production was expected to be seen between 4 -5 h in the glucose-treated culture with subsequent effects on cell growth (4) however peak production was at 6 h (Fig. 5 & 6) with no observable effects on either cell density or concentration. Even though the graphs show a trend of sorts, these results are inconclusive for three main reasons.

First, AI-2 levels were expected to be minimal or undetectable at the beginning of the experiment whereas the results show 30-50% for the different growth conditions. This suggests that there may have been residual luminescence or autoinducer remaining in the overnight culture that affected results. As well, the light intensities of the samples were below the required minimum 200 relative light units (Helena H. Wang, personal communication), thus it is difficult to determine the accuracy of the results of the assay.

Second, the microtitre plate was covered with a plastic seal to prevent contamination between the wells under the conditions of incubation. This may have resulted in low oxygen tension and subsequently cell death since *V. harveyi* is aerobic and requires sufficient aeration for growth and luminescence. This may account for the subsequent reduction in light intensity readings over the three hours of luminescence measurements (see Appendix).

Third, previous studies suggest that AI-2 production is only induced in the presence of a sugar that can be transported via the phosphotransferase system (11). Experiments previously conducted showed that there was no production when the quorum sensing strain was grown in LB broth. Production occurred only when grown in LB supplemented with a carbon source such as glucose. As a result, our control was AB1157 in LB only and no AI-2 was expected to be present in the cell-free extracts therefore there shouldn't have been any light production. However, this result can also be attributed to the residual activity from the overnight *V. harveyi* culture.

This assay also depends on cell density. Light production in *V. harveyi* occurs when cell density is at a threshold concentration (7). It is possible that this threshold was not attained in the length of time that the assay was carried out for some of the reasons previously mentioned. It is also possible that the transfer from an overnight culture in stationary phase to fresh media would have required an initial adjustment period before active growth occurred. In addition, there is uncertainty as to whether the culture provided was a pure culture of *V. harveyi* that would respond to AI-2 since some species of Vibrio are insensitive to AI-2 (4). If this were the case, then the assay results would have been affected.

The hypothesis that glucose as opposed to glycerol would result in maximal AI-2 production and subsequent alteration of the growth rate was not confirmed. Our results show no correlation between autoinducer production and growth. Two possible conclusions can be drawn from this; either significant error was accumulated over the course of the experiment rendering the results inconclusive or AI-2 does not influence AB1157 growth.

FUTURE EXPERIMENTS

The structure of the AI-2 molecule is unknown; hence designing assays to detect its presence are difficult at best. The best course of action to undertake for future studies is to first characterize its structure then proceed with analyses of function. Current information is non-specific and only states failure to have the autoinducer bind to ion exchange columns; molarity of elution fluids and column specifics are not given (11). Attempts to purify it using differential salt fractionation, ion exchange and size exclusion chromatography would enable size and charge data to be determined. Alternatively, thin layer chromatography may be used to isolate this molecule. Precipitation by an organic solvent could provide a pure sample for functional studies. It would also be useful to identify strains other than AB1157 that produce AI-2 that can be used to continue this study. Current availability of the *V. harveyi* BB170 and BB152 reporter strains will enable the use of the assay to detect AI-2.

Although knowledge of the structure and properties of the AI-2 molecule would have been valuable to our experiment in terms of designing an assay, it would not have solved our problems in methodology. The first difficulty encountered was the inability of AB1157 to grow in minimal media supplemented with a carbon source. This strain is auxotrophic for several amino acids and requires rich media or minimal media with the necessary supplements. We think it is not an ideal strain to use in growth variation studies therefore a broader range of *E. coli* should be examined to identify strains that are more suitable for growth experiments. The second difficulty encountered was in the assay to detect for presence of AI-2. Only one assay is currently available. This assay requires the use of *V. harveyi* BB170 that can only detect and respond to AI-2. This response results in the production of light which can be then be correlated to AI-2 levels present. Unfortunately we were unable to acquire the desired mutant strain and had to use a wild type organism. The wild type strain might not have responded to AI-2 in the same way as *V. harveyi* BB170 since the presence of endogenous AI-2 and AI-1 might have masked the results.

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APPENDIX

Sample Calculations:
1. Plate counts
Colony forming units (CFU)/ml = # of colonies counted x final plated dilution factor

2. Luminescence readings

1) (Well Reading) – (Positive control (*V. harveyi c*ells alone)
Example: (70 rlu for LB time 0 – 55 cells alone) = 15rlu corrected value

2) Average reading for each well = (Reading from Plate 1+ reading from Plate 2)/2
Example = (LB time 0 15rlu + 40 rlu)/2 = 27.5 Raw value

3) Set the Values to a Normalized Percent = Call the Highest Value 100%.
= Average Reading/ Highest Reading X 100%
Example = (27.5 rlu for LB time zero/ 50 rlu Maximum value) X 100% = 55%

3. Error calculations for Table II

Absolute Error for each Count:

$$=\sum \frac{\text{Readings from the Empty wells}}{\text{Total Number of Empty Wells}}$$

Absolute Error for the Average of the 2 Counts:

$$= [(\text{Error From Count 1})^2 + (\text{Error from Count 2})^2]^{1/2}$$

Normalized Error:

1) Error Count1/ Count 1 Value X 100%
2) Error Count 2/Count 2 Value X 100%
3) Total error = $[(\text{Normalized Error Count1})^2 + (\text{Normalized Error Count 2})^2]^{1/2}$

Table 1: Readout from the Luminescence Plate Count

1	2	3	4	5	6	7	8	9	10	11	12		
0	20	5	5	5	5	5	0	WT SN 65	70	60	110	A
0	5	10	5	0	15	0	5	WT 55	90	75	80	B
5	0	0	5	0	0	5	5	MB 0	125	105	75	C
5	5	5	5	0	10	10	5	10	75	70	85	D
10	0	0	0	10	5	10	5	0	60	65	80	E
0	5	0	5	0	0	0	0	10	65	130	65	F
5	0	5	5	5	5	5	5	5	95	60	55	G
0	5	0	5	0	5	0	5	0	5	10	5	H
									controls	LB	Glu	Gly	

Plate	Repeat	End time	Start temp.	End temp.	BarCode
1	2	11:19:01 AM	29.3	30	N/A

CPS (0.2s)
(CPS)
0

0	5	5	5	0	5	10	0	45	50	35	15	A
0	5	0	5	5	10	0	0	10	15	35	15	B
0	5	5	5	10	0	0	0	0	35	15	15	C
5	10	0	5	0	10	0	0	0	15	40	15	D
5	0	0	5	0	0	0	5	0	40	30	40	E
5	5	0	5	0	0	0	0	0	45	35	50	F
0	5	0	10	0	0	5	0	0	35	35	45	G
0	0	0	0	0	0	0	0	0	0	0	0	H

KEY
Wells A12 to G12 represent glucose-supplemented sample time points 1 – 8 (Glu)
Wells A11 – G11 represent glycerol-supplemented sample time points 1 – 8 (Gly)
Wells A10 – G10 represent control time points 1 – 8 (LB)
A9 : *V. harveyi* cells with cell free extract (WT SN = wildtype supernatant)
B9: *V. harveyi* cells in MB only (Positive control) (WT)
C9 : Marine Broth only (negative control) (MB)

AB Media

17.5 g NaCl
6.02 g $MgSO_4$
2.0 g Casamino acids, vitamin free

Make up to 960 ml with dH_2O
Adjust to pH 7.0 with KOH. Autoclave. Cool to RT and aseptically add the following sterile solutions
10.0 ml 1M K_3PO_4, pH 7.0
10.0 ml L-Arginine
20.0 ml 50% glycerol