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Plasmid Variance and Nutrient Regulation of Bioluminescence Genes

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Summary

Bioluminescence, the natural production of light by living organisms, may one day compete with electrical lighting as a form of light production. The limited gene expression of bioluminescent proteins is one hindrance preventing the widespread application of bioluminescence. The purpose of this experiment is to increase expression of *Aliivibrio fischeri* bioluminescence genes, study the effects of increased expression, and examine the dependence of bioluminescence on nutrient concentration. Discovering the connection between nutrient dependency and light production with *A. fischeri* could open new possibilities to receive maximal bioluminescence.

Expression of *A. fischeri* genes from a high copy plasmid is expected to increase the gene expression and bioluminescence of *Escherichia coli*. We hypothesize that because of an increase in the gene expression of the *A. fischeri* lux operon, the production of bioluminescence should be more dependent on nutrients, which provide cofactors and substrates for the bioluminescence reaction. After transfer of the *A. fischeri* genomic fragment to the high copy plasmid, the bioluminescence of the host *E. coli* increased significantly. Furthermore, bacterial growth seen in *E. coli* containing the high copy plasmid decreased. A complex pattern of regulation was seen after exposing the bioluminescent *E. coli* to various nutrients. Collectively, these findings demonstrate that the higher expression of bioluminescence genes, achieved with a plasmid with a higher copy number, did not render bioluminescent *E. coli* more dependent on nutrients for bioluminescence.

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Introduction

Bioluminescence, the natural production and emission of light by living organisms, is a phenomenon that is important to many organisms, as it allows various species to camouflage themselves, communicate with others, and mimic different creatures. Marine animals, arthropods, and fungi all include bioluminescent species, but luminous bacteria are the most widely distributed light-emitting organisms (1). Most of these organisms exist in saltwater and form symbiotic relationships with marine animals. *A. fischeri* is a luminous bacterium that has a symbiotic relationship with the Hawaiian bobtail squid; the bacteria feed from the squid's ciliated cells while the squid uses the bacteria's light to eliminate its shadow, protecting it from predators (1). The overall purpose of this research was to discover ways to increase bioluminescence in *A. fischeri* genes.

The first concept in this research is altering expression of the *lux* operon by changing the plasmid expression level. All of the genes required for the chemical reactions generating bioluminescence are contained in a single genomic fragment of the *A. fischeri* genome. The position of these genes and their roles are described in **Figure 1 and Table 1**. The *A. fischeri* genomic fragment contains two operons: the left operon, containing the *luxR* gene, and the right operon, which has the rest of the *lux* genes (*luxICDABEG*). The *luxA* and *luxB* genes code for the subunits of the bacterial luciferase, while *luxCDE* code for the fatty acid reductase polypeptides. The *luxI* gene encodes the enzyme that synthesizes the autoinducer molecule, and *luxR* encodes the receptor protein for the autoinducer. The receptor-autoinducer complex binds to a specific sequence of DNA in front of the right operon and activates the expression of the lux structural genes (2). The two luciferase operons are naturally found on

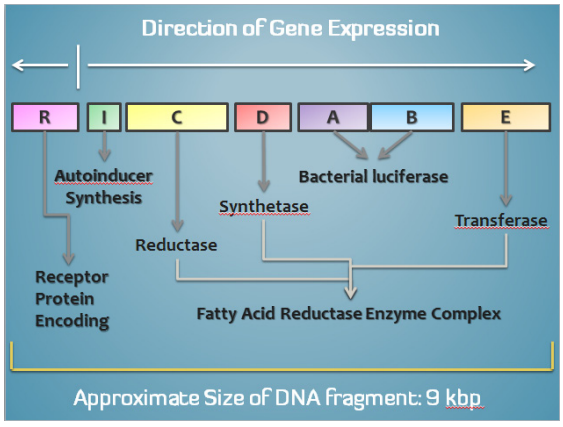

Figure 1: A map of the *lux* operons in the *A. fischeri* 9 kb fragment. Adapted from Lin and Meighen, 2009 (1).

Gene	Function
luxR	This gene codes for the receptor protein that binds the chemical autoinducer. When complexed with the autoinducer the receptor increases transcription of the *luxA, B, C, D, E* and *I* genes.
luxI	This gene encode the enzyme that synthesizes the autoinducer.
luxCDE	These genes encode the reductase, synthase and transferase enzymes, that make up the fatty acid reductase complex. That complex converts an activated fatty acid to a long chain aldehyde which becomes a direct substrate for the luciferase reaction.
luxAB	These genes encode the alpha and beta polypeptides, which create the bacterial luciferase. The luciferase catalyzes the bioluminescence reaction in *A. fischeri*.

Table 1: A table indicating the role that each *lux* operon gene found in *A. fischeri* plays in the production of bioluminescent light.

the chromosome of *A. fischeri*, but previous studies have used plasmids to study the expression of these genes. In this study we transferred this operon from a low copy plasmid used in a previous study (3) to a high copy plasmid. The bacterial bioluminescence was compared between the bacteria containing either high or low copy numbers of the bioluminescence operons (4). Greater expression of the *lux* operon was achieved after transfer to the high copy plasmid as the light production increased by a factor of 12.

In addition, the effect of supplemental nutrients riboflavin, galactose, glucose, and glycerol on bioluminescence expression was determined in this research. Vitamin B-2, also known as riboflavin, is a precursor for flavin, a key component of the bioluminescence reaction (5). Galactose, glucose, and glycerol provide reduced carbon sources that can be metabolized to fatty aldehydes and act as substrates in the bioluminescence reaction (6). Our hypothesis was that if the gene expression of the *A. fischeri lux* operon is increased in *E. coli* by increasing the plasmid copy number, then bioluminescence will be more dependent on nutrients, like vitamin B-2 and carbon sources to produce bioluminescence. Using a higher copy number plasmid increases the number of DNA copies per cell, leading to more RNA and higher levels of bioluminescent protein, (7). Consequently, the bioluminescence proteins may require more cofactors to render the bacteria more bioluminescent. A similar nutrient dependency has been shown previously using hemoprotein expression in *E. coli* (8). However, a greater nutrient dependence was not shown in the high copy plasmid compared to the low

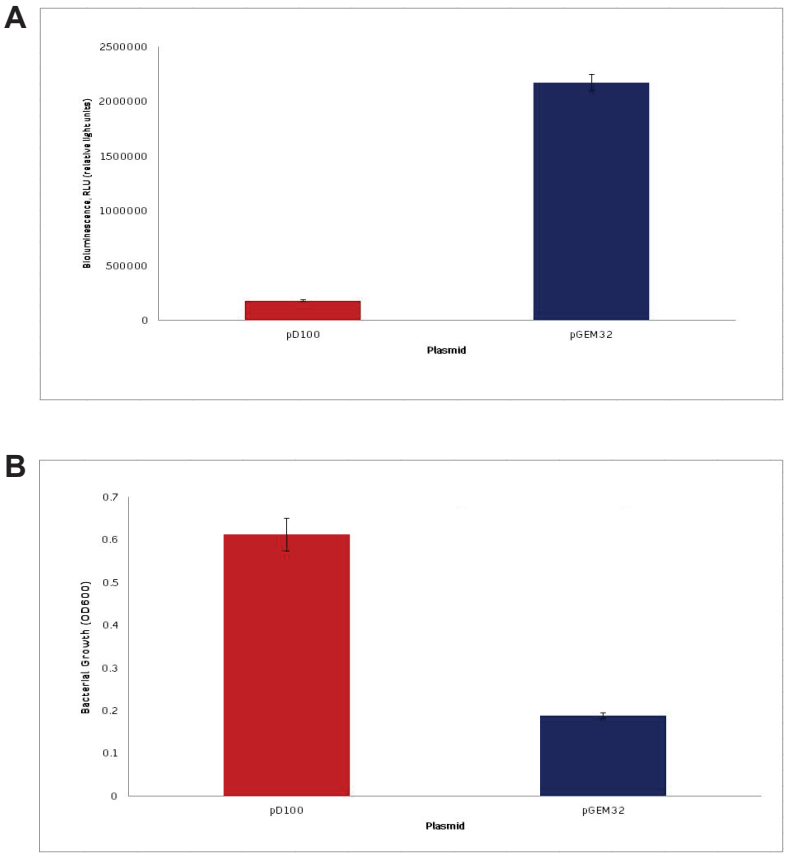

Figure 2: A) Comparison of the bioluminescence of DH5a/pD100-*lux* and DH5a/pGEM3Z-*lux* with the *A. fischeri* bioluminescence gene fragment. B) Comparison of the bacterial growth of DH5a/pD100-*lux* and DH5a/pGEM3Z-*lux* with the *A. fischeri* bioluminescence gene fragment.

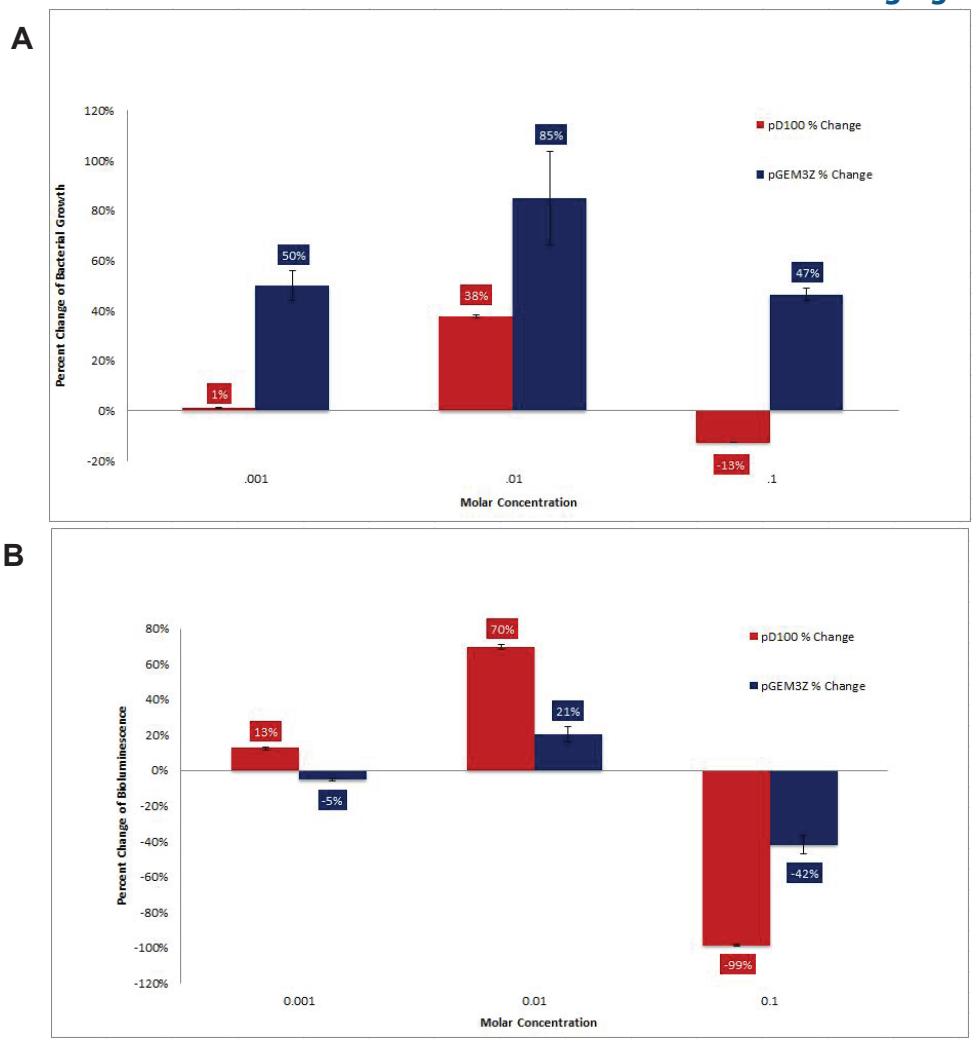

Figure 3: A) Percentage change for bacterial growth of DH5a/pD100-*lux* and DH5a/pGEM3Z-*lux* after exposure to galactose at different concentrations. B) Percentage change for bioluminescence of DH5a/pD100-*lux* and DH5a/pGEM3Z-*lux* after exposure to galactose at different concentrations. Bioluminescence data was normalized for the OD_{600} bacterial growth readings to represent cellular bioluminescence.

copy plasmid.

Results

The initial pD100 plasmid vector contains the entire *lux* genomic fragment, including the natural luciferase operon promoter sequences responsible for all gene expression monitored in these experiments. This plasmid was derived from pACYC184, a low copy number plasmid. The genomic fragment containing the bioluminescence genes was transferred into the pGEM3Z plasmid vector, which is maintained at a higher copy number than the pD100 vector (4). The *A. fischeri* genes were transferred into the pGEM3Z vector by isolating the genomic fragment from the pD100 plasmid, ligating the genomic fragment into the high copy plasmid, and finally transforming the plasmid into DH5a *E. coli* bacteria. Individual clones were characterized by restriction digestion and screening for bioluminescence. *E. coli* bacteria were exposed to nutrients by adding various concentrations of each nutrient to liquid cultures of the bacteria. The bacterial growth was observed after 12 hours of overnight growth in liquid culture at 37 ºC. Liquid assays of the bacterial culture were analyzed to measure bioluminescence as well as bacterial growth.

E. coli cultures transformed with the high copy plasmid had 12-fold high bioluminescence in comparison to the low copy plasmid, increasing from 172,000 RLU (relative light units) to 2,200,000 RLU (**Figure 2A**). Simultaneously, bacterial growth decreased to less than one-third of DH5a/pD100-*lux* (the bacteria containing the pD100 plasmid), from 0.613 OD_{600} units to 0.189 OD_{600} units (**Figure 2B**).

We next examined the effect of nutrient addition on bioluminescence and bacterial growth. The addition of

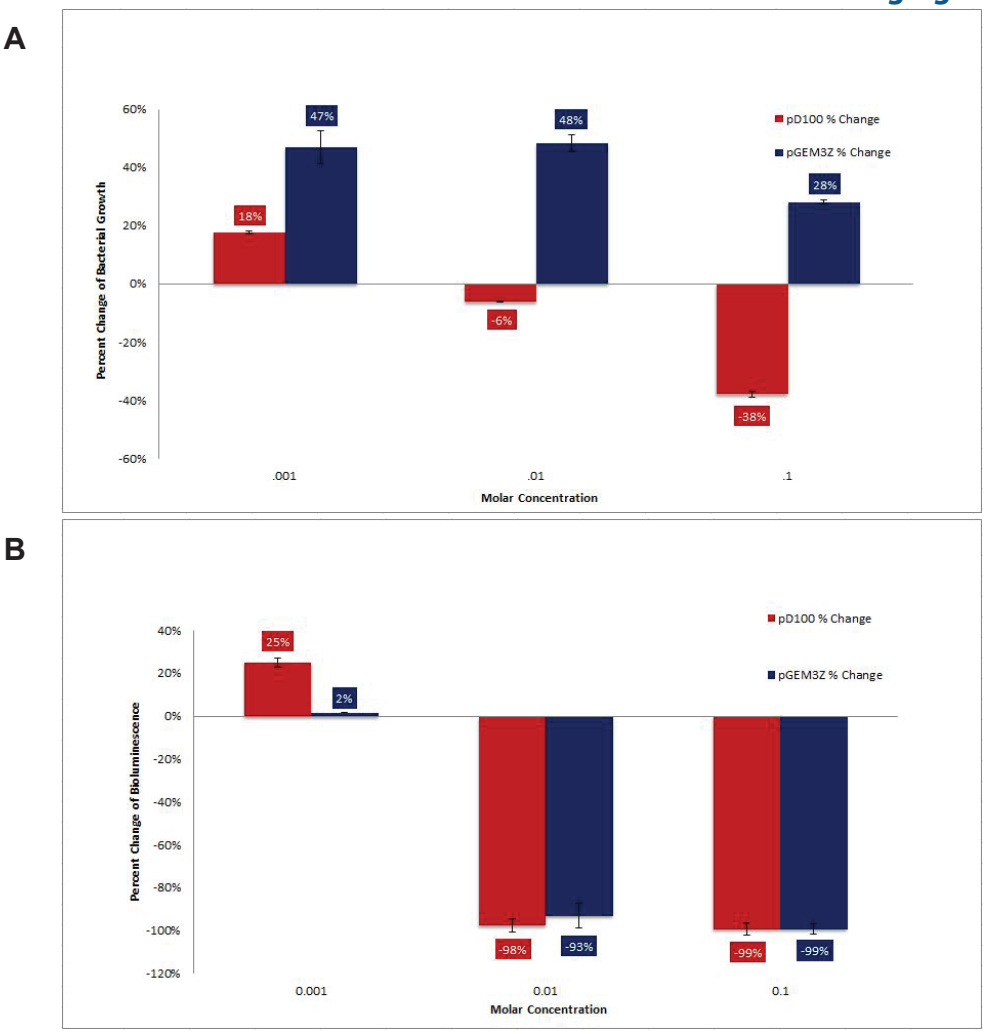

Figure 4: A) Percentage change for bacterial growth of DH5a/pD100-*lux* and DH5a/pGEM3Z-*lux* after exposure to glucose at different concentrations. B) Percentage change for bioluminescence of DH5a/pD100-*lux* and DH5a/pGEM3Z-*lux* after exposure to glucose at different concentrations. Bioluminescence data was normalized for the OD_{600} bacterial growth readings to represent cellular bioluminescence.

galactose caused an increase in bioluminescence and bacterial growth, both most pronounced at 10 mM, followed by an inhibitory effect on those two variables at 100 mM. However, the inhibitory effect on DH5a/pGEM3Z-*lux* (the bacteria containing the pGEM3Z plasmid) was less severe than for DH5a/pD100-*lux* (**Figure 3A and B**). The addition of glucose caused an increase in bioluminescence and bacterial growth up to a concentration of 1 mM, followed by an inhibitory effect in DH5a/pD100-*lux* at 10 and 100 mM. However, in pGEM3Z-lux, the inhibitory effect for bacterial growth was negligible (**Figure 4A and B**). Supplementation with glycerol showed an increase for DH5a/pD100-*lux*'s bioluminescence followed by a sharp decline at 100 mM, similar to the other two carbon sources. DH5a/pGEM3Z-*lux* exhibited a minor inhibitory effect on bioluminescence after exposure to glycerol at 1, 10 and 100 mM. Bacterial growth remained relatively constant for both plasmids despite the addition of nutrients (**Figure 5A and B**). Vitamin B-2 had a small stimulatory effect on the bioluminescence and the bacterial growth of both plasmids present in DH5a *E. coli* bacteria (**Figure 6A and B**). Overall, the nutrient dependence of DH5a/pGEM3Z-*lux* for bioluminescence was not greater than DH5a/pD100-*lux*'s same dependence, despite the increase in bioluminescence for DH5a/pGEM3Z-*lux*.

Discussion

As predicted, we found that transferring the bioluminescent operon to a higher copy plasmid did result in higher bioluminescence. However, the experimental results examining the effects of nutrient regulation did not support our second hypothesis. Regulation of bioluminescence by carbon sources was complex for DH5a/pD100-*lux* and DH5a/pGEM3Z-*lux*. Contrary to our hypothesis, DH5a/pGEM3Z-*lux* did not

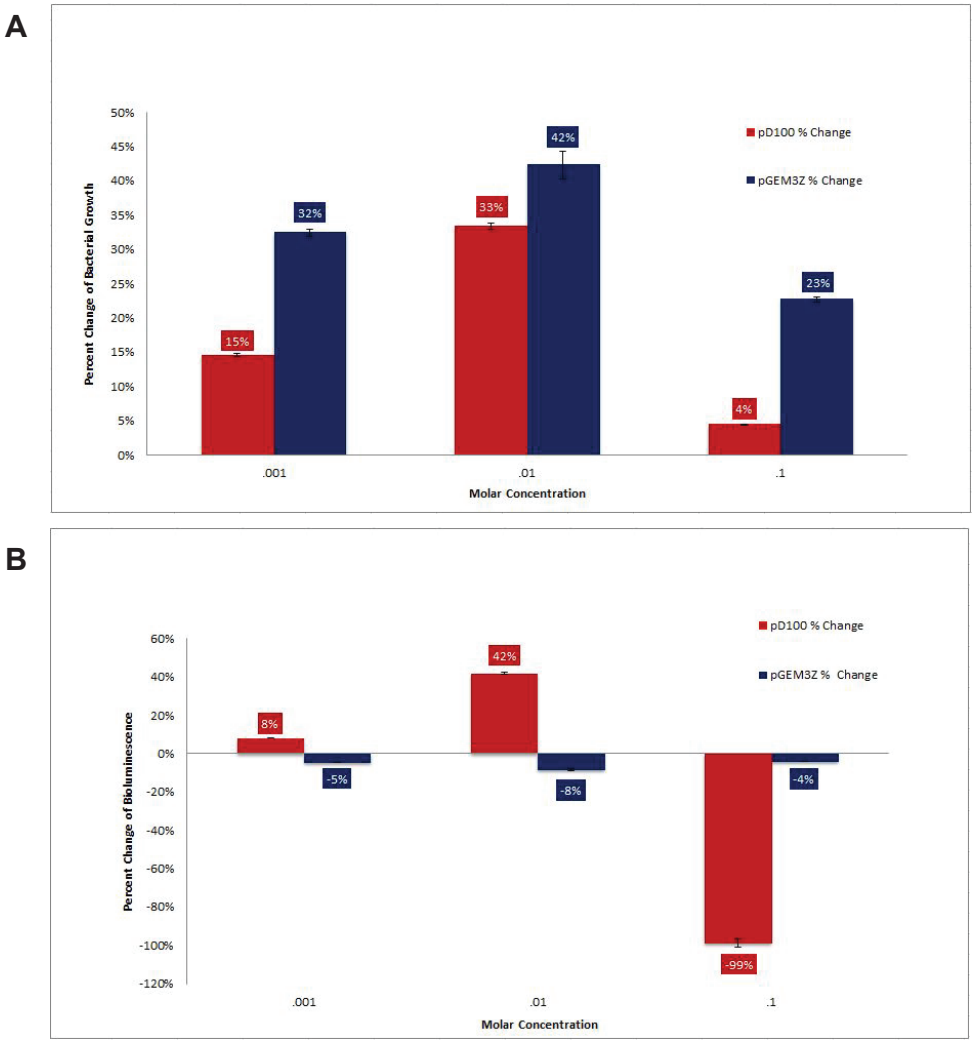

Figure 5: A) Percentage change for bacterial growth of DH5a/pD100-*lux* and DH5a/pGEM3Z-*lux* after exposure to glycerol at different concentrations. B) Percentage change for bioluminescence of DH5a/pD100-*lux* and DH5a/pGEM3Z-*lux* after exposure to glycerol at different concentrations. Bioluminescence data was normalized for the OD_{600} bacterial growth readings to represent cellular bioluminescence.

exhibit a greater dependence on nutrients. Also, vitamin B-2 had a minimal effect on DH5a/pD100-*lux* and DH5a/pGEM3Z-*lux*. These results were not consistent with previous research (8) on nutrient expression in *E. coli.* The most likely reason for the lack of nutrient dependence on carbon sources is that *luxR* protein concentration is shown to be repressed when *A. fischeri* is exposed to a high glucose concentration (2).

We observed a decrease in bacterial growth and bioluminescence with the addition of 100 mM riboflavin to the media. The toxicity of riboflavin is one possible explanation for this observation. Research indicates that riboflavin can be toxic for bacteria at a concentration of 0.1% (9). Therefore, riboflavin toxicity may be responsible for lower bioluminescence and inhibition of bacterial growth at higher concentrations.

In future experiments, it may be useful to examine each specific *lux* gene of the bioluminescent system in order to identify their roles within the system. After proper examination, altered expression of various genes may achieve greater bioluminescence. Dividing the bioluminescent genes into manipulable units would be a more effective way to analyze the roles that the bacterial strain, plasmid, and nutrients each play in light production. Alternately, in further experiments, we could employ bacteria, such as *E. coli* Rosetta to enhance translational efficiency of the *lux* operon genes (11). Furthermore, the introduction of cyclic AMP (cAMP) and AHL (the autoinducer) could produce increases in bioluminescence. Previous research indicates that cAMP is a vital part of the bioluminescence production by *A. fischeri* genes, and is also required to have proper control of the production (3). High glucose in cells leads to low cAMP levels, which could explain the inhibited

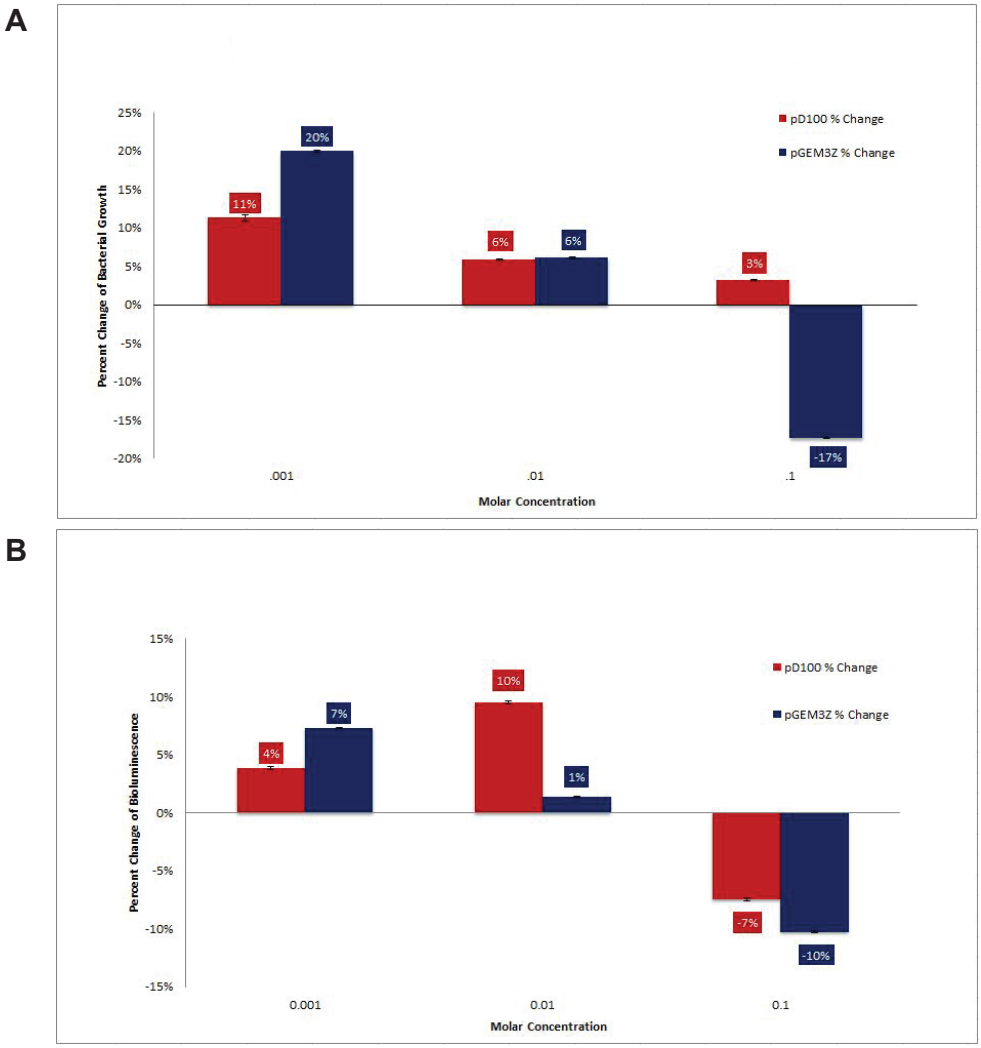

Figure 4: A) Percentage change for bacterial growth of DH5a/pD100-*lux* and DH5a/pGEM3Z-*lux* after exposure to vitamin B-2 at different concentrations. B) Percentage change for bioluminescence of DH5a/pD100-*lux* and DH5a/pGEM3Z-*lux* after exposure to vitamin B-2 at different concentrations. Bioluminescence data was normalized for the OD_{600} bacterial growth readings to represent cellular bioluminescence.

bioluminescence with increased levels of glucose (**Figure 4B**). AHL is an important part of quorum sensing, a phenomenon where population density changes are used to regulate gene expression in bacteria. Quorum sensing in *A. fischeri* relies on bacteria producing enough AHL to bind to LuxR and cause an increase in the expression of *luxA* and *luxB*. This increased expression leads to significant light production, and the cycle starts again, this time with more AHL within each bacterial cell (10). Direct introduction of cyclic AMP or AHL are two possibilities to receive an even greater bioluminescence output from *A. fischeri* genes.

The results show that greater expression of the *A. fischeri* genomic fragment is achievable with a change in plasmid copy number, and that exposure to different nutrients can affect expression of the bioluminescence genes. This finding strengthens the idea that bioluminescent organisms may be used as a substitute for electrical lighting. Further experimentation could be conducted by exploring greater optimization of bioluminescent gene expression or by transferring the genes to a different organism. For example, a bioluminescent houseplant would be a useful, renewable light source that would not threaten the health of its owners. The research findings described here indicate that the enzymes encoded on the *A. fischeri* genomic fragment may be useful for further development of bioluminescence.

Materials and Methods

Bacterial Growth and Bioluminescence Measurement

Bioluminescence and bacterial growth were measured in this experiment. Bioluminescence was measured with a plate luminometer (BMG Labtech,

Germany), while bacterial growth was measured by reading the light scattering of the bacterial culture with a spectrophotometer (Eppendorf, Germany). The assays were performed with the same amount of bacterial culture in a 96-well plate, while the bacterial growth readings used the same volume of liquid culture. The two different plasmid vectors, four different nutrients, and three different molar concentrations of the nutrients were controlled in the experiment. The nutrients were added to the liquid cultures during their growth and then incubated for a constant time (16 hours), while the molar concentrations used were the same for each nutrient. All measurements were taken from the same sample at the same point in time during the stationary phase of growth, recorded in triplicate, averaged, and the standard deviation between the measurements was determined.

Plasmid Extraction

Initially, a large-scale (500 mL of bacteria) plasmid preparation of the pD100 plasmid with the *A. fischeri* genes was performed using the GenElute HP Plasmid Maxiprep Kit (Sigma-Aldrich), in order to extract the plasmid from the *E. coli* bacteria. The plasmid preparation was performed according the protocol provided with the Sigma-Aldrich kit.

DNA Fragment Isolation

A restriction digest of the DNA was the next procedure used in this experiment. 8 µL of DNA, along with 1 µL of SalI-HF restriction endonuclease (New England Biolabs), 1 µL of buffer and 2 µL of water were added to a plastic 1.5 mL tube. After mixing, the tube was incubated at 37 ºC for an hour. The DNA resulting from the restriction digest was electrophoresed on a 1.1% agarose gel. 2 µL of 10x stop buffer (50% glycerol, 5% sodium dodecyl sulfate and 0.05% bromophenol blue) were added to the digest and the mixture loaded onto a 1.1% agarose gel in 1XT buffer solution, along with a sample of ladder for comparison. The gel was exposed to 100 V in an electrophoresis apparatus until the ladder and digest ran almost to completion as indicated by the bromophenol blue dye. The gel was stained in 5 ug/ml ethidium bromide and visualized on a 300 nm ultraviolet light box. The gel containing the DNA fragment was excised with a razor blade.

DNA Purification

Purification of DNA from the gel slice was the next step, and was completed using the QIAquick Gel Extraction Kit from Qiagen. The DNA purification was performed using the protocol provided with the Qiagen kit.

DNA Ligation

Following purification, the DNA was ligated into the high copy plasmid, pGEM3Z. A 5:1 dilution of vector DNA into water was created. Two samples of one microliter of the dilution were added to two 1.5 mL tubes. 3 µL of insert and the 9 kb SalI fragment were added to one tube, while 3 µL of water was added to the second tube, along with 0.5 µL of DNA ligase and 0.5 µLof buffer. The ligation was left at room temperature for an hour or overnight.

Bacterial Transformation

Lastly, the vector and insert were transformed into *E. coli* bacteria. The ligation-competent cells, and several empty Falcon 2059 test tubes were placed on ice. 200 µL of competent cells were added to the ligation, mixed, and transferred to the 2059 tubes. The tubes were kept on ice for 15 minutes; heat shocked at 42 ºC for one minute, and put on ice for 30 seconds. 1 µL of SOC media from Sigma-Aldrich was added to the tubes, which were then put in a shaking incubator at 37 ºC for one hour. After an hour, the contents of the tubes were transferred to 1.5 mL tubes and spun at 5000 rpm for 2 minutes. The supernatant in the tubes was removed and 70 µL of SOC media was added to the tubes. The mixture was then spread onto an agar plate with ampicillin and incubated at 37 ºC until colonies were visible.

Recombinant Plasmid Validation

A smaller plasmid preparation screened bacterial colonies for the presence of the 9 kb insert in the pGEM3Z. This procedure is possible with the Zyppy Plasmid Miniprep Kit from Zymo research, and the procedure was performed with the protocol from the same kit.

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