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# Identification, Cloning and Characterization of Van Operon in Cyanobacterium Synechococcus sp. IU 625

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# IDENTIFICATION, CLONING AND CHARACTERIZATION OF VAN OPERON IN CYANOBACTERIUM *Synechococcus* sp. IU 625

**By**

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**Submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology from the Department of Biological Sciences of Seton Hall University**

**May 2011**

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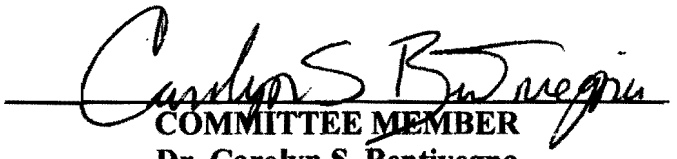

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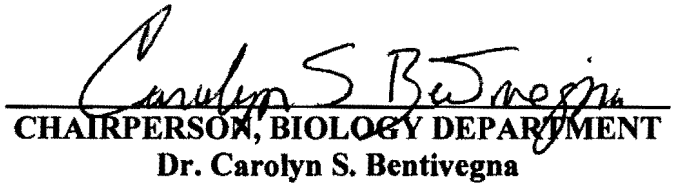

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## ABSTRACT

Freshwater harmful algal blooms remain a modern day concern due to freshwater eutrophication. To understand how those harmful algal blooms are capable of forming, especially in the presence of EPA target heavy metals, this thesis has undertaken the design of vanillate inducible gene expression vectors to provide a set of molecular tools to understand gene regulation and expression. Vanillate is a byproduct of saprotrophic digestion of freshwater plant lignin. This thesis explores the utilization of vanillate as a sole carbon source with the model cyanobacterium *Synechococcus* sp. IU 625 (*S.* IU 625) when deprived of light for photosynthesis. Other species of bacteria such as the freshwater oligotroph *Caulobacter crescentus* (*C. crescentus*) possess enzymes which are capable of utilizing vanillate as a sole carbon source. Evidence shown here indicates that *S.* IU 625 is capable of utilizing vanillate as a sole carbon source at 0.5 mM and 1.0 mM concentrations when photosynthesis is not available. Using the known sequence from *C. crescentus* BLAST searches were carried out, and using a closely related species Cyanobacterium *Synechococcus elongatus* PCC 7942, primers were designed for regions which showed high homology. Evidence of upregulation of one vanillate subunit, VanA, has been experimentally shown using qPCR. Using the sequence from the isolated operon region, several vanillate based expression vectors have been created as molecular tools. These expression based vectors, pSM1039-1041 have been designed to create inducible gene expression of metallothionein (*smtA*). SmtA is a cysteine rich protein which preliminary evidence suggests plays a role in heavy metal response within *S.* IU 625. Using *S.* IU 625 as a model cyanobacterium, this thesis explores the potential use of vanillate inducible gene expression to understand the role *smtA* plays in *S.* IU 625 survival when exposed to heavy metal stress. Use of these vectors allows the expression of *smtA* to be linked to the presence or absence of vanillate, thus we are allowed to see three unique situations governing *smtA* expression: wild-type using wild-type *S.* IU 625, pseudo knock-out using vector transformed cells with no vanillate exposed, and over expression using transformed cells exposed to vanillate. A fourth vector, pSM1116 was designed containing a truncated section of *smtA* and will recombine in the SmtA operon

region, interrupting normal gene expression of *smtA*. Using all these molecular tools designed in this project, a goal to better understand cyanobacterial heavy metal processing has been established. Understanding these interactions is vital to understanding the physiological response enabling cyanobacteria to bloom in heavy metal contaminated environments.

# INTRODUCTION

Heavy metal regulation within cyanobacteria remains poorly understood. Cyanobacteria (formally known as blue-green algae) are photosynthetic prokaryotes of great importance in many ecological settings. They affect water quality, and play a huge role in global biogeochemical cycles (Oliver and Ganf, 2000). Harmful algal blooms (HAB) due to eutrophication have been reported in nearly every industrialized nation (Paerl et al., 2001, Slonczewski and Foster, 2008). *Synechococcus* sp. IU 625 is a freshwater unicellular cyanobacterium which has been reported to cause nontoxic HAB (Beardall, 2008).

Cyanobacteria can produce toxins which can remain in water after the HAB has disappeared (Codd et al., 2005). These cyanotoxins have been implicated in spikes of liver cancer as well as other forms of cancer in industrialized nations (Carmichael et al., 2001). It also appears that long-term small dose exposure to cyanotoxins has dermotoxic, neurotoxic, and hepatotoxic effects (Barros et al., 2009). Cleanup of cyanotoxins involves costly filtration through silica gel and does not treat the underlying issue (Tsuji et al., 1994).

Eutrophication in freshwater appears to be enhanced by the presence of certain heavy metals. Research has shown that a natural predator of cyanobacteria, cyanophages, is inhibited by the presence of high concentrations of heavy metals (Chu et al., 2009). Cyanophages are important in regulating the growth of cyanobacteria (Mühling et al., 2005). Heavy metals appear to further alter their environment by altering the pH,

changing the oxidative state of nutrients, and in cases like mercury, will bioaccumulate in the respective food web. This alteration of the environmental is creating a niche for heavy metal resistant cyanobacteria to flourish and potentially bloom (Thajuddin and Subramanian, 2005).

Heavy metals such as iron, copper, nickel, cobalt, and manganese are important trace nutrients and are often added to fertilizers to enhance plant growth (Küpper et al., 1995). These heavy metals are also released by unregulated industrial waste water effluent (Audry et al., 2004). Several of these heavy metals have been designated by the US Environmental Protection Agency (EPA) as potential threats. These EPA targeted heavy metals include zinc, nickel, cobalt, iron and manganese.

Several species of cyanobacteria possess proteins which respond and sequester heavy metals. One such protein is known as metallothionein (SmtA). SmtA is a low molecular weight, cysteine rich protein whose purpose in cyanobacteria is to bind up excessive levels of cytoplasmic zinc, and evidence exists that other divalent cations can elicit expression of *smtA*, indicating multi usage (Morby et al., 1993). Mutations, which change the regulation of *smtA*, can potentially allow mutants, which can tolerate much higher concentrations of heavy metals.

In order to understand the role that SmtA plays in survival of heavy metal stressed cyanobacteria, this thesis has undertaken the usage of a unicellular cyanobacterium *Synechococcus* sp. IU 625 (*S.* IU 625). *S.* IU 625 is a unicellular, gram-negative, bacillus shaped cyanobacteria which has been previously implicated it causing non-toxic

freshwater algal blooms (Beardall, 2008). *S.* IU 625 can be used as a model organism to study the effects of the EPA target heavy metals on cell growth, and can be genetically manipulated using common laboratory techniques such as inducible and knock-out plasmid vectors.

*S.* IU 625 interactions with EPA target heavy metals has been previously characterized (Lee et al., 1991, Lee et al., 1992, Lee et al., 1993, Lee et al., 1994., Lee et al., 1996, Lee et al., 1999., Lee et al., 2000., Lee et al., 2002). The heavy metal response genes in *S.* IU 625 for mercury have been previous characterized (Chu et al., 2005, Chu et al., 2011). Metallothionein in *S.* IU 625 has been sequenced and the operon has been proposed (Chu et al., 2007). Previous reports showed the expression of *S.* IU 625's *smtA* is zinc concentration dependent (Perez, 2010).

Cyanobacteria (formally called blue-green algae) were originally generally considered obligate photoautotrophs (Kratz and Myers, 1955). Evidence provided in this thesis and other experimental studies shows that *S.* IU 625, and other cyanobacteria, can be photoheterotrophs under certain conditions (Anderson and McIntosh, 1991). Photoheterotrophic growth of cyanobacteria can be used a potential tool for molecular studies of cyanobacteria much similar to that of other bacteria such as lactose based expression in *E. coli* and vanillate based expression in *C. crescentus*.

This study will focus on identification, cloning and characterization of Van operon in *S.* IU 625. Potential heterotrophic growth by utilizing vanillate has been

investigated in this study. Quantitative real-time PCR assays have also been carried out to study the expression of vanA gene in *S.* IU 625.

## MATERIALS AND METHODS

### Growth conditions of *Synechococcus* sp. IU 625

The unicellular cyanobacterium *Synechococcus* sp. IU 625 was obtained from American Type Culture Collection (ATCC; Manassas, VA) and was maintained in sterile Mauro's Modified Medium (3M) at a pH 7.9. The cells were grown at 26°C, with constant fluorescent light and continuous agitation at 100 rpm in a Gyromax 747R incubator shaker (Amerex Instruments; Lafeyette, CA). Growth of culture was measured by turbidity study using an Ultraspec III (Pharmacia LKB; Sweden) at $OD_{750nm}$. Direct counts of total cells/ml were measured using a hemocytometer (Hausser Scientific; Blue Bell, PA). Stock cultures were maintained on 3M with 2% Bacto™ Agar (BD; Sparks, MD). Stock liquid cultures of *Synechococcus* sp. IU 625 were maintained by passage into sterile media when the $OD_{750nm}$ was greater than 1.5, by doing a 1:20 (Culture to 3M) dilution, or when the growth reaches stationary phase. *Synechococcus* sp. IU 625 was maintained in sterile 250 ml Erlenmeyer flasks containing no more than 100 ml of media and cells (Chu et al., 2010).

*Synechococcus elongatus* PCC 7942 (Pasture Culture Collection # 7942), was maintained in unmodified sterile 1X BG-11 (Sigma-Aldrich; St. Louis, MO). Growth conditions, including temperature and shaking conditions, were followed as stated above for *S.* IU 625.

**Primer Design**

PCR primers were designed with National Center for Bioinformatics (NCBI) Primer BLAST software and were analyzed using Integrated DNA Technology's (IDT) OligoAnalyzer. A closely related species of cyanobacterium, *Synechococcus elongatus* PCC 7942, whose sequence is known, was used as a template. Primers were obtained from Eurofins MWG-Operon (Huntsville, AL), and were resuspended in sterile di$H_2O$ to a final concentration of 100 μM following manufactures recommendations. Primers were designed to encompass the entire hypothetical vanillate operon (*vanR*, *vanA*, and *vanB*), the size of the amplicons ranging from 300-800 bp. No primer was designed to amplify more than 850 bp. Each oligo was designed to be under 30nt in length with melting temperatures of the oligos 60°C or above.

**Real Time PCR Primer Design**

Real Time PCR (qPCR) primers were designed using IDT's RealTime Oligo tool, as well as Applied Biosystems Primer Express 3.0 software. Oligos were designed to have no more than 5 G or C nucleotide residues at the 5' or 3' ends per manufactures recommendations. Primers were designed to amplify a short 110 nt region on the hypothetical *vanA* gene. Oligos were ordered using the *vanA* region sequenced from *Synechococcus* sp. IU 625.

**PCR-based assay and Sequencing of hypothetical Vanillate Operon**

Polymerase Chain Reactions (PCR) were carried out using genomic DNA isolated using common lab protocol. The cells were digested with lysozyme (final conc. 2mg/ml) overnight, then separated using Chloroform:Isoamyl Alcohol (24:1). The aqueous phase

was harvested by centrifugation at 16,000 rpm in a microcentrifuge for 10 minutes. The DNA was then precipitated with 100% ethanol overnight at -20°C and then digested with RNaseA and Proteinase K. The DNA pellet was resupended in 50 μL of sterile di$H_2O$.

1 μL of genomic DNA was added to 2X Taq PCR Master Mix (Denville Scientific; Denville, NJ). 1 μL of each primer, forward and reverse were added to a final concentration of 1 μM. 2 μL of DMSO was added to the reaction and the final volume was brought up to 25 μL in sterile di$H_2O$ The general run method of reaction was activation of the Hot Start polymerase at 94°C for 3 minutes, followed by denaturation for 30 seconds, lowest Tm of primer group for 30 seconds, 72°C for 30 seconds, for 35 cycles. A final extension step was done at 72°C for 7 minutes.

PCR products were run on 1% Agarose Gels in 1X TAE at 100 volts. The gels were visualized using EtBr under high-wavelength UV, and the photograph of the gel was taken with a UVP Imager (UVP; Upland, CA). Sequencing of the PCR product was conducted at GeneWiz laboratories (South Plainfield, NJ). The sequence assembly was conducted using the bioinformatics tool CodonCode (www.codoncode.com).

**Growth of *Synechococcus* sp. IU 625 with Vanillate**

50 mM stocks of vanillate (Sigma) were prepared in sodium hydroxide and sterile filtered through a 0.2 μm filter. Eight sterile flasks were autoclaved and labeled accordingly. A stock culture whose $OD_{750nm}$ was approximately 0.9 was selected; and 5 ml was removed and placed into each of the eight flasks. 95 ml of sterile 3M was pipetted into each flask so the final volume was 100 ml. Vanillate was then immediately placed into 100 ml of early logarithmically growing cells in duplicates of 0.0 mM

(control), 0.5 mM, and 1 mM final concentrations. A set of three flasks containing every concentration was grown using standard growth conditions; and another set was wrapped completely with aluminum foil (in the dark setting) to block out the fluorescent lighting. After 26 days, the aluminum foil was removed and the entire remaining volume was spun down at 17,500 rpm in a Sorvall RC-5 (Thermo Scientific; Madison, WI), 4°C for 10 minutes. The supernatant was removed and the cell pellets were washed with 10 ml of sterile 3M. This process was repeated three times, and on the final wash, the resuspended cells were transferred to sterile flasks, and 3M media was added up to 100 ml. The cells were incubated following typical growth conditions, with samples being taken as stated below.

**Sample collection and pre-processing**

Twice a week for 32 consecutive days, aliquots were obtained from each of the challenge flasks. 4.5 ml of sample was removed at each collection day; 1.5 ml of each sample was placed into three sterile microfuge tubes. Two microfuge tubes were spun down at 16,000 rpm in a microfuge (Denville Scientific; Denville, NJ) for ten minutes. Once the spin stopped, the supernatant was discarded and the pellets were resuspended in 500 μL of sterile 10% glycerol. Each set of tubes was combined into one tube containing 1 ml of resuspended cells in 10% glycerol. The tubes were stored at -80°C until post processing.

1mL of the remaining microfuge tube was removed and placed into a clean plastic cuvette. The $OD_{750nm}$ was taken using an Ultraspec III (Pharmacia LKB; Sweden) and recorded. 160 μL was removed from the microfuge tube and placed into another

microfuge tube containing 40 µL of 5X 12.5% formaldehyde buffered in 150 mM sodium phosphate, pH 7.5(5X fixative), for post processing image analysis. The remaining aliquot was spun down at 16,000 rpm in a microfuge (Denville Scientific, Denville, NJ) for 10 minutes. 2 µL of supernatant and the concentration of vanillate in the media was measured at $OD_{286}$ nm using a Nanodrop ND1000 (Thermo Scientific; Madison, WI). Sterile di$H_2O$ was used as a blank.

From the 1 ml cuvette, 10 µL was removed and placed onto both halves of a hemocytometer (Hausser Scientific; Blue Bell, PA). Using the small internal grids of 25 4 by 4 boxes, the cells/ml were calculated using the following formula:

(Average # Cells per 5 boxes of 4*4) x 25 x $10^4$ = Total Cells/ml

**Total RNA Extraction**

Total RNA was extraction from sample aliquots using the commercially available Trizol Max Bacterial RNA Isolation Kit (Invitrogen; Carlsbad, CA.). Procedure was followed with the manufacturer's recommendation, modified only during initial incubation with lysis reagent. Five minutes are used instead of four to provide additional lysing to increase yield. RNA pellets were resupended in 50 µL of sterile RNase free di$H_2O$, and stored at -20°C for cDNA synthesis. RNA samples were checked for purity and quantity using a Nanodrop ND1000 (Thermo Scientific; Madison, WI) measuring $A_{260}/A_{280}$.

**cDNA Synthesis (rtPCR)**

Using 10 µL of RNA extracted from samples, cDNA was made using the commercially available High Capacity cDNA Reverse Transcription Kit (Applied

Biosystems; Foster City, CA). Random primers were used to synthesize first strand cDNA from total RNA. 10 µL of RNA isolated from each collection point was used to standardize total amount of RNA in cDNA reaction. cDNA reaction followed the following steps, 25°C for 10 minutes to activate the reverse transcription enzyme, 37°C for 2 hours, followed by enzyme inactivation of 85°C for 5 minutes to stop the reverse transcription reaction, and a final soak at 4°C. Approximate concentration of cDNA was made following the reaction using a Nanodrop ND1000. The cDNA was stored at -20°C until used in further downstream applications.

**qPCR**

Quantative PCR was conducted using an ABI Step One Plus (Applied Biosystems; Carlsbad, CA), with Fast SYBR Green Master Mix (Applied Biosystems; Carlsbad, CA). 10 µL Fast SYBR Green, 1 µL of forward qPCR Primer, 1 µL of reverse qPCR Primer and 3 µL of sterile nuclease free were added to form a master mix. 5 µL of cDNA was added to the master mix inside the wells. PCR was carried out in optically clear plates, and in triplicates. The sigma subunit of the 16s rRNA gene (rpoD) was used a homogenous control. The cycling conditions were as follows: 95°C for 20 seconds to activate the Taq polymerase, then 95°C for 3 seconds, and 60°C for 30 seconds. This was conducted for 40 cycles. A melt curve was conducted at the end to ensure the purity of the products formed. The data was analyzed using the ABI StepOne Plus software.

**Transformation of vectors**

*Synechococcus elongatus* PCC 7942 was used as a host for the vector designed in this project. *Synechococcus elongatus* PCC 7942 is naturally transformable. The

following procedure was used to transform vectors into *Synechococcus elongatus* PCC 7942: The cells were grown in sterile 1X BG-11 until an $OD_{750nm}$ of 0.7 was reached. 80 ml of cells were aseptically transferred into two 40 ml centrifuge bottles. The cells were pelleted using a Sorvall RC-5 at 3000rpm for 20 minutes. The supernatant was then decanted and the pellets were resuspended in 10 mM NaCl. The resuspended cells were pelleted again at 3000rpm for 15 minutes, with the supernatant again discarded. One pellet was resuspended in sterile 1X BG-11, and then used to resuspend the 2nd pellet. 300 µL aliquots were taken from the tube and placed into sterile microfuge tubes. 10 µL of Plasmid DNA were added to the tubes wrapped in aluminum foil, and incubated at 30°C, 100rpm overnight. 100 µL was plated onto 1X BG-11+ 2% Agar plates, 100 µL was plated onto 1X BG-11 + 2% Agar + 5 µg/ml Kanamycin (Denville Scientific; Denville, NJ). The remaining 100 µL was transferred to a sterile 15 ml test tube and 4.9 ml of 1X BG-11 + 5 µg/ml Kanamycin were added to the tubes. The test tubes were incubated at 26°C and 100rpm. Once single colonies appeared, they were selected using a sterile inoculation loop and transferred onto another 1X BG-11 plate containing 5 µg/ml Kanamycin.

**Statistical Analysis**

All results were calculated and presented as the mean and standard deviation for each control or experimental group. One-Way ANOVA performed statistical comparisons between the means of different experimental groups with a Bonferroni's post-test. Statistical analysis was performed with GraphPad Prism 5 and statistical significance defined as $p<0.05$.

## RESULTS

### Homology of Vanillate Operon between *Caulobacter crescentus* NA1000 and *Synechococcus* sp. IU 625

Prior published work has shown that the freshwater oligotroph *Caulobacter crescentus* (*C. crescentus*) possesses the genes necessary to utilize vanillate as a sole source of carbon (Thanbichler, 2007). Preliminary bioinformatic analysis showed two genes which possessed homology to the *vanA* and *vanB* in *C. crescentus* in a closely related species of cyanobacteria, *Synechococcus elongatus* PCC 7942. Figure 1 shows the BlastP of the *vanA* and *vanB* gene in *C. crescentus* into the completely sequenced *Synechococcus elongatus* PCC 7942 highlighted two potential genes which are relatively conserved. Figure 2 highlights the partial order alignment of VanA from several species, which the vanillate gene has been sequenced, and function shown to exist, in a visual form (POAVIZ). *Synechococcus elongatus* PCC 7942 gene SynPCC7942_2035 was used to compare its relationship. Based on the results from the BlastP and BlastN of the *C. crescentus* vanillate operon into the chromosome of *Synechococcus elongatus* PCC 7942, 16 sets of primers were designed for the regions of high similarity. Based on the information from Figure 2, there is are several areas of homology between conserved between several bacteria whose *vanA* has been shown experimentally to exist, and the hypothetical VanA sequence from *Synechococcus elongatus* PCC 7942, indicating a potential uniform function.

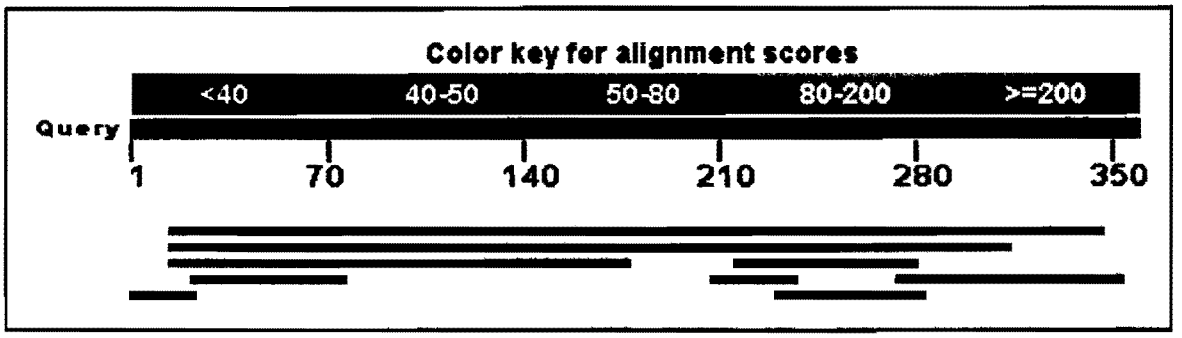



**Figure 1.** BlastP results for VanA proteins. BlastP matches are shown using the VanA protein sequence from *C. crescentus* into the genome of *Synechococcus elongatus* PCC 7942. The top two results are SynPCC7942_2035 and SynPCC7942_2036 respectively. SynPCC7942_2035 has a query coverage of 92% and an E value of $2 * 10^{-28}$. SynPCC7942_2036 has a query coverage of 83% and an E value of $7 * 10^{-26}$.

* Block Label: the length of the sequence
SEQ ID 0: Caulobacter (crescentus)
SEQ ID 1: Ralstonia (solanacearum GMI1000)
SEQ ID 2: Rhodoferax (ferrireducens T118)
SEQ ID 3: Halomonas (elongata DSM 2581)
SEQ ID 4: Acinetobacter (baumannii ATCC 17978)
SEQ ID 5: Marinomonas (sp. MWYL1)
SEQ ID 6: Leptothrix (cholodnii SP-6)
SEQ ID 7: Azospirillum (sp. B510)
SEQ ID 8: Variovorax (paradoxus EPS)
SEQ ID 9: Xanthomonas (vesicatoria ATCC 35937)
SEQ ID 10: Bradyrhizobium (sp. BTAi1)
SEQ ID 11: Xanthomonas (albilineans GPE PC73)
SEQ ID 12: Acinetobacter (calcoaceticus PHEA-2)
SEQ ID 13: Streptosporangium (roseum DSM 43021)
SEQ ID 14: Corynebacterium (glutamicum ATCC 13032)
SEQ ID 15: Klebsiella (pneumoniae subsp. rhinoscleromatis ATCC 13884)
SEQ ID 16: Granulibacter (bethesdensis CGDNIH1)
SEQ ID 17: Synechococcus (sp. WH 8102)
SEQ ID 18: Synechococcus (elongatus PCC 7942)

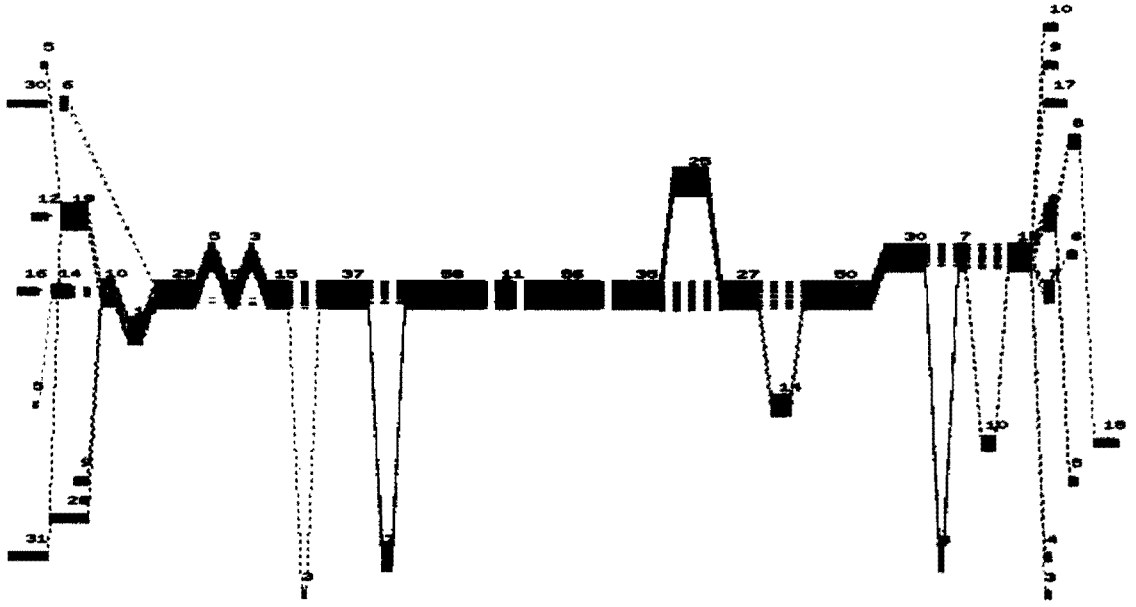


**Figure 2.** POAVIZ of known VanA with *Synechococcus elongatus* PCC 7942 (Smooth: 2)

**Van operon primer design**

Using the BlastP results from *Synechococcus elongatus* PCC 7942, a comprehensive set of primers was designed using PrimerBlast and PCR-based assays were carried out. Gel electrophoresis was carried out for the PCR products. Pure PCR products were sent out for sequencing. Figures 3 and 4 are representative gel photos from the PCR products using designed primers to prime the genomic DNA isolated from *S.* IU 625 and primers designed to amplify the hypothetical vanillate region. Figure 5 is a gel image showing the amplification size of the primers used for quantitative PCR (qPCR). The result indicated that all the designed primers successfully primed the *S.*IU 625 DNA. The amplicons sizes are all close to the expected sizes.

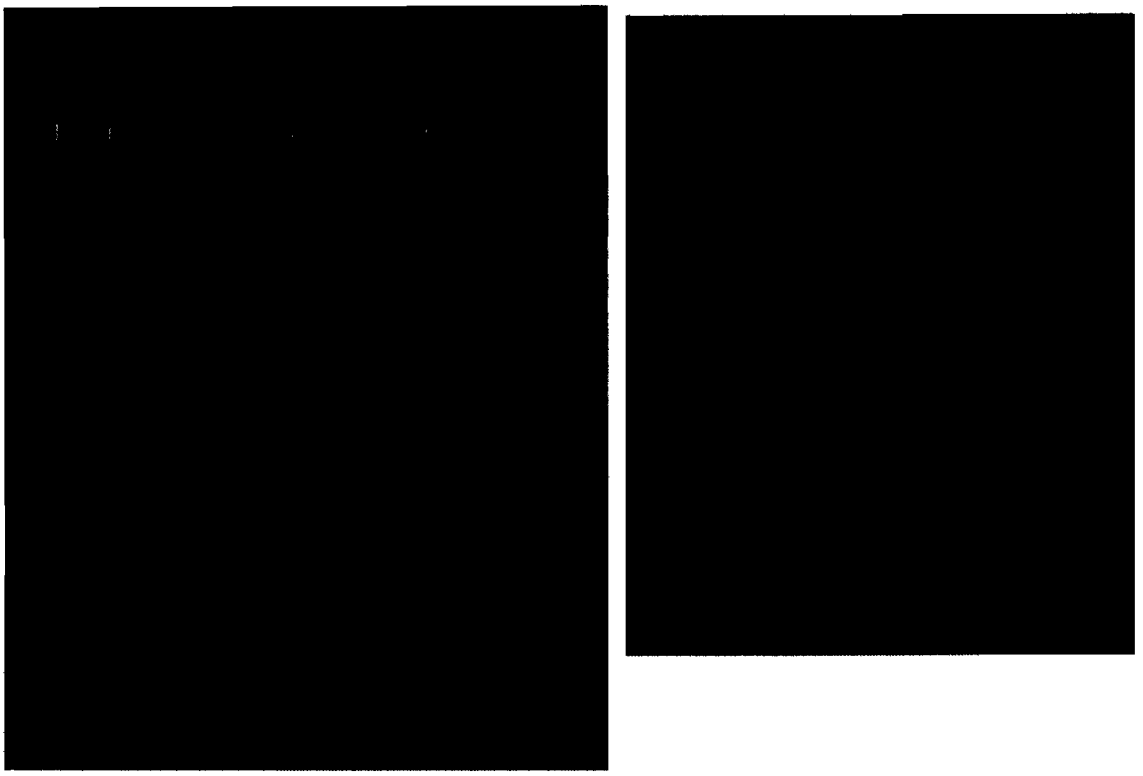


**Figure 3.** Gel image of PCR products of OVanR and VanA. The primer name and expected amplicon size are listed next to the image.

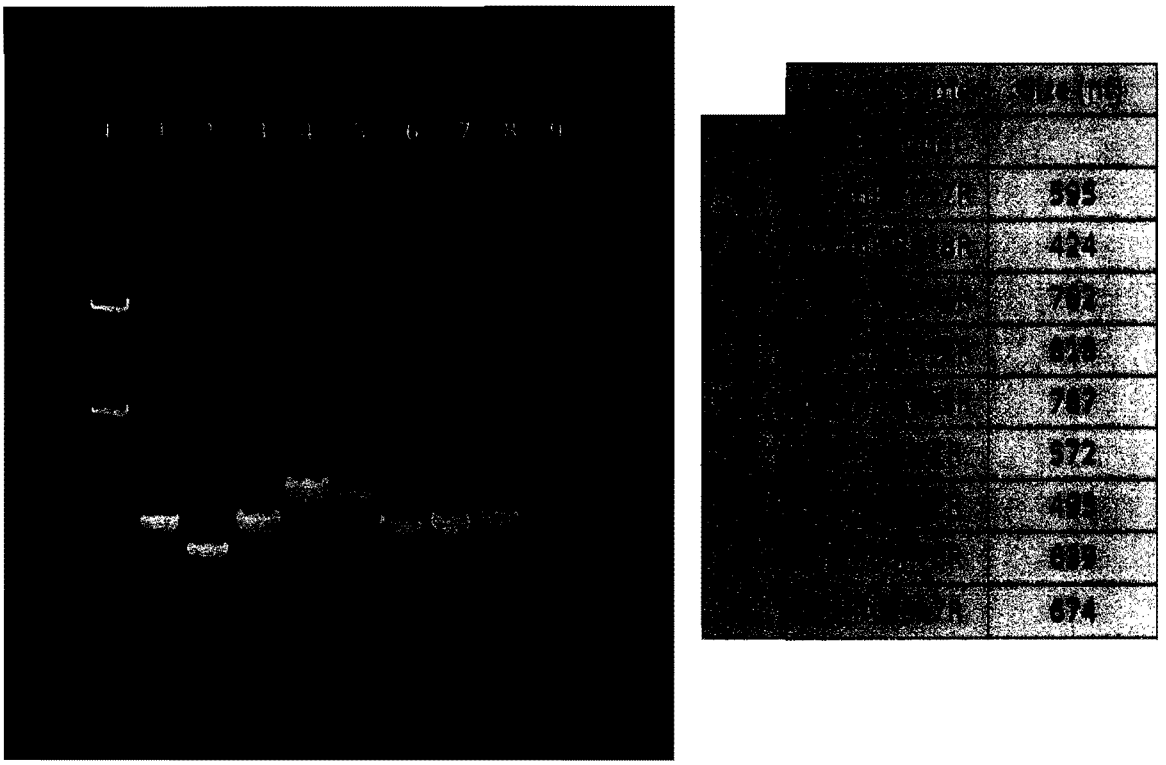



**Figure 4.** Gel image of PCR products of OVanR, NVanR, VanB & VanA. The primer name and expected amplicon size are listed next to the image.

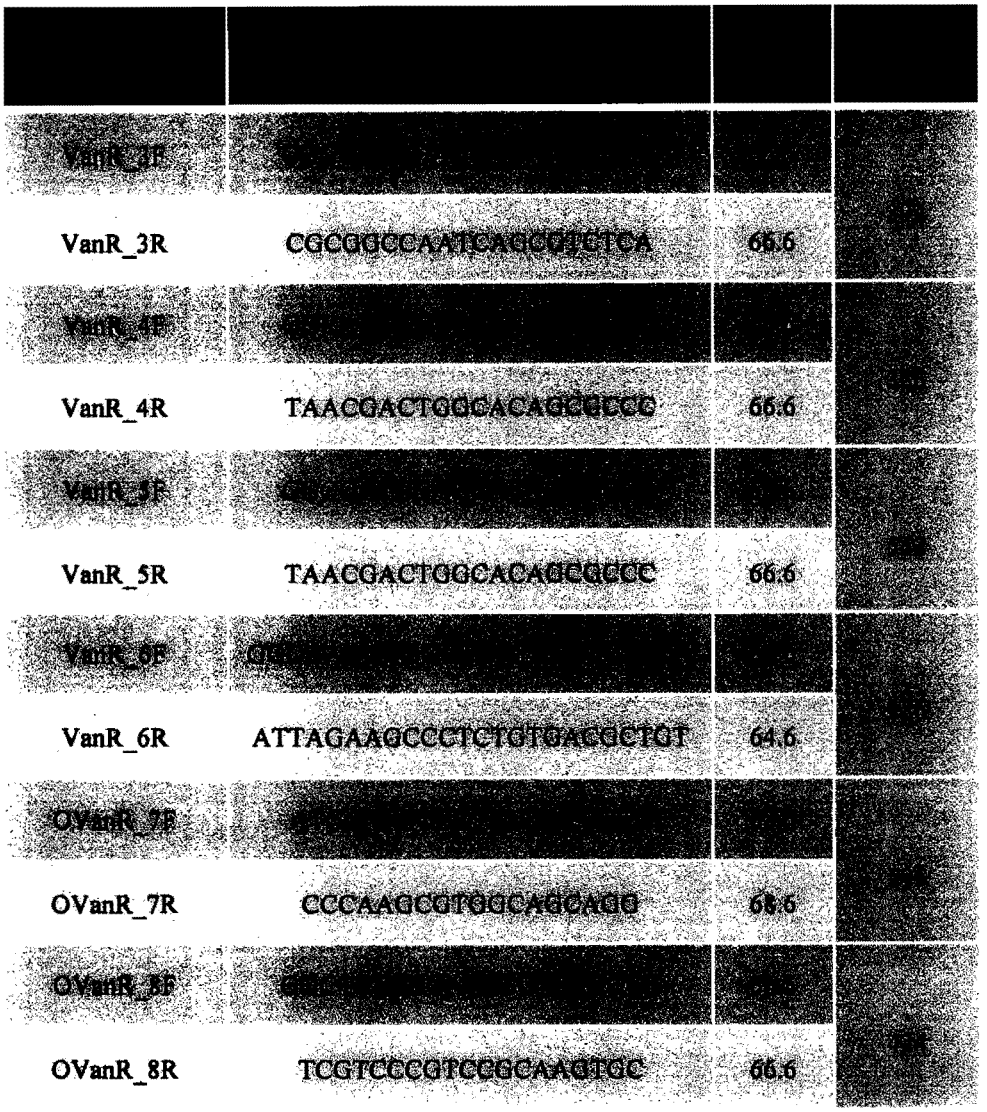
**Figure 5.** Gel image of PCR products of *vanA* with qPCR primers. The PCR was performed on genomic DNA isolated from *S.* IU 625. The primer name and expected amplicon size are listed in the table to the right of the gel image

**Vanillate Operon Sequencing**

Based on the above bioinformatic analysis, primers were ordered to primer the regions of high homology of the Van operon of *Caulobacter crescentus* to *Synechococcus elongatus* PCC 7942. For VanA, five sets of primers were ordered. For VanB, two sets of primers were ordered. For the region which closely matched the reported VanR from *Caulobacter crescentus,* six sets of primers were ordered. A gene which is in succession of the hypothetical *vanA* and *vanB* was then sequenced. A total of three sets of primers were ordered to sequence this gene. This gene was designated *vanR*.

The nucleotide sequence obtained using the primers from Tables 1-4 shows a 100% homology to a closely related species of cyanobacterium *Synechococcus elongatus* PCC 7942. The hypothetical *vanA* gene is a 1038 nt long sequence which codes for a protein that is 345 a.a. long. Table 1 is a list of the primers used in this study to sequence a gene which has homology to *C. crescentus vanR*, their Tm, and the total expected nucleotide size of the amplicon is listed. The obtained sequence is shown in Figure 6. Table 2 is a list of primers used in this study to sequence *vanA*, their Tm, and the total expected nucleotide size of the amplicon is listed. The obtained sequence is shown in Figure 7. Table 3 is a list of primers used in this study to sequence *vanB*, their Tm, and the total expected nucleotide size of the amplicon is listed. The obtained sequence is shown in Figure 8. Table 4 is a list of primers used in this study to sequence *vanR*, their Tm, and the total expected nucleotide size of the amplicon is listed. The obtained sequence is shown in Figure 9.

Once the hypothetical operon region was sequenced, the orientation and spacing of the genes was undertaken. This was done using CodonCode alignment software, which separated the region into two contigs. The two contigs were the VanR region and the VanAB region. There is a 27 nt overlap between *vanR* and *vanA*, and a 4 nt gap between *vanA* and *vanB*. Based on bioinformatic analysis the entire proposed operon consists of 3526 nt, with three coding domains. The entire region is 100% homologous to *Synechococcus elongatus* PCC 7942.



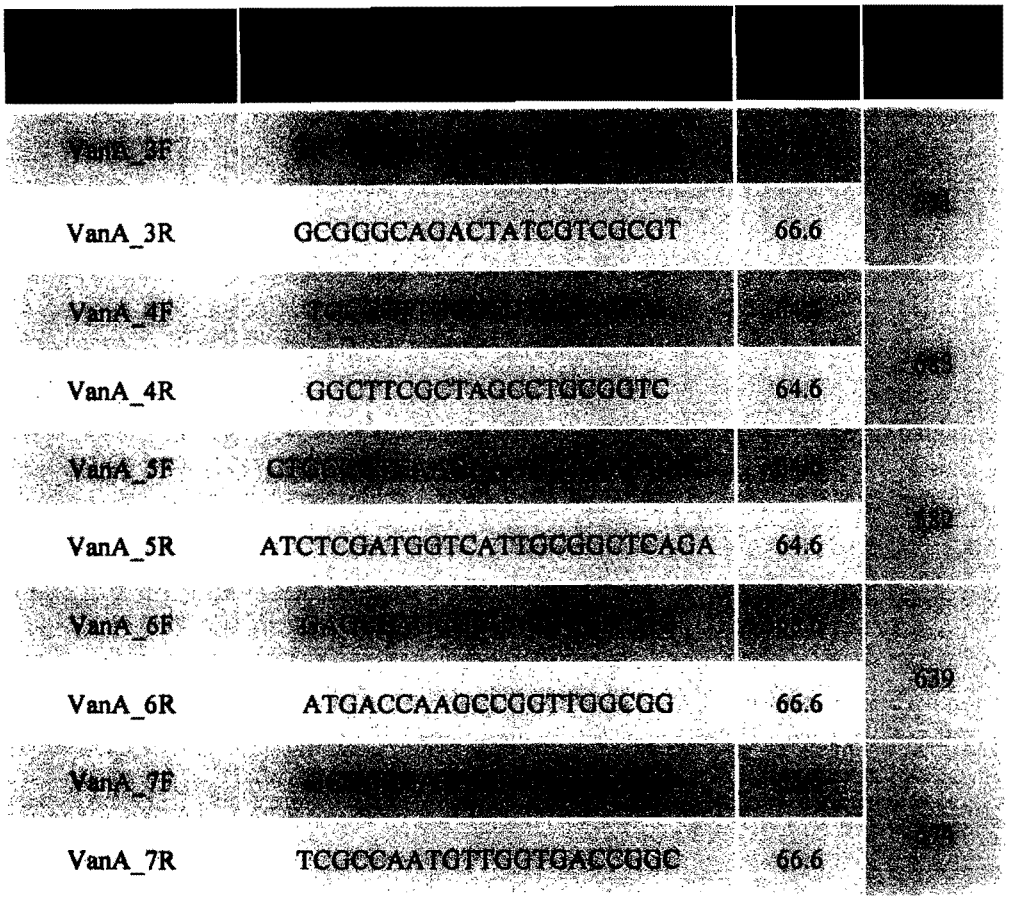
| | | | |
|---|---|---|---|
| VanR_3F | | | |
| VanR_3R | CGCGGCCAATCAGCGGTCTCA | 66.6 | |
| VanR_4F | | | |
| VanR_4R | TAACGACTGGCACAGCGCCC | 66.6 | |
| VanR_5F | | | |
| VanR_5R | TAACGACTGGCACAGCGCCC | 66.6 | |
| VanR_6F | | | |
| VanR_6R | ATTAGAAGCCCTCTGTGACGCTGT | 64.6 | |
| OVanR_7F | | | |
| OVanR_7R | CCCAAGCGTGGCAGCAGG | 66.6 | |
| OVanR_8F | | | |
| OVanR_8R | TCGTCCCGTCGGCAAGTGC | 66.6 | |

**Table 1.** Old *vanR* Primer sets designed in this study. The primers were used to amplify and sequence the region of the chromosome which has homology to *Caulobacter crescentus* VanR.

>Hypothetical Transcriptional Repressor Sequence 5' →3' (606 nt)

CTAACTGGGATACCAAAACATGCCCTTGATGCCCTCGGGGTCGAGCACAAAGCCAAGACGCCGATAGAAATCAACCAC
ATGGGGATCGGCAAAGAGGGTGATGTTGGGAATCTCAGCTTGTTGCAGCTGAGCAATCACGTATTCCATCAAAACGCG
CCCTAAACCCTGTCCTTGGAAGCTGGGGTTGATCACCACATCCCAAATCGTGGCATTGAACGCGTGGTCAGAAGTGGCA
CGGGCAAAGCCGATCAGCCGCCGCTGTTGAGCCAACTGTTCCCAAAGGGAAACAACTAAAAAGCTGTTGTCGAGGGCA
CGGCGCACTTTGCGGACGGGACGACGTGACCAACCGACAGCGTCACAGAGGGCTTCTAATTCTTGCAGATCGATGTCG
CGACGGGTGCTGAGAATGAGACGCTGATTGGCCGCGATCGCGGGGGTGATCTCCACAGCTTGGGGGCCATAGCCTGCC
AAGGGATCATCTGCAGGAACTGAGCTGGGAGCTGCAACAGGTGTATCTAGCGCAGAGAATAAACTCTTCCAAAACCCC
ATTCCTGCTGCCACTCGCTTGGGCGCTGTGCCAGTCGTTAGTATATCCTGCAGGCTCAA

**Figure 6.** Complete sequence of OVanR similar to *vanR* in *C. crescentus.*



| | | | |
|---|---|---|---|
| VanA_3F | [illegible] | [illegible] | [illegible] |
| VanA_3R | GCGGGCAGACTATCGTCGCGT | 66.6 | [illegible] |
| VanA_4F | [illegible] | [illegible] | [illegible] |
| VanA_4R | GGCTTCGCTAGCCTGGGGTC | 64.6 | [illegible] |
| VanA_5F | [illegible] | [illegible] | [illegible] |
| VanA_5R | ATCTCGATGGTCATTGCGGGCTCAGA | 64.6 | [illegible] |
| VanA_6F | [illegible] | [illegible] | [illegible] |
| VanA_6R | ATGACCAAGCCGGTTGGCGG | 66.6 | [illegible] |
| VanA_7F | [illegible] | [illegible] | [illegible] |
| VanA_7R | TCGCCAATGTTGGTGACCGGC | 66.6 | [illegible] |

**Table 2.** *vanA* primer sets designed in this study. The primers were used to amplify and sequence the region of the chromosome which has homology to *Caulobacter crescentus* VanA.

>Vanillate A complete sequence (1,038 nt)

CTACTTAGCGGCGGGCACCAGTTCCTGATCGGCTGGCTGAGATTCGGCAATTGCCCAACCGCGATCGCGGGCTTGTCGC
AGCAAGTTGCGGTAGCCGATTTGTAGGGCATCAGCGGCGACGTGGGTTTCACTGCGATCGTCCAAGGGAATCATCTTGG
GACGCTGGGTTTCGACGATCCGTTTGTCCTCTTCGATGATTTTGAGACTGCGGCGCACCGTGTCGCCATCCGCCCAAGC
ACCGGTAAAGAAGGTGCGCACGGTCAGCCAAAAGGTCTTGGTGGTCTCGGCATCGATCGGCAGATTGGCACCGTAGCC
AATGAAGCGGAAGCCGTTGCCTAAGTCCGTTTCGGTGCGGACGATGCAGGGCATGTAAAGGGTGATTGTGGTGGTGAC
GGTACGTGGGGCATCGCCACGATTGAACAGCTTCCAAATGCCGCGATAGGCCGATTTGGTGAACTGGGTTTGGGCGCG
TAGACCGTCTGGAAGTTGTTCGACTTTGTAAGGCTCAATTAATGGATCGGACGCTGCCCCAAACGAACCCGAGTGGGTG
AAGGGTGCATGGGCGAAATCCATCAGGTTTTCGGTGACGCGGGTGTAGTGGGTGGTGTAGGTCACTTCACCCTGCACG
GTGCGCCAGGCTGGATCGTCGTATTCCGGCAAGGGCGGCAAGGGTGGACGATCCGCTTCAGGTAGATCGCCGTAGAAC
AGCCAGACTAGGCCGTAGCGTTCGATCGCATCATAGTGGGGAACTTTGGCTGCAAAGGGAATGCGCTGTTGCTCCGTAT
TGGCAGGAATTTTGCGGCAGTGCCCTTGGGCGTCGTACTGCCAACCGTGGTACGGACAGACGAGACAGTCATTTTCAAC
CCAGCCGCCGGAAAGTGCTGCACCCCGATGCACACAGCGATCGCTCAAGGCGTGAATTTGACCCGCACTGTCGCGGTA
GAGGGCGATCGCTTCACCGCAGAGAGTGACAGACAGGGGGCGATCGCCGACCTTCTGAGCCAGCGCCACGGGATACC
AGTAGTTCTTGAGAAACAT

**Figure 7.** Complete sequence of *vanA*.

| | | | |
|---|---|---|---|
| VanB_1F | [redacted] | [redacted] | [redacted] |
| VanB_1R | GATCGTCCACCCTTGCCGCC | 68.6 | |
| VanB_2F | [redacted] | [redacted] | [redacted] |
| VanB_2R | GCGCTGGCTCAGAAGGTCGG | 66.6 | |

**Table 3.** *vanB* primer sets designed in this study. The primers were used to amplify and sequence the region of the chromosome which has homology to *Caulobacter crescentus* VanB.

>Vanillate B complete sequence (1,005 nt)
TCAGCCTTGGGCGACGAGGGAGGAATGGTGCTGTTGCCAGAGCTGGCGGTAGTAGAGTTGCAGGCGATCGGAGGCAAC
CGAAATTTCATTGCGATCGCGCAGAGGAGCAATCCCCTCAATGATCTTTTGATCTTCCTGAAAAAGCTTGGCGGTGCGT
TTGCGTGTCCCACCATCCGCCCAGCCTGCCGTCAAGAAATTGCGGACATGGAGCCAGTGACTACGGGTCGTTGTGGCCG
AGATCGGCTGGTGAATACCGACCAAGATCAAATGAAAGTTGCCGAATTCAATATCGATACGAGTGATGTTCGGTAAGT
GAAAGGCTGCCCCCGATCGCCCTGCCTTGGGGGTTTCTTGCTTGCGTCCTAGCAGGTTGAAGCTGCCACGATAGTTCGC
CTTCGGCTCGATCGGAATCCAGATTTGGGCGCCGAGATCGCTCTTTTCCAACTCAAAATCGGCGATCACTTCATTGCCG
CTAGCACCAAAGGAATTGGCATGCACGAAGGGTGAATGCGCCACATCCATGCCATTTTCGGTAACGCGACGATAGTCT
GCCGCCCAATCGAACTGACCTTGAACCACCCGCCAACCGGCTTGGTCATATTCCGGCAGTTCCCACAACGGCGTTTGTG
CCGCCCGATCGCGATCGCCGGTGAACACCCAAATCAAGCCATAGCGCTCCTGGACAGGATAGCTCGCCACTTTTGCCGC
TTGGGGAATTCGTGCCGAAGCCTGCTGAGATGGAATCTGAGCGCAATGACCATCGAGATCAAATTGCCAACCGTGATA
GGGACAGGCAATCGCGTTGCCCTTGACCTGACCGCAGGCTAGCGAAGCCCCACGGTGGGGGCAGCGATCGTCGAGCAC
CACAACTTGTCCAGCAGTAGTGCGATAGACCACCAGCTGTTGATCGAGTAGCGACGCTGCGATCGGTGTGGAACCCAG
ATCAGTACTGGCAGCGATCGCGTACCAGTGATTCGGTAGCAGCGCGACGTCTAGATTAGCCAT

**Figure 8.** Complete sequence of *vanB*.

| | | | |
|---|---|---|---|
| [redacted] | | | |
| [redacted] | | | |
| NVanR_1R | GCGGAAACCAAGGGCTCGCA | 66.6 | |
| [redacted] | | | |
| NVanR_2R | AGTTCTGCGACCCGCTTGCC | 66.6 | |
| [redacted] | | | |
| NVanR_3R | AGTGAAACCCACGTCGCCGC | 66.6 | |

**Table 4.** *vanR* primer sets used in this study. The primers were used to amplify and sequence the region of the chromosome which contains the potential transcriptional repressor VanR.

**>VanR complete sequence (1,239 nt)**

ATGCGGATTGTCATTCTGACGATTGGGACGCGCGGTGACGTCCAACCGTTTATGGCGCTGGGTTTGGGGCTGAAAGCGG
CGGGCTATGAGGTGGCGATCGCCACGCAAGCCAACTATCAGTCGATGGTGGAAGGGCTGGGGCTGGAGTTTCGGTTGC
TGGCGGGTGATCCCCAAGGGGTGCAGCAGCAATCAGGCGCTTACTCCAAGGAAACGGTCGCGGCGGCAGCGCAGCTGC
TAGGCCAGATTCTCAAGGATTCTTGGGCGGCTTGTCAGGATGCGGATGCGATCGTGGCTTCGCCGAATGCGCGGGGTGC
GACTCATATTGCCGAAGCGCTGAAGATTCCTTGCTTTCTGGGATCGCCCACGCCCTACGGGTTTACCCAAGCCTTTGCG
AGCCCTTGGTTTCCGCCGAACTTCATGCTGGGAGGTGGCTGGGGCAATTGGCTCAGTCACTATGCCGTCGATAAATTGC
TCTGGGTGGCGACTCGCAAGACGGTCAACGAGTGGCGCATTTCTGATCTAGGACTGAAGCCCTTGAGTTGGAGCAGTCC
TTACAAACAGCTGGTGCGCAGAGGGCAAGTCTTCTTGCATCCACTCAGTGAAGTGACCTTGCCGAAACCTGCAGACTGG
CCAGAGCAAGCGCATCTGACGGGTTATTGGCTGCTACCGGAAGCTGAGGCAACGCTCTCACCCGAACTGGAAGCCTTT
CTAGCAGCGGGTGAGCCGCCGGTGTTCATTGGCTTTGGCAGCATGGTCGACCAAGAACCGGAGCGGTTGACCGCGATC
GCAGTCGAAGCGCTGCAGAAAAGTAATCAGCGGGGCATTTTGCTAGCAGGCTGGAGCCGGATCGACCGCTCTCAGCTA
CCAGACACGGTGTTTCCACTAGAGTCCGCGCCCTTTGGCCTGCTGTTTCCGCGCCTCGCAGCGGCAGTGCATCACGGTG
GTTGTGGTACCACGGCAGCGAGTTTGCAGGCAGGGTTGCCAACAATCATCACGGCCTACGGCAATGACCAAGCCTTTTG
GGGCAAGCGGGTCGCAGAACTAGGGGCAGGGCCATCCCCAATTACCCGCGAGGGTTTGACGGCTGAGACTCTGGCGAC
TGCGATCGCCCAAGCCGTCAGCGATCCGCAAATGCGATCGCGGGCGCAGGCGATCGGGGAACGGCTACGGGCAGAGA
ATGGGGTTTCTAAAGCAGTGAAACTGCTGGGTGACTACTTAGCGGCGGGCACCAGTTCCTGA

**Figure 9.** Complete sequence of *vanR*.

| | | |
|---|---|---|
| SynPCC7942_2034 | ATGCGGATTGTCATTCTGACGATTGGGACGCGCGGTGACGTCCAACCGTTTATGGCGCTG | 60 |
| SIU625 - VanR | ATGCGGATTGTCATTCTGACGATTGGGACGCGCGGTGACGTCCAACCGTTTATGGCGCTG | 60 |
| SynPCC7942_2034 | GGTTTGGGGCTGAAAGCGGCGGGCTATGAGGTGGCGATCGCCACGCAAGCCAACTATCAG | 120 |
| SIU625 - VanR | GGTTTGGGGCTGAAAGCGGCGGGCTATGAGGTGGCGATCGCCACGCAAGCCAACTATCAG | 120 |
| SynPCC7942_2034 | TCGATGGTGGAAGGGCTGGGGCTGGAGTTTCGGTTGCTGGCGGGTGATCCCCAAGGGGTG | 180 |
| SIU625 - VanR | TCGATGGTGGAAGGGCTGGGGCTGGAGTTTCGGTTGCTGGCGGGTGATCCCCAAGGGGTG | 180 |
| SynPCC7942_2034 | CAGCAGCAATCAGGCGCTTACTCCAAGGAAACGGTCGCGGCGGCAGCGCAGCTGCTAGGC | 240 |
| SIU625 - VanR | CAGCAGCAATCAGGCGCTTACTCCAAGGAAACGGTCGCGGCGGCAGCGCAGCTGCTAGGC | 240 |
| SynPCC7942_2034 | CAGATTCTCAAGGATTCTTGGGCGGCTTGTCAGGATGCGGATGCGATCGTGGCTTCGCCG | 300 |
| SIU625 - VanR | CAGATTCTCAAGGATTCTTGGGCGGCTTGTCAGGATGCGGATGCGATCGTGGCTTCGCCG | 300 |
| SynPCC7942_2034 | AATGCGCGGGGTGCGACTCATATTGCCGAAGCGCTGAAGATTCCTTGCTTTCTGGGATCG | 360 |
| SIU625 - VanR | AATGCGCGGGGTGCGACTCATATTGCCGAAGCGCTGAAGATTCCTTGCTTTCTGGGATCG | 360 |
| SynPCC7942_2034 | CCCACGCCCTACGGGTTTACCCAAGCCTTTGCGAGCCCTTGGTTTCCGCCGAACTTCATG | 420 |
| SIU625 - VanR | CCCACGCCCTACGGGTTTACCCAAGCCTTTGCGAGCCCTTGGTTTCCGCCGAACTTCATG | 420 |
| SynPCC7942_2034 | CTGGGAGGTGGCTGGGGCAATTGGCTCAGTCACTATGCCGTCGATAAATTGCTCTGGGTG | 480 |
| SIU625 - VanR | CTGGGAGGTGGCTGGGGCAATTGGCTCAGTCACTATGCCGTCGATAAATTGCTCTGGGTG | 480 |
| SynPCC7942_2034 | GCGACTCGCAAGACGGTCAACGAGTGGCGCATTTCTGATCTAGGACTGAAGCCCTTGAGT | 540 |
| SIU625 - VanR | GCGACTCGCAAGACGGTCAACGAGTGGCGCATTTCTGATCTAGGACTGAAGCCCTTGAGT | 540 |
| SynPCC7942_2034 | TGGAGCAGTCCTTACAAACAGCTGGTGCGCAGAGGGCAAGTCTTCTTGCATCCACTCAGT | 600 |
| SIU625 - VanR | TGGAGCAGTCCTTACAAACAGCTGGTGCGCAGAGGGCAAGTCTTCTTGCATCCACTCAGT | 600 |
| SynPCC7942_2034 | GAAGTGACCTTGCCGAAACCTGCAGACTGGCCAGAGCAAGCGCATCTGACGGGTTATTGG | 660 |
| SIU625 - VanR | GAAGTGACCTTGCCGAAACCTGCAGACTGGCCAGAGCAAGCGCATCTGACGGGTTATTGG | 660 |
| SynPCC7942_2034 | CTGCTACCGGAAGCTGAGGCAACGCTCTCACCCGAACTGGAAGCCTTTCTAGCAGCGGGT | 720 |
| SIU625 - VanR | CTGCTACCGGAAGCTGAGGCAACGCTCTCACCCGAACTGGAAGCCTTTCTAGCAGCGGGT | 720 |
| SynPCC7942_2034 | GAGCCGCCGGTGTTCATTGGCTTTGGCAGCATGGTCGACCAAGAACCGGAGCGGTTGACC | 780 |
| SIU625 - VanR | GAGCCGCCGGTGTTCATTGGCTTTGGCAGCATGGTCGACCAAGAACCGGAGCGGTTGACC | 780 |
| SynPCC7942_2034 | GCGATCGCAGTCGAAGCGCTGCAGAAAAGTAATCAGCGGGGCATTTTGCTAGCAGGCTGG | 840 |
| SIU625 - VanR | GCGATCGCAGTCGAAGCGCTGCAGAAAAGTAATCAGCGGGGCATTTTGCTAGCAGGCTGG | 840 |
| SynPCC7942_2034 | AGCCGGATCGACCGCTCTCAGCTACCAGACACGGTGTTTCCACTAGAGTCCGCGCCCTTT | 900 |
| SIU625 - VanR | AGCCGGATCGACCGCTCTCAGCTACCAGACACGGTGTTTCCACTAGAGTCCGCGCCCTTT | 900 |
| SynPCC7942_2034 | GGCCTGCTGTTTCCGCGCCTCGCAGCGGCAGTGCATCACGGTGGTTGTGGTACCACGGCA | 960 |
| SIU625 - VanR | GGCCTGCTGTTTCCGCGCCTCGCAGCGGCAGTGCATCACGGTGGTTGTGGTACCACGGCA | 960 |
| SynPCC7942_2034 | GCGAGTTTGCAGGCAGGGTTGCCAACAATCATCACGGCCTACGGCAATGACCAAGCCTTT | 1020 |
| SIU625 - VanR | GCGAGTTTGCAGGCAGGGTTGCCAACAATCATCACGGCCTACGGCAATGACCAAGCCTTT | 1020 |
| SynPCC7942_2034 | TGGGGCAAGCGGGTCGCAGAACTAGGGGCAGGGCCATCCCCAATTACCCGCGAGGGTTTG | 1080 |
| SIU625 - VanR | TGGGGCAAGCGGGTCGCAGAACTAGGGGCAGGGCCATCCCCAATTACCCGCGAGGGTTTG | 1080 |
| SynPCC7942_2034 | ACGGCTGAGACTCTGGCGACTGCGATCGCCCAAGCCGTCAGCGATCCGCAAATGCGATCG | 1140 |
| SIU625 - VanR | ACGGCTGAGACTCTGGCGACTGCGATCGCCCAAGCCGTCAGCGATCCGCAAATGCGATCG | 1140 |
| SynPCC7942_2034 | CGGGCGCAGGCGATCGGGGAACGGCTACGGGCAGAGAATGGGGTTTCTAAAGCAGTGAAA | 1200 |
| SIU625 - VanR | CGGGCGCAGGCGATCGGGGAACGGCTACGGGCAGAGAATGGGGTTTCTAAAGCAGTGAAA | 1200 |
| SynPCC7942_2034 | CTGCTGGGTGACTACTTAGCGGCGGGCACCAGTTCCTGA | 1239 |
| SIU625 - VanR | CTGCTGGGTGACTACTTAGCGGCGGGCACCAGTTCCTGA | 1239 |

| | | |
|---|---|---|
| SynPCC7942_2035 | CTACTTAGCGGCGGGCACCAGTTCCTGATCGGCTGGCTGAGATTCGGCAATTGCCCAACC | 60 |
| SIU625 – Van A | CTACTTAGCGGCGGGCACCAGTTCCTGATCGGCTGGCTGAGATTCGGCAATTGCCCAACC | 60 |
| SynPCC7942_2035 | GCGATCGCGGGCTTGTCGCAGCAAGTTGCGGTAGCCGATTTGTAGGGCATCAGCGGCGAC | 120 |
| SIU625 – Van A | GCGATCGCGGGCTTGTCGCAGCAAGTTGCGGTAGCCGATTTGTAGGGCATCAGCGGCGAC | 120 |
| SynPCC7942_2035 | GTGGGTTTCACTGCGATCGTCCAAGGGAATCATCTTGGGACGCTGGGTTTCGACGATCCG | 180 |
| SIU625 – Van A | GTGGGTTTCACTGCGATCGTCCAAGGGAATCATCTTGGGACGCTGGGTTTCGACGATCCG | 180 |
| SynPCC7942_2035 | TTTGTCCTCTTCGATGATTTTGAGACTGCGGCGCACCGTGTCGCCATCCGCCCAAGCACC | 240 |
| SIU625 – Van A | TTTGTCCTCTTCGATGATTTTGAGACTGCGGCGCACCGTGTCGCCATCCGCCCAAGCACC | 240 |
| SynPCC7942_2035 | GGTAAAGAAGGTGCGCACGGTCAGCCAAAAGGTCTTGGTGGTCTCGGCATCGATCGGCAG | 300 |
| SIU625 – Van A | GGTAAAGAAGGTGCGCACGGTCAGCCAAAAGGTCTTGGTGGTCTCGGCATCGATCGGCAG | 300 |
| SynPCC7942_2035 | ATTGGCACCGTAGCCAATGAAGCGGAAGCCGTTGCCTAAGTCCGTTTCGGTGCGGACGAT | 360 |
| SIU625 – Van A | ATTGGCACCGTAGCCAATGAAGCGGAAGCCGTTGCCTAAGTCCGTTTCGGTGCGGACGAT | 360 |
| SynPCC7942_2035 | GCAGGGCATGTAAAGGGTGATTGTGGTGGTGACGGTACGTGGGGCATCGCCACGATTGAA | 420 |
| SIU625 – Van A | GCAGGGCATGTAAAGGGTGATTGTGGTGGTGACGGTACGTGGGGCATCGCCACGATTGAA | 420 |
| SynPCC7942_2035 | CAGCTTCCAAATGCCGCGATAGGCCGATTTGGTGAACTGGGTTTGGGCGCGTAGACCGTC | 480 |
| SIU625 – Van A | CAGCTTCCAAATGCCGCGATAGGCCGATTTGGTGAACTGGGTTTGGGCGCGTAGACCGTC | 480 |
| SynPCC7942_2035 | TGGAAGTTGTTCGACTTTGTAAGGCTCAATTAATGGATCGGACGCTGCCCCAAACGAACC | 540 |
| SIU625 – Van A | TGGAAGTTGTTCGACTTTGTAAGGCTCAATTAATGGATCGGACGCTGCCCCAAACGAACC | 540 |
| SynPCC7942_2035 | CGAGTGGGTGAAGGGTGCATGGGCGAAATCCATCAGGTTTTCGGTGACGCGGGTGTAGTG | 600 |
| SIU625 – Van A | CGAGTGGGTGAAGGGTGCATGGGCGAAATCCATCAGGTTTTCGGTGACGCGGGTGTAGTG | 600 |
| SynPCC7942_2035 | GGTGGTGTAGGTCACTTCACCCTGCACGGTGCGCCAGGCTGGATCGTCGTATTCCGGCAA | 660 |
| SIU625 – Van A | GGTGGTGTAGGTCACTTCACCCTGCACGGTGCGCCAGGCTGGATCGTCGTATTCCGGCAA | 660 |
| SynPCC7942_2035 | GGGCGGCAAGGGTGGACGATCCGCTTCAGGTAGATCGCCGTAGAACAGCCAGACTAGGCC | 720 |
| SIU625 – Van A | GGGCGGCAAGGGTGGACGATCCGCTTCAGGTAGATCGCCGTAGAACAGCCAGACTAGGCC | 720 |
| SynPCC7942_2035 | GTAGCGTTCGATCGCATCATAGTGGGGAACTTTGGCTGCAAAGGGAATGCGCTGTTGCTC | 780 |
| SIU625 – Van A | GTAGCGTTCGATCGCATCATAGTGGGGAACTTTGGCTGCAAAGGGAATGCGCTGTTGCTC | 780 |
| SynPCC7942_2035 | CGTATTGGCAGGAATTTTGCGGCAGTGCCCTTGGGCGTCGTACTGCCAACCGTGGTACGG | 840 |
| SIU625 – Van A | CGTATTGGCAGGAATTTTGCGGCAGTGCCCTTGGGCGTCGTACTGCCAACCGTGGTACGG | 840 |
| SynPCC7942_2035 | ACAGACGAGACAGTCATTTTCAACCCAGCCGCCGGAAAGTGCTGCACCCCGATGCACACA | 900 |
| SIU625 – Van A | ACAGACGAGACAGTCATTTTCAACCCAGCCGCCGGAAAGTGCTGCACCCCGATGCACACA | 900 |
| SynPCC7942_2035 | GCGATCGCTCAAGGCGTGAATTTGACCCGCACTGTCGCGGTAGAGGGCGATCGCTTCACC | 960 |
| SIU625 – Van A | GCGATCGCTCAAGGCGTGAATTTGACCCGCACTGTCGCGGTAGAGGGCGATCGCTTCACC | 960 |
| SynPCC7942_2035 | GCAGAGAGTGACAGACAGGGGGCGATCGCCGACCTTCTGAGCCAGCGCCACGGGATACCA | 1020 |
| SIU625 – Van A | GCAGAGAGTGACAGACAGGGGGCGATCGCCGACCTTCTGAGCCAGCGCCACGGGATACCA | 1020 |
| SynPCC7942_2035 | GTAGTTCTTGAGAAACAT | 1038 |
| SIU625 – Van A | GTAGTTCTTGAGAAACAT | 1038 |


**Figure 10.** Blast2 alignments of *vanR, vanA,* and *vanB* between *S.* IU 625 and *S. elongatus* PCC 7942. Using the sequence obtained from *S.* IU 625 to the genome of *Synechococcus elongatus* PCC 7942 to show homology.

**Sequence analysis**

Thirty-two sequences were obtained from sequencing the PCR products and were aligned using CodonCode software. Once the sequences were aligned based on homology, and contigs generated, sequences were compared to *Synechococcus elongatus* PCC 7942. Figure 10 shows the Blast2 alignment between the novel *vanR, vanA,* and *vanB* sequences from *S.* IU 625 and the corresponding region in *S. elongatus* PCC. The hypothetical VanA was then compared to several species whose VanA protein is sequenced and function is known. Figure 11 shows the visualized alignment result (POAVIZ) with Partial Order Alignment (POA). Once the sequence was obtained, a proposed operon was then created. Figure 13 shows the primer map with relative position based on the region primed.

Figure 12 shows the proposed vanillate operon based on bioinformatic analysis and the sequence data obtained from the sequencing reactions. The size and orientation of the vanillate response genes are shown compared to *Synechococcus elongatus* PCC 7942 and *Caulobacter crescentus*. The vanillate operon consists of three genes: *vanR,* a transcriptional repressor, tasked with inhibiting the expression of *vanA* and *vanB*. VanA is the alpha subunit of the Vanillate degradation complex. VanB is the beta subunit of the Vanillate degradation complex. VanA and VanB together form a holoenzyme capable of utilizing vanillate for carbon. In *Caulobacter crescentus*, VanA is a terminal oxygenase and VanB is a ferredoxin-like reductase. *vanA-vanB* are typically transcribed together.

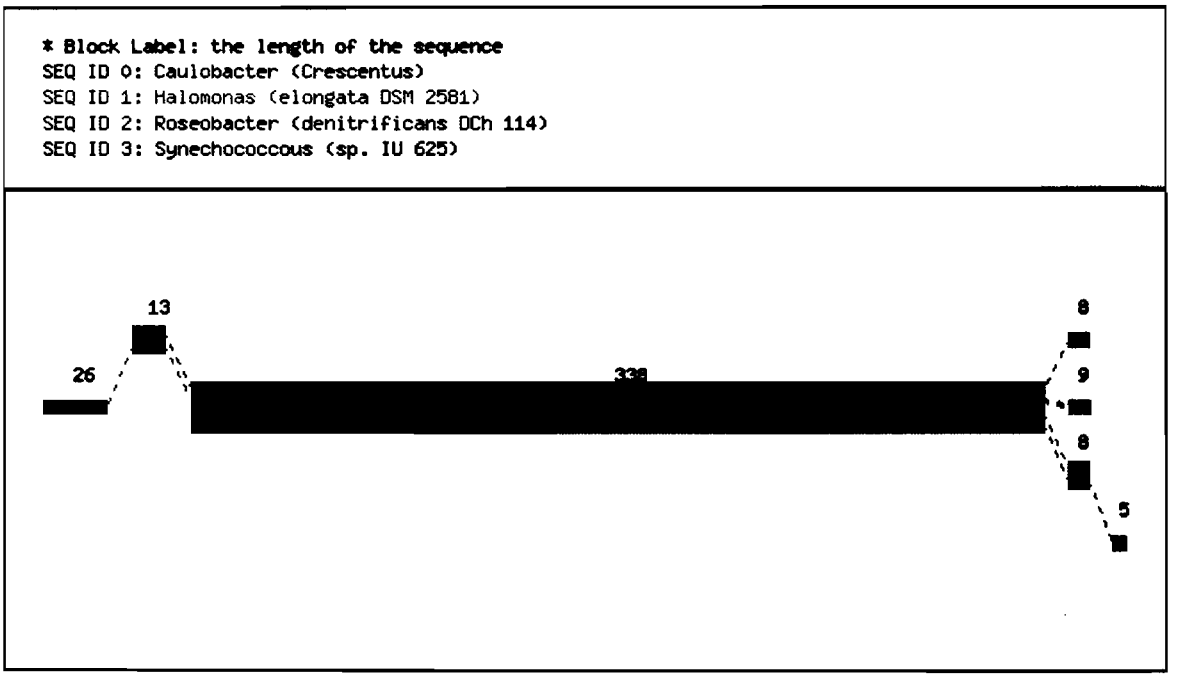



**Figure 11.** POAVIZ results of *vanA* sequence obtained from *S.* IU 625 and another 3 species of bacteria. (Smooth: 3)

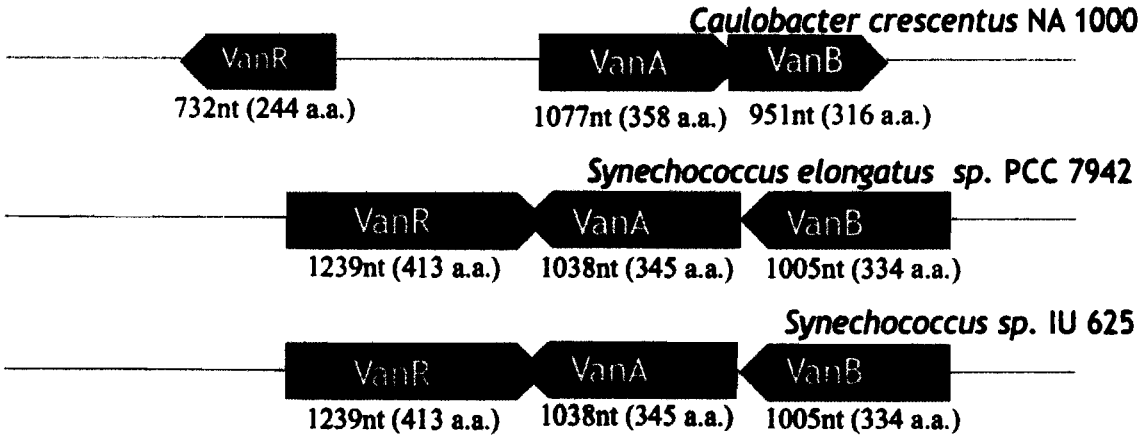



**Figure 12.** Proposed Van operon.

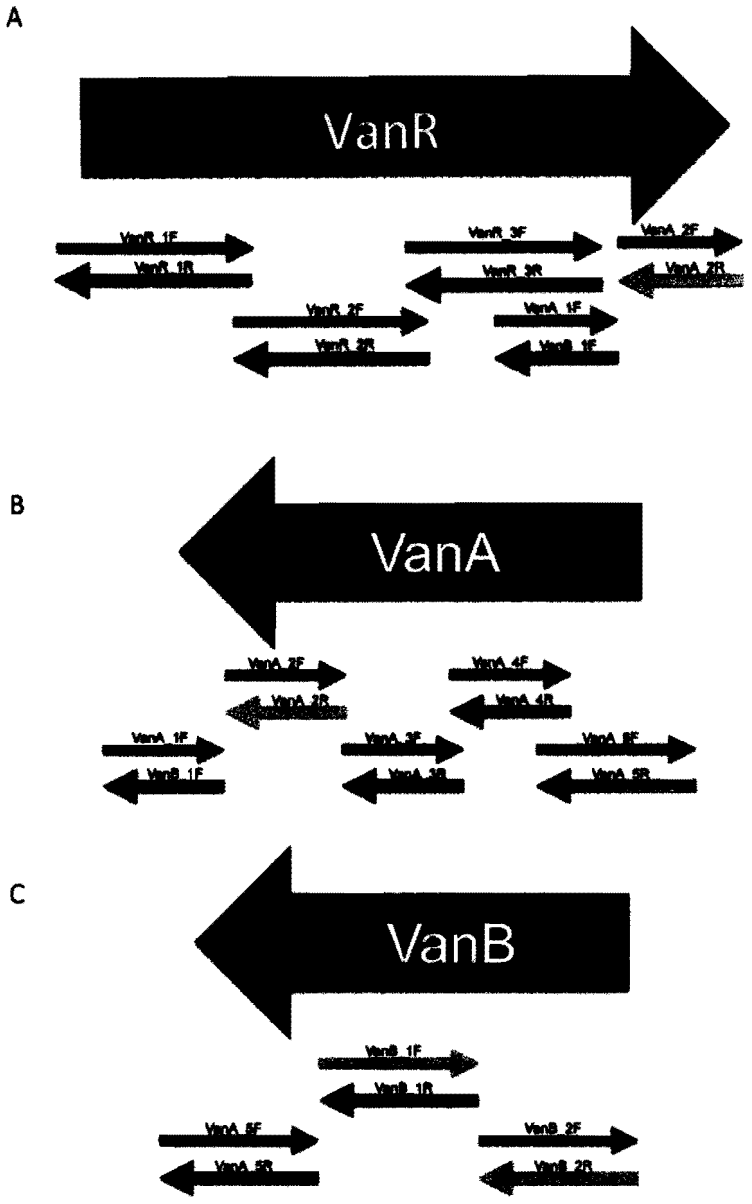
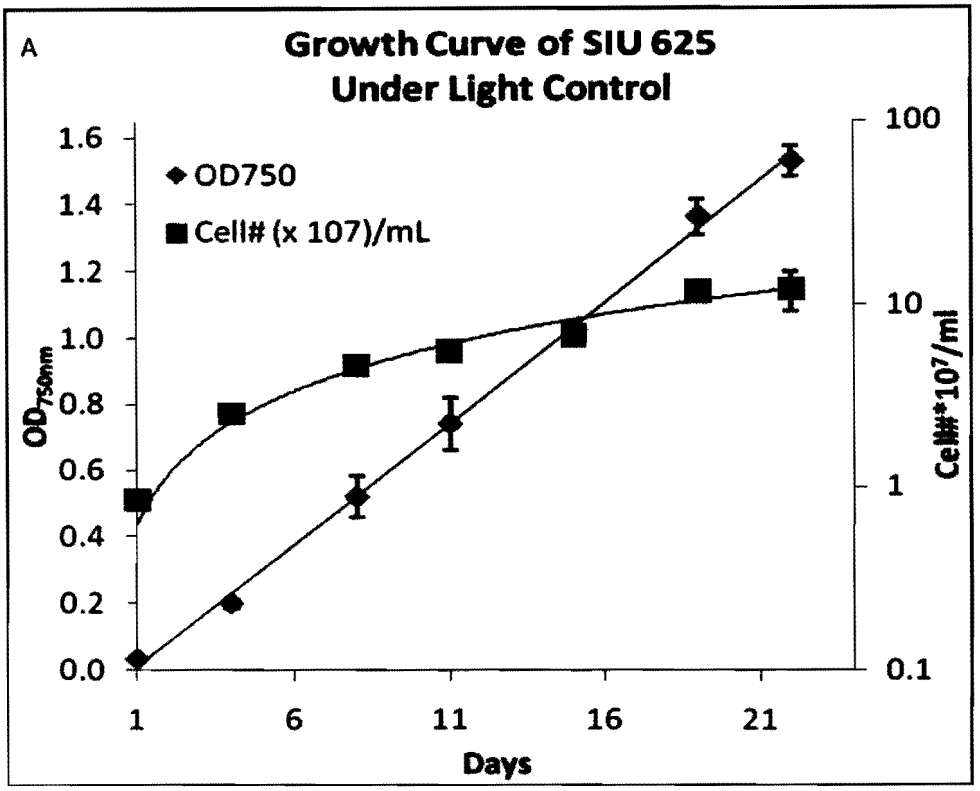


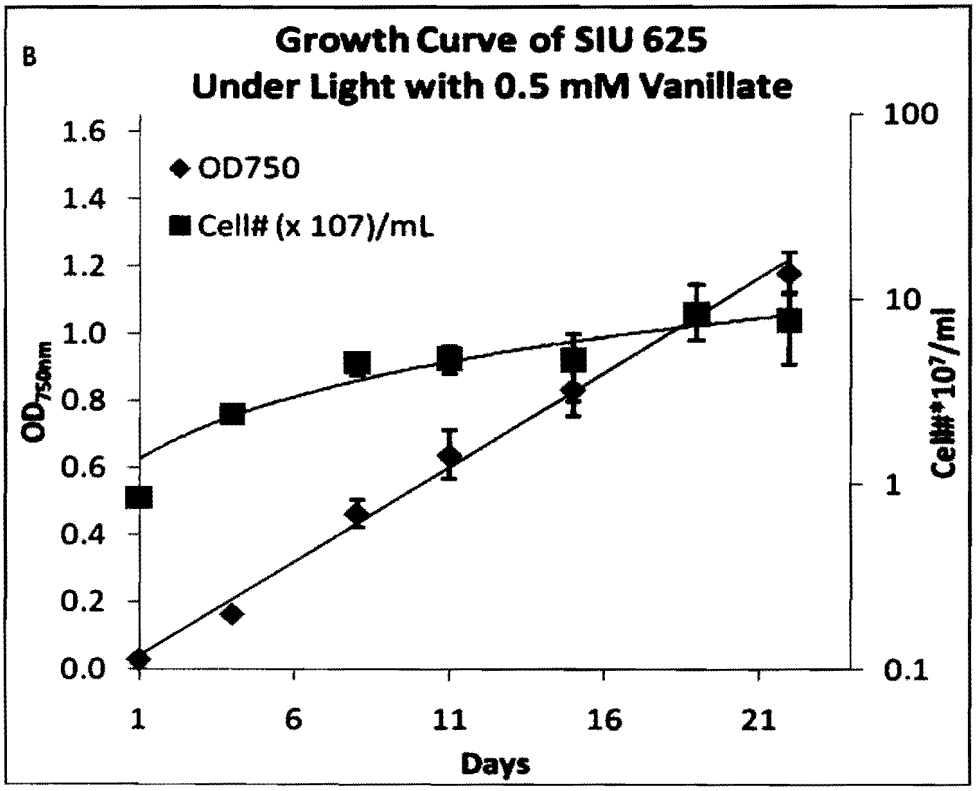

**Figure 13.** Primer map with corresponding positions. A) Primers used to prime the *vanR* region. B) Primers used to prime the *vanA* region. C) Primers used to prime the *vanB* region.

**Vanillate utilization in *Synechococcus* sp. IU 625**

Experimental design was used to determine the ability of *S.* IU 625 to utilize vanillate as a sole carbon source when photosynthesis was not available. 250 ml Erlenmeyer flasks were sterilized in an autoclave prior to usage. The flasks were allowed to cool down then inoculated with a 1:20 (cells to 3M) log stage culture of *S.* IU 625. Over the course of 32 days, the flasks were monitored for cell growth by measuring the optical density with wavelength of 750 nm, as well as total direct cell number with a hemocytometer at 400x magnification. After 22 days the cells were washed with sterile 3M media and allowed to recover in light conditions. Figure 14 shows the growth of the cells grown in the light conditions. Figure 15 shows the growth of the cells grown in the dark conditions.

After day 22 the cells were spun down and washed with sterile 3M media. After day 22 the dark set of cells were allowed to grow in light conditions. Exponential growth is then seen as the cells begin to switch from heterotrophic to photoautotrophic growth.


A
Growth Curve of SIU 625
Under Light Control
OD750
Cell# (x 107)/mL
OD750nm
Cell#*10^7/ml
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
100
10
1
0.1
1
6
11
16
21
Days


B
Growth Curve of SIU 625
Under Light with 0.5 mM Vanillate
OD750
Cell# (x 107)/mL
OD750nm
Cell#*10^7/ml
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
100
10
1
0.1
1
6
11
16
21
Days

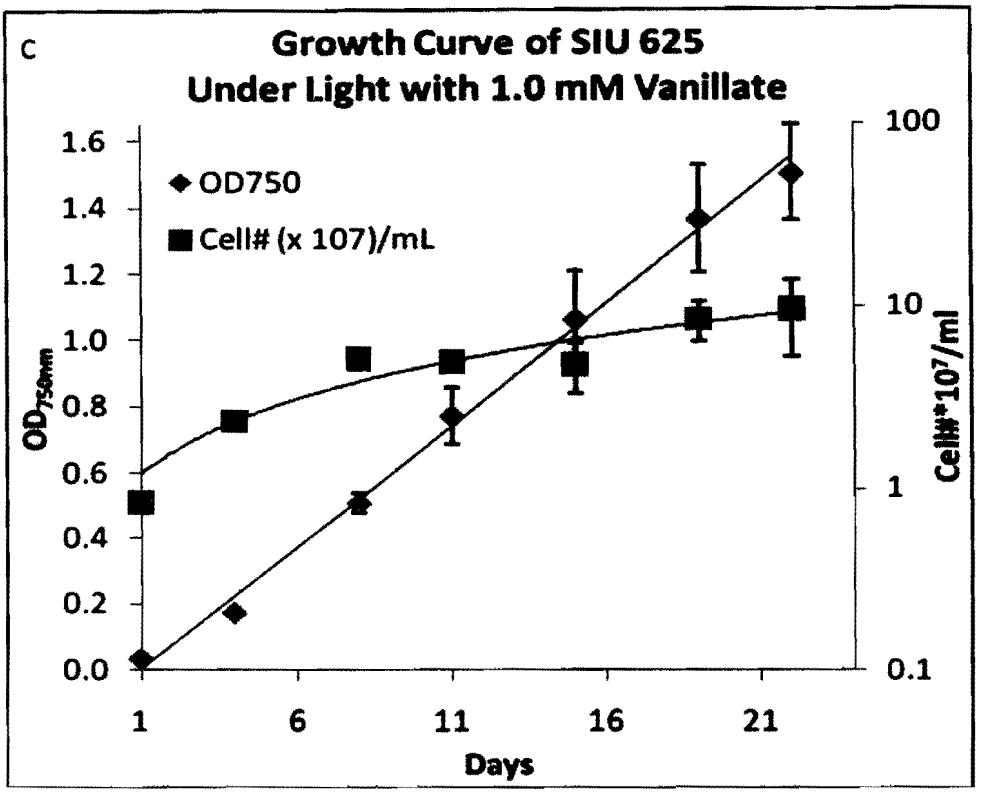
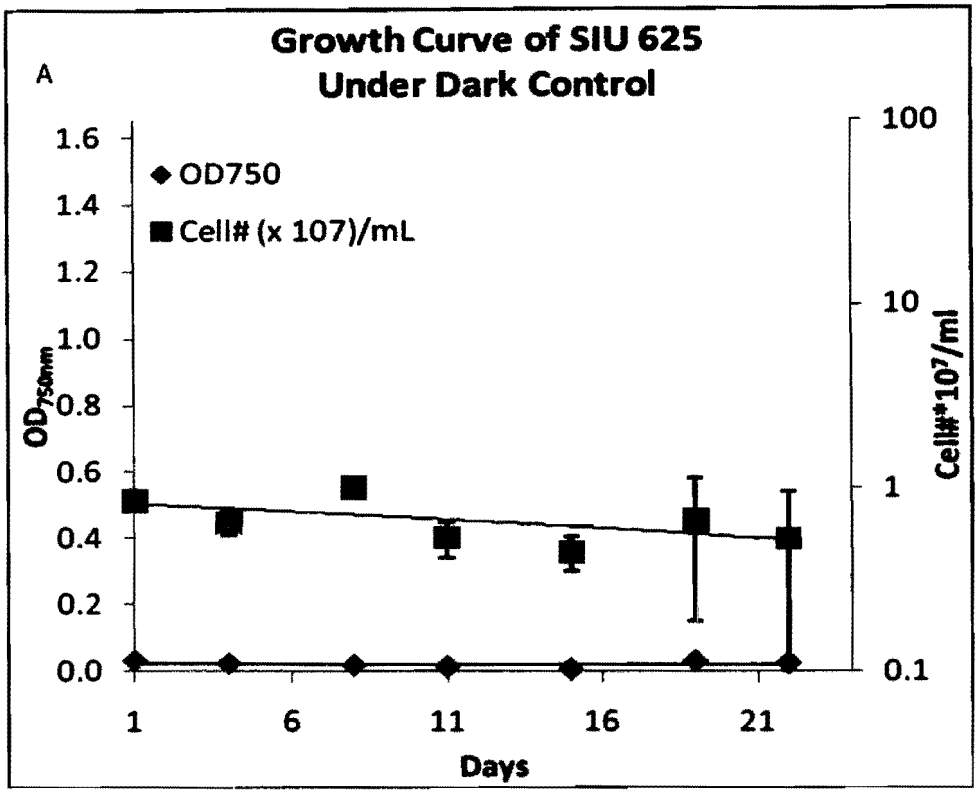
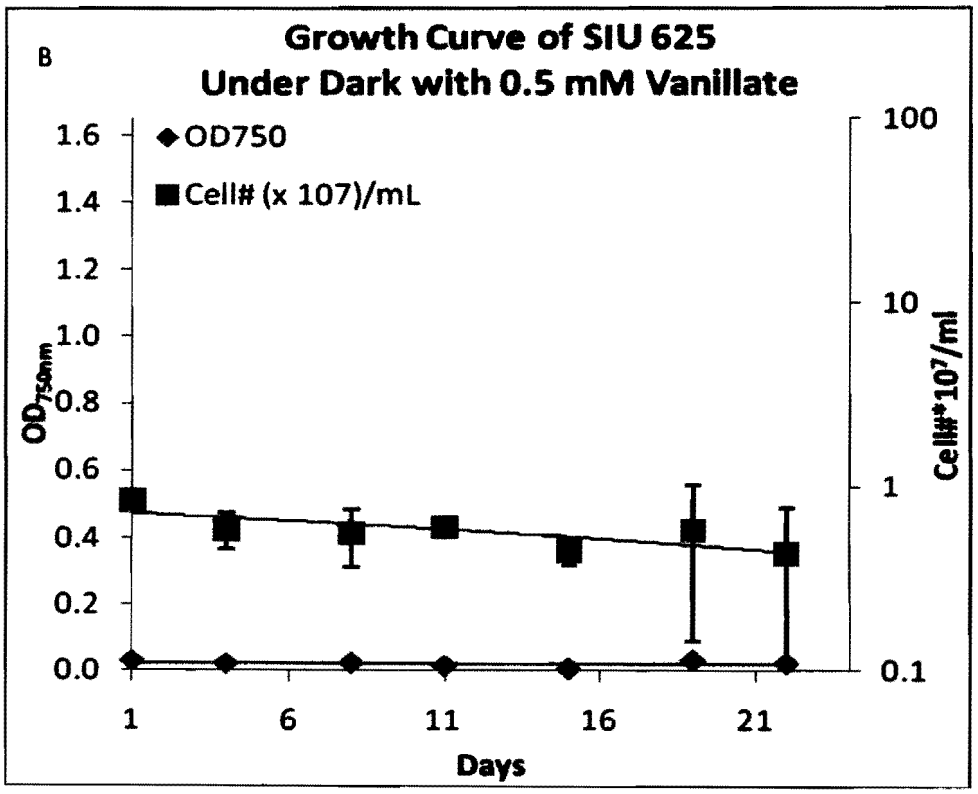



**Figure 14.** The growth curves of *S.* IU 625 with 0, 0.5 and 1.0 mM vanillate under light conditions. A) represents the growth curve obtained from the cells grown in light conditions with no vanillate. B) represents the growth curve obtained from the cells grown in the light condition and exposed to 0.5 mM Vanillate. C) represents the growth curve obtained from the cells grown in the light condition and exposed to 1.0 mM Vanillate. Turbidity was measured with spectrophotometer at $OD_{750nm}$ on the primary vertical axis and cell numbers were measured with hemacytometer on the secondary vertical axis, both plotted against time in days.


A
Growth Curve of SIU 625
Under Dark Control
OD750
Cell# (x 107)/mL
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD750nm
100
10
1
0.1
Cell#*10⁷/ml
1
6
11
16
21
Days


B
Growth Curve of SIU 625
Under Dark with 0.5 mM Vanillate
OD750
Cell# (x 107)/mL
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD750nm
100
10
1
0.1
Cell#*10⁷/ml
1
6
11
16
21
Days

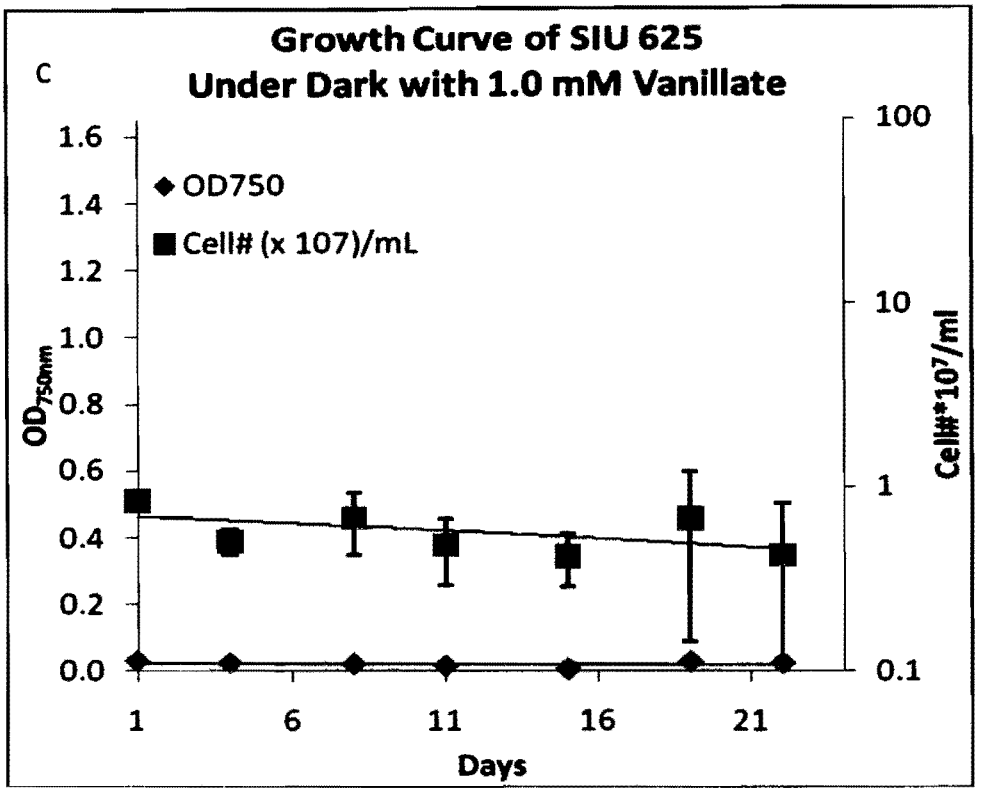



**Figure 15.** The growth curves of *S.* IU 625 with 0, 0.5 and 1.0 mM vanillate under dark conditions. A) represents the growth curve obtained from the cells grown in dark conditions with no vanillate. B) represents the growth curve obtained from the cells grown in the dark condition and exposed to 0.5 mM Vanillate. C) represents the growth curve obtained from the cells grown in the dark condition and exposed to 1.0 mM Vanillate. Turbidity was measured with spectrophotometer at $OD_{750nm}$ on the primary vertical axis and cell numbers were measured with hemacytometer on the secondary vertical axis, both plotted against time in days.

**Evidence of vanillate utilization by *Synechococcus* sp. IU 625**

Utilization of vanillate by *S.* IU 625 was measured by the absorbance at $OD_{286nm}$. Based on work in Thanbichler (2007), this was the measured non-interfering absorbance of vanillate in the supernatant. Figure 16 highlights the distinct difference between the experimental sets after the first spin of the wash step on Day 22. The three tubes on the left hand side show the set grown in light conditions. L1 is the standard control set; L2 is the 0.5 mM vanillate experimental set; L3 is the 1.0 mM vanillate experimental set. The three tubes on the right hand side show the set grown in dark conditions. D1 is the standard control grown in the absence of light; D2 is the 0.5 mM vanillate experimental set grown in the absence of light D3 is the 1.0 mM vanillate experimental set grown in the absence of light. The dark brown color indicates the cells were not actively utilizing vanillate. The absence of brown coloration is indicative that *Synechococcus* sp. IU 625 is capable of utilizing vanillate. Figure 16 shows the degradation of vanillate (0, 0.5 and 1.0 mM conditions) under light and dark conditions in the absence of cells. There were no observable degradation in any of the samples. Figure 17 shows the $OD_{286nm}$ over the time course study between the light set and the dark set. The light set has a statistically higher $OD_{286nm}$ compared to the dark set. Vanillate has a distinct brown coloration compared to the clear coloration of the media. To verify the vanillate was not being altered by the media, a set of flasks placed in both conditions, light and dark at 0.5 mM and 1.0 mM concentrations, were also set up.

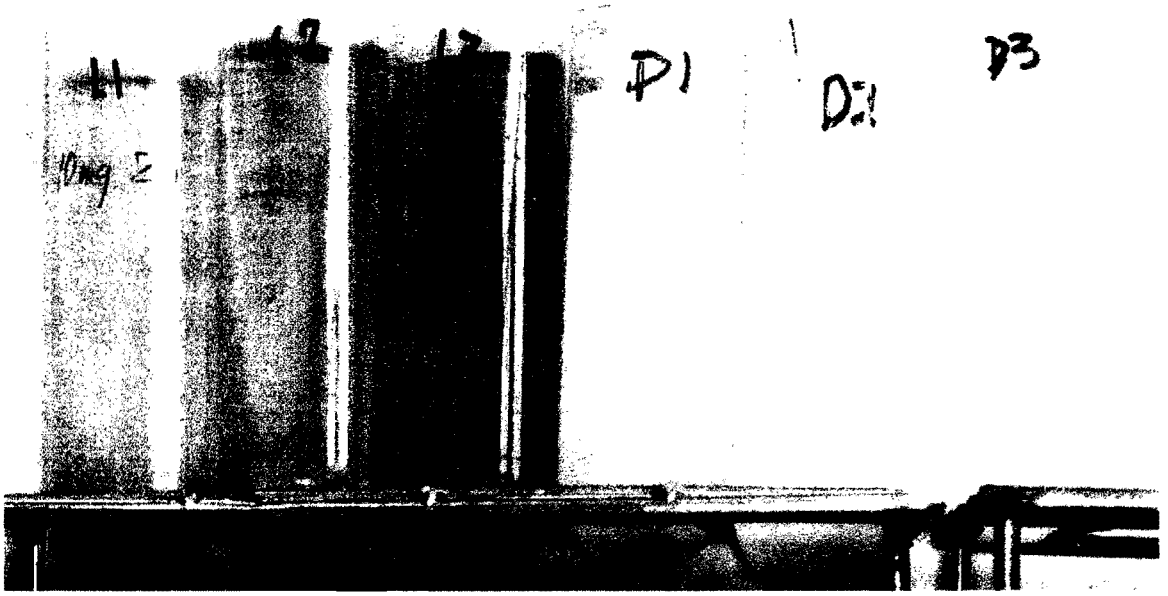



**Figure 16.** Supernatant of each sample after centrifugation at day 22.

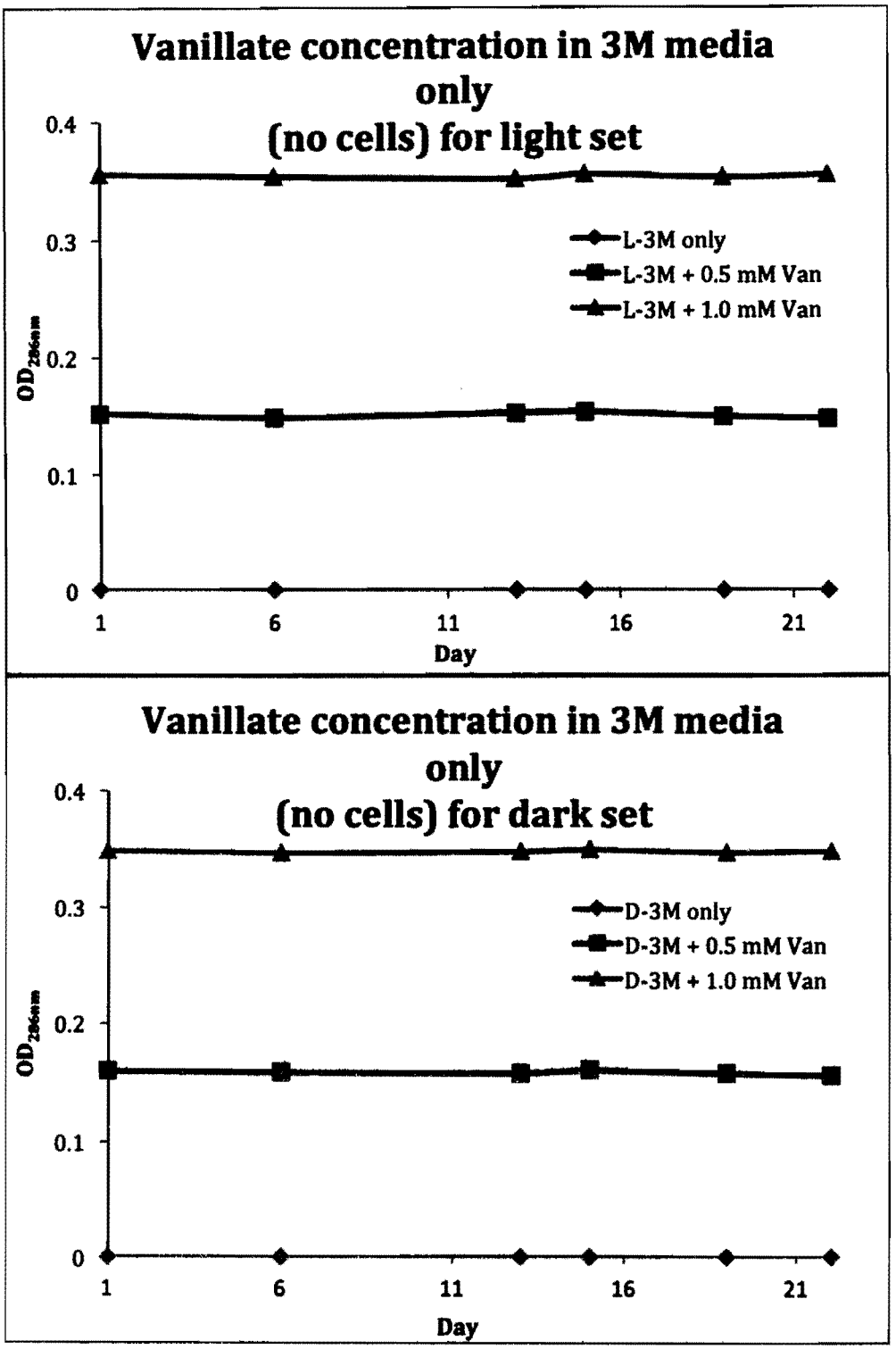



**Figure 17.** Degradation of vanillate (0, 0.5 and 1.0 mM conditions) under light and dark conditions in the absence of cells.

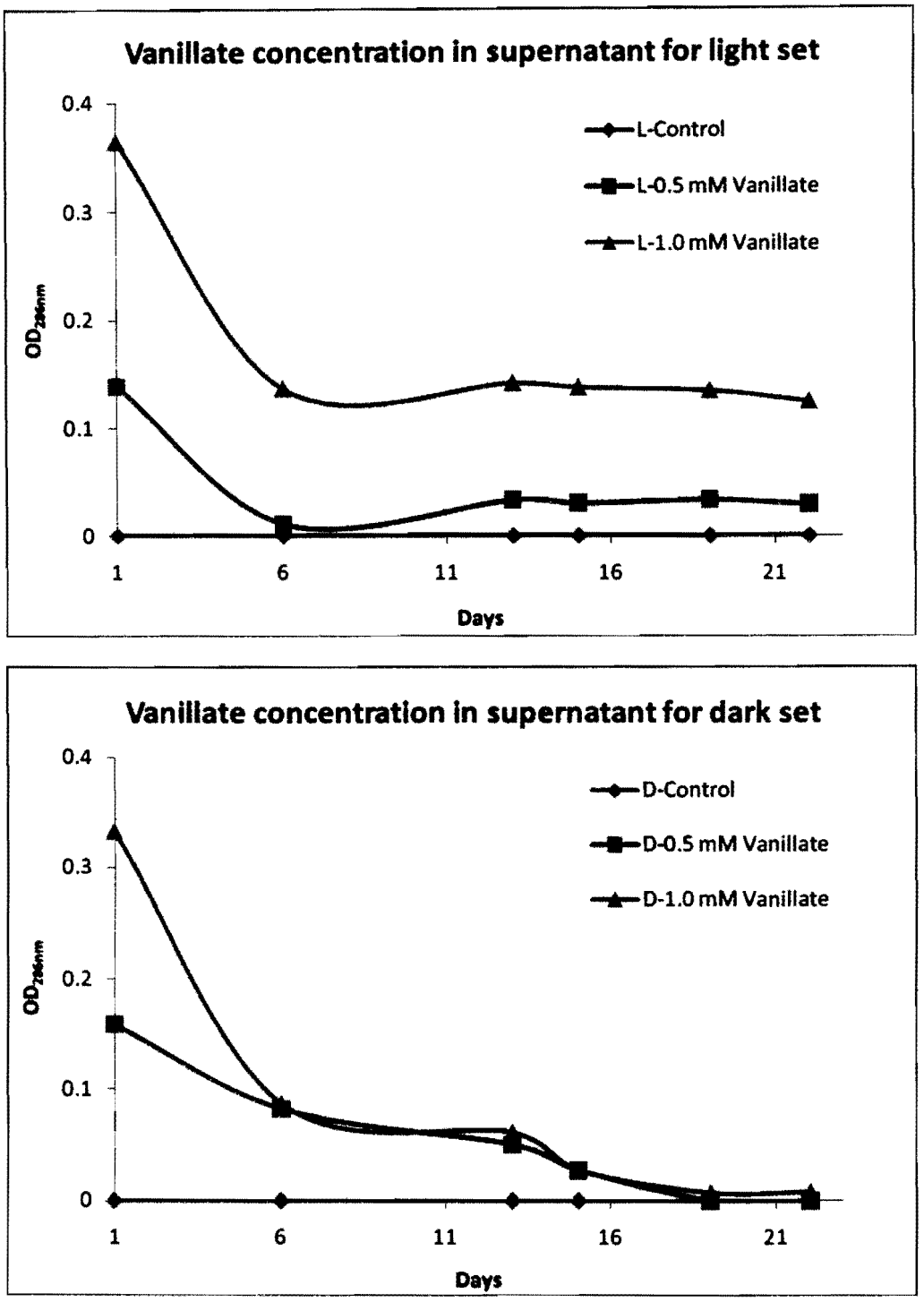



**Figure 18.** Degradation of vanillate (0, 0.5 and 1.0 mM conditions) under light and dark conditions. *S.* IU 625 cells were given 0.5 mM vanillate and 1.0 mM vanillate. Cell supernatants were collected at the time points listed above. A wash to remove all vanillate was conducted at Day 22.

**Reverse transcription and qRT-PCR analysis of *vanA* gene expression**

Quantitative real-time PCR (qRT-PCR or qPCR) analysis was used to measure the expression levels of the hypothetical alpha subunit of the vanillate degradation complex. Total RNA was extracted from samples collected during the vanillate challenge using Bacterial Enhanced Trizol and cDNA was synthesized using the High Capacity Reverse Transcription cDNA synthesis kit, following manufactures recommendations using random primers to prime all RNA present. qPCR analysis was used to measure the expression levels of the hypothetical alpha subunit of the vanillate degradation complex. Differences of expression were compared to a uniformly expressed gene, *rpoD*. *rpoD* is the sigma subunit of the 16s rRNA, and sequence is known in *Synechococcus elongatus* PCC 7942. Table 5 lists out the sequences and Tm of the primers used in this study. Comparative $C_T$ analysis was performed with SYBR Green. Levels of expression are presented as $C_T$ values compared to the expression of the *rpoD* gene. Changes in expression level of the *vanA* gene in the light set are shown in Figure 19. Only day 4, 0.5 mM Van sample showed statistically significance ($p < 0.05$) compared with the control. Changes in expression level of the *vanA* gene in the dark set are shown in Figure 20. Only day 8, 0.5 mM Van sample showed statistically significance ($p < 0.05$) compared with the control. Day 1 exposed to no vanillate is used as control to establish basal level expression of *rpoD* and *vanA*. Expression of genes is compared to the basal level expression. Data is presented as the means of triplicates with standard deviation.

| | | | |
|---|---|---|---|
| Syn7942_rpoD_F | [illegible] | [illegible] | 210 |
| Syn7942_rpoD_R | CGTAAGCCCACCGAGCAAGAGA | 60.6 | |
| VanA_RTF3 | [illegible] | [illegible] | 200 |
| VanA_RTR3 | TGCTAGCGGCAATGAAGTGA | 60.4 | |

**Table 5.** qPCR primer sets designed and used in this study.

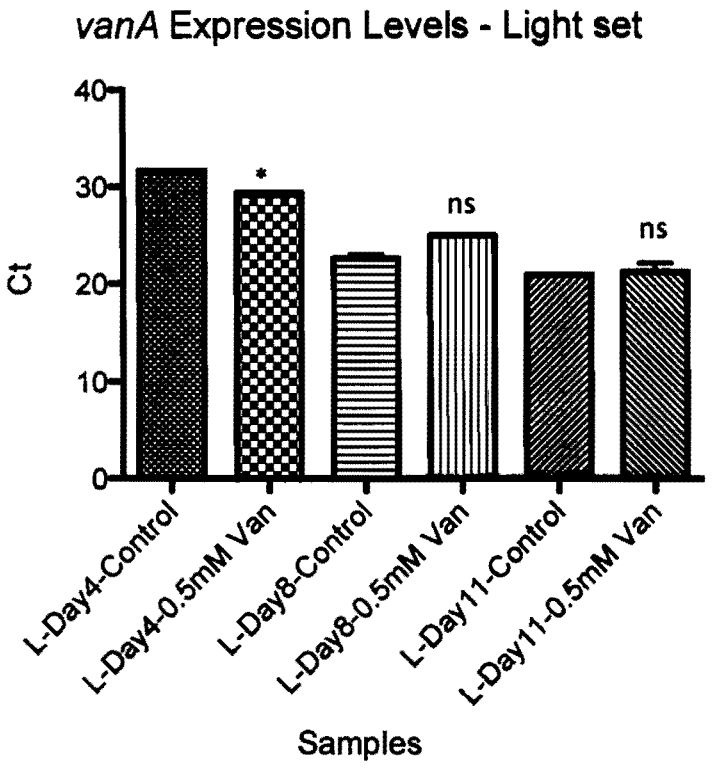



**Figure 19.** qPCR results ($C_T$) of *vanA* gene expression under light conditions. * above bar indicate statistical difference in expression compared to control levels. Statistical significant defined as $p < 0.05$

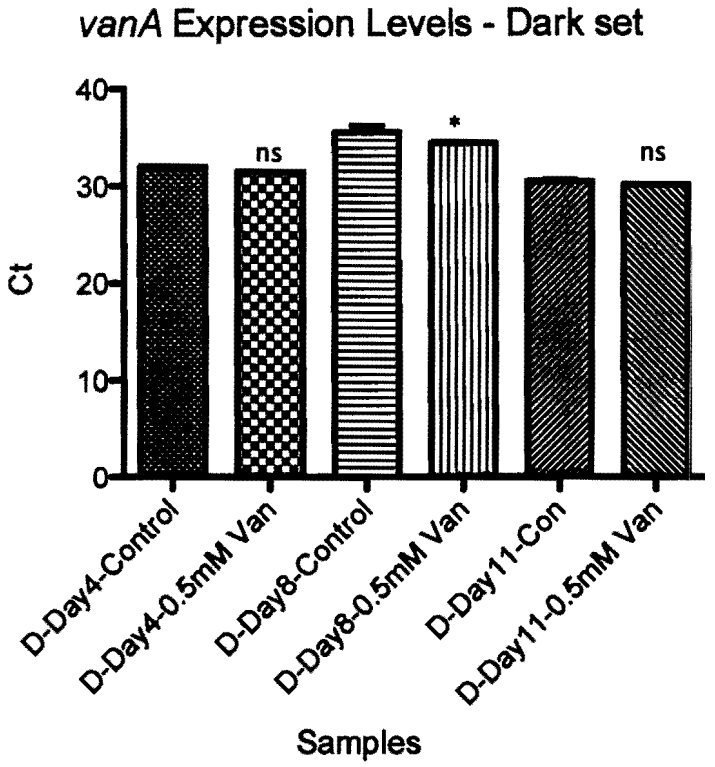



**Figure 20.** qPCR results ($C_T$) of *vanA* gene expression under dark conditions.
* above bar indicate statistical difference in expression compared to control levels.
Statistical significant defined as $p < 0.05$

**Vector Design and Transformation**

Using the isolated sequence from the vanillate operon, several novel vectors were designed to measure gene expression and reporting in *Synechococcus* sp. IU 625. A set of vectors has been made for this study. These vectors link the expression of SmtA to the presence of carbon source, vanillate. Vanillate is the byproduct of the saprotrophic digestion of plant lignin (Brunel and Davidson, 1988). It is a phenolic compound, which can be, used a sole carbon source in several prokaryotic species. Typically the vanillate operon (Van) consists of three genes: *vanA*, a monooxygenase; *vanB*, a phenolic demethylase; and *vanR*, a transcriptional repressor (Thanbichler et al., 2007). Work has previously done in *Caulobacter crescentus* NA1000, which has characterized and cloned the promoter from the Van region, and a set of molecular vectors has been released linking the expression of a gene of interest to the Van promoter (Thanbichler et al., 2007). These vectors are presented in figure 21, and were carried in DH5α, a dam+ strain of *E. coli.* Another knock-out vector which will homologously recombine into *S.* IU 625 has been created for *smtA* and its vector map is shown in figure 22. This will allow studies to show the response to heavy metal stress without *smtA* being expressed. Bioinformatic analyses of Van gene were carried out in our lab, and plasmids were constructed by Dr. Sean Murray at CSU: Northridge. The constructed plasmid vectors were then characterized in Dr. Chu's lab at SHU.

Using the Van operon sequence obtained from this study, three vectors were designed to link the expression of *smtA* to the presence or absence of vanillate. In two of

the designed vectors (pSM1040 and pSM1041), the C' terminus of *smtA* was fused to a green fluorescent protein (GFP), so that real time expression of *smtA* can be monitored by fluorescence microscopy. GFP has been shown to be a viable molecular tool in previous cyanobacterial studies (Kunert and Erdmann, 2000). This study is the first to use a vanillate inducible system in cyanobacteria.

Plasmids were made so that a homologous recombination event would occur and that genetic knock ins/outs could be established. pSM1039, pSM1040, and pSM1041 were designed link expression of metallothionein (*smtA*) to the presence or absence of vanillate. pSM1116 contains a truncated region of *smtA*, which when recombined into the chromosome, would create a knockout of *smtA*. All the vectors contain *sacB*, a sucrose sensitive gene. SacB converts sucrose to a toxin. It serves as a counter-selectable marker.

Transformation was carried using three methods: 1) to verify if *Synechococcus* sp. IU 625 was naturally transformable as its close related species, *Synechococcus elongatus* PCC 7942; 2) chemical alteration of the cell wall using calcium shock, heat shock; and 3) electroporation. The cells were plated out onto media containing 5 μg/ml kanamycin sulfate, and allowed to grow for five days before integration screening was conducted.

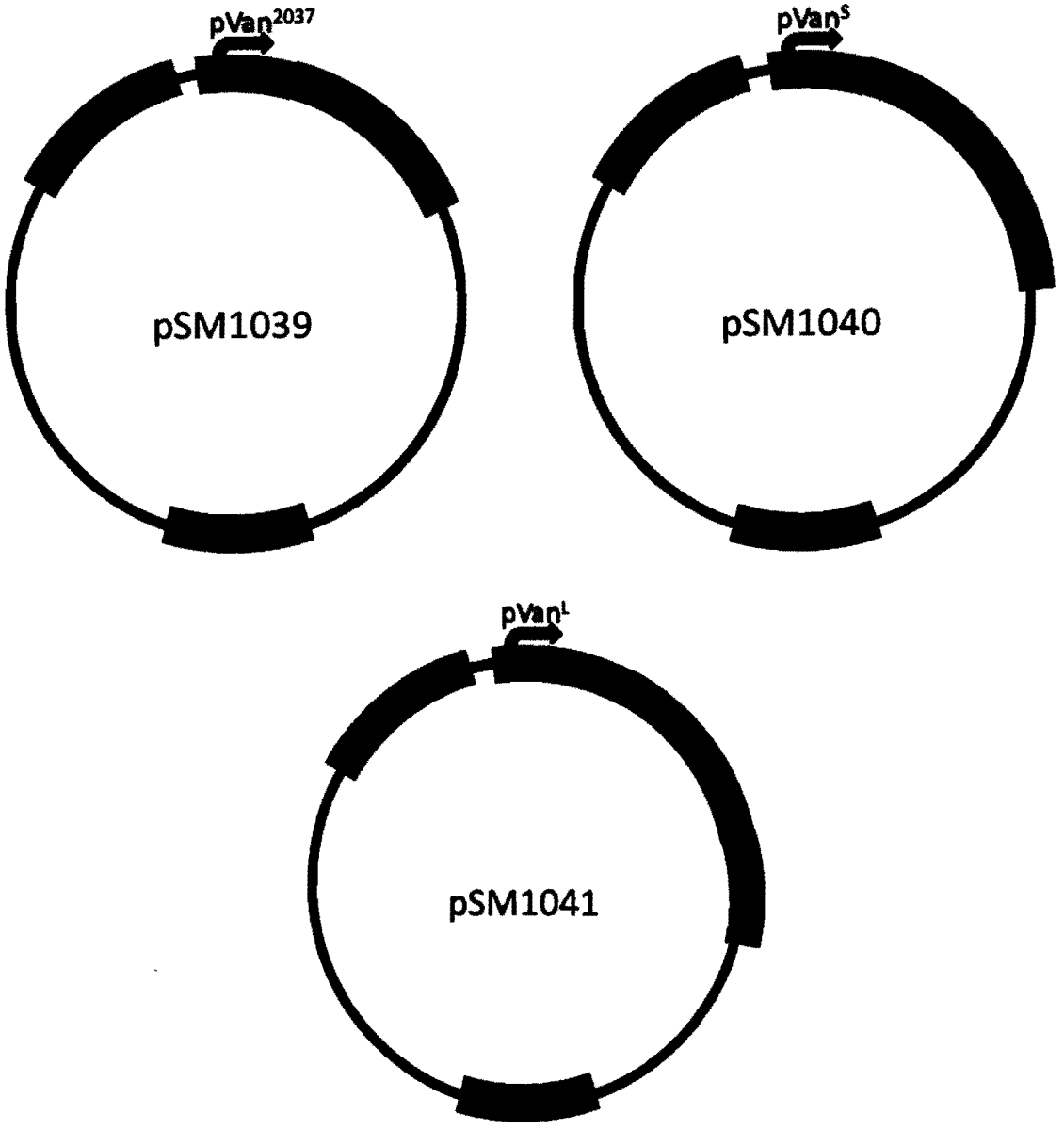



**Figure 21.** Constructed inducible plasmid vectors containing Van operon regions.

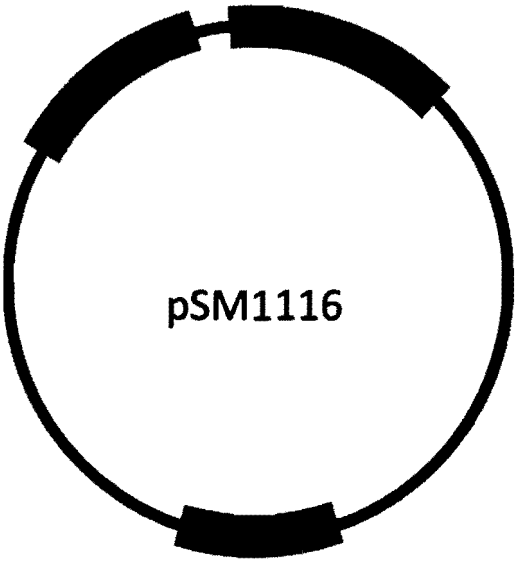



**Figure 22.** Constructed knockout plasmid vector for *smtA*.

## DISCUSSION

Vanillate has been experimentally shown to encourage heterotrophic growth of *Synechococcus* sp. IU 625. This is a novel study indicated that the obligated photoautroph *Synechococcus* sp. IU 625 can also survive and grow in the dark condition with supplemented vanillate. Since experimental evidence exist which show the ability of *S.* IU 625 to utilize vanillate as a sole carbon source, a cluster of genes for its regulation and processing must exist.

In our experiments we show that a high homology between vanillate response genes in other freshwater oligotrophs, such as *Caulobacter crescentus*, has homology to a cluster of genes in *S.* IU 625. Sequencing of the operon, which was undertaken using *Synechococcus elongatus* PCC 7942 as a template for design, gave insight into the genome of *S.* IU 625 not previously seen. One potential purpose of the genes might be to allow the cell to survive when nutrient deprived or in an area where sunlight is blocked by natural foliage. The ability for *S.* IU 625 to utilize vanillate is an important finding since it allows a better understanding of how cyanobacteria respond to external sources of nutrients. Unpublished data shows that this cluster of genes exists in several other species of cyanobacteria. Based on the work in Thanbichler, 2007, vanillate is degraded by the enzyme complex VanAB. The VanAB enzyme breaks down vanillate to pyruvate. The exact mechanism is not known in *S.* IU 625 and is an interest for further studies for this project.

Cells that were grown in the light set were shown to incorporate some of the vanillate into the cells, but not all vanillate was utilized. Figures 15 and 16 highlighted

this trend. qPCR analysis showed that expression of *vanA* increased as time increased when cells were exposed to 0.5 mM vanillate, and that the gene appears to be always expressed. One of these genes in particular, *vanA*, has been shown to experimentally increase expression, even when not being utilized. This indicates that the promoter which regulates the genes is not strictly regulated, or that the gene responsible for oxidation of vanillate is a general purpose enzyme, which is constantly expressed. Further experimentation would be needed to determine the structure and other functions of the genes in the proposed operon.

Figures 18 and 19 show the qPCR results of *vanA* expression from days 4 to day 11. No discernable trend can be seen from these results. A statistically insignificant trend can be seen from the *vanA* expression in the dark set of cells. Since in *Caulobacter crescentus vanA* and *vanB* are transcribed and translated together, the same trend might not be true in *S.* IU 625. Further analysis of the true mechanism of the operon is needed, and further qPCR analysis can be done using primers designed for *vanB* to see if a difference in expression levels are detected.

This study sought to explore the use vanillate inducible gene expression to measure gene expression. By cloning the hypothetical promoter from the vanillate operon, we sought to make a molecular switch, similar to the LacZ based promotion in *E. coli.* This work has been previously described in great detail in *Caulobacter crescentus*, and a set of vanillate inducible plasmids exists based on work by Thanbichler, 2007.

However, work is not limited to understanding *smt*A, as shown in the vector maps, in *S.* IU 625. Due to the nature of the constructs in figure 20 any particular gene of interest can be substituted in place. Although similar work has not been described in great detail for cyanobacteria, several of the key concepts used in other bacteria were taken into account during the creation of these vectors. These vectors are similar to the xylose inducible vectors created for *Staphylococci* spp. in Wieland et al., 1995. Work in other organisms such as *Bacillus* spp. show that carbon based inducible gene expression can be used to regulate gene expression *in vivo* (Charpentier et al., 2004).

Use of a carbon based inducible vector has not been shown previously for cyanobacteria. This study represents the first of its kind to show that cyanobacteria are capable of both heterotrophic and phototrophic growth. Based on the results seen in Figures 14 and 15, the cyanobacteria are capable of surviving up to 22 days when supplemented with vanillate. However, vanillate degradation is not an optimal source of energy to sustain the continued growth of *S.* IU 625.

## FUTURE STUDIES

Characterizing heavy metal responses is one of the overall goals of this laboratory. Previous research from this laboratory and its collaborators show a variety of studies on the effects of heavy metal toxicity in *S.* IU 625. In particular the response of the cell to zinc has been of interest. Zinc exists as a divalent cation ($Zn^{2+}$) in solution, and in excessive cytoplasmic amounts acts as a weak lewis acid, potentially denaturing cytoplasmic proteins, and can cause DNA damage by antagonizing the phosphodiester bond which stabilizes and keeps DNA as a double helix. Cyanobacterial response to heavy metal stress is of important concern to developing nations. While *S.* IU 625 is a non-toxin forming strain, many other freshwater strains form toxins (cyanotoxins) which remain long after the bloom as been reduced (Carmichael, 2001). Understanding how these freshwater cyanobacteria are able to not only survive, but thrive when exposed to EPA target heavy metals is a critical goal.

This study sought to explore the use vanillate inducible gene expression to measure the overall response of type II metallothionein (MT) in *S.* IU 625. SmtA is a cysteine rich protein which is believed to be the key response protein to keeping zinc homeostasis in the cell. Previous work in this laboratory has shown that *smtA* can respond to other EPA target heavy metals aside from zinc (Chu, unpublished work). To better understand the role *smtA* plays in the survivability of *S.* IU 625 in heavy metal stress situations we sought to change its expression to substrate based. By cloning the hypothetical promoter from the vanillate operon, we sought to make a molecular switch,

similar to the *lacZ* based promotion in *E. coli*. This work has been previously described in great detail in *Caulobacter crescentus*, and a set of vanillate inducible plasmids exists based on work by Thanbichler, 2007.

It appears however, that just by the presence of vanillate that expression of the *vanA* gene will increase over time. This indicates that a promoter which responds to vanillate may be in the region near by. Cloning of this promoter will allow linkage of expression of a gene of interest to the presence or absence of vanillate. This information provides a new set of powerful molecular tools to study gene expression in cyanobacteria, and provides additional insight into the nature of cyanobacteria not previously described.

Vanillate has been experimentally shown to encourage the heterotrophic growth of *S.* IU 625. By using this information, the region responsible was first analyzed with bioinformatics tools, and then sequenced. The sequence was used to construct vectors which could control expression of the heavy metal response gene *smtA* based on the presence or absence of the substrate vanillate. The future study of this project was to use a set of molecular vectors that have constructed to study the effect of knockout or over-expression of a specific gene such as *smtA* on the cell physiology in *S.* IU 625. In addition, physiological responses of cyanobacteria to various environmental stresses such as heavy metals will also be studied using these vectors. By using these vectors, we hope to gain a better insight into whether *smtA* is the sole response gene for heavy metal response, and how these adaptations might lead to an increase of freshwater harmful algal blooms.

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