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INCIDENCE OF A BRIGHTER VARIANT OF *VIBRIO HARVEYI* STRAIN N2 UNDER CONTINUOUS LAB CULTURING

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ABSTRACT

A brighter variant of a lab cultured strain of *Vibrio harveyi* was observed under routine lab culturing procedures and was investigated for differences in luminescence characteristics and protein profile as compared to the wild type parent. A lysate of the variant and wild type strain was subjected to BCA protein analysis and proteins were separated on PAGE to elucidate differences. Luminescence studies revealed a faster growing, 20% brighter variant that had a clear difference in protein profiles when subjected to quantitative and qualitative protein analyses. The bright variant of N2 had 0.21µgm/µl more total protein content as compared to the wild type and exhibited darker protein bands for some of the proteins separated on PAGE.

KEY WORDS: Brighter varient, *Vibrio harveyi, Strain N2,* luminescence characteristics, protein profile.

INTRODUCTION

Bacteria have evolved through time by adapting to any changes that have occurred in their environment. The course of evolution has posed many environmental and physiological challenges to the bacterial cells, but these microbial entities have surfaced each time by altering their genetic makeup which renews their physiology for better survival. In recent times, when biological science is employing bacteria in every niche of research, bacteria are faced with a plethora of stress conditions owing to lab culturing conditions. The adaptive response of bacteria may be through a gain or loss of function, where loss of function adaptation is more common as compared to a gain of an appropriate function for better adaptation (Hottes *et al*., 2013).

Bioluminescence in bacteria is under the control of the *lux* operon, which mainly consists of the *lux* structural genes *luxCDABE* (Dunlap, 2014) and a regulatory element which is usually the *luxR* (Showalter *et al*., 1990) gene. The structural genes are responsible for production of the luciferase enzyme and its aldehyde substrate. Despite the physiological differences among different luminous species of bacteria, all share homologous biochemical machinery for the production of light. The signaling pathways that control induction and repression of the *lux* operon are

essentially common with a few differences amongst bacterial species (Meighen, 1988). The biochemical reaction responsible for emission of light involves the participation of the luciferase enzyme, the luciferase substrates i.e. flavin monoucleotide molecule, molecular oxygen and fatty aldehyde molecule (Dunlap, 2014). Functionally, the luciferase oxidizes the reduced Flavin Mononucleotide molecule ($FMNH_2$) along with the Fatty Aldehyde synchronous to the reduction of molecular oxygen. The excess energy is released as blue green light at a wavelength of 490nm (Abu-Soud *et al.,* 1992). Other genetic elements at play in luminescence production in *Vibrio harveyi* are *luxLM* genes for autoinducer-1(AI-1) synthesis (Bassler *et al.,* 1993), *luxN* gene synthesizes AI-1 membrane bound sensor kinase protein (Bassler *et al*., 1993), *luxS* gene synthesizes the AI-2 molecule (Surette *et al*., 1999), *luxPQ* for AI-2 sensor proteins (Bassler *et al*., 1994a), *luxU* (Freeman and Bassler, 1999) and *luxO* (Bassler *et al*., 1994b) are responsible for phosphor-relay proteins. Bioluminescence in *Vibrio harveyi* is modulated by three separate quorum sensing mechanism which function together to produce light. The first system is the HAI-1 system which detects the signal of a homoserine lactone molecule also designated as AI-1, the second system is the AI-2 system which is induced by the AI-2 molecule identified as a furanosyl borate diester. The third system was identified in *Vibrio cholera* and is known as CAI-1 system which recognizes the cholera autoinducer 1 (CAI-1) which is a (Z)-3-aminoundec-2-en-4-one molecule encoded by the *cqsA* gene. Signals from all three systems aided by a variety of small RNA molecules and RNA chaperone molecules that aid in signal transduction, function together to induce an optimum level of bioluminescence in *Vibrio harveyi*.

Bioluminescence is a high energy consuming function which gorges about 20% of the total cell's energy (Nackerdien *et al*., 2008; Kozakiewicz *et al*., 2005). However the character of bioluminescence has been retained by different luminous bacterial species as the advantages gained by the property of light production outweigh the high energy utility of the luminescence function (Meighen, 1993; Czyz *et al*., 2000). Variations in bacterial luminescence due to state of medium have been documented in recent literature, where light produced by the same strain differs in intensity upon liquid and agar solidified medium (Lyell *et al*., 2010). Bright variants of luminous bacteria have occured spontaneously in nature and lab procedures and these mutants usually harbor a mutation of the *luxO* or the *luxU* gene as a result of which the phospho-relay mechanism is disrupted and *lux* repression is lifted, this gives a constitutive bright phenotype regardless of growth phase or cellular density of culture (Lenz *et al*., 2005). However these mutants have been described to constitutively

produce light with intensity equal to the wild type bacteria (Henke and Bassler, 2004). Disruption in the RNAs and RNA related molecules involved in the regulation and expression of luminescence via quorum sensing manifest themselves in the form of a lower or higher intensity of light production as compared to the wild type. Mutants of the RNA chaperone molecule Hfq exhibit a constitutively bright phenoltype which is however indistinguishable from the LuxO mutant (Lenz *et al*., 2004). LuxN mutants have been studied to display a difference in expression levels of the Qrr (Quorum regulated RNAs) genes, a mutation in the *luxN* reading frame resulted in a dark phenotype with high levels of *qrr4* gene or a constitutive bright phenotype with low levels of Qrr4 (Swem *et al*., 2008).

Insertional mutations in various genes of *Vibrio fischeri* ES114, like *arcA* and *arcB* (redox responsive regulators), *pstA* (phosphate transport protein), *pstC* (phosphate ABC transporter), *ainS* (*Vibrio fischeri* autoinducer synthase), certain genes for tRNA synthesis and *phoQ* (Ca+ and Mg+ sensor proteins) produce a 2-1,000 fold brighter mutants as compared to the wild type ES114 strain (Lyell *et al*., 2010). The conversion towards a brighter phenoltype in *Vibrio fischeri* is a pleiotropic effect that naturally occurs in the host light organ. This favors the symbiosis between the luminous bacterium and the host squid with a preferable selection towards the brightest variant of *Vibrio fischeri* (Foster *et al*., 2004; Bose *et al*., 2008; Lyell and Stabb, 2013). In general a loss of repression over the *lux* operon regulation in a luminous bacterial species has been documented to result in a constitutive and a brighter luminescence as compared to the parental wild type strain.

Studies at protein level for defining the disparity between wild type and brighter variants of luminous bacterial strains have illustrated distinctions in the outer membrane proteins and soluble cell protein extracts when fractionated on SDS gel. *Vibrio fischeri* strain ES114 was studied to present a differentiation in the protein band profiles of a brighter variant of the strain (Dunlap *et al*., 1995). An attribute suggested for the bright variant in the study conducted by Dunlap et al., (1995) pointed towards a possible tolerance to and utilization of acids produced during cellular growth.

The present study investigated a bright variant of a lab cultured luminous *Vibrio harveyi* strain N2 to understand the difference in physiological characteristics between the wild type culture and the bright variant at protein level.

MATERIALS AND METHODS

Bacterial strain, culture media and conditions: Luminescent bacterial strain *Vibrio harveyi* N2 previously isolated from coastal areas of Karachi was employed in the study (Nawaz and Ahmed, 2011). Culture medium LSW70 (Ast and Dunlap, 2004) was used in all of the experimental

procedures as broth and agar solidified medium, the medium composed of 10 g tryptone, 5 g yeast extract, 700 ml of artificial sea water and 300ml of distilled water. Artificial sea water was made with NaCl 23g/l, $MgCl_2$. $6H_2O$ 5g/l, $CaCl_2$ 1.1g/l, KCl 0.66g/l, H_3BO_3 0.026g/l, $SrCl_2$ 0.024g/l, which was dissolved in distilled water (dH_2O) and autoclaved at 121°C for 20 minutes (Nawaz and Ahmed, 2011). The pH of the medium was adjusted to 8.09 with concentrated HCl and buffered using a 1M Tris solution at final concentration of 25mM (Scheerer *et al*., 2006).

Luminescence characteristics:

Luminescence was documented with respect to time. Overnight cultures of N2 wild type (N2wt) and N2 bright variant (N2bv) were inoculated in 50mL of fresh LSW70 such that the starter O.D was 0.05 at an absorbance of 600nm. 200µL of the freshly inoculated culture was then transferred to wells of a sterile 96 well white microtiter plate in triplicates. The plate was then placed in a dark box fitted with a sophisticated photon imaging camera (PHOTEK) (Tauriainen *et al*., 1998). The camera was set up in a time lapse to take photon count images of the plate every 30 minutes for 24hrs The photon count data obtained was processed in an excel worksheet that calculated the standard error of means of the triplicate data. Values of means were plotted against time with error bars indicating the standard error values.

Quantitative protein analysis: To get a vivid picture of the difference in proteins at the cellular level for N2wt and N2bv, total proteins were extracted by cell lysis. Overnight cultures were grown in 50mL of LSW70, cells were harvested from the cultures in a refrigerated centrifuge at 3000g for 10mins at 10°C, supernatant was removed and the cells were suspended in 2mL of Lysis buffer (Burley *et al*., 2014). The suspension was mixed thoroughly by vigorous vortexing and then kept on ice for 1hr. The lysate was centrifuged at 13,000g for 15mins at 4°C, the supernatant was carefully extracted using a micropipette and transferred to a clean and sterile eppendorf tube.

The BCA Protein Assay Kit by Pierce was used to quantify the total proteins of the Wild type and bright variant cells of N2, as previously described (Ceradini *et al*., 2004). The reagents were prepared according to the instructions given by the manufacturer. Bovine Serum Albumin (BSA) standard solutions were made at concentrations of 0.5mg/mL, 1mg/mL, 2mg/mL, 4mg/mL, 6mg/mL, 8mg/mL and a blank vial at 0mg/mL of BSA (just dH_2O). 200µL of the reagent mix was added to 10µL of the standard solutions and 10µL of the protein solution extracted from the strain N2, in a sterile 96 well microtiter plate. The plate was then wrapped in aluminum foil and incubated at 37°C for 30mins. The bacterial lysates were diluted 1:1 with lysis buffer as the samples showed a high protein content that was out of range to get a proper reading. After incubation, the absorbance of each

well was measured using a microtiter plate reader at 560nm (Ceradini *et al*., 2004). A standard curve was plotted using the absorbance value against the standard protein concentration, sample protein concentrations were determined by deducing formula from the standard curve and calculating the amount of protein.

PAGE analysis: A 10% running gel consisting of 6.9ml sterile dH_2O, 4.0ml 30% acrylamide Solution, 3.8ml of 1.5M Tris Solution, 0.15ml 10% SDS Solution, 0.15ml 10% APS Solution, and 9µl TEMED and a 5% stacking gel comprising of 3.4ml Sterile dH_2O, 0.85ml 30% acrylamide Solution, 0.63ml 1.0M Tris Solution, 0.05ml 10% SDS Solution, 0.05ml 10% APS Solution and 10µL TEMED were prepared between vertical gel casting glass plates. 1X running buffer, diluted from a 10X Tris Glycine SDS running buffer from Sigma, was used to run the gel. 10µL of Precision Plus Protein All Blue Standards (#161-0373) was loaded as a standard ladder. A calculated volume of the samples, which equaled a concentration of 30µgm/mL, was loaded in the wells of the SDS-PAGE gel. The gel was electrophoresed at 120 Volts for 70mins and then removed from the casting plates and stained with Coommasie brilliant blue for 1hr. The stained gel was then de-stained overnight with a solution of methanol, acetic acid and dH_2O with periodic agitation to remove the excess stain from the gel. The de-stain solution was refreshed whenever the solution became saturated with the Coommasie blue dye as described previously (Sasse and Gallagher, 2009). The de-stained gel was visualized for the difference in protein profile of each sample and scanned on an HP digital scanner to record the results. SDS-PAGE gels were stored immersed in sterile dH_2O at 4°C.

RESULTS

Luminescence characteristics: Visually the luminescence of N2wt and N2bv was clearly different, where N2bv showed an increased light emission as compared to N2wt in LSW70 broth and agar plates (Figure 1).

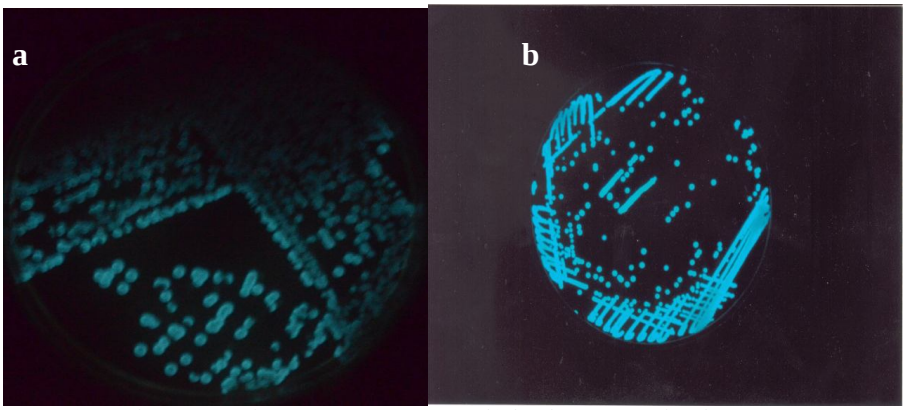

Figure 1: a) Luminescence of N2wt on LSW70 agar, b) luminescence of N2bv on LSW70 agar

Photon count data revealed a marked difference in the light emission characteristics of N2wt and N2bv (Figure 2). Data showed an early induction of light for N2bv at the 3rd hour of culture incubation as compared to induction of light at 7th hour of culture incubation for N2wt. Luminescence for N2bv increased exponentially and maxed up to 10 hours into growth which was followed by a decline in light emission up to 24hrs into growth. In contrast, N2wt light emission maxed at 13hrs into growth followed by a comparatively gradual decrease in luminescence up to 24hrs under culture. The variant N2bv was assessed to be 20% more bright as compared to the wild type N2wt. Growth characteristics were not assessed for this study.

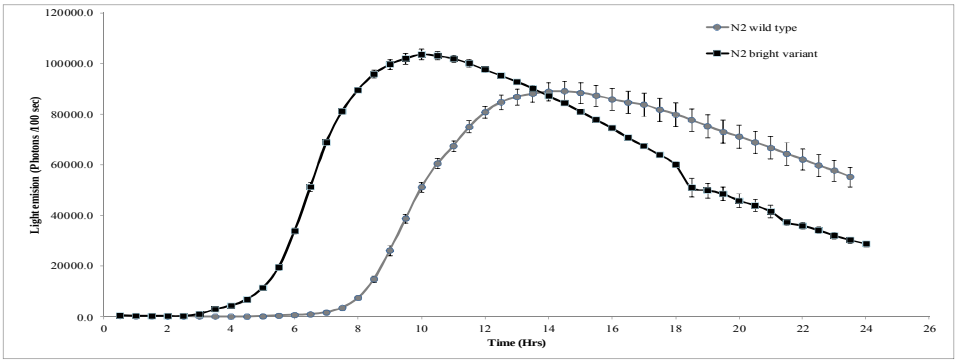

Figure 2: Photon count data plotted against time for N2wt and N2bv luminescence. Data recorded for 24hrs with each reading taken after a lapse of 30mins, each reading represents photon counts per 100secs. The bars indicate the standard deviation of means of the triplicate data for each data point.

Quantitative protein analysis: BSA quantification of the protein samples extracted from the N2wt and N2bv cells generally revealed that the total protein content was different under the tested conditions, where the N2wt culture showed a lower total protein content as compared N2bv culture. For N2wt culture a total protein of 4.03µgm/µL was recorded while N2bv presented a total protein content of 4.24µgm/µl.

Poly-AcrylAmide Gel Electrophoresis: As estimated from the BSA protein quantification, an amount of 30µL was loaded in to the wells of the PAGE gel which equaled an amount of 7.4µgm/µL of total proteins for N2wt and 7.1 µgm/µL total proteins for N2bv. The Coomasie blue stained gels revealed a banding pattern as seen in Figure 3. A clear difference was seen in the protein banding pattern of N2wt and N2bv where concurrent with the protein quantification results, the N2bv culture showed denser protein bands which indicated a higher concentration of

protein as compared to the N2wt culture. The difference in the protein profiles of the N2wt and N2bv cultures offered a window into the change that had occurred causing the strain to luminesce brighter than its wild type (Figure 3).

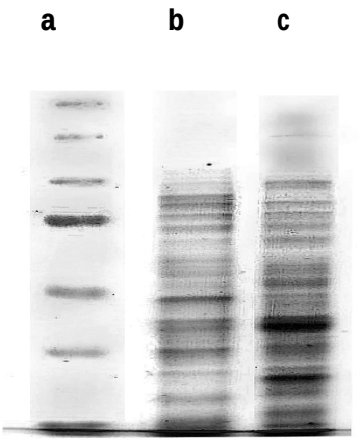

Figure 3: Coommasie blue stained PAGE gel showing protein bands for N2wt and N2bv culture. Lane a): Biorad Precision Plus Protein All blue Standard, lane b) Total protein extract of N2wt culture, lane c) Total protein extract of N2bv culture.

DISCUSSION

During the course of repeated lab culturing, a change in the lumine-scence character of *Vibrio harveyi* N2 was observed that displayed a bright variant of the parental wild type strain. Bacteria are known to mutate under stress conditions or continuous culture conditions (Dunlap *et al*., 1995), and it is possible that N2 adapted under the changing nutritional stress of continuous lab sub-culturing, giving rise to a variant strain that showed a markedly brighter luminescence as compared to its wild type ancestor, a phenomenon previously discussed by Dunlap *et al*., (1995) for a lab cultured strain of *Vibrio Fischeri* ES114.

Luminescence characteristic studies reveal that the light in N2bv is induced earlier than the parent N2wt strain. The light intensity of N2bv maximizes at an earlier point but declines at a faster rate as compared to the parental strain. Having the inducible character of luminescence established for N2bv, it can be postulated that the light is under control of the cell density dependant quorum sensing system. A study into the relationship between quorum sensing and growth documented that

presence of each of the three autoinducers (HAI-1, AI-2, CAI-1) found in *Vibrio harveyi* corresponds to a different stage of growth, and different levels of each of the auto-inducer can be inferred from the expression of bioluminescence of the bacterium with time (Anetzberger *et al*., 2012). Keeping in view, in absence of a growth curve data the lumine-scence expressed by N2wt and N2bv essentially corresponds to their respective stages of growth. It could be inferred that in comparison N2bv has a faster growth rate with a high expression of luminescence. It has been argued that brighter strains of luminous bacteria are slow growing as compared to their dark mutant counterparts owing to the energy expensive nature of bioluminescence production and an energy sink hypo-thesis has been put forward which describes a competition between energy utilization of growth and bio-luminescence production (Nackerdien *et al*., 2008). If this holds true then N2bv is an ambiguity that possesses a faster growth rate along with a brighter luminescence phenotype. It has been reported in various studies that mutants that yield a brighter than wild type luminescence are slow growing owing to the energy burden of high intensity luminescence (Bose *et al*., 2008; Lyell *et al*., 2010).

When total soluble protein extracts of N2wt and N2bv were compared, N2bv showed more protein levels indicating either production of inducible stress proteins or over expression of any of the proteins that would result in luminescence-up phenotype. The phenomenon of over expression could be a probable explanation as most of the bright mutants defined in literature harbor a *luxO* or *luxU* null mutation (Lenz *et al*., 2005) where the repression over *lux* operon expression is lifted and a constitutive bright phenotype is observed. However, as evident from studies of luminescence characteristics of N2bv the light is under inducible control rather than a cell density independent constitutive luminescence that results from null mutation of the repressor elements of the quorum controlled *lux* system.

The PAGE gel obtained indicated that a clear difference was present in the protein profiles of the N2wt and the N2bv, proving that a change in the general cell physiology had occurred in the strain under experimental conditions. The banding pattern of N2bv shows presence of few proteins that are not visible in the N2wt profile, indicating a probable production of stress proteins, a fact previously explained in recent literature (Ron, 2013; Foster, 2005; Boor, 2006). Most of the protein bands for N2bv are observed to be denser than their parental counterparts, these dense protein bands could either be indicative of over expression of protein that results in a brighter phenotype or a combination of different proteins of which some could be parental proteins while others may be stress induced proteins.

The present study is an initial investigation into the exciting phenomenon of spontaneous bright variants that occur in lab culturing and requires further experimentation towards identifying the proteins that exhibited a difference between N2wt and N2bv through protein sequencing analysis. The genetic elements involved in the bright variant phenotype also requires a detailed consideration

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