Study of cellular location preferences of *Salmonella typhimurium* along with gene expression analysis

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***Abstract*— Salmonella enterica serovar typhimurium a hyper-replicating population, exists inside the cytoplasm of infected epithelial cells. This population expresses genes belonging to the Salmonella Pathogenicity Island - 1 (SPI-1) after exiting from the Salmonella Containing Vacuole (SCV) (Knodler, et al., 2010) (Beuzon, et al., 2002).**

SCV provides a protective niche to Salmonella typhimurium and the reasons for its escape into the cytoplasm are not known. We wanted to isolate cytoplasmic as well as SCV contained Salmonella typhimurium, so as to understand their cellular location preferences and to study the gene expression and regulation. In order to achieve this, we wanted to make use of the fact that vacuolar bacteria have their Salmonella Pathogenicity Island - 2 (SPI-2) genes turned on, while those that exit from the vacuole switch off this system and express SPI-1 genes(Knodler, et al., 2010).

Thus, a time based expression tracking of SPI-1 and SPI-2 in epithelial Salmonella typhimurium would help us to determine their cellular location and using fluorescent reporters as trackers they would be separated with the help of FACS (Fluorescence Activated Cell Sorting). This gave rise to the objective of my work: Creation of a molecular tool to study temporal gene expression in bacteria.

***Keywords*—** *SCV- Salmonella Containing Vacuole, T3SS- Type Three Secretory System, Tsp- Tail Specific Potease, SPI- Salmonella Pathogenicity Island, RFP- Red Fluorescent Protein, HBI 4 - (p-hydroxybenzylidene) imidazolidin-5-one*

I. INTRODUCTION

A. Salmonella: An Overview

Salmonella is a genus belonging to the family of enterobacteriaceae. It consists of Gram-negative bacilli of about 0.7-1.5µm in diameter and 2-5µm in length. This genus comprises of non-spore forming bacteria which are facultative anaerobes.

B. Clinical Implications

Salmonella infection mainly results in two forms of diseased conditions: Typhoid (enteric) Fever and Gastroenteritis. Focal infections of the vasculature (endocarditis), bone (osteomyelitis), joints (arthritis) and many other organs may occur, but this is mostly seen in immune compromised individuals. Enteric fever is caused by *S.enterica* ssp. *enterica* serovars Typhi and Paratyphi that demonstrate strict human specificity. Other serotypes are associated with specific animal populations, such as *S.enterica* ssp. *enterica* serovar Dublin (cattle) and *S.enterica* ssp. *enterica* serovar Choleraesuis (swine), but may also cause disease in humans. The two predominant agents of human gastroenteritis, *S. typhimurium* and *S. enteritidis,* infect a wide range of zoonotic hosts, including poultry, cattle, sheep, pigs, horses, rodents and primates (Groisman, 2001).

C. Entry Into Host Cell

Salmonella infects a variety of host cells including macrophages, dendritic cells, neutrophils, epithelial cells, enterocytes, etc. It enters the human host via gastro-intestinal route and crosses the epithelial layer in the small intestinal region, to avoid encounter with IgA and antimicrobial peptides in the intestinal lumen. Salmonella prefers entry into the Microfold (M) cells which are involved in the sampling of intestinal antigens. (Haraga, et al., 2008)

Through the M cells, Salmonella gains access to underlying Peyer's Patches which are rich in lymphocytes. Sensing the phagocytic environment, Salmonella (Typhi/Paratyphi) mediates its own uptake by macrophages and then gains access to other organs of the reticuloendothelial system. This is the mode of infection in enteric fever, where Salmonella causes a systemic infection.

For entry into non-phagocytic epithelial cells, Salmonella makes use of the Type Three Secretory System (T3SS) encoded in SPI-1. SPI-1 effectors SopB, SopE and SopE2 activate host cell Rho GTPases (GTP: Guanosine triphosphate) - Cdc42, Rac1 and RhoG (Hardt, et al., 1998). They cause endoskeletal re-organization and membrane ruffling which leads to bacterial uptake by macropinocytosis. SopE and SopE2 activate the GTPases by acting as Guanine Nucleotide Exchange Factors (GEF) for the three aforementioned Rho GTPases (Stender, et al., 2000) (Friebel, et al., 2001). SopB is known to activate only Cdc42 and RhoG, indirectly, through its phosphoinositide phosphatase activity (Patel & Galan, 2006). Other effectors also involved in entry process are SipA and SipC. They mediate uptake by causing host cell membrane's actin remodeling (Zhou, et al., 2001). (Haraga, et al., 2008).

D. Salmonella Containing Vacuole (SCV)

After *Salmonella* has entered the epithelial cells, some of its effector proteins return epithelial cell membrane back to the normal state (without ruffles). SptP which acts on Rho

GTPases - Cdc42 and Rac1, is one such protein. SspH I and SptP proteins act to bring down the host cell innate immune responses generated during the invasion process.

Salmonella undergoes a lot of surface remodeling so as to shut down markers responsible for its recognition by immune system, which also includes the SPI-1 genes. (Haraga, et al., 2008)

At first, when it enters the host cells, (both macrophages and epithelial cells), *Salmonella* is surrounded by an envelope called as the spacious phagosome. The phagosome then shrinks in size and gets acidified within some hours to give rise to a vacuole called as SCV. A lot of changes take place in bacterial cell membrane during its stay inside SCV, which help it to adjust to the acidic environment. Inside SCV, *Salmonella* secretes different SPI-2 effectors like SifA, SseG, SseF, SopD2, SseJ, etc. The secretion of SPI-2 effectors by *Salmonella* is necessary for the placement of SCV in a juxtanuclear region and also the formation of *Salmonella* induced filaments; which are tubule like structures spreading from the SCV surface.

E. Exit From The Cell

Even though majority of *Salmonella* exist inside SCV, there have been reports of their existence in the cytoplasm of epithelial cell. Cytoplasmic existence would subject the bacteria to high oxidative stresses inside macrophages, but it has been shown to support their survival in epithelial cells (Beuzon, et al., 2002)(Knodler, et al., 2010)(Kale, et al., 2011). It has been proposed that the cytoplasmic presence of *Salmonella* might lead to the apoptosis of these epithelial cells, leading to their removal from the layer, and thus the dissemination of *Salmonella* in the intestinal lumen (Knodler, et al., 2010). Fluorescent Reporter Proteins

A reporter is a certain form of a signaling agent used in molecular biology studies to detect the presence of a molecule of interest like a gene product or to carry out gene expression studies. There are a number of reporters available, which include GUS, *lacZ*, *lux*, Chloramphenicol Acyl Transferase, etc. Fluorescent reporter proteins are also being increasingly used in molecular biology applications due to the ease of detection. In my project, I have made use of two fluorescent proteins namely GFPmut3b (mutant of Green Fluorescent Protein (GFP))(Andersen, et al., 1998) and mCherry as reporters. Their sources and properties are mentioned in the forthcoming paragraphs.

F. Green Fluorescent Protein

Green Fluorescent Protein is a 238 amino acid long protein first isolated from jellyfish *Aequorea victoria*. It exhibits bright green fluorescence on being exposed to light in the blue to UV range (Tsien, 1998). The GFP from *A. victoria* has a major excitation peak at a wavelength of 395 nm and a minor one at 475 nm. Its emission peak is at 509 nm (Prendergast & Mann, 1978)(Tsien, 1998).

G. GFP Fluorescence Mechanism

The chromophore of GFP has three important residues known to play a role in its fluorescence namely Ser65, Tyr66 and Gly67. In nature, Aequorin on interaction with calcium ions emits light in the blue color range. These waves then excite the electron dipoles of GFP which has absorbance maxima at 460nm. This energy transfer which takes place via Fluorescence Resonance Energy Transfer mechanism leads to the emission of green colored fluorescence by GFP (Cormack, et al., 1995).

H. Mutant Forms of GFP

Engineered mutations in the sequence of GFP have lead to the creation of its many different forms. These forms possess different properties like different excitation maxima, different emission maxima, different stabilities, different fluorescence intensities, etc. Some of the variants of GFP include Yellow Fluorescent Protein, Cyan Fluorescent Protein, Blue Fluorescent Protein, and Enhanced Green Fluorescent Protein. Each of these has a different emission range due to which they appear differently colored (Labas, et al., 2002). The wild type GFP has excitation maxima at 395nm, but for application in FACS, which has a blue laser exciting at 488nm; mutants which can be suitably excited at 488nm have also been created. Mutants of GFP like GFPmut1, GFP mut2, GFP mut3, GFP mut3b, etc, have been created, which have increased fluorescence intensities as compared to their wild type counterpart (Crameri, et al., 1995).

I. Red Fluorescent Protein (RFP) and Derivatives

In addition to GFP variants, scientists were trying to find out fluorescent protein molecules having an emission range different from that of GFP. This was to increase the number of factors that can be monitored at one time. The search led to the discovery of DsRed, which had its excitation and emission peaks at 558 nm and 583 nm respectively (Baird, et al., 2000). However, DsRed being a tetramer took a long time to mature and its maturation seemed to be a hindrance for the expression of a protein tagged to its N terminus (Shaner, et al., 2004). Thus, mutation studies were carried on to overcome this problem. mRFP1 was the first mutant monomeric form of DsRed which showed faster maturation and an extension of DsRed's peak excitation and emission by 25 nm (Bevis & Glick, 2002) (Campbell, et al., 2002) (Shaner, et al., 2004).
A further mutation study by Shaner, et.al., (Shaner, et al., 2004) to reduce the sensitivity of mRFP1 to N-terminal fusions and improve its brightness led to the creation of mCherry. The chromophore of mCherry is N-[(E)-(5-hydroxy-1*H*-imidazol-2yl) methylidene] acetamide. The photoactivated chromophore acts as an oxidant to release a carbon dioxide molecule from Glu-215 of mCherry. The final effect of this decarboxylation is the extension of the π-conjugation between the phenolic ring of Tyr-67, the imidazolone, and the N-acylimine. This leads to the formation of mCherry's red fluorescent chromophore.(Subach, et al., 2009)

The key residues involved in mCherry fluorescence are Met66, Tyr67 and Gly68 (Shaner, et al., 2004). mCherry has been widely used in the fields of molecular and cell biology due to its improved properties over the parent.

II. REVIEW AND LITERATURE

Fluorescent reporters have revolutionized the fields of molecular and cell biology. They have been used successfully as gene fusions in gene expression studies (Hautefort, et al., 2003) and protein fusions in protein based studies (Gordon, et al., 2000). Fluorescent reporters tagged to antibodies are being largely used in microscopic studies as well as single cell based analysis techniques like FACS (Hautefort, et al., 2003). Although traditional *lacZ* (Mason, et al., 1988) and *lux* (Kragelund, et al., 1995) gene based reporter systems have proven to be efficient, they face a number of shortcomings.

They can be used for assaying uniform cell suspensions, where distribution of substrates required for the assay system is even. However, in a heterogeneous system, such an assay will not be accurate. These assays are invasive in nature and represent the bacterial population as a whole and not at single cell level. Fluorescent reporters help to overcome these problems, as they allow both in-situ and single cell analysis. Their variety in terms of color makes them a suitable choice as reporter system for studying multiple genes at one time.

However, when expressed inside cells, fluorescent reporters like GFP have been shown to be highly stable, having a half life of more than a day (Andersen, et al., 1998). Thus, a temporal gene expression study would not be possible using them, as they might lead to false positive signals. Andersen, et al., (Andersen, et al., 1998) have shown that tagging fluorescent reporter GFPmut3b at its 'C' terminus with an eleven peptide tag having the consensus sequence 'AANDENYALAA', subjects it to the action of certain tail specific proteases present inside bacterial cells, and results in its faster degradation (Keiler & Sauer, 1996) (Andersen, et al., 1998). This C-terminus tag is denoted by the last three amino acids present in its consensus sequence, example 'LAA' tag. Tags varying at last three amino acids namely LVA, AAV and ASV had been attached by Andersen, et al.,(Andersen, et al., 1998) to the C terminus of GFPmut3b, and they have shown that depending on the variant attached, the degradation rate of GFPmut3b varies, the lowest half life being that of the LAA and LVA tags (40 min for each) (Andersen, et al., 1998). The AAV and ASV tags reduce the half life of GFPmut3b to 60 min and 110 min respectively(Andersen, et al., 1998). Thus, this system of 'peptide tag recognizing' cellular proteases was made use of in my study to achieve a time based gene expression study.

A. SsrA – SmpB System

In eubacteria, there exists an mRNA (mRNA: Messenger Ribonucleic Acid) molecule termed as SsrA (Small Stable RNA A), which shows structural similarity to alanine specific t-RNA. This mRNA has been shown to have a role in the degradation of aberrant proteins inside the bacterial cell by causing co-translational addition of a non polar peptide tag to the 'C' terminus of such proteins. The consensus sequence of the non polar peptide tag, AANDENYALAA, is probably recognized by certain proteases present in the bacterial cytoplasm, namely ClpAP, ClpXP and FtsH or by periplasmic Tail Specific Protease-Tsp (Prc), which then degrade the protein (Karzai, et al., 2000).

SsrA secondary structure shows regions similar to t-RNA like an amino acid acceptor region, T stem and a D loop (without stem). There are also two knot like regions which do not show similarity to t-RNA structure. One of these regions encodes the non polar degradation tag's sequence followed by a stop codon. SmpB (Small Protein B) is a protein molecule known to be involved in the attachment of SsrA to 70S subunit of the ribosome.

The ribosomes, on encountering an mRNA without a stop codon during translation, get stalled on the mRNA. The stalling of ribosomes occurs as the release of the translated peptide chain does not happen, unless a stop codon is encountered. This holds up the cellular ribosomes and there is also a risk associated with releasing an aberrant peptide into the cell cytoplasm.

This problem is overcome by SsrA mediation. When a ribosome is stalled on an mRNA, SsrA RNA attaches an alanine residue to the already constructed peptide chain, by a general trans-peptidation reaction. The SsrA RNA then encodes the rest of the peptide chain, attaching the signature 'C-terminal tag' to the peptide chain. The original mRNA is displaced in this process and thus the ribosomal machinery is now freed to translate other mRNAs (Keiler, et al., 1996) (Karzai, et al., 2000).

B. Dual Intracellular Lifestyle of Salmonella Typhimurium

Salmonella typhimurium upon entry into its host cell exists within the SCV. However, it has been reported that a hyper replicating population of this bacteria exists in the cytoplasm of epithelial cells, instead of residing in SCV (Beuzon, et al., 2002)(Knodler, et al., 2010) (Kale, et al., 2012). This population expresses SPI-1 encoded genes and shows the presence of flagella on its surface. Due to the expression of flagella, it has been postulated that the exit of *Salmonella* Typhimurium from the SCV is probably a step in its release into intestinal lumen. The cytoplasmic bacteria result in apoptosis of the infected epithelial cell and its release from the monolayer (Knodler, et al., 2010) However, this lifestyle of *Salmonella* Typhimurium significantly contributes to its intracellular replication (Kale, et al., 2012). The primary aim of starting this project was to study in detail this cytoplasmic class of intracellular bacteria.

As it is known that *Salmonella* Typhimurium inside the SCV switches on its SPI-2 genes, the bacteria which have been modified to contain a red fluorescent reporter fused to a SPI-2 promoter would give a red signal, upon entry into SCV and during their stay there. SPI-1 genes are switched on by *Salmonella* Typhimurium during its entry into non phagocytic epithelial cells. On entering the SCV, these genes are then switched off. SPI-1genes are again turned on when bacteria exit from SCV into cytoplasm (Knodler, et al., 2010).

Now, if *Salmonella* contains both a destabilized reporter like GFPmut3b LVA fused to a SPI-1 promoter and a destabilized reporter like mCherry-LVA fused to a SPI-2 promoter, then, inside the cells, bacteria giving a green signal after a significant time of infection represent the cytoplasmic population and those giving red signal represent the SCV population. Using these two signals in FACS, the two intracellular populations of *Salmonella* can thus be successfully separated.

Attaining this objective needed the creation of a molecular tool that would allow the temporal study of SPI-1 and SPI-2 genes, with different reporters, but together in the same bacterial cell. We thought that pZEP08 vector would prove to be a helpful tool as it has been already used to study gene expression in *Salmonella* Typhimurium by Hautefort, et al., (Hautefort, et al., 2003). Also, as maintenance of plasmid inside the cell might pose a problem in the study, we planned to modify pZEP08 so that it can be used to do a dual gene temporal study and then knock-in that part of the vector, into the *Salmonella* Typhimurium genome. The modification strategy of pZEP08 is mentioned in detail in the 'Cloning Strategy' section. We also had to validate whether the addition of the LVA degradation tag to fluorescent proteins GFPmut3b and mCherry resulted in their faster degradation. This was to be done using a time based degradation analysis of both of these proteins, compared to their untagged counterparts.

III. MATERIALS AND METHODS

A. *Materials: Vectors used in the study*

- pTrc99C: addgene
- pZEP08: A kind gift from Dr. Jay C.D. Hinton belonging to the School of Genetics and Microbiology, Trinity College, Ireland
- pJBA116: A kind gift from Dr. Soren Molin belonging to the Centre for Sytems Biology, Technical University of Denmark.
- pFPV-mCherry: addgene

B. *Strains of Cells*

- *E.coli* Dh5α cells
  Genotype: F– Φ80*lac*Z ΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1
- *Salmonella* enterica ssp. enterica serovar Typhimurium (ATCC: 14028)

C. *Media Components*

- Agar Powder: Himedia
- Calcium Chloride: MERCK
- Casein Enzyme Hydrolysate Type I: Himedia
- Dextrose: Ualigens
- Luria Broth (LB): Himedia
- Sodium Chloride: Ualigens
- Yeast Extract: Himedia
- Di-Sodium Hydrogen Phosphate: MERCK
- Potassium Di-Hydrogen Phosphate: MERCK
- Ammonium Chloride: MERCK
- Magnesium Sulfate: MERCK

D. *Other Experimental Reagents*

- Agarose: Himedia
- Sodium Hydroxide: PURE
- Sodiumdodecylsulfate: BDH Lab
- IPTG: Sigma
- Glycerol: SRL Chemicals
- Phenol Tris Saturated and Water Saturated: SRL Chemicals
- Isoporopanol: Fischer Scientific
- Ethanol: SRL Chemicals
- Lysozyme: Sigma
- Trizol: Ambion
- Chloroform: SRL Chemicals
- Ethylene-diamine-tetra-acetic-acid (EDTA): Sigma

E. *Antibiotics*

- Ampicillin (Stock: 100mg/ml, Working Concentration: 100µg/ml): Ranbaxy
- Chloramphenicol (Stock: 40mg/ml, Working Concentration: 20µg/ml): Himedia
- Kanamycin (Stock: 100mg/ml, Working Concentration: 50µg/ml): Himedia

F. *PCR Reagents*

- Deoxyribonucleotide triphosphate (dNTPs) (10 mM): New England Biolabs
- Primers (10 µM Working Stock): EuroFins and IDT
- DyNazyme^{TM} (Taq Deoxyribonucleic acid (DNA) Polymerase)
- DyNazyme^{TM} buffer (10X)
- MilliQ^{TM} Water
- Random Hexamers(0.2µg/µl): Fermentas

G. *Enzymes and Buffers*

- *NcoI* (10U/µl) : Fermentas
- *XbaI* (10U/µl) : Fermentas
- *NotI* (10U/µl) : Fermentas
- *EcoRV* (10U/µl) : Fermentas
- *BamHI* (10U/µl) : Fermentas
- T4 DNA Ligase (2U/µl): New England Biolabs
- T4 DNA Ligase Buffer (10X): New England Biolabs
- 10X Tango Buffer: Fermentas (For double digestion with BamHI and XbaI , NcoI and XbaI and EcoRV and XbaI)
- 10X Buffer O: Fermentas (For double digestion of *NotI and XbaI*)
- DNase Enzyme (2U/µl): Fermentas
- 10X DNase Buffer: Fermentas
- Reverse Transcriptase (2U/µl): Fermentas
- 10X Reverse Transcriptase Buffer: Fermentas

H. *Methods: Preparation of LB Broth*

2 g of LB was dissolved in 100ml of Reverse Osmosis (RO) treated Water.

I. Preparation of LB Agar

Reagents were added in the following composition for 100 ml of LB Agar

- Casein Enzyme Hydrolysate Type I: 1g
- Sodium Chloride: 0.5g
- Yeast Extract: 0.5g
- Agar Powder: 1.5g

J. Preparation of 10X M9 Media (1000ml)

Reagents were added in the following composition for 100 ml of LB Agar

- Casein Enzyme Hydrolysate Type I: 1g
- Sodium Chloride: 0.5g
- Yeast Extract: 0.5g
- Agar Powder: 1.5g

K. Preparation of 10X M9 Media (1000ml)

Reagents were added in a Schott Duran bottle in the following amounts:

- Na_2HPO_4: 60g
- $KH_2PO_{4:}$ 30g
- NaCl: 5g
- NH_4Cl: 10g
- The pH was adjusted to 7.0-7.5 and the volume was made up to 1000ml with RO water.

This comprised the Salt Stock.

For 1 Litre of M9 Media the following reagents were added in the mentioned amounts:

- 10X Salt Stock: 100ml
- 1M $MgSO_{4:}$ 1ml (Autoclaved)
- 10mM $CaCl_2$: 10ml (Autoclaved)
- 20% Dextrose: 10ml (Filter Sterelized)
- RO Water: 879ml

L. Preparation of Reagents for Plasmid Isolation

Solution I: Reagents were added in the following proportion:

- 50mM Glucose
- 25mM Tris-Cl (pH 8.0)
- 10mM EDTA (pH 8.0)

They were then autoclaved and stored at 4 degrees.

Solution II (Prepared Fresh): Reagents were added in the following proportion:

- 0.2 N NaOH
- 1% w/v SDS

Solution III (For 100ml): Reagents were added in the following proportion:

- 5M Potassium Acetate: 60 ml
- Glacial Acetic Acid: 11.5ml
- RO Water: 28.5ml

Solution was made and stored at 4 degrees.

M. Plasmid Isolation By Alkaline Lysis Protocol

- Single colony of bacteria was inoculated in 5ml of LB broth (with suitable antibiotic) and incubated overnight at 37 degrees.
- The culture was poured in a 1.5ml microfuge tube and centrifuged at 12000 rpm for one minute. Supernatant was discarded. (Centrifugation was done in Kubota 3400)
- 200µl of Solution I was added to dissolve the pellet. The tube was then vortexed and kept on ice for 5min.
- 400µl of Solution II was added to the tube and the contents were mixed gently by inverting the tube several times. The tube was then kept on ice for 5min.
- 300µl of Solution III was added to the same tube and it was inverted several times. It was then kept on ice for 10 min.
- The tube was then centrifuged at 13000 rpm, 4 degrees for 10 min.
- The supernatant was transferred to another tube and added with 5µl of RNase. Tube was then incubated at 37 degrees for 2hrs.
- After incubation, an equal volume of Phenol:Chloroform (1:1) solution was added to the supernatant. The organic and aqueous phases were mixed gently and the tube was centrifuged at 12500rpm for 10min.
- To the supernatant equal volume of chloroform was added and mixed gently. Tube was then centrifuged at 13000rpm for 5min.
- The supernatant was transferred to a fresh tube and added with an equal volume of Isopropanol. The tube was incubated at - 20 degrees for 15min.
- The tube was then centrifuged at 4 degrees and 14000rpm for 15min.
- The supernatant was discarded and the pellet was added with 500µl of 70% Ethanol. It was then centrifuged at 13000 rpm for 5min.
- All the ethanol was removed and the tube was allowed to dry for 10min at room temperature.
- 30µl of MilliQ water was added to the tube and tube was gently vortexed to dissolve the plasmid in water.
- The plasmid was then run in a 0.8% Agarose Gel in an electrophoresis unit at 100V. It was then visualized under UV light in a FluorChem GelDoc. Quantification was done using NanoDrop™.

N. Restriction Digestion Protocol

The following reagents were added in a double digestion reaction for a 20µl reaction:

- DNA: 2µg
- Respective Enzymes: About 0.3µl for an enzyme concentration of 10U/µl.
- 1X Enzyme Buffer
- Remaining volume was made up with MilliQ™ water.

The reaction was carried out in 200µl maximum volume vials and incubated at 37 degrees for minimum 4 hrs. Elongated periods were used depending on the digestion status. The digested vector backbone was purified using HiPurA gel elution kit.

O. Ligation Protocol

This reaction was carried in a Thermo ligation bath for 12hrs at 16 degrees. The reagents were added in the following proportion for a 10µl reaction:

- Vector Backbone (Digested vector): 40ng
- Insert: Amount to be added was calculated as per the vector backbone size and amount in-order to maintain the vector to insert ratio as 1:3.
- T4 DNA Ligase: 1X Concentration
- T4 DNA Ligase Enzyme: 1U

P. Transformation Protocol

E.coli Dh5α cells were transformed with the ligation mixture in the following manner:

- The *E.coli* Dh5α competent cells (200µl in 25% Glycerol) were allowed to thaw on ice for 10 min. They were then added with the ligation mixture and kept on ice for 30min.
- The cells were then subjected to a heat shock at 42 degrees for 90 seconds and then kept on ice for 5 min. They were then added with 800µl of LB and allowed to grow at 37 degrees for 40 min.
- The cells were then centrifuged at 4700g for 10 min and then 900µl of LB supernatant was discarded. The cells were suspended in the remaining 100µl and plated onto LB agar plates with appropriate antibiotics to select the transformants. The plates were incubated for 12hrs at 37 degrees to allow the growth of transformants.

Q. Colony PCR Protocol and Restriction Confirmation

Putative transformant colonies were taken and dissolved in 10µl of MilliQ and used as DNA source for PCR reaction. PCR reaction mix (25µl) was made by using the below mentioned reagents in the given proportion:

- dNTP : 0.2mM
- Forward and Reverse Primers (each): 0.2µM
- Enzyme: 1U
- Enzyme Buffer: 1X
- DNA: 400ng
- Milli Q: To make up the volume.

The mixture was then processed through a PCR cycle with the following design:

- Initial Denaturation: 95 Degrees for 10 min. (Once)

The set of steps mentioned below were repeated for about 30 cycles.

- Denaturation: 95 degrees for 25 sec.
- Annealing: For 30 sec and a temperature characteristic of the annealing temperature of the primers.
- Extension: At 72 degrees and a time span depending on the product length.
- (The processivity of Taq polymerase is 1000bp/60sec)

Final Extension: 72 degrees for 10 min (Once)

The reaction tubes were then maintained at 4 degrees. The PCR processed mixtures were then loaded in an agarose gel (1%) and subjected to electrophoresis at 100V. The gel was visualized under UV light and a positive transformants were detected by the presence of a band of the desired product's size. The positive colonies were then screened by a restriction confirmation test, in which plasmid was extracted from them and subjected to restriction with the same restriction enzymes as used in the cloning procedure. The release of the desired insert of appropriate size confirms the transformation.

R. Fluorescent Reporter Degradation Study

- A colony of *E.coli* having pTrc99C with a fluorescent reporter, a colony of *E.coli* having pTrc99C with a fluorescent reporter tagged to LVA, and a colony of empty pTrc99C containing *E.coli* were inoculated in three different tubes with 5ml LB broth and incubated overnight at 37 degrees. Ampicillin (100µg/ ml) was added to all the three sets.
- The cultures were then sub-cultured into fresh LB (5ml) broth containing tubes in the ratio 1:100.
- The cells were allowed to grow till an OD of 1 at 600nm and then induced with 0.2mM IPTG for 3.5hrs.
- After the induction period, the cells were subjected to centrifugation at 4700g. The supernatants were discarded.
- The pellets were given a wash with M9 media, twice, by repeating the above step and using M9 media as suspension medium. They were finally suspended in 5ml of M9 media and allowed to grow at 37 degrees, in presence of Ampicillin (100µg/ml).
- Aliquots were taken out after 15min, 30min, 45min, 60min, 80min, 100min and 120min and they were subjected to a fluorimetric study. It involved exciting the aliquots at wavelengths characteristic of the fluorescent reporter protein and collecting emissions signals at its emission maxima.
- Plotting a graph of the fluorescent signals with time, one can determine the half life of the fluorescent reporter protein, and thus verify if there is any reduction in its half life due to LVA tag.

S. PCR Amplification of mCherry, mCherry LVA, GFPmut3b and GFP mut3b LVA

The design of PCR cycle and proportion of PCR reagents used for the 25µl reaction was the same as mentioned in section 4.2.9. For mCherry and mCherry LVA, pFPV-mCherry was used as the template. For GFPmut3b and GFP mut3b LVA, pJBA116 was used as the template. About five 25µl reactions were set up for each insert.

The primers were designed with the help of Sequence Manipulation Suite(Anon., 2000) and vector sequences form addgene(Anon., n.d.). Their annealing temperatures were also determined using Sequence Manipulation Suite(Anon., 2000).

The annealing temperature was found to be 55 degrees for mCherry and mCherry-LVA set and 58 degrees for GFPmut3b and GFP mut3b LVA set (experimental observation). The extension time was kept as 48 sec.

T. Purification of Inserts

The PCR amplified inserts were subjected to a purification process, as in section 4.2.5., after the RNase treatment of the supernatant obtained after Solution III treatment. The inserts were then suspended in 10µl of MilliQ^{TM} water, and quantified using NanoDrop^{TM}.

U. Restriction digestion of Inserts

The purified inserts were subjected to digestion with appropriate enzymes as mentioned previously in this section. The digested inserts were again purified and used for the ligation reaction, as mentioned previously in this section. They were then quantified using NanoDrop^{TM}.

V. RNA Isolation

RNA isolation was done specifically to detect whether transcription of mCherry LVA gene takes place after 3.5 hrs of 0.2mM IPTG induction (From cells transformed with pTrc99C containing mCherry LVA insert). The protocol used was as follows.

- The culture was taken in 1.5ml eppendorf tubes (RNase Free) and centrifuged at 5000rpm for 5min at 4 degrees (In Kubota 6500).
- The supernatant was discarded and 200µl of 20mg/ml of Lysozyme was added to the cells. The cells were mixed with Lysozyme by pipetting and then incubated at room temperature for 30min.
- 800µl of Trizol was then added to the cells and gently mixed.
- 200µl of chloroform was then added to the eppendorfs and then centrifuged at 13000rpm for 15min, at 4 degrees.
- The aqueous layer was aspirated out and added with an equal volume of Iso-propanol.
- The mixture was mixed properly and then subjected to centrifugation at 13000rpm for 30 min at 4 degrees.
- The supernatant was discarded and the pellet was added with 800µl of 70% ethanol.
- It was then subjected to centrifugation at 13000rpm for 10 min at 4 degrees.
- Supernatant was discarded and pellet was allowed to air dry.
- The pellet was suspended in 15µl of Diethyl Polycarbonate (DEPC) treated water and then quality of RNA was checked by running it on a 1.5% agarose gel in an electrophoresis unit at 100V.

The RNA was then subjected to DNase treatment by setting up a 20µl reaction containing

- RNA: 10µg
- DNase: 3µl
- DNase Buffer: 1X Concentration
- DEPC treated water was used to make up the volume.

W. cDNA (Complementary DNA) Synthesis protocol

Reagents were mixed in the following proportion:

- Random Hexamer Primers: 1 µl
- dNTP mix: 1 µl
- RNA: 10 µl
- This mixture was kept at 70 degrees for 10min.
- It was then transferred immediately to ice.
- 4µl of 5X Reverse Transcriptase Buffer was added to this mixture and the volume was made to 20µl with DEPC treated water.
- Mixture was then added with 0.1µl of Reverse Transcriptase enzyme and incubated at 40 degrees for 1hr and then at 65 degrees for 10 minutes.
- This lead to the formation of cDNA, which was stored at -20 degrees.

IV. RESULTS AND DISCUSSION

The results are divided into three groups, based on the three steps in my study. They are

- Cloning mCherry LVA and GFPmut3b LVA into pZEP08
- Cloning GFPmut3b and GFPmut3b LVA into pTrc99C for degradation study
- Cloning mCherry and mCherry LVA into pTrc99C for degradation study

A. Cloning mCherry LVA and GFPmut3b LVA into pZEP08

a) Isolation of Vectors

The different vectors to be used in the study were isolated and visualized using a 0.8% Agarose gel under UV.

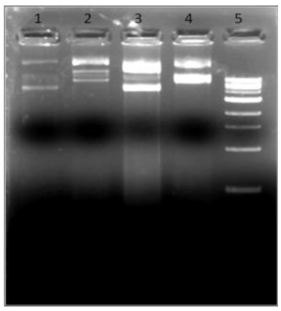

Fig. 1. Isolated Vectors

Lane 1: pFPV-mCherry, Lane 2: pJBA116, Lane3: pTrc99C, Lane 4: pZEP08, Lane 5: 1Kb Marker

b) Digestion of pZEP08

The next step in cloning procedure was to digest pZEP08 with *NotI* and *XbaI* enzymes, so as to remove the Kanamycin resistance cassette which would be replaced by mCherry LVA. The digested vector was visualized on a 1% Agarose gel under

UV and the backbone was extracted by gel elution procedure. The Gel Doc image of the digested vector is shown in Figure 2:

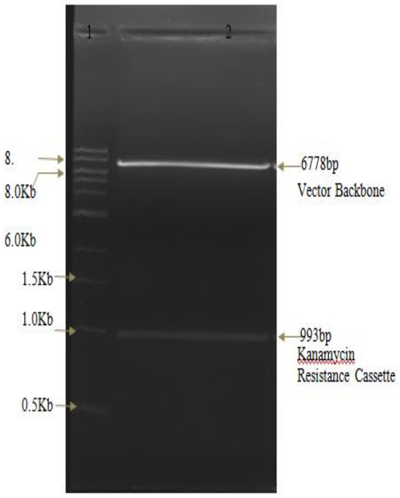

Fig. 2. pZEP08 digested with NotI and XbaI

Lane 1: 1Kb Marker, Lane 2: pZEP08 Digested with NotI and XbaI

c) PCR amplification of mCherry LVA and GFPmut3b LVA inserts

pFPV-mCherry was the template used to amplify mCherry LVA and pJBA116 was the template used to amplify GFPmut3b LVA. The inserts were purified, subjected to digestion with *NotI-XbaI* for mCherry LVA and *EcoRV- XbaI* for GFPmut3b LVA. The digested inserts were again purified and visualized under UV in a 1% Agarose gel, as shown in Figure 3.

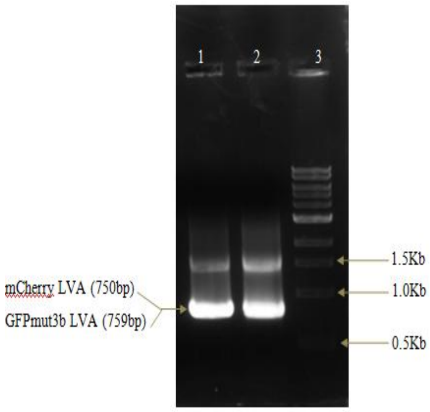

Fig. 3. mCherry LVA and GFPmut3b LVA Digested Inserts

Lane 1: mCherry LVA, Lane 2: GFPmut3b LVA, Lane 3: 1Kb Marker

The vector backbone of pZEP08 was ligated to mCherry-LVA insert. *E.coli* Dh5α cells were then transformed with this mixture. The cells were plated onto LB agar plates containing chloramphenicol and the colonies which grew after 12hrs of incubation were assessed by a colony PCR.

d) Colony PCR for cloning mCherry LVA into pZEP08

A single colony which grew on the LB agar plate containing chloramphenicol was tested for Colony PCR and it displayed a band corresponding to the size of mCherry LVA, as seen in Figure 4:

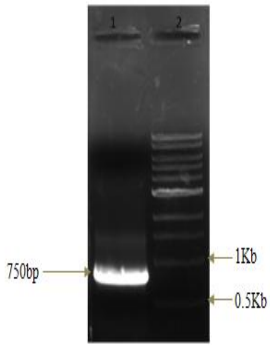

Fig. 4. Colony PCR

Lane1: Band of mCherry LVA cassette, Lane 2: 1Kb Marker

e) Restriction Confirmation

The pZEP08 vector contained in the putative transformant was isolated and its double digestion with *NotI* and *XbaI I* enzymes was carried out. The vector upon digestion released an insert of about 750bp size, corresponding to that of mCherry LVA cassette.

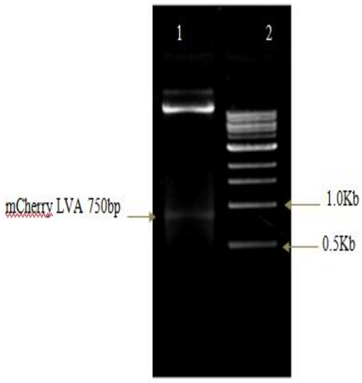

Fig. 5. Restriction Confirmation

Lane 1: mCherry LVA Insert Release, Lane 2: 1Kb Marker

The cloning of mCherry LVA into pZEP08 was thus completed successfully. The validation of this cassette would be done with help of a *HilA* promoter (SPI-1) which will be cloned upstream of mCherry LVA and an *SseJ* (SPI-2) promoter which will be cloned upstream of gfp^{+}. The whole cassette would be knocked into *S.enterica ssp. enterica* serovar Typhimurium strain 14028 genome and the expression would be monitored with the help of confocal microscopy.
The cloning of GFPmut3b LVA into the modified pZEP08 is also in progress.

B. Degradation study of GFPmut3b LVA

a) Digestion of pTrc99C

Vector pTrc99C was digested with *BamHI* and *XbaI* as shown in Figure 6. The digested backbone was then extracted by gel elution.

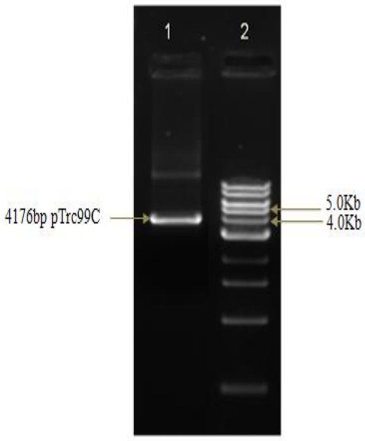

Fig. 6. pTrc99C Digested with BamHI and XbaI

Lane 1: Digested pTrc99C, Lane 2: 1Kb Marker

b) Obtaining GFPmut3b and GFPmut3b LVA inserts

GFPmut3b and GFPmut3b LVA inserts were obtained using vector pJBA116 as template. The PCR products were purified and subjected to digestion with *BamHI* and *XbaI*. They were again purified and ligated to the gel eluted vector backbone of pTrc99C. This mixture was used to transform *E.coli* Dh5α cells and they were plated onto LB agar containing ampicillin for selecting transformants.

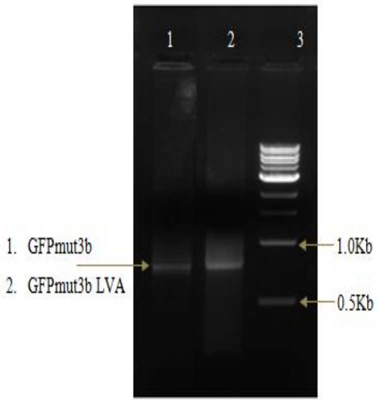

Fig. 7. GFPmut3b and GFPmut3b LVA Inserts

Lane1: GFPmut3b (720bp), Lane2: GFPmut3b LVA (750bp),

Lane 3: 1Kb Marker

c) Colony PCR for cloning GFP mut3b into pTrc99C

A colony PCR was performed to identify putative transformants. Results are depicted in Figure 8.

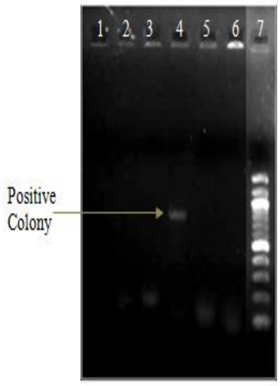

Fig. 8. Colony PCR

Lane 1- 6: Putative Clones, Lane 7: 100bp Marker

Lane 4: Putative Clone positive for the presence of GFPmut3b (720bp)

d) Restriction confirmation for cloning of GFPmut3b into pTrc99C

Vector pTrc99C was isolated from the colony showing a positive signal in colony PCR and subjected to digestion with *BamHI* and *XbaI*. The digestion resulted in the release of GFP mut3b insert from pTrc99C as seen in Figure 9. The cloning of GFPmut3b into pTrc99C was thus completed successfully.

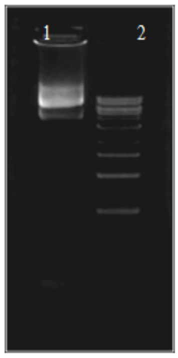

Fig. 9. Vector pTrc99C Isolated from Positive Colony

Lane 1: Isolated pTrc99C, Lane2: 1Kb Marker

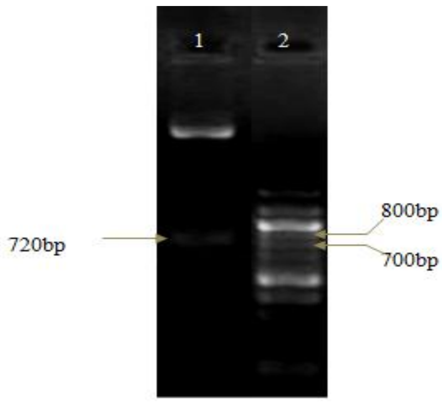

Fig. 10. Restriction Confirmation

Lane 1: Digested pTrc99C Showing Release of GFPmut3b (720bp)

Lane 2: 100bp Marker

e) Colony PCR for cloning GFPmut3b LVA into pTrc99C

A colony PCR was done to assess putative clones for cloning GFPmut3b LVA into pTrc99C. The result is shown in Figure 11.

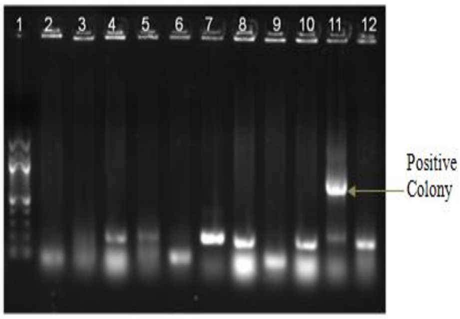

Fig. 11. Colony PCR

Lane 1: 100bp Marker, Lane 2-12: Putative Clones, Lane 11: Positive Colony

f) Restriction confirmation for cloning of GFPmut3b LVA into pTrc99C

Vector pTrc99C was isolated from the positive colony and was then subjected to digestion with BamHI and XbaI. The digestion resulted in the release of GFPmut3b LVA insert from pTrc99C as seen in Figure 12

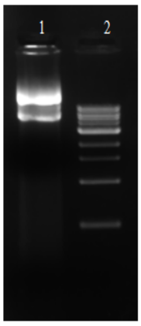

Fig. 12. Vector pTrc99C Isolated from Positive Colony.

Lane 1: Isolated Vector pTrc99C, Lane 2: 1Kb Marker

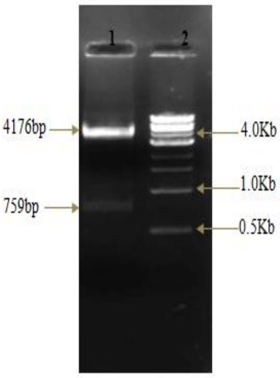

Fig. 13. Restriction Confirmation

Lane 1: Vector pTrc99C Showing Insert release of GFPmut3b LVA (759bp), Lane 2: 1Kb Marker

Thus, the cloning of GFPmut3b LVA into pTrc99C was successfully completed. The expression of GFPmut3b and GFPmut3b LVA after being induced by 0.2mM IPTG was confirmed by Fluorimetry. The degradation study GFPmut3b LVA is in progress.

g) Degradation study of mCherry LVA: Isolation of pTrc99C and its Digestion

Vector pTrc99C was isolated and digested with *NcoI* and *XbaI* as shown in Figure 14. The digested backbone was then extracted by gel elution and used for the ligation reaction.

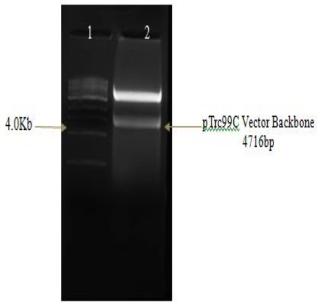

Fig. 14. Digestion of Vector pTrc99C

Lane 1: 1Kb Marker, Lane 2: Digested pTrc99C

h) Colony PCR for cloning mCherry LVA into pTrc99C

The vector backbone of pTrc99C was ligated with mCherry LVA (digested with NcoI and XbaI) and this mixture was used to transform E.coli Dh5α cells. The colonies were selected using ampicillin as selection marker. The putative clones were then assessed by Colony PCR. The results obtained are seen in Figure 15.

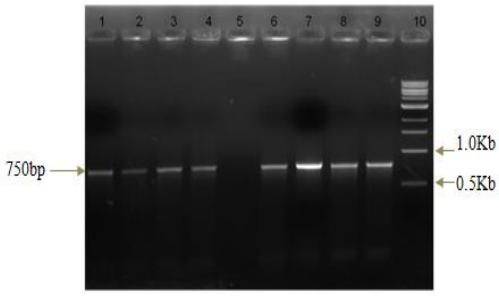

Fig. 15. Colony PCR

Lane 1-9: Putative Clones, Lane 10: 1 Kb Marker

i) Restriction confirmation for cloning of mCherry LVA into pTrc99C

Vector pTrc99C was isolated from positive colonies and subjected to digestion with *NcoI* and *XbaI*. The digestion resulted in the release of mCherry LVA insert from pTrc99C as seen in Figure 16.

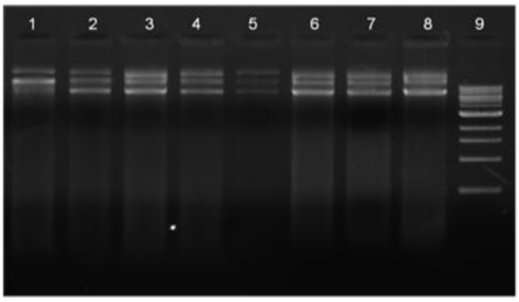

Fig. 16. Vector pTrc99C Isolated from Positive Colonies

Lane 1 - 8: Vector pTrc99C from Positive Colonies, Lane 9: 1Kb Marker

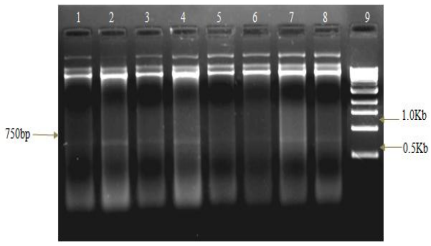

Fig. 17. Restriction Confirmation

Lane 1- 8: Digested pTrc99C showing mCherry LVA Insert Release (759bp), Lane 9: 1Kb Marker

The cloning of mCherry LVA into pTrc99C was thus successfully completed. However, the clones failed to show any expression of mCherry LVA upon IPTG induction. I therefore decided to obtain the sequence of the inserted fragment. The sequence obtained showed that the inserted fragment's sequence was considerably similar to mCherry LVA sequence. The analysis was done using BLAST program at NCBI website.

Score	Expect	Identities	Gaps	Strand	Frame
1260 bits(682)	0.0()	702/712(99%)	0/712(0%)	Plus/Plus	

Features:

```
Query  50   TGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCG  109
Sbjct  1    TGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCAAGATCGAGGGCG  60
Query  110  AGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTG  169
Sbjct  61   AGGGCGAGGGCCGCCCCTACGAGGGCACCCAAACCGCCAAGCTGAAGGTGACCAAGGGTG  120
Query  170  GCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCT  229
Sbjct  121  GCCCCCTGCCCTTCGCCGGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCT  180
Query  230  ACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCA  289
Sbjct  181  ACGTGAAGCACCCCGCCAACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCA  240
Query  290  AGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCT  349
Sbjct  241  AGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCT  300
Query  350  CCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCG  409
Sbjct  301  CCCTGCAGGACGGCAAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCG  360
Query  410  ACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACC  469
Sbjct  361  ACGGCCCCGTAATGCAAAAAAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACC  420
Query  470  CCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCC  529
Sbjct  421  CCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAAAGGCTGAAGCTGAAGGACGGCGGCC  480
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Sbjct  481  ACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCG  540
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Sbjct  601  AACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGA  660
Query  710  GGCCTGCAGCAAACGACGAAAACTACGCTTTAGTAGCTTAATTCTAGAATTA  761
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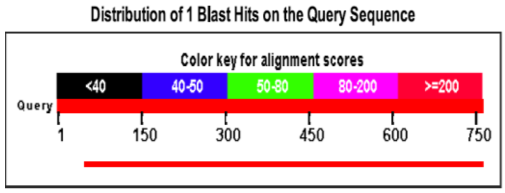
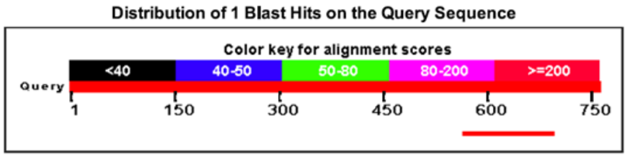


Fig. 18. BLAST Results Comparing Sequence from Forward Primer



Score	Expect	Identities	Gaps	Strand	Frame
231 bits(125)	1e-65()	129/131(98%)	0/131(0%)	Plus/Minus	

Features:

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Query  624  CCACAACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCAC  683
Sbjct  71   CCACAACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCAC  12
Query  684  CGGCGGCATGG  694
Sbjct  11   CGGCGGCATGG  1
```

Fig. 19. BLAST Results Comparing Sequence from Reverse Primer

As the sequence of the mCherry LVA inserted into pTrc99C was considerably similar to the originally planned construct, I speculated that there might be some hindrance occurring at transcriptional level. Thus, RNA was isolated from these clones after they were induced with 0.2mM IPTG for 3.5hrs. The results obtained in this study demonstrated that mCherry LVA was probably not getting transcribed in these cells, due to which there was no expression. The absence of transcription may be due to the formation of some DNA secondary structures, which would hinder transcription. The RNA was isolated and run on a 2% Agarose gel, whose Gel Doc image is shown in Figure 20.

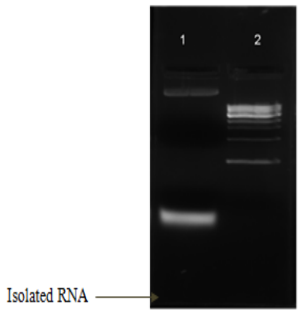

Fig. 20. RNA Isolation

Lane 1: Isolated RNA, Lane 2: 1Kb Marker

The RNA was then treated with DNase enzyme to convert it into cDNA. A PCR was then performed with primers specific to mCherry LVA and conditions similar to those used in its amplification, using cDNA as the template. Detection of the presence of 16sRNA by using its specific primers acted as a positive control. Figure 21 shows the PCR results. As seen, no amplification was obtained for mCherry LVA.

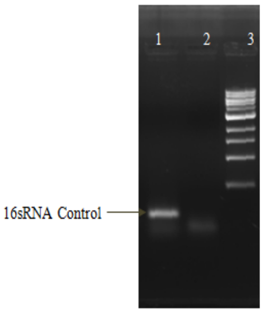

Fig. 21. PCR to Detect RNA Specific to mCherry LVA

Lane 1: 16sRNA control, Lane 2: mCherry LVA RNA Presence Test,

Lane 3: 1Kb marker

V. Acknowledgment

I would like to express my heartfelt thanks to Dr. J. Tamilselvan, Assistant professor, Department of Biotechnology, Anna University,chennai for his continuous guidance and suggestions throughout the course of the project. I wish to express my sincere gratitude to Mr. Senthil Raja, for the support he extended throughout the period of my project work.

I am also indebted to my family and friends for providing a constant motivation to approach this project as a service to society.

I would like to express my sincere thanks to Anand R.S, Dhasarathan G. for the valuable guidance that enabled me to learn the techniques. Specially, I m obliged to Mr.Arvind Mishra (Scientific officer in BARC Mumbai) for all their help, support, interest and timely suggestions whenever needed.

I cannot end without thanking my beloved parents and friends, who were there in difficult times motivating me to keep going. It is to them that I would like to dedicate this work.

Dinesh Kumar Singh

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