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Research Article

Development of a Cloning Vector for Biologicals and Therapeutics

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Abstract

Molecular cloning is a technique used to manipulate an organism's genome using biotechnology. This provides a way of overcoming barriers to gene transfer between species. The aim of this study was to remove a gene from one organism and transfer it to another so that the gene is expressed in the new host. A Nuclear inclusion (NIa) protease gene coding for an autocatalytic protease obtained from Cowpea aphid-borne mosaic virus (CABMV) was fused with the green fluorescence protein gene in a pUC57 plasmid. The fusion was then cloned into a pUC18 expression vector via the *Sma*I site. *Escherichia coli* (*E. coli*) NM522 cells were transformed by this recombinant vector. Protein expression was induced using Isopropyl β-D-thiogalactopyranoside (IPTG). The protein sample was analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis which showed that the protein was successfully expressed and cleaved out of the fusion vector system by the protease.

Keywords: Gene Cloning; Autocatalytic Protease; Cowpea Aphid-Borne Mosaic Virus; Protein Expression; Therapeutic

Introduction

Gene Cloning

Genetic engineering, also known as molecular cloning or gene technology, is a technique used to manipulate an organism's genome using biotechnology. This manipulation changes the genetic makeup of cells. The aim of genetic engineering is to remove a gene or set of genes from one organism and insert it into a foreign organism so that the gene of interest is expressed in the new host. Two types of gene cloning techniques are known, namely *in vivo* cloning and *in vitro* cloning. *In vivo* cloning involves excising a fragment of DNA using restriction enzymes and ligating it into a vector which can be amplified inside a foreign host cell. *In vitro* gene cloning involves the use of polymerase chain reaction (PCR) to generate copies of DNA fragments [1]. DNA of interest can be inserted into the host genome by isolating and copying the gene sequence of interest by molecular cloning a DNA sequence or synthesizing the DNA and then inserting the designed construct into the host genome. The altered DNA containing lengths of nucleotide from different organisms is called recombinant DNA (rDNA). The organism that expresses the recombinant DNA is termed a transgenic organism. Genetic engineering has been widely applied in fields such as research, medicine, industrial biotechnology and agriculture [2].

Gene technology has many benefits. This technique enables altering of an organism's genome at a much quicker rate than obtained by selective breeding. It is possible to transfer a gene or set of genes for the desired qualities between unrelated organisms that may be from different kingdoms. Furthermore, the gene transfer does not affect the bulk of the recipient's genetic makeup, enabling it to survive in its natural environment. Products specific to humans can be made using the technique of genetic engineering, enabling the removal of similar products obtained from other mammals for example, in the production of insulin by gene technology among other human proteins [3]. Another benefit of gene technology is for high throughput commercial production of enzymes which can be used in the food industry e.g. rennin; and in biological washing powders e.g. lipases and proteases. Recent developments could lead to the possibility of gene therapy in humans to treat genetic disorders such as cystic fibrosis. This can be achieved by inserting normal genes into patients with defective genes so that the protein required for normal functions is produced *in vivo* as would be the case in a person without the genetic disorder. Other proteins, albumin and vaccines can also be safely produced commercially using gene technology [4].

However, despite the above-mentioned benefits, gene technology has its share of potential hazards. Due to the genetic alterations, the transgenic organism produces many substances that are not normally produced in the unmodified organism. The greatest concern is that when transgenic organisms carrying genes for antibiotic-resistance come into contact with the wild-type organism, the recombinant gene may be transferred, adding to the problem of antibiotic-resistant pathogen strains. Up to date, there is little evidence of any debilitating adverse effects of transgenic organisms on the environment. However, these organisms are not readily accepted in some communities because they are seen as unnatural and intrinsically unsafe. Genetic engineering is a relatively new technology and as a result, public experience and knowledge of this development is minimal. This technique has developed rapidly in the scientific world, first being introduced in 1982 in the form of a genetically engineered animal and now transgenic organisms are standard research tools and also used in pharmaceutical production [5].

Vectors

In gene cloning, a vector is a molecule of DNA which is used as a vehicle for carrying foreign DNA into another host cell for amplification and/or expression. There are four main types of vectors namely, plasmids, lambda phage vectors (viral vectors), cosmids and expression vectors. Of the four, the most common vectors are plasmids.

A plasmid is a cloning vector which can carry approximately 10 kb of DNA. These cloning vectors vary their sizes with some extending beyond 200 kb in size. Plasmids are circular pieces of extrachromosomal DNA which are found in bacterial cells e.g. *Escherichia coli* (*E. coli*) [6], and sometimes in eukaryotic cells such as *Saccharomyces cerevisiae* [7]. Plasmid DNA is self-replicating and therefore independent of the host cell's replication system [8]. However, in some cases, the plasmid may depend on some host enzymes to catalyze its replication [9]. There are three common features to all engineered vectors: origin of replication, a multiple cloning site, and a selection marker.

Host Cells and Transformation

The recombinant DNA is introduced into a host cell by a process known as transformation. This foreign DNA is replicated along with the DNA of the host cell. Strains of *E. coli* are the most commonly used host cells due to their ease of transformation by plasmids [10]. Transformation is facilitated by divalent cations under cold conditions, making the host cell competent. The plasmids are then introduced into the host cell either by electroporation or chemical transformation methods [10]. Electroporation involves applying a brief electrical pulse to the solution of host cells and recombinant DNA fragments. In chemical transformation, the solution undergoes a short heat shock which induced uptake of DNA into the host cells.

Transformed cells can be distinguished from untransformed cells using different methods. One simple way is by using vectors with a selection marker for antibiotic resistance. As a result, only bacterium carrying the recombinant plasmid vector will grow on agar plates in the presence of the antibiotic.

Blue-white screening is a method that can be used to select for clones that have been transformed with vectors containing the desired insert. This screening is a non-destructive histochemical method used to detect enzymatic activity of β-galactosidase in transformed host cells [10]. The plasmid vector should contain unique restriction sites carrying a marker gene. Incorporation of DNA will lead to the disruption of the marker genes. The β-galactosidase will not be produced if the lacZ gene in the plasmid is disrupted. Plasmid without an insert contains an intact lacZ gene and therefore continues to produce the β-galactosidase enzyme [1]. A synthetic sugar, X-gal, is cleaved by β-galactosidase to produce galactose and glucose [11]. One of these sugar components is blue and as a result, colonies transformed by a vector without the insert will turn blue while colonies transformed by a vector containing the insert will remain white [1].

Gene cloning involves excising a specific gene from a larger chromosome and ligating it into a plasmid vector. The recombinant DNA is then transformed into host cells. The transformed host cells are grown in culture and the recombinant DNA molecule is replicated along with the host cell DNA. The cells are then grown on agar plates containing the appropriate antibiotic. To identify clones carrying the recombinant plasmid, with the insert of the gene of interest, colonies are picked and grown in liquid culture containing the appropriate antibiotic before the plasmid DNA is extracted and purified.

Cowpea Aphid-Borne Mosaic Virus Genome

Cowpea aphid-borne mosaic virus (CABMV) is a potyvirus from the family *Potyviridae* [12]. This virus has a wide host range but with distinct virus infection in cowpea. The genome of the Zimbabwe CABMV isolate was sequenced and found to be 9 465 nucleotides long with a single open reading frame. The open reading frame encodes a large polyprotein. This polyprotein can be auto-catalytically cleaved into 10 mature proteins as can be seen in figure 1 below.

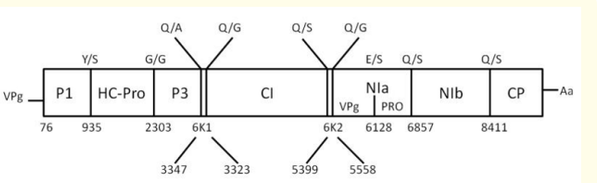

Figure 1: The proposed map of the CABMV genome. The bar represents the open reading frame. Cleavage sites are indicated by the vertical lines. Amino acids on either side of each cleavage site are shown above the vertical lines. Numbers below the map indicate the position of the first nucleotide of each gene. Ten mature proteins are predicted from the polyprotein, namely, N-terminal proteinase (P1), Helper component proteinase (HC-Pro), cofactor proteinase (P3), genome amplification protein (6K1), Cytoplasmic inclusion protein (CI), intracellular transport protein (6K2) Nuclear inclusion proteins a and b (NIa, NIb) and the coat protein (CP). VPg is the viral encoded protein. [13].

The NIa gene coding for protease activity is responsible for cleavage of the polyprotein into 10 smaller proteins [13].

Green Fluorescent Protein

Green fluorescent protein (GFP) is a 714-nucleotide long protein of size 26.9 kDa. This protein was originally isolated from the jellyfish *Aequorea victoria* [14]. The protein fluoresces bright green under ultraviolet light. GFP has two excitation peaks, a major peak at 395 nm and a minor peak at 475 nm. The emission peak for GFP is at 509 nm [15]. The fluorescent properties of GFP make it an essential tool in cell and molecular biology research as intracellular sensors and for visualising protein location. The protein can synthesise the fluorescent chromophore internally without any chemical reactions except for the use of molecular oxygen [16].

Several mutant forms of GFP have been engineered to adapt to the widespread and evolving needs in the scientific field of research. Some of these mutations include increased fluorescence, photo-stability and even mutations in the visible colour ranging from cyan blue to yellow, orange and even red. These fluorescent proteins find wide application as reporter genes in molecular biology research [17], fluorescence microscopy [18] and macrophotography [19].

The rationale behind this study was to develop a cloning vector which can be used to safely produce antibodies, vaccines, enzymes, pigments and other important substances in Zimbabwe by using genetic engineering. Some of these products are obtained from other countries and can prove to be uneconomical for routine use. Therefore, it is important to develop a less costly yet equally efficient method to produce these much-needed products locally.

Materials and Methods

Generating gene sequence

Three constructs were designed as indicated in the figures below.

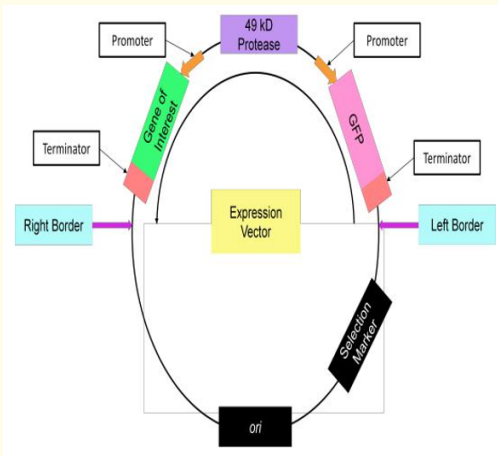

Figure 2: Plasmid construct, vector would contain the 49 kDa NIa protease gene, the gene of interest, reporter Green Fluorescent Protein each with their own promoter and terminator sequence and an antibiotic selection marker.

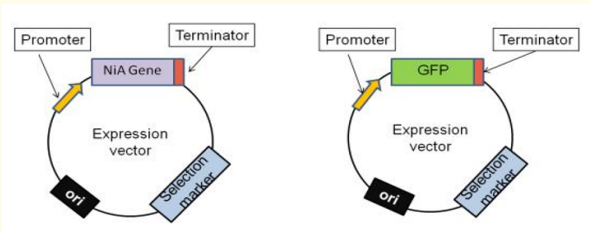

Figure 3: Two plasmids. One contains the 49 kDa NIa protease gene while the other plasmid contains the reporter GFP gene for sake of proving the concept. The GFP gene can be replaced by the gene of interest. In this setup, the protease would auto-catalytically cleave in trans.

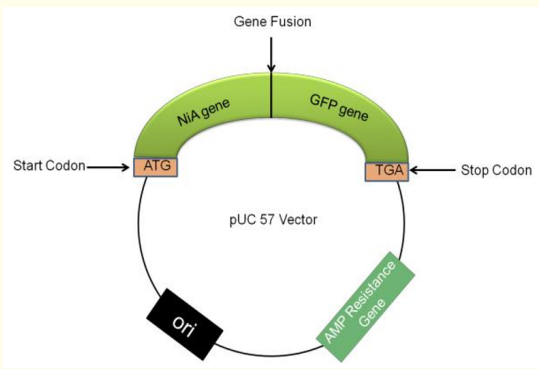

Figure 4: pUC57 plasmid vector containing a fusion of the protease gene and GFP reporter gene. The fusion was at a glutamic acid/serine amino acid sequence. The protease would auto-catalytically cleave and at the same time release one side of GFP. The stop codon in GFP would enable the whole GFP protein to be released.

We designed the gene fusion insert sequence and had it synthesized by gene synthesis (GeneWiz). The gene fusion insert was then cloned into pUC57 plasmid by GeneWiz.

Resuscitation of bacterial cells

The NM522 bacterial cells were rejuvenated on LB agar at 37^0C overnight. An NM522 cell culture was prepared by inoculating LB broth with NM522 colonies from what had been plated the previous day. The cell culture was incubated at 37^0C and shaking at 150 RPM. The cell culture was grown to mid-log phase at which the optical density at 660 nm was 0.4 units. At mid-log phase, cell growth was slowed down by placing the cell culture on ice.

Preparation of competent cells

The cell culture from the previous step was placed in cold falcon tubes and centrifuged at 3500 RPM for 10 minutes to collect the NM522 cells. The supernatant was discarded. The pellet was re-suspended in 8 ml of ice cold $CaCl_2$ by vortexing. The re-suspended mixture was centrifuged for 10 minutes and the $CaCl_2$ was discarded. The pellet was re-suspended again in ml of $CaCl_2$. To this re-suspended mixture, 1.6 ml of sterile 50 % glycerol was added and mixed thoroughly.

M13 PCR of pUC57-NIaGFP

M13 Polymerase chain reaction was used to amplify the fusion insert cloned in the pUC57 vector to screen for the insert size and to generate several copies of the fusion insert in the amplicons. The M13 forward primer sequence was 5'-(TGT AAA ACG ACG GCC AGT) - 3', and the sequence for the reverse primer was 5'-(CAG GAA ACA GCT ATG ACC) - 3'.

The PCR reaction was carried out in a total volume of 25 µl per reaction. The PCR reaction mixture contained 2.5 µl of 10X standard PCR buffer; 1.5 µl of 25 mM $MgCl_2$; 2.5 µl of 2 mM dNTPs; 1 µl of each of the forward and reverse primer; 15.25 µl of DNAse free PCR grade water; 0.25 µl of *Taq* DNA polymerase and 1 µl of the recombinant pUC57 plasmid of concentration 4 ng/µl.

The PCR reaction was run in an Applied Biosystems model 2720 thermal cycler under the following conditions for each cycle: pre-denaturation temperature of 94^0C for 5 minutes; denaturation at 94^0C for 30 seconds; primer annealing at a temperature of 56^0C for 30 seconds; elongation at 72^0C for 90 seconds and then final elongation for a further 5 minutes at 72^0C. The PCR reaction was run for 35 cycles. After the PCR was completed, 10 µl of the PCR product was run on a 0.8 % Agarose gel for 30 minutes and visualized in a UV transilluminator. The remaining PCR product was stored at -20^0C.

Plasmid digestion

The pUC18 plasmid was digested with *Sma*I restriction enzyme. The digestion was carried out in a 1.5 ml microcentrifuge tube. The digestion reaction mixture contained 17 µl of plasmid DNA, 2 µl of Tango buffer and 1 µl of *Sma*I (10 U/µl) restriction enzyme. Digestion was carried out at 25^0C for 2 hours.

The pUC57 plasmid containing the fusion insert was digested with the restriction enzyme *EcoR*V. The digestion was carried out in a 1.5 ml microcentrifuge tube. The digestion reaction mixture contained 68 µl of the pUC57+fusion M13 PCR product, 8 µl of the 10X enzyme buffer and 4 µl of *EcoR*V (10U/µl) restriction enzyme. Digestion was carried out in a 37^0C water bath for 2 hours

Gel purification

The pUC57 *Eco*RV digested product was run on a 0.8% Agarose gel against a λ/*Hind* III molecular weight marker for 30 minutes at a voltage of 150 V and current of 400 mA.

Purification of the fusion DNA was carried out using the Thermo Scientific Gene Jet gel extraction kit (CA, USA).

Ligation

The purified NIaGFP fusion was ligated into the *Sma*I-digested pUC18 plasmid. T4 DNA ligase enzyme was used to catalyse this process. The reaction mixture contained 9 µl of the purified fusion DNA; 8 µl of pUC18 plasmid digest; 2 µl of 10X enzyme buffer and 1 µl of T4 DNA ligase enzyme. The ligation mixture was left to stand at room temperature (≈ 25^0C) for 2 hours.

Transformation of competent cells

From the NM522 competent cells, two 200 µl aliquots were thawed on ice. One of the aliquots was labelled T (transformation tube) and the other C (control). To tube T, 5 µl of the pUC18+NIaGFP ligation was added and mixed gently. The tubes were incubated on ice for 30 minutes. The reaction tubes were then placed in a 42^0C water bath for exactly 90 seconds and then quickly transferred back to the ice and incubated for a further 2 minutes. The cells were transferred to two sterile tubes and 800 µl of LB broth was added to each tube without shaking. The tubes were incubated in a 37^0C water bath for 45 minutes. After incubation, the cells were plated in aliquots of 50 µl, 100 µl and 150 µl on LB agar plates containing 0.1 g/ml ampicillin and left to grow overnight at 37^0C.

The next day, cell cultures of the colonies of transformed cells were grown in LB broth containing ampicillin overnight at 37^0C with shaking at 150 RPM.

Protein Induction

In a one litre glass bottle, 250 ml of LB broth was prepared. The bottle was stoppered with a foam bung with a silicone tube passing through it. The silicone tubing was suspended above the level of the liquid media. The bottle, bung and tubing were wrapped in aluminium foil and autoclaved for 20 minutes. After autoclaving, the silicone tube was aseptically pushed so that the end of the tube was below the culture media surface. The sterile bottle was then equilibrated in a 37^0C water bath and the other end of the silicone tube was connected to a 0.2 µm ISOvent filter which was then connected to an air pump.

From the cell culture of NM522 transformed cells, 100 µl was aseptically pipetted into the sterile LB broth in the glass bottle. The culture was grown at 37^0C until turbid (when optical density at 600 nm = 0.6 - 0.8). The optical density was determined spectrophotometrically. At O.D of approximately 0.6, protein expression was induced by adding 50 µl of 1 M IPTG into the cell culture. The cell culture was left to grow overnight with bubbling. After 16 hours, the reaction was stopped by placing the cell culture at 4^0C.

The bacterial culture was the pelleted in 2 ml microcentrifuge tubes. The supernatant was discarded, and the pellet was re-suspended in 200 µl Tris-EDTA (TE) buffer of concentration 10 mmol. An aliquot of the cell suspension was viewed in the dark under UV against a tube of untransformed *E. coli* cell culture.

Analysis by SDS-PAGE

The mini vertical polyacrylamide gel electrophoresis apparatus was assembled according to the manufacturer's instructions.

The 8% separating gel solution was prepared as follows: to a 250 ml glass beaker, add 4 ml of 30% acrylamide-bis solution; 3.75 ml of 4X TrisCl-SDS at pH 8.8; 7.25 ml distilled water; 50 µl of 10% ammonium persulfate solution and 10 µl of TEMED. The mixture was swirled gently. The separating gel solution was carefully pipetted into the glass sandwich until the level was approximately 4 cm from the top. The top of the separating gel was covered with running gel overlay (1X TrisCl-SDS, pH 8.8 saturated with 2-butanol). The gel was left to polymerise for 60 minutes at room temperature. After 60 minutes, the running overlay was poured off and the gel was rinsed with 1X TrisCl-SDS, pH 8.8.

The 4.5% stacking gel was prepared as follows: to a 100 ml glass beaker, add 1.5 ml of 30% acrylamide-bis solution; 2.5 ml of 4X TrisCl-SDS at pH 6.8; 5.9 ml of distilled water; 90 µl of 10% ammonium persulfate solution and 10 µl of TEMED. The mixture was gently swirled and then pipetted over the separating gel in the glass sandwich mould until the solution was approximately 0.5 cm from the top of the plates. A 1 mm comb was inserted into the stacking gel solution. Additional stacking gel solution was added to fill the spaces in the comb completely. The stacking gel was left to polymerise for 30 minutes. After polymerisation, the upper buffer chamber containing the glass mould was placed into the lower buffer chamber and filled with 1X running buffer.

A 100 µl aliquot of the protein sample to be analysed was diluted with 2X sample buffer in a ratio of 1:1. The mixture was then divided into two separate 1.5 ml microcentrifuge tubes. The sample in one tube was boiled for 5 minutes at 100^0C. The samples were then loaded into the wells in the stacking gel against an untransformed *E. coli* cell culture sample as the control and a Pierce pre-stained protein marker from Thermo Scientific. The electrophoresis was run at 100 V until the samples reached the separating gel and then voltage was increased to 200 V and the gel was left to run for 120 minutes.

The gel was then removed from the running buffer. The glass plate sandwich was carefully disassembled. The gel was rinsed in distilled water three times for 15 minutes each. After rinsing the gel was stained for 60 minutes in EZ-Run stain containing Coomassie brilliant blue stain. The gel was then de-stained in distilled water three times for 10 minutes each. The gel was viewed on a white tile.

Results and Discussion

M13 PCR Product of pUC57-NIaGFP

The M13 PCR product was run on a 0.8 % Agarose gel and viewed using ethidium bromide stain under UV.

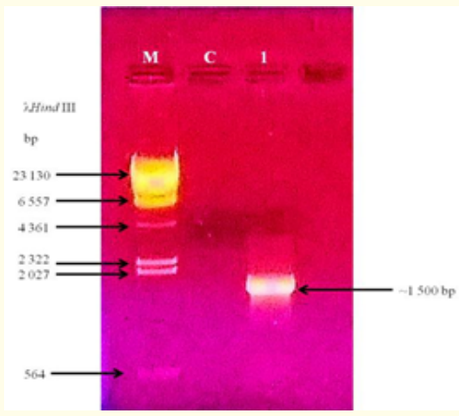

Figure 5: Agarose gel for M13 PCR. The well labelled M contained the molecular marker λHind III; well C contained the negative control so no bands were seen; well 1 contained the PCR product and the amplified NIaGFP fusion insert band can be seen at an approximate size of 1 500 base pairs.

Plasmid Digestion

The pUC57-NIaGFP plasmid was digested using the blunt end cutter EcoRV to cleave out the NIaGFP fusion insert of approximately 1500 base pairs. The pUC18 plasmid DNA was digested with SmaI blunt end cutter to give a linearised conformation of size 2690 base pairs. Aliquots of the digestion products were run on a 0.8% Agarose gel and visualised with ethidium bromide under UV.

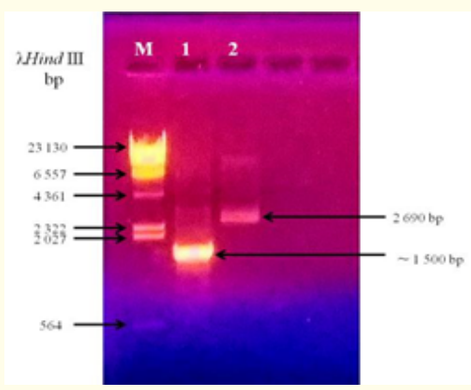

Figure 6: Agarose gel showing the digestion products. Well M contained the molecular weight marker λHind III; well 1 contained the pUC57-NIaGFP digestion product and an intense band of the fusion insert can be seen at ~1 500 base pairs and smears of the remaining digested plasmid can also be seen; well 2 contained thepUC18 digestion product and the linear form of the plasmid can be seen at 2 690 base pairs.

Gel Extraction and Insert Purification

The remaining pUC57-NIaGFP digestion product was run on a separate 0.8% Agarose gel. The bands obtained were cut out of the gel for DNA purification.

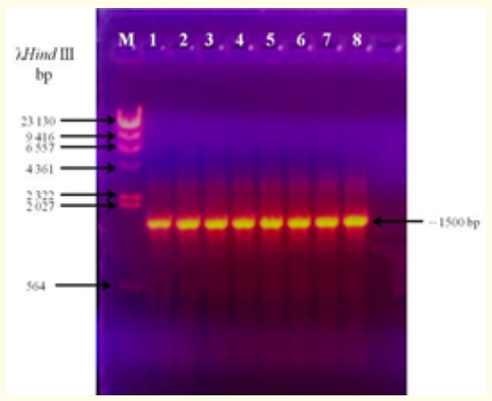

Figure 7: Agarose gel of pUC57-NIaGFP digestion using EcoRV. Well M contained the molecular weight marker λHind III; wells 1 to 8 contained the digestion product. Intense bands of the fusion insert that was cleaved out of the plasmid can be seen at ~1 500 base pairs. These intense bands were cut out for DNA purification of the NIaGFP fusion.

Cell Transformation

The competent NM522 cells were transformed using the recombinant pUC18 and fusion ligation. The transformed cells were grown overnight at 37^0C on LB-Amp agar plates. The control plate had no growth because the cells were not transformed and had no antibiotic resistance so they were unable to grow on the media inoculated with ampicillin. The plates with 50 µl and 100 µl of transformed cells had little bacterial growth but not distinct colonies. The plate with 150 µl of transformed cells had distinct bacterial colonies growing on it.

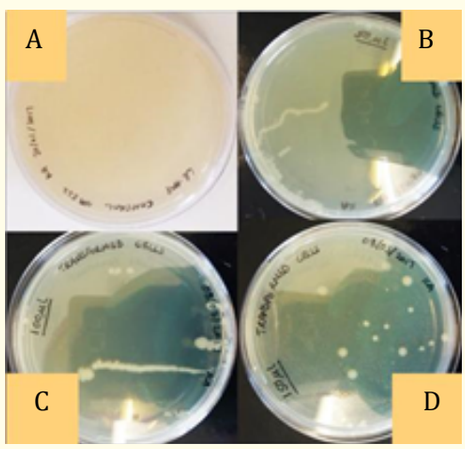

Figure 8: LB-Amp agar plates containing transformed NM522 cells grown overnight. Plate A was the control plate of untransformed NM522 competent cells. Plate B, C and D were plates of transformed cells containing 50 µl, 100µl and 150 µl respectively.

Fluorescence Visualisation

After protein induction with IPTG was complete, the cell culture was visualised under UV light in the dark. The tube containing the induced transformed cells produced fluorescence. The control tube containing untransformed E. coli cell culture did not show any fluorescence.

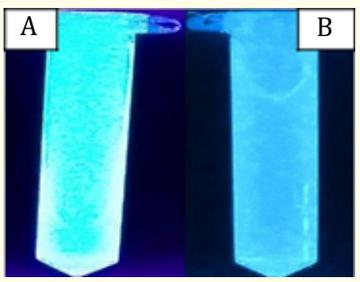

Figure 9: Microcentrifuge tubes containing cell cultures. Tube A contained the induced culture of transformed cells. Tube B contained untransformed cell culture working as a control.

Protein Analysis with SDS-PAGE

The pellet from the cell culture was run on a protein gel (SDS-PAGE). One sample was boiled and another sample was not boiled. Bands were obtained at ~ 49 kDa and at ~ 27 kDa showing expression of the protease and GFP protein respectively.

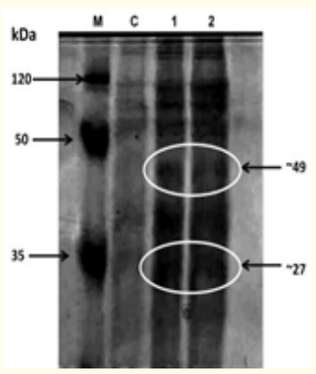

Figure 10: SDS-PAGE gel showing protein separation based on size and mass-to-charge ratio. Well M contained the Pierce pre -stained protein marker; well C contained boiled untransformed *E. coli* cell culture; well 1 contained boiled sample of transformed cells; well 2 contained an unboiled sample of transformed cells.

Discussion

Initially, three construct models were designed as illustrated in figures 2, 3 and 4. However, due to unavailability of adequate resources, only the plasmid vector containing the protease and GFP gene fusion was synthesised. The construct was designed with an ATG added to the beginning of the NIa gene serving as the start codon for translation of the protease. The GFP gene contained a stop codon since the whole GFP sequence had been synthesised into the fusion. As a result, when the NIa protease gene was translated into a functional protease, the protease auto-catalytically cleaved out of the vector system at the cleavage target where the amino acids glutamic acid and serine were next to each other. This cleavage also resulted in the release of the 5'- terminal of the GFP protein. Once the GFP translation was completed and the stop codon reached, the GFP protein was released from the vector system. One limitation with this design is that the footprint of the NIa gene remains on the protein of interest in the form of an extra amino acid, serine. In this study using GFP, the extra amino acid did not affect the function of the protein as can be seen by the presence of fluorescence in tube 'A' in figure 9. However this extra amino acid could affect function of other proteins expressed using this system [20]. Each of the two vectors will contain resistance to separate antibiotics. The advantage of using the second vector system design is that each time a new protein needs to be expressed, instead of synthesising a fusion gene, only the gene of interest needs to be synthesised on a vector. The vector containing the protease gene will already be in hand and just needs to be cloned in the laboratory. As a result, the cost is also reduced, making the in trans vector cleavage system more economical.

The synthesised gene fusion insert was placed in pUC57 plasmid vector in the *EcoR*V cloning site. Upon transformation and induction, the protein was not expressed. When the plasmid sequence was inspected, it was discovered that the sequence was out of frame in pUC57. As a result, another expression vector was necessary into which the fusion insert could be cloned. The plasmids available in the laboratory were pUC18 and pUC19 plasmids. Of these two vectors, pUC19 was also found to be out of frame. However, the sequence was in frame in pUC18. Due to the challenge faced with pUC57, it became necessary to cut the fusion out of pUC57 by running a single digest using *EcoR*V. This produced blunt end fragments. The pUC18 plasmid was also digested with the blunt-end cutter *Sma*I to linearise the plasmid as can be seen in figure 6. Purified gene fusion was then ligated into pUC18 using T4 DNA ligase. The pUC18 expression system was not the best as it is "leaky" [21]. However, at the time it was the only vector available. The vector can be replaced with a pMAL vector which gives a higher protein output [22].

DNA purification of the gene fusion was necessary because shot-gun cloning would not produce the necessary amount of ligation into the target pUC18 vector. In the gel extraction process, it was necessary to ensure that the size of the gel was minimized to remove extra agarose and make purification of the DNA fragments easier. After the gel was dissolved, the pH was adjusted to ≤ 7.5. This step was necessary because the adsorption of DNA to the membrane in the quickspin column is efficient only at pH ≤ 7.5. The addition of 1 volume of isopropanol increases the yield of DNA fragments. The second addition of binding buffer to the column membrane was to remove all traces of Agarose to ensure purity of the DNA for use in direct sequencing, microinjection or *in vitro* transcription. The elution buffer provided was at pH 8.5. The reason for this pH value was because elution efficiency depends on the pH. Maximum elution is achieved at pH values between 7.0 and 8.5 [23]. When the transformed cells were grown overnight on LB-Amp agar plates, the plate on which the 150 µl

aliquot was grown produced distinct bacterial colonies as seen in plate D in figure 8. The colonies from this plate were used to proceed to growing the cell culture and inducing protein expression.

In the pUC18 expression vector system, the GFP gene was under the control of the lac operon. Isopropyl β-D-thiogalactopyranoside (IPTG) is a molecular analogue of allolactose which is responsible for triggering lac operon transcription. Induction with IPTG is therefore used when working with recombinant protein expression. The cell is unable to metabolize IPTG due to the chemical bond formed by its sulphur atom [24]. As a result, the concentration of IPTG remained the same despite the 16 hour long induction period and the expression of protein genes controlled by the lac operon were not inhibited in the duration of the experiment. When the cell culture containing the protein, sample was analysed using SDS-PAGE, bands were observed as can be seen in figure 10. The band at 49 kDa indicated the protease which was expressed by the NIa gene. The 27 kDa band indicated expression of GFP. The presence of both bands indicated that the experiment worked, and the protease cleaved out of the vector system and also cleaved out the GFP protein. Furthermore, presence of fluorescence as seen in tube A in figure 9 also indicates successful expression of GFP.

The successful expression of GFP in this system enables us to explore expression of other proteins using this cloning and expression vector system. A potential protein would be an enzyme for example xylanase. Sequences of xylan-degrading enzymes have been isolated from microorganisms such as bacteria and fungi and expressed in *E. coli* [25]. During the research period, *B. subtilis* was grown and its genomic DNA was isolated. Xylanase primers were designed and synthesised. A xylanase PCR was performed on the *B. subtilis* DNA. From the results obtained for the PCR reaction, *B. subtilis* was found to contain the xylanase gene. The next step would have been to sequence this xylanase gene and then clone it into this vector system for enzyme expression. However due to lack of adequate funds at the time, the xylanase gene was not sequenced. When funds are available, this would be a good place to begin cloning of enzyme genes into this vector system.

Conclusion

In conclusion we have constructed a vector that is capable of producing a fusion protein that can be cleaved into individual protein components that are functional as predicted. Furthermore, this vector facilitates purification of the expressed proteins downstream without a yield penalty once it is isolated as opposed to expression systems that require cleavage of the protein after purification. It is envisaged that the vector will be used to express proteins and other biological molecules of diverse functions.

Bibliography

1. Passarge E. "Color Atlas of Genetics". Thieme, Stuttgart (2001).
2. Thyer R and Ellefson J. "Synthetic biology: New letters for life's alphabet". *Nature* 509.7500 (2014): 291-292.
3. Goeddel D., *et al*. "Expression in Escherichia coli of chemically synthesized genes for human insulin". *Proceedings of the National Academy of Sciences of the United States of America* 76.1 (1979): 106-110.
4. Andrianantoandro E., *et al*. "Synthetic biology: new engineering rules for an emerging discipline". *Molecular Systems Biology* 2 (2006): 246.
5. Capecchi MR. "Generating mice with targeted mutations". *Nature Medicine* 7.10 (2001): 1086-1090.
6. Alberts B., *et al*. "Molecular Biology of the Cell". 4th Edition. Garland Science (2002): 212-214.
7. Brown T. "Vectors for gene cloning: Plasmids and Bacteriophages". *Gene Cloning and DNA Analysis* 6 (2010): 730.
8. McPherson MJ and Moller SG. "PCR: The basics from background to bench". Bios Scientific Publishers Ltd. (2000).
9. Ellwell LP and Shipley PL. "Plasmid mediated factors associated with virulence of bacteria to animals". *Annual Microbiology Review* 34 (1980): 465-496.
10. Sambrook J and Russell DW. "Molecular Cloning: A Laboratory manual". Cold Spring Harbour Laboratory Press (2001).
11. Nelson DC and Cox MM. "Lehninger Principles of Biochemistry". Worth, New York (2002).
12. Goldbach R. "Molecular evolution of plant RNA viruses". *Annual Review of Phytopathology* 24 (1986): 289-310.
13. Mlotshwa S., *et al*. "The helper component proteinase of cowpea aphid-borne mosaic virus". *Archives of Virology* 5 (2000): 283-297.
14. Ormo M., *et al*. "Crystal structure of the Aequorea victoria green fluorescent protein". *Science* 273.5280 (1996): 1392-1395.
15. Prendergast FG and Mann KG. "Chemical and physical properties of aequorin and the green fluorescent protein isolated from Aequorea forskalea". *Biochemistry* 17.17 (1978): 3448-3453.
16. Stepanenko OV., *et al*. "Fluorescent proteins as biomarkers and biosensors: throwing colour lights on molecular and cellular processes". *Current Protein and Peptide Science* 9.4 (2008): 338-369.
17. Arun KH., *et al*. "Green fluorescent proteins in receptor research: an emerging tool for drug discovery". *Journal of Pharmacological and Toxicological Methods* 51.1 (2005): 1-23.
18. Yuste R. "Fluorescence microscopy today". *Nature Methods* 2.12 (2005): 902-904.
19. Baker SS., *et al*. "An epifluorescent attachment improves whole plant digital photography of Arabidopsis thaliana expressing red-shifted green fluorescent protein". *AoB Plants* 1.3 (2012): 853-859.
20. Goda S., *et al*. "Effect of extra N-terminal residues on the stability and folding of human lysozyme expressed in Pichia pastoris". *Protein Engineering, Design and Selection* 13.4 (2000): 299-307.
21. Mullerller-Hill B., *et al*. "Mutants that make more lac repressor". *Proceedings of the National Academy of Sciences of the United States of America* 59.4 (1968): 1259-1264.
22. Riggs P. "Expression and purification of recombinant proteins by fusion to maltose-binding protein". *Molecular Biotechnology* 15.1 (2000): 31-63.
23. QIAquick Spin Handbook (2001): 23-24.
24. Marbach A and Bettenbrock K. "Lac operon induction in Escherichia coli: systematic comparison of IPTG and TMG induction and influence of the transacetylase LacA". *Journal of Biotechnology* 157.1 (2012): 82-88.
25. Asadi-Farsani K., *et al*. "Cloning and sequencing of xylanase gene from Bacillus subtilis". *Research Opinions in Animal and Veterinary Sciences* 5.5 (2015): 215-218.

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