Subcloning of the Bacteriophage B3 terminase gene into pUCP20

A senior project by

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February 2002

Abstract

The purpose of this project was to clone the terminase gene from bacteriophage B3 into pUCP20 in order to express functional wild-type terminase protein. The known terminase gene sequence from the B3 genome was amplified using terminase specific primers and cloned into pCR2.1 (Invitrogen). Following an *EcoRI* digestion of the pCR2.1 terminase gene clones, the terminase gene was subcloned into pUCP20. The correct orientation of the terminase gene within pUCP20 was mandatory for proper expression of the terminase protein. The correct alignment was determined by using *Sal I* digestion of pUCP20 terminase vector followed by confirmation with agarose gel electrophoresis. Proper orientation of the terminase gene into pUCP20 was further confirmed by genetic sequencing. Induction of the cloned pUCP20 vector in *E.coli* was carried out using IPTG. A SDS-polyacrylamide gel analyzed the expression of the terminase protein.

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Phage packaging systems are a new tool for controlled delivery of desired DNA. The goal of this experiment was to create a phage packaging system from B3, a bacteriophage of *Pseudomonas aeruginosa* strain PAO1 (Holloway 1960). *Pseudomonas aeruginosa* was chosen for several reasons. It is a versatile organism and can survive in many environmental conditions. Another feature of *P. aeruginosa* is that the genome has been fully sequenced, which allows for the complete access of the genetic code for investigations into PAO1 (Stover 2000). Finally, multiple plasmids have been constructed which are stable in *E.coli* as well as *P.aeruginosa*. This allows for bacterial cloning and direct screening of recombinants in *P. aeruginosa* (Schweizer 1991).

According to Schweizer (1991) pUCP20 is an ideal vector because it has a *P. aeruginosa* origin of replication, a multiple cloning site, an easily detectable reporter gene, and primer sites suitable for sequencing. The plasmid pUCP20 contains a PAO1 origin of replication to maintain itself within a *Pseudomonas* cell. Additionally, pUCP20 has a multiple cloning site (MCS) that contains multiple restriction sites for dozens of enzymes useful for cloning. Transformation success was easily detected with blue and white screening due to the *lac Z* gene. Lastly, pUCP20 has M13 forward and reverse priming sites that are necessary for the genetic sequencing.

The function of the terminase protein is also important for the production of a phage packaging system. In the bacteriophage Mu, and presumably in B3, the terminase protein is responsible for cleaving one length of the phage genome and packaging it into phage capsids.

Bacteriophage B3 displays similarities to the bacteriophage Mu in *Escherichia coli* (Krylov 1980). The B3 virus displays random genome integration patterns (Houde 2000). Bacteriophage B3 has a terminase-like protein that functions to cleave and package headfuls of phage DNA. Likewise, bacteriophage Mu is a transposable phage and inserts itself into the host genome at various locations (Madigan 1997). Also, Mu shows terminase protein assistance in packaging measured lengths of phage DNA into capsids (Madigan 1997).

In this experiment, the terminase gene of the B3 phage was cloned into pUCP20, a plasmid stable in *E.coli* and PAO1. The terminase gene was recently sequenced and

identified (Braid 2000). The B3 open reading frame 31 was identified as the terminase gene by homology with bacteriophage Mu. The gene was primed, amplified, cloned and transformed into competent *E.coli*. In theory, the cloned terminase gene would be capable of producing functional wild-type terminase protein.

Previously, a *P. aeruginosa* double recombinant mutant was generated to facilitate the construction of a phage packaging system. After recombination of the viral DNA with the PAO1 genome the terminase gene was knocked-out through the use of a transposon mutation. Ultimately, this generated a recombined *P. aeruginosa* cell that yielded viral particles that were not viable—due to the production of defunct terminase protein.

To aid in the production of a phage packaging system an anti-sense repressor vector was cloned using pUCP20 and an anti-sense repressor sequence useful for binding and inhibiting the repressor sequence. As a result, the vector was expected to control the lytic state of the recombined B3 phage within PAO1.

The present research was performed using pUCP20 that contained the wild-type terminase gene. This project presented conclusive evidence that the terminase gene from the B3 bacteriophage was properly cloned into the pUCP20, a plasmid with a PAO1 origin of replication. The next step in the creation of a phage packaging system will be to complement the recombined PAO1 mutant with the wild-type terminase expression vector and anti-sense repressor vector to generate a biological tool capable of controlled expression. More research needs to be done to determine length of DNA accepted by the virus as well as induction of anti-sense repression.

Materials and Methods

B3 Genome Extraction

A PAO1 culture, ATCC 15692, was inoculated and grown overnight in 10 ml of TSB (Tripticase Soy broth) at 37 °C in a shaker incubator at 250 rpm. After incubation, two samples were prepared with each containing a 300 µl aliquot of PAO1 mixed with a 100 µl aliquot of a 10^{-3} dilution B3 phage lysate and incubated for 30 minutes at 37 °C in sterile 15 ml test tubes. The two tubes were mixed with 5 ml of melted and cooled top

agar (tryptone 8 g/L, NaCl 5 g/L and agar 7.5 g/L). The solution was poured onto two separate TSA (Tripticase Soy Agar) plates and grown overnight (~22 hours) in a 37 °C incubator. After incubation, the TSA plates that contained plaques were used for B3 phage isolation, purification, and genome extraction.

The top agar from the plaque containing plates was scraped off with a sterile spatula and placed into two sterile 50-ml falcon tubes. The phage DNA was isolated by chloroform precipitation of DNA from top agar and cellular debris. Phage purification was performed by the addition of DNase (7.4 µg/µl) and RNase (10 mg/ml) to the solution in order to degrade PAO1 genomic DNA. Phage DNA extraction included the addition of lysis Buffer (100 mM tris pH 8.0, 0.3 M NaCl, 20 mM EDTA) and 10% SDS and proteinase K (20 mg/ml) with incubation at 37 °C for ~80 minutes. After incubation, a phenol: chlorophorm: isoamyl solution (25:24:1) solution was used to extract DNA followed by the addition of 100% ethanol to precipitate the phage DNA.

Amplification of the Terminase gene

The B3 terminase gene extends from 14788 bp to 16245 bp in the phage genome. Primer Select software was used to construct upper and lower primers for gene amplification (Lasergene Suite). The upper primer was composed of 23 bases: 5' TGGCGTCAGCGGCAAAGAACATC 3' and the lower primer which was a 25-mer: 5' CACCGCCGGCTTCCACCTTCCACTC 3'. The PCR mixture was composed of 5 µl 10X buffer, 4 µl dNTP's (10 mM), 5 µl BSA (20 µg/ml), 4 µl $MgCl_2$ (25 mM), 5 µl forward primer (100 nmol), 5 µl reverse primer (100 nmol), 1 µl *Taq* and 16 µl PCR water. The PCR conditions included: 95 °C for 60 seconds for molecular denaturation, 70 °C for 60 seconds for annealing of primers, and 72 °C for 60 seconds for primer elongation. The number of cycles was 40 and the reaction volume was 50 µl per sample. The amplification of the terminase gene was confirmed by performing a 1.0% agarose gel electrophoresis.

Enzyme Digestion

The two enzymes used for digestion were *EcoR1* and *Sal1*. The digestion procedure for each enzyme was a standard 20 µl reaction including 10 µl plasmid DNA

(100 ng/µl), 1 µl enzyme (1000 U/µl), 2 µl 10X buffer, and 7 µl water. The volume was then incubated in a water bath at 37 °C for 3 hours followed by 20 minutes at 65 °C. Once the incubation was complete, the digest results were analyzed using gel agarose electrophoresis.

Cloning

The terminase gene was cloned into pCR2.1 and transformed into TOP10F' *E.coli* cells following manufacturer's protocol (Invitrogen). Insertion of the cloned sequence was confirmed by a 1.0% agarose gel electrophoresis.

The terminase gene was later sub-cloned into pUCP20 following the cloning procedure provided by Invitrogen. The ligation reaction consisted of 1.5 µl digested pUCP20 DNA (35 ng/µl), 6 µl digested terminase DNA (18 ng/µl), 0.5 µl ATP, 1.0 µl T4 DNA ligase (Invitrogen), 1.0 µl 10X buffer (Invitrogen). The reaction was incubated overnight in a 2400 Applied Biosystems PCR instrument at 14 °C. Following overnight incubation the reaction was transformed into XL10-Gold Ultracompetent *E.coli* cells (Stratagene).

Gel Extraction

The terminase insert was isolated from the pCR2.1 by restriction digest using *EcoRI*. Two 45 µl samples were loaded into agarose gel wells along with 4 µl of digested sample and 4 µl 1Kb+ DNA linear standard. The samples were separated on a 1.0% agarose gel electrophoresis. The gel was then stained with ethidium bromide and prepared for UV light visualization. Parafilm was used to prevent UV light from mutating the DNA and a straight edge was used to remove the band at ~2000 bp from the gel. The recovered gel was melted and processed with a gel purification kit (UltraClean GelSpin by MoBio). The terminase gene, which contained *EcoRI* overhanging ends, was completely separated and isolated from the plasmid DNA after the gel extraction.

Sequencing

A Big Dye Terminator sequencing amplification was performed using the pUCP20 terminase clone. A 20 µl reaction volume was amplified. The reaction tube

consisted of 8 µl Big Dye dNTPs (Beckman Coulter DTCS), 4 µl M13 forward primer, and 8 µl DNA. A separate reaction tube had 8 µl Big Dye dNTPs (Beckman Coulter DTCS), 4 µl M13 reverse primer, and 8 µl DNA. Sequencing parameters included a 96 °C hold for two minutes followed by 40 cycles of the following series: 96 °C for 20 second, dropping to 50 °C for 20 seconds, and finally increasing to 60 °C for four minutes. After the sequencing event, 50 µl of cold 95% ethanol and 1 µl of sodium acetate (pH 4.6) were added to the sample. The sample was then incubated at –4 °C for 30 minutes, followed by centrifugation for 30 minutes at 3490 rpm to pellet the DNA. The ethanol was decanted and 100 µl of 70% cold ethanol was added to the sample. Next, the sample was centrifuged for 15 minutes at 3490 rpm. The ethanol was removed by decanting and the tubes were inverted onto a paper towel and centrifuged for 1 minute at 700 rpm to completely dry the DNA pellet. The sample was then loaded into the CEQ2000 (Applied Biosystems) for genetic analysis.

Induction of Terminase gene Expression in E.coli

E.coli was grown in 100 ml of LB broth containing ampicillin (150 µg/ml) at 37 °C in a 225 rpm shaker incubator for ~17 hrs. After incubation, the cells were washed three times with 0.85% saline. After the saline washes, the cells were centrifuged and the pellet was transferred to a 2.0 µl microfuge tube and sonicated to lyse the cells. The lysed cells were centrifuged at 55,000 rpm for one hour to separate cellular protein from cellular debris. A Bradford protein quantification assay using Bio-Rad protein assay solution was performed to quantify the protein solution in each sample. Samples were diluted to (0.5 mg/ml) and loaded into pre-cast 4-12% SDS-NuPage gel (Invitrogen) and run at 100 amps for 4.5 hours.

Two separate induction procedures were performed. The first required 50 µl of 1 M IPTG to be added before incubation of *E.coli* containing the pUCP20 clone. This sample was compared to two other samples; one containing transformed pUCP20 clone in *E.coli* without the presence of IPTG and the other was transformed pUCP20 without insert in *E.coli* and without the presence of IPTG.

The second induction protocol required 50 µl of 1 M IPTG to be added to transformed pUCP20 clones in *E.coli* before incubation and 1 hour after harvesting cells.

The sample was compared to three other samples: *E.coli* without pUCP20, *E.coli* with pUCP20 clone without IPTG, *E.coli* with pUCP20 without IPTG. All the samples were incubated for 17 hours at 225 rpm and 37 ºC.

Results

Amplification of the Terminase Gene

The terminase gene from bacteriophage B3 genome was amplified using terminase forward and reverse primers. A 0.8% agarose gel confirmed the presence of the amplified terminase gene after PCR. The expected band size was 1888 bp. Figure 1 displays the amplified terminase gene.

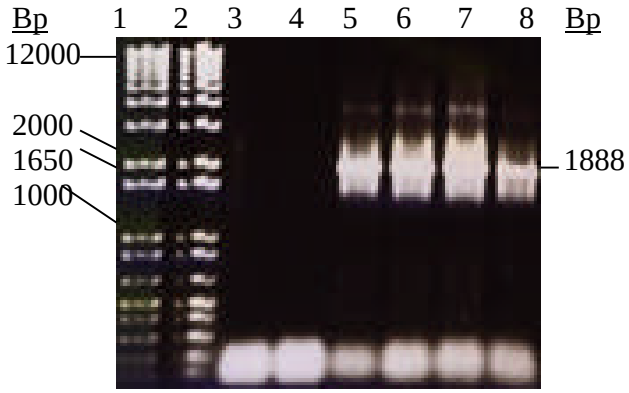


Figure 1. An agarose gel with 1 KB linear DNA ladder standard and terminase gene amplified product after staining with ethidium bromide and visualization with UV light. Lanes 3 and 4 indicate the negative open and closed controls.

Cloning

The amplified terminase gene was cloned into pCR2.1 and transformed into TOP10F' *E.coli* cells (Invitrogen). The plasmids were isolated and purified using the MoBio Miniplasmid Prep DNA purification kit. A 1.0% agarose gel electrophoresis confirmed successful cloning and transformation. The known size of the pCR2.1 was 3.9 Kb and the expected band size for pCR2.1 containing the terminase gene was ~6 Kbs.

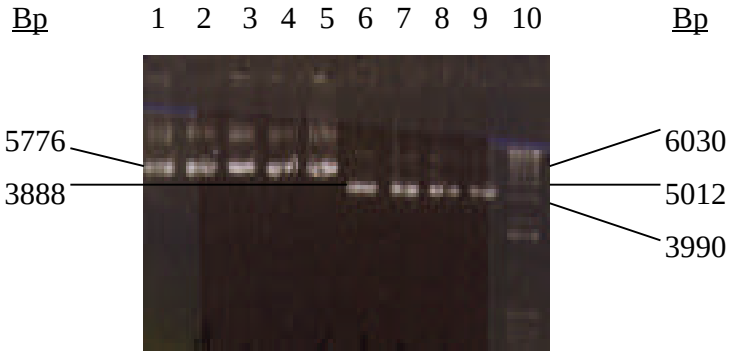


Figure 2. An agarose gel with 1 Kb DNA supercoiled ladder standard, isolated pCR2.1 clones and isolated pUCP20 without insert. Lanes 1-4 show the bands corresponding to the pCR2.1 with the insertion. The bands in lanes 5-6 correspond to the isolated pUCP20 without the insert.

After confirmation of the cloned pCR2.1, the plasmid was digested with *EcoRI* to separate the vector from the terminase sequence. The terminase gene was isolated from PCR2.1 by gel extraction and was sub-cloned into the plasmid pUCP20. After transformation, the plasmids were extracted and isolated. Figure 3 shows the gel results.

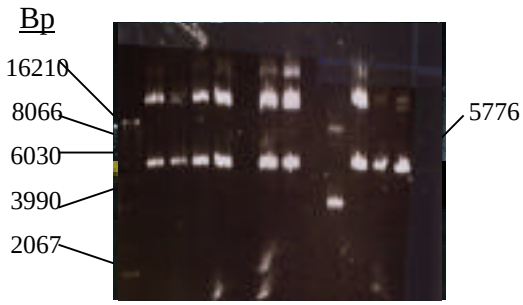


Figure 3. A 1.0% agarose gel with 1 Kb DNA supercoiled ladder and the thirteen isolated pUCP20 clones. Nine of the thirteen samples appeared to display a band corresponding to the predicted size of the cloned vector--5776 bp.

The nine cloned pUCP20 plasmids were screened for correct alignment of the terminase gene by *Sal 1* digestion and analyzed by a 1.0% agarose gel electrophoresis. The enzyme cleaved the sub-cloned pUCP20 at three locations. For correct gene orientation the expected sequence lengths were 4249 bp, 1061 bp, and 466 bp, whereas the expected lengths for incorrect orientation were 4959 bp, 466 bp, and 361 bp. Figure 4 shows a gel photo with plasmid banding patterns.

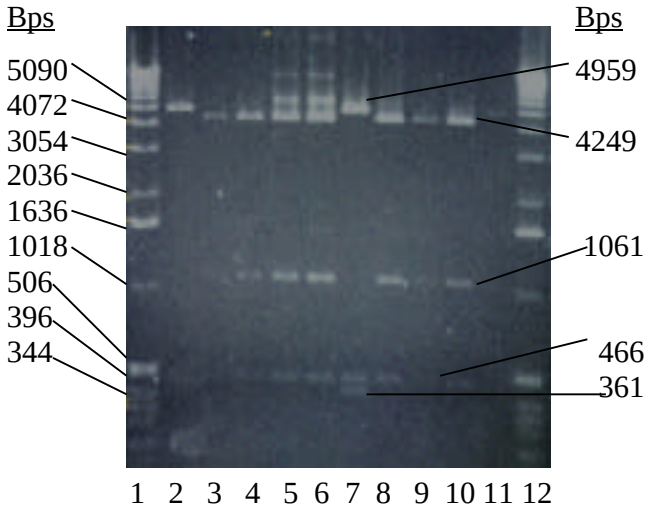


Figure 4. An agarose gel with 1 Kb+ DNA linear ladders standard and Sal I digestion of pUCP20 clones visualized by UV light. Lanes 4, 5, 9, 10, and 11 illustrate banding patterns similar to the expected sequence lengths with the terminase gene in correct orientation. However, the banding pattern shown in lane 7 indicated incorrect insert orientation.

Sequencing

Genetic sequencing of the terminase gene within the plasmid provided additional evidence that the gene was in the correct orientation. The terminase gene was sequenced using M13 forward and reverse primers.

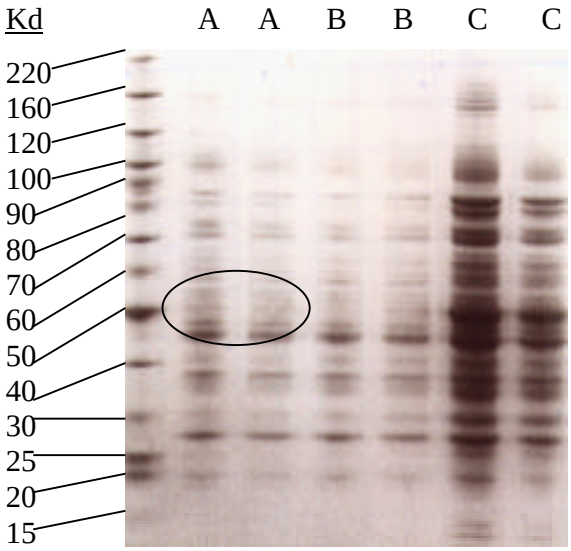
The resulting sequences using M13 forward and reverse primers were ~500 bp in length. The sequences were then compared to the terminase gene sequences from the B3 phage in the 5' to 3' direction. The forward and reverse sequence contigs were aligned appropriately with the terminase gene contig. Therefore, the genetic sequencing

confirmed that the terminase gene was in the correct orientation within pUCP20. Obtaining clones in the correct orientation was mandatory for the production of functional terminase gene protein.

SDS-PAGE

The terminase gene protein size was unknown. Therefore the gene sequence was analyzed by Seqman software (Lasergene Suite) and the molecular weight of the terminase protein determined to be 54.3 Kd.

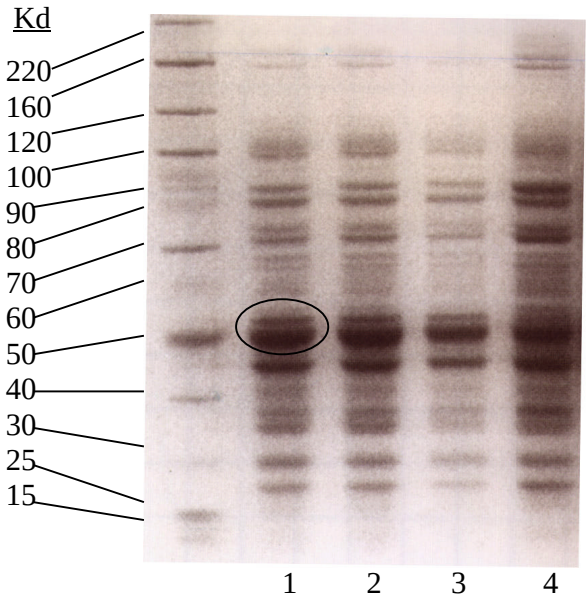
Two separate PAGE gels were performed with two separate protocols in order to verify expression of the terminase gene product. The first protocol required the addition of IPTG to one of the three samples of *E.coli* before incubation. The cells were grown overnight, lysed, and centrifuged. The supernant was loaded into a SDS-NuPage pre-cast gel. Figure 5 indicates the outcome.



Lane	Sample	IPTG
A	*E.coli* with pUCP20 clone	+
B	*E.coli* with pUCP20 clone	-
C	*E.coli* with pUCP20	-

Figure 5. A 4-12% SDS-NuPage gel with 220Kd protein ladder showing induction of the terminase gene in E.coli*. The expected size of the terminase protein band was 54.3 Kd and was expected to appear in Sample A.*

The second protocol required the addition of IPTG to the cloned pUCP20 within *E.coli* both before and one hour after harvesting the cells. Figure 6 shows banding.



Lane	Sample	IPTG
1	*E.coli* with pUCP20 clone	+
2	*E.coli*	-
3	*E.coli* with pUCP20 clone	-
4	*E.coli* with pUCP20	-

Figure 6. A SDS-NuPage gel with a 220 Kd protein standard shows induction of the terminase gene in E.coli. A band at 54.3 Kd was expected in Sample 1.

Discussion

The results indicated that the terminase gene from bacteriophage B3 genome was amplified and cloned into pCR2.1. Subsequent *EcoR1* digestion and gel extraction produced isolated terminase gene sequences. The terminase gene was successfully cloned into pUCP20 based on the results of the *Sal I* digested clones and genetic sequencing. The banding of the strain expressing terminase gene product was similar to strains that did not contain the terminase sequence. There are two possible explanations for this observation. First, the product could be produced but not visible as it was masked by a large band in all samples at ~50 Kd. Second, the protein might not have been produced at all.

Further studies are needed to confirm terminase gene expression. An experiment to prove expression would be to complement the recombined B3 terminase knock-out mutant with the terminase pUCP20 clone. Another would be to analyze the mRNA produced by the terminase pUCP20 clone using RT-PCR.

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