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The *dim2* ORF from *Peltigera membranacea*:

Amplification and expression cloning

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Spring 2010

Acknowledgement

I wish to express my gratitude to Professor Ólafur S. Andrésson for giving me the opportunity of performing this project in his group of work and also for his help and his quality instructions.

I want to say thank you to Sheeba Santhimi Manoharan for her intense work in the laboratory and for being always available to explain me everything, dedicating her time to me.

I wish also to thank Sunna Helgadóttir for her help in the experiments of ligation and transformation.

Thank you everyone for this fantastic semester in Iceland, it has been an authentic pleasure to meet you all.

Abstract

The objective of this project was to clone the *dim-2* ORF (open reading frame) from *Peltigera membranacea* and express it in *E. coli* in order to produce protein and try to purify it for its study.

The *dim-2* gene encodes a methyltransferase enzyme, responsible for DNA methylation.

The *dim-2* ORF was amplified by PCR from the genomic DNA of the lichen *P.membranacea*.

Two oligonucleotides with homology to the ends of the *dim-2* ORF were designed including suitable restriction targets (*NcoI* and *NotI*) for amplification and subcloning in a pET28a expression vector.

The resulting amplified fragment was purified from an agarose gel and treated with the restriction enzymes *NcoI* and *NotI*. The pET28a vector was cut with the same restriction enzymes.

Ligation reactions were performed in a vector insert ratio of 1:3 and transformed into competent *E.coli*, selecting of kanamacyn plates.

Among the positive transformants, sixteen were selected for PCR analysis using primers contained in the sequence of the insert. This screening was negative and therefore all 700 colonies were screened in 20 pools; again with negative results.

Since no pET28a vector with the *dim-2* ORF was recovered, it was not possible to carry out experiments for purification and characterization of the protein.

Table of Contents

1. Introduction

1.1. General aspects of *Peltigera membranacea*

The specie *Peltigera membranacea* is a lichen belonging to the family *Peltigeraceae*.
It is a symbiotic association between a fungus (mycobiont) and a cyanobacteria (photobiont) (1).

The genus *Peltigera* contains both bipartite (one mycobiont and one photobiont) and tripartite lichens (one mycobiont and two photobionts) (2).

Morphological characteristics

This lichen has grey lobes covered with dense white hairs on the lower side, called rhizines. Along the edge of the thallus, it has some orange-red structures, these are the fruiting bodies from the fungal portion of the lichen, which eventually release spores (2).

It can also propagate itself through breakage and distribution of a part of the thallus to the new environment.

Habitat

It grows on various substrates: mosses, rocks, soil, or bark, and mostly in the damp grassy vegetation (2).

1.2. Molecular Cloning

1.1.1. What is cloning? Definition and Steps

'Cloning a gene' means isolating an exact copy of a single gene from the entire genome of an organism (3).

The procedure for cloning a gene consists in the introduction of a single recombinant DNA molecule, composed of a vector plus an inserted DNA fragment (the gene), into a host cell.

The inserted DNA is reproduced along with the vector, producing large numbers of recombinant DNA molecules that include the fragment of DNA originally linked to the vector (4).

The steps involve in the molecular cloning are:

1. Fragmentation
2. Ligation
3. Transformation
4. Screening/selection

1) Fragmentation

The DNA fragment to be cloned is generated by using restriction endonucleases.

The same restriction enzyme must be used to cut both the vector and the DNA sample.

2) Ligation

The DNA fragment (gene) produced by digestion with restriction enzymes is ligated to the vector.

3) Transformation

The recombinant DNA molecule is transferred to a host cell. Within the host cell, the recombinant DNA molecules replicate producing dozens of identical copies known as clones.

4) Screening

As the host cells replicate, the recombinant DNA is passed on to all progeny cells, creating a population of identical cells, all carrying the cloned sequence.

Finally, the cloned DNA segments can be recovered from the host cell, purified and analyzed in various ways (5).

1.3. *dim-2* gene

The *dim-2* gene is the only eukaryotic gene currently known in which mutations appear to eliminate DNA methylation.

The *dim-2* gene has been studied in the fungus *Neurospora crassa* and it is known that encodes a 1454 amino acid protein including a C-terminal domain homologous to the known DNA methyltransferases (MTase) a novel N-terminal domain. This protein is responsible for all DNA methylation in vegetative tissues of Neurospora.(6)

DNA methylation

The methylation of DNA is an epigenetic process involved in the regulation of the gene expression in two ways, directly by preventing the binding of transcription factors, and indirectly by promoting the "closed structure" of the chromatin (7).

The enzyme involves in this process is a DNA methyltransferase which, during the replication of the DNA, add methyl groups to the 5 carbon of cytosines of the newly synthesized chain.

In eukaryotes, methylation is almost exclusively found at the C5 position of selected cytosines and in many organisms, nearly all 5mC is found in symmetrical sites, such as the sequence 5'CpG/3'GpC. This is thought to reflect the operation of a 'maintenance DNA methyltransferase (MTase)' that preferentially recognizes and methylates hemimethylated sites.
Methylation patterns are consistently maintained, but it is common to find cytosines that are 'partially' methylated.

It is also known that DNA methylation can indirectly interfere with transcription initiation or transcription elongation, and it is involved in some epigenetic phenomena, such as X chromosome inactivation, genomic imprinting and silencing of selfish DNA.

DNA methylation is required for normal development of both mammals and plants (6).

1.4. pET28a vector

1.4.1. What is a vector?

'Vector' is an agent that can carry a DNA fragment into a host cell.
If it is used for expressing certain gene in the DNA fragment it is called an 'expression vector'.

One of the most commonly used vectors is a plasmid.

Plasmid

Plasmids are circular, double-stranded DNA molecules that exist in bacteria and in the nuclei of some eukaryotic cells. They can replicate independently of the host cell (4).

The typical plasmid vector contains:

- an origin of replication (ORI): recognized by the host cell that allows its replication
- a selection marker: an antibiotic resistance gene to select the bacterias with the plasmid
- a multiple cloning site (the polylinker region): a number of unique target sites for the restriction endonucleases

1.4.2. pET28a vector (from *Novagen*)

pET28a is an expression vector which carries:

- T7 promoter
- T7 transcription start
- N-terminal and C-terminal His-Tag coding sequence
- T7-Tag coding sequence
- Multiple cloning sites
- *lacI* coding sequence
- kan coding sequence
- pBR322 origin
- f1 origin

pET28a plasmid is under the control of the strong bacteriophage T7 transcription and translation signals. The expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is so selective and active that almost all of the cell´s resources are converted to target gene expression.

The antibiotic *Kanamycin* inactivates translation by interfering with the ribosome function.

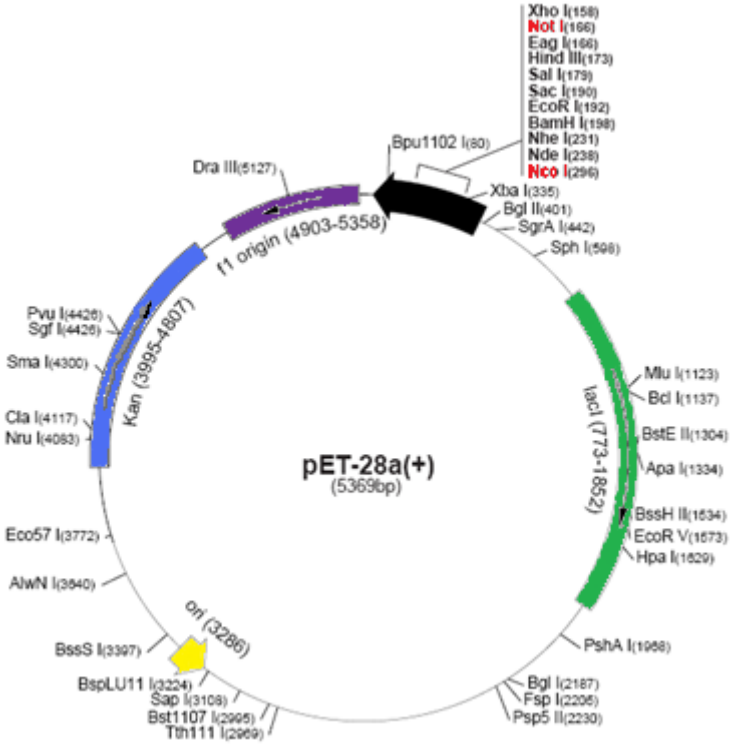

Figure 1: Cloning vector pET28a(+). Circular map where are shown the elements of the vector, including the polylinker region with the target sequences for the restriction enzymes used in this work in red color.

1.5. Restriction Endonucleases Enzymes

Restrictions enzymes are enzymes isolated from bacteria that recognize specific sequences in DNA and then cut the DNA to produce fragments, called restriction fragments.The play a very important role in the construction of recombinant DNA molecules (8).

The restriction enzymes used in this work were *NcoI* and *NotI*.

2. Aims

The *dim-2* gene has been studied before in the fungus *Neurospora crassa*.

In this project it was tried to study the protein encoded by the *dim-2* ORF in the lichen *Peltigera membranacea*.
It is known that the *dim-2* protein is a methyltransferase, which is involved in the process of DNA methylation.

To address this work, the following objectives were considered:

- Clone the *dim-2* ORF (open reading frame) in a pET28a expression vector
- Expression of the protein in *E.coli*
- Purification and study of the protein encoded by the *dim-2* ORF.

3. Materials and Methods

3.1. Lichen Sample: Processing and DNA Extraction

Genomic DNA from *Peltigera membranacea* thalli was extracted and used as a template for amplifying the *dim-2* ORF.

Thallus was directly collected from the field in Reykjavik (Iceland) and processed in the laboratory.

The processing consisted of several washes with deionized distilled water. All the small impurities were removed under a stereoscope using forceps and then the thallus was cut. Approximately 2 grams of dried cleaned thallus fragments were ground separately in a cold mortar with liquid nitrogen.

Finally, DNA was extracted from the resulting lichen powder (see protocol in Appendix).

3. 2. Primers

Specific primers were designed based on the annotated nucleotide sequence of the *dim-2* gene (Sheeba Santhini Manoharan, pers. comm.).

The sequences of the primers used in this work are shown in the table 1 below.

Primers **1F-*NotI*** and **1R-*NcoI*** were designed to amplify the coding region of *dim2* by PCR and to introduce the coding sequence into expression vector pET28a.

Primers **4F** and **1R-*NcoI*** were used to confirm the results of the transformation by PCR screening.

Primer	**Nucleotide Sequences (5´→ 3´)**
1F-NotI-pET28a-dim2	5'- GCCACGCCATGGCTCCTCATATGTTGTATCCC -3'
1R- NcoI-pET28a-dim2	5'- TATATAGCGGCCGCCGGCCTTGCATTCAGGAT - 3'
4F	5'- TAAATTGCCCAGCCCGAAGACGGGGATGGGT -3'

Table 1:Primers used in this project. The blue color shows the targets for restriction enzymes (*NotI* for the forward primer and *NcoI* for the reverse primer). The initiation and termination codons were deleted. The first six nucleotides were added to facilitate the recognition of the sequence by endonucleases.

3.3. DNA Amplification by Polymerase Chain Reaction (PCR)

The *dim-2* ORF was isolated and amplified using the PCR technique.

The pair of primers 1F-*NotI* and 1R-*NcoI*,containing the target sequences for the restriction endonucleases *NotI* and *NcoI*, were used.

PCR reactions were performed in a 50 µl mixture containing:

Total volume	50µl
Water,nuclease-free	26,4
10X Pfu Buffer without MgSO4	5
dNTP Mix (2mM each)	5
Forward Primer:1F-NcoI	1,5
Reverse Primer:1R-NotI	1,5
Template DNA T1 (1:10)	5
25mM MgSO4	5
Pfu DNA Polymerase(recombinant)	0,6

Table 2:Reagents used for amplification of the *dim-2* ORF from genomic DNA of *Peltigera membranacea*.

The program used for the amplification was:

Primers 1F_NcoI-1R_NotI	Temp, °C	Time	n°of cycles
Initial Denaturation	94	2min	1
Denaturation	94	20sec	25
Annealing	55	20sec	
Extension	72	07:10min	
Final Extension	72	10min	1
Holding	12	Infinity	

Table 3:Amplification conditions.

3µl of PCR product were loaded and separated by electrophoresis on a 1% agarose gel in order to confirm its size (~3350 bp). The resulting band was visualized under UV light.

3.4. Gel purification - Crystal violet

This technique is used to visualize and purify PCR products by agarose gel electrophoresis using crystal violet, avoiding ethidium and UV light damaging the DNA.

The remaining volume from a PCR amplification was run on a 0.8% agarose gel with a 0.6 µg/ml crystal violet and run at 80 volts.

The DNA band of interest, which was visible with a naked eye with the help of a visible light transilluminator, was excised from the gel with a razor blade and transferred to a microcentrifuge tube.

DNA was purified directly using the GenElute Gel Extraction kit (Sigma) and following the gel purification protocol (see Appendix).

3.5. Isolation of the plasmid from Bacteria

pET28a plasmid was isolated from an E. coli DH5α, using the Qiagen Plasmid Isolation Protocol (see Appendix).

3.6. Digestion of DNA with restriction enzymes

Both pET28a plasmid and the DNA fragment were digested with the same restriction endonucleases *NcoI* and *NotI*.

Restriction digestion was performed in two separate reactions.

In the first digestion the restriction enzyme *NcoI* was used, in a final volume of 20 µl for the plasmid and 40 µl for the insert, containing:

	Plasmid: pET28a	Insert:*dim-2* ORF
distilled H_20	5.3µl	1.7 µl
DNA	12µl (1µg)	36µl (360ng)
10XTango Buffer	2µl (2X)	2µl (2X)
Enzyme: *NcoI*	0.7µl (7units)	0.3µl (7units)
Total volume	20µl	40µl

Table 4: Digestion of isolated pET28a plasmid and *dim-2* ORF with restriction enzyme *NcoI*.

Digestion reactions were incubated at 37ºC for 3 hours followed by a drop dialysis to assure the complete cleavage of the DNA by the restriction endonuclease and to increase the efficiency of the subsequent ligation.

3µl from each reaction were run on 0.8% agarose gel in order to check the digestion and the remaining volume was used in the second digestion with the restriction endonuclease *NotI*:

	Plasmid: pET28a	Insert:*dim-2* ORF
distilled H_20	0.8µl	0.5 µl
DNA	16µl (1µg)	37 µl
10X NEB 3 Buffer	2µl (2X)	2µl (2X)
BSA	0.4 µl	0.2 µl
Enzyme: *NotI*	0.7µl (7units)	0.3µl (7units)
Total volume	20µl	40µl

Table 5: Digestion of pET28a plasmid and *dim-2* ORF with restriction enzyme *NotI*.

The tubes were incubated at 37ºC for 3 hours and then they were heat inactivated at 65ºC for 20 minutes.

3µl of double digestion were run on 0.8 %agarose gel electrophoresis and DNA fragments were visualized by ethidium bromidium staining under UV light.

Dephosphorylation of the plasmid

In order to reduce the self-ligation of the plasmid, pET28a was dephosphorylated by treating with the enzyme alkaline phosphatase, which removes the terminal 5'-phosphate from the plasmid preventing its recircularization (2).

The reaction mixture contained 2.5 µl of10X Antartic Phosphatase Buffer, 5 units of Antartic Phosphatase Enzyme and 5 µl of the digested plasmid. The reaction was incubated at 37ºC for 15minutes and then heat inactivated at 65ºC for 5minutes.

3.7. Ligation

The insert was mixed together with dephosphorylated or nondephosphorylated vector in a ligation reaction of 20 µl, containing 2 µl of 10X T_4 DNA Buffer, 2.5 units of DNA T_4 ligase and distilled water. Also a background of the uncut plasmid was treated with DNA T_4 ligase to check the efficiency of the ligation.

The molar ratio vector/insert used was 1:3.

The ligation reaction mixture was incubated at 22ºC for 30 minutes and then heat inactivated at 65ºC for 7 minutes.

3.8. Transformation

Escherichia coli DH5α chemically competent cells were transformed with ligation products. For that, 100 µl of competent cells were mixed with the ligation reaction and this mix was kept on ice for 30 minutes. After this time a heat shock at 42ºC for 90 seconds was given and then the tubes were cooled on ice for 1 minute.
After the transformation, 1 ml of SOC medium was added to the tubes with the transformed cells and this suspension was incubated at 37ºC for 1 hour to allow transformed cells to express resistance to kanamycin (see protocol in Appendix).

Alternatively, the process of electroporation was also used to drive the DNA into electrocompetent *Electromax DH10B* E. coli cells with a pulse of 1800 Volts in a cuvette with a 1 mm gap.

Selection of transformants

For the selection and cultivation of the strains transformed with plasmids, aliquots of 100 µl of the suspension were plated on a selective LB medium (Bacto Yeast Extract 5g/l, Peptone 10g/l, NaCl 10g/l, pH 7.0). The antibiotic *kanamycin* was added to the medium with a final concentration of 50 µg/ml, since the plasmid confers resistance to this antibiotic; and the plates were incubated at 37ºC overnight.

3.9. Analysis of the Transformants

PCR Screening of Transformation colonies

To amplify the DNA from the whole transformed cells, they were taken with a sterile toothpick from the colony of interest and resuspended directly in the following PCR reaction:

- 16.44 µl distilled water
- 0.24 µl of 10 mM dNTP
- 5 µl 10X PCR Taq buffer with $MgS0_4$
- 0.6µl of each primer forward and reverse (10 µM each)
- 0.12 µl Taq DNA polymerase (5 u/µl)

The DNA of the transformed cells was amplified with the pair of primers 4F and 1R-NcoI, which are listed in table 1.
A positive control with the original genomic DNA diluted in SOC medium was used.

The amplification conditions were: an initial denaturation of 94ºC for 2 minutes, followed by 33cycles of 94ºC for 20 seconds, 50ºC for 20s, and 72ºC for 45sec, with a final extension of 72ºC for 10 minutes.

PCR products were separated by electrophoresis on a 1.2% agarose gel and the bands were visualized with UV light.

Purification of plasmid DNA from *E.coli*

To analyze the plasmid DNA from the transformants clones, 10ml of liquid LB media with 30µg/ml kanamycin were inoculated with each transformant selected and incubated at 37ºC overnight.
The culture cells were collected by centrifugation at 14000rpm for 1 minute in microcentrifuge tubes and DNA plasmid was purified using the QIAprep Spin Miniprep Kit (see protocol in Appendix).
Afterwards, they were digested with appropriate restriction enzyme and the resulting DNA fragments were separated by electrophoresis on 0.7% agarose gel stained with ethidium bromide.

Analyzing the size of the DNA fragments obtained was determined whether the transformants contained the desired plasmid construction.

4. Results and Discussion

4.1. Amplification of *dim-2* ORF

The specific primers allowed to amplify a PCR product of the expected size (~3350bp), corresponding to the *dim-2* ORF.

The size was confirmed by electrophoresis on a 1% agarose gel with ethidium bromure (see fig.2).

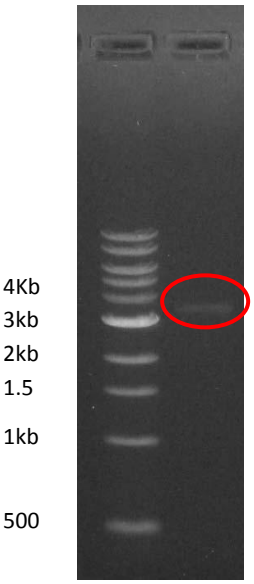

Figure 2: Agarose gel electrophoresis of DNA fragment generated by amplification of *dim-2* ORF from *P. membranacea* genomic DNA using specific primers 1F-*NcoI* & 1R-*NotI*.

4.2. Digestion with restriction enzymes *NcoI* & *NotI*

pET28a circular plasmid was cut with both enzymes *NcoI* and *NotI*, creating a linear plasmid as well as *dim-2* ORF, to be joined in a ligation reaction.

The fragment produced by digestion of pET28a plasmid was checked by electrophoresis, resulting in a band of the corresponding size (~5.4Kb).

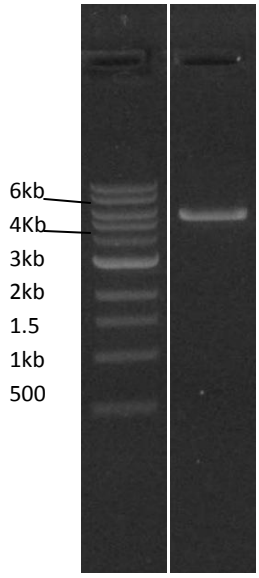

Figure 3: A 0.8% agarose gel electrophoresis of digestion of pET28a plasmid with both restriction endonucleases *NcoI* and *NotI*.

4.3. Transformation

Bacterial strains resulting from transformation were count, showing a low efficiency of the transformation.

The amplified fragments by PCR analysis of sixteen positive transformed cells were separated electrophoretically in a 1.2% agarose gel. Results are shown in figure 4.

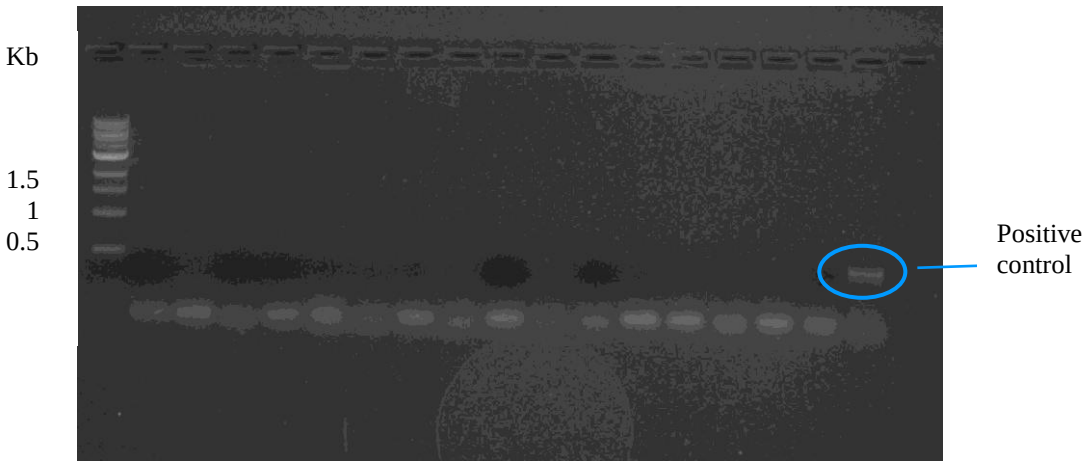

Figure 4: PCR amplification of DNA fragments from 16 positive transformed cells. On the left side shows the molecular weight marker.

Of all the sixteen isolated clones, none showed a product of amplification of 450bp size.

Nevertheless, 10 of the colonies were selected for plasmid DNA purification and they were analyzed by digestion and electrophoresis.

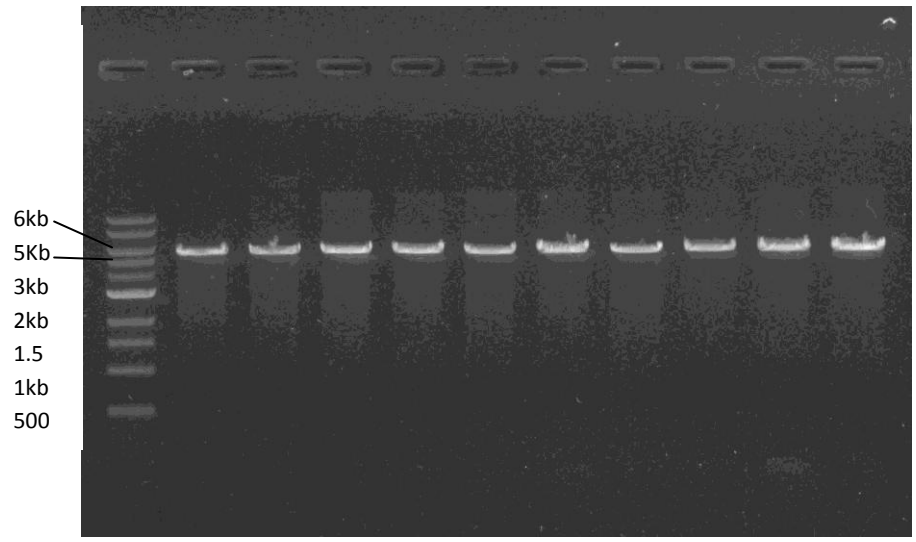

Figure 5: Amplification of DNA plasmid purified from 10 selected transformants clones.

In this case, all the DNA fragments had the same size as the linearized plasmid after the digestion (~5.4Kb).

Either, the PCR screening of the remaining 700 colonies didn't show any band with the suitable expected size for the plasmid construction (~8.7Kb).

These results confirmed the absence of the desired dim-2 ORF in the plasmid construction.
No sucessfully positive clones were detected and no pET28 plasmid with the dim-2 ORF was recovered.

Due to these unexpected negative results from the transformation, the amplified DNA fragments couldn't be sequenced to analyze. Further, the expression of the protein and the subsequent experiments of purification and the characterization study of the protein weren't able to be performed.

The proposed aims at the beginning could not be followed as it was expected and it was not possible to finish the present study.

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Appendix

Protocol for Lichen DNA Isolation (*Peltigera membranacea*)

1. Sample of interest is collected from the field and washed running tap water for several times and finally it is rinsed with deionized water.
2. Sample is cleaned under microscope to remove the debris from the field.
3. Clean lichens (about 2 grams) are grounded with liquid nitrogen in a cold morter. The powder material is collected into pre- cooled 50 ml tubes.
4. Add 25 ml of Lysis buffer (50mM EDTA, 100 mM NaCl, 10mM Tris HCl pH 8.0, 1% SDS). Buffer temperature should be 65°C prior to use.
5. Tubes are kept in the 65 °C water bath for 20 minutes, during the incubation the tubes are shaken for many times.
6. After the incubation, the soup is collected from the tubes and distributed evenly in 2ml EP tubes.
7. Tubes are spinning it 4k for 4 minutes.
8. The pellet is discarded and supernatant will distribute in new 2 ml tubes, approximately 1.25 ml each. Then, add 535 µl of 10.5M of NH4Ac and mix well. Tubes are kept in the ice for 20 minutes.
9. Tubes are spinning it 4k for 4 minutes and the peller is discarded.The supernatant is collected in the new 2ml tubes for next step.
10. Add 800 µl of Isopropanol into supernatant and inver the tubes for several times. The tubes are kept in ice for 20 minutes.
11. Tubes are spinning it 14k for 4 minutes. The supernatant is discarded and the pellet is saved for the next step.
12. Add 100 µl of TE buffer with RNAase to the pellet of each tube (stock concentration of RNAase 10 mg/ml. It should be 4 µl for 1 ml of solution)
13. Then the tubes are kept in the thermomixer at 50° C for 20- 60 minutes until pellet get dissolved.
14. The tubes go for short spinning to bring down the condensed waters during the time of incubation.
15. The supernatant is pooled into 1.5 ml tubes (4 samples per tube)
16. Add equal volume of Phenol:Chloroform (1:1) to the tubes and mixed well
17. Spinning it for 14k for 4 minutes. The top phase of the solution is pooled out without disturbing the interphase.
18. The equal volume of saturated chloroform is added to the supernatant and mixed
19. Spinning it for 14k for 4 min. After spinning, top layer is pooled out or proceed with next steps (if needed it can be confirmed through 0.6% agarose gel for DNA)
20. Add (1:10) of 3M NaAc. (i.e.) If supernatant was 500 µl means add 50 µl
21. Add double amount of 100% ETOH to the solution and mixed well. Keep the tubes in ice for 3 minutes.
22. Spinning it for 14k for 4 minutes. Supernatant is discarded and pellet is preserved for next step.
23. Pellet is washed with 70% ETOH.Approximately 500 µl.
24. Spinning it 14K for 4 minutes, discard the supernatant and pellet goes for air dry to make sure ethanol gets evaporated.
25. Add 50 µl of TE buffer to the pellet and keep it in the thermomixer at 50ºC for 3 minutes to dissolve the pellet. Then the tubes go for short spinning.
26. Load 5 µl of the sample into a 0.6% of agarose gel to confirm the DNA bands.
27. Store the tubes in the freezer.

Gel- purifying PCR products

Preparing the gel: 0,8 % agarose gel

1. Mix 0,8g of agarose and 100ml of 1X TAE buffer in a clean glass flask.
2. Place flask in the microwave and heat until just boiling.
3. Remove from the microwave and allow to cool for 3 minutes.
4. Add 30µl of the 2mg/ml crystal violet solution to the agarose and swirl to mix. The agarose should be light to medium purple in color.
5. Pour the gel in the gel box and set the comb.

Loading and Running the gel

6. Add 8µl of 6X crystal violet loading buffer to 40µl of the PCR amplification and load onto the gel.
7. Run the gel at 80 voltios for 45-50 minutes.

Excising PCR products

8. Using a new razor blade, carefully excise the desired purple band from the gel.
9. Weigh the gel as follow:
    a. weigh the empty eppendorf tube
    b. weigh the eppendorf tube with the gel
    c. calculate the real weigh of the gel

Solubilize the gel

10. Add 3 gel volumes of the Gel Solubilization Solution to the gel slice.
    Ex: for every 100mg of agarose gel add 300µl of Gel Solubilization Solution.
11. Incubate the gel mixture at 50°C for 10 minutes (the gel slice will be dissolved)
    While the gel mixture is incubating at 50°C, invert the tubes several times to check the gel slice is dissolving.

Prepare Binding Column

12. Add 500µl of the Column Preparation Solution to each binding column.
13. Centrifugate at 12000rpm for 1minute and discard flow-through liquid.
14. Centrifugate again without any additional liquid.

Check the color of the mixture

Make sure the color of the mixture is yellow, similar to fresh Gel Solubilization Solution.

Add Isopropanol

15. Add 1 gel volume of 100% isopropanol and mix until homogenous by inverting.

Bind DNA

16. Load the solubilized gel solution mixture into the corresponding column in 700µl portions.
17. Centrifuge at 12000rpm for 1minute after loading the column each time. Discard flow-through liquid.
    It is normal to see an occasional color change from yellow to red.

Wash Column

18. Add 700µl of Wash Solution to the binding column.
19. Centrifuge at 12000rpm for 1minute.
20. Remove the binding column from the collection tube and discard the flow-through liquid.
21. Place the binding column back into the collection tube and centrifuge again without any additional wash solution to remove excess ethanol.

Elute DNA

(For efficient recovery, preheat the elution buffer to 50°C for 2-3 minutes)
22. Transfer the binding column to a fresh collection tube.
23. Add 50µl of Elution Solution to the center of the membrane and incubate for 2 min at room temperature.
24. Centrifuge at 14000rpm for 1minute.

Measure the concentration of DNA in the Nanodrop

25. Load 1µl of the sample in the Nanodrop to measure the concentration of DNA.

Run an Electrophoresis Gel

26. Load 4µl of sample with 1.5 µl of loadding dye to check the correct band.

Recovering plasmid DNA

1. Prepare liquid LB with the appropriate antibiotic, in this case *kanamycin*.
2. Using a sterile pipette tip, touch a single colony of bacteria from the agar plate.
3. Inoculate the liquid LB by swirling the tip in it.
4. Grow bacterial culture for ~16 hours in a 37^{o}C shaking incubator.
5. In the morning, bacterial growth should be visible. Centrifuge the culture to pellet the bacterial cells, then proceed with DNA preparation.

Plasmid DNA Isolation from Bacteria: QIAprep Spin Miniprep Protocol (using a microcentrifuge)

1. Cell lysis:
    a) Pour bacterial culture into labeled 2ml microcentrifuge tubes and spin cells down in microcentrifuge on highest setting for 1 minute.Repeat this step in same tube to get bigger cell pellet.
    b) Resuspend pelleted bacterial cells in 250µl Buffer P1 – The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain. (Ensure that Rnase A has been added to Buffer P1).
    c) Add 250µl Buffer P2 and mix gently by inverting the tube 4-6 times. Do not vortex and do not allow the lysis reaction to proceed for more than 5 minutes.
    d) Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times. To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. The solution should become cloudy.
2. Clarification of the lysate: Centrifuge for 10 min at 13,000 rpm in a microcentrifuge. A compact white pellet will form on the side of the tube wall.
3. Bind DNA:
    a) Pour the supernatants from step 2 into labeled and cleaned QIAprep spin columns.
    b) Centrifuge at 14,000rpm for 1min. Discard the flow-through. The DNA is bound on the silica gel in the column.
    c) Wash QIAprep spin column by adding 0,70 ml Buffer PE and centrifuge at 14,000rpm for 1min (Wash buffer is added to remove the salts to avoid problems during electroporation).
    d) Discard the flow-through, and centrifuge for an additional 1min to remove residual wash buffer.
    Important: Residual wash buffer will not be completely removed unless the flow- through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.
4. Elute DNA
    a) Place the QIAprep column in a clean 1.5 ml microcentrifuge tube.
    b) To elute DNA, add 50µl Buffer EB (10mM tris-Cl, pH 8.0) to the center of each QIAprep spin column (don't touch gel matrix with tip), let stand for 1min, and centrifuge for 1min at 14000rpm.

The flow-through contains your plasmid DNA. Label tube with plasmid name, your initials, date and store it at 4ºC.
Determine concentration using the Nanodrop machine.

LB Agar Plates

LB media is a generic rich media suitable for growing many aerotolerant species of bacteria and different yeasts.

Ingredients (per liter):

- 5g Bacto Yeast Extract: source of additional proteins, vitamins and minerals and some sugars, necessary for growth.
- 10g Peptone: tryptic digest of proteins to release small peptides and amino acids that bacteria can use for food and/or protein synthesis.
- 10g NaCl
- 15g Sterile powdered agar (polymer made up of subunits of the sugar galactose)
-1 L distilled water

Preparation of LB media:

1. Take a 2L Erlenmeyer flask with 1L of distilled water (The volume of the flask should be at least twice the volume of the media to be prepared).
2. Measure all dry ingredients except the agar and place into the flask.
3. Shake/stir vigorously to get most of the dry material into solution.
4. Check the pH of the solution. The pH should be adjusted to 7.
5. Add the agar and continue mixing gently. This action is necessary to insure even distribution of the agar in the media, else it often remains denser near the bottom.
6. Cover the opening of the flask with tin foil and place it in an autoclave/standard pressure cooker with some water in the bottom and cook it for approximately 30 minutes with a maximum pressure.
7. After autoclaving, let it cool. The media needs to *temper* before it is poured into plates. Don't agitate the solution too much, to prevent the formation of bubbles near the surface.
8. Once the media has tempered, additives such as antibiotics, carbohydrates such as glucose or other carbon sources, may be added to the media. Be certain to gently swirl the media after adding components to distribute the materials within the agar.
9. Get your plates ready (the empty Petrie plates should be sterile and packaged in a plastic bag)
10. The bottom half of the plate should be ~1/2 full, approximately 25mL of media per plate. Plates poured singly generally solidify within 1/2 hour at room temperature.
11. Store them at 4ºC upside down. This keeps condensation which forms in the lid from dropping onto and disrupting the bacteria growing surface.

Transformation Protocol using Heat Shock

1. Transfer 100µl of competent E.coli DH5α cells to prechilled tube and add 50ng of plasmid.
2. Mix the contents by swirling gently.
3. Incubate on ice for 30mins.
4. Heat shock in a preheated waterbath at 42°C and store for 90sec. Do not shake the tubes.
5. Transfer to an ice bath. Allow to cool for 1-2 min.
6. Add 1ml of SOC medium.
7. Incubate at 37°C for 1 hour.
8. Spread about 100µl of the resulting culture on LB plates (with appropiate antibiotic) and grow overnight.
9. Pick colonies about 12-16 hours later.

Drop Dialysis

1. Pour milli Q water into a petri plate.
2. Float a filter paper or millipore membrane with the shiny side up on the water.
3. Allow the floating filter to wet completely (~5 minutes) before proceeding. Make sure there are no air bubbles trapped under the filter.
4. Pipette the DNA droplet carefully onto the center of the filter. Don't cover the petri plate.
5. Carefully retrieve the DNA droplet with a micro-pipette and place in the corresponding tube.