Cloning and expression of *lec-1* from *Peltigera membranacea*

Eva Hauksdóttir

Líf- og umhverfisvísindadeild
Háskóli Íslands
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10 eininga ritgerð sem er hluti af
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Leiðbeinandi
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Líf- og umhverfisvísindadeild
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Abstract

Lichens are organisms that have emerged from the symbiosis of a fungus and a green alga and/or cyanobacterium. They can be found far and wide because of their great ability to endure extreme environments. *Peltigera membranacea* is a terricolous foliose lichen, the symbiosis of a heterotrophic ascomycete and a *Nostoc* species.

Among the many proteins encoded by *P. membranacea* is LEC-1, which is a mycobiont lectin. Lectins are a group of proteins that may play role in symbiosis, e.g. by recognizing their symbiont partner. In order to analyse the protein, the corresponding gene was cloned into an *E. coli* expression host. Expression from the pasmid vector was induced by lactose or IPTG. To verify the expression of the LEC-1 protein, fractionations were performed and electrophoresed on SDS-PAGE gels. The results showed the protein, although it proved a bit larger than expected.

Útdráttur

Fléttur eru lífverur sem verða til vegna sambýlis svepps og grænþörungs og/eða blágrænnar bakteríu. Útbreiðsla þeirra er gífurleg vegna aðlögunar þeirra að jaðarskilyrðum. *Peltigera membranacea* er runnaflétta sem samanstendur af ófrumbjarga sveppi og blágrænnar bakteríu af ættkvíslinni *Nostoc*.

P. membranacea myndar ýmis prótein, þar á meðal LEC-1 sem er lectin myndað af svepphluta fléttunnar. Lectin eru stór hópur próteina sem gætu gegnt hlutverk í samlífi t.d. með því að þekkja sambýlislífveruna. Til að greina próteinið var samsvarandi gen klónað í efnafræðilega hæfar frumur. Í þeirri tjáningarferju sem notuð var, er hægt að tjá LEC-1, líkt og önnur prótein, með því að bæta laktósa eða IPTG í ræktina. Til að sannreyna framleiðslu LEC-1 voru framkvæmdar próteineinangranir og þær rafdregnar á SDS-PAGE gelum. Niðurstöður tilraunarinnar sýndu fram á myndun próteinsins, en það virtist aðeins stærra en búist hafði verið við.

Lichen

Boulder in the meadow, world in the world,
And lichen upon it: field in a field.
On the smooth top there, death's grey grain
Creeping by centuries, circular, small:
Whosoever watches, still but a stain
Spreading in one place, waiting to be all.

Oh, the cold time yet, oh, the slow years
Till granite is covered, and sleep overpowers.
All the great rock there, grim as the world,
Whitened with death's wheat, motionless, mute:
Whatsoever wind, no leaf uncurled;
Whatsoever rain, no sweet green shoot.

Not that it hates this, tightening hold
On the hair of the stone, on the scalp of the world.
Ready for ice cap, ready under drought,
Ready now for nothing or for all, it lies,
Putting incisibly its feelers out
For the last great changes. Lichen is wise.

- Mark Van Doren

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Abbreviations

A_{260} = light absorption at 260 nm
A_{600} = light absorption at 600 nm
AB = Applied Biosystems
APS = Ammonium persulfate
ATP = adenosine triphosphate
BL21 (DE3) = chemically competent *Escherichia coli*
BSA = bovine serum albumin
DE3 = lysogen that encodes T7 TNA polymerase. Used to induce expression in T7-driven expression systems (Life technologies, n.d.)
dmc = abolishes endogenous cytosine methylation at CCWGG sequences (Life technologies, n.d.)
DNA = deoxyribonucleic acid
EDTA = Ethylene-diamine-tetra acetid acid
endA = mutation in the non-specific endonuclease Endonuclease I (Life technologies, n.d.)
EtBr = Ethidium bromide
F^- = A self-transmissible, low-copy plasmid used for the generation of single-stranded DNA when infected with M13 phage (Life technologies, n.d.)
galK = galactokinase mutation blocks catabolism of galactose. Cells that are galK minus cannot grow on media that contain galactose as the sole carbon source (Life technologies, n.d.)
galU = glucose-1-phosphate uridylyltransferase mutation blocks ability to use galactose. Cells that are galU minus cannot grow in the presence of galactose as the sole carbon source (Life technologies, n.d.)
GFP = green fluorescent protein
His = histidine
hsd = mutations in the system of methylation and restriction that allow *E. coli* to recognize DNA as foreign. The hsd genotype allows efficient transformation of DNA generated from PCR reactions (Life technologies, n.d.)
IPTG = isopropyl-1-thio-β-D-galactopyranoside
kan = kanamycin
kb = kilo base
kDa = kilo Dalton
lacZΔM15 = Element required for β-galactosidase complementation when plated on X-gal (Life technologies, n.d.)
LB = lysogeny broth (Bertani, 2004)
Met = methionine
mRNA = messenger RNA
mcrA/mcrBC/mrr
NEB = New England Biolabs
NPI = native purification imidazole
nupG = mutation for the transport of nucleosides (Life technologies, n.d.).
ompT = indication that the *E. coli* lack an outer membrane protease (Life technologies, n.d.)
pET28a = plasmid vector

PCR = polymerase chain reaction
recA = mutation in a gene responsible for general recombination of DNA (Life technologies, n.d.)
RNA = ribonucleic acid
rpm = revolutions per minute
rpsL = confers resistance to streptomycin (Life technologies, n.d.)
RSB = reducing sample buffer
RT-PCR = reverse transcription polymerase chain reaction
SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoreses
SOC = super optimal broth with added glucose
TAE = Tris base, acetic acid, and EDTA
TOP10 = chemically competent *Escherichia coli*
UTR = untranslated region
YFP = yellow fluorescent protein

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1 Introduction

Many may think that lichens are odd organisms that live on rocks as molds, while others do not know these lifeforms even exist. They are not large and therefore it is understandable if people pass them by unknowingly. Lichens are, however, very interesting and complicated organisms. They are strong survivors in this world as they have adapted to extreme environments and are widespread, from dry land to intertidal zones (Raven, Evert & Eichhorn, 1999). In 2012 the estimated number of described lichen species in the world was 17000 (IUCN Red List, 2012). In Iceland, however, there are only about 660 species (Starri Heiðmarsson, 2000).

Compared to other organisms, lichens have not been the topic of many research projects. This is changing, however, as every year the number of lichen studies increases. It is important to understand and identify all aspects of lichens because, like other organisms, they may hold the answers to many questions. They could, for example, contain the key substances to cure some illnesses.

1.1 Lichens

Lichens have existed on Earth for at least 400 million years, judging from lichen fossil finds. One fossil, for example, was found in the Rhynie chert in Scotland, which dates to the early Devonian era (416 to 359 million years ago) (Taylor, Hass, Remy & Kerp, 1995).

Studies have shown that existing lichens did not evolve only once from a common ancestor, but rather five seperate times. This was discovered by using sequences of a small subunit ribosomal RNA in comparison between lichens and various fungi. Each evolution thus comprises a distinct phylogenetic group (Gargas, DePriest, Grube, & Tehler, 1995).

A lichen is a symbiotic relationship between a fungus and a green alga or cyanobacterium or, on rare occasions, both (Raven et al., 1999). Where there is either a green alga or a cyanobacterium in the symbiosis, the lichen is said to be a bimembered association. If there are two photobionts, however, it is called trimembered (Miadlikowska & Lutzoni, 2000). In cases where a green alga is involved, it is most often *Trebouxia*, *Pseudotrebouxia* or *Trentepohlia*. On the other hand, the most frequent encountered cyanobacteria are of the genus *Nostoc*. These organisms live mutualistically, that is both or all parties benefit from this association (Raven et al., 1999). The fungus plays a protective role in this symbiotic relationship, as it shelters the alga and/or bacteria, which protects them from dehydration or damage from solar radiation. In turn, the fungus obtains sugars that the alga or bacterium produces (Starri Heiðmarsson, 2000).

Although these two/three organisms are joined as one, each still maintains its own role in this symbiosis and is named in order to prevent misunderstanding. The fungal part of a lichen is called the mycobiont and the photosynthetic part (the green alga and/or cyanobacteria) is called the photobiont (primary and secondary if need be) (Raven et al., 1999 & O´Brien, Miadlikowska & Lutzoni, 2005). The names of the partners derive from Greek, as myco- (Gk. mykés) stands for fungus, photo- (Gk. phóto) for light, and biont (Gk. bios) for life. The lichen´s scientific name is based only on the name of the fungus (Raven et al., 1999). Their classification is also based on the fungus (table 1.1), though the

classification within a genus and/or species, e.g. same fungus with different photobiont, is often based on the photobiont (Miadlikowska & Lutzoni, 2000).

*Table 1.1 – Classification of **Peltigera membranacea** (Xavier, Miao, Jonsson & Andresson, 2012 & as cited in Miadlikowska & Lutzoni, 2000).*

Kingdom	Fungi
Division	Ascomycota
Class	Lecanoromycetes
Order	Lecanorales
Suborder	Peltigerineae
Family	Peltigeraceae
Genus	Peltigera
Specie	*Peltigera membranacea*

1.1.1 Structure

There is no rule as such that states which fungus can live with a particular green alga or cyanobacterium. Many fungal species can live in symbiosis with number of species of green algae or cyanobacteria, and vice versa (Raven et al., 1999).

The morphology of lichens can be different between species, but the inner structure is always the same: Thallus, rhizines, and apothecia. There are, however, exceptions, as the exception proves the rule. The thallus is the basal sheet of the lichen. This is the central part of the symbiosis, as this is the part of the lichen where the fungus and its photosynthetic partner are located. It has four layers, in the following order: Upper cortex, photobiont layer, medulla, and lower cortex (figure 1.1). The upper and lower cortex protect the inner layers, as they are formed by a dense layer of fungal tissue. The medulla contains loose hyphae that take part in the formation of rhizines. The photobiont layer has photobiont cells that are intertwined with the fungal hyphae, and there photosynthesis takes place (Miao, Manoharan, Snaebjarnarson & Andresson, 2012).

The rhizines are tiny root-like features of the lichen. They are located below the thallus and are made up only of fungus. They help the lichen to attach itself to its substrate (Miao et al., 2012).

The apothecia are fruiting bodies on the surface of the thallus. They grow on the end of the thallus lobes. The colour of the apothecia differs between species. They contain asci, which contribute to sexual reproduction (see 1.1.3 Reproduction). The apothecia contain only fungal cells, and in fact the greater part of nearly all lichens is made up of the mycobiont because the thallus is made up mostly the fungus (Miao et al., 2012 & Raven et al., 1999).

The various morphologies of lichens are many, but the three major structures are crustose, foliose and fruticose. Crustose lichens are flat and firmly attached to their substrate. They are thought to have a „crusty“ appearance, leading some to confuse them with molds (figure 1.2 a) (Raven et al., 1999). Foliose lichens are said to be leaf-like (figure 1.2 b) while fruticose lichens are erect and most often branched, and so are therefore thought of as bush-like (figure 1.2 c) (Raven et al, 1999). Most of the species that are found in Iceland are of the crustose appearance (Starri Heiðmarsson, 2000).

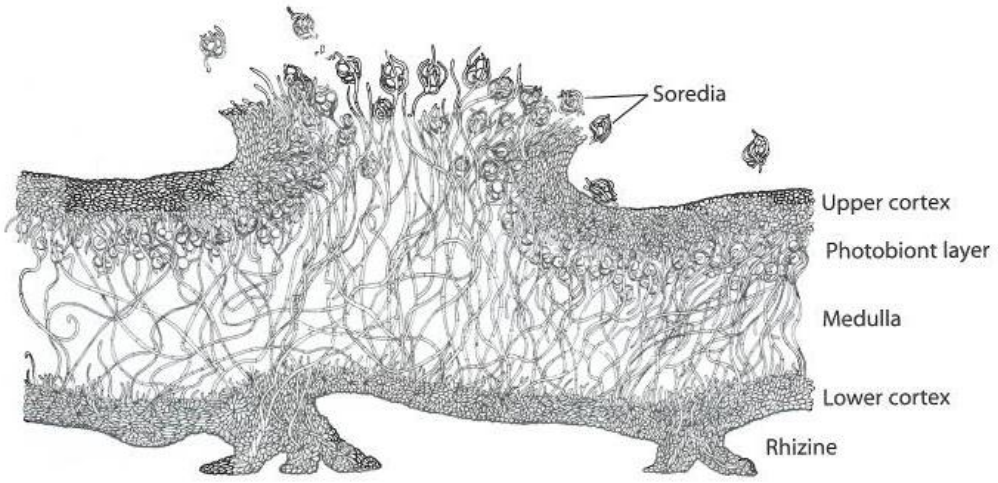

*Figure 1.1 – A cross section of the lichen **Lobaria verrucosa**. Its thallus has distinct layers, they are: upper cortex, photobiont layer, medulla and lower cortex. Rhizines are located underneath the thallus. The lichen is releasing soredia (Raven et al., 1999).*

1.1.2 Reproduction

Lichens can reproduce in two different ways, asexually and sexually. Asexual reproduction is a common way for lichens to propagate. They do so either by spreading soredia or isidia. Soredia are powdery propagules (figure 1.1), and isidia are small outgrowths (Raven et al., 1999). Both of these contain a little of both mycobiont and photobiont. Therefore they can produce a new lichen wherever they settle (O´Brien et al., 2005). This is called vertical transmission, as both parts of the lichen disperse together (see, Scheidegger, C., & Werth, S., 2009).

The sexual reproduction of lichens, however, is a process in which only the fungal part of the lichen participates. The asci of the apothecia produce fungal spores, there is no production of any photobiont (Raven et al., 1999). The dispersed spores must then reunite with free living green algae or cyanobacteria in order to form a new lichen (O´Brien, Miadlikowska & Lutzoni, 2013). This is called horizontal transmission, as the mycobiont disperses independently and forms a new generation (see, Scheidegger, C., & Werth, S., 2009).

It would therefore seem that asexual reproduction is a more practical way for a lichen to reproduce. Although practical not all lichens seem to disperse by asexual means, some genera, e.g. *Peltigera,* appear to prefer sexual reproduction (O´Brien, Miadlikowska, Lutzoni, 2009). The reason lichens prefer sexual reproduction is that the spores are small and can travel far, so the species can disperse over a wider range. The soredia and isidia are, however, larger then the spores, and therefore they do not travel as far, resulting in a more limited dispersal (as cited in O´Brien et al., 2009).

a)

b)

c)

Figure 1.2 – Differences between lichen structures.
a) Crustose, ***Lecidea atrobrunnea*** *(Clair, n.d.).*
b) Foliose, ***Pseudocyphellaria rainierensi*** *(Dillman, n.d.).*
c) Fruticose, ***Evernia mesomorpha*** *(Pope, n.d.).*

Peltigera membranacea

The terricolous foliose lichen *Peltigera membranacea* was the subject of this study (figure 1.3 & table 1.1). The mycobiont of *P. membranacea* is a heterotrophic ascomycete, and the photobiont, as with most lichens of the order Peltigerales, is a cyanobacterium of the genus *Nostoc* (Manoharan, Miao & Andresson, 2012 & O´Brien et al., 2005). *P. membranacea* is therefore a bimembered association.

*Figure 1.3 – **Peltigera membranacea**. The thallus surface of **P. membranacea** (Ditchburn, n.d.).*

The rhizines are described as mostly squarrosely branched and isolated, while the spores are long and narrow, and the thallus is thin (Martinez & Burgaz, 1996).

1.1.3 Effects and usage

The lichen symbiosis gave the component organisms (fungi, cyanobacteria, and green algae) the ability to pioneer new environments that had been uninhabitable for plants (Taylor et al., 1995). This ability, though enabling lichens to live in extreme environments, is first and foremost an evolutionary adaption, as lichens have a hard time when competing with plants for niches (Starri Heiðmarsson, 2000). During their lifetime lichens produce secondary metabolites which enrich the surrounding soil, and some even prevent mold from infesting them (Raven et al., 1999 & Nash, 1996). Also, when in symbiosis with cyanobacteria, lichens can release nutrients to the soil, as the bacteria can fix atmospheric dinitrogen (Raven et al., 1999). The benefit of this adaption is that lichens can live in a new, untouched niche, but the disadvantage is that they enrich the soil, and therefore plants can start to grow which in turn can lead to a loss of lichens.

Humans have found many uses for lichens, for pleasure, sensation or for medicinal purposes. Lichens have been used for dyes because of their diverse colours. People have been dyeing fabrics since at least 1100 A. D. and even earlier, and throughout the centuries colours have played important roles in peoples lives (Casselman, 2001). Lichens have also been used to provide compounds for perfumes, and can range from 1% to 12% of the final product (Nash, 1996). Most recently, lichens have been investigated for anti-tumour compounds (Raven et al., 1999).

1.2 Lectins

Lectins were first discovered in plants, but are now said to be found in all eukaryotic organisms. Lectins are a group of diverse single or multi-domained glycoproteins. They bind to sugars through specific interactions of sugar recognition domains, with high specificity and decent affinity (Lehninger, Nelson & Cox, 2008 & as cited in Brown et al., 2007). Lectins can play different roles in cells, e.g. in signaling or cell-cell recognition

(Lehninger, Nelson & Cox, 2008). They have been thought to participate in symbiosis by recognizing sugars on the surfaces of other organisms (as cited in Manoharan et al., 2012).

Peltigera species have been used as models in some studies on lectins, and it has been shown that some mycobiont lectins can distinguish between strains of their symbiotic photobiont and cultured *Nostoc* (Petit, Lallemant & Savoye, 1983 & Diaz, Vicente-Manzanares, Sacristan, Vicente & Legaz, 2011).

1.2.1 Lectin in Peltigera membranacea

The gene in question, *lec-1*, is a mycobiont gene that was identified in the *P. membranacea* whole genome sequence project (Xavier et al., 2012). It is known to be of mycobiont origin because of studies that showed gene expression in the thallus, rhizines and apothecia, but not in lichen-derived photobionts. The gene itself is divided into two sequences by a 77 nucleotide intron. The sequences are 469 and 38 nucleotides long. Surrounding the gene are 5´ and 3´ UTR. The 3´ UTR is 400 nucleotides long, while the 5´ UTR is 162 nucleotides in length, and has a 56 nucleotide intron at its centre (figure 1.4) (Miao et al., 2012).

Studies performed in order to estimate the expression of *lec-1* showed great expression of *lec-1* from thallus samples, while expression was far less in the samples from rhizines and apothecia. The reason for this might be that the protein LEC-1 has a role in recognizing ligands produced by the *Nostoc*, and therefore the expression is less in the fungal tissues (Miao et al., 2012).

a)

b)

```
161                                                                 240
TGTTATGCTGACTTCTTAAGCAGAAGAATCACATAACCACTACATCATCATGGCCAGCCAGTGGATTTTTCAGAAGATCA
    (M  L  T  S  *  A  E  E  S  H  N  H  Y  I  I) M  A  S  Q  W  I  F  Q  K  I

241                                                                 320
ACGACAAACCTACGAAATTGGCTAGGCCACTCGTCGCGGGTTCATCCATCTTCTTCACGTCAACCTCGTTTGATCCAAAG
N  D  K  P  T  K  L  A  R  P  L  V  A  G  S  S  I  F  F  T  S  T  S  F  D  P  K

321                                                                 400
CCCGATCCAAGTGCTGCCATTGATAACTCGAGTTTCAATCTGGAGGATGAAAAAAAATGTCCTTCTTCATATCTCTACCCG
 P  D  P  S  A  A  I  D  N  S  S  F  N  L  E  D  E  K  N  V  L  L  H  I  S  T  R

401                                                                 480
TCGAGGTAAGGGTGAGATCGTACTAGACACCAAGCGGGGCGATACCTGGGACAACAAGCTTCAAAAGATCGATCTCAAAG
  R  G  K  G  E  I  V  L  D  T  K  R  G  D  T  W  D  N  K  L  Q  K  I  D  L  K

481                                                                 560
ACATCTCCACCGGATCTGACCTGGTCATCAGGGTCACTGTGACTGCTAAGTCGTACGACATCTCATTCAATAATAGCCCC
D  I  S  T  G  S  D  L  V  I  R  V  T  V  T  A  K  S  Y  D  I  S  F  N  N  S  P

561                                                                 640
AAGATATATACCTTCGACAAGAGGATCGAGGCGAACGCGACAGCCGTATCCTACGGCTTGAATAAGGGCCAAAGAGCCGT
 K  I  Y  T  F  D  K  R  I  E  A  N  A  T  A  V  S  Y  G  L  N  K  G  Q  R  A  V

641                                                                 720
CTATTCCAATCCAGTGGTCATTCAGGTTTTTGATAATGGTGTGTTTTATCTATCCGTCCCCCATCCACCCCCCGTTGTGT
  Y  S  N  P  V  V  I  Q  V  F  D  N ----------------------(intron)-------------

721                                                                 720
GAACCATTTTCCTGACATCTCTTGCGCTTCCAAAGGGACTGCGGCCGGACCTCCCACTGTATCCGTCGAATAAGCCAGGA
---------------------------------- G  T  A  A  G  P  P  T  V  S  V  E  *
```

*Figure 1.4 – Map of **lec-1**. a) The map shows an intron (white) in the gene (grey) and in the UTRs (thick lines). b) Sequence of bases in **lec-1** with corresponding codons. The part that is located inside the brackets is homologous to an N-terminus of another **Peltigera** lectin, but has a stop codon (*). Therefore the **lec-1** reading frame is thought to start at the Met codon (M) after the bracket (adapted from Miao et al., 2012).*

2 Materials and methods

2.1 Lichen RNA

The *P. membranacea* samples used in this study were collected in Iceland at Keldur (63° 49‘ 21‘‘ N, 20° 04‘ 30‘‘ W). The samples were processed, and both DNA and RNA isolations were performed. For this study, DNA can not be directly used for generating an open reading frame, since there is an intron in the *lec-1* gene. Therefore mRNA (RNA id TW G1, G2, and G3) and total RNA (RNA id TW-4 28/9´12 G) were used. These RNAs were isolated from the thallus.

2.2 Plasmid and bacteria strains

In order to clone the synthesized cDNA there must be a vector in which it can be cloned. In this study, the plasmid pET28a was used (Appendix, figure A). It is resistant to kanamycin and has an optional C-terminal His tag sequence, which is necessary in order to isolate the cloned protein (for more detailed description see 2.5 Insertion of *lec-1* and YFP into pET28a).

Colonies of TOP10 containing pET28a were cultivated in order to amplify the plasmid. Cell cultivations in this study were performed at 37°C, 150-300 rpm in Innova® 44 from New Brunswick Scientific and all isolation procedures followed a spin column protocol (QiaGen, 2004).

In order to analyze whether the isolated plasmid constructs were correct, they were digested with the double cutter *AvaI* (from NEB), and in that reaction NEBuffer 4 (from NEB) was used. The absorption of the isolated DNA was then measured using a Nanodrop 1000 spectrophotometer from Thermo Scientific. This method was performed for all DNA isolations during the project.

Two different types of chemically competent cells were used in this study, TOP10 and BL21 (DE3) (table 2.1). Both of these cell types are strains of *Escherichia coli*. TOP10 was used for amplification steps (non-expression host), while the role of BL21 (DE3) was to produce the protein, LEC-1. BL21 (DE3) cells were made chemically competent by using the One-step procedure developed by Chung, Niemela and Miller (1989). TOP10 were made chemically competent as described in Current Protocols in Molecular Biology (1.8.1) (Seidman, Struhl, Sheen & Jessen, 2003).

The plasmids were able to penetrate the cells by use of a standard *E. coli* chemical transformation. Deviations from the protocol were the following:

- In the heat step for TOP10, a temperature of 41°C was used instead of 42°C and the duration was 60 seconds instead of 90.
- Instead of keeping the BL21 (DE3)+plasmids mix on ice for 30 minutes they were kept for 15 minutes.
- LB medium (table 2.6) was used for the BL21 (DE3) transformation with *lec-1*, while SOC was used for *lec-1*-YFP.

- The only comparisons for the plasmid-containing BL21 were BL21 with an uncut vector (for *lec-1*-YFP) and BL21 with vector + insert without ligase (for *lec-1*).

Table 2.1 – Genotypes of BL21 (DE3) and TOP10 (Life technologies™, n.d.).

Bacterial strain	Genotype
BL21 (DE3)	F^- ompT $hsdS_B$ (r_B^-, m_B^-) gal dcm (DE3)
TOP10	F^- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG

2.3 RT-PCR

Reverse transcriptase (from AB) was used in this RT-PCR, along with 10x Reverse transcriptase buffer (from AB), random primers (from AB), and 25x dNTP (from Fermentas). In order to detect whether there had been any DNA contamination, a negative control was performed as well. This reaction (table 2.2) took place in a DNA Engine Tetrad 2 from MJ Research.

Table 2.2 – cDNA reaction.

Temperature	Time
25°C	10 minutes
37°C	120 minutes
85°C	5 minutes
12°C	Forever

A negative control for a RT-PCR contains all the same ingredients as a normal RT-PCR sample should, except for reverse transcriptase. Thus if there is any amplification after a PCR, which often takes place after a RT-PCR, it is evidence of DNA contamination. No amplification would take place in a PCR that contains only RNA.

2.4 PCR

In order to amplify and analyse the *lec-1* cDNA, *Onetaq*® 2x MM with standard buffer (from NEB) was used in the reactions. 25 mM MgCl (from Fermentas) was added in addition to the 1.8 mM Mg concentration of the buffer, making the final Mg++ concentration 6.9 or 27 mM, depending on whether the reaction´s final volume was 15 µl or 25 µl. Primers for this reaction were Lec-1 NcoI F, Lec-1 EcoRI R, and Lec-1 EcoRI Rcor (table 2.4). Lec-1 EcoRI Rcor was created especially for this study, in order for the plasmid pET28a to place the open *lec-1* reading frame in the correct phase needed to yield a 6X His tail. PCR was also used for amplification and analysis of ligated products; the same ingredients were used for those reactions, except for the primers (see 2.5 Insertion of *lec-1* and YFP into pET28a).

All PCR reactions (table 2.3) performed in this study were performed in a DNA Engine Tetrad 2 from MJ Research. After the reactions the PCR products were electrophoresed on agar gels, prepared from agarose and TAE+EtBr. The gels were then photographed with a FOTO/Convertible setup from FOTODYNE Incorporated.

Table 2.3 – PCR reaction.

Temperature	Time
94°C	1 minute
94°C	20 seconds
53°C	20 seconds
72°C	50 seconds
72°C	10 minutes
12°C	Forever

2.5 Insertion of *lec-1* and YFP into pET28a

In order to insert *lec-1* into a plasmid of the type pET28a, both parts required double digestion. The restriction enzymes that were used were *Nco*I (20 u/µl) and *Eco*RI (20 u/µl) from NEB. The buffer was 10x EcoRI buffer, also from NEB. Before the samples were ligated, they were heated in order to inactivate the restriction enzymes. For the ligation process, a 10x Tango buffer and T4 DNA ligase (from Thermo Scientific) were used, along with 0.5 mM ATP (from Fermentas). The ligation process took place at room temperature for 50 minutes, overnight at 4°C, and then 30 minutes at room temperature. In order to ascertain whether insertion had taken place, a PCR was used with primers NcoI and EcoRI.

After LEC-1 had been produced in BL21 (DE3), plasmids with *lec-1* were isolated and YFP was inserted behind *lec-1* on pET28a. In order to do so, the plasmids were double digested with *Hind*III (20 u/µl) and *Xho*I (20 u/µl), both from NEB. Also present in the reaction were NEBuffer 2 and 100x BSA (also from NEB). Before the samples were ligated, they were heated in order to inactivate the restriction enzymes. The YFP had already been amplified with primers pH3GFPcor and RJ-GFP (table 2.4), to ensure the correct reading frame of *lec-1*, with the plasmid´s 6X His tail. It was also digested with the same enzymes as the plasmids. Both actions were performed by students in the Molecular Genetics course of 2013 at the University of Iceland. For the ligation process a T7 DNA ligase and buffer (from Fermentas) were used. The ligation process took place at room temperature for 15 minutes. In order to analyze whether the insertion had succeeded, a PCR was used with the primers pH3GFPcor and RJ-GFP.

Table 2.4 – Primers used in this study.

Name	Sequence	Manufacturer
Lec-1 NcoI F	5´-GCAGACCCATGGCCAGCCAGTGGATTTTTCAGAAGA-3´	Microsynth
Lec-1 EcoRI R	5´-GCACGTGAATTCTTATTCGACGGATACAGTGGGAGG-3´	Mcrosynth
Lec-1 EcoRI Rcor	5´-CGCACGTGAATTCGGGTATTCGACGGATACAGT GGGAGG-3´	Microsynth
pH3GFPcor	5´-CAGCTGAAGCTTCGCTACGCTGCAGGTCGACG-3´	Microsynth
RJ-GFP	5´-GGAGCATGCTCGAGTTTGTACAATTCATCCATACCATGGGT-3´	Microsynth

2.6 SDS-PAGE analysis for LEC-1

BL21 (DE3) containing either pET28a or pET28a-*lec-1*, were cultivated and grown in LBkan (50) for four and a half hour at 300 rpm. From those broths zero samples (#0) were taken. 10 µl of each broth was added to a 20 ml lactose medium (table 2.5) and incubated overnight at 150 rpm, after which the second samples (#2) were taken. Samples were spun down for 3 minutes at 14000 rpm in a 5417R centrifuge from Eppendorf and the pellets were collected. RSB was added to dissolve the pellets, and the samples were boiled for six minutes before being electrophoresed on a 12% SDS-PAGE gel.

All SDS-PAGE gel electrophoresis in this study (table 2.5) followed the protocol described in Current Protocols in Molecular Biology (10.2A.4) (Gallagher, 2003).

Table 2.5 – SDS-PAGE gels. Different percentages of resolving gels and a stacking gel, volume for two gels.

15% Resolving gel	3 ml H_2O 2 ml Lower buffer 3 ml 40% Acrylamide 8 µl TEMED 48 µl 10% APS
12% Resolving gel	3.6 ml H_2O 2 ml Lower buffer 2.4 ml 40% Acrylamide 8 µl TEMED 40 µl 10% APS
Stacking gel	2.5 ml H_2O 1 ml Upper buffer 0.5 ml 40% Acrylamide 16 µl TEMED 32 µl 10% APS

However, the 15% SDS-PAGE gels that were used in „Production of LEC-1“ and „Comparison of methods“ had additional 20% Bis-acrylamide to the 40% acrylamide to ensure the correct percentage of the gels.

The SDS-PAGE gels were dyed in commassie blue overnight after being electrophoresed, and then decoloured by the use of a destaining buffer (methanol (30%), acetic acid (10%) and d.H_2O).

Table 2.6 – Culture medium.

LB medium	5 g Bacto yeast extract 10 g NaCl 10 g Peptone H_2O until volume reaches 1 L	
Lactose medium	18.56 ml LB medium	
	20 µl 1000x metalmix	2.86 g/L H_3BO_3 1.81 g/L $MnCl_2$ * $4H_2O$ 0.222 g/L $ZnSO_4$ * $4H_2O$ 0.079 g/L $CuSO_4$ * $5H_2O$ 0.050 g/L $COCl_2$ * $6H_2O$ 0.391 g/L $NaMoO_4$ * $2H_2O$
	20 µl $MgSO_4$	24.65 g $MgSO_4$ * $7H_2O$ 100 ml H_2O filter sterilized
	0.4 ml 50x 5052	25 g glycerol 2.5 g glucose 10 ‹ lactose 100 ml H_2O filter sterilized
	1 ml 20x NPS	6.6 g $(NH_4)SO_4$ 13.6 g KH_2PO_4 17.86 g Na_2HPO_4 * 2H2O 100 ml H_2O filter sterilized
	40 µl kanamycin (Sigma)	

2.7 His-tag protein purification

BL21 (DE3) containing pET28a-*lec-1* were cultivated in lactose medium overnight at 300 rpm. The broth was spun down for 3 minutes at 14000 rpm in a 5417R centrifuge from Eppendorf. The pellet was collected and resuspended by adding 630 µl lysis buffer (NPI-10 (table 2.7)) and 70 µl Lysozyme (10 mg/ml) (from USB, Amersham Life Science). The sample was kept on ice for 10 minutes, and then sonicated and centrifuged at 12000 x g for 25 minutes at 4°C. The supernatant was collected, and in order to further isolate the protein, the supernatant was placed on a column containing His-select® nickel affinity gel (from SIGMA). The column was then washed with buffers containing different amounts of imidazole (NPI-20 and NPI-500 (table 2.7)), in order to release the protein from the column. Samples from the supernatant, flowthrough, and elution were electrophoresed on a 15% SDS-PAGE gel.

Table 2.7 – Buffers used for protein purification

Buffer	Ingredients	pH
NPI-10 (lysis)	50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole	8.0
NPI-20 (wash)	50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole	8.0
NPI-500 (elution)	50 mM NaH2PO4, 300 mM NaCl, 500 mM imidazole	8.0

2.8 His-tag protein denaturation

BL21 (DE3) containing pET28a-*lec-1*-YFP were cultivated in LBkan (50) overnight at 300 rpm. The broth was then diluted (1/20) in the same medium and incubated under the same circumstances, until the A_{600} measured 0.4. Then 100 mM IPTG (from Fermentas) was added to the broth in the ratio 1/200 and cultivated for two hours at 250 rpm. The broth was spun down at 3000 rpm for 15 minutes in a Sorvall® RT 6000D, and the pellet was collected. The pellet was dissolved in 700 µl Buffer B (table 2.8) and 15 units of DNAse I (650 u/mg, 10 mg/ml), from Fermentas. The sample was kept at room temperature with mild shaking and then centrifuged for 15 minutes at 20000 x G at room temperature. The supernatant was collected, and in order to further isolate the protein, the supernatant was placed on a column containing His-select® nickel affinity gel. The column was then washed with buffers containing different pH (buffers C and E (table 2.8)), in order to release the protein from the column. Samples from the flowthrough, first wash, and first and second elution were electrophoresed on a 12% SDS-PAGE gel.

Table 2.8 – Buffers used for protein denaturation.

Buffer	Ingredients	pH
B (lysis)	7 M urea, 0.1 M NaH2PO4, and 0.1 M Tris HCl	8.0
C (wash)	8 M urea, 0.1 M NaH2PO4, and 0.1 M Tris HCl	6.3
E (elution)	8 M urea, 0.1 M NaH2PO4, and 0.1 M Tris HCl	4.9

2.9 Production of LEC-1

BL21 (DE3) containing either *lec-1*, *lec-1*-YFP or the negative control (pET28a) were cultivated in LBkan (50) overnight at 250 rpm. The broths were then diluted (1 ml broth/24 ml LBkan (50)) and 100 mM IPTG was added, making the final concentration 0.75 mM. The broths were then cultivated at the same settings as before, and samples taken every hour for five hours. The first sample was taken immediately after the IPTG had been added. The A_{600} value of the samples was measured at the time of collection, and the samples were then frozen until all samples had been taken, except for samples 1 and 4, which were kept at 4°C for an hour, after which their A_{600} value was measured along with the next sample. They were then spun down for 3 minutes at 14000 rpm and pellets collected. RSB was added to dissolve the pellets, and the samples were incubated at 99°C for 10 minutes, before being electrophoresed on SDS-PAGE gels. The volumes of the samples that were put on the gels were adjusted, so there would be a similar amount of cell material in each lane. This was done because of the different growth rates (figure 3.11). The *lec-1* samples, along with negative control samples, were electrophoresed on a 15% SDS-PAGE gel, while *lec-1*-YFP with negative control samples were electrophoresed on a 12% SDS-PAGE gel.

2.10 Comparison of methods

Here, a traditional lysation method (see 2.9 His-tag protein purification) was compared to a urea lysis method (see 2.10 His-tag protein denaturation) using BL21 (DE3) containing pET28a-*lec-1* or the negative control, pET28a. Two different broth volumes, 0.5 ml and 1.5 ml were used for this analysis. Both supernatants and pellets were electrophoresed on a

15% SDS-PAGE gel, in order to determine whether there was any difference between these methods.

3 Results

3.1 Reverse transcription

After the RNA (both total RNA and mRNA) had been reverse transcribed into cDNA and then amplified, the PCR products were electrophoresed on a 1% agar gel along with a 1 kb ladder (figure 3.1).

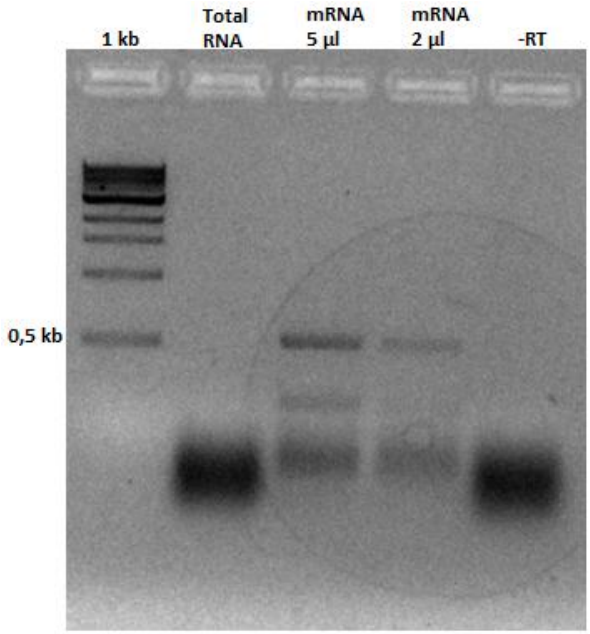

Figure 3.1 - PCR products after reverse transcription, electrophoresed on a 1% agar gel along with a 1 kb ladder. Different mRNA volumes had been used as templates in the PCR, 5 µl and 2 µl. –RT = negative control.

Bands the size of 500 bases appeared for reverse transcribed mRNA, and in all the lanes bands of unknown sizes appeared at the bottom of the ladder gradient.

What was left of the mRNA RT-PCR products was pooled and electrophoresed on a 1% gel with 1 kb as a ladder (figure 3.2).

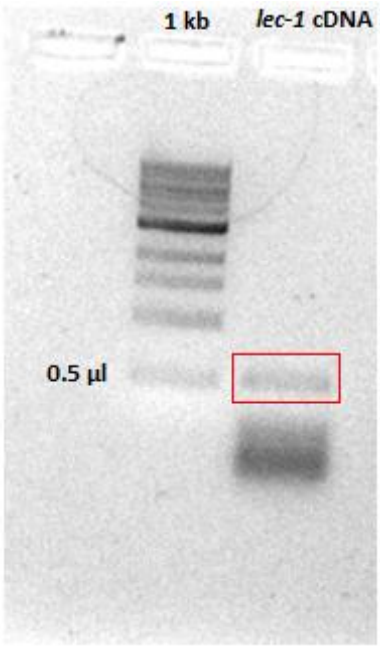

Figure 3.2 - PCR products electrophoresed on a 1% agar gel along with a 1 kb ladder. Marked band was used for further studies.

The *lec-1* cDNA sample showed a 0.5 kb band and band of an unknown size, they are identical to the bands in figure 3.1.

The 500 base band that appeared was pricked, and the gel frozen and thawed in order to elute the *lec-1* sample. Another PCR was applied to the newly amplified *lec-1* cDNA, as the primer (Lec-1 EcoRI R) used in the former PCR had been incorrect. The PCR products were then analysed on a 1.5% agar gel with 1 kb as a ladder (figure 3.3).

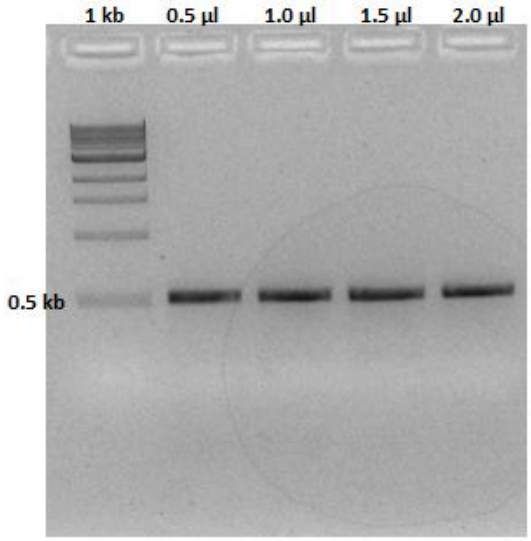

Figure 3.3 - PCR products electrophoresed on a 1.5% agar gel along with a 1 kb ladder.

The samples were all very pure, and were all of the same size of 0.5 kb. The samples 0.5 µl and 1.0 µl were pooled (*lec-1*-1), as were those of 1.5 µl and 2.0 µl (*lec-1*-2). The A_{260} of the new samples was then measured in order to determine their DNA concentration (table 3.1).

*Table 3.1 – Absorption results for **lec-1**-1 and **lec-1**-2.*

Sample	ng/µl	A260	A280	260/280	260/230
lec-1-1	20.01	0.400	0.239	1.67	1.39
lec-1-2	18.84	0.377	0.215	1.76	1.06

3.2 pET28a

Only six colonies grew on two plated LBkan (50) agar plates, five were cultivated in LBkan (50) medium, and four of the broths were miniprepped in order to isolate the plasmids. The DNA concentration of the isolated samples was measured (table 3.2).

Table 3.2 – Absorption results for pET28a.

Sample	ng/µl	A260	A280	260/280	260/230
pET28a 1	53.78	1.076	0.514	2.09	3.56
PET28a 2	44.60	0.892	0.596	1.50	3.16
PET28a 3	30.99	0.620	0.210	2.95	9.42
PET28a 4	38.90	0.778	0.421	1.85	4.15

All four of the isolated samples and a positive control (G3) were digested with *AvaI* and electrophoresed on a 1.5% agar gel with 1 kb serving as a ladder (figure 3.4).

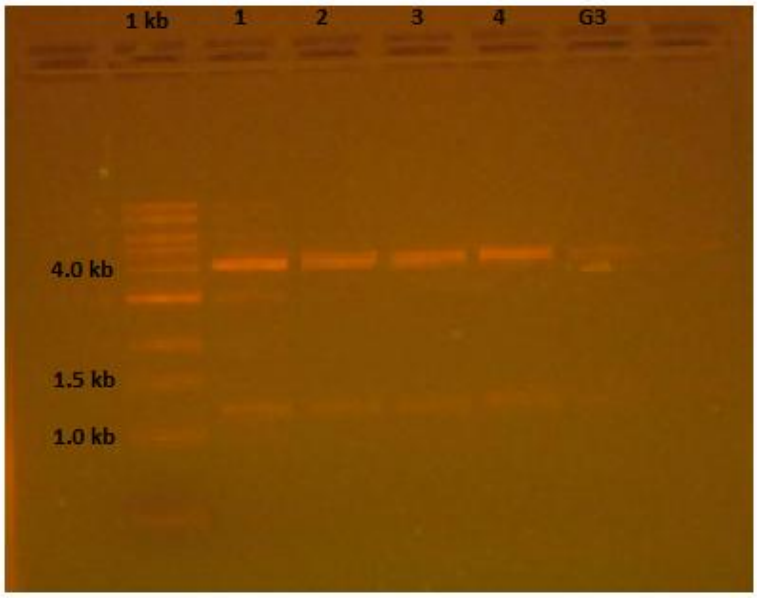

Figure 3.4 – Verification of pET28a samples. The samples had been digested with AvaI and then electrophoresed on a 1.5% agar gel along with a 1 kb ladder and a positive control (G3).

Bands the size of roughly 4.0 and 1.2 kb appear for every sample, and also very faintly for the positive control.

3.3 Insertion of *lec-1* into pET28a

After the double digestion the plasmids (pET28a 1) and *lec-1* (*lec*-1-1), the A_{260} of the samples was measured in order to determine their DNA concentration (table 3.3), and the amounts adjusted in order to have a fivefold amount of insert to vector in the ligation.

Table 3.3 – Absorption results for double digested samples.

Sample	ng/µl	260	280	260/280	260/230
lec-1	31.94	0.639	1.033	0.62	0.17
pET28a	195.31	3.906	5.875	0.66	0.65

The ligated product was inserted into TOP10 cells and plated. Of four plates, 10 colonies grew. Seven of these underwent a PCR analysis to check whether the ligation truly had succeeded. The PCR products were electrophoresed on a 1.5% agar gel with 1 kb as a ladder (figure 3.5).

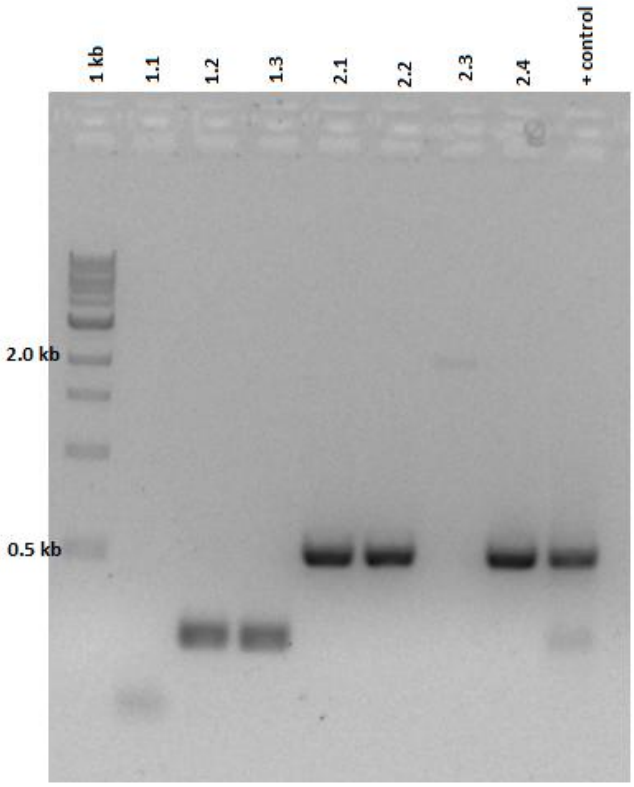

*Figure 3.5 – Verification of ligated samples (pET28a-**lec-1**). Along with seven samples, a positive (+) control and 1 kb ladder were electrophoresed on a 1.5% agar gel as comparison.*

Three of the seven samples have bands the size of 500 bases, and the other 4 samples have either a 2.0 kb band or band of unknown size compared to the ladder.

The three colonies corresponding to the samples with the 500 base bands were cultivated in LBkan (50) medium and the plasmids were isolated. To determine whether these plasmids really did have *lec-1* as an insert, they were double digested with the same restriction enzymes with which they had been digested previously (*Nco*I and *Eco*RI). The digested products were then electrophoresed on a 1.5% agar gel with 1 kb as ladder (figure 3.6).

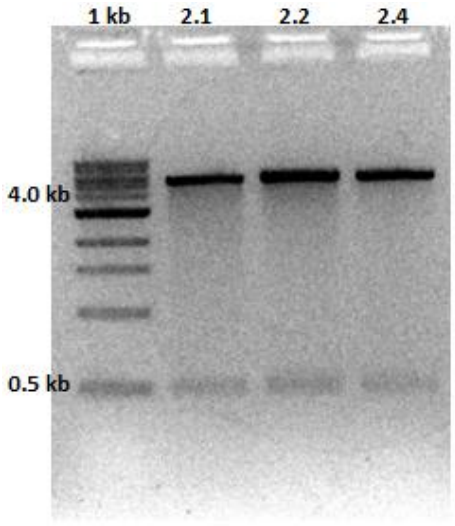

*Figure 3.6 – Double digested samples (pET28a-**lec-1**) electrophoresed on a 1.5% agar gel along with a 1 kb ladder.*

All three of the samples show bands of 0.5 kb and larger than 4.0 kb.

3.4 SDS-PAGE gel analysis for LEC-1

All the correct pET28a-*lec-1* samples (2.1, 2.2. and 2.4) were inserted into BL21 (DE3), and each culture was plated on two LBkan (50) agar plates (table 3.4).

*Table 3.4 – Number of colonies grown from cell transformation with pET28-**lec-1**.*

Plate	Colonies		
	2.1	2.2	2.4
1	≈ 200	≈ 156	≈ 164
2	≈ 204	≈ 220	≈ 132

One colony from each sample (2.1, 2.2 and 2.4) and a negative control (pET28a) were cultivated in LBkan (50) medium and then in lactose medium. Samples were taken from both types of broth and electrophoresed on a 12% SDS-PAGE gel in order to determine the production of LEC-1 (figure 3.7).

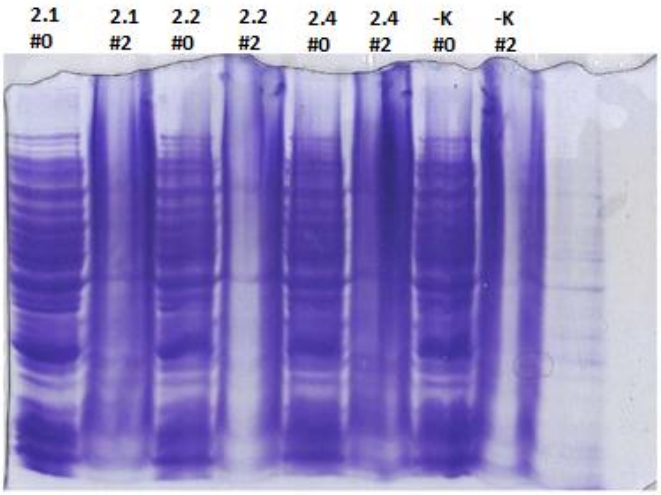

*Figure 3.7 – Samples from lysed BL21 (DE3) containing pET28a-**lec-1**, that had been cultivated in LBkan (50) and then lactose medium, electrophoresed on a 12% SDS-PAGE gel. Three different colonies were cultivated, 2.1., 2.2 and 2.4. #0 = samples from LBkan (50) and #2 samples = samples from lactose medium.*

The #2 lanes are all smudged and are unreadable. The bands at #0 lanes are rather thick, and it is difficult to distinguish one from the other.

3.5 His-tag protein purification

After placing the supernatant (from the cell lysis) on a column in order to further isolate LEC-1, three samples were collected. These samples were the flowthrough of the supernatant, first wash, and elution. The samples were electrophoresed on a 15% SDS-PAGE gel, with a BenchMark protein ladder (from Invitrogen), so as to examine where the protein would come off the column (figure 3.8).

a) b)

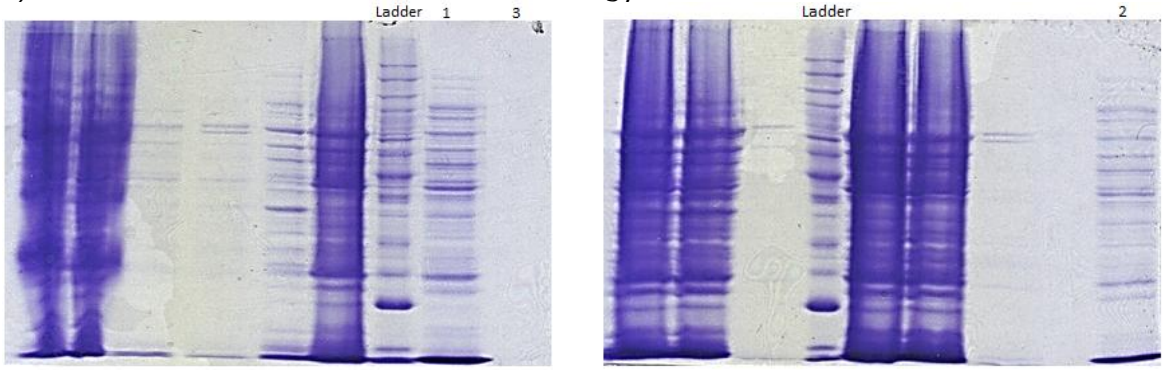

Figure 3.8 – Purified protein samples collected from a column containing His-select® nickel affinity gel, electrophoresed on a 15% SDS-PAGE gels (a and b) with a BenchMark protein ladder. The samples are from the supernatant (1), wash (2), and elution (3). Unmarked samples are irrelevant.

Bands appeared in the lanes of samples 1 and 2, which seem similar, but none for sample 3.

3.6 Insertion of YFP into pET28a-*lec-1*

After the plasmids had been digested and ligated with YFP, they were inserted into TOP10 and BL21 (DE3) cells, as was a negative control (pET28a-*lec-1*), and plated (table 3.5).

Table 3.5 – Number of colonies grown, containing ligation product or negative control, in TOP10 and BL21 (DE3) cells.

Plate	Cell type	Insertion	Number of colonies
-	TOP10	pET28a-*lec-1*	40
1 (T1)	TOP10	pET28a-*lec-1*-YFP	≈ 200
2 (T2)	TOP10	pET28a-*lec-1*-YFP	≈ 400
-	BL21 (DE3)	pET28a-*lec-1*	0
-	BL21 (DE3)	pET28a-*lec-1*-YFP	0

15 colonies from TOP10 plates 1 and 2 were chosen at random to undergo PCR, along with a positive control, so as to analyze whether the ligation product truly contained YFP. The PCR products were electrophoresed on a 1.5% agar gel with a 1 kb ladder (figure 3.9).

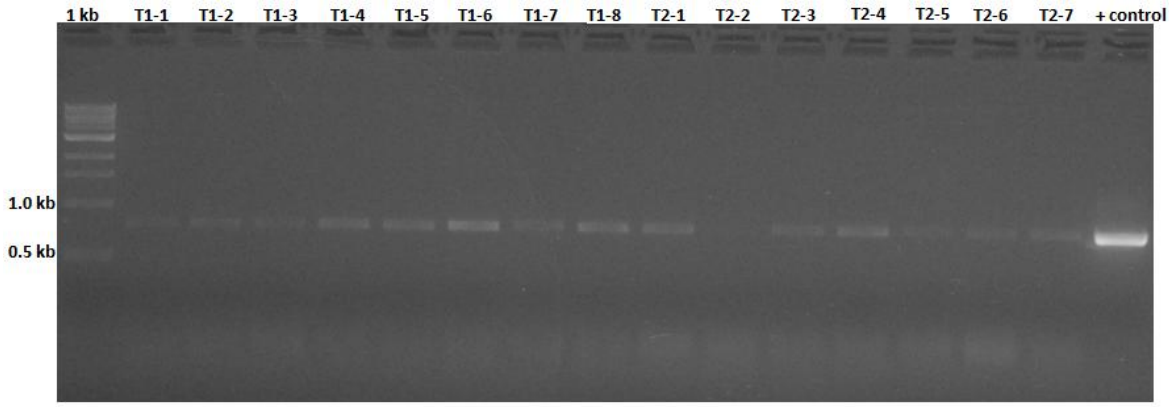

*Figure 3.9 – Verification of ligated samples (pET28a-**lec-1**-YFP). Along with 15 samples, a positive control and 1 kb ladder were electrophoresed on a 1.5% agar gel for comparison.*

Every sample, apart from T2-2, shows a band a little under 1.0 kb in size, the same size as shown by the positive control.

Two colonies (T1-6 and T1-8) were cultivated in LBkan (50) medium, the plasmids were isolated, and their DNA concentration measured (table 3.6).

Table 3.6 – Absorption results for ligation product.

Sample	ng/µl	A260	A280	260/280	260/230
T1-6	38.79	0.776	0.475	1.63	4.62
T1-8	30.34	0.607	0.392	1.55	5.84

3.7 His-tag protein denaturation

After the supernatant (from the cell lysis) had been placed on a column so as to further isolate LEC-1-YFP, four samples were collected. These samples were the flowthrough of the supernatant, first wash, first elution, and a second elution. The samples were electrophoresed on a 12% SDS-PAGE gel, with a BenchMark protein ladder, in order to determine where the protein would be released from the column (figure 3.10).

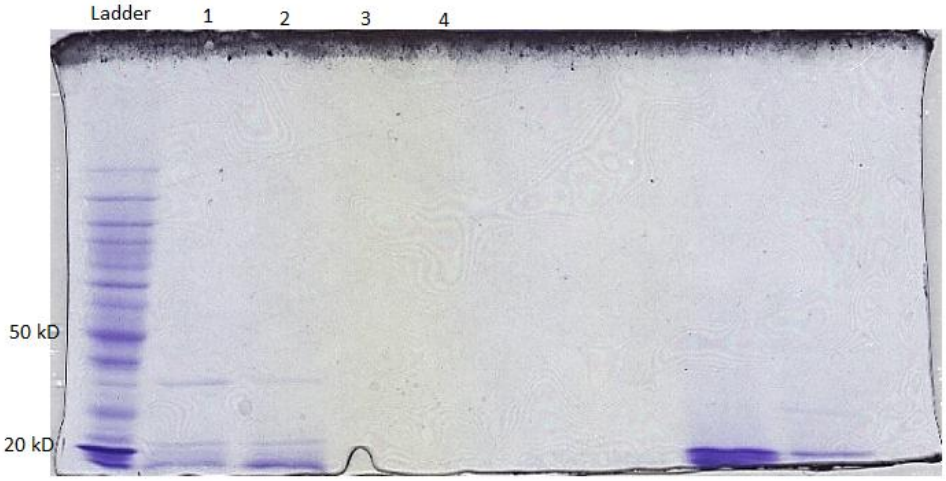

Figure 3.10 – Denatured protein samples collected from a column containing His-select® nickel affinity gel, electrophoresed on a 12% SDS-PAGE gel with a BenchMark protein ladder. The samples are from the supernatant (1), first wash (2), first elution (3), and second elution (4). Unmarked samples are irrelevant.

Bands only appeared in samples 1 and 2, they are very faint and very few, or a total of four. No bands appeared in the lanes where samples 3 and 4 are located.

3.8 Production of LEC-1

Samples were taken every hour for five hours after the BL21 (DE3) were induced, to produce LEC-1 by addition of IPTG to the broths. A_{600} measurements were made for every sample (figure 3.11).

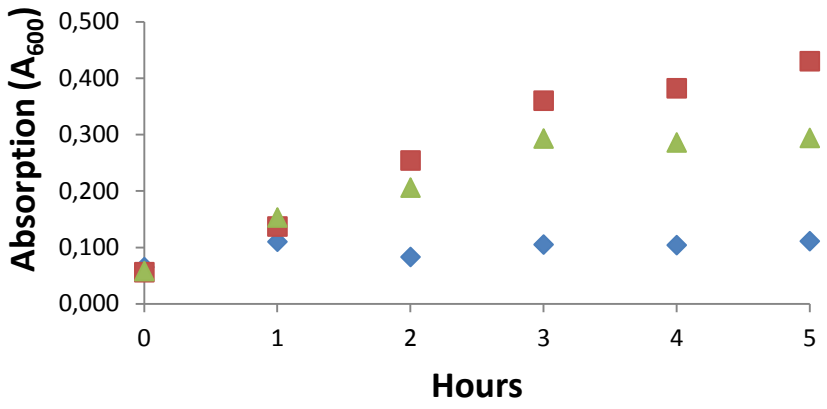

*Figure 3.11 – Light absorption of different samples hourly. The samples were BL21 (DE3) broths, that contained either pET28a (◆), pET28a-**lec-1** (■) or pET28a-**lec-1**-YFP (▲).*

The broth containing pET28a-*lec-1* increased the most in density, followed closely by pET28a-*lec-1*-YFP. The broth containing the negative control (pET28a) increased least.

All samples were lysed and electrophoresed on a SDS-PAGE gel, in order to determine the difference in LEC-1 production. pET28a-*lec-1* samples along with the negative control were electrophoresed on a 15% SDS-PAGE gel (figure 3.12). The pET28a-*lec-1*-YFP samples, however, were electrophoresed on a 12% SDS-PAGE gel (figure 3.13). Both gels were run with a BenchMark protein ladder.

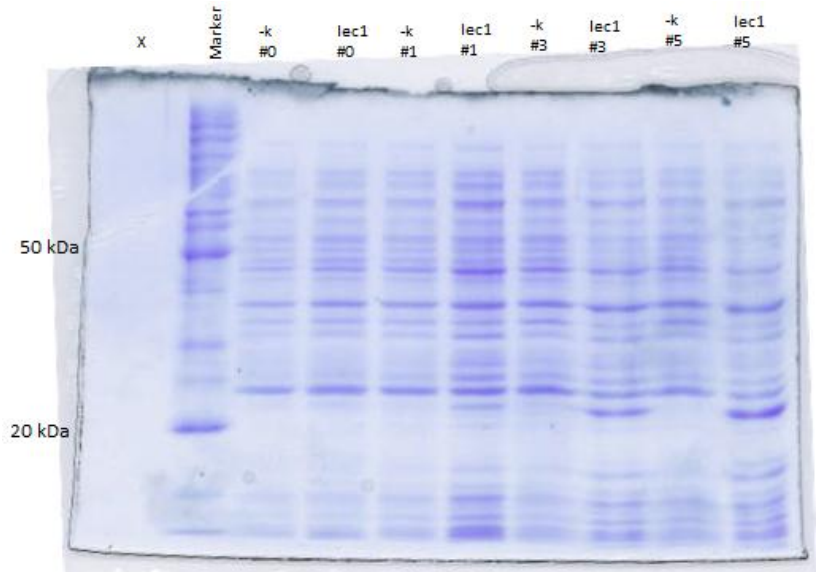

*Figure 3.12 – Supernatant from lysed BL21 (DE3) cells containing either pET28a-**lec-1** or pET28a electrophoresed on a 15% SDS-PAGE gel. Benchmark protein ladder was used as a marker on the gel. Samples were taken hourly for five hours, and are marked correspondingly, #1 = sample taken after one hour. lec1 = pET28a-**lec-1** and –k = pET28a (negative control).*

Nearly all of the protein bands are identical between samples. In lanes lec-1 #1, #3 and #5, however, a band can be seen. This band is a little larger than the 20 kDa band of the ladder. There is not a corresponding band in the negative control lanes.

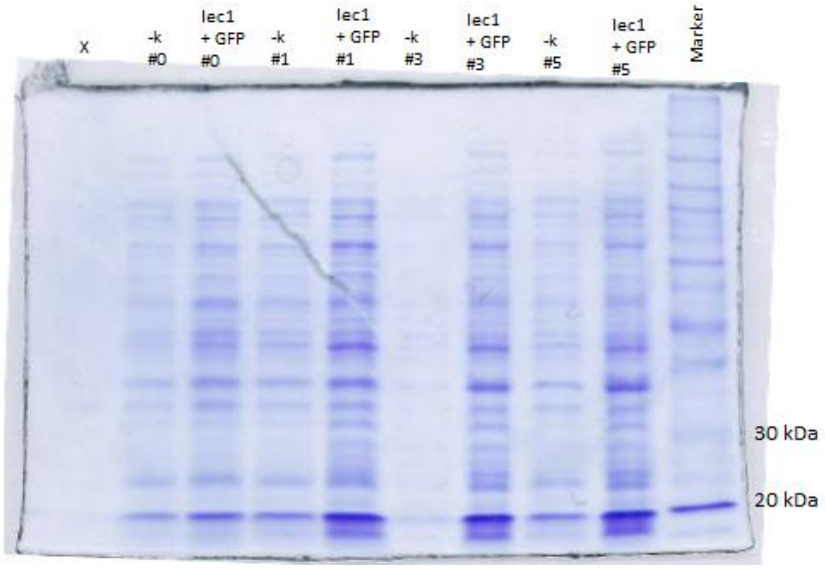

*Figure 3.13 – Supernatant from lysed BL21 (DE3) cells containing either pET28a-**lec-1**-YFP or pET28a electrophoresed on a 12% SDS-PAGE gel. Benchmark protein ladder was used as a marker on the gel. Samples were taken hourly for five hours, and are marked corrispondingly, #1 = sample taken after one hour. lec1+GFP = pET28a-**lec-1**-YFP and –k = pET28a (negative control).*

Most of the protein bands are identical for all samples. There are, however, some bands that can only be found in samples lec1-GFP #1, #3, and #5. These bands are located directly below and slightly above the 20 kDa band of the marker, and between the 20 and 30 kDa marker bands.

3.9 Comparison of methods

The pellet and supernatant of lysed BL21 (DE3), containing either pET28a-*lec-1* or pET28a (negative control) were electrophoresed on a 15% SDS-PAGE gel along with a Benchmark protein ladder as a marker. Samples from the same volume were electrophoresed together (figures 3.14 and 3.15).

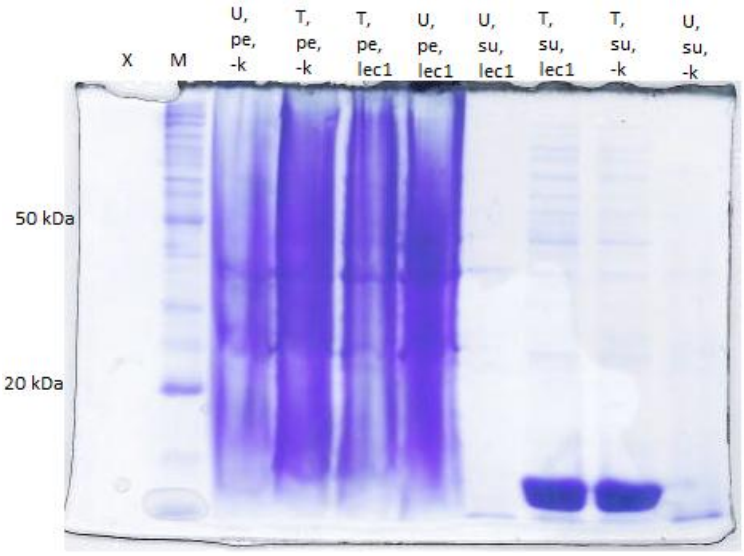

*Figure 3.14 – Lysed samples from 1.5 ml broth volume were electrophoresed on a 15% SDS-PAGE gel. Benchmark protein ladder was used as a marker (M). U = urea method, T = traditional method, pe = pellet, su = supernatant, -k = negative control (pET28a), and lec1 = samples that contained pET28a-**lec-1**.*

The lanes where the pellets are located are smudged, and there seem to be only two bands visible in all four lanes. There appear to be two bands at the lec1 and negative control, urea method supernatant, though very faint. More bands appear for both lec1 and the negative control, traditional method supernatant, though these are all faint as well, except for the ones near the end of the gel, which are extremely strong.

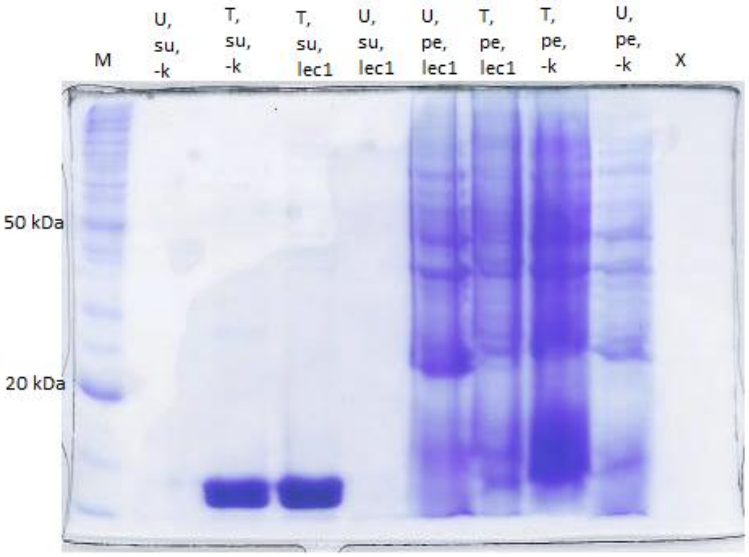

*Figure 3.15 – Lysed samples from 0.5 ml broth volume were electrophoresed on a 15% SDS-PAGE gel. Benchmark protein ladder was used as a marker (M). U = urea method, T = traditional method, pe = pellet, su = supernatant, -k = negative control (pET28a), and lec1 = samples that contained pET28a-**lec-1**.*

The samples from the pellets are slightly smudged, but some bands can be seen, and these appear to be identical. There seems to be a band in the lec1, traditional method pellet lane, slightly above the 20 kDa marker band, which cannot be seen in the other pellet lanes. No bands can be seen in the supernatant lanes, except for identical bands in lec1 and negative control, traditional method lanes, which are located near the end of the gel.

4 Discussion

4.1 Reverse transcription

After the RNA was reverse transcribed and amplified, bands the size of 526 bases were expected on the 1% agar gel. The gene itself is only 502 bases, but with the addition of primers there was an increase of 24 bases (each primer has 12 bases located outside of the gene). According to figure 3.1, only bands of the expected size appear in the mRNA lanes. In all lanes (total RNA, mRNA, and negative control), there were bands of unknown size near the end of the gel. These are expected to be primer dimers. Because of these results the study was continued using the cDNA that had been reversed transcribed from the mRNA.

Although the amplified RT-PCR products were purified by pricking the *lec-1* band, the absorbance results show that the pooled samples are below the accepted ratio for 260/280 (1.8) (Thermo scientific, 2013) as *lec-1*-1 is 1.67 and *lec-1*-2 is 1.76 (table 3.1). These results do not necessarily mean that the samples are bad or contaminated. At this time the cDNA were not isolated and there were still enzymes and nucleotides in the samples, which could have affected the measurements.

4.2 pET28a

Very few, or a total of six, colonies grew after plating of the transformation mix. Of these, two were clear, unlike a typical *E. coli* colony, which has a cream colour appearance. They were, therefore, not used further in this study.

The isolated plasmids A_{260} were measured and the results (table 3.2) show that all but one have a 260/280 value higher than 1.8. Their 260/230 measurements are also above the accepted ratio of 2.0 (Thermo scientific, 2013). The samples pET28a 1, 3, and 4 are, therefore, all very pure samples.

In order to verify whether the isolated plasmids were in fact pET28a, all four samples were digested with *Ava*I (figure 3.4). The bands that were expected are of the sizes 1229 and 4140 bases. All the samples showed bands of those sizes; brighter even than the positive control showed. Thus, it was possible to use these samples of pET28a in this study.

4.3 Insertion of *lec-1* into pET28a

The A_{260} results of double digestion samples (table 3.3) showed very low 260/280 and 260/230 values, but they can be written off due to the presence of restriction enzymes.

The colony growth was again very low, or only ten colonies. To verify that the ligation had been successful, seven colonies were examined in order to determine whether they indeed contained *lec-1* (figure 3.5). Bands of the size 529 bases were expected, and three of the seven samples (2.1, 2.2 and 2.4) showed those results. Two of the other four

samples (1.2 and 1.3) showed primer dimers, while 2.3 showed a very faint 2.0 kb band, and 1.1 showed a band of smaller size than a primer dimer. I was unable to explain the results of samples 2.3 and 1.1.

Samples 2.1, 2.2 and 2.4 underwent another test to ensure that the ligation was true. They were digested again (figure 3.6) and were expected to show two bands, 512 and 5265 bases. These appeared, confirming that the ligation had been a success.

4.4 SDS-PAGE gel analysis for LEC-1

A good number of colonies grew following the insertion of pET28a-*lec-1* into BL21 (DE3) (table 3.4). The number of colonies ranged from 130 to 220 colonies per plate.

To identify LEC-1 on a SDS-PAGE gel, negative control samples were run with samples containing pET28a-*lec-1* (figure 3.7). The samples taken after LEC-1 was induced (#2) were of such large cell volumes (90 µl cells) that the lanes in the gel were all smudged and no bands were noticeable. The samples taken before LEC-1 was induced (8 µl cells) do have bands, but these are so wide that many merge together. These results are unusable, since nothing can be read from the gel.

4.5 His-tag protein purification

When isolating and purifying LEC-1 using a column with His-select® nickel affinity gel, the protein was expected to be released from the nickel with the elution buffer. That is, the imidazole should have competed with the protein for ligation with the nickel. Considering figure 3.8, the result is not what was expected. LEC-1 was expected to come down off the column in sample 3, the elution stage, but there no bands were present on the gel. Bands did, however, appear from samples 1 (flowthrough) and 2 (wash).

It appears that the protein did not bind to the nickel in the column, and may have come off the column with the flowthrough. The reason for these results may be that the protein was in its natural form (aggregated), and therefore the 6X His tail was unable to bind to the nickel.

4.6 Insertion of YFP into pET28a-*lec-1*

A good number of TOP10 with insert grew, but no BL21 (DE3) (table 3.5). It was not thought unusual that BL21 (DE3) did not grow, as the plasmids had apparently not been able to penetrate the cells, and therefore the cells could not have survived in broth containing kanamycin.

In order to verify that the ligation truly took place, colonies were tested for whether they contained YFP (figure 3.9). Comparing the samples to the positive control, all but one sample contained YFP. Two of the colonies containing YFP were cultivated, and the plasmids isolated. Their 260/280 value were below the accepted ratio, though their 260/230 value was very good (table 3.6).

4.7 His-tag protein denaturation

When isolating LEC-1-YFP, using a column with His-select® nickel affinity gel, the protein was expected to be released off the nickel when the elution buffer was added to the column. The low pH level of the elution buffer should have converted the negative charge of the protein´s 6X His tail into a positive charge. This in turn would have a repelling affect for LEC-1-YFP and the nickel, because nickel has a positive charge. Considering figure 3.10, the result is not what was expected. No protein was eluted or washed from the column. The only proteins present in the supernatant came off the column in the flowthrough. Therefore, it is unlikely that there was any LEC-1-YFP in the supernatant, since it´s denatured appearance should have bound to the nickel. Thus, another study was performed, "Comparison of methods", in order to determine whether the protein might have been spun down into the cell pellet.

4.8 Production of LEC-1

There was a difference in growth rate for BL21 (DE3) containing pET28a, pET28a-*lec-1*, or pET28a-*lec-1*-YFP (figure 3.11). The slowest growth was that of the negative control (pET28a), which appears to have entered it´s stationary phase after the first hour. The cells containing pET28a-*lec-1*-YFP and pET28a-*lec-1* grew faster, and after the last sample it seemed that *lec-1* was still growing, while *lec-1*-YFP seems to have entered it´s stationary phase after the third hour.

The SDS-PAGE gels were expected to show a band for LEC-1 and LEC-1-YFP not corresponding to the negative control. The result from the 15% SDS-PAGE gel (figure 3.12) shows identical bands between samples, except for three bands. These bands are a bit larger than 20 kDa, and are located at samples lec1 #1, #3, and #5. The band becomes clearer every hour, indicating an increasing amount of the protein. This is very likely LEC-1, since it´s not present in the negative control, even though the protein was expected to be 18.5 kDa (Miao et al., 2012).

The result from the 12% SDS-PAGE gel (figure 3.13) shows matching bands between LEC-1-YFP and the negative control. There are, however, bands that only appear in the lanes of lec1-GFP #1, #3 and #5. These bands are located directly below, slightly above the 20 kDa band of the marker, and between the 20 and 30 kDa marker bands. Of these three bands, LEC-1 might be located near the end of the gel (below 20 kDa). The other bands do not correspond to the location of LEC-1 in figure 12. Also the band at the end of gel seems to become stronger every hour. Those bands might therefore be degraded protein, but LEC-1 contains six rare arginine codons, which are known to hinder translation and lead to degradation (Hayes, Bose & Sauer, 2002).

4.9 Comparison of methods

Two different volumes were used for this study, and it is difficult to read the 1.5 volume 15% SDS-PAGE gel (figure 3.14). All the pellet samples seem to have clogged the lanes, so they are completely covered in coomassie blue dye. A few bands are noticeable, but they are of no use because they are identical. The supernatant samples on the other hand have really very faint bands which appear to be identical. There is, however, a band at the

end of the gel in lanes lec1 and negative control, traditional method, which are extremely strong. No LEC-1 band can be read from this gel.

The pellet lanes are also clogged in the 0.5 ml 15% SDS-PAGE gel (figure 3.15), but not nearly so much as in the other gel. There are actually comparable bands and sample lec1, urea method has a band slightly above 20 kDa, which does not appear in the other pellet lanes. No bands are noticeable in the supernatant lanes, except for two very strong bands at the end of the gel at samples lec1 and negative control, traditional method.

Samples lec1 and negative control in both gels show a singular strong band at the end of the gels. Corresponding bands can not be found when comparing figures 3.14 and 3.15 to figure 3.12. The likely explanation is that these are degradated proteins as can be found in figure 3.13 (for more details, see 4.8 Production of LEC-1).

It is very likely that the extra band at sample lec1 pellet, urea method (figure 3.15) is the LEC-1 protein. This would explain why no protein was present in the “His-tag protein denaturation” study, since there only the supernatant was tested (see 3.7 His-tag protein denaturation, figure 3.10).

5 Conclusion

Overall, the project results were acceptable. All the ligations were successful, although cell growth on the LBkan (50) plates and liquid medium varied. It was only during the final stage of the project that the results became unclear. What is believed to be LEC-1 on the SDS-PAGE gels is larger than what was expected. The samples should, therefore, be electrophoresed on a SDS-PAGE gel one more in order to confirm these results. Precautions must be taken in order not to overload the gels, which can lead to useless results.

It is most important, when conducting protein isolation and analysis, that after cells have been lysed the experiment continue to examine both the supernatant and pellet. As the results from this project showed, target proteins can be found in pellets after lysis. By examining the pellet as well as the supernatant, a possible loss of target proteins could be prevented, this could ruin time consuming experiments.

In order to obtain even clearer results it is possible to try to increase the protein yield by cultivating the expression cells in higher density growth medium (along with IPTG) than LB. This would increase the total amount of protein-producing cells, and therefore the yield of LEC-1. The yield should also increase by using cells supplemented with tRNAs recognizing rare codons as an expression cell. This may prevent the degradation of LEC-1 caused by the rare arginine codons (Expression Technologies Inc., 2003).

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Appendix

pET-28a(+) sequence landmarks	
T7 promoter	370-386
T7 transcription start	369
His•Tag coding sequence	270-287
T7•Tag coding sequence	207-239
Multiple cloning sites	
(*Bam*H I - *Xho* I)	158-203
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	773-1852
pBR322 origin	3286
Kan coding sequence	3995-4807
f1 origin	4903-5358

The maps for pET-28b(+) and pET-28c(+) are the same as pET-28a(+) (shown) with the following exceptions: pET-28b(+) is a 5368bp plasmid; subtract 1bp from each site beyond *Bam*H I at 198. pET-28c(+) is a 5367bp plasmid; subtract 2bp from each site beyond *Bam*H I at 198.

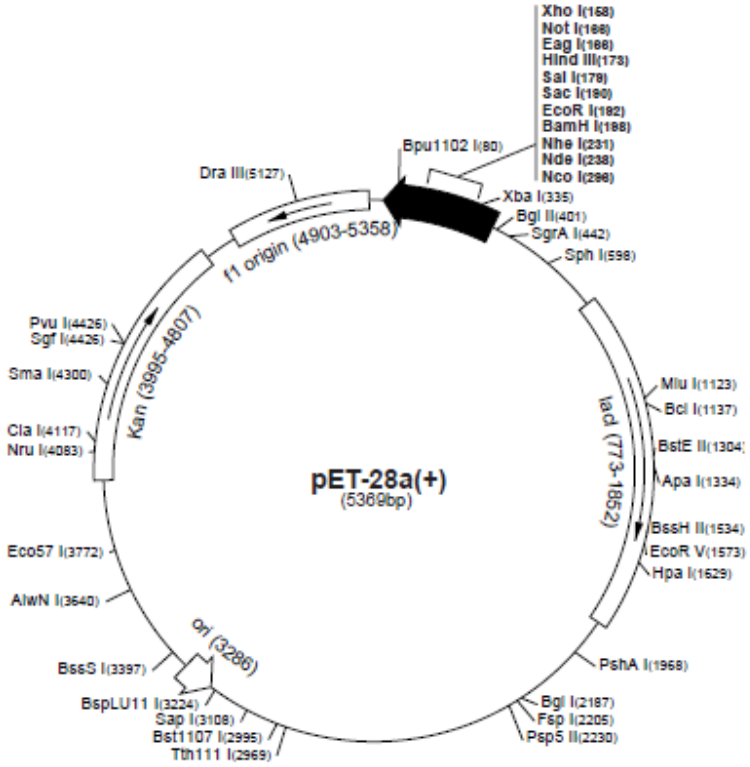

Figure A – pET28a. A circular map of the plasmid, list of sequence landmarks and detailed cloning/expression regions (Novagen, n.d.).