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Evaluation of the *Pm*/XylS expression system for production of IFN-alpha-2b in *Cupriavidus necator* and *Escherichia coli*

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Preface

This thesis concludes my degree Master of Science in Chemical Engineering and Biotechnology at the Norwegian University of Science and Technology (NTNU) in Trondheim. The thesis was written at the Department of Biotechnology in collaboration with Vectron Biosolutions AS. The experimental work presented herein was conducted during the fall of 2015 and spring 2016 at the Department of Biotechnology's laboratories.

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Abstract

Recombinant bacteria are used for production of human proteins such as the medical important cytokine Interferon-α-2b (IFN-α-2b). In the present study, *Escherichia coli (E.coli)* and *Cupriavidus necator (C.necator)* have been used as expression hosts for production of human IFN-α-2b. The *Pm*/XylS expression system, originating from the *Pseudomonas putida* TOL plasmid, is used for recombinant gene expression. A set of expression vectors based on the broad-host-range plasmids RK2 or pBBR1, in combination with the *Pm*/XylS expression cassette, was constructed. Previous research has identified high-expression variants of regulatory elements of the *Pm*/XylS expression cassette, combined these variants and successfully obtained high protein expression. In order to evaluate the expression of IFN-α-2b, a high-expression variant of the *Pm*/5'UTR element in combination with a codon-optimized version of the IFN-α-2b gene was compared with the wild-type *Pm*/5'UTR variant in combination with a codon-optimized IFN-α-2b gene. In addition, the expression of IFN-α-2b in the presence/absence of the signal peptide *pelB* was evaluated. Soluble and insoluble IFN-α-2b fractions were observed with both *E.coli* and *C.necator* as expression host. However, it was generally detected higher expression of IFN-α-2b in *E.coli* than *C.necator*. Strong expression cassette elements without *pelB* were favorable for the expression of soluble IFN-α-2b, while strong expression cassette elements in combination with *pelB* promoted the expression of insoluble IFN-α-2b. For both of the expression hosts, a larger fraction of the protein was found as insoluble than soluble IFN-α-2b. As far as one knows, detectable expression of soluble IFN-α-2b is not previously been reported in neither *E.coli* nor *C.necator*. This study has reported clearly detectable levels of soluble IFN-α-2b in both of these strains.

Sammendrag

Rekombinante bakterier brukes til produksjon av humane proteiner, blant annet kan cytokinet Interferon-α-2b (IFN-α-2b) uttrykkes rekombinant i bakterier. I dette studiet har *Escheriachia coli (E.coli)* og *Cupriavidus necator (C.necator)* blitt brukt som produksjonsverter for ekspresjon av det humane proteinet IFN-α-2b. Ekspresjonssytemet *Pm*/XylS, med opprinnelse fra *Pseudonomas putida* TOL plasmid, er brukt for ekspresjon av rekombinant protein. Det ble konstruert en samling av ekspresjonsvektorer basert på plasmidene RK2 og pBBR1, som begge er funnet til å fungere i mange bakteriearter, kombinert med ekspresjonskassetten *Pm*/XylS. Tidligere forskning har identifisert varianter av *Pm* og 5'UTR av ekspresjonskassetten *Pm*/XylS som gir sterkere ekspresjon, kombinert disse elementene, og raportert om vellykket protein produksjon. For å evaluere ekspresjonen av IFN-α-2b i de to vertsorganismene, ble varianten av *Pm*/5'UTR elementet som gir sterk ekspresjon kombinert med en kodonoptimalisert versjon av IFN-α-2b genet, og sammenlignet med villtype varienten av *Pm*/5'UTR kombinert med en kodonoptimalisert versjon av IFN-α-2b genet. I tillegg ble det testet for ekspresjon ved tilstedeværelse av signalpeptidet *pelB*. Det ble detektert produksjon av løselig og ikke-løselig IFN-α-2b fraksjoner i både *E.coli* og *C.necator*. Det ble imidlertid detektert høyere produksjon av IFN-α-2b i *E.coli* enn *C.necator*. Sterk ekspresjonskassett uten *pelB* ble funnet til å gi høyest andel løselig protein, mens sterk ekspresjonskassett med *pelB* ble funnet til å gi høyest andel ikke-løselig protein. Den største fraksjonen av protein ble funnet som ikke-løselig IFN-α-2b, noe som var gjeldene for begge produksjonsvertene. Så vidt man vet er det til nå ikke raportert om detekterbar produksjon av løselig IFN-α-2b i hverken *E.coli* eller *C.necator*. Denne studien har raportert om produksjon av detekterbare mengder løselig IFN-α-2b i begge de nevnte produksjonsvertene.

Contents

Nomenclature

Amp	Ampicillin
Amp^r	Ampicillin resistance
Bp	Base pair
Cm	Cloramphenicol
Cm^r	Chloramphenicol resistance
dH_2O	Distilled H_2O
DNA	Deoxyribonucleic acid
IB	Inclusion bodies
IFN-α-2b	Interferon-alpha-2b
LA	Luna Bertani Agar Medium
LB	Luna Bertani Medium
mRNA	messenger ribonucleic acid
OD_{600}	Optimal density measured at a wavelength of 600 nm
ORF	Open reading frame
PCR	Polymerase chain reaction
SD	Shine Dalgarno
tRNA	transfer ribonucleic acid
UTR	Untranslated region
α	alpha
β	beta

1 Introduction

1.1 Recombinant DNA technology

Biotechnology is defined as the use of any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use (definition by United Nations). Since the early days of biotechnology, humans have been manipulating the natural environment to improve the food supplies, housing and health [13]. Traditional biotechnological products such as bread, cheese, wine and beer have been made worldwide for centuries using microorganisms [13]. Today, applications of biotechnology can be divided into several branches, such as blue (marine), green (agricultural), white (industrial) and red biotechnology (medical), respectively. Medical biotechnology is especially important for the work presented in this thesis. This branch may include the use of molecular biology and genetic engineering to utilize more specific and compatible medicines.

Recombinant DNA technology includes the techniques that are used to separate and recombine DNA segments to create recombinant DNA, where the purpose is production of a desired substance for the harvesting or for the benefit of the host organism itself. The production of recombinant proteins in bacteria is an important technique within recombinant DNA technology. This technique has experienced major improvements the past decades, allowing us to design plasmid-based vectors by combining DNA elements from different plasmids to produce recombinant proteins. The use of engineered bacteria, is increasing productivity and cost-efficiency. It may also improve the protein purity. The use of recombinant bacteria is beneficial within research, therapy and diagnostics.

1.2 *E.coli* as an expression platform for recombinant protein production

1.2.1 *E.coli* as a host

Escherichia coli (*E.coli*) was discovered in 1885 by Theodor Escherich, and is today widely used as a bacterial host. It is frequently used as a model organism because of its well-characterized metabolic, genetic and morphological features. Other advantages achieved by using *E.coli* as a host are simple media requirement and fast growth rate. Many expression systems have been developed for *E.coli*, enabling a more efficient production of recombinant proteins.

However, not all recombinant genes are expressed efficiently in *E.coli*. The following factors may all result in less efficient protein expression; mRNA instability, differences in codon usage, toxicity of gene products, inappropriate protein folding, lack of post-translational modifications, degradation of the product by the host, and lack of secretion mechanisms [38]. The choice of bacterial host and expression system may improve some of these problems. An optimal expression system will have tight control of the expression at all relevant levels, cheap inducer agents which are not metabolized by the host and that do not require an uptake system, and be stable during scale up like high-cell-density cultivation [12]. It is also advantageous if the expression system works across species barriers.

1.2.2 Formation of inclusion bodies

High-level expression of human proteins in *E.coli* often lead to the formation of inclusion bodies (IB). Formation of IB may occur due to partially unfolded recombinant proteins which accumulate in the cytoplasm [46]. When being a part of an inclusion body the protein is neither soluble nor active[46] [22]. Factors which lead to IB formation may be high protein concentration due to overproduction, reducing environment in the cytoplasm, lack of post-translational modifications and improper interactions with enzymes involved in protein folding [22].

The formation of IB are common when proteins are overexpressed [22], which is a problem

in recombinant protein expression if the copy number is increased. To be functional the proteins have to be soluble and biologically active. Methods exist to recover the inclusion bodies to soluble, active proteins. This involves denaturation and solubilization, plus renaturation [22]. However, these methods are often inefficient and expensive.

1.2.3 Recombinant proteins to be produced

Expression of recombinant proteins by using *E.coli* as a host, is demonstrated by the production of Interferon-α-2b (IFN-α-2b) in this study. IFN-α-2b belongs to the interferon family of cytokines. Cytokines are proteins which function as cell signal molecules and they are part of the human immune response. IFN-α-2b has antiviral, anti-proliferative and immunomodulatory activities against many mammalian viruses [21]. The protein is used in the treatment of hepatitis B and C, as well as some types of cancer, such as hairy cell leukemia [6]. Previous research shows that IFN-α-2b has been expressed recombinantly in *E.coli*, but with very low expression levels and only insoluble fractions were detected [50].

mCherry is used as a reporter gene. Reporter genes are often used as an indication of successful uptake and expression of a certain gene. mCherry is a fluorescent protein used to visualize expression from the bacteria in vitro [33]. The expression of mCherry is shown by a pink color of the culture, which easily indicate successful uptake of the plasmid carrying *mCherry* and functional expression from this plasmid, i.e. it demonstrates a functional expression system.

1.3 Bacterial gene expression

The expression of a gene occurs through transcription and translation. These two processes mainly regulate the gene expression, and tightly control of their components may result in a gene expression system with the preferred characteristics. The important DNA elements of an expression system are illustrated in Figure 1. This section will give a brief explanation of how genes are expressed in prokaryotes.

1.3.1 The transcriptional process

Transcription may be divided into three major steps: initiation, elongation and termination. During the initiation step, RNA polymerase recognizes the promoter sequence which is the start site of the transcription process. In some cases, transcription factors may be needed to bind the promoter region before the RNA polymerase will recognize it. RNA polymerase uses the template strand of the DNA to synthesis a complementary mRNA strand in a 5' to 3' direction, through a process called elongation. The RNA polymerase synthesis mRNA until it reaches a termination signal which triggers the termination step. Transcription may be regulated by activator and repressor proteins. These proteins are then bound to the promoter region, and either stimulate (positive regulation) or block (negative regulation) the binding of RNA polymerase [13].

1.3.2 The translational process

The translation process may also be divided into three major steps, namely initiation, elongation and termination. Downstream from the promoter sequence is the 5' untranslated region (5' UTR). This region is not translated into a protein, but it contains translational regulatory binding sites, like the Shine-Dalgarno (SD) sequence. The SD sequence is the ribosomal binding site in bacteria, and it is approximately 9 bp upstream from the translational start codon.

An open reading frame (ORF) is a DNA sequence that do not contain a translational stop codon, and hence code for a protein. During the initiation step, the ribosome and translation factors bind to the SD sequence. The translation starts at the first initation codon after the SD sequence, which often is AUG. In the elongation step, the mRNA moves through the ribosome, tRNA recognize complementary codons, and amino acids are added to a growing chain. The elongation continues until the RNA polymerase reaches the translational stop codon. In the termination step, release factors bind to the stop codon and stimulate the release of the polypeptide chain, the ribosome assembly dissociates and may be reused for translation of another mRNA [13].

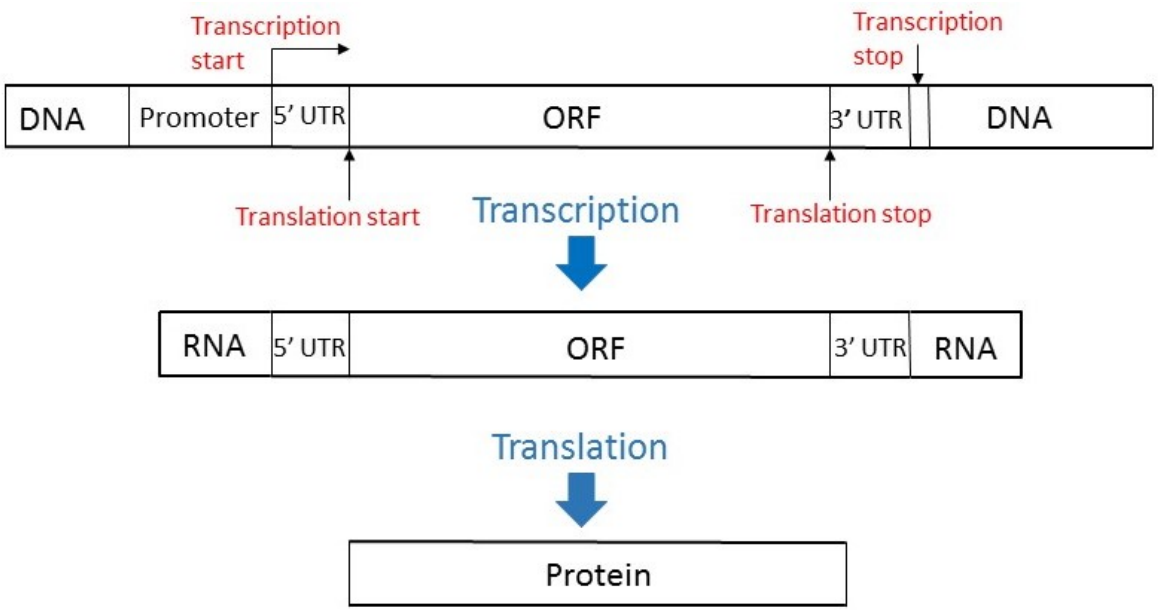

Figure 1: Overview of the gene expression process in prokaryotes. Briefly explained; DNA is transcribed to give RNA, and RNA is translated into a protein. The figure is adapted from Clark (2012) [13].

1.4 The RK2 plasmid

The RK2 plasmid is a broad-host-range plasmid, capable of replicating in a large number of bacterial species [54]. Research has shown that it replicates in many Gram negative species [47][Vectron unpublished results]. The most essential regions in the RK2 replicon are the origin of vegetative replication (oriV) and the gene encoding the replication initiator protein (TrfA) [43]. The TrfA protein binds to iterons in oriV initiating replication of RK2. Specific mutations in the *trfA* gene are found to increase the copy number of the plasmid [20] [26]. The copy-up mutation which is used in this study is termed cop271C, and results in a copy number of approximately 20 [20] [27]. In fact, copy-up mutations can increase the copy number up to 24-fold for some plasmids [55]. Changes are that the higher the copy number of the plasmid is, the higher the protein expression will be [38].

1.5 The *Pm*/XylS expression cassette

1.5.1 The TOL plasmid and its transcription factors

The *Pm*/XylS expression system originate from the toulene-degradative (TOL) plasmid pWW0 of *Pseudomonas putida* [24]. The expression system is found to function in a wide range of bacterial species [9] [19] [10]. The TOL plasmid encodes a pathway for catabolism of xylenes and toluene [59]. The genes involved are grouped into the upper- and lower(meta)-pathway operons, positively regulated by XylR and XylS respectively [12]. XylS belongs to the AraC/XylS family of transcription factors, which are positive regulators for recombinant gene expression control in bacteria. The expression of XylS in the lower-pathway is controlled by two individually regulated promoters, *Ps1* and *Ps2*. *Ps2* is constitutively expressing low levels of XylS protein monomers. In the presence of inducer molecules such as benzoic acid derivatives (m-toluate), XylS interacts with the activator, forming dimers. This complex binds the *Pm* operator, which initiate transcription from the meta-operon promoter *Pm* [40]. Dimerization occurs both in the absence and the presence of an inducer, but to a greater extent in its presence [18]. The fraction of XylS monomers converted into dimers are unknown. There exist a maximum concentration level of active dimers inside the cell, and XylS will form inactive aggregates if the concentration increases beyond this threshold value [61]. Thus, at very high concentrations of XylS inside the cell, the concentration of dimers will be the same in the presence and the absence of an inducer. *Ps1* is activated by XylR resulting in transcription of XylS in the presence of pathway substrates (toluene/xylene). XylR also stimulates transcription from the upper-operon promoter *Pu* in the presence of pathway substrates. As noted above, expression from *Pm* can also be stimulated independently of the effector molecules if XylS is present in high concentrations. Overproduction of XylS usually occurs by xylene-activated XylR stimulation of *Ps1* [1]. A simplified representation of the *Pm*/XylS expression system is illustrated in Figure 2.

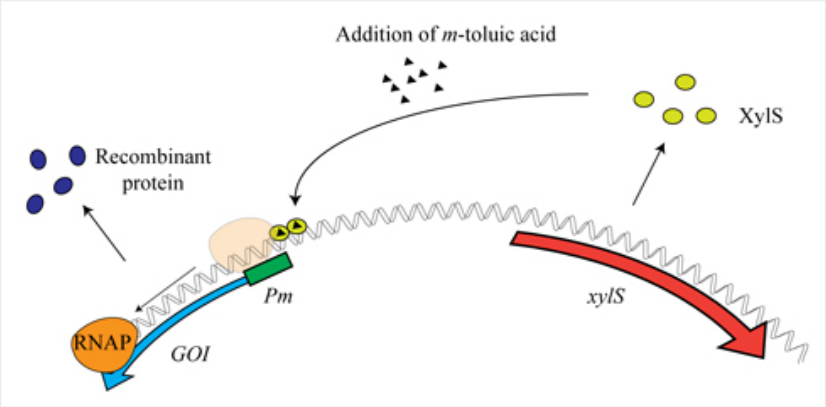

Figure 2: A simplified figurative presentation of the *Pm*/XylS expression system showing the transcription process, but not translation. XylS and m-toluic acid form a complex that binds the *Pm* operator which results in transcription of a given gene and recombinant protein production. (Obtained from Vectron Biosolutions).

1.5.2 pJB658-based expression vectors

When combining elements from the TOL plasmid with the RK2 mini replicon, a broad-host-range vector with a versatile expression cassette is established. This is illustrated in Figure 3 as pJB658 (also termed pVB1, Vectron unpublished), which was constructed in 1997 by Blatny and colleagues [11]. It is a modified version of pJB653, with a more well defined expression system for the use in Gram-negative bacteria. pJB653 was modified such the position of any cloned gene relative to the promoter and ribosome binding site was optimized (see Figure 3). The bases that enclosed the ATG (start codon) were changed by PCR so that new genes to be expressed could be inserted directly into the ATG downstream of the *Pm* by digesting with NdeI [11]. The plasmid also contain the *bla* gene. This gene produces β-lactamase which lead to bacterial resistance of ampicillin [37]. The system is especially useful because of its flexible regulation of the *Pm* promoter combined with the broad-host-range RK2 plasmid which can function in many Gram negative bacteria. Moreover, the effectors act in a concentration-dependent manner and their uptake is based on passive diffusion [49]. The effectors are not metabolized by most bacteria, which makes the system more useful across species barriers [49].

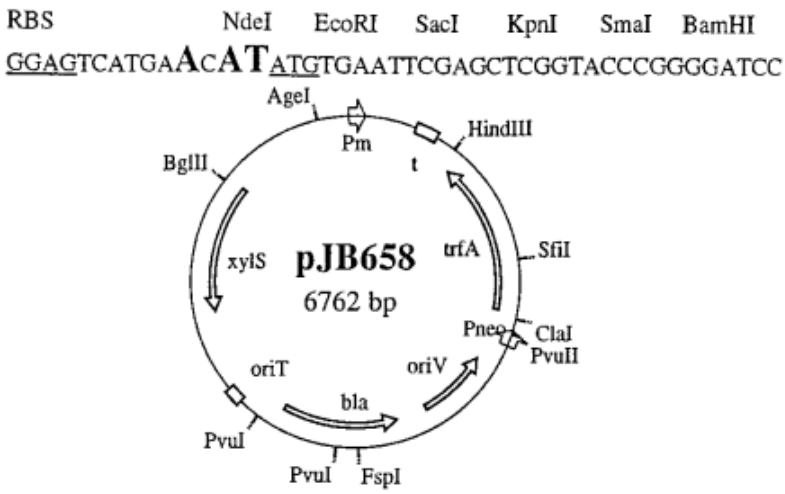

Figure 3: Illustration of pJB658, obtained from Blatny (1997) [11]. The plasmid consists of the *Pm* promoter upstream from the gene, the *xylS* gene, the *trfA* gene, oriV and the *bla* gene.

pJB658 has been modified further in later studies [50][Vectron unpublished results]. pIFN30SpelB (also termed pAT64) is a pJB658-based vector expressing the codon-optimized IFN-α-2b protein with *pelB* signal sequence [50]. The entire gene sequence (originating from pIFN30pelB) was completely redesigned through complete gene synthesis, resulting in a codon-optimized gene without any rare codons and a higher GC content than its parental version [50]. The synthetic gene is 80.4% identical to its parental version, still maintaining the original sequence of the protein product [50].

1.5.3 The 5'-untranslated region, the *Pm* promoter and the *xylS* gene

Important elements of the *Pm*/XylS expression cassette are *Pm*, 5'UTR and *xylS*. As previous described, transcription from the meta-operon promoter *Pm* is stimulated by the activator protein XylS encoded by the *xylS* gene. The 5' untranslated region is critical for efficient translation and is found to contain the SD sequence. The *Pm*/XylS promoter system has been found to be very useful due to its flexible regulation. The *Pm* promoter can be regulated by several cheap and harmless effectors, mutations in *xylS*, 5'UTR and *Pm* are found to affect the expression level, and so are different cell growth temperatures [24] [29] [49].

DNA elements, which are part of the expression system, such as *Pm*, *xylS* and 5'UTR,

have been mutated and selected for the version with the highest expression. The *Pm* version that lead to the highest expression is a variant called ML2-5 [5], also termed comP in another study [60]. This variant is found to stimulate higher transcription level, in addition to increase the final protein expression [60]. A high-expression variant of 5'UTR is found to be a variant called LIII-3 [7], also termed comU in another study [60]. This mutation is found to stimulate higher translation of a given gene. The high-expression variant of *xylS* is named StEP-13 [57], also termed comX in another study [60]. This mutation is found to act at the level of transcription, in addition to increasing the final protein expression.

Increased expression level may also be obtained by combining different evolved DNA control elements of the expression cassette [60]. The variants with the highest expression (described above), were combined to determine if a combination would lead to further improvements [60]. A combination of the two high-expression variants of *Pm* (comP) and 5'UTR (comU) in the same expression system (comPU), gave further stimulation of translation and resulted in higher protein expression. A similar result was obtained for a combination of the high-expression variant of *xylS* (comX) and 5'UTR. However, the comXP combination did not lead to further improvements [60].

The effect of the improved StEP-13 variant of *xylS* on the expression level has been evaluated in several studies [57] [61]. Improved variants of XylS have been obtained by a combination of error-prone PCR and DNA shuffling procedures which resulted in identification of 13 unique shuffled *xylS* genes [57]. One of these mutant variant is StEP-13, which contain five amino acid substitutions (F3Y, I50T, F97L, E195G, M196T). In the work done by Aune and colleagues, the *bla* gene for ampicillin tolerance under transcriptional control of the *Pm* promoter functioned as a reporter gene to test for StEP-13 stimulation on the expression level of β-lactamse [57]. The StEP-13 mutant XylS protein was found to increase the ampicillin tolerance about ninefold compared to wild-type XylS under induced conditions (1 mM m-toluic acid) [57]. The ampicillin tolerance in the absence of the inducer was about ninefold higher than wild-type XylS. The production level of the host-toxic antibody fragment scFv-phOx expressed from *Pm* with improved XylS variant, was about nine times higher than when the wild-type XylS was part of the

expression cassette in the presence of inducer (0.1 mM m-toluic acid) [57].

However, the StEP-13 mutation has later been found only to affect the expression level from *Pm* for low and moderate inducer concentrations [61]. Most likely, StEP-13 is not able to form higher concentrations of active dimers than wild type XylS, but it reaches the maximum level at lower inducer concentrations. Thus, the maximum concentrations of active dimers will be unchanged. However, the dimers will form aggregates at lower XylS concentrations for StEP-13 than for wild type XylS [61].

1.5.4 N-terminal secretion signal sequence

A signal peptide (also called a signal sequence) is an amino acid sequence present at the N-terminus of a protein. It is usually composed of a short positively charged amino-terminal domain, followed by a hydrophobic core and neutral amino acids [3] [41]. A more polar segment is located preceding the cleavage site [3] [41]. The presence of a signal peptide will facilitate translocation of the protein to the periplasm or the extracellular medium. The presence of a signal peptide is found to provide several advantages, such as correct folding, enhanced biological activity, higher product stability and solubility, and a simplified downstream purification process [38]. On the other hand, accumulation of protein in the periplasm can also provide reduced folding and formation of inclusion bodies [38]. Some proteins, including human IFN-α-2b, have a native signal sequence which naturally translocate the protein when being expressed [25]. IFN-α-2b is secreted by human lymphocytes during virus infections, and its native secretion sequence of 23 amino acids directs its secretion from the lymphocytes [25].

In addition to work as a fusion partner which facilitate translocation, the presence of a N-terminal secretion signal peptide has been found to increase the stimulation of the total expression levels of recombinant proteins in *E.coli* A frequently applied signal sequence is the *pelB* signal sequence, which is the one used in this study. The *pelB* gene encode pectate lysate B, originated from *Erwinia carotovora* EC [35]. When expressed recombinantly in *E.coli*, researchers found that a 22-amino-acid leader peptide had been removed (*pelB* signal peptide) [35]. pelB are targeted to the Sec translocation pathway in

E.coli, which is the most common secretion pathway in this organism [39]. The choice of signal sequence for high production level and efficient translocation is found to be highly protein specific and apparently unpredictable in *E.coli* [49] [50].

A previous study has examined the presence of signal sequences, including *pelB*, at the N-terminus of scFv-phOx, GM-CSF and IFN-α-2b when using the pBJ658-based expression system when producing these proteins recombinantly in *E.coli*. [50]. No expression of IFN-α-2b from pIFN30pelB was detected. Thus, a codon-optimized version the IFN-α-2b gene was introduced into the vector (pIFN30SpelB), which resulted in detectable production of the protein (40 mg/L). However, all recombinant IFN-α-2b product was present as inclusion bodies [50]. The presence of pelB was found to increase the expression of scFv-phOx and GM-CSF as well (without codon-optimized genes).

Not all fusion partners used to stimulate recombinant protein production are protein translocation signals, such as pelB. For instance, a short DNA sequence corresponding to the 5' end of the *celB* gene, whose protein product is cytosolic, has been found to be an efficient fusion partner for the expression of IFN-α-2b in *E.coli* [32]. Kucharova and colleagues have found that celB can functionally replace pelB as a 5' fusion partner for efficient expression of IFN-α-2b. celB of various lengths were tested, and a minimum of eight codons were found to stimulate expression more efficient than fusion of pelB [32]. Hence, this suggests that translocation may not be required for efficient expression of IFN-α-2b. However, the underlying mechanism of how fusion partners work are not completely clear [32].

1.6 The pBBR1 MCS plasmid

pBBR1 MCS is a 4.7 bp broad-host-range expression vector derived from pBBR1, illustrated in Figure 4. pBBR1 was originally isolated from *Bordetella bronchisptica* and is found to replicate in a variety of Gram-negative bacteria [2]. Its incompatibility group is not known [34]. pBBR1 is frequently used for the construction of expression systems such as pBBR1 MCS and its derivatives. pBBR1 MCS is carrying chloramphenicol resistance, but there exists antibiotic resistance derivatives which increase the usefulness of this vec-

tor in Cm^r bacteria [31]. pBBR1 MCS backbone combined with the *Pm/XylS* expression system is a potentially valuable expression vector for a wide range of Gram-negative bacteria, including *C.necator*.

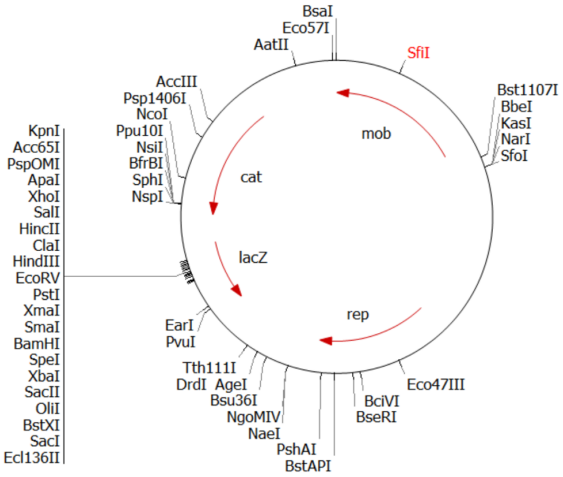

Figure 4: Illustration of the pBBR1 MCS vector carrying the chloramphenicol (cat) gene. Restriction sites, mobilization sequences (mob), and the *rep* gene encoding the replication initiator protein are shown. Made in Clone Manager 9.

1.7 *Cupriavidus necator*

1.7.1 Characteristics and metabolic features

Cupriavidus necator (*C.necator*) (previous named *Ralstonia eutropha* and *Alcaligenes eutrophus*) is an alternative bacterial species which possibly can overcome some of the disadvantages of using *E.coli* as recombinant protein expression host. *C.necator* is a nonpathogenic Gram-negative bacteria described as a peritrichously flagellated rod. [56]. *C.necator* has high metabolic diversity and has attracted great interest due to its versatile metabolic features. One of these features is the ability to efficiently produce polyhydroxybutyrate (PBH), which is of industrial interest [48]. In addition, *C.necator* has the ability to grow lithoautotrophically, meaning it can utilize CO_2 and H_2 as sole carbon and energy sources in the absence of organic substrates [44] [53]. Moreover, *C.necator* can degrade chloroaromatic and chemically related pollutants [53] [44]. Thus, there exists a well-established starting point for further development of *C.necator*, which may be a potentially valuable organism for molecular engineering.

1.7.2 *C.necator* as a host

One frequently used strain for molecular engineering is *C.necator* H16. Up until now, a number of different expression systems are known to be active in *C.necator* H16. For instance prokaryote expression systems based on well-known promoters is known to be active in *C.necator* H16. However, they were lacking the ability to be induced due to inability of *C.necator* H16 to take up the effectors, and/or they exhibited weak activity [17] [23]. Autonomously replicating expression vectors based on the broad-host-range plasmids RP4 (identical to RK2), RSF1010, pBBR1 and pMOL28 are all found to function in *C.necator* H16. However, significant plasmid loss for all the expression vectors during fermentation in *C.necator* H16 have been reported [31] [52] [36]. This illustrates the need for developing an improved expression vector system that fully function in *C.necator*.

A previous study accomplished by Srinivasan and colleagues, shows that the model protein organophosphohydrolase (OPH), which traditionally has been expressed as insoluble protein in *E.coli*, has been produced at high yield as soluble protein in the correct folded form in *C. necator* [52]. Moreover, the *Pm*/XylS expression system has been found to be functional in *C. necator* H1 in the work done by Bi and colleagues [9]. The study used a pBBR1 MCS-based expression vector combined with a P_{BAD} promoter, which successfully was transconjugated into *C.necator* H16 [9]. The P_{BAD} was replaced by the *Pm*/XylS expression cassette. Red fluorescent protein (RFP) was used as a reporter gene and expression of RFP from *Pm* in the presence and the absence of an inducer was tested. The expression in presence of inducer was found to be high both for the plasmid carrying P_{BAD} and *Pm*/XylS respectively [9]. This is the first demonstration that the *Pm*/XylS expression system is functional in *C.necator*.

1.8 The aim of this study

The aim of this study was to establish functional expression systems to be used in *E.coli* and *C.necator* respectively, in order to utilize these organisms as expression host for production of the therapeutic protein IFN-α-2b. A set of expression vectors was therefor designed, based on the broad-host-range plasmids RK2 or pBBR1, all harboring the

Pm/XylS expression cassette. The protein production in both *E.coli* and *C.necator* was evaluated to find favorable expression vectors, as well as evaluating the two host organisms as suitable expression host for the production of soluble IFN-α-2b.

2 Material and Methods

2.1 Media and solutions

2.1.1 Antibiotics

Final concentration of antibiotics:

100 mg/mL Ampicilin (PanReac AppliChem)
20 mg/mL Chloramphenicol (Sigma)

2.1.2 Growth media

Luria-Bertani (LB) medium
5.0 g Tryptone (OXOID)
2.5 g Yeast extract (OXOID)
5 g NaCl (Merck)
Up to 500 mL dH_2O
Autoclaved

LB Agar (LA) medium
5.0 g Tryptone (OXOID)
2.5 g Yeast extract (OXOID)
5 g NaCl (Merck)
7.5 g Agar (OXOID)
Up to 500 mL dH_2O
Autoclaved

PSI medium
2.5 g Yeast extract (OXOID)
10 g Tryptone (OXOID)
5.12 g $MgSO_4 \cdot 7H_2O$ (VWR)
Up to 500 mL dH_2O
pH adjusted to 7.6 using KOH before autoclaving.

Super Optimal Broth (SOB) medium
0.5 g Yeast extract (OXOID)
2 g Tryptone (OXOID)
0.058 g NaCl (Merck)
0.019 g KCl (Merck)
0.50 g $MgSO_4 \cdot 7H_2O$ (VWR)
Up to 100 mL dH_2O
Autoclaved

Super Optimal Catabolite-repression (SOC) medium
100 mL SOB media
2 mL 20% glucose solution (VWR)

Basis medium 1+Yeast extract Hi (Hi+YE Basis medium)

Basis medium 1:
The volumes used to make the stock solutions are given in Appendix B.
- 8.6 g $Na_2HPO_4 \cdot 2H_2O$
- 3 g KH_2PO_4
- 1 g NH_4Cl
- 0.5 g NaCl
- 10 mL Fe(3) citrate hydrate
- 0.1 mL H_3BO_3
- 1.5 mL $MnCl_2 \cdot 4H_2O$
- 0.1 mL EDTA$\cdot 2H_2O$
- 0.1 mL $CuCl_2 \cdot 2H_2O$
- 0.1 ml $Na_2Mo_4O_4 \cdot 2H_2O$
- 0.1 mL $CoCl_2 \cdot 6H_2O$
- 2 mL $Zn(CH_3COO)_2 \cdot 2H_2O$
- Up to 900 mL dH_2O

Autoclaved

Yeast extract Hi
- 10 g yeast extract (OXOID)
- Up to 100 mL dH_2O

Autoclaved

Basis medium 1 and Yeast extract HI were mixed to make 1 L Hi+Ye Basis medium after autoclaving.

Nutrient Broth:
- 2.5 g Peptone
- 1.5 g meat extract
- Up to 500 mL dH_2O

pH adujusted to 7.2. Autoclaved.

Nutrient Broth Agar:
- 2.5 g Peptone
- 1.5 g meat extract
- 7.5 g agar
- Up to 500 mL dH_2O

pH adujusted to 7.2. Autoclaved.

Tryptone soya broth
- 30 g Tryptone soya broth (Oxoid) in 1L of dH_2O.

Autoclaved.

2.1.3 Other solutions

TFB1, super competent cells:
0.588 g KAc (Potassium acetate) (Merck)
2.42 g RbCl (Rubidium chloride) (ACROS Organics)
0.389 g $CaCl_2 \cdot 2H_2O$ (Calcium chloride) (Merck)
3.146 g $MnCl_2 \cdot 4H_2O$ (Manganese chloride) (J.T Baker)
30 mL Glycerol (VWR)
Up to 200 mL dH_2O
pH adjusted to 5.8 using diluted acetic acis. Sterilized by filtration.

TFB2, super competent cells
0.21 g MOPS (Fisher Scientific)
1.1 g $CaCl_2 \cdot 2H_2O$ (Merck)
0.121 g RbCl (ACROS Organics)
15 mL Glycerol (VWR)
Up to 100 mL dH_2O
pH adjustedd to 6.5 using diluted NaOH. Sterilized by filtration.

1M $MgSO_4 \cdot 7H_2O$, expression of recombinant proteins
12.3 g $MgSO_4 \cdot 7H_2O$ (VWR)
Up to 50 mL dH_2O

Glucose solution, expression of recombinant proteins
11.35 g Glucose (VWR)
Up to 50 mL dH_2O
Autoclaved

Glycerol solution, expression of recombinant proteins
50.15 g Glycerol (VWR)
Up to 100 mL dH_2O
Autoclaved

3×sample buffer, SDS-PAGE
1.5 mL 2M Tris (Sigma)
7.54 g 30% glycerol (VWR)
1.20 g 6% SDS (Merck)
0.06 g 0.3% bromophenolblue (Sigma)
0.93 g 300 mM dithiothereitol (DTT) (VWR)
Up to 20 mL dH_2O

2×sample buffer, SDS-PAGE
2× Laemmli Sample Buffer, no1610737 (BioRad)
465 mg DTT (VWR)

De-staining solution, SDS-PAGE

400 mL MeOH (VWR)
500 mL dH_2O
100 mL concentrated AcOH (VWR)

Tris-Buffered Saline (TBS), Western blotting

40 mL 2M Tris base (Sigma)
35.2 g NaCl in 2 L volume (VWR)
pH adjusted to 7.5

TBST, Western blotting

TBS with 0.05% Tween20 (VWR) (500 mL TBS + 250 μL Tween20)

Blotto, Western blotting

3 % skim milk powder (OXOID) in TBS (3 g in 100 mL TBS for each membrane)

Blotting buffer, Western blotting

25 mL 2M Tris base (Sigma)
28.8 g Glycine (VWR)
400 mL Methanol (VWR)
1 g SDS in 2 L volume (Merck)
pH adjusted to 7.5 with HCl

50xTris-acetate-EDTA (50xTAE) buffer, gel electrophoresis

242 g Tris-base (Sigma)
57.1 mL acetic acid (VWR)
100 mL 0.5M EDTA, pH 8 (VWR)
Up to 1000 mL dH_2O
Autoclaved

0.8% Agarose solution, gel electrophoresis

3.2 g Agarose (Lonza)

400 mL 1xTAE

2.2 Bacteria strains and plasmids

The different bacteria strains and plasmids that were used or constructed in this study are described in Table 2.2. An overview explaining the vector system is given in Table 2.1. All growth media and solutions used in this study are listed in section 2.1. *E.coli* DH5α was used as a general cloning host. *E.coli* DH5α was cultivated in Luria Bertani (LB) medium or LB Agar medium added a selective antibiotic. Bacteria cultivated in LB were incubated at 37 °C and 225 rpm, while bacteria cultivated in LA were incubated at 37 °C and no shaking. The selective antibiotic was chosen based on antibiotic resistance of the plasmids.

C. necator CCUG 13796, used as an expression host, was cultivated in Nutrient Broth at 37 °C and 225 rpm or in Nutrient Broth Agar at 37 °and no shaking added appropriate antibiotic. Wild-type *C. necator*, which is not harboring recombinant genes, was cultivated without antibiotic.

Table 2.1: A guidance explaining the vector system created by Vectron Biosolutions and their references.

DNA element	**Properties**	**Reference(s)**
Backbone	1: pIFN30SpelB/pAT64	Sletta (2007) [50]
	2: pBBR1 MCS	Kovach (1994) [30]
Pm/5'UTR	A: WT	
	B: comPU	Bakke (2009) [5], Berg (2009) [7], Zwick (2013) [60].
Signal sequence	0: no signal sequence	
	1: *pelB*	Sletta (2007) [50]
trfA gene	B: *cop271C*	Durland (1990) [20], Haugan (1995) [27]
	E: not analyzed	
xylS gene	0: WT	
	1: WT	
	2: StEP-13	Aune 2009 [57]

Table 2.2: Bacteria strains and plasmids used or constructed in this study.

Strain or plasmid	Description	Source or reference(s)
E. coli DH5α	General cloning host	BRL
E.coli BL21(DE3)	Carries chromosomal gene for T7 RNA polymerase	NEB
C.necator CCUG 13796	Expression host	CCUG
pBBR1 MCS	Broad-host-range expression vector,Cm^r, 4.7 kb	Kovach (1994) [30]
pVB1 (pJB658)	RK2-based expression vector harboring *Pm/xylS* regulatory promoter system for expression of cloned genes. Amp^r.	Blatny (1997) [11]
pVB-1B0B1-IL-1RA	RK2-based vector expressing the codon-optimized IL-1RA-c-*myc*-His6 fusion protein from the *Pm/xylS* promoter system; *Pm* variant ML2-5, 5'UTR variant LIII-3, Amp^r, 8.8 kb.	Vectron unpublished
pVB-1A0B1-mCherry	RK2-based vector expressing the codon-optimized mCherry-c-*myc*-His6 fusion protein from the *Pm/xylS* promoter system; Amp^r, 9.1 kb.	Vectron unpublished
pVB-1A1B1-mCherry	RK2-based vector expressing the codon-optimized pelB-mCherry-c-*myc*-His6 fusion protein from the *Pm/xylS* promoter system; Amp^r, 9 kb.	Vectron unpublished
pVB-1B0B1-mCherry	RK2-based vector expressing the codon-optimized mCherry-c-*myc*-His6 fusion protein from the *Pm/xylS* promoter system; *Pm* variant ML2-5, 5'UTR variant LIII-3, Amp^r, 7.8 kb.	Bakka (2015) [4]
pVB-1B1B1-mCherry	RK2-based vector expressing the codon-optimized pelB-mCherry-c-*myc*-His6 fusion protein from the *Pm/xylS* promoter system; *Pm* variant ML2-5, 5'UTR variant LIII-3, Amp^r, 9.1 kb.	Vectron unpublished
pVB-1A0B1-IFN-α-2b	RK2-based vector expressing the codon-optimized IFN-α-2b-c-*myc*-His6 fusion protein from the *Pm/xylS* promoter system; Amp^r, 8.9 kb.	This study
pVB-1A1B1-IFN-α-2b	RK2-based vector expressing the codon-optimized pelB-IFN-α-2b-c-*myc*-His6 fusion protein from the *Pm/xylS* promoter system; Amp^r, 9 kb.	Vectron unpublished
pVB-1B0B1-IFN-α-2b	RK2-based vector expressing the codon-optimized IFN-α-2b-c-*myc*-His6 fusion protein from the *Pm/xylS* promoter system; *Pm* variant ML2-5, 5'UTR variant LIII-3, Amp^r, 8.9 kb.	Bakka (2015) [4]
pVB-1B1B1-IFN-α-2b	RK2-based vector expressing the codon-optimized pelB-IFN-α-2b-c-*myc*-His6 fusion protein from the *Pm/xylS* promoter system; *Pm* variant ML2-5, 5'UTR variant LIII-3, Amp^r, 9 kb.	Vectron unpublished
pVB-1B1B2-IFN-α-2b	RK2-based vector expressing the codon-optimized pelB-IFN-α-2b-c-*myc*-His6 fusion protein from the *Pm/xylS* promoter system; *Pm* variant ML2-5, 5'UTR variant LIII-3, XylS variant StEP.13, Amp^r, 8.9 kb.	This study
pVB-1B0B2-IFN-α-2b	RK2-based vector expressing the codon-optimized IFN-α-2b-c-*myc*-His6 fusion protein from the *Pm/xylS* promoter system; *Pm* variant ML2-5, 5'UTR variant LIII-3, XylS variant StEP.13, Amp^r, 8.8 kb.	This study
pVB-2A0E0-IFN-α-2b	pBBR1–based expression vector expressing the codon-optimized IFN-α-2b-c-*myc*-His6 fusion protein from the *Pm/xylS* promoter system; 7.5 kb, Cm^r.	Vectron unpublished
pVB-2B1E0-IFN-α-2b	pBBR1–based expression vector expressing the codon-optimized pelB-IFN-α-2b-c-*myc*-His6 fusion protein from the *Pm/xylS* promoter system; *Pm* variant ML2-5, 5'UTR variant LIII-3, 9.1 kb, Cm^r.	Vectron unpublished
pVB-2A1E0-IFN-α-2b	pBBR1–based expression vector expressing the codon-optimized pelB-IFN-α-2b-c-*myc*-His6 fusion protein from the *Pm/xylS* promoter system; 7.5 kb, Cm^r.	Vectron unpublished
pVB-2B0E0-IFN-α-2b	pBBR1–based expression vector expressing the codon-optimized IFN-α-2b-c-*myc*-His6 fusion protein from the *Pm/xylS* promoter system; *Pm* variant ML2-5, 5'UTR variant LIII-3, 9.1 kb, Cm^r.	Vectron unpublished

2.3 Plasmids constructed

2.3.1 pVB-1B0B1-mCherry (Bakka, 2015)

E.coli DH5α pVB-1B0B1-IL-1RA and *E.coli* DH5α pVB-1A0B1-mCherry were cultivated and plasmids isolated using Miniprep DNA Purification system (Promega). Both vectors were digested with NdeI and NotI. The 714 bp NdeI/NotI fragment of plasmid pVB-1B0B1-mCherry was used to substitute for the corresponding fragment of plasmid pVB-1B0B1-IL-1RA, yielding pVB-1B0B1-mCherry (expressing mCherry).

2.3.2 pVB-1B0B1-IFN-α-2b (Bakka, 2015)

E.coli DH5α pVB-1B0B1-mCherry and *E.coli* DH5α pVB-1A1B1-IFN-α-2b were cultivated and plasmids isolated using Miniprep DNA Purification system (Promega). Both vectors were digested with NdeI and NotI. The 501 bp NdeI/NotI fragment of plasmid pVB-1A1B1-IFN-α-2b was used to substitute for the corresponding fragment of plasmid pVB-1B0B1-mCherry, yielding pVB-1B0B1-IFN-α-2b (expressing IFN-α-2b).

2.3.3 pVB-1A0B1-IFN-α-2b

E.coli DH5α pVB-1A0B1-mCherry and *E.coli* DH5α pVB-1B0B1-IFN-α-2b were cultivated and plasmids isolated using Miniprep DNA Purification system (Promega). Both vectors were digested with NdeI and NotI. The 501 bp NdeI/NotI fragment of plasmid pVB-1B0B1-IFN-α-2b was used to substitute for the corresponding fragment of plasmid pVB-1A0B1-mCherry, yielding pVB-1A0B1-IFN-α-2b (expressing IFN-α-2b).

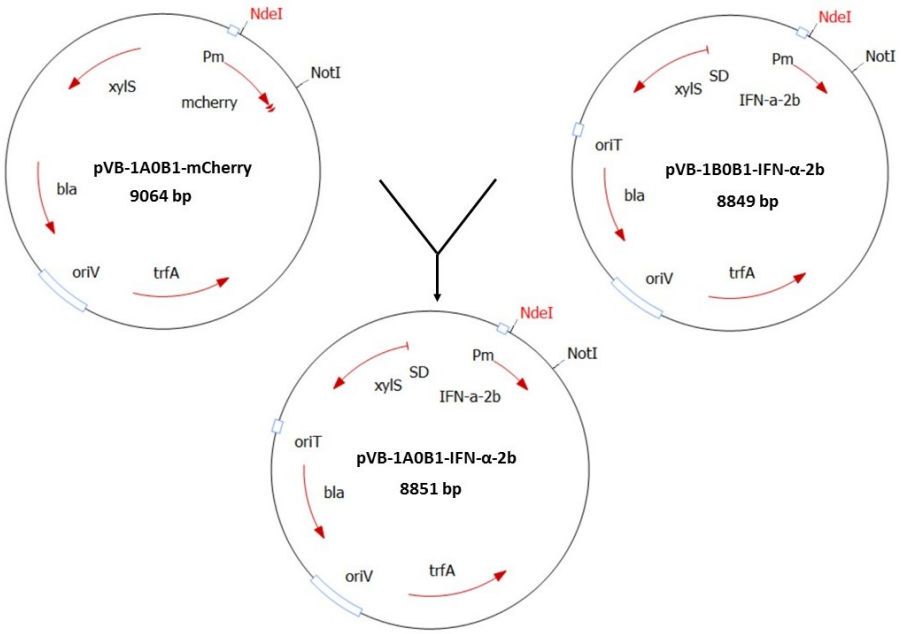

Figure 5: Cloning procedure for construction of pVB-1A0B1-IFN-α-2b. pVB-1A0B1-mCherry and pVB-1B0B1-IFN-α-2b are digested with NdeI and NotI, generating the backbone fragment 1A0B1 and the IFN-α-2b gene, respectively. The fragments are ligated to generate the vector pVB-1A0B1-IFN-α-2b.

2.3.4 pVB-1B1B2-IFN-α-2b

E.coli DH5α pVB-1B1B2-IL-1RA and *E.coli* DH5α pVB-1B0B1-IFN-α-2b were cultivated and plasmids isolated using Miniprep DNA Purification system (Promega). Both vectors were digested with NdeI and NotI. The 501 bp NdeI/NotI fragment of plasmid pVB-1B0B1-IFN-α-2b was used to substitute for the corresponding fragment of plasmid pVB-1B1B2-IL-1RA, yielding pVB-1B1B2-IFN-α-2b (expressing IFN-α-2b).

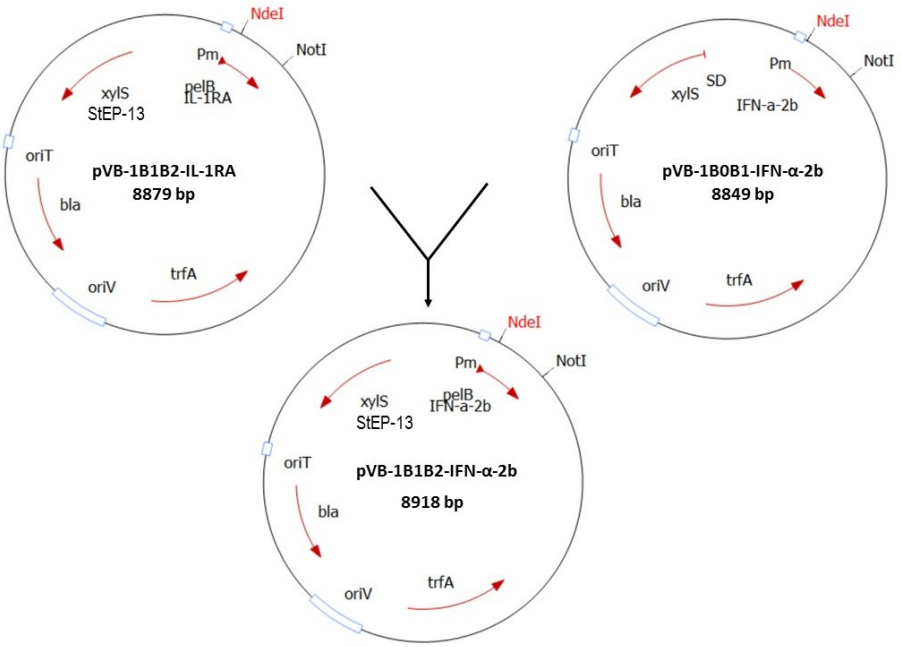

Figure 6: Cloning procedure for construction of pVB-1B1B2-IFN-α-2b. pVB-1B1B2-IL-1RA and pVB-1B0B1-IFN-α-2b are digested with NdeI and NotI, generating the backbone fragment 1B1B2 and the IFN-α-2b gene, respectively. The fragments are ligated to generate the vector pVB-1B1B2-IFN-α-2b.

2.3.5 pVB-1B0B2-IFN-α-2b

E.coli DH5α pVB-1B1B2-IL-1RA and pVB-1B0B1-IL-1RA were cultivated and plasmids isolated using Miniprep DNA Purification system (Promega). Both vectors were digested with XbaI and NotI. The 590 bp XbaI/NotI fragment of plasmid pVB-1B0B1-IL-1RA was used to substitute for the corresponding fragment of plasmid pVB-1B1B2-IL-1RA, yielding pVB-1B0B2-IL-1RA (expressing IL-1RA). *E.coli* DH5α pVB-1B0B2-IL-1RA and *E.coli* DH5α pVB-1A0B1-IFN-α-2b were cultivated and plasmids isolated. Both vectors were digested with NdeI and NotI. The 501 bp fragment of plasmid pVB-1A0B1-IFN-α-2b was used to substitute for the corresponding fragment of plasmid pVB-1B0B2-IL-1RA, yielding pVB-1B0B2-IFN-α-2b.

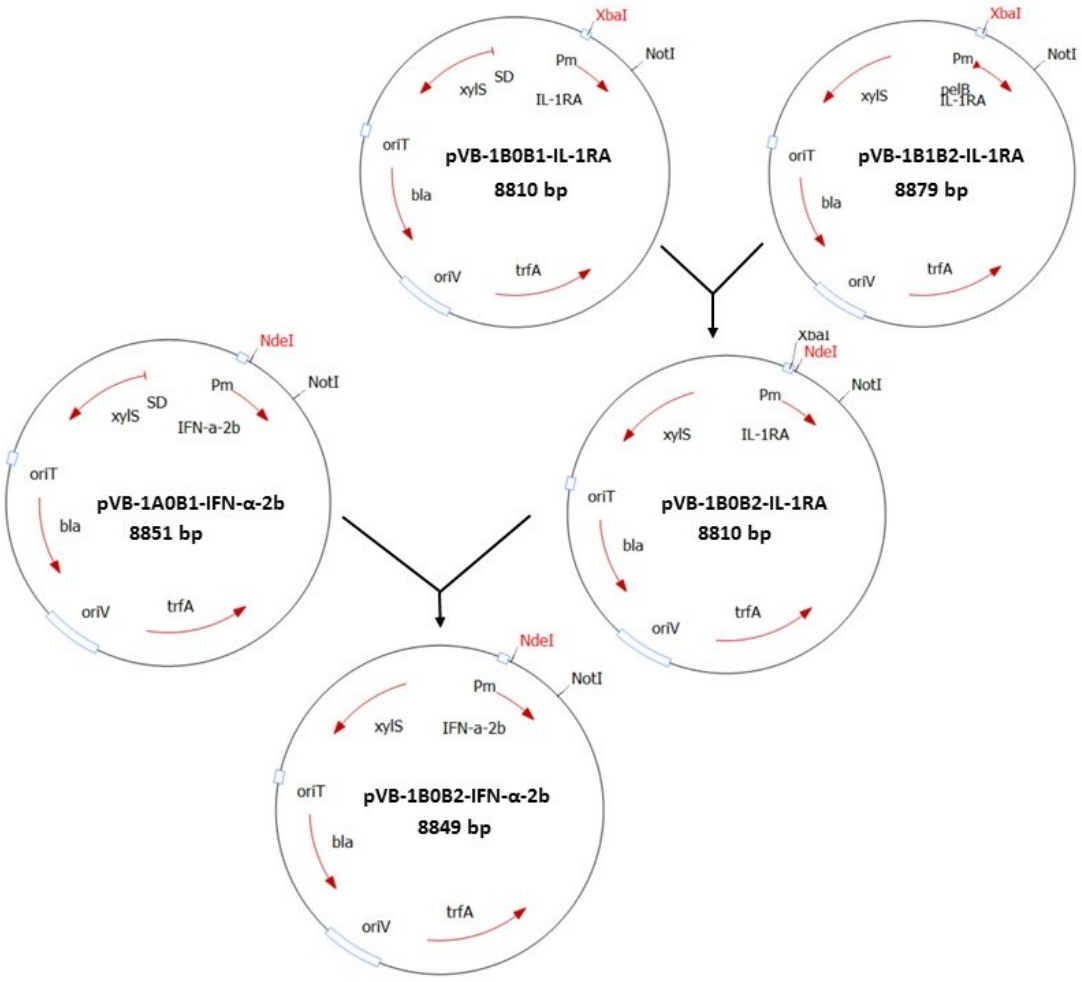

Figure 7: Cloning procedure for construction of pVB-1B0B2-IFN-α-2b. pVB-1B1B2-IL-1RA and pVB-1B0B1-IL-1RA are digested with XbaI and NotI, generating the backbone fragment 1B0B2 and the IL-1RA gene, respectively. The fragments are ligated to generate the vector pVB-1B0B2-IL-1RA. The vectors pVB-1B0B2-IL-1RA and pVB-1A0B1-IFN-α-2b are digested by NdeI/NotI, yielding the backbone fragment 1B0B2 and the IFN-α-2b gene. The fragments are ligated to generate the vector pVB-1B0B2-IFN-α-2b.

2.3.6 pVB-2A1E0-IFN-α-2b

E.coli DH5α pVB-2A0E0-IFN-α-2b and *E.coli* DH5α pVB-1A1B1-IFN-α-2b were cultivated and plasmids isolated using Miniprep DNA Purification system (Promega). Both vectors were digested with XbaI and NdeI. The 130 bp XbaI/NdeI fragments of plasmid pVB-1A1B1-IFN-α-2b was used to substitute for the corresponding fragment of pVB-2A0E0-IFN-α-2b, yielding pVB-2A1E0-IFN-α-2b.

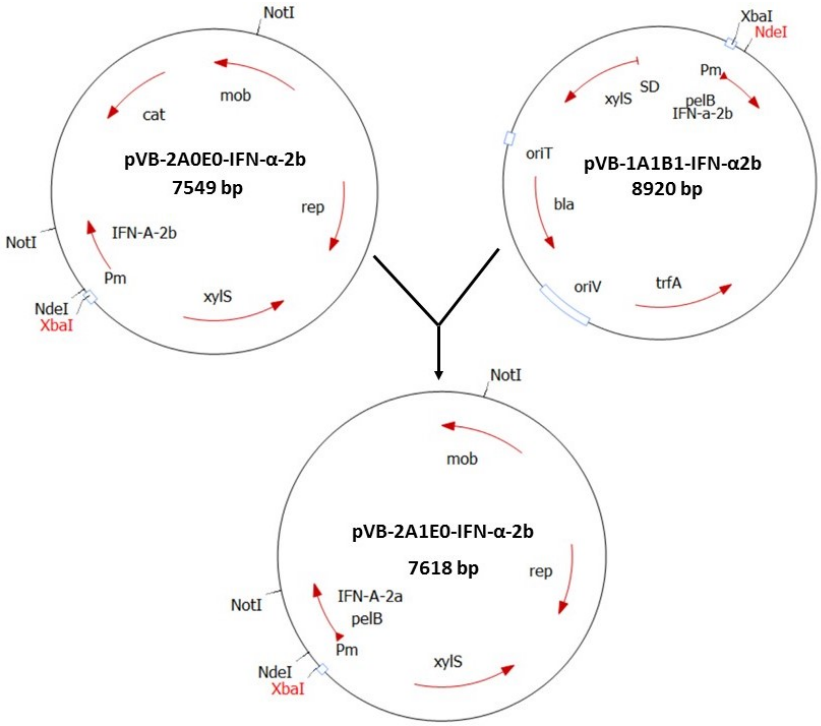

Figure 8: hei Cloning procedure for construction of pVB-2A1E0-IFN-α-2b. pVB-2A0E0-IFN-α-2b and pVB-1A1B1-IFN-α-2b are digested with XbaI and NotI, generating the backbone fragment 2A0E0 and the *pelB* signal sequence, respectively. The fragments are ligated to generate the vector pVB-2A1E0-IFN-α-2b.

2.3.7 pVB-2B0E0-IFN-α-2b

E.coli DH5α pVB-2B1E0-IFN-α-2b and *E.coli* DH5α pVB-1B0B1-IFN-α-2b were cultivated and plasmids isolated using Miniprep DNA Purification system (Promega). Both vectors were digested with XbaI and NdeI. The 59 bp XbaI/NdeI fragments of plasmid pVB-1B0B1-IFN-α-2b was used to substitute for the corresponding fragment of pVB-2B1E0-IFN-α-2b, yielding pVB-2B0E0-IFN-α-2b.

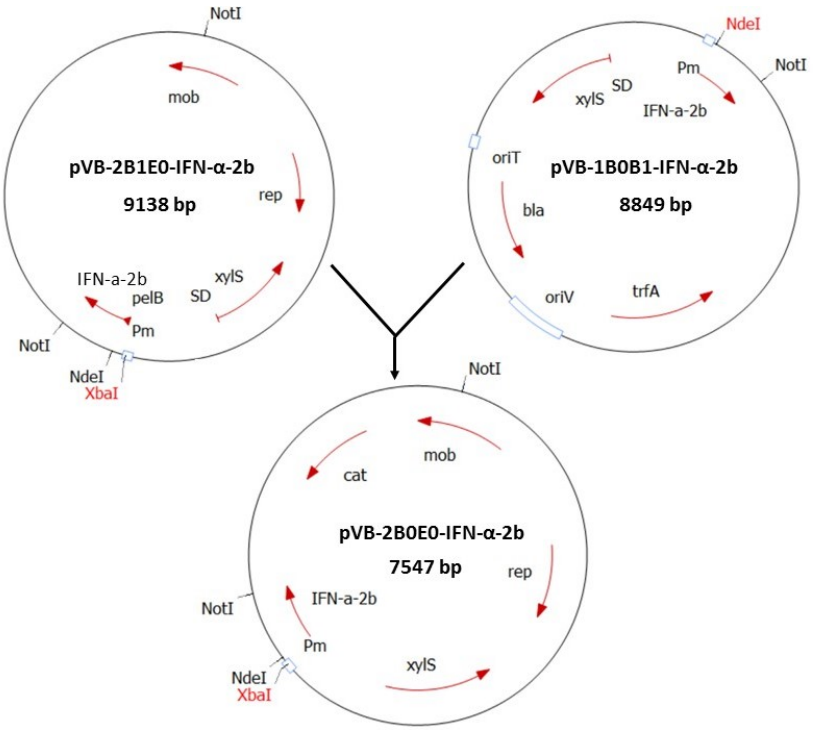

Figure 9: hei Cloning procedure for construction of pVB-2B0E0-IFN-α-2b. pVB-2B1E0-IFN-α-2b and pVB-1B0B1-IFN-α-2b are digested with XbaI and NotI, generating the backbone fragment 2B1E0 and the "no-pelB" fragment, respectively. The fragments are ligated to generate the vector pVB-2B0E0-IFN-α-2b.

2.4 Methods used for plasmid construction

2.4.1 Super competent cells

Competent cells are treated in a way so they are able to take up naked DNA, through a process called transformation [8]. Treatment with calcium chloride ($CaCl_2$) has been used to obtain competent cells for a long time [14] [16]. DNA binds to the cell surface, followed by uptake through the membrane when $CaCl_2$ are present in high concentration [58] [14]. Treatment with rubidium chloride (RbCl) is an improved method to make chemically competent cells. In this study, TFB1 and TFB2 are used as treatment solutions (see section 2.1). A temperature shock from 0 to 42 degrees, in combination with $CaCl_2$ or other salts, are found to be an efficient transformation method [8]. The following procedure is a modified version of the work done by Dagert and Ehrlish [16].

Competent *E.coli* DH5α cells were incubated in 4 mL LB medium (without antibiotic) at 37°C, 225 rotation per minute (rpm) overnight. It was made two parallels. The next day 0.5 mL of the overnight cultures were used to inoculate 50 mL Psi medium. The cultures were grown at 37°C, and OD_{600} was measured several times to obtain $OD_{600}= \sim$ 0.4 (Unicam Helios epsilon). After about 2 hours, the OD_{600} was found to be about 4 for both of the parallels. The samples were incubated on ice for 15 minutes to stop further growth. Each parallel was transferred to a 50 mL tube and centrifuged at 4500 rpm at 4°C for 5 minutes (Eppendorf Centrifuge 5804R). The supernatants were discarded and the pellets resuspended in 20 mL cold TFB1, before incubation on ice for 15 minutes. The centrifugation was repeated (Eppendorf Centrifuge 5424) and the cells were resuspended in 1.5 mL cold TFB2. The samples were aliquoted in Eppendorf tubes on ice (100 μL per tube) and immediately frozen in liquid nitrogen before storage at minus 80°C.

2.4.2 Transformation of *E.coli* DH5α

Transformation is the process where competent cells take up naked DNA, as described in the past section. The following procedure describes the heat-shock transformation process used to transform *E.coli*. The procedure is adapted from the work done by Dagert and Ehrlich. [16].

One tube (100 μL) of competent *E.coli* DH5α cells were thawed on ice. 10 μL of ligation mix or circular plasmid was added to the thawed cells and gently mixed. The tube was incubated on ice for 15-30 minutes, the bacteria were heat-shocked in a water bath at 42°C for 45 seconds, and then transferred to ice and incubated for two minutes. 900 μL pre-warmed SOC medium (37°C) was added before the cells were incubated at 37°C for 1 hour. After incubation, the cells were diluted and plated on LB agar containing a selective antibiotic for the plasmid. The plates were incubated overnight at 37°C.

2.4.3 Isolation of plasmid

Isolation of plasmids were performed by using Wizard Plus SV Miniprep DNA Purification System (Promega) or Wizard Plus Midiprep DNA Purification System (Promega).

Wizard Plus SV Miniprep DNA Purification System:

1. 4 mL ON culture was centrifuged for 5 minutes at 6000xg (Eppendorf Centrifuge 5804R)

2. The pellet was resuspended with 250 μL Cell Resuspension Solution and transferred to an Eppendorf tube

3. 250 μL Cell Lysis Solution was added, and the tube was inverted 4 times to obtain mixing.

4. 10 μL Alkaline Protease Solution was added, the tube was inverted 4 times and incubated for 5 minutes at room temperature. Alkaline protease inactivates endonucloases and other proteins released during the lysis of cells. These proteins may reduce the quality of isolated plasmid if they don't get inactivated(Promega).

5. To neutralize the solution, 350 μL Neutralization Solution was added, then the tube was inverted 4 times and centrifuged at 13000 rpm for 10 minutes at room temperature (Eppendorf Centrifuge 5424).

6. A spin column was insertet into a collection tube, and the cleared lysate from the centrifuged tube was decanted into the spin column.

7. The tube was centrifuged at 13000 rpm for 1 minute at room temperature (Eppendorf Centrifuge 5424). The flow through was discarded and reinserted into the collection tube.

8. 750 μL Wash Solution was added to the spin column before centrifuged at 13000 rpm for 1 minute (Eppendorf Centrifuge 5424). The flowthrough was discarded and reinsertedd into the collection tube. The washing procedure was then repeated with 250 μL Wash Solution. Then the tube was centrifuged at 13000 rpm for 2 minutes at room temperature.

9. The spin column was carefully transfered to a sterile 1.5 mL microcentrifuge tube without transfering any of the Column Wash Solution with the spin column. 100 μL Nuclease free water was added to the spin column, and the column was centrifuged at 13000 rpm for 1 minute at room temperature (Eppendorf Centrifuge 5424).

10. The column was discarded and the DNA in the tube was stored at -20°C

Wizard Plus Midipreps DNA Purification System:

1. 10-100 mL of cells were harvested by centrifugation at 10,000×g (Eppendorf Centrifuge 5804R)for 10 min at 4°C. The tube was blotted upside down on a paper to remove excess liquid.

2. The cell pellet was completely resuspended in 3 mL of Cell Resuspension Solution

3. 3 mL of Cell Lysis Solution was added and mixed by inverting the tube four times.

4. 3 mL Neutralization Solution was added and mixed by inverting the tube four times.

5. The tube was centrifuged 17 14,000×g (Eppendorf Centrifuge 5804R) for 15 minutes at 4°C. Pellet for another 15 minutes if the pellet has not formed by the end of centrifugation.

6. The supernatant was carefully decanted to a new centrifuge tube, avoiding the white precipitate.

7. 10 mL of resuspended Wizard Midiprep DNA Purification Resin was added to the DNA solution and mixed by swirling.

8. For each Midiprep, one Midicolumn was used. The Midicolumn tip was inserted into the vacuum manifold port (A self-made vacuum pump was used due to no access to Vac-Man).

9. The resin/DNA mixture was transferred into the Midicolumn. The sample was vacuumed until all of the sample had passed through the column.

10. 15 mL Column Wash Solution was added to the Midicolumn and a vacuum was applied to draw the solution through the Midicolumn. The step was repeated.

11. The resin was dried by continuing vacuuming for 30 seconds after the solution had been pulled through the column. The Midicolumn was removed from the vacuum source. The Reservoir was separated from the Midicolumn by cutting with a scissors. The Midicolumn was transferred to a 1.5 mL microcentrifuge for 2 minutes to remove any residual Column Wash Solution. The Midicolumn was transferred to a new microcentrifuge tube.

12. 300 μL of preheated (60-70°C) Nuclease-Free Water to the Midicolumn, before waiting for 1 min. The DNA was eluted by centrifuging the Midicolumn at 10,000$\times$g for 20 seconds (Eppendorf Centrifuge 5424). The Midicolumn was removed and discarded.

13. The sample was centrifuged at 10,000$\times$g for 5 minutes to separate residual resin pellet from the final eluate. The DNA-containing supernatant was carefully transfered to a clean microcentrifuge tube.

14. The plasmid DNA was stored at -20°C

2.4.4 Measurement of concentration

The plasmid concentrations were determined with Nanodrop Spectrophotometer ND-1000 (Saveen and Werner) by measuring the absorbance at 260 nm.

2.4.5 Digestion of plasmid with restriction enzymes

A restriction enzyme is an endonuclease that recognizes a specific nucleotide sequence in the DNA and cleave both the DNA strands at that site [51]. A restriction enzyme is site-specific and only cleave DNA at that restriction site. The restriction enzymes used in this study are NdeI, NotI and XbaI. NotI and NdeI are originally produced by the bacteria *Nocardia ototodis* [51]. Restriction sites for all three restriction enzymes are given in Figure 10. As illustrated in the figure, the cleaving results in sticky ends (5' overhang). The procedure used in this study is adopted from New England Biolabs (NEB).

In biotechnology, restriction enzymes are used to isolate specific DNA fragments, such as a gene. The specific DNA fragment is ligated into a vector, which are cleaved with the same restriction enzyme(s). The DNA fragments may be further analyzed by gel electrophoresis.

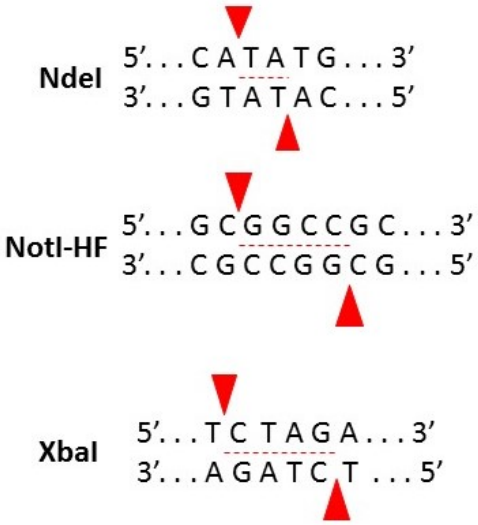

Figure 10: The restriction sites for NdeI, NotI-HF and XbaI. Digestion with both NdeI, NotI-HF and XbaI result in sticky ends. (Adapted from NEB).

Each vector was digested with appropriate restriction enzymes. The digestion mixes used in this study are listed in Table 2.3. For each vector variant, all the reagents were pipetted into an Eppendorf tube and gently mixed. The samples were incubated at 37°C for 1 hour. After incubation, 6× loading dye was added to each sample to a final concentration of 1×. The loading dye is found to stop the enzyme reaction, and in addition give brighter nucleotide bands (NEB). The DNA fragments were separated by gel electrophoresis.

Table 2.3: A general digestion mix, using NdeI, NotI-HF or XbaI as restiction enzyme(s).

Component	**Volume digestion**	**Volume control digestion**	**Reference**
10x CutSmart Buffer	5 μL	1 μL	NEB
Restriction enzyme 1	0.5 μL	0.5 μL	NEB
Restriction enzyme 2	0.5 μL	0.5 μL	NEB
Vector	20-50 μL	8 μL	
dH_2O	Up to 50 μL		

2.4.6 Gel electrophoresis

Gel electrophoresis is a method used to separate DNA molecules by size [13]. The gel is placed in a buffer-filled tank that has a positive and a negative electrode at opposite ends, and the DNA samples are loaded into wells in the gel. DNA are negatively charged molecules, and they will move from the negative electrode toward the positive electrode when being exposed to an electric current. Thus, larger DNA fragments will move slower than smaller DNA fragments due to different size [13]. A molecular weight standard with known band sizes, is used as a reference to determine the size of the DNA fragments. Figures of the molecular weigh standards used in this study is given in Appendix C. The gel in this study is made of 0.8% agarose solved in 1xTAE buffer, and added GelRed for visualizing of the DNA. The gel was run at 90 V for about 90 minutes. GelRed binds to DNA by intercalation. When exposed to UV light, GelRed will fluoresce [15], and thus DNA fragments bound to GelRed will be seen as bright bands.

2.4.7 Extraction of DNA from agarose gel

The DNA fragments were excised from the gel and purified by using Zymoclean Gel DNA Recovery Kit (Nordic BioSite).

1. The DNA fragment was excised from the agarose gel using a scaple and transferred into a 1.5 mL microcentrifuge tube.
2. 3 volumes of ADB to each volume of agarose excised from the gel were added to the tube.
3. The tube with the solution was then incubated at 55°C for 5-10 minutes until the gel slice was completely dissolved.
4. The melted agarose solution was transferred to a Zymo Spin Column in a Collection Tube and centrifuged for 30-60 seconds (Eppendorf Centrifuge 5424). The flow-through was discarded.
5. 200 μL Wash Buffer was added to the column and centrifuged for 30 seconds (Eppendorf Centrifuge 5424). The flow-through was discarded and the washing procedure repeated.
6. 6-10 μL DNA Elution Buffer was added directly to the column matrix. The column was transferred into a 1.5 mL tube and centrifuged for 30-60 seconds to elute DNA (Eppendorf Centrifuge 5424).

2.4.8 Ligation

Ligation of vector a fragment and insert DNA is the last step before construction of the recombinant plasmid is completed. The enzyme T4 DNA ligase is used to catalyze the reaction where the vector fragment and the insert DNA are joined together. This is done by T4 DNA ligase catalyzing linkage between the 3'-OH of one strand and the 5'-PO_4 of the other [13]. The following protocol is based on Ligation Protocol with T4 DNA ligase from NEB.

A ligation mix with molar ratio 1:3 vector DNA to insert DNA, is found to be most optimal for successful ligation. An online ligation calculator was used to calculate the molar ratios based on the indicated size of the DNA fragments ($http://www.insilico.uni-duesseldorf.de/Lig_Input.html$). The components in the ligation mix were set up in a microcentrifuge tube on ice where T4 DNA ligase was added last. It was also made a negative control reaction without insert DNA. The reaction was gently mixed by pipetting up and down and briefly micro-centrifuged. The reaction was incubated at room temperature for 1-2 hours, or alternatively at 16°C overnight. After incubation, the reaction was chilled on ice, and 10 μL of the reaction was added into 100 μL competent cells. The cells were transformed by the heat-shock method, as described in section 2.4.2.

Table 2.4: Components and volumes used for ligation and re-ligation.

Component	**Liagation**	**Ligation control**	**Reference**
10× T4 ligase Buffer	1 μL	1 μL	NEB
T4 ligase	1 μL	1 μL	NEB
Digested insert	x μL	No insert	
Digested vector	y μL	y μL	
dH_2O	Up to 10 μL	Up to 10 μL	

2.4.9 Transformation of *C.necator*

Electroporation utilizes high-voltage electric shock to introduce DNA into the cells. The method was first performed by Wong and Neumann in 1982, and the technique has been generalized and further developed since then [45]. When the cell membrane is exposed to a high-voltage electric field, it causes a temporary breakdown of the membrane and the formation of pores, so that DNA can enter the cell [45]. Electroporation of *C.necator* was used for uptake of both RK2-based and pBBR1-based expression vectors. The following procedure is derived from Aneja and colleagues for expression of recombinant protein in *C.necator* [1]. The method is further adapted by Vectron Biosolutions.

1. *C.necator* was directly inoculated from -80°C and grown in 3 mL Nutrient Broth overnight at 37°C and 225 rpm.

2. 2 mL of this culture was used to inoculate 48 mL Nutrient Broth in a 250 mL E-flask. The cultures were grown for approximately 3 hours at 37°C and 225 rpm until $OD_{600} = 0.4$ was obtained.

3. 25 mL × 2 of this culture was centrifuged at 6,000×g at 4°C for 15 minutes (Eppendorf Centrifuge 5804R). The cells were kept on ice after this step.

4. The harvested cells were resuspended twice (10 mL, then 5 mL by 6,000×g at 4°C for 15 minutes (Eppendorf Centrifuge 5804R)) in a prechilled, sterile 0.3 M sucrose solution (filtered), and the pellet was resuspended in 400 μL 0.3 M sucrose solution.

5. The concentrated cell suspension (100 μ) was mixed with 5 μL transforming plasmid in an ice-cold Eppendorf tube and incubated on ice for 10 minutes. Note that the concentration should not exceed 10% of total volume.

6. The transformation mix was transferred to a prechilled electroporation cuvette (0.1 cm gap width). 100 μL concentrated cell suspension was used as a negative control. Electroporation was performed at 2.5 kV, 25 μF, and 200Ω settings in a Gene Pulser II electroporator equipped with a Pulse Controller Plus module (BioRad).

7. The cells were well distributed in the cuvette, and the outside of the cuvette was dried off before it was placed in the electroporator and the "pulse-button" was pushed. The time constant was recorded (about 4.7 to 4.9)

8. 900 μL pre-warmedd SOC medium was added immediately after electroporation and the cells were transferred to an Eppendorf tube before incubation for 2 hours at 37°C and 225 rpm.

9. The transformants were selected by spreading 100 μL and 900 μL (13000 rpm, 1 min, supernatant removed, 100 μL used for spreading) of the cell suspension on Nutrient Agar added appropriate antibiotic, followed by 1-2 days incubation at 37°C.

2.5 Expression of recombinant proteins in *E.coli* BL21(DE3)

The production host *E.coli* BL21(DE3) was transformed to take up vectors harboring the IFN-α-2b and mCherry genes, respectively. Cultivation of *E.coli* BL21(DE3), is under optimal conditions better suited for recombinant protein production than the strain *E.coli* DH5α. The following procedure, adapted from Sletta and colleagues, was used to recombinantly express mCherry and IFN-α-2b in *E.coli* BL21(DE3) [49] [50]. The procedure splits up the total protein content of the cell into soluble and insouble protein fractions.

1. An overnight (ON) culture of BL21(DE3) harboring a recombinant plasmid was grown in LB containing appropriate antibiotic at 37°C and 225 rpm.

2. The OD_{600} value of each culture was determined (Unicam Helios epsilon). The OD_{600} measurements were used to calculate the amount of culture needed to give Hi+YE Basis medium a starting OD_{600} of 0.05.

3. 600 μL glycerol solution, 240 μL glucose solution and 30 μL ampicilin were added to 30 mL Hi+YE Basis medium. The amount of culture needed to obtain a starting OD_{600} of 0.05, was added to Hi+YE Basis medium. It was executed two parallels of each culture.

4. The cultures were incubated a 30°C until OD_{600} around 2-2.5 was obtained. OD_{600} above 2 was obtained for all the cultures after 4.5-5 hours incubation.

5. 120 μL 0.5M m-toulate (ALDRICH) was added to one parallel of each culture, resulting in one induced and one uninduced parallel of each culture. The cultures were incubated at 25°C for 16-18 hours.

6. The next day, OD_{600} of each culture were measured to confirm growth.

7. Each culture was transferred to a 50 mL sterile tube and centrifuged at 10000xg at 4°C for 10 minutes (Eppendorf Centrifuge 5804R). The supernatants were removed by vacuuming, and the tubes were weighted to calculate the wet weight of each pellet.

8. The pellets were transferred to ice and 1 mL/100 mg pellet 0.9% NaCl was added to each tube. 1 mL of each culture was transferred into a 1.5 mL tube. The samples

were centrifuged at 13 000 rpm for 5 minutes at 4°C (Eppendorf Centrifuge 5424). The supernatants were removed by vacuuming.

9. 500 μL LyticM lysis buffer (Sigma) was added to each sample to resuspend the pellet. The samples were incubated at room temperature for 1 hour at 100 rpm.

10. The samples were centrifuged at 13000 rpm for 8 minutes (Eppendorf Centrifuge 5424) to separate the soluble protein fraction and the insoluble protein fraction.

11. 400 μL supernatant (soluble fraction) was transferred into a clean 1.5 mL tube. The remaining supernatant was removed by carefully pipetting. The pellet (insoluble fraction) was resuspended in 500 μL running buffer (SDS non-reducing running buffer, C.B.S. Scientific).

2.5.1 Sample preparation for SDS-PAGE

Proteins are usually smaller than DNA molecules, hence polyacrylamide gel electrophoresis (PAGE) is better suited to separate proteins by size. Acrylamide has smaller pores than agarose and is therefor a better choice fir separation of proteins. Moreover, proteins are not negatively charged and they need to be treated with the negative sodium dodecyl sulfate (SDS) to obtain a negative charge. SDS unfolds the protein and coat it with negatively charged SDS molecules. As for agarose gel electrophoresis, the samples are loaded into the wells, and an electric current is used so that the proteins migrate through the gel. Then the separated proteins are visualized by Coomassie Blue Staining Solution. The visual protein bands are compared to a molecular weight standard to determine their molecule weight (see Appendix D). The following steps was preformed to prepare the samples for SDS-PAGE [50] [49].

1. The soluble fraction was diluted 2-fold with SDS-running buffer, and the insoluble fraction was diluted 5-fold with SDS running buffer (SDS non-reducing running buffer, C.B.S. Scientific).

2. 30 μL diluted sample was combined with 15 μL 3x sample buffer (3xDTT loading buffer)/30 μL 2x sample buffer (2xDTT loading buffer), and boiled at 99°C for 5 minutes.

3. The gel was loaded (5 μL soluble sample, 10 μL insoluble sample and 5 μL molecular weight standard), and the SDS-PAGE was run at 130 V for 90 minutes.

4. After SDS-PAGE, the gel was stained in BioRad Coomassie stain solution for 30 minutes and then destained overnight.*

5. The next day, the membrane was rehydrated in dH_2O for 30 minutes.

*Alternatively, the gel was stained in InstantBlue (a coomassie based staning solution, Expedoen) for 1 hour and destained in dH_2O.

2.5.2 Western-blotting

Western blotting is a method used to detect specific proteins in a sample by using antibodies that binds to the target protein [13]. It also confirms detection of a protein, and it can be used to estimates the protein expression level. After separation of the proteins by SDS-PAGE, the proteins are being transfered from the gel to a membrane. The membrane is positively charged so that the negatively charged proteins will bind to its surface [13]. The membrane will be exposed to a primary antibody that is used to locate the target protein. However, areas of the membrane without proteins may bind non-specifically to the antibody. Non fat milk is often used to block these sites so that the membrane only can bind target proteins. The secondary antibody is used to recognize and bind to the primary antibody [13]. Also, the secondary antibody has a tag than easily can be detected. This study use Anti-myc (Invitrogen) as its primary antibody and Rabbit-anti-mouse Ig HRP (Daco) as its secondary antibody. The genes to be expressed in this study are marked with a *c-myc*-His6 tag recognized by the primary antibody. The following protocol was based on the work done by Sletta and colleagues [49] [50].

1. SDS-PAGE was preformed as described above.

2. The gel, filter paper and blotting sponges were incubated in blotting buffer for 30 minutes. The membrane was incubated in methanol for 15 seconds followed by 2 minutes in dH_2O and 10 minutes in blotting buffer.

3. The set-up for the transfer from gel to membrane was build, blotting buffer added and the chamber containing the set-up was placed on ice. Then the protein was transferred from the gel to the membrane with electroblotting at 90 V for 3 hours.*

4. The membrane was washed with TBS for 1 minute. Then the blotted membrane was blocked in freshly prepared Blotto (30 mL) for 1 hour at room temperature with constant agitation. Blotto contain skim milk powder that masks the areas of the membrane without proteins so that they don't bind the antibody.

5. 3μL primary antibody was diluted in 30 mL Blotto. The membrane was then incubated overnight in Blotto added primary antibody at 4°C with constant agitation.

6. The next day, the membrane was washed with TBST for 10 minutes 3 times to remove unbound primary antibody. After washing, the membrane was incubated in 30 mL Blotto added 15μL secondary antibody for 1 hour in room temperature with constant agitation.

7. The membrane was washed with TBST for 10 minutes. This step was repeated 2 times before the membrane was rinsed with TBS for 10 minutes.

8. The blot was placed in a clean container and 3 mL substrate Tetramethylbenzidine (TMB) was added before incubation at room temperature for 1-10 minutes.

9. The blot was rinsed with Mili-Q water to stop the reaction, and the membrane was scanned.

*Alternatively: The Trans-Blot Transfer System (BioRad) was used for transferring the protein from the gel to the membrane. (Program used: Mixed molecular weight, 7 minutes).

2.6 Expression of recombinant proteins in *C.necator*

The following procedure was used for the expression of IFN-α-2b in *C.necator*. It is adapted from the work done by Sletta and colleagues [49]. The method differ from expression in *E.coli* (Section 2.5) by changes in incubation time and temperature, plus composition of growth media. Sample preparation for SDS-PAGE and Western blotting are executed as described Section 2.5.

1. *A*n ON culture of *C.necator* harboring a recombinant plasmid was cultivated in Nutrient Broth containing appropriate antibiotic at 37 °C and 225 rpm.

2. The OD_{600} value of each culture was determined (Unicam Helios epsilon). The OD_{600} values were used to calculate the amount of culture needed to give Tryptone Soya Broth Basis medium a starting OD_{600} of 0.1 or 0.2.

3. 600 μL glycerol solution, 240 μL glucose solution and 5 μL chloramphenicol in were added to 30 mL Tryptone Soya Broth. The calculated amount of culture was added to Tryptone Soya Broth. It was executed two parallels of each culture.

4. The cultures were incubated a 37°C until OD_{600} around 2-2.5 was obtained. OD_{600} above 2 was obtained for all the cultures after approximately 4.5 hours.

5. 120 μL 0.5M m-toulate (ALDRICH) was added to one parallel of each culture, resulting in one induced and one uninduced parallel of each culture. The cultures were incubated at 25°C for 16-18 hours.

6. The next day, OD_{600} values of each culture was measured to confirm growth.

7. Each culture was transferred to a 50 mL sterile tube and centrifuged at 10000xg at 4°C for 10 minutes (Eppendorf Centrifuge 5804R). The supernatants were removed by vacuuming, and the tubes were weighted to calculate the wet weight of each pellet.

8. The pellets were transferred to ice and 1 mL/100 mg pellet 0.9% NaCl was added to each tube. 1 mL of each culture was transferred into a 1.5 mL tube. The samples were centrifuged at 13 000 rpm for 5 minutes at 4°C (Eppendorf Centrifuge 5424). The supernatants were removed by vacuuming.

9. 500 μL LyticM lysis buffer (Sigma) was added to each sample to resuspend the pellet. The samples were incubated at room temperature for 1 hour at 100 rpm.

10. The samples were centrifuged at 13000 rpm for 8 minutes (Eppendorf Centrifuge 5424) to separate the soluble protein fraction and the insoluble protein fraction.

11. 400 μL supernatant (soluble fraction) was transferred into a clean 1.5 mL tube. The remaining supernatant was removed by carefully pipetting. The pellet (insoluble

fraction) was resuspended in 500 μL running buffer (SDS non-reducing running buffer, C.B.S. Scientific).

2.7 mCherry activity

mCherry activity was determined by using an Infinite 200 Pro multifunctional microplate reader (Tecan) that measures the amount of fluorescent per unit (PFU). *E.coli* BL21(DE3), containing vectors expressing mCherry or the negative control pVB1, was cultivated in LB medium added appropriate antibiotic. SDS sample preparation was performed as described in Section 2.5 to obtain induced and uninduced cultures of soluble and insoluble protein fractions. Each well was loaded with 200 μL culture with excitation and emission wavelengths of 584 nm (9 nm bandwidth) and 620 nm (20 nm bandwidth) respectively, and normalized against OD_{600}.

2.8 Quantification of production level

The software GelQuant.net was used for quantification of the production level of IFN-α-2b.. The software measures the intensity of bands on SDS-PAGE by drawing a selection rectangle around a segment of the band of interest. By comparing the band intensity of the molecular mass standard (information on protein amount in the different bands are given by BioRad) and the band of interest, the total amount of protein in the band can be calculated. Further, the total amount of mg protein per liter can be estimated, see Appendix E.

3 Results

3.1 Construction of expression vectors

Plasmids constructed in this study are described in detail in Section 2.2 and Section 2.3. The intention of vector construction in this study is to examine different combinations of DNA elements of the expression cassette, such as the *Pm*/5'UTR element, the signal sequence *pelB* and the *xylS* gene, to find the best high-expression variant in *E.coli* and *C.necator* respectively. A series of vectors based on pAT64 [Vectron unpublished] was used as backbone for the construction of vectors for use in *E.coli*, while pBBR1 MCS [30] was used as starting point for construction of vectors for use in *C.necator*.

3.1.1 Construction of pVB-1A0B1-IFN-α-2b

pVB-1A0B1-mCherry and pVB-1B0B1-IFN-α-2b were digested and correctly separated by gel electrophoresis to achieve the NdeI/NotI backbone fragment (8350 bp) and the NdeI/NotI IFN-α-2b fragment (501 bp) respectively. The fragments were ligated and successfully taken up by *E.coli* DH5α by transformation. The transformants were cultivated, plasmids isolated and digested by NdeI/NotI and separated by gel electrophoresis. The restriction analysis gave the expected separation of 8350 bp and 501 bp. Transformation of the expression host *E.coli* BL21(DE3) succeeded in uptake of the constructed vector.

3.1.2 Construction of pVB-1B1B2-IFN-α-2b

pVB-1B1B2-IL-1RA and pVB-1B0B1-IFN-α-2b were digested and correctly separated by gel electrophoresis to achieve the NdeI/NotI backbone fragment (8417 bp) and the NdeI/NotI IFN-α-2b fragment (501 bp) respectively. The fragments were ligated and successfully taken up by *E.coli* DH5α by transformation. The transformants were cultivated, plasmids isolated and digested by NdeI/NotI and separated by gel electrophoresis. The restriction analysis gave the expected separation of 8417 bp and 501 bp. Transformation of the expression host *E.coli* BL21(DE3) succeeded in uptake of the constructed vector.

3.1.3 Construction of pVB-1B0B2-IFN-α-2b

pVB-1B1B2-IL-1RA and pVB-1B0B1-IL-1RA were digested and correctly separated by gel electrophoresis to achieve the XbaI/NotI backbone fragment (8289 bp) and the XbaI/NotI *pelB*-IL-1RA fragment (521 bp) respectively. The fragments were ligated and successfully taken up by *E.coli* DH5α by transformation. The transformants were cultivated, plasmids isolated and digested by XbaI/NotI and separated by gel electrophoresis. The restriction analysis gave the expected separation of 8289 bp and 521 bp. The constructed plasmid pVB-1B0B2-IL-1RA and the plasmid pVB-1A0B1-IFN-α-2b were digested and correctly separated by gel electrophoresis to achieve the NdeI/NotI backbone fragments (8348 bp) and the NdeI/NotI IFN-α-2b fragment (501 bp). The fragments were ligated and successfully taken up by *E.coli* DH5α by transformation. The transformants were cultivated, plasmids isolated and digested by NdeI/NotI and separated by gel electrophoresis. The restriction analysis gave the expected separation of 8348 bp and 501 bp. Transformation of the expression host *E.coli* BL21(DE3) succeeded in uptake of the constructed vector.

3.1.4 Construction of pVB-2A1E0-IFN-α-2b

pVB-2A0E0-IFN-α-2b and pVB-1A1B1-IFN-α-2b were digested and correctly separated by gel electrophoresis to achieve the XbaI/NdeI backbone fragment (7488 bp) and the XbaI/NdeI *pelB* fragment (130 bp) respectively. The fragments were ligated and successfully taken up by *E.coli* DH5α by transformation. The transformants were cultivated, plasmids isolated and digested by XbaI/NdeI and separated by gel electrophoresis. The restriction analysis gave the expected separation of 7488 bp and 130 bp. High nucleotide concentration was required for successful construction due to small DNA fragments. Transformation of the expression host *C.necator* succeeded in uptake of the constructed vector.

3.1.5 Construction of pVB-2B0E0-IFN-α-2b

pVB-2B1E0-IFN-α-2b and pVB-1B0B1-IFN-α-2b were digested and correctly separated by gel electrophoresis to achieve the XbaI/NdeI backbone fragment (7547 bp) and the XbaI/NdeI "no-*pelB*" fragment (59 bp) respectively. The fragments were ligated and successfully taken up by *E.coli* DH5α by transformation. The transformants were cultivated, plasmids isolated and digested by XbaI/NdeI and separated by gel electrophoresis. The restriction analysis gave the expected separation of 7547 bp and 59 bp. High nucleotide concentration was required for successful construction due to small DNA fragments. Transformation of the expression host *C.necator* succeeded in uptake of the constructed vector.

3.2 Transformation of *E.coli*

E.coli was transformed using the heat-shock method for uptake of expression vectors (see Section 3.1), cultivated and selected by its ability to grow on the appropriate antibiotic. Transformation of *E.coli* DH5α and *E.coli* BL21(DE3) with the vectors pVB-1A0B1-IFN-α-2b, pVB-1B1B2-IFN-α-2b, and pVB-1B0B2-IFN-α-2b all resulted in small, white colonies. A negative control (not containing the selective gene) was executed for all transformations and resulted in none or few colonies.

3.3 Growth curve *C.necator*

A typical bacterial growth curve consists of four phases; lag phase, exponential (log) phase, stationary phase and death phase. In order to calculate the generation time of *C.necator*, the exponential phase was used to calculate the number of times the population doubles during a time interval. *C.necator* was cultivated over night and inoculated in Nutrient broth the next day. OD_{600} was measured each hour for about nine hours. The calculations were executed in three parallels, before finding the average generation time. Based on this experiment, the generation time was calculated to be 1 hour . A semi-log plot of the OD_{600} measurements as a function of time are given in Figure 11.

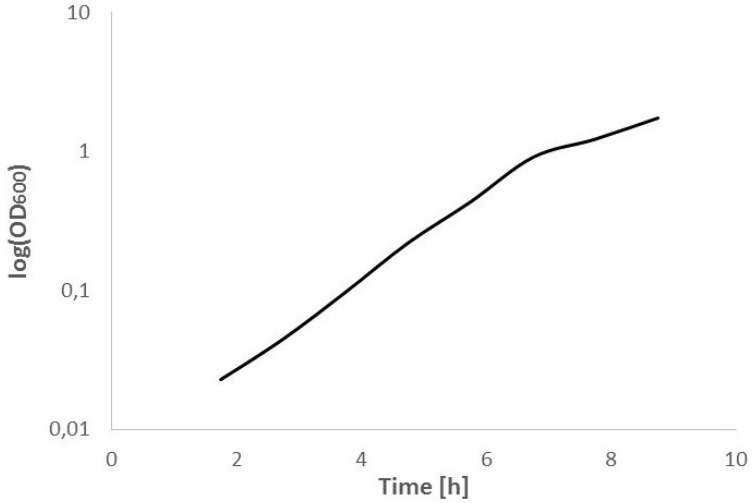

Figure 11: Wild-type *C.necator* CCUG 13796 was cultivated over night in Nutrient Broth without antibiotic and inoculated the next day. OD_{600} was measured every hour for about nine hours. Based on the OD_{600} measurements, a semi-log plot of OD_{600} as a function of time, was generated. The growth curve illustrates the exponential growth phase. OD_{600} was not measured at the starting time of the experiment, hence is the lagging phase not included in the figure.

3.4 Transformation of *C.necator*

C.necator was transformed using the electroporation method (Section 2.4.9) to take up pAT64-based vectors (pVB-1B1B1-IFN-α-2b, pVB-1B0B1-IFN-α-2b, pVB-1A1B1-IFN-α-2b, pVB-1A0B1-IFN-α-2b), cultivated and selected by its ability to grow on the appropriate antibiotic. Transformation resulted in no visible colonies.

Due to not successful uptake of pAT64-based vectors, electroporation of *C.necator* to take up pBBR1-based vectors (pVB-2A1E0-IFN-α-2b, pVB-2B0E0-IFN-α-2b) was tested. *C.necator* was transformed using the electroporation method to take up the given vectors, cultivated and selected by its ability to grow on the appropriate antibiotic. Transformation resulted in very small, almost invisible colonies. Extended incubation time (two days in total) resulted in better growth and small, white colonies.

3.5 Expression of IFN-α-2b and mCherry in *E.coli* BL21(DE3)

3.5.1 Expression of IFN-α-2b

E.coli BL21(DE3) was used as expression host for the production of IFN-α-2b in shake flask cultures. Expression in both the presence and the absence of inducer (2 mM m-toulate) was tested. Different combinations of expression cassette elements were combined to find the best high-expression variant. It was also examined how the presence and the absence of the signal peptide *pelB* impact the expression level of IFN-α-2b. Expression from the following vectors, also presented in Table 3.1, was evaluated; pVB-1A0B1-IFN-α-2b (*Pm* wild-type, 5'UTR wild-type, no *pelB*, *xylS* wild-type), pVB-1A1B1-IFN-α-2b (*Pm* wild-type, 5'UTR wild-type, *pelB*, *xylS* wild-type), pVB-1B0B1-IFN-α-2b (*Pm* ML2-5, 5'UTR LIII-3, no *pelB*, *xylS* wild-type), pVB-1B1B1-IFN-α-2b (*Pm* ML2-5, 5'UTR LIII-3, *pelB*, *xylS* wild-type). See Table 2.1 in Section 2.2 for a more thorough explanation of the vector system. pVB1 was used as a negative control. Expression from the vectors pVB-1B1B2-IFN-α-2b and pVB-1B0B2-IFN-α-2b was not tested due to time shortage.

Table 3.1: pAT64-based vectors used for expression of IFN-α-2b in *E.coli* BL21(DE3).

Expression vector
pVB-1A0B1-IFN-α-2b
pVB-1A1B1-IFN-α-2b
pVB-1B0B1-IFN-α-2b
pVB-1B1B1-IFN-α-2b

The expressed proteins were separated into soluble and insoluble fractions, which were evaluated separately by SDS-PAGE and Western blot analysis (methods described in Section 2.5.1 and Section 2.5.2). The results are given in Figure 12 and Figure 13 respectively. The codon-optimized IFN-α-2b-*c-myc*-His6 fusion protein is found to have a mass of 22.3 kDa. The substrate (TMB) used for protein detection in Figure 12 and Figure 13 was not adopted for use on membranes, and would therefor not stick properly to the membrane, resulting in blurry pictures.

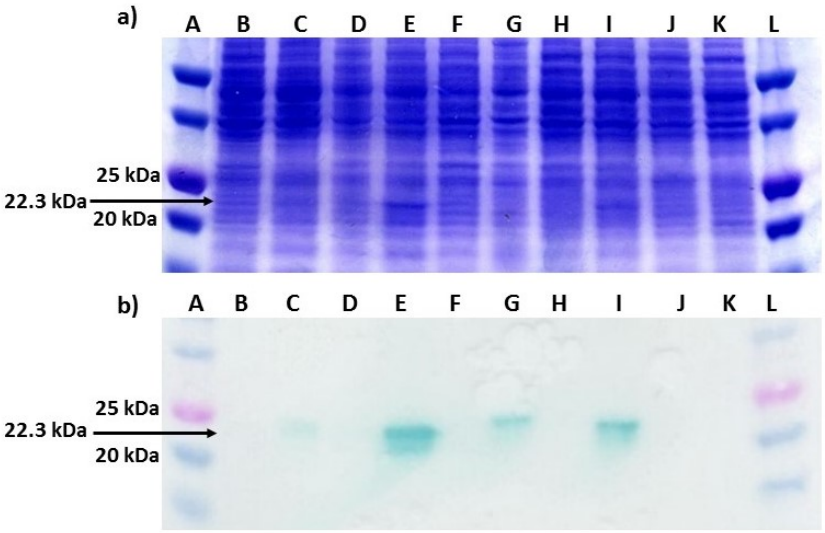

Figure 12: SDS-PAGE (a) and Western blot analysis (b) of soluble IFN-α-2b samples obtained from expression in *E.coli* BL21(DE3). 5 μL sample were loaded into each well. Lane A: molecular mass standard; lane B: uninduced pVB-1B1B1-IFN-α-2b; lane C: induced pVB-1B1B1-IFN-α-2b; lane D: uninduced pVB-1B0B1-IFN-α-2b; lane E: induced pVB-1B0B1-IFN-α-2b; lane F: uninduced pVB-1A1B1-IFN-α-2b; lane G: induced pVB-1A1B1-IFN-α-2b; lane H: uninduced pVB-1A0B1-IFN-α-2b; lane I: induced pVB-1A0B1-IFN-α-2b; lane J: uninduced pVB1; lane K: induced pVB1; lane L: molecular mass standard.

SDS-PAGE of the soluble fractions (Figure 12) indicates high expression of IFN-α-2b from the vector pVB-1B0B1-IFN-α-2b using *E.coli*BL21(DE3) as expression host. Expression of IFN-α-2b is aso observed from the vectors pVB-1A0B1-IFN-α-2b and pVB-1A0B1-IFN-α-2b. In order to verify the expression of IFN-α-2b, Western blot analysis was applied. The results confirm highest expression from pVB-1B0B1-IFN-α-2b, as well as expression from pVB-1A0B1-IFN-α-2b and pVB-1A1B1-IFN-α-2b. The absence of *pelB* signal sequence seems to have a positive impact on the production of soluble IFN-α-2b, which seems to be further increased in combination with a strong expression cassette.

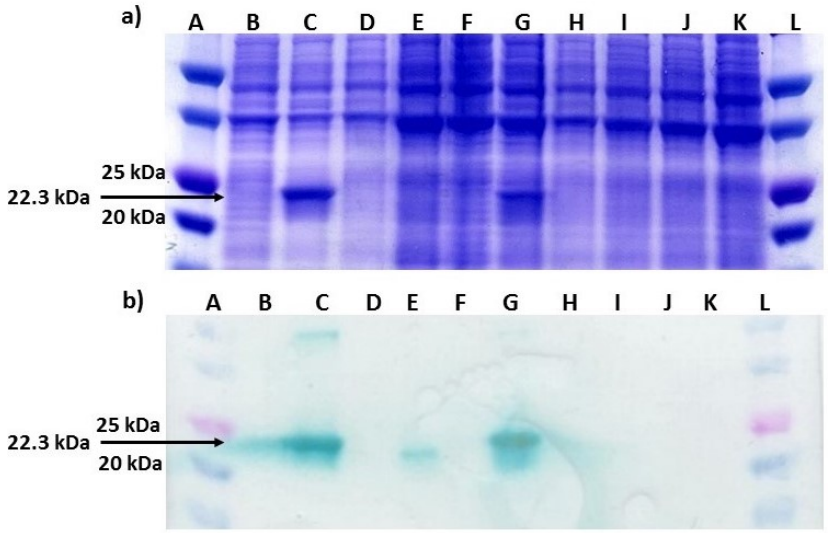

Figure 13: SDS-PAGE (a) and Western blot analysis (b) of insoluble IFN-α-2b samples obtained from expression in *E.coli* BL21(DE3). The samples were diluted 5 fold, and 10 μL were loaded into each well. Lane A: molecular mass standard (5 μL); Lane B: uninduced pVB-1B1B1-IFN-α-2b; lane C: induced pVB-1B1B1-IFN-α-2b; lane D: uninduced pVB-1B0B1-IFN-α-2b; lane E: induced pVB-1B0B1-IFN-α-2b; lane F: uninduced pVB-1A1B1-IFN-α-2b; lane G: induced pVB-1A1B1-IFN-α-2b; lane H: uninduced pVB-1A0B1-IFN-α-2b; lane I: induced pVB-1A0B1-IFN-α-2b; lane J: uninduced pVB1; lane K: induced pVB1; lane L: molecular mass standard.

Regarding the insoluble fractions (Figure 13), there was obtained a slimy layer together with the pellet, making it hard to load the samples into the wells. Thus, the slime was removed in the cases where this were possible. However, the removal gave some differences in the total amount of protein loaded on the gel. The SDS-PAGE of the insoluble fractions indicates high expression of IFN-α-2b from the vectors pVB-1B1B1-IFN-α-2b and pVB-1A1B1-IFN-α-2b. Western blot analysis was applied and the analysis confirms high expression from the same vectors. Some expression of IFN-α-2b is also detected from the vector pVB-1B0B1-IFN-α-2b. The presence of the *pelB* signal sequence seems to promote expression of IFN-α-2b, where the expression seems to further increase combined with a strong expression cassette.

The expression of IFN-α-2b in BL21(DE3) in shake flask cultures was repeated to confirm the results. The experiment was repeated under the same conditions, but no control (i.e. uninduced cultures) was included. No slime was removed from the insoluble fraction, giving more comparable bands. The SDS-PAGE and Western blot analysis are given in Figure 14.

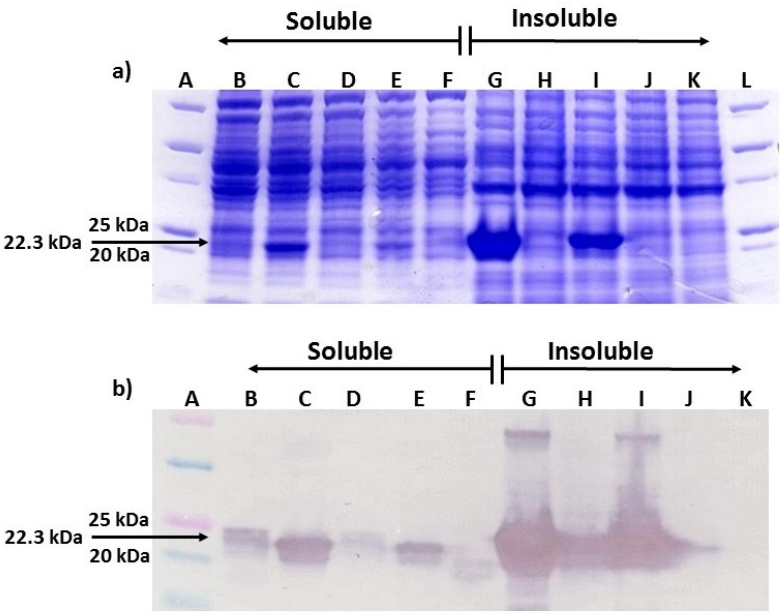

Figure 14: SDS-PAGE (a) and Western blot analysis (b) of IFN-α-2b samples obtained from expression in *E.coli* BL21(DE3). All samples are obtained from induced cultures. Lane B-F: soluble fractions (5 μL); lane G-K: insoluble fractions (10 μL). Lane A: molecular mass standard; lane B: pVB-1B1B1-IFN-α-2b soluble fraction; lane C: pVB-1B0B1-IFN-α-2b soluble fraction; lane D: pVB-1A1B1-IFN-α-2b soluble fraction; lane E: pVB-1A0B1-IFN-α-2b soluble fraction; lane F: pVB1 soluble fraction; lane G: pVB-1B1B1-IFN-α-2b insoluble fraction; lane H: pVB-1B0B1-IFN-α-2b insoluble fraction; lane I: pVB-1A1B1-IFN-α-2b insoluble fraction; lane J: pVB-1A0B1-IFB-α-2b insoluble fraction; lane K: pVB1 insoluble fraction; lane L: molecular mass standard.

The repeated SDS-PAGE analysis (Figure 14a) is in accordance with the previous obtained results. The new results show high expression of soluble IFN-α-2b from pVB-1B0B1-IFN-α-2b, and high expression of insoluble IFN-α-2b from pVB-1B1B1-IFN-α-2b and pVB-1A1B1-IFN-α-2b. The repeated Western blot analysis is also in accordance with the previous results, and is consistent with the repeated SDS-PAGE. The combination of a strong expression cassette and no signal sequence seems most likely to be favorable for high expression of soluble IFN-α-2b, while the combination of a strong expression cassette and *pelB* signal sequence seems very likely to trigger expression of insoluble IFN-α-2b in *E.coli* BL21(DE3).

3.5.2 Expression of mCherry

E.coli BL21(DE3) was used as host for the expression of mCherry in shake flask cultures. Expression in both the presence and the absence of inducer (2 mM m-toulate) was tested. The expression of mCherry is expected to give the culture a pink color due to fluorescent characteristics. Thus, *mCherry* was used as a reporter gene to easily verify a functional expression system. Different combinations of expression cassette elements were combined to find the best high-expression variant. It was also examined how the presence and absence of *pelB* impact the expression level of mCherry. Expression from the following vectors, also presented in Table 3.2, was evaluated; pVB-1A0B1-mCherry, pVB-1A1B1-mCherry, pVB-1B0B1-mCherry and pVB-1B1B1-mCherry (see Section 3.5.1 for vector description). The expressed proteins were separated into soluble and insoluble fractions, which were evaluated by SDS-PAGE. The results are given in Figure 16. The codon-optimized mCherry-*c-myc*-His6 fusion protein is found to have a mass of 26.7 kDa. The vector pVB1 was used as a negative control.

Table 3.2: pAT64-based vectors used for expression of mCherry in *E.coli* BL21(DE3).

Expression vectors
pVB-1A0B1-mCherry
pVB-1A1B1-mCherry
pVB-1B0B1-mCherry
pVB-1B1B1-mCherry

An illustration of the pink color obtained when expressing mCherry is given in Figure 15.

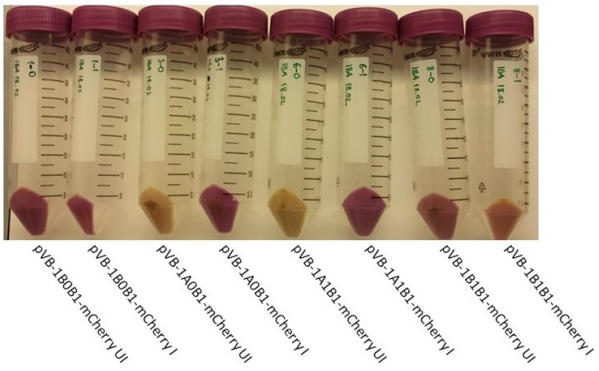

Figure 15: Harvested cells from the expression of mCherry in *E.coli* BL21(DE3). Illustrates the pink color obtained when mCherry is being expressed. The expression vector is given below each tube. UI: uninduced, I: induced.

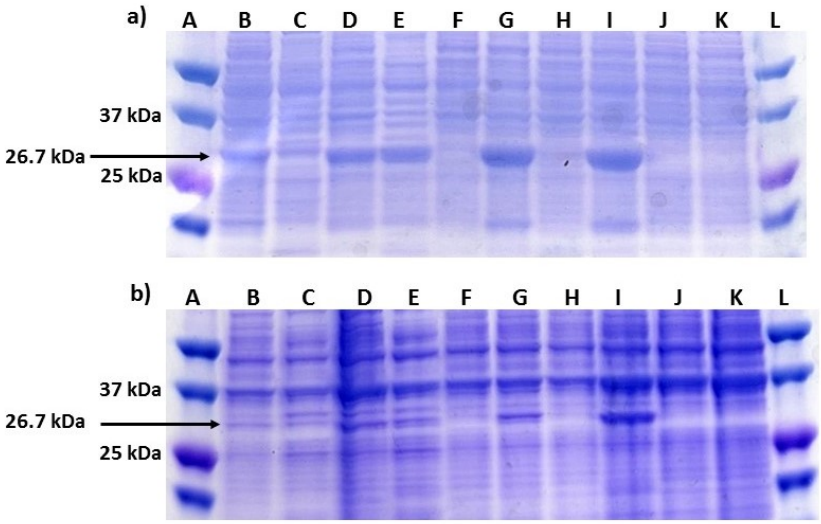

Figure 16: SDS-PAGE analysis of soluble mCherry (a) and insoluble mCherry (b) from expression in BL21(DE3) shake flask cultures. For soluble fraction; 5 μL sample loaded into each well. For insoluble fraction; 5 fold diluted, 10 μL sample loaded into each well. Lane A: molecular mass standard; lane B: uninduced pVB-1B1B1- mCherry; lane C: induced pVB-1B1B1-mCherry lane D: uninduced pVB-1B0B1-mCherry; lane E: induced pVB-1B0B1-mCherry; lane F: uninduced pVB-1A1B1-mCherry; lane G: induced pVB-1A1B1-mCherry; lane H:uninduced pVB-1A0B1-mCherry; lane I: induced pVB-1A0B1-mCherry; lane J: uninduced pVB1; lane K: induced pVB1; lane L: molecular mass standard.

The SDS-PAGE (Figure 16) gave easily detectable bands, which most likely denote expression of mCherry. Figure 15 presents the cultures after harvesting the cells, where the pink color clearly indicates expression of mCherry. Hence, Western blot analysis was not necessary to execute to confirm protein expression. Interestingly, *E.coli* BL21(DE3) containing pVB-1A1B1-mCherry (*Pm* wild-type, 5'UTR wild-type, *pelB*) and pVB-1A0B1-mCherry (*Pm* wild-type, 5'UTR wild-type, no *pelB*) in the presence of inducer gave the highest expression level in both soluble and insoluble fractions. Relatively strong bands were also obtained when pVB-1B0B1-mCherry was expressing mCherry (both under uninduced and induced conditions), while expression from pVB-1B1B1-mCherry gave less detectable bands. Thus, the wild type *Pm*/5'UTR element seems to give the highest expression of mCherry in *E.coli* BL21(DE3). This differ from expression of IFN-α-2b, where the strong *Pm*/5'UTR element gave highest expression level. Addition of *pelB* signal sequence does not seem to be favorable for expression of mCherry.

3.6 Expression of IFN-α-2b in *C.necator*

C.necator was used as host for the expression of IFN-α-2b in shake flask cultures. Expression in both the presence and the absence of inducer (2 mM m-toulate) was performed. As described in Section 3.5, different combinations of expression cassette elements and the signal peptide *pelB*, were combined to find the best high-expression variant. The expression from the vectors pVB-2A0E0-IFN-α-2b (*Pm* wild-type, 5'UTR wild-type, no *pelB*, *xyls* wild-type) and pVB-2B1E0-IFN-α-2b (*Pm* ML2-5, 5'UTR LIII-3, *pelB*, *xyls* wild-type) was evaluated. The expressed proteins were separated into soluble and insoluble fractions and evaluated by SDS-PAGE and Western blot analysis. The results are given in Figure 17 and Figure 18. Expression from the vectors pVB-2A1E0-IFN-α-2b and pVB-2B0E0-IFN-α-2b was not performed du to lack of time.

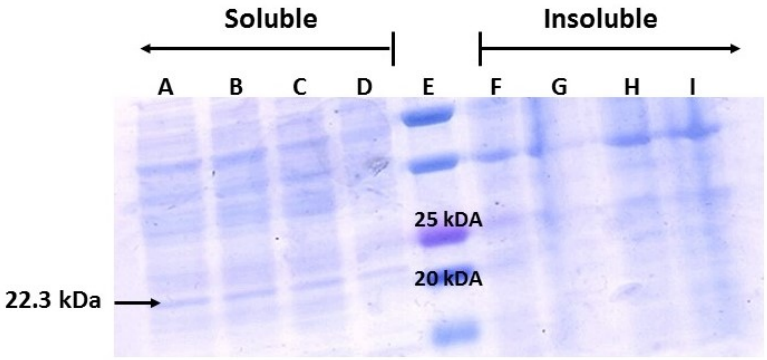

Figure 17: SDS-PAGE analysis of IFN-α-2b expression in *C.necator* shake flask cultures. The cultures had a starting OD_{600} of 0.1. Soluble fractions; lane A-D, 5 μL sample loaded into each well. Insoluble fractions; lane F-I, 10 μL sample loaded into each well. Lane A: uninduced pVB-2A0E0-IFN-α-2b; lane B: induced pVB-2A0E0-IFN-α-2b; lane C: uninduced pVB-2B1E0-IFN-α-2b; lane D: induced pVB-2B1E0-IFN-α-2b; lane E: molecular mass standard; lane F: uninduced pVB-2A0E0-IFN-α-2b; lane G: induced pVB-2A0E0-IFN-α-2b; lane H: uninduced pVB-2B1E0-IFN-α-2b; lane I: induced pVB-2B1E0-IFN-α-2b.

Figure 17 presents the SDS-PAGE of *C.necator* containing the vectors pVB-2A0E0-IFN-α-2b and pVB-2B1E0-IFN-α-2b. The cultures were inoculated to a starting OD_{600} of 0.1, and were then incubated at 37 °C until OD_{600} reached 2.0. The growth was considerably slower compared to cultivation of *E.coli* BL21(DE3) within the same conditions, even though the incubation temperature was increased. This is most likely due to longer doubling time (see Figure 11). As presented in Figure 17, there are weak but detectable bands of soluble IFN-α-2b, while it is hard to observe any detectable bands of insoluble

IFN-α-2b.

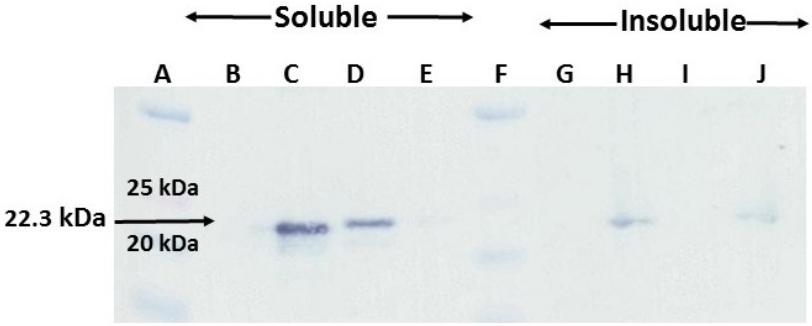

Figure 18: Western blot analysis of IFN-α-2b expression in *C.necator* shake flask cultures. The cultures had a starting OD_{600} of 0.1. Soluble fractions; lane B-E, 5 μL sample loaded into each well. Insoluble fractions; lane G-I, 10 μL sample loaded into each well. Lane A: molecular mass standard; lane B: pVB-2A0E0-IFN-α-2b uninduced; lane C: pVB-2A0E0-IFN-α-2b induced; lane D: pVB-2B1E0-IFN-α-2b uninduced lane E: pVB-2B1E0-IFN-α-2b induced; lane F: molecular mass standard; lane G: pVB-2A0E0-IFN-α-2b uninduced; lane H: pVB-2A0E0-IFN-α-2b induced; lane I: pVB-2B1E0-IFN-α-2b uninduced; lane J: pVB-2B1E0-IFN-α-2b induced.

To verify the expression of IFN-α-2b, Western blot analysis was performed (Figure 18). The results show detectable expression of soluble IFN-α-2b from the vectors pVB-2A0E0-IFN-α-2b (induced) and pVB-2B1B1-IFN-α-2b (uninduced). In addition, the induced samples from the same vectors confirm expression of insoluble IFN-α-2b. It is important to note that the insoluble fractions are diluted 5 fold and 10 μL is loaded into each well, while soluble fractions are undiluted and 5 μL is loaded into each well.

3.7 Comparison of expression in *E.coli* BL21(DE3) and *C.necator*

To better evaluate and compare production of IFN-α-2b in *E.coli* BL21(DE3) and *C.necator*, it was executed new SDS-PAGE and Western blot analysis, where samples from both *E.coli* and *C.necator* were included. The expression experiment of IFN-α-2b from *C.necator* in shake flask cultures was repeated due to low growth in the previous experiment (Section 3.6). The starting OD_{600} was set to 0.2, and wild type *C.necator* was used as a negative control, as this strain does not harbor the IFN-α-2b gene. Expression from *E.coli* BL21(DE3) was not repeated, and induced samples obtained from previous experiment were used to load the gels (Section 3.5, Figure 14). As previous described, different combinations of expression cassette elements were evaluated to find the best high-expression variant. In addition, comparison of *E.coli* and *C.necator* as suitable expression hosts for the production of IFN-α-2b were evaluated. RK2- and pBBR1-based expression vectors harboring the same version of expression cassette elements were compared against each other. The expression from the following vectors, also listed in Table 3.3, was tested; pVB-2A0E0-IFN-α-2b, pVB-2B1E0-IFN-α-2b, pVB-1A0B1-IFN-α-2b and pVB-1B1B1-IFN-α-2b (see Section 3.5 and Section 3.6 for descriptions). The expressed proteins were separated into soluble and insoluble fractions and evaluated by SDS-PAGE and Western blot analysis. The results are given in Figure 19 (soluble fractions) and Figure 20 (insoluble fractions).

Table 3.3: Vectors utilized for comparison of IFN-α-2b production in *E.coli* BL21(DE3) and *C.necator*.

Strain	**Expression vector**
C.necator	pVB-2A0E0-IFN-α-2b
C.necator	pVB-2B1E0-IFN-α-2b
E.coli BL21(DE3)	pVB-1A0B1-IFN-α-2b
E.coli BL21(DE3)	pVB-1B1B1-IFN-α-2b

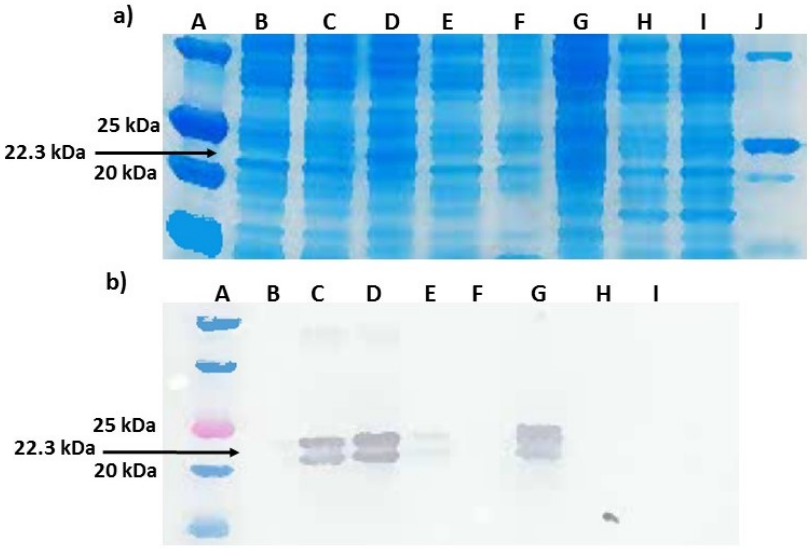

Figure 19: SDS-PAGE (a) and Western analysis (b) of soluble IFN-α-2b samples obtained from expression in *C.necator* shake flask cultures, plus vectors expressed in *E.coli* BL21(DE3), harboring the same expression cassette elements. 5 μL sample were loaded into each well. Starting OD_{600} of 0.2. Lane A: molecular mass standard; lane B: uninduced pVB-2A0E0-IFN-α-2b; lane C: induced pVB-2A0E0-IFN-α-2b; lane D: induced pVB-1A0B1-IFN-α-2b; lane E: uninduced pVB-2B1E0-IFN-α-2b; lane F: induced pVB-2B1E0-IFN-α-2b; lane G: induced pVB-1B1B1-IFN-α-2b; lane H: uninduced *C.necator* wild-type (not expressing IFN-α-2b); lane I: induced *C.necator* wild-type (not expressing IFN-α-2b); lane J: molecular mass standard.

As shown in Figure 19, it is hard to confirm expression of soluble IFN-α-2b based on the SDS-PAGE, as all the bands representing IFN-α-2b have low intensity. However, the Western blot analysis indicates expression of IFN-α-2b from the vectors pVB-2A0E0-IFN-α-2b, pVB-1A0B1-IFN-α-2b and pVB-1B1B1-IFN-α-2b. The highest expression level seems to be obtained from pVB-1A0B1-IFN-α-2b.

SDS-PAGE (Figure 20a) of insoluble IFN-α-2b shows very high expression of IFN-α-2b from the vector pVB-1B1B1-IFN-α-2b. Moreover, the Western blot analysis verifies high expression from pVB-1B1B1-IFN-α-2b. In addition, expression from the vectors pVB-2B1E0-IFN-α-2b and pVB-1A0B1-IFN-α-2b was confirmed.

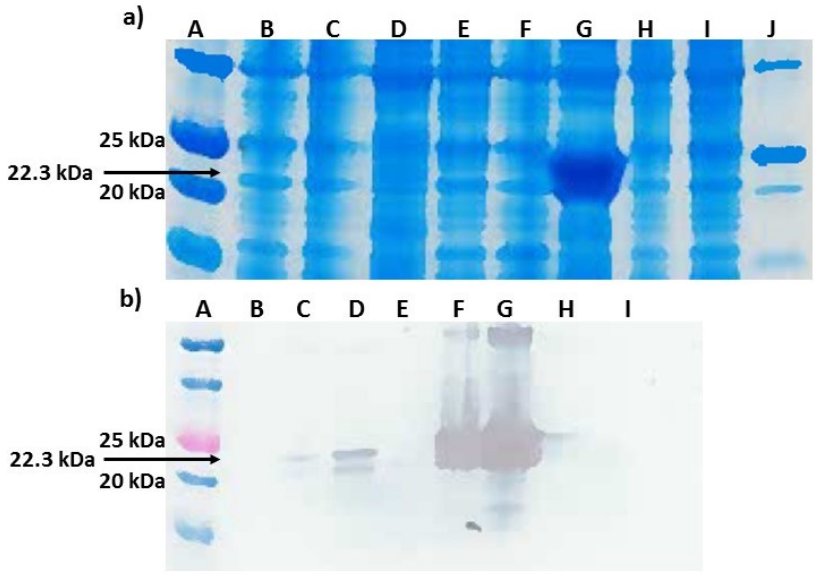

Figure 20: SDS-PAGE (a) and Western analysis (b) of insoluble IFN-α-2b samples obtained from expression in *C.necator* shake flask cultures, plus vectors expressed in *E.coli* BL21(DE3) harboring the same expression cassette elements. 10 μL sample were loaded into each well. Starting OD_{600} of 0.2. Lane A: molecular mass standard; lane B: uninduced pVB-2A0E0-IFN-α-2b; lane C: induced pVB-2A0E0-IFN-α-2b; lane D: induced pVB-1A0B1-IFN-α-2b; lane E: uninduced pVB-2B1E0-IFN-α-2b; lane F: induced pVB-2B1E0-IFN-α-2b; lane G: induced pVB-1B1B1-IFN-α-2b; lane H: uninduced *C.necator* wild-type (not expressing IFN-α-2b); lane I: induced *C.necator* wild-type (not expressing IFN-α-2b); lane J: molecular mass standard.

Based on the results presented in this section, it seems to be higher expression of IFN-α-2b in *E.coli* than *C.necator* when they are containing vectors with the same *Pm*/XylS expression cassette elements. Moreover, it seems to generally be higher protein expression in *E.coli* than *C.necator*, which might, among other reasons, be caused by lower growth (see Figure 11). Among the results presented in this section, the highest expression of soluble IFN-α-2b is obtained from *E.coli* containing pVB-1A0B1-IFN-α-2b, while the highest expression of insoluble IFN-α-2b is obtained from *E.coli* containing pVB-1B1B1-IFN-α-2b. Still, the expression of IFN-α-2b in *E.coli* and *C.necator* has consistent trends. The absence of the signal sequence *pelB* seems to be favorable for the expression of soluble IFN-α-2b in both *E.coli* and *C.necator*, while the expression of insoluble IFN-α-2b seems to increase in the presence of *pelB*. Note that *E.coli* containing pVB-1B0B1-IFN-α-2b resulted in higher level of soluble protein than pVB-1A0B1-IFN-α-2b. However, the combination of strong expression cassette and no *pelB* was not tested in *C.necator*.

3.8 Quantification of production level

One objective of this study was to compare the expression of IFN-α-2b from the individual vectors and between the two expression hosts. In order to compare the expression of IFN-α-2b from the individual vectors, quantifications of the bands representing IFN-α-2b were accomplished. The results obtained in previous sections are hard to quantify due to very different expression levels. Therefore, the samples were diluted and run on SDS-PAGE to obtain more comparable results. The results are given in Figure 21, Figure 22 and Figure 23. The calculated production levels are given in Table 3.4. The software GelQuant.net is used to measure the intensity of SDS-PAGE bands. The area intensity is used to calculate the total number of mg protein per liter. This is done by comparing the band intensity of the molecular mass standard (with known protein amounts, information on protein amount given by BioRad) and the band of interest. It is advantageous that the two bands are close in mass weight (kDa) and have comparable band intensities. For each band used to estimate a production level of IFN-α-2b, the protein amount was calculated in three parallels before finding the average production level. A more detailed description of the method is given in Section 2.8 and Appendix E.

3.8.1 Production level of IFN-α-2b in *E.coli* BL21(DE3)

Figure 21 shows SDS-PAGE and Western blot analysis for expression of IFN-α-2b in *E.coli* BL21(DE3). Each sample is diluted to a given level based on previous results (see Figure 14). As seen in Figure 21, only some of the bands are diluted to the same level as the molecular mass standard and hence only these bands are quantified. It would have been favorable to repeat the experiment with more dilutions to get more accurate quantifications and also quantify more of the samples. However, this was not done due to lack of time.

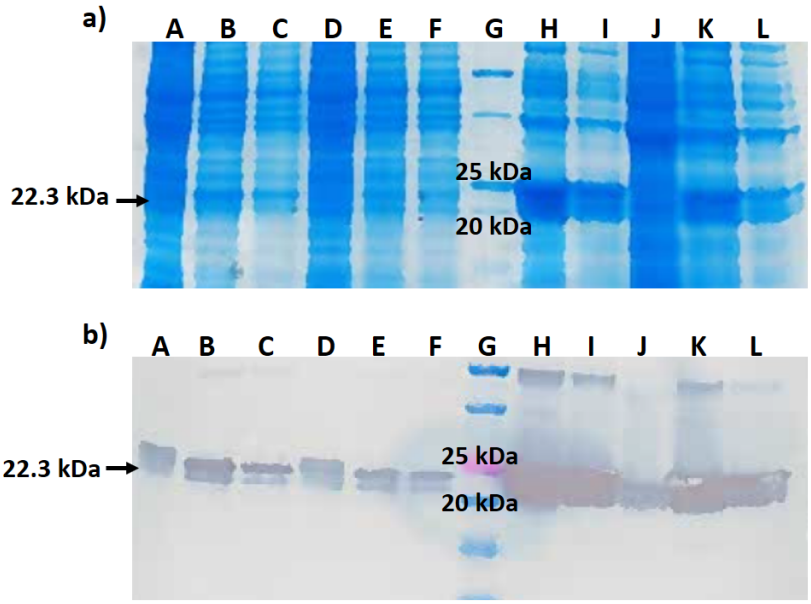

Figure 21: SDS-PAGE (a) and Western blot analysis (b) for quantification of IFN-α-2b expression in *E.coli* BL21(DE3) shake flask cultures. Soluble fractions; lane A-F, 5 μL sample loaded into each well. Insoluble fractions; lane H-L, 10 μL sample loaded into each well. The Precision Plus Unstained Standard (BioRad) was used as the molecular mass standard for the SDS-PAGE. Lane A: pVB-1B1B1-IFN-α-2b undiluted; lane B: pVB-1B0B1-IFN-α-2b 2×diluted; lane C: pVB-1B0B1-IFN-α-2b 5×diluted; lane D: pVB-1A1B1-IFN-α-2b undiluted; lane E: pVB-1A0B1-IFN-α-2b undiluted; lane F: pVB-1A0B1-IFN-α-2b 2×diluted; lane G: molecular mass standard; lane H: pVB-1B1B1-IFN-α-2b 25×diluted; lane I: pVB-1B1B1-IFN-α-2b 50×diluted; lane J: pVB-1B0B1-IFN-α-2b 5×diluted; lane K: pVB-1A1B1-IFN-α-2b 10×diluted; lane L: pVB-1A1B1-IFN-α-2b 25×diluted.

The protein bands used for quantification of the production level of IFN-α-2b in *E.coli* BL21(DE3), are presented in Figure 21a. The intensity of the bands representing soluble IFN-α-2b in lane B and C (expressed from pVB-1B0B1-IFN-α-2b) and lane E and F (expressed from pVB-1A0B1-IFN-α-2b) was measured using GelQuant. The total amount of IFN-α-2b per liter was calculated, as demonstrated in Appendix E. The protein production was calculated to be about 300 mg per liter for both pVB-1B0B1-IFN-α-2b and pVB-1A0B1-IFN-α-2b, respectively.

The protein bands visualized by Western blotting are compared in relation to each other (Figure 21b). The highest production of IFN-α-2b is observed when it is expressed from pVB-1B1B1-IFN-α-2b (strong expression cassette and *pelB*). Even when diluted 50 times, the protein sample obtained from this strain shows the highest intensity (Figure 21b lane I). In general, it is hard to distinguish the band intensities due to few dilutions and hence

hard to determine an accurate ratio. Also note that there is high uncertainty in the method, and the calculated amounts are hence only an estimate. Still, the best vector expressing soluble IFN-α-2b in *E.coli* BL21(DE3) was found to be pVB-1B0B1-IFN-α-2b, and the best vector expressing insoluble IFN-α-2b in *E.coli* BL21(DE3) was found to be pVB-1B1B1-IFN-α-2b.

3.8.2 Comparison of the production level of IFN-α-2b in *C.necator* and *E.coli* BL21(DE3)

In order to compare the expression of IFN-α-2b between the two hosts *C.necator* and *E.coli*, SDS-PAGE and Western blot analysis, with samples from both of the hosts, were performed. The expression of IFN-α-2b from pVB-2A0E0-IFN-α-2b and pVB-2B1E0-IFN-α-2b in *C.necator*, and vectors harboring the same combination of expression cassette elements expressed in *E.coli* (i.e pVB-1A0B1-IFN-α-2b and pVB-1B1B1-IFN-α-2b), was quantified and compared in relation to each other. The SDS-PAGE and Western blot analysis of soluble and insoluble fractions are presented on separate gels in Figure 22 and Figure 23.

The SDS-PAGE given in Figure 22a is used to estimate the production level of soluble IFN-α-2b in both *C.necator* and *E.coli* BL21(DE3). Expression of soluble IFN-α-2b represented in lane B and C (expressed from pVB-2A0E0-IFN-α-2b in *C.necator*) and lane I and J (expressed from pVB-2B1E0-IFN-α-2b in *C.necator*) was estimated to be about 6 mg/L and 3 mg/L respectively. *E.coli* BL21(DE3) containing pVB-1A0B1-IFN-α-2b was estimated to express about 20 mg/L, which is significant more than the expression level in *C.necator*. Moreover, when *E.coli* BL21(DE3) is the expression host, production of IFN-α-2b from the vector pVB-1A0B1-IFN-α-2b was estimated to be about 20 mg/L and about 300 mg/L based on separate SDS-PAGE gels (Figure 21 and Figure 22 respectively). Most likely, the real expression level lies somewhere between these two values.

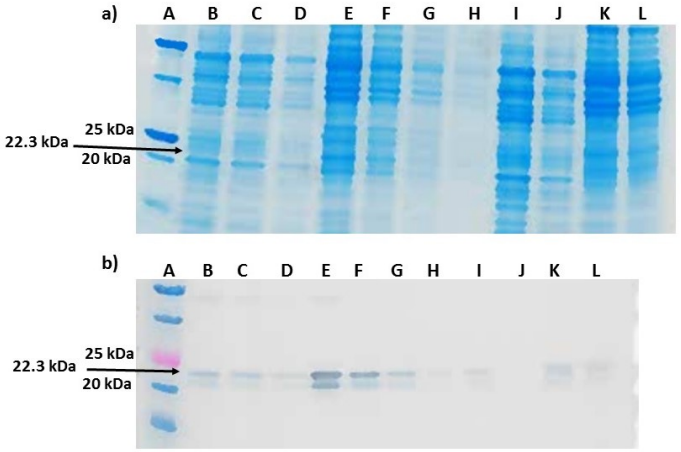

Figure 22: SDS-PAGE (a) and Western blot analysis (b) for quantification of soluble IFN-α-2b expression in *C.necator* shake flask cultures, plus vectors expressed in *E.coli* BL21(DE3) harboring the same expression cassette elements. All samples are obtained from induced cultures except lane I and J, and all lanes are loaded 5 μL. The Precision Plus Unstained Standard (BioRad) was used as the molecular mass standard for the SDS-PAGE. Lane A: molecular mass standard; lane B: pVB-2A0E0-IFN-α-2b 2×diluted; lane C: pVB-2A0E0-IFN-α-2b 5×diluted; lane D: pVB-2A0E0-IFN-α-2b 10×diluted; lane E: pVB-1A0B1-IFN-α-2b 2×diluted; lane F: pVB-1A0B1-IFN-α-2b 5×diluted; lane G: pVB-1A0B1-IFN-α-2b 10×diluted; lane H: pVB-1A0B1-IFN-α-2b 20×diluted; lane I: uninduced pVB-2B1E0-IFN-α-2b undiluted; lane J: uninduced pVB-2B1E0-IFN-α-2b 2×diluted; lane K: pVB-1B1B1-IFN-α-2b 2×diluted; lane L: pVB-1B1B1-IFN-α-2b 5×diluted.

The Western blot analysis given in Figure 22b, is comparing the visualized protein bands in relation to each other. The highest production of soluble IFN-α-2b is observed when it is expressed from pVB-1A0B1-IFN-α-2b in *E.coli*, which is in agreement with the quantification analysis. The comparison of soluble fractions indicates that there is 5-10 times higher production level obtained from *E.coli* pVB-1A0B1-IFN-α-2b than from *C.necator* pVB-2A0E0-IFN-α-2b.

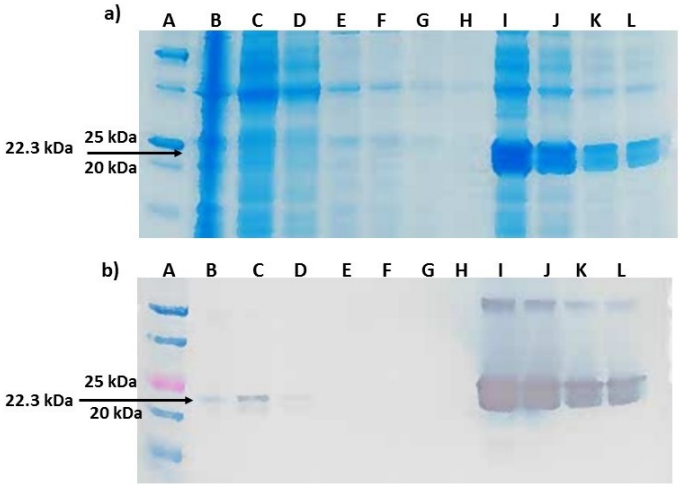

Figure 23: SDS-PAGE (a) and Western blot analysis (b) for quantification of insoluble IFN-α-2b expression in *C.necator* shake flask cultures, plus vectors expressed in *E.coli* BL21(DE3), harboring the same expression cassette elements. All samples are obtained from induced cultures and loaded 5 μL. The Precision Plus Unstained Standard (BioRad) was used as the molecular mass standard for the SDS-PAGE. Lane A: molecular mass standard; lane B: pVB-2A0E0-IFN-α-2b 5×diluted; lane C: pVB-1A1B1-IFN-α-2b 10×diluted; lane D: pVB-1A1B1-IFN-α-2b 25×diluted; lane E: pVB-2B1E0-IFN-α-2b 25×diluted; lane F: pVB-2B1E0-IFN-α-2b 50×diluted; lane G: pVB-2B1E0-IFN-α-2b 75×diluted; lane H: pVB-2B1E0-IFN-α-2b 100×diluted; lane I: pVB-1B1B1-IFN-α-2b 25×diluted; lane J: pVB-1B1B1-IFN-α-2b 50×diluted; lane K: pVB-1B1B1-IFN-α-2b 75×diluted; lane L: pVB-1B1B1-IFN-α-2b 100×diluted.

The SDS-PAGE given in Figure 23a is used to estimate the production level of insoluble IFN-α-2b in both *C.necator* and *E.coli* BL21(DE3). The expression of insoluble IFN-α-2b represented in lane E (expressed from pVB-2B1E0-IFN-α-2b in *C.necator*) and lane J and K (expressed from pVB-1B1B1-IFN-α-2b in *E.coli* BL21(DE3)) was estimated to be about 80 mg/L and 340 mg/L respectively.

Comparison of insoluble IFN-α-2b based on the Western blot analysis, indicates at least 100 times higher expression in *E.coli* pVB-1B1B1-IFN-α-2b than in any other of the expression vectors presented in Figure 23b. As previously known, a larger fraction of IFN-α-2b is found as insoluble than soluble. Thus, it is expected to obtain higher production level of insoluble than soluble IFN-α-2b in both *C.necator* and *E.coli* BL21(DE3).

An overview of the estimated production levels of IFN-α-2b is listed in Table 3.4.

Table 3.4: Estimate of the production levels of recombinant IFN-α-2b protein obtained in shake flasks cultures of *E.coli* BL21(DE3) and *C.necator* respectively.

Strain	**Plasmid**	**Fraction**	**Production level [mg/liter]**
E.coli BL21(DE3)	pVB-1B0B1-IFN-α-2b	Soluble	300*
E.coli BL21(DE3)	pVB-1A0B1-IFN-α-2b	Soluble	150**
E.coli BL21(DE3)	pVB-1B1B1-IFN-α-2b	Insoluble	340
C.necator	pVB-2A0E0-IFNα-2b	Soluble	6
C.necator	pVB-2B1E0-IFNα-2b	Soluble	3
C.necator	pVB-2B1E0-IFNα-2b	Insoluble	80

*Probably too high due to high uncertainty in the method.
**A mean of two production levels obtained from separate gels are calculated to give a more reliable estimate.

3.9 Spectrophotometry analysis of mCherry

A spectrophotometer (Tecan) was used to measure relative fluorescence units (RFU) of samples obtained from *E.coli* containing vectors expressing mCherry or the negative control plasmid pVB1. One expects induced samples to have greater activity than their corresponding uninduced samples. As described in Section 3.5.2, mCherry was expressed from the vectors pVB-1A0B1-mCherry, pVB-1A1B1-mCherry, pVB-1B0B1-mCherry, and pVB-1B1B1-mCherry. Spectrophotometry analysis of soluble and insoluble protein fractions are presented in Figure 24 and Figure 25 respectively.

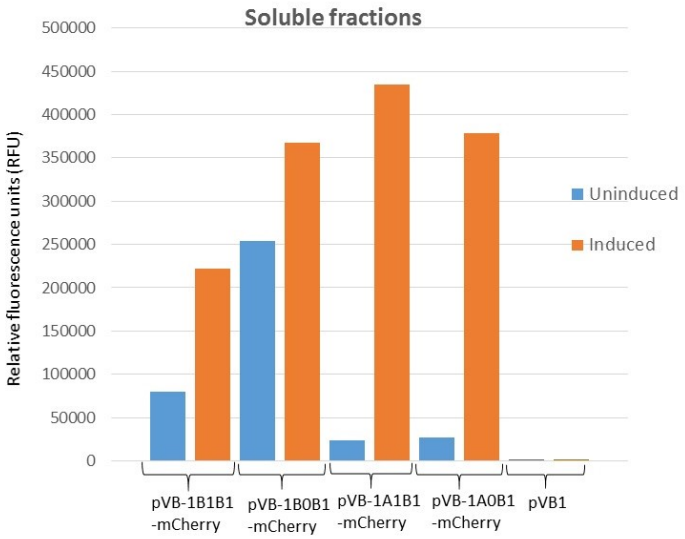

Figure 24: Activity of soluble mCherry measured by relative fluorescence units.

Spectrophotometry analysis of soluble fractions show that all of the induced samples have greater activity than their corresponding uninduced samples (Figure 24). The greatest activity was obtained from *E.coli* containing pVB-1A1B1-mCherry, while the lowest activity was obtained from *E.coli* containing pVB-1B1B1-mCherry. The results are in agreement with the SDS-PAGE from Section 3.5.2, where cultures containing pVB-1B1B1-mCherry generate lowest expression, and hence the lowest activity. Cultures containing pVB-1B0B1-Cherry, pVB-1A1B1-mCherry or pVB-1A0B1-mCherry all have activity measurements close to each other, which are in agreement with the SDS-PAGE (Figure 16). However, the greatest activity is observed from the vector pVB-1A1B1-mCherry.

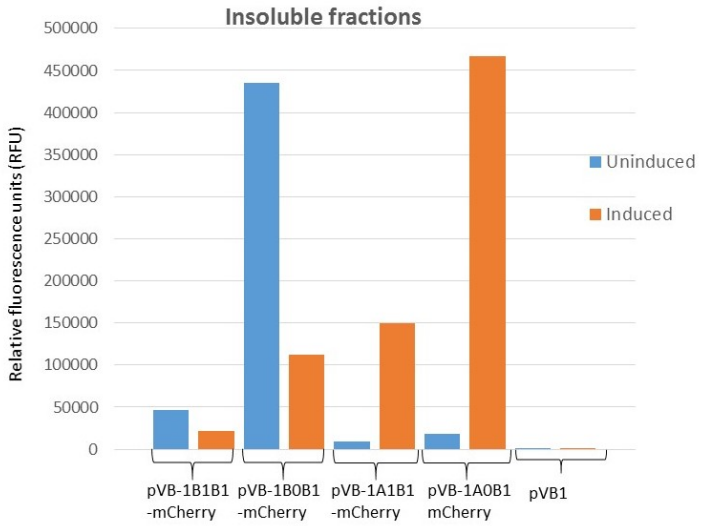

Figure 25: Activity of insoluble mCherry measured by relative fluorescence units.

Spectrophotometry analysis of insoluble mCherry fractions show varying results, as presented in Figure 25. It was obtained higher activity of induced samples than uninduced samples of *E.coli* containing pVB-1A1B1-mCherry and pVB-1A0B1-mCherry, which is exactly as expected. However, it was obtained higher activity of uninduced cultures containing pVB-1B1B1-mCherry and pVB-1B0B1-mCherry than induced cultures containing the same vectors. Thus, it seems like the improved *Pm*/5'UTR element has a negative impact on the mCherry expression, which is in agreement with the SDS-PAGE (Figure 16).

It is not known why the strong *Pm*/5'UTR element has a negative impact on the expression of mCherry, but it might be due to overexpression, which again might cause stress and eventually cell lysis. Interestingly, it was observed that the induced culture containing pVB-1B1B1-mCherry had an intense pink color before cell harvesting, but the resulting protein pellet was observed to be less pink (see Figure 15). The experiment was repeated to examine if it might be released protein into the supernatant due to cell lysis, especially in the cases with strong expression cassette elements. Only induced cultures were tested. Figure 26 represent the results when the activity was measured in soluble and insoluble fractions, and the supernatant. Figure 27 represent the activity measurement only for the supernatant.

As shown in Figure 26, the activity of the supernatants are relatively low compared to

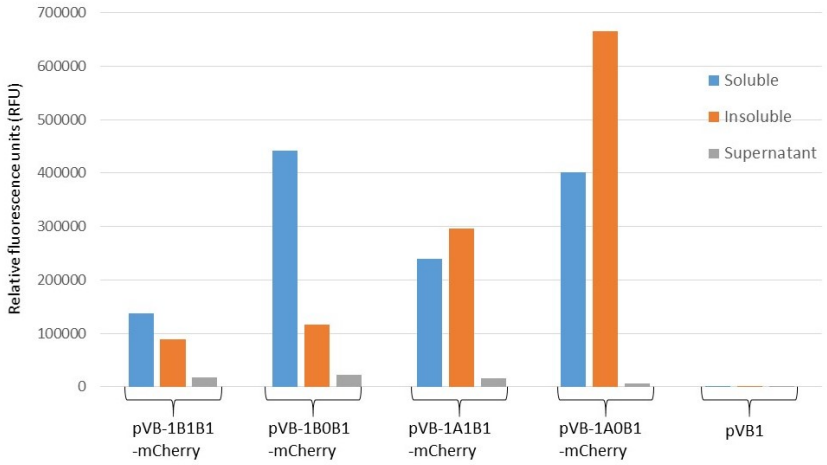

Figure 26: Activity of soluble and insoluble mCherry, and the supernatants obtained after cell harvesting, measured by relative fluorescence units.

the activity of soluble and insoluble mCherry fractions. Figure 27 better illustrates the internally differences between the supernatants. It is observed highest activity in the supernatants obtained from cultures containing vectors with strong expression cassette elements, i.e. the *Pm* variant ML2-5 and the 5'UTR variant LIII-3. However, the activity of the supernatant obtained from the culture containing pVB-1A1B1-mCherry is also found to have an activity above 15000 RFU. Induced cultures containing pVB-1A0B1-mCherry are found to have the lowest activity of the supernatant The expression from this vector does also generate high activity of soluble and insoluble mCherry fractions, which most likely makes this vector the favorable choice for expression of mCherry.

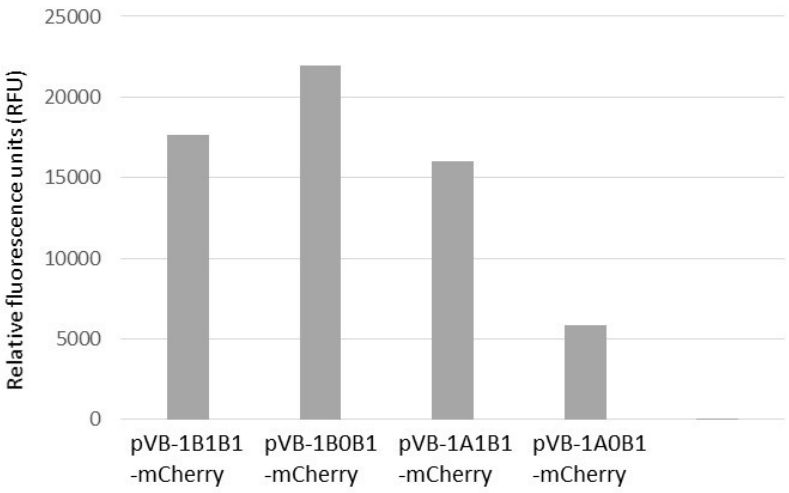

Figure 27: Activity of the supernatants obtained after harvesting the cells. The activity is measured by relative fluorescence units.

4 Discussion

4.1 Evaluation of cloning strategies in *C.necator*

Previous studies have successfully transformed *C.necator* by transconjugation [9] or biparental-mating [52]. This study tested electroporation of *C.necator* uptake of RK2-based expression vectors, which resulted in no visible colonies. For further work it is suggested that alternative methods like transconjugation and biparental-mating are executed to test for uptake of RK2-based vectors by *C.necator*. Vectron Biosolutions has previously constructed pBBR1-based expression vectors and successfully electroporated *C.necator* for uptake of these [Vectron unpublished results]. Therefore, several attempts of construction of pBBR1-based expression vectors were performed, which finally resulted in successful construction. Due to time shortage, expression of these vectors in *C.necator* was not accomplished. For further work it is suggested to test expression of IFN-α-2b from the pBBR1-based vectors in *C.necator*.

4.2 Expression of IFN-α-2b in *E.coli* BL21(DE3)

It was obtained detectable expression of both soluble and insoluble IFN-α-2b in *E.coli* BL21(DE3). In order to express high levels of IFN-α-2b, combinations of different expression cassette elements and presence/absence of the signal peptide *pelB* were tested. The presence of the *Pm*/5'UTR variant comPU was found to give higher expression than the presence of the corresponding wild-type variant, regarding both insoluble and soluble fractions. This is in accordance with a previous study by Zwick and colleagues (2103) that obtained high expression of β-lactamase when combining high-expression variants of regulatory elements of the *Pm*/XylS expression cassette [60]. The study states that the combination of the *Pm* variant ML2-5, which gives further stimulation of transcription [5], and the 5'UTR variant LIII-3, which gives further stimulation of translation [7], together (termed comPU) gives further stimulation of translation and thus higher expression levels than for the improved *Pm* and 5'UTR individually [60].

While the presence of *pelB* signal peptide does not seem to promote higher expression of soluble IFN-α-2b, its presence seems to greatly promote higher expression of insoluble

IFN-α-2b. The greatest expression of insoluble IFN-α-2b is achieved when *pelB* is present and in combination with the strong *Pm*/5'UTR element. Previous research by Sletta and colleagues (2007) found that the codon-optimized IFN-α-2b gene lead to high production level for IFN-α-2b in *E.coli* RV308, but only when *pelB* was fused to the 5'-terminal end of the coding region [50]. They obtained a production level of 40 $\pm$ 12 mg IFN-α-2b per liter, but the protein was only present as insoluble fractions [50]. This study is consistent with the work done by Sletta and colleagues regarding expression of insoluble protein, where the presence of *pelB* promote high expression of IFN-α-2b. Moreover, the production level detected in this study is much higher than reported in previous studies. As far as one knows, detectable levels of soluble IFN-α-2b in *Ecoli* is not reported before. This study has detected a production level of soluble IFN-α-2b at about 300 mg/L from the vector harboring the strong *Pm*/XylS expression cassette element without *pelB*. Note that the estimated level of 300 mg/L most likely is higher than the real production level due to high uncertainty in the quantification method. The software GelQuant.net is used to estimate the production level by drawing a selection rectangle around a image segment, hence calculating the area intensity a SDS-PAGE band. However, the calculated intensity varies according to the size of the selection rectangle set by the user itself, which gives inaccurate results.

As described above, the presence of *pelB* signal sequence facilitate translocation of IFN-α-2b to the periplasm in both of the expression hosts. Hence, expression vectors harboring *pelB* is expected to give two bands on the SDS-PAGE and Western blot gels. One band represents the expression of IFN-α-2b, while the other band represents the cleaved *pelB* signal peptide. The expression from vectors harboring the *pelB* is consistent with the expected results. However, there is also observed two protein bands from expression vectors which do not harbor the *pelB* signal peptide. It is not known what causes the appearance of the unexpected band. For future work it is suggested to investigate what may cause the additional band. For instance, it is suggested to run a sequence alignment of the codon-optimized and the human IFN-α-2b genes to identify differences. It is believed that the native signal sequence of IFN-α-2b is removed in the codon-optimized version. However, this may also be confirmed by the sequence alignment.

Vectors combining the high-expression *Pm*/5'UTR element and the high-expression variant of the activator protein gene StEP-13 (also termed comX) were constructed. In cooperation with Vectron Biosolutions, expression from these vectors were tested in *E.coli* BL21(DE3). The *Pm* variant ML2-5, the 5'UTR variant LIII-3 and the *xylS* variant StEP-13 were combined and expression tested in presence and absence of *pelB* [Vectron unpublished results]. However, the presence of StEP-13 did not further increase the expression of IFN-α-2b. This is not in agreement with results obtained by Zwick and colleagues (2013), which found that combination of all three high-expression variants lead to further improvement of protein expression [60]. It might be likely that the presence of a strong expression cassette may cause an overproduction rate that the cell does not tolerate, meaning that the cell can not produce more protein above a given level. This might explain the results obtained by Vectron Biosolutions. Moreover, the StEP-13 mutation has been found only to affect the expression level from *Pm* for low and moderate inducer concentrations [61]. The inducer concentration used by Vectron Biosolutions is 2 mM, which is regarded as a relatively high concentration.

4.3 Expression of IFN-α-2b in *C.necator*

It was obtained detectable expression of both soluble and insoluble IFN-α-2b in *C.necator*. As mentioned above, not all expression vectors were tested in *C.necator* due to time shortage. The expression of IFN-α-2b from the vector harboring wild-type expression cassette elements without *pelB* and the vector harboring strong expression-cassette element with *pelB* was evaluated. Wild-type expression cassette elements without *pelB* resulted in the highest expression of soluble IFN-α-2b, while strong expression cassette elements with *pelB* gave the highest expression of insoluble IFN-α-2b. Previous research have demonstrated that the *Pm*/XylS expression cassette function in *C.necator*, and that expression in *C.necator* has resulted in protein in the correct folded way [52] [9] [Vectron/Sintef unpublsihed results]. This is also demonstrated in this study, showing that *C.necator* has the potential to function as an efficient expression host.

4.4 Comparison of *C.necator* and *E.coli* as suitable expression hosts

The comparison of expression in *C.necator* and *E.coli* BL21(DE3), shows that the production levels in *C.necator* are significant lower than production levels obtained in *E.coli* BL21(DE3). However, the expression trends in the two hosts are consistent, showing that the combination of the same expression cassette elements promote expression of IFN-α-2b in both *C.necator* and *E.coli* BL21(DE3). Note that *E.coli* is a more well-characterized organism than *C.necator*, and further characterization of *C.necator* may lead to improvement of *C.necator* as a host organism.

4.5 Conclusion

The production of IFN-α-2b in soluble form in both *C.necator* and *E.coli* BL21(DE3) was achieved. Strong expression cassette elements without *pelB* signal sequence were favorable for expression of soluble IFN-α-2b. Insoluble fractions of IFN-α-2b were also obtained, where strong expression cassette elements in presence of *pelB* gave the highest expression of IFN-α-2b. A larger fraction of the protein was found as insoluble than soluble IFN-α-2b. Generally, it is obtained higher expression of IFN-α-2b in *E.coli* BL21(DE3) than *C.necator*.

4.6 Further work

In the future work it is suggested to test alternative transformation methods of *C.necator*, for instance transconjugation or biparental-mating, for uptake of RK2-based vectors. It is also suggested to test expression from pBBR1-based vectors in *C.necator* that not were tested in this study. In addition, testing of more expression cassettes and other signal peptides, plus optimization of growth conditions such as medium and temperature, may open up for potential future improvement of expression in *C.necator*. As previous stated by Kuscharova and colleagues, the *celB* fusion partner is found to stimulate expression of IFN-α-2b in *E.coli* without translocation of the protein to the periplasm [32]. Hence, it would be interesting to test fusion of *celB* to the codon-optimized version of the IFN-α-2b gene used in this study, and how this impact expression of IFN-α-2b in both *E.coli* BL21(DE3) and *C.necator*. Further investigation of *C.necator* and future work toward

even tighter gene expression in this organism may result in a functional expression platform useful for expression of many proteins, including IFN-α-2b.

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A The codon-optimized IFN-α-2b gene

A codon-optimized version of the human IFN-α-2b gene was to used to express IFN-α-2b-*c-myc*-His6 in this study. The codon-optimized version is obtained from Vectron Biosolutions and the sequence is given in Figure 28.

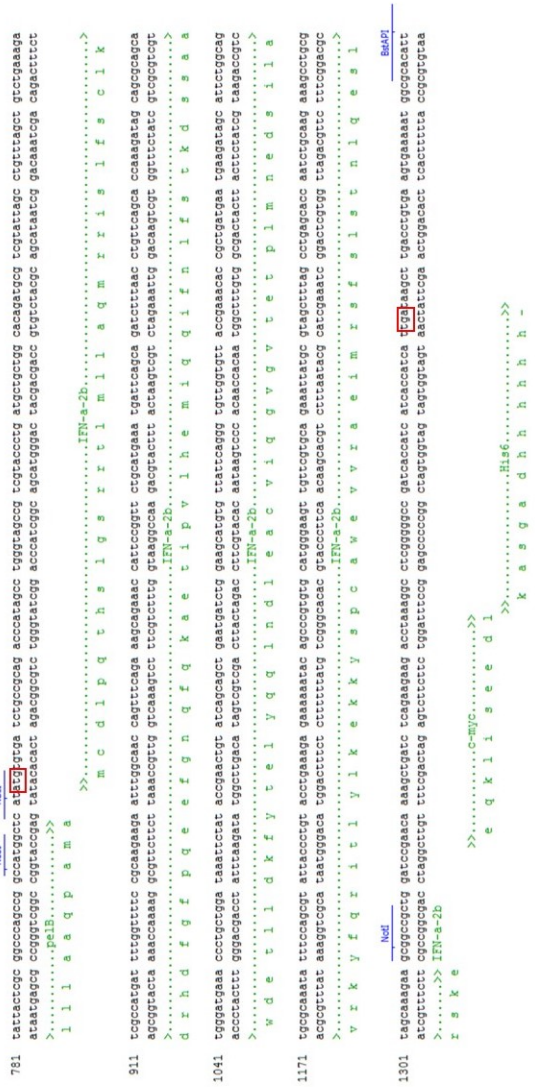

Figure 28: Nucleotide sequence of codon-optimized IFN-α-2b, plus the protein sequence given in green letterrs. The *c-myc*-His6 tag is presented after the IFN-α-2b gene. Start codon (ATG) and stop codon (TGA) is indicated by red boxes. The figure is obtained from the software Clone Manager 9.

B Stock solutions Hi+YE Basis medium

Stock solutions used to make Hi+YE Basis medium for expression of recombinant proteins in *E.coli* BL21(DE3) is given in Figure 29.

Stock solutions

Component	**g/l**	**pr 0,5 l**
Fe(III) citrate hydrate	6	3
Distilled, ion free water	1000	500

Component	**g/l**	**pr 25 ml**
H_3BO_3	30	0.75
Distilled, ion free water	1000	25

Component	**g/l**	**pr 250 ml**
$MnCl_2*4H_2O$	10	2.5
Distilled, ion free water	1000	250

Component	**g/l**	**pr 25 ml**
$EDTA*2H_2O$ (titriplex)	84	2.1
Distilled, ion free water	1000	25

Component	**g/l**	**pr 25 ml**
$CuCl_2*2H_2O$	15	0.375
Distilled, ion free water	1000	25

Component	**g/l**	**pr 25 ml**
$Na_2Mo_4O_4*2H_2O$	25	0.625
Distilled, ion free water	1000	25

Component	**g/l**	**pr 25 ml**
$CoCl_2*6H_2O$	25	0.625
Distilled, ion free water	1000	25

Component	**g/l**	**pr 250 ml**
$Zn(CH_3COO)_2*2H_20$	4	1
Distilled, ion free water	1000	250

Figure 29: Stock solutions used to make Hi-YE Basis medium. All stock solutions are pre-made from Vectron Biosolutions (unknown reference).

C Molecular weight standard for gel electrophoresis

Molecular weight standards with known band sizes were used to determine the size of DNA fragments separated by gel electrophoresis. Figure 30 and Figure 31 present the molecular weight standards used in this study. These are from NEB and GeneRuler respectively.

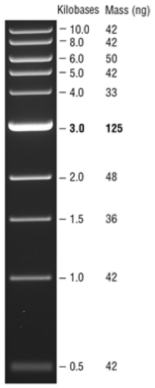

Figure 30: 1 kb DNA ladder (NEB) visualized by GelRed staining on a 0.8% TAE agarose gel.

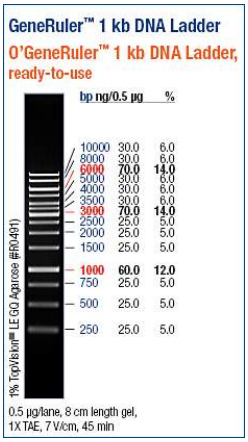

Figure 31: 1 kb DNA ladder (GeneRuler) visualized by GelRed staining on a 0.8% TAE agarose gel.

D Molecular weight standard for SDS-PAGE

Molecular weight standards with known band sizes were used to estimate the molecular weight of the protein bands obtained from SDS-PAGE gel and Western blot, see Figure 32 and Figure 33.

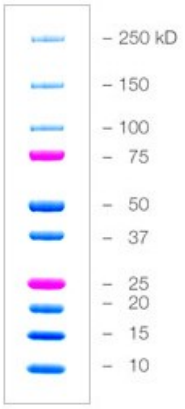

Figure 32: The Precision Plus Protein Standards Dual Color (Bio Rad) was used as a molecular weight standard for SDS-PAGE and Western blot analysis.

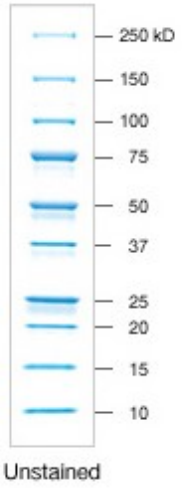

Figure 33: The Precision Plus Protein Unstained Standards (Bio Rad) was used as a molecular weight standard for SDS-PAGE analysis.

E Quantification of production level

The Software GelQuant.net was used to measure the area intensity of SDS-PAGE bands. The samples were diluted to obtain similar band intensities as bands of the molecular weight standard within the same mass range. IFN-α-2b has a mass of 22.3 kDa. Precision Plus protein amount of the 20 kDa band is given to be 150 ng/10 μL, and the protein amount of the 25 kDa band is given to be 800 ng/10μL. The Precision Plus protein amount per 5 μL is 75 ng for the 20 kDa band and 400 ng for the 25 kDa band, respectively. The area-intensity-ratio between the band of the molecular weight standard (20 kDa or 25 kDa) and the band of interest (IFN-α-2b) was calculated. The ratio was multiplied with the given protein amount in the molecular weight standard to find the total protein amount [ng/5μL] of the band of interest.

ng per 5μL was multiplied by 200 to obtain the protein amount in μg per liter production culture, before multiplying by 0.001 to obtain the protein amount in mg per liter, as illustrated below.

$\frac{ng}{5\mu L} \times 1000\frac{\mu L}{mL} = \frac{ng}{\mu L} \times 200\frac{ng}{mL} = \frac{ng}{mL} = \frac{\mu g}{L}$

$\frac{\mu g}{L} \times 0.001\frac{mg}{\mu g} = \frac{mg}{L}$

The following factors were considered when finding the original protein amount per liter production culture

- NaCl concentrating factor X: amount mL NaCl $\times$ X = 30 mL production culture, giving a concentrating factor
  X = 30 mL/amount mL NaCl. The protein amount was divided by X.
- 1 mL harvested cells diluted in NaCl was centrifuged and the pellet concentrated in 500 mL lysis buffer. Thus the protein amount was divided by 2.
- The protein amount was multiplied by a dilution factor Y (Y fold dilution of the sample), which is individual for each sample.
- Regarding dilution of 30 μL sample in 30 μL sample buffer, the protein amount was multiplied by 2.

F Alternative method: construction of vectors for use in *C.necator*

In addition to the construction of pBBR1-based expression vectors by utilizing the digestion/ligation method, the expression vectors pVB-2A1E0-IFN-α-2b and pVB-2B0E0-IFN-α-2b were also tried constructed by using the Polymerase chain reaction method.

F.1 Polymerase chain reaction (PCR)

PCR is a technique used to amplify the number of copies of a DNA segment *in vitro*. Two synthetic oligonucleotides (primers) are prepared, complementary to sequences on opposite strands of the target DNA (template) at positions defining the ends of the segment to be amplified [42]. The thermostabele *Taq* DNA polymerase, originating from *Thermus aquaticus*, is used for DNA synthesis [42]. The primers used in this study, their sequence and the complementary templates are listed in Table F.1. The template pBBR1 and the primers Vf-pBBR1/Vr-pBBR1 are utilized for construction of the backbone fragment. The templates pVB-1A1B1-IFN-α-2b and pVB-1B0B1-IFN-α-2b are used to create the "insert", containing the *Pm*/XylS expression cassette with/without the *pelB* signal sequence, respectively.

Table F.1: PCR primers, their sequences and the corresponding templates used in construction of pBBR1-based expression vectors.

PCR primer	**Primer sequence 5'- 3'**	**Template**
Vf-pBBR1	AGCTGTTTCCTGTGTGAATTG	pBBR1
Vr-pBBR1	GCGTTAATATTTTGTTAAAATTCGCGTTA	pBBR1
1f-pVB-1A0B1-o(	CGCAATTTTAACAAAATATTAACGCTGCC AACCCATCCCTTCT	pVB-1A1B1-IFN-α-2b/ pVB-1B0B1-IFN-α-2b
1r-pVB-1A0B1-o(	AATTTCACACAGGAAACAGCTCAAGCTTG CTAGAGGGTCAG	pVB-1A1B1-IFN-α-2b/ pVB-1B0B1-IFN-α-2b

PCR can be divided into the following major steps, which usually are repeated 25-30 times:

- **Denaturation**: Separation of the DNA template strand by heating, usually up to 94-98°C.
- **Annealing of primers**: The temperature is lowered to about 50-56°C. Single stranded DNA template is annealed to synthetic oligonucleotide primers.
- **DNA synthesis**: The temperature is increased to about 75°C, allowing *Taq* DNA polymerase to synthesis DNA in a 5' to 3' direction.

The following PCR mix was used, given in Table F.2 All reagents were pipetted into a PCR tube.

Table F.2: PCR mix used for used for construction of pBBR1-based expression vectors.

Component	**1x**
5× Q5 buffer	10 μL
Q5 Polymerase	0.5 μL
5× GC enhancer	10 μL
10 mM dNTP	1 μL
dH_20	22.5 μL
Primer 1	2.5 μL
Primer 2	2.5 μL
Template	1 μL

The following PCR programs were used for amplification of the backbone fragment and insert fragments, see Table F.3.

Table F.3: PCR programs used for denaturation of the DNA template, annealing of primers and DNA synthesis by DNA polymerase.

Program Backbone		
Temperature	**Time**	
98°C	30 sec	
98°C	30 sec	Repeated
54°C	30 sec	35×
72°C	4 min	
72°C	5 min	
4°C	hold	

Program Insert		
Temperature	**Time**	
98°C	30 sec	
98°C	10 sec	Repeated
50°C	30 sec	35×
72°C	2 min	
72°C	5 min	
4°C	hold	

Separation of the PCR products by gel electrophoresis, resulted in the correct separation. The DNA fragments were excised from the gel and assembled using the SLIC protocol, followed by regular heat-shock transformation.

F.2 One-step sequence- and ligation-independent cloning (SLIC)

The following procedure was performed to assemble the DNA fragments obtained by PCR [28]. The vector and insert mixture is given in Table F.4. Even though correct the fragments were obtained when separating the PCR products, the SLIC protocol did not lead to successful assembling. It is not known why the assembling was unsuccessful.

Table F.4: Mixture for assembling of backbone vector and insert. The amount of vector and insert is determined by using a ligation calculator.

Components	**Volume added**
Linearized vector	x μL
Insert	y μL
10× NEB Buffer 2.1	1 μL
dH_20	Up tp 10 μL

SLIC protocol:

1. The linearized vector and insert were mixed at a molar ratio of 1:2 in a 1.5 mL tube, as given in Table F.4.

2. 0.2 μL of T4 DNA polymerase (NEB) was added to the mixture and incubated at room temperature for 2.5 minutes.

3. The reaction mixture was immediately put on ice to stop the reaction. The mixture was incubated on ice for 10 minutes.

4. Chemically component *E.coli* cells were thawed on ice for approximately 10 minutes.

5. For single fragment cloning, the cells were gently mixed with 1-2 μL of the reaction mixture. For multiple fragments cloning, the cells were gently mixed with with 3-5 μL of the reaction mix.

6. The cells were incubated on ice for 20 minutes.

7. The cells were heat-shocked at 42°C for 45 seconds.
8. The cells were incubated on ice for 2 minutes.
9. 900 μL LB broth was added to 100 μL of the cells and transferred to a 15 mL round-bottom tube.
10. The cells were incubated at 37 °for 1 hour.
11. The cells were plated on agar plates containing suitable antibiotics and incubated at 37°overnight, before the colonies were analyzed.