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Development of Expression Systems and Cultivation Conditions for Production of Heterologous Proteins in Pseudomonas

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Biotechnology
Submission date: May 2011
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Preface

The work presented in this study was performed for the Institute for Biotechnology, NTNU, Trondheim and SINTEF Materials and Chemistry, Department for Biotechnology, Trondheim. It was part of the project "Development of versatile bacterial expression systems for use in recombinant protein production, metabolic engineering, and systems biology", a collaboration between NTNU, SINTEF (Department for Biotechnology) and SU (Saarland University, Germany). The work was done under the leadership of Trond E. Ellingsen (Professor II at NTNU and Research Director at SINTEF, Department of Biotechnology). I appreciate your advice.

I would like to thank my supervisor Håvard Sletta (Senior Scientist at SINTEF, Department of Biotechnology) and Anne Tøndervik (Research Scientist at SINTEF, Department of Biotechnology). I am grateful to you for your guidance and enthusiasm and for our motivating conversations.

I would also like to thank Randi Aune, Tone Haugen and Vu To (Engineers at SINTEF, Department of Biotechnology) for always taking the time to answer my questions during my laboratory experiments.

Last but not least I would like to thank Marte Valde for being my good friend and lunch partner, my family for being supportive and my fiancé Johnny for always taking the time to listen to all my concerns.

May 2011, Trondheim

Tonje Husby Haukaas

Content

Content

Abstract

This study has been part of the project "Development of versatile bacterial expression systems for use in recombinant protein production, metabolic engineering, and systems biology", a collaboration between NTNU, SINTEF (Department for Biotechnology) and SU (Saarland University, Germany). The major goal of both the project and this study was to develop and apply advanced biological tools for control of gene expression.

Recombinant DNA, created by artificially producing genetic sequences, can be transferred to microorganisms and change their properties. One new possible property is the production of specific recombinant proteins, which have potential for use in both industry and medicine. The most intensely studied and attractive heterologous (recombinant) protein producer is to date *E. coli.* Although there are several advantages using *E. coli,* some of its related disadvantages can cause low volumetric yield of specific proteins. When this occurs, there is a need for alternative producers and therefore a gene expression system that functions in diverse bacterial species. The *Pm/XylS* expression cassette, which has proven useful for industrial level production of recombinant protein, is used as a basis for this study's expression system.

Expression vectors harbouring *Pm/XylS* and genes for the human proteins IFN-α2b and GM-CSF were constructed. Protein expression from these vectors was evaluated in *Pseudomonas* species under different cultivation conditions. During cultivations of *P. fluorescens* SBW25 in shake flasks, instability of the relevant expression plasmids was detected. Evaluation of alternative *Pseudomonas* strains revealed that the same plasmids were stable in *P. putida* KT2440. Furthermore it was found that *P. putida* KT2440 was easy to cultivate in both rich and minimal media, and it was therefore chosen for further use in this study.

Production of IFN-α2b and GM-CSF from KT2440 was obtained under optimized shake flask experiments and fed batch fermentations, but in low quantities. To further examine KT2440s production potential, the expression plasmids was genetically engineered. This was done by incorporating a copy number mutation and codon optimizing target genes and signal sequence *pelB*. Exchange of *trfA* (the gene for replication protein TrfA) with *trfAcop271C* yielded approximately 3.5-fold increase of plasmid number in KT2440, the same as previously reported for *E. coli*. After this modification, the production of both model proteins was estimated to have increased 3.5-fold or more. Additionally, soluble IFN-α2b was detected,

Abstract

which was not reported for *E. coli* under the same conditions in a previous study. Codon optimization of the target genes and signal sequence did not have expected effects on protein production in KT2440 under conditions tested, when compared to wild type copy number expression plasmids. Combination of codon optimized sequences and increased copy number had negative effect under the conditions tested.

Further evaluation of the genetically modified expression plasmids was performed in fed batch fermentations of KT2440. Plasmid stability was found to be high, but the protein production obtained was lower than expected from results in the previous shake flask experiment. During fed batch fermentations, an observed increase in metabolism at induction indicated that the inducer was consumed. Since induction here is performed when substrate is limited, in contrast to the conditions in shake flask experiments, it is possible that the inducer is metabolized instead of inducing protein production. This would explain the observed differences in production and should therefore be tested.

 P. putida KT2440 have through this study proved as a potential industrial protein producer based on its growth properties and the fact that simple genetic modifications of expression plasmids proved to positively affect the production of model proteins.

1 Introduction

1.1 The history of recombinant DNA in biotechnology

The *term* biotechnology was first used in 1917 by Karl Ereky to describe the large-scale breeding of pigs (Glick et al., 2010). The *use*, on the other hand, has a longer history. Domestication of plants and animals can be traced back as far as 10 000 years ago. The use of microbes for food fermentation has also excised for a long time, and many of the techniques for beer, wine and cheese production have developed from old biotechnology (Parekh, 2004). Today biotechnology can be defined as technology using, for the most part microorganisms (Glick et al., 2010), but also plant-, animal- and human cells (Regjeringen.no).

In 1944, Avery, MacLeod and McCarty discovered that the genetic information is not stored in proteins, as previously believed, but in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). For many, this discovery marks the beginning of the recombinant DNA (rDNA) technology that would forever change biotechnology. From time-consuming screening of microbial mutants, the biotechnology today has efficient tools for creating microorganisms with specific genetic attributes (Glick et al., 2010). This is done by the use of rDNA molecules which consist of DNA fragments, either from the same or from different sources, artificially joined together (Børresen-Dale, 2009).

In addition to discovery of the genetic material, several other important milestones are worth mentioning in the history of rDNA technology. In 1953 the structure of DNA was determined by Watson and Crick and in 1966 the genetic code was established (Emery and Malcolm, 1995). Even though the previous events were crucial, many consider the following to be the starting point for rDNA technology (Emery and Malcolm, 1995). In the early 1960s' Werner Arber discovered bacteria's restriction endonucleases ability to cleave foreign DNA at nonspecific sequences (Nelson and Cox, 2008), and in 1970 Hamilton Smith detected sequence-specific restriction endonucleases (Emery and Malcolm, 1995). After experiments with these enzymes done by Daniel Nathans in 1969, Werner Arber, Daniel Nathans and Hamilton Smith were awarded with the Nobel Prize in Physiology or Medicine in 1978. The basis for this award was *"for the discovery of restriction enzymes and their application to problems of molecular genetics"* (Nobelprize.org).

Introduction

The restriction endonucleases, also called restriction enzymes, cut DNA molecules to protect the bacteria against viruses. When viruses attack, their DNA or RNA is imported into the cell. The viruses start controlling metabolism and the host begins to produce viral proteins that can form new viruses. These new reproduced viruses exit the cell and attack new ones (Henriksen and Bøvre, 2010). The restriction enzymes could protect the bacteria against such virus attacks by digesting the viral DNA. When, among other contributors, Nathans published that restriction enzymes cut at specific nucleotide sequences in viral DNA, methods for inserting specific fragments into other DNA molecules were soon developed. It now became possible to clone specific DNA sequences and make microorganisms produce proteins from specific genes. These proteins are referred to as recombinant proteins because they originate from rDNA. Figure 1 illustrates how recombinant DNA plasmids, circular DNA, can be made by the use of restriction enzymes.
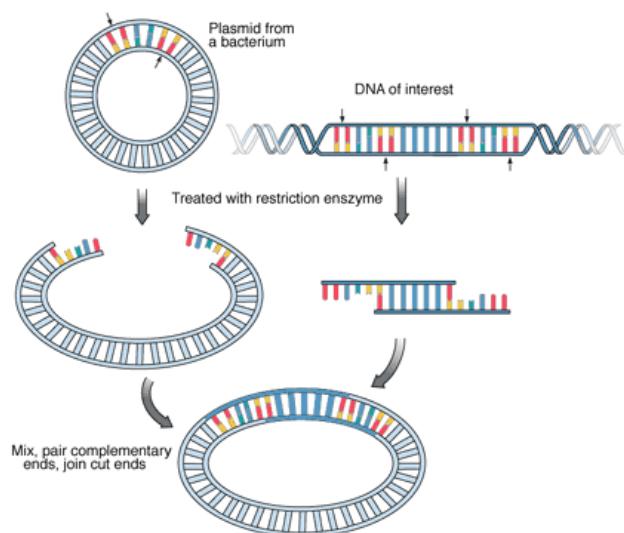
Figure 1: Illustration of how restriction enzymes cuts at specific sites in bacterial plasmids (top left) and DNA of interests (top right) to join the fragments in recombinant DNA (bottom) (Learning, 2002)

1.2 Recombinant DNA technology in biotechnology today

The knowledge and techniques within recombinant DNA and biotechnology has since the 1970s evolved at rapid rate. Some of the important milestones over these years are the construction of a recombinant plasmid in 1972 (Jackson et al., 1972) development of sequencing techniques in 1976, production of human insulin in *E. coli* in 1978, and completion of sequencing of the human genome in 2001, to name a few (Glick et al., 2010). The work and research done based on these technologies have enabled us to develope efficient methods for sequencing, modifying, cloning and synthesis of genes and gene fragments (Berg and Mertz, 2010).

One of the new fields arisen from the new knowledge and techniques is synthetic biology. The fact that it is possible to make synthetic DNA fragments has emerged in the goal to make synthetic life and in 2010 researchers at the J. Craig Venter Institute (JCVI) manage to create the first self-replicating synthetic bacteria cell (Gibson et al., 2010). Synthetic biology tries to describe biological networks and regulation with the help of several fields. The goal for this work is to engineer biological networks, or circuits, for manipulation of the cellular control and thereby building novel bimolecular components, networks and pathways. This constructs can then be used to rewire and reprogram organisms. The potential of this field is large and many researchers believe that these re-engineered organisms will change our lives in the future by leading to cheaper drugs, eco-friendly fuels and therapies for diseases (Khalil and Collins, 2010).

1.2.1 Applications of Recombinant DNA Technology in medicine and industry

In medicine and industry, rDNA technologies have given opportunities to solve existing or prevent upcoming problems. One good example was made in 1977 when the human neurotransmitter somatostatin was first produced. By chemically synthesizing the gene for this protein, fusing it inside a circular DNA (plasmid) and transferring this to *E. coli* cells, expression of the protein was made possible. Somatostatin was believed to have therapeutic values in, among other diseases, acute pancreatitis (Itakura et al., 1977). Soon after the production of somatostatin, human insulin was made replacing the need for insulin extracted from pigs and cows (Watson et al., 1992).

Production of recombinant proteins has become one of the main applications for rDNA technology. The production of these proteins is explained in the next section, but to shed light on the importance of recombinant protein production, some of the different applications of recombinant proteins are illustrated in Figure 2.

Introduction
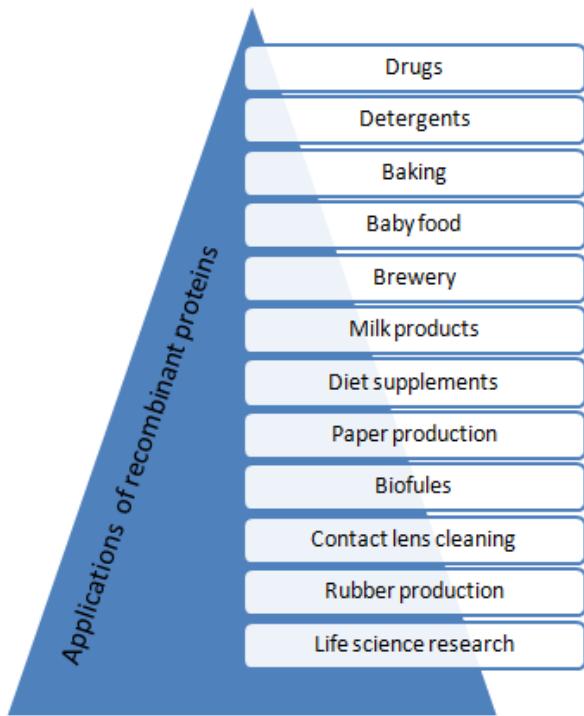
Figure 2: Illustration of some applications of recombinant proteins. (Reproduced from presentation after permission from Trond Erik Vee Aune, Vectron Biosolutions)

1.2.2 Protein synthesis

One of the main functions for rDNA molecules is the production of recombinant proteins as in the examples with somatostatin and insulin. Mechanisms for protein synthesis consist of the same elements, whether the protein is recombinant or not. The transcription of DNA to messenger RNA (mRNA) is the first step. DNA is the genetic material in both prokaryotic and eukaryotic cells. It is made up by individual units for nucleotides linked together in a long strand. Each nucleotide consists of a five-carbon sugar, a phosphate group and a nitrogen base which is either cytosine (C), adenine (A), guanine (G) or thymine (T). Two polynucleotide strands form a double helix by hydrogen bonds between the nitrogen bases. The interactions between the bases are specific (C binds to G and A binds to T) and called base pairs (bp). During transcription, one strand of the double stranded DNA is used to synthesize a complementary single strand of mRNA (Glick et al., 2010).

The transcription and composition of genes and genetic elements differ between prokaryotic and eukaryotic cells, however some parts are equal. This includes the general mechanism for mRNA synthesis, which is done by the enzyme RNA polymerase (RNAP), and the mechanism for transcription of mRNA to proteins performed by the ribosomes.

Introduction

In prokaryotes, protein synthesis can be generalized as shown in Figure 3. RNAP binds to the promoter region (p), a nucleotide sequence upstream the gene sequence. The protein coding sequence of the synthesised mRNA transcript is called the structural gene. Downstream this region is a transcription termination region (t) where transcription is terminated.
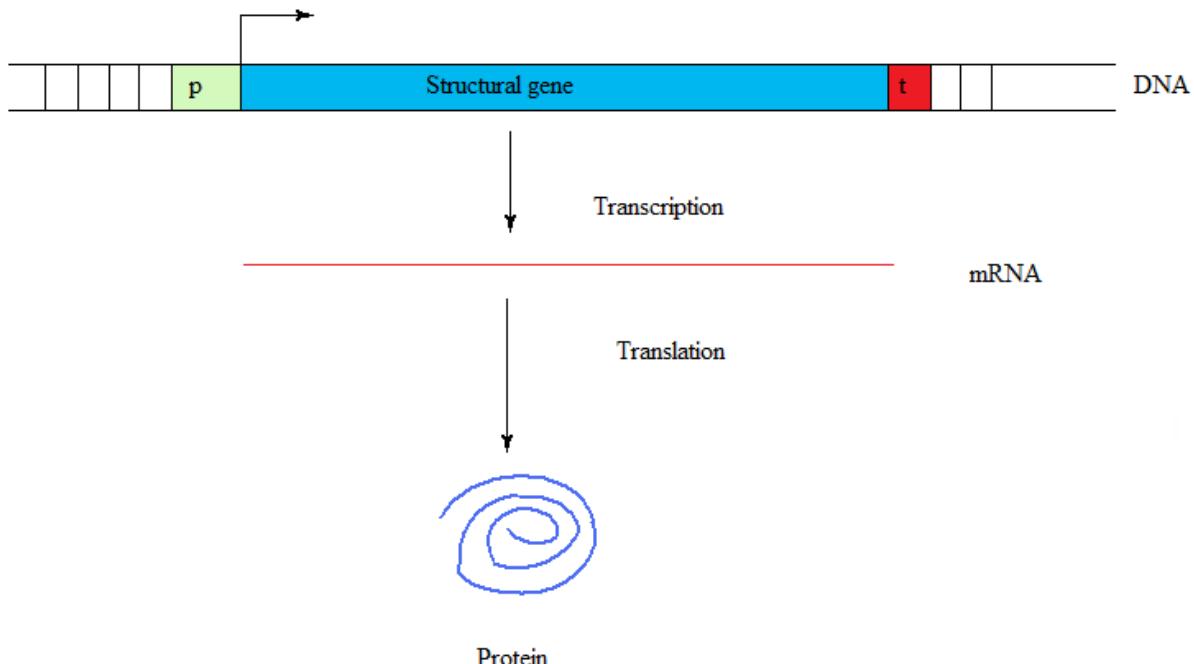
Figure 3: Schematic representation of a prokaryotic structural gene. The promoter region (p), the site of initiation and direction of transcription (the right-angled arrow), and termination sequence for RNAP (t) are depicted. A prokaryotic structural gene is transcribed into mRNA and then directly into protein. Figure adapted from (Glick et al., 2010)

Since prokaryotes have no nucleus and no spatial separation of the protein synthesis events, translation of the mRNA starts while the transcription is still ongoing. During translation, ribosomes read three bases at the time called codons. Transfer RNA molecules (tRNA), with complementary sequences to these codons, transfer a specific amino acid to the poly peptide chain (protein) forming. Established of the genetic code (Figure 4) in 1966 (Chapter 1.1) revealed what each of the 64 codons coded for, an amino acid or transcription termination.

Introduction
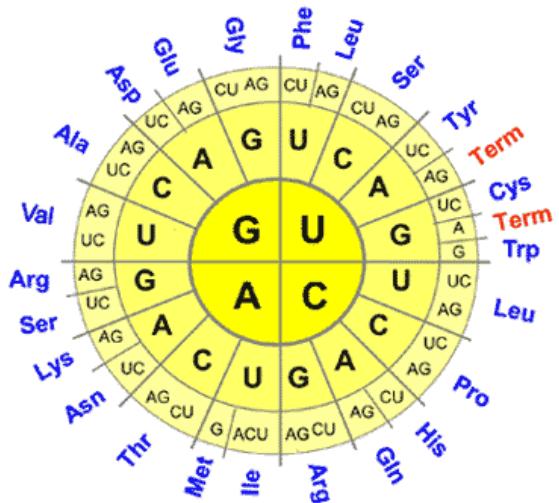
Figure 4: The genetic code. The inner circle is the first base of the codon, thereafter the second and the third in the following circles. The outer circle shows the amino acid or termination sequences coded by the sequence. Figure adapted from (Merkel and Budisa, 2006)

Gene expression in prokaryotes can be regulated at many levels, but transcriptional regulation is the most common. This is performed by regulator proteins and effector molecules. These two elements either facilitate the binding of RNAP or prevent it and therefore determine if the structural gene shall be transcribed. If the regulator protein is required for gene expression, it is said to be under positive control mechanisms. If the regulator proteins shuts off the expression, the control mechanisms is called negative (Snustad and Simmons, 2010).

Many of the prokaryotic structural genes encoding proteins that are required for a single metabolic pathway are arranged as contiguous genes in the chromosome. These genes are under control of the same promoter and transcription will generally yield a large mRNA molecule. This arrangement of genes is called an operon (Glick et al., 2010).

In contrast to prokaryotic cells, the genetic material in eukaryotes is present in a nucleus. While transcription and translation are coupled in prokaryotes, this location separates the events because RNAP is part of the nucleus environment while ribosomes are not. The details in eukaryotic gene regulation will not be discussed here since the main focus of this study is on prokaryotes and their protein production. The structural gene of eukaryotes, shown in Figure 5, usually consists of both protein coding regions, called exons, and non-coding regions, called introns. The entire structural gene is transcribed by RNAP into a primary transcript often called pre-mRNA. Before the transcript is transported out of the nucleus, the introns are removed and a methylated G-cap is added to the 5' end and poly-A-tail to the 3'

Introduction

end. The excision of introns enables alternative splicing because some exons may be excised together with the introns. After the introns are deleted, the functional transcript is translated in the cytoplasm or on the endoplasmic reticulum (ER) (Glick et al., 2010).
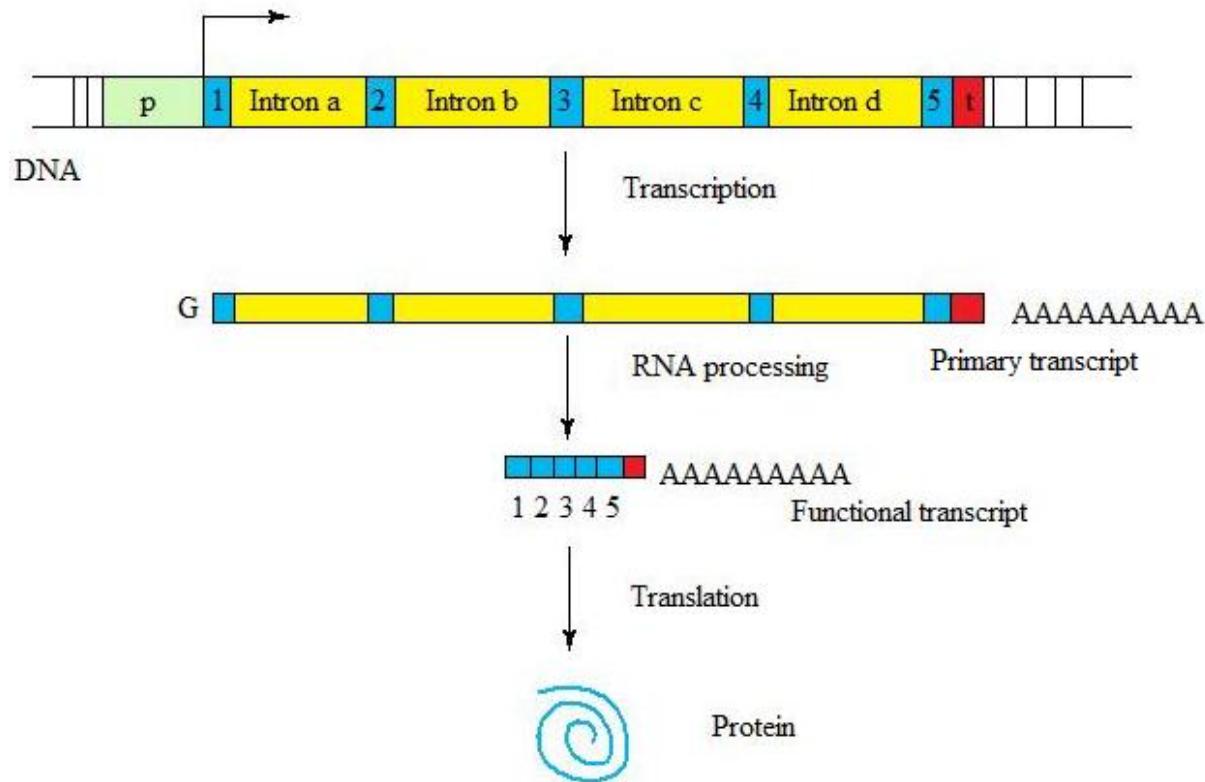

Figure 5: Schematic representation of a eukaryotic structural gene. The promoter region (p), the site of initiation and direction of transcription (the right-angled arrow) and the termination sequence for RNAP (t) are depicted. The numbers 1 to 5 mark the exons of the structural gene, and the introns a to d mark the introns. The primary transcript is polyadenylated at the 3' end and capped with a modified G (G) nucleotide at the 5' end. Processing of the primary transcript removed the introns. The functional RNA is translated into protein. Figure adapted from (Glick et al., 2010)

One other element of eukaryotic protein synthesis important mentioning is posttranslational modifications. The proteins produced by ribosomes connected to ER are either inserted into its membrane or secreted into the lumen, where they are processed further. The addition of sugar molecules to specific amino acids (glycosylation) is one example of modification that may be performed. Another example is the formation of disulfide bonds inside the protein. Gram negative bacteria (prokaryotes) also have the capacity of forming these bounds if the protein is sent to the intracellular compartment called periplasm (Glick et al., 2010). There have also been reports that prokaryotic glycosylation is performed by more bacteria than previously thought (Schaffer et al., 2001).

Introduction

Modifications of the proteins are often necessary for their activity or stability. A good example is proteins produced as inactive precursor polypeptides only active after being cleaved by proteases at specific sites. This can be a control mechanism to ensure that the active form of the protein only is present at specific locations. Correct folding of the protein is essential for both protein activity and protection of regions that proteases otherwise would recognize Chaperones, which exists in both prokaryotes and eukaryotes, assists in this modification. Additional control mechanisms for eukaryotic proteins is performed in the Golgi apparatus or outside the cell, making sure that only correctly folded proteins are released (Glick et al., 2010).

1.2.3 Heterologous protein expression platforms

The single cell is the basic unit in every living organism. Characteristic for a living organism, whether it is single-celled or multi-cellular, is its ability to reproduce and metabolize components in the environment. Viruses do not have this ability, are by some still defined as living organisms (Godal et al., 2010). One essential feature for reproduction and metabolism is the production of proteins, which is described in Chapter 1.2.2. The similar mechanisms between protein synthesis in prokaryotic and eukaryotic cells have been utilized in the development of expression platforms. Expression platforms are cells or organisms used for heterologous (recombinant) protein production.

The need for new suitable expression platforms increases with the number of gene-targets for various industrial branches. Expression platforms already in use range from bacteria, yeast and filamentous fungi to cells of higher eukaryotes. When choosing among these cells, both economic and qualitative aspects have to be considered. Industrial production relies on the use of inexpensive medium components and high anticipated economic yield from the product. The quality of the produced compound is also essential, especially in medicine where human pharmaceuticals production is regulated under strict safety aspects (Gellissen, 2005).

Prokaryotic platforms are widely used to produce single subunit recombinant proteins. These platforms can easily be manipulated genetically by well-known methods and cultivated in low costing medium. Prokaryotic cells can be grouped in gram-positive and gram-negative cells, based on their cell wall structure. Both of these groups have well established representatives

in the field of recombinant protein production. For gram-negative bacteria, *Escherichia coli* is the most studied, while *Bacillus subtilis* is a well known gram-positive producer of recombinant proteins (Watson et al., 1992). There are several challenges using prokaryotic cells for protein expression. Most of them will be discussed in greater detail later, but protein insolubility and low expression levels (especially mammalian proteins) or post-translational modifications are some of the most challenging problems (Gellissen, 2005).

In theory, prokaryotic hosts can express any gene, but in practice the proteins produced do not always have the desired biological activity or stability. In addition, toxic components from the bacteria may contaminate the final product. When producing a protein intended for medical use, all properties of this protein must be identical to the natural protein. Eukaryotic cells share many molecular, genetic and biochemical features, making them an alternative to prokaryotic expression of proteins. Today, a variety of fungal expression platforms are available, but initially the baker's yeast *Saccharomyces cerevisiae* was most widely used. Like most fungi, *S. cerevisiae* have the same growth advantages as prokaryotes (rapid growth in low cost medium). In addition it has been widely used as a model organism for cell function research making its biochemistry, genetics and cell biology well known. Today, a number of proteins have been produced using this host, including proteins for use as vaccines, pharmaceutical products and for diagnostics (Glick et al., 2010).

Protein producing mammalian cell lines, like CHO (Chinese Hamster Ovary), BHK (Baby Hamster Kidney) and NS0 (Mouse myeloma), have an advantage in the production of human proteins because they are able to glycosylate proteins in the same pattern as higher eukaryotes. Since this can result in recombinant proteins identical to their human counterparts, the possibility for producing a functioning protein increases. Still there are some disadvantages leading to the need for alternative producers. Mammalian cell lines are demanding in needing specialized medium that may lead to high costs compared to prokaryotic host. Furthermore in fermentations, their growth kinetics are slow, they have low cell densities and are sensitive to stress (Gellissen, 2005).

Introduction

1.2.3.1 Escherichia coli as host

One of the most intensely studied and attractive heterologous protein producer is *E. coli*. *E. coli* is a gram negative rod-shaped bacterium, which in humans and animals normally is found in the intestine. This bacterium is part of the Enterobacteriaceae family and is facultative anaerobe meaning it is capable of living without oxygen. Its optimum temperature is $37\,°C$ (Sussman, 1997) .

The reasons for the many studies and broad usage of this bacterium are the many advantages. One important feature of *E. coli* is its short generation time. Under optimal conditions, *E. coli* is capable of dividing every $20 - 30$ minutes (Snustad and Simmons, 2010). It is also capable of living on inexpensive substrates and can grow to high cell densities. These features enable possibilities for high yields and low costs when *E. coli* is used for recombinant protein production. Because of the advantages associated with *E. coli,* it has often been chosen for recombinant production of proteins and for genetic studies. This has lead to new knowledge and development of many tools such as controllable expression systems specific for *E. coli* with the best known example being the *lac* operon (Gellissen, 2005).

There are also problems related to the use of *E. coli* as production host. Some recombinant proteins end up as part of an insoluble fraction in the cytoplasm. These proteins are often called inclusion bodies (IB) and consist of inactive protein aggregations which have to be treated to form the right folded proteins. An additional treatment leads to higher costs and may affect the proteins activity. It is therefore desirable to use hosts where IB are avoided (Leonhartsberger, 2006). For production of therapeutic proteins, accumulation of endotoxins (LPS), which are pyrogenic in humans, is another problem related to *E. coli.* As for IB, this cause additional steps with purification before the recombinant protein can be used for its original purpose. Other possible problems are plasmid or mRNA instability, toxic recombinant protein or translational problems caused by difference in codon usage (Terpe, 2006).

The problems described above may also occur in other hosts, but by having different bacterial expression systems one can choose the strain most suitable for each case. The ultimate goal would be to have a universal system that functioned in a variety of organisms, but several reasons suggest that this will be impossible to accomplish. Control elements are known to

behave different among species (Terpe, 2006), inducers of inducible systems may cause problems (metabolized, not taken up or causing physiological changes) and it is likely that host dependent differences in translation will occur.

1.2.3.2 Pseudomonas species as host

One alternative gram-negative production host might be one of the members of *Pseudomonas* species. The *Pseudomonas* species are normally found in the nature in soil and water. They are rods without spores and with one or several flagellum. Most of the members of the species are considered aerobic, but some can live under anaerobic conditions in the presence of nitrate (Degré et al., 2004). Some of the *Pseudomonas* species have the ability to synthesize alginate which is a commercially used component in food, clothes and medicaments (Onsøyen, 1996).

Of all *Pseudomonas* species, *Pseudomonas aeruginosa* is the most studied pathogen for humans. It is opportunistic, but can cause severe infections in immunocompromised hosts (Cornelis, 2008). *Pseudomonas fluorescens* is another member of the family. This strain has been proven to function as a recombinant protein producer. It can be cultivated to high cell densities and have several times been proven to produce soluble proteins when *E. coli* produces insoluble (Squires et al., 2004). As an example, it was recently reported that *P. fluorescens* was able to produce soluble fractions of the human granulocyte stimulation factor (G-CSF) in high quantities. This protein is often synthesized as IB in *E. coli* (Jin et al., 2011).

1.2.3.2.1 Pseudomonas putida KT2440

The results from expression experiments with *P. fluorescens* provides confidence in alternative expression host solving problems with *E. coli* and contributing in production of recombinant proteins important for human health. The curiosity of which strain or strains that will become new key producers of recombinant proteins enables research on unknown as well as established prokaryotes. Because of the features of *P. fluorescens,* this study chose to explore the possibilities lying in *Pseudomonas species*. One of the interesting strains here is *Pseudomonas putida.*

Phenotypic the *Pseudomonas* species can be divided in to two groups based on their ability to produce pigments that fluoresce under UV radiation with low wave length (around 260 nm). *P. putida* is part of the group that has this ability (Cornelis, 2008). The *putida* strain KT2440 was sequenced in 2002 and found to have a G+C content of 61.6 % (Nelson et al., 2002). It is now a well characterized saprophytic laboratory Pseudomonad. KT2440 is a plasmid free

derivative of the toluene-degrading strain *P. putida* mt-2 which original was designed *Pseudomonas arvilla* strain mt-2. KT2440 is certified as a host-vector biosafety (HV1) which means that it is a vector system that provides moderate level of containment (Cornelis, 2008). KT2440 has recently been reported to produce yields up to 3.5 mg/l of pure, soluble, active antibody fragment scFv (Dammeyer et al., 2011) proving its potential as production host.

1.2.4 Expression of recombinant proteins

Heterologous protein production in eukaryotic and prokaryotic hosts requires different regulatory elements combined with the gene of interest (GOI). While the basics of these elements are similar, they have to be adapted to the host of choice. This includes the transcriptional promoter, transcriptional and translational stop sequences and selectable marker genes. If the vector, where all of these elements are gathered, is designed for integration into the chromosomal DNA, complementary sequences to DNA have to be part of the vector. Elsewhere, when the vector is designed for extra-chromosomal replication, an origin (*ori*) of replication has to be integrated in the vector. This sequence will initiate DNA synthesis so that the expression vector is replicated. In addition, poly-linker sequences have to be assembled in the expression vector to facilitate insert of a GOI (explained later) (Glick et al., 2010).

Eukaryotic host cells have the advantage of post-translational modifications) of the produced proteins (Chapter 1.2.2. The eukaryotic expression vectors are therefore poised with A-codons which will be attached to the pre-mRNA strand. Because it is technically difficult to perform rDNA procedures in eukaryotic cells, the vectors have often an additional *ori* of replication suitable for a prokaryote host, typically *E. coli*. These vectors are called shuttle vectors and can replicate in two different organisms. Examples of eukaryotic expression vectors are the yeast 2 μm plasmid and yeast artificial chromosomes (YAC) (Glick et al., 2010).

Introduction

One example of a prokaryote expression vector functioning as an expression system is the plasmid shown in Figure 6.
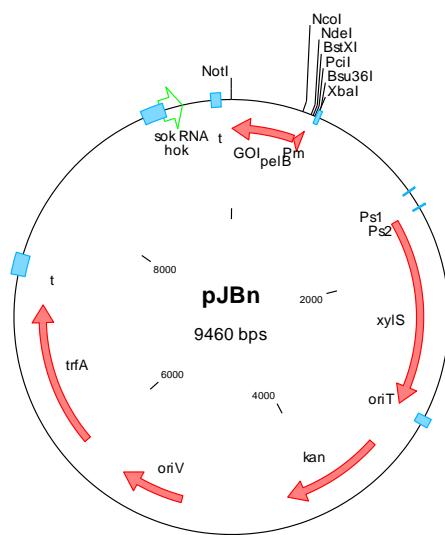
Figure 6: Illustration of a typical expression vector in a prokaryotic expression platform. *oriV*: origin of vegetative replication, *trfA*: replication initiation protein: t: transcription termination, sok RNA and hok: potential killing mechanism, *GOI*: recombinant gene of interest, *pelB*: signal sequence, Pm, Ps1, Ps2: promoters, *xylS*: transcription regulator, *oriT*: origin of transfer, *kan*: kanamycin resistance. The different elements are described in Chapter 1.2.4.

As already mentioned expression vectors have several basic elements, but three are often considered as essential. The first of these features is the poly-cloning site. This is a short sequence where several restriction enzymes have sites for digestion. The function of this poly-cloning site is to enable cloning of a broad range of genes. By having a large number of possible enzymes for digestion of both gene and vector the possibility of finding a match of enzymes increases. Ligation of fragments cut with the same enzymes yield complementary single strands. These will join together under appropriate renaturation conditions with the help of DNA ligase (Snustad and Simmons, 2010). An example of a poly-cloning site is shown in Figure 6 where restriction sited is marked with their corresponding enzyme names.

Another feature considered essential is a selectable marker gene. This gene facilitates selection of cells containing the vector versus cells without it. Usually this is done by an antibiotic resistance gene. By adding the antibiotic into the growth media or plates, only

Introduction

bacteria carrying the plasmid will grow. In Figure 6 the gene *kan* encodes a protein leading to kanamycin resistance.

The last essential feature is the origin of replication which is needed for the plasmids ability to replicate (Snustad and Simmons, 2010). *Ori V* (origin of vegetative replication) shown in Figure 6 has its origin in the broad host plasmid called RK2. While some replicons function is single or related bacteria, this replicon have the ability to replicate in unrelated species. This is one of two feature making the plasmid called RK2 broad host functioning where *trfA* (described later in this chapter) is the last one (Perri et al., 1991).

There are also other elements in the pJBn vector in Figure 6 worth describing. The hok – sok system is a plasmid stabilization system ensuring that only daughter cells containing the expression plasmid will survive. The *hok* gene is a potent killing protein while the *sok* gene codes for an anti-sense complementary to *hok* mRNA and function as the antidote by eliminating *hok* mRNA. The mRNA from the *hok* gene is stable and will be transferred from mother to daughter cells. The anti-sense *sok* mRNA on the other hand is unstable and will not be transferred. If the daughter cells not receive the expression plasmid, they will be killed when mother *hok* mRNA is expressed (Gerdes, 1988).

While *oriV* as described is the origin of vegetative replication, *ori T* is the origin of transfer and enables transferring of the vector during conjugation. The process of conjugation is described under Chapter 2.4.8.3. In addition to *oriV*, *trfA* is part of the two necessary elements for broad host replication originating from RK2. This gene codes for a replication initiation protein called TrfA. RK2s copy number is regulated by the interaction of these two necessary elements and have been determined to be 2 to 3 per bacteria cell in *Pseudomonas* and $4 - 6$ in *E. coli* (Funnell and Phillips, 2004). Different mutations in *trfA* have been proven to elevate the plasmids copy number in mutations called copy-up. The mutation named trfAcop271C cause an amino acid substitution in the TrfA protein primary sequence leading to an 3.5-fold increase of copy number in *E. coli* (Durland et al., 1990).

The *Pm* sequence is a promoter where RNAP attach. The promoter is usually upstream of the gene and the sequence will align the RNAP so that the transcription will initiate at a specific nucleotide (Glick et al., 2010). *Pm* is a constituent of the *Pm-xylS* cassette described later, but there are also other examples of promoters and regulatory systems used for expression of

recombinant proteins. The *lac* system regulated by the T7 promoter is a good example. The expression vector of this system contains a T7 promoter which is transcribed by a T7 RNAP. This RNAP is supplied in form the host and not part of the expression vector. The polymerase is either supplied by infecting the host with a T7 phage or by using the phage λDE3(Gellissen, 2005).

The last two elements part of the general expression vector pJBn in Figure 6 is *pelB* and *GOI*. *pelB* is a signal sequence connected to the *GOI* to export the recombinant protein from the inner cell membrane to the periplasm of the bacteria. This process is called translocation. The signal sequence is often an N-terminal extension that will be cleaved off the protein during the translocation process. There are several reasons for wanting proteins to be exported to periplasm. As described earlier it is only here disulfide bridges can be formed, which for some proteins is necessary for correct folding. Other proteins need to be protected from the many proteases present in the cytoplasm or need the cleavage of the signal sequence in periplasm to remove the methionine amino acid encoded by the start codon (Gellissen, 2005). Secretion to the periplasm also makes its purification less costly and easier to perform, because there are fewer proteins located there than inside the cytoplasm (Glick et al., 2010).Other examples of signal sequences are *ompA* and synthetic CSP. Even though the presence of a signal sequence does not guarantee a high rate of secretion, the choice of signal sequence could influence the production and translocation of the recombinant protein (Sletta et al., 2007).

GOI is the DNA sequence of the recombinant protein. The different synonymous of codons (Chapter 1.2.2, Figure 4) do not have the same frequencies between different organisms and even internal in a genome (Grantham et al., 1980). Recombinant genes may therefore have "foreign" codon usage leading to lower expression of the protein. Today, one can analyse this in different databases. By inserting the nucleotide or amino acid sequence and the new host strain, the data base will give you an estimated nucleotide sequence specialized for the host. The amino acid sequence will of course be the same, but the codons may have changed.

1.2.4.1.1 The Pm/XylS cassette

The *Pm/XylS* expression cassette is derived from the *Pseudomonas putida* TOL plasmid pWW0 which was first described in 1974 by Williams and Murray. This plasmid consists of two operons responsible for the degradation of aromatic hydrocarbons. The upper operon encodes the enzymes responsible for oxidizing toluene/xylenes to benzoate/toluates while the other operon, called the *meta-* cleavage pathway operon, encodes enzymes that oxidize the produced benzoates or toluates. The genes or gene products of these operons and the genes for the regulator proteins have the prefix *xyl*. The two regulator genes *xylS* and *xylR,,* which are located at the 3'end of the *meta-* cleavage pathway operon, are transcribed from divergent promoters called *Ps1/Ps2* and *Pr* respectively. The *Ps2* promoter ensures constitutively low expression of XylS regulator, while the *Ps1* promoter is stimulated by active *XylR* regulator. The transcription of this regulator is auto-regulated. Activation is achieved when toluenes/xylenes, the substrates for the upper operon, are part of the environment. In addition to increasing the *XylS*-production, activated *XylR* leads to transcription from the *Pu*-promoter and therefore enable oxidization of the toluenes/xylenes to benzoates. Since this is the substrate for meta-operon, it activates *XylS* regulator and leads to stimulation of the *Pm* promoter and transcription of the *meta*-cleavage pathway genes (Marqués and Ramos, 1993).

XylR regulates both the upper operon and the expression of *XylS*. When *XylR* is active and both *Ps1* and *Ps2* are stimulated, *XylS* is hyper-expressed. As a response to this, the XylS overfload leads to activation of the *Pm* promoter even in the absence of the benzoate effector (Marqués and Ramos, 1993).

The *Pm/XylS* cassette consists of the *Pm* promoter and *xylS* coding for the positive regulator transcription protein *XylS*. By placing the recombinant gene downstream the *Pm*-promoter, transcription of this gene can be controlled by the presence of substrates for XylS benzoates like m-toluic acid. When this is present, *XylS* is activated and binds to *Pm* stimulating transcription by recruiting RNAP. The induction of recombinant protein production based on *Pm/XylS* system is illustrated in Figure 7.

Introduction
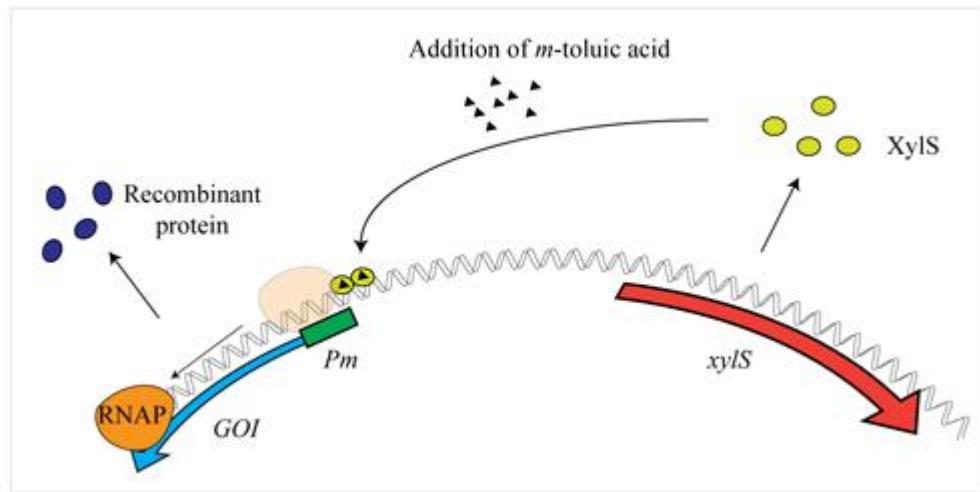
Figure 7: Illustration of the *Pm/XylS*- expression cassette. The regulation of the *Pm* promoter and transcription of the recombinant gene (here *GOI*) by RNAP is controlled by addition of *m*-toluic acid to the growth medium. Figure created by (Aune, 2008).

The *Pm/XylS*-expression cassette was chosen for the present study because of previous studies and advantages. As discussed, the fact that few alternative prokaryotic hosts have been developed as an alternative to *E. coli* limits the possibilities to produce functional recombinant proteins. One of the most important features is the fact that this cassette has been proven to function in a broad range of gram negative bacteria including, *Aeromonas hydrophila, Aerobacter aerogenes*, *Serratia marcescens*, *Erwinia carotovora*, *E. coli* and most importantly for this thesis, *Pseudomonas* subspecies (Mermod et al., 1986).

The inducers of *Pm/XylS* expression systems are carboxylic acids in the form of benzoates. m-Toluic acid is often chosen because it is a non-toxic and a relatively inexpensive inducer inactively taken up by the cells in the undissociated form (Marques et al., 1994). Other examples of inducers are o-toluic acid and 2-chloro-benzoic acid (Ramos et al., 1990). pH has been observed to effect is ability to induce the *Pm/XylS* system. For expression in an extended period, pH 6.8 is been reported as preferred (Winther-Larsen et al., 2000b).

Even though this study emphasises on the need for an alternative system to *E. coli,* this strain in many cases will still be the host giving the highest recombinant protein yield compared with alternatives. Thus, the results achieved here should be compared to production in *E. coli* so that the optimal platform is chosen for each case. The *Pm/XylS*- cassette has been proven to produce high levels of different recombinant proteins in *E. coli,* including GM-CSF and IFNα-2b (Sletta et al., 2007).

1.2.5 Cultivation for production of recombinant proteins

After genetic fusing the GOI into a vector and transferring it a host organism, the expression of recombinant protein has to be evaluated. This is often done in a small-scale usually carried out using shake flasks of 250-ml to 1-litre capacity. These small-scale experiments can be used to determine optimal conditions for growth and production of recombinant proteins. While these experiments are performed with low cell densities, the next step for developing a new recombinant-DNA-derived product is to scale-up the process. Ideally the protein production is proportional with the amount of cells. These up-scaled experiments will therefore involve high cell density cultivation (HCDC) using the conditions that optimized growth and production in the small-scale. The first stage here might be a 1- to 3-litre bioreactor where the cultures and different features of the organism are closely monitored. Examples are oxygen requirement, growth rates and consummation of nutrients (Doran, 1995).

There are several terms used to describe different processes that may be used as a system for the bioreactor. A *batch process* describes a closed system where all material is added at the start, while a *semi-batch* either allows input or output. During a *continuous process*, there is flow of material in and out of the system during the entire process (Doran, 1995). The last term is *fed-batch* processes, the kind of process used for this study. This process allows supplement of one or more nutrients while the cells and products remain inside the fermentor. A relatively low concentration of substrate (typically the carbon source) is present before input is started and this will avoid high growth rates. The purpose of this is to not create an oxygen demand higher than can be supplied and the fact that high concentrations of substrate may switch on undesirable metabolic pathways or be inhibitory for growth. Fed-batch cultivations are therefore designed to be beneficial to the productivity and yield of the desired product (Lee et al., 1999) and are often used for protein production (Sletta et al., 2007, Sletta et al., 2004, Brautaset et al., 2010).

Typical fed-batch fermentations can be divided in three phases. Initially, the culture experience free growth following the equation:

Introduction

$$\mu = \frac{\ln\left(\frac{x_2}{x_1}\right)}{t_2 - t_1} = \frac{\ln\left(\frac{x_2}{x_1}\right)}{\Delta t} \qquad \text{Eq. (1)}$$

Where μ is the growth rate (h^{-1}), t_1 and t_2 are the culture times for optical density (OD) measurements at $600 - 660$ nm x_1 and x_2 respectively.

The next phase is initiated when the carbon source initially added is completely utilized. Exponential substrate feeding is started controlling the cultures growth rate. This exponential feeding can be continued until oxygen transfer becomes a problem and the third phase starts. From now on feeding is constant. During this period, the cells will still be growing but their specific growth rate will decrease.

1.3 IFN-α2b and GM-CSF: Important therapeutic model proteins

Since the 1950s the research in human health and disease has been pinpointed towards naturally produced proteins that have obvious therapeutic applications by being part of the human immune system. The potential of these proteins have been rendered impractical because of their tiny quantities in which they are naturally produced, but since the advent of recombinant DNA technology this problem and many others have diminished. Source availability and product safety have been overcome and it provides an alternative to direct extraction from inappropriate sources. In addition it facilitates engineered therapeutic proteins that may have advantages over the native protein (Walsh, 2007).

Examples of naturally produced proteins with therapeutic potential are the cytokine group interferones and growth factors called colony-stimulation factors. Cytokines function as chemical communicators by binding to specific receptors located on different cells and triggering various intracellular events. The interferons (IFNs) of this group are substances secreted by virally infected cells and the biological effect is among other induction of cellular resistance to viral attack and regulation of growth and differentiation of many cell types. Humans produce at least three different kinds of interferons named IFN-α, IFN-β and IFN-γ (Walsh, 2007).

Introduction

The growth factors, which also might be called cytokines, are the most important regulators of differentiation, growth and division for many cell types. They have a mitogenic effect on a characteristic range of cells meaning that they promote cell division. One important group of growth factors getting attention from the pharmaceutical industry is the haematopoietic growth factors. Haematopoietic stem cells are the origins of all blood cells and therefore an important part of the human immune system by the development of white blood cells. Granulocyte macrophage colony-stimulation factor (GM-CSF) is one example of haematopoietic growth factors (Walsh, 2007). GM-CSF is also able to act on macrophage populations, reflecting an important role in inflammatory processes (Hamilton et al., 1980).

Because of their natural importance, human cytokines have become important tools for treatment of microbial and virus infections and vaccination against cancer. IFN-α2b have mainly been used to treat hepatitis C, but also some types of cancer (Neves et al., 2004) and GM-CSF have been proven to reduce the risk associated with bone marrow transplantation (Metcalf, 1991). Both IFN-α2b and GM-CSF have been produced recombinant in *E. coli* RV308 by fusion with N-terminal secretion signal sequences. This gave volumetric yields from high –cell-density cultivations of 1.7 and 0.6 g/litre respectively (Sletta et al., 2007).

1.4 Objectives and strategy of the present study

Most of the research focusing on recombinant protein production is executed in *E. coli*. When this host fails to produce, alternatives have to be tested. If there existed an expression vector that, with little necessary genetic modification, could function in different platforms, the optimal production host could easily be chosen. This principle is illustrated in Figure 8.
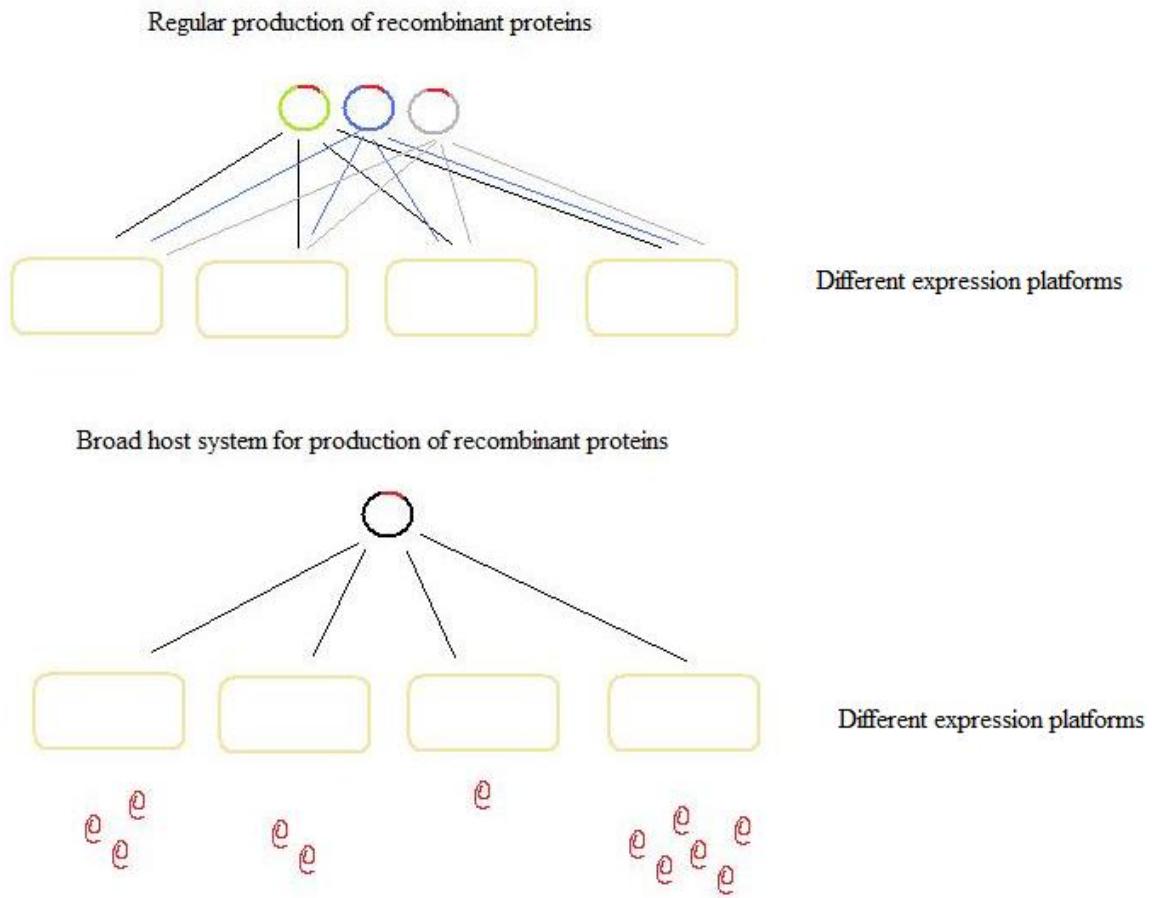
Figure 8: Illustration of how the broad host system would simplify the search after the optimal prokaryote platform for protein production compared to regular protein production.

In regular protein production, different expression vectors are tested in different platforms. This may be time consuming because of the many possible combinations of platform and vector. The goal of the broad host system is to have an expression vector that function in different platforms, and therefore will decrease the possible combinations.

In this study, the expression cassette *Pm/XylS* and RK2 plasmids broad host elements has been used for production of the two model proteins IFN-α2b and GM-CSF. The platforms chosen for this study was the *Pseudomonas* strains *P. fluorescens* and *P. putida*.

The plasmid pDI-8_MV-ifna2 was the starting point and the expression vectors pIFNpelB and pGMpelB (see Chapter 2.2) with wild type copy number was the first plasmids constructed by replacing the *ifn-α2* gene with *ifn-α2b* and *gm-csf* respectively. The vectors was transferred to SBW25 (*P. fluorescens*), NCIMB (*P. fluorescens*) and KT2440 (*P. putida*) before growth and

Introduction

production is evaluated. The *Pseudomonas* strain that showed the highest potential for the production was selected for further study.

When cultivation conditions had been adapted to the chosen stain, genetic modification to the vectors will be made. This modification was chosen to be copy-up mutations of the *trfA* gene and codon optimization of gene targets and signal sequence for the chosen host. In addition, different combinations of these modifications were evaluated. After optimizing the different parameters, high density cultivations was performed to compare the system generated against previously tested *E. coli* RV308 (Sletta et al., 2007). A sketch of the execution is illustrated in Figure 9.
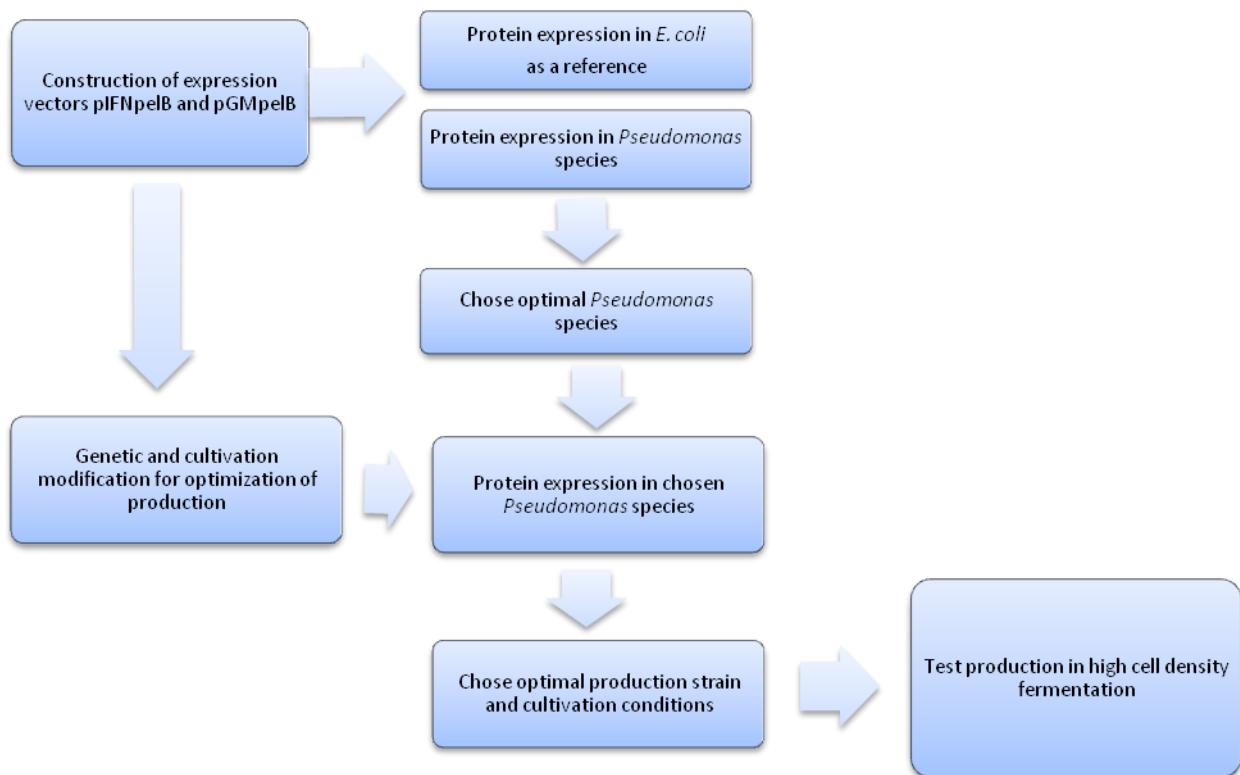
Figure 9: Sketch of the execution of the study.

2 Materials and methods

2.1 Media and supplements

<u>Luria-Bertani (LB) medium</u>

       10 g/l NaCl (Merck)

       10 g/l Trypton (Bacto)

       5 g/l Yeast extract (Oxoid)

Autoclaved at 120°C for 20 minutes

<u>Luria-Bertani agar (LA)</u>

       10 g/l NaCl (Merck)

       10 g/l Trypton (Bacto)

       5 g/l Yeast extract (Oxoid)

       20 g/l Agar (Oxoid)

Autoclaved at 120°C for 20 minutes and kept at 50°C until distributing on petri dishes (VWR)
with 9 or 14 cm diameter

<u>PIA- medium</u>

       20 g/l Pepton (Oxoid)

       1.4 g/l $MgCl_2 \cdot 6H_2O$ (Riedel deHaën)

       10 g/l K_2SO_4 (Riedel deHaën)

       5 g/l NaCl (Apotekerproduksjon)

       23.5 g/l Glycerol (Sigma)

Autoclaved at 120°C for 20 minutes

<u>PIA-10GLU-medium</u>

The same concentration of pepon, $MgCl_2 \cdot 6H_2O$, K_2SO_4 and NaCl as in PIA-medium

       10 g/l Glucose (Apotekerproduksjon)

Autoclaved at 120°C for 20 minutes

Materials and methods

PIA agar– *Pseudomonas Isolation Agar*

    45 g/l PIA (BD Difco)

    20 ml Glycerol bidistilled 99.5 % (VWR) in 1000 ml water

Autoclaved at 120°C for 20 minutes and kept at 50°C until distributing on petri dishes

Kanamycin stock (kan) (40 mg/ml)

    40 mg/ml Kanamycinsulfat (Sigma-Aldrich)

Dissolved in distilled water. Sterile filtered with 0.2 μl filter (Sarstedt). Stored at – 20°C.

Used to give final concentrations 40 μg/ml.

Ampicillin stock (amp) (100 mg/ml)

    100 mg/ml Ampicillin Natriumsalz (AppliChem)

Dissolved in distilled water. Sterile filtered with 0.2 μl filter (Sarstedt). Stored at – 20°C.

Used to give final concentrations 100 μg/ml.

500 mM m-toluic acid

    68.25 g m-Toluic acid (Fluka) per litre ethanol (Kemetyl)

Stored at 4°C

500 mM o-toluic acid

    68 g m-Toluic acid (Sigma) per litre ethanol (Kemetyl)

Stored at 4°C

500 mM 2-chloro-benzoic acid

    78.25g 2-Chlorobenzoic acid (Sigma) per litre ethanol (Kemetyl)

Stored at 4°C

Psi-medium

    5 g/l Yeast extract (Oxoid)

    20 g/l Trypton (Bacto)

    10.24 g/l $MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich)

pH adjusted to 7.6 with 1 M KOH

Autoclaved at 120°C for 20 minutes

Materials and methods

TFB1

 2.94 g/l (30 mM) Porasium acetate (Merck)

 12.1 g/l (100 mM) Rubidiumcloride (Aldrich)

 1.945 g/l (10 mM) Kalsiumcloride $\cdot 2H_2O$ (VWR)

 15.73 g/l (50 mM) Mangan(II)cloride $\cdot 4H_2O$ (Riedel-deHäen)

 150 ml (15 v/v %) Glycerol bidistilled 99.5 % (VWR)

About 180 ml distilled water was added before adjusting pH to 5.8 with diluted acetic acid.

The solution was then added distilled water to at total volume of 200 ml

Sterile filtered with Stericup-GP 0.22 μm (Millipore). Stored at 4°C

TFB2

 2.1 g/l (10 mM) MOPS (AppliChem)

 11.0 g/l (75 mM) Kalsiumklorid $\cdot 2H_2O$ (VWR)

 1.21 g/l (10 mM) Rubidium Chloride (Aldrich)

 150 ml (15 v/v %) Glycerol bidistilled 99.5 % (VWR)

pH adjusted to 6.5 with NaOH or HCl. Distilled, ion free water added to a total volume of
1000 ml. Sterile filtered with Stericup-GP 0.22 μm (Millipore). Stored at 4°C

SOC-medium

 20 g/l Trypton (Bacto)

 5 g/l Yeast extract (Oxoid)

 0.5 g/l NaCl (Merck)

 25 g/l KCl (Merck)

 3.6 g/l Glucose (Apotekerproduksjon)

 10.8 g/l $MgCl_2 \cdot 6\ H_2O$ (Merck)

Autoclaved at 120°C for 20 minutes

Materials and methods

M9 medium

      6.0 g/l Na_2HPO_4 (Sigma-Aldrich)

      3.0 g/l KH_2PO_4 (Riedel-deHäen)

      1.0 g/l NH_4Cl (Riefel-deHäen)

      0.5 g/l NaCl (Apotekerproduksjon)

Autoclaved at 120°C for 20 minutes and thereafter added the following sterile stock solutions:

      1 ml/l TMS

      1 ml/l of stock solution $CaCl_2$

      1 ml/l of stock solution $MgSO_4$

      0.8 % (w/v) of stock solution Glucose

Stock solution for M9 medium

      TMS

          0.5 g/l $ZnSO_4 \cdot 7H_2O$ (VWR International)

          0.5 g/l $FeSO_4 \cdot 7H_2O$ (Riedel-deHäen)

          0.5 g/l $MnSO_4 \cdot 7H_2O$ (Riedel-deHäen)

      14.71 g/l $CaCl_2$ (Sigma-Aldrich)

      246.47 g/l $MgSO_4 \cdot 7H_2O$ (Riedel-deHäen)

      250 g/l Glucose (Apotekerproduksjon)

Sterile filtered with Stericup-GP 0.22 μm (Millipore).

M9-10GLU medium

M9 medium with glucose stock concentration increased to 1.0 % (w/v)

0.8 % Agarose gel

      3.2 g Agarose (SemKem LE Agarose)

      400 ml TAE buffer

Heated in microwave oven at 650 W for 10 minutes or until all agarose is dissolved. Kept at 65°C

Materials and methods

2.1.1 SDS-PAGE

<u>3 x Sample Buffer (20 ml)</u>

        1.50 ml 2M Tris, pH 6.8

        7.54 g Glycerol (AnalaR Normapur)

        1.2 g Sodium dodecyl sulphate (SDS) (GPR RECTAPUR)

        0.06 g Bromophenol Blue (Sigma)

        0.93g Ditiotreitol (DTT) (VWR)

<u>Blotting buffer (Transfer buffer)</u>

        12.5 ml/l Tris-HCl stock solution

        14.4 g/l Glycine (Sigma)

        200 ml/l Methanol (Sigma Aldrich)

        5 ml/l SDS stock solution

Distilled water to total volume 2 L

Stored at 4°C

<u>TBS</u>

        10 ml/l 2 mM Tris-HCl, pH 7.5 (stock solution)

        8.8 g/l NaCl (Apotekerproduksjon)

<u>Stock solution blotting buffer and TBS</u>

        315.2 g/l Tris-HCl (Sigma), pH adjusted to 7.5

        100 g/l Sodium Dodecyl Sulphate (GPR Rectapur)

<u>TBST</u>

        500 ml TBS

        250 µl Tween 20 (Sigma Aldrich)

<u>Blotto</u>

        10 g/l Skim milk (Oxoid)

        1000 g/l TBS

        5 ml/l SDS stock solution

Materials and methods

Stock solution Blotto

      200 g/l Sodium Dodecyl Sulphate (GPR Rectapur)

2.1.2 DNA gel electrophoresis

0.8 % Agarose gel

      8 g/l SeaKem® LE Agarose (Lonza) in TAE buffer

Dissolved by heating. Stored at 65°C.

0.8 % Agarose gel + 20 or 30 µl GelRed

      400 ml 0.8 % Agarose gel was added 20 or 30 µl GelRed (Biotium)

TAE buffer (premade by the laboratory staff)

      40 mM Tris

      20 mM Acetic Acid

      1 mM Na_2EDTA

pH adjusted to 8.0

Loading buffer

      400 g/l Glycerol (Sigma)

      2.5 g/l Bromophenol blue (Sigma)

2.1.3 Diverse

Tris-EDTA (TE) Buffer

      10 ml 1M Tris-Cl

      2.0 ml 0.5M EDTA pH 8.0

0.9 % NaCl

      9 g/l NaCl (Apotekerproduksjon)

Materials and methods

100 mg/ml lysozyme

      100 mg Lysozyme from chicken egg white - lyophilized powder (Sigma)

      or

      200 mg Lysozyme from chicken egg white - dialyzed, lyophilized, powder (Fluka)

Diluted in 1 ml TBS

DNaseI

      for 1 mg/ml: 2 mg (Sigma) in 1 ml of stock solution with

            10 mM Tris-HCl (pH 7.5)

            150 mM NaCl

            1 mM $MgCl_2$

      Dissolved before adding 1 ml Glycerol (Sigma).

Stored at $-20°C$

Stock solution for DNaseI

      1.58 g/l Tris-HCl (Sigma) diluted in e.g. 200 ml ion free water and pH adjusted to 7.5

      0.58 g/l NaCl (Apotekerproduksjon)

      0.21 g/l $MgCl_2 \cdot 6H_2O$ (Riedel-deHäen)

10 mg/ml Benzonase

      0.05 ml Benzonase (Sigma)

Diluted in 1 ml TBS

Sucrose stock solution

      400 g/l Sucrose (Sigma)

      100 ml/l 0.5 M Tris-HCl, pH 8 (autoclaved)

      100 ml/l 2 mM EDTA (Triplex) (VWR), pH 8 (autoclaved)

2 M Tris-HCl

      315.2 g/l Tris-HCl (Sigma)

pH adjusted to wanted value

Materials and methods

<u>0.5 M Tris-HCl</u>

      78.8 g/l Tris-HCl (Sigma)

pH adjusted to wanted value

2.2 Bacteria strains and plasmids

Table 1: Bacterial strains used in experiments of this study with relevant genotype information and source

Bacteria	Description	Source
E. coli DH5α	General cloning host	Bethesda Research Laboratories
E. coli RV308	Production strain	ATCC 31608
E. coli S17.1	Strain used for conjugation	(de Lorenzo et al., 1993)
E. coli ER2925	Cloning host, *dam⁻ , dcm⁻*	New England BioLabs
P. fluorescens SBW25	Wild type	(Rainey et al. , 1996)
P. fluorescens NCIMB10525	Nonmucoid *Pseudomonas fluorescens* wild type	NCIMB
P. putida KT2440	mt-2 *hsdR*	Achieved from J. L Ramos

Materials and methods

Relevant characteristics for the plasmids used in this study are summed up in Table 2. Genes marked with "s" after the genes name indicate that the sequence has been codon optimized for *P. putida* KT2440.

Table 2: Bacterial plasmids used in experiments of this study with genotype information and source

Plasmids	Relevant characteristics	Source
pDI-8_MV_ifna2	Used as vector after deletion of *ifn-α2*, *trfA*, *Pm/xylS*, Kan[r]	Roman Netzer, SINTEF Dep. of Biochemistry
pAT64	Source of the gene *ifn-α2b, trfAcop271C*, Amp[r]	Sletta et al. 2007 (here called pIFN30SpelB)
pMV11	Source of the gene *gm-csf, trfAcop271C*, Amp[r]	Sletta et al. 2007 (here called pGM29pelB)
pHH100	Used for plasmid stability evaluation, *Kan[r]*	(Homberset, 2003) Here called pIB6-wtleader-luc
pJB861	Expression vector, *Pm/xylS*, Kan[r]	Blatny et al., 1997
pIFNpelB	*ifn-α2b, pelB, trfA, Pm/xylS*, Kan[r]	This study
pGMpelB	*gm-csf, pelB, trfA, Pm/xylS* , Kan[r]	This study
pCR.1-IFNspelBs	Harbours *ifn-α2b* codon optimized for *P. putida* KT2440, Amp[r]	Eurofins MWG Operon
pCR2.1-GMspelBs	Harbours *gm-csf* codon optimized for *P. putida* KT2440, Amp[r]	Eurofins MWG Operon
pIFN271pelB	*ifn-α2b, pelB, trfAcop271C, Pm/xylS*, Kan[r]	This study
pGM271pelB	*gm-csf, pelB, trfAcop271C, Pm/xylS*, Kan[r]	This study
pIFNs271pelB	*ifn-α2bs, pelB, trfAcop271C, Pm/xylS*, Kan[r]	This study
pGMs271pelB	*gm-csfs, pelB, trfAcop271C, Pm/xylS*, Kan[r]	Not successfully constructed
pIFNs271pelBs	*ifn-α2bs, pelBs, trfAcop271C, Pm/xylS*, Kan[r]	This study
pGMs271pelBs	*gm-csfs, pelBs, trfAcop271C, Pm/xylS*, Kan[r]	This study

2.3 Construction of plasmids for protein production in Pseudomonas

In this study, two *Pseudomonas* strains are evaluated as alternative expression platforms to *E. coli*. For the evaluation of recombinant protein production, IFN-α2b and GM-CSF are chosen as model proteins. These proteins have the size 23.7 kDa and 19.7 kDa respectively.

The plasmid pDI-8_MV_ifna2 (Figure 10) was used as a basis for construction of expression vectors for IFNα2b and GM-CSF. This plasmid was chosen because it already harbours the *kan* gene which allows for selection on kanamycin. In addition it contains the *Pm-XylS* system proven as a functioning expression cassette in different hosts. pDI-8_MV_ifna2 originally harbours a version called *ifn-α2*.
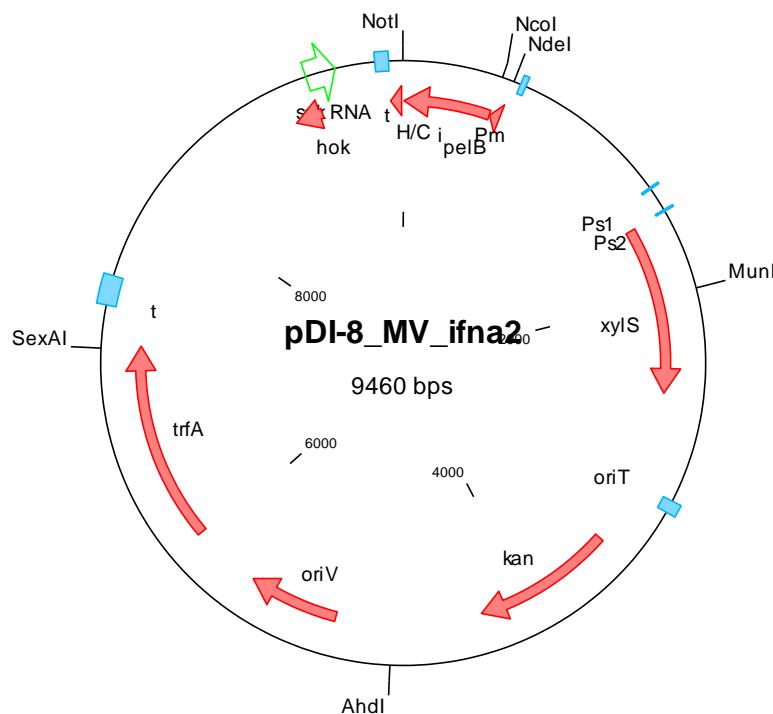

Figure 10: Physical map of the vector pDI-8_MV_ifna2 used for construction of expression vectors in this study. oriV: origin of vegetative replication, trfA: replication initiation protein: t: transcription termination, sok RNA and hok: potential killing mechanism, H/C: his6/c-myc, I: recombinant gene, pelB: signal sequence, Pm, Ps1, Ps2: promoters, xylS: transcription regulator, oriT: origin of transfer, kan: kanamycin resistance. For details se text in Chapter 1.2.4

Detailed information about the different components of the vector is given in Chapter 1.2.4.

Materials and methods

The restriction sites surrounding the gene and signal sequence (here *pelB*) shown in Figure 10 enable the possibility both exchange and deletion of signal sequence. More details can be seen in Figure 11.
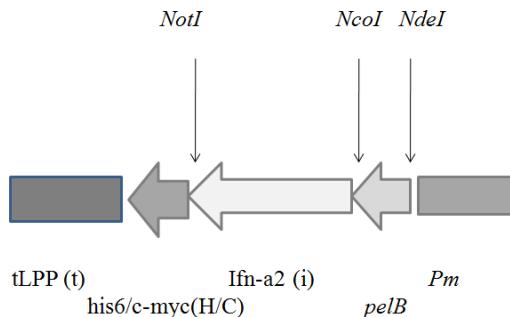
Figure 11: Physical map of part of the expression cassette in pDI-8_MV_ifna with relevant restriction sites for changing the signal sequence and the recombinant gene, here represented by *pelB* and *Ifn-a2* respectively. tLPP (t), transcription terminator, his6/c-myc (H/C) and *Pm*, promoter. Figure based on illustration from (Sletta et al., 2007)

The two proteins IFN-α2b and GM_CSF are encoded by the plasmids pAT64 and pMV11 as shown in Figure 12.
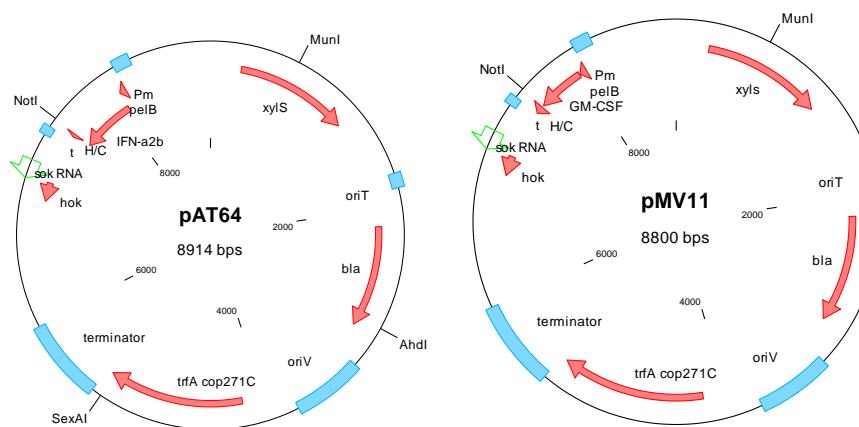
Figure 12: Source of IFN-α2b, pAT64 (pIFN30SpelB) and source of GM-CSF, pMV11

Both of the plasmids pAT64 and pMV11 contained the *bla* gene. This gene codes for resistance against the antibiotic ampicillin (amp).

The genes *ifn-α2b* and *gm-csf* were cloned into the plasmid pDI-8_MV_ifna2 by digestion with *NotI* and *MunI*. Thereafter, the strategy for modifications of the genes and expression vector is illustrated in Figure 13.

36

pIFNpelB and pGMpelB and modified plasmids were constructed with the same strategy. pIFNpelB will therefore function as an example for what was further done, but the same will account for pGMpelB, pGM271pelB and pGMs271pelBs, only then with the *gm-csf* gene

Materials and methods

Step 1: Construction of wild type copy number plasmids

pIFNpelB originating from ligation of *NotI* and *MunI* site from pAT64 (1987 bp) in pDI-8_MV11_ifna2 (7473 bp)
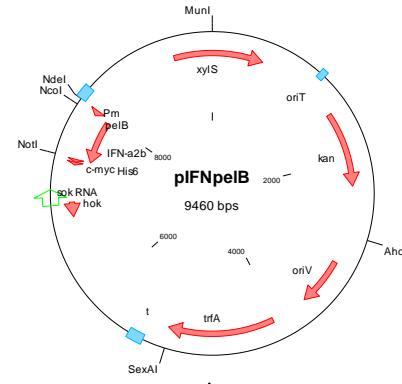
Step 2: Exchange of *trfA* with *trfAcop271C*

Ligation of *SexAI* and *AhdI* site from pAT64 (2368 bp) and from pIFNpelB (7092 bp) yielding pIFN271pelB
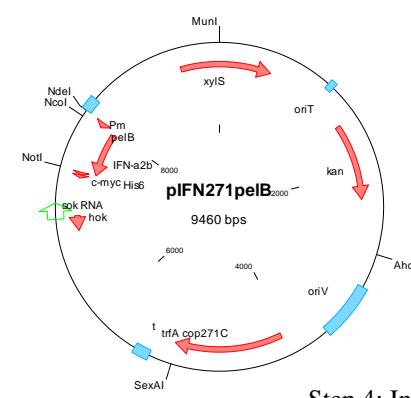
Step 3: Insertion of codon optimized sequence

Ligation of *NcoI* and *NotI* site from pIFN271pelB (8956 bp) and codon optimized IFNspelBs (504 bp) from pCR.1-IFNspelBs
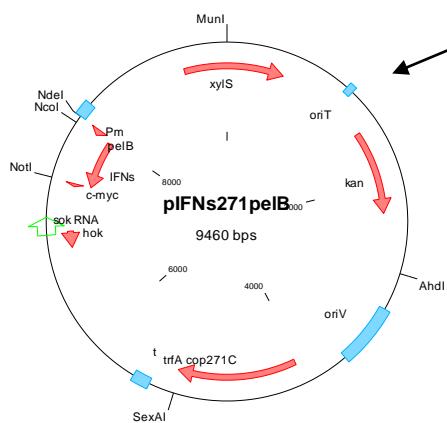
Step 4: Insertion of codon optimized gene and signal sequence

Ligation of *NdeI* and *NotI* site from pIFN271pelB (8894bp) and codon optimized IFNspelBs (566 bp) from pCR.1-IFNspelBs
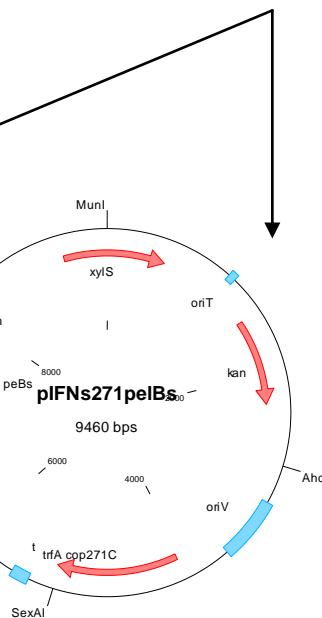
Figure 13: Strategy for construction for construction of expression plasmids relevant for this study. For details see text

2.4 Methods

2.4.1 Growth of bacteria

Growth of precultures from colonies on plates, culture or frozen in stocks

A sterile tooth picker was used to pick a colony on plate or from a cell stock. This was transferred to the desired volume of LB medium (or the medium of interest) in sterile tubes or sterile shake flasks with baffles. Antibiotics were added to the medium depending on the wanted selection.

As mentioned in the introduction, plasmids can contain genes for resistance against certain antibiotics. This can be used to select bacteria which have the plasmid versus bacteria without. In the cases where selection was wanted the following concentrations of antibiotics were made:

> 40 µg/ml kanamycin
>
> 100 µg/ml ampicillin

Unless otherwise is stated, all cultivation of bacteria was done in medium containing antibiotics following the genotype in Chapter 2.2.

The shake flasks were placed in a shaking incubator (Infors) for 200 rpm overnight.

The temperature in the incubator depended on the bacteria strain; *E. coli* cultures were incubated at 37°C while Pseudomonas cultures were incubated at 25 - 30°C.

Growing bacteria on plates

The agar medium was made as described under Media and supplements (Chapter 2.1). After autoclavation the medium was cooled to 50 – 60°C while standing on magnet stirrer. Before pouring the medium into petri dishes (VWR) with wanted size, and if selection was wanted the medium was added antibiotic.

Unless otherwise is stated, all cultivation of bacteria was done in medium containing antibiotics following the genotype in Chapter 2.2.

Materials and methods

Overnight cultures or cell samples were added 16 – 20 % sterile glycerol before incubating on ice for 30 minutes. The cells were stored at – 80°C.

2.4.2 Growth and pH measurements

The cell density was measured by optical density at wave length 600 or 660 nm. It is shown that OD_{660}=3-4 equals to 1 g dry weight cells per litre *P. putida* (Attachment F, Table F- 23). The spectrophotometer (Shimadzu UV160A) in use has a linear area from OD = 0.1 to 0.3, so most of the cultures had to be diluted with medium or 0.9 % NaCl to this area.

Cultures and solutions pH were measured on PHM210 Standard pH Meter (Radiometer analytical) after validated calibration.

2.4.3 Plasmid isolation

Plasmid isolation: Wizard Plus SV Minipreps DNA Purification System (Promega)

Plasmid isolation was used to isolate the initial plasmids and to isolate ligated plasmids.

- 1.5 ml of the overnight cultures was transferred to sterile 1.5 ml tubes (Eppendorf) and centrifuged at 10 000 rpm for 5 minutes (Heraeus). The supernatant was removed and cells resuspended in 250 µl Cell Resuspension Solution

- To lysis the cells and bring the plasmids into solution, 250 µl Cell lysis buffer was added before inverting the tubes 4 times. The mixture was incubated for 1.5 minutes until the cell suspension appeared clear

- 10 µl Alkaline Protease Solution was added before the tubes again were inverted 4 times. This was incubated for 5 minutes in room temperature. Alkaline Protease inactivates proteins released from the bacterial cells and endonucleases.

- To neutralize the protease, 350 µl Neutralization Solution was added before the cell lysate was centrifuged for 15 minutes, 13 000 rpm.

- The lysate was transferred to Spin Column in 2 ml Collection tubes and centrifuged for 1 minute at 13 000 rpm (Heraeus)

- The flow-through was discarded before adding 750 µl Column Wash Solution and centrifuged at 13 000 rpm for 1 minutes (Heraeus)

- The column was washed again by removing the flow-through and adding 250 µl Column Wash Solution before it was centrifuged for 2 minutes, 13 000 rpm (Heraeus)

- To eluate the DNA molecules the Spin Columns were transferred to sterile 1.5 ml tubes (Eppendorf) and added 30 µl sterile water and centrifuged at 13 000 rpm for 1 minutes

- After elution the Spin Columns were removed and plasmid preparations stored at – 20°C

2.4.4 Restriction digestion

As described in Chapter 1.1, bacteria use restriction enzymes as protection against viral attacks. Specific restriction enzymes have specific recognition sites in the nucleotide sequence. Most of the sequences are four to six nucleotides long, but there are examples of enzymes with longer recognition sites. The patterns of the cut also differ between enzymes. The cut can produce either blunt or sticky ends. Blunt ends is made if the nucleotide strand is cut at the same nucleotide pair, while sticky ends is made if offset cuts are made (Klug et al., 2009).

Restriction digestion was used to select the right gene fragments and vector. In addition it was used to verify the ligation and transformation of produced plasmids.

The following restriction mixtures were made:

 8 µl isolated plasmid (concentration ranging from 20 – 150 µg/µl)

 1 µl enzyme (NEB)

 0. 5 µl BSA (if necessary) (NEB)

 2 µl 10x concentrated buffer (1-4, specified for each enzyme) (NEB)

 Sterile water to a total volume of 20 µl

If double digestion was of interest and the buffers of the two enzymes agreed, 1 µl of each enzyme were added in the same mixture.

The restriction mixture was incubated 37°C for 1. 5 hours. The restriction enzymes used in this study is shown in Attachment B

2.4.5 Separation of DNA fragments by gel electrophoresis

Electrophoresis is a technique that can be used to separate small molecules, like DNA fragments, from each other with the help of an electrical field. When a mixture of molecules is placed in a porous substance and influenced of the electrical field, it forces the molecules to migrate different lengths determined by the different sizes and net charge of the molecules. If two molecules have the same charge but different size, the porous substance, for example a gel, will separate them based on the size. Smaller molecules will migrate faster than the larger molecules (Klug et al., 2009).

2 – 3 µl loading buffer was added to the restriction mixtures. Separation of DNA fragments was then executed in Model B1A or B2 (Thermo Scientific) and with Power Pac 200 (BioRad). UV-light from Gel Doc 2000 (BioRad) was used to visualize the gel fragments after one of the following staining procedures. The program Quantity One 4.1.1 (BioRad) was used to handle the pictures.

2.4.5.1 Ethidium bromide (EtBr) staining

The most commonly used staining method for DNA fragments separated in agarose gels use the fluorescent dye ethidium bromide. It forms a complex with the DNA which can be visualized by UV-light (Sambrook and Russell, 2006).

Samples were run in a 0.8 % Agarose gel at 90 – 110 V for 50 – 80 minutes depending on the fragment- and gel sizes. The standard used was 1 kb DNA Ladder (Attachment C, Figure C-1). The gel was transferred to a bath containing 5 µg/ml EtBr (Merck) for 10 minutes. Afterwards, decolorization for 25 minutes.

2.4.5.2 GelRed staining

EthBr is toxic (ECOonlineAS, 2007). An alternative for staining gel was therefore adopted during the study. This method uses GelRed (Biotium) which have been shown to be nonmutagenic and noncytotoxic. GelRed (and GelGreen) DNA-binging stain is developed to reduce genotoxicity by preventing the dye to enter living cells (Biotium, 2009).

Samples were run in a 0.8 % Agarose gel containing 20 - 30 µl GelRed at 90 – 110 V for 50 – 80 minutes depending on the fragment- and gel sizes. The standard used was GeneRuler™ DNA Ladder Mix 0.5 µg/µl 50 µg (Attachment C, Figure C- 2).

2.4.6 Extraction of DNA fragment from agarose gel

After identifying the desired fragments, they were cut out from the gel using a clean scalpel. The gel fragments were kept in a 1.5 ml tubes (Eppendorf) at 4°C until extraction of DNA.

Isolation of plasmids from gel fragments: QIAquick (Qiagen)

- 300 µl QG buffer was added to the 1.5 ml tubes (Eppendorf) and incubated at 50°C for 10 minutes or until the gel was completely dissolved
- 100 µl isopropanol was added and mixed together by flicking the tubes. This will precipitate the DNA
- QIAquick spin columns were placed into collection tubes before the Agarose/DNA/isopropanol mixtures were transferred to the columns and centrifuged for 2 minutes 13 000 rpm (Heraeus)
- The DNA was now bound to the column material. The flow-through was removed before adding 500 µl QG buffer and centrifuge as above
- The flow-through was removed before adding 750 µl of the wash PE buffer. The tubes were then let standing for 5 minutes before spinning for 2 minutes (13 000 rpm) (Heraeus). The flow-through was discarded.
- To remove the ethanol completely ,the columns before spinning for 2 minutes at 15 000 rpm (Heraeus)
- The columns were moved to sterile 1.5 ml tubes (Eppendorf) and DNA was eluated with sterile water and left for 5 minutes before centrifugation at 15 000 rpm for 2 minutes (Heraeus)

QIAEX II Agarose Gel Extraction Protocol (Qiagen)

- 450 µl of the solubilisation and binding buffer QX1 and 300 µl sterile H_2O was added to the gel fragments
- The QIAEX II suspension was resuspended by vortexing for 30 minutes before adding 10 µl into each tube. QIAEX II suspension consists of silica gel particles which selectively absorbs nucleic acids
- The mixtures were incubated for 20 minutes to solubilise the agarose and bind the DNA. To keep the QIAEX II in suspension, the mixtures were vortexed every 2 minutes

Materials and methods

- The samples were centrifuged at 13 000 rpm for 1 minute (Heraeus). The supernatant was removed
- The pellet was washed with 500 µl Buffer QX1 before centrifuging at 13 000 rpm for 1 minute (Heraeus)
- After removing the supernatant, the pellet was washed twice with 500 µl wash buffer PE (13 000 rpm for 1 minute (Heraeus))
- The pellet was air-dried for 15 minutes or until the pellet became white
- Eluting the DNA was accomplished by adding 20 µl sterile H_2O, centrifuging as above and carefully pipetting the supernatant to a clean 1.5 ml tube (Eppendorf)

2.4.7 Ligation of DNA fragments

As described earlier restriction enzymes make specific cuts in DNA strands. If two DNA fragments with complementary sticky ends are mixed together in the right proportions, they can anneal by base pairing their single strands. This situation can be made by using the same enzyme on two different DNA strands. By adding a DNA-ligase in the solution, covalent linkages will form between the fragments and give a recombinant DNA molecule as product (Klug et al., 2009).

The following ligations were made:

2 µl   Vector DNA

15 µl   Insert DNA

1 µl   DNA-ligase T4 (NEB)

2 µl   T4 DNA Ligase Reaction Buffer (NEB)

The ligation mixture tubes was left at the top of a box containing ice overnight

2.4.8 Transformation of bacteria

Bacteria have the ability to transfer and take up DNA in three ways; transformation, transduction and conjugation. During transformation, DNA from the environment is taken up by the cell. Transduction is uptake from a bacteriophage and conjugation is DNA transfer between two bacteria. Several laboratory methods utilize these biological principles for production of recombinant strains. To ensure the uptake selection based on resistance against a certain antibiotic can be used (Klug et al., 2009).

2.4.8.1 Transformation

Transformation is the bacterial uptake of extracellular DNA through receptor sites at the cell surface. The bacteria containing these receptor sites are called competent. When DNA binds to the sites, it is passed into the cytoplasm in an active process. (Klug et al., 2009)

Competent cells

- An 1 % inoculum was made from an overnight culture in Psi medium and incubated to $OD_{600} = 0.400$
- The cells were incubated on ice for 15 minutes and centrifuged at 4 500 rpm for 5 minutes (Heraeus)
- The supernatant was removed and the pellet resuspended in 40 ml cold TFB1
- The cells were incubated on ice for 5 minutes and centrifuged (4 500 rpm, 5 minutes (Heraeus))
- The supernatant was removed and pellet resuspended in 3 ml cold TFB2
- The resuspantion was distributed in sterile 1.5 ml tubes (Eppendorf) (0,1 ml per tube) and quickly frozen in a mixture of ethanol and dry ice
- Stored at $-80°C$

Transformation of plasmid to competent cells

- 10 µl ligation mixture or 3 µl plasmid preparation was added to 100 µl competent cells
- The mixture was kept on ice for 30 minutes
- The tubes were incubated in a 42°C water bath for precisely 35 seconds, then placed back on ice for 2 minutes
- 1 ml preheated (37°C) SOC was added and incubated for 60 minutes at 37°C
- The transformation mixture was plated out on LA with selection and incubated overnight

2.4.8.2 Electro transformation

Electro transformation is a quick and efficient method for introducing molecules into a cell. A quick electric pulse is used to make cells competent i.e. the membrane permeable for DNA (Eynard and Teissié, 2000).

Electro competent cells

- An 1 % (v/v) inoculum in100 ml pre-warmed LB was made from an overnight culture in 10 ml LB
- The culture was grown for 2.5 hours before kept on ice for 15 – 30 minutes.
- The cells was harvested by centrifuging (5000 rpm, 15 minutes) (Eppendorf)
- The medium was removed and the cells washed 3 times in 100, 50 and 10 ml 10 % sterile glycerol. Between each wash, the cells were centrifuged as above. After the last wash the cells was resuspended in 0.5 – 1.0 ml 10 % glycerol
- 40 µl of the competent cells was frozen in a mixture of ethanol and dry ice. The cells were kept at – 80 ∘C

Transforming plasmids to electro competent cells

- The following setting was made on a BIO RAD Gene Pulser XCell:

  Voltage: 2.5 kV

  Capacitance: 25 microF

  Resistance: 200 Ω

  Time constant: 5.6 – 5.8 mSec

  Cuvette (mm):   2

- Competent cells made for electro transformation was kept on ice (40 µl per tube) and added 2 µl DNA. This was transferred to a cold and clean optical cell.
- The cells were given pulse and cell-DNA mixture before adding and resuspending in 1 ml LB. The mixture was then moved to a sterile Eppendorf tube.
- The culture was grown at 30 ∘C for 1 – 2 hours or overnight before plating out on LA with selection

2.4.8.3 Conjugation

Conjugation is a process where genetic information is transferred from one bacterium to another. For conjugation to be possible the plasmid has to be conjugative, i.e. encode genetic information that enables cell-to-cell contact. The host bacteria need to have the gene coding a

pili structure. These pili can attach to an OmpA protein on the surface of another bacterium that does not contain the plasmid. Connection between pili-OmpA lead to a cell-to-cell contact where the DNA from one bacterium can be transferred to the other with the help of an enzyme which starts to digest in *oriT*. This starts the "rolling circle"; a DNA replication at the 3' end. After the conjugation, both bacteria have the plasmid (Degré et al., 2004).

- LB overnight cultures of bacteria with plasmids of interest were used to make 1 % (v/v) inoculums in LB. The receiver strain culture was inoculated 2 hours earlier than the plasmid harbouring strains. After this, the cultures were left for 2 hours.
- 2 ml of plasmid strains was mixed with 2 ml of the receiver strains and spun at 4000 rpm for 5 minutes. The supernatant was removed and the cell pellet was resuspended in 100 μl LB
- The resuspention was placed in a droplet on LA plates. The droplets were not plated out but left as one deposit and incubated at 30°C overnight
- The droplets were harvested and resuspended in 1 ml LB. 10^{-2}, 10^{-4} and 10^{-6} dilutions of this was plated out on suitable agar with selection and incubated overnight at preferred temperature

2.4.9 Production of proteins in HCDC (fermentation)

HCD cultivations are used to increase the production of proteins from established expression platforms. If the cultivation in a bench top bioreactor is successful in terms of economic perspectives and quality of produced proteins, further up-scaling to *pilot-scale bioreactors* and perhaps *industrial-scale operation* can be performed. Fermentations in a bench top bioreactor gives the opportunity to measure and adjusting temperature, pH, dissolved-oxygen concentration, stirrer speed, nutrition and other process variables (Doran, 1995).

HCDC and production of model proteins was examined in 1-litre (Dasgip) and 3-litre (Applicon) fed batch bench top fermentors with 0.35 and 0.75 litres media start volume respectively. The temperature was automatically regulated to 30°C.

2.4.9.1 Preparing inoculums for fermentation

- Cultures to be fermented were plated out on LA with selection overnight at optimal temperature
- A sterile scoop was used inoculate 100 ml inoculum LB medium (see Attachment E for details) with one colony. The culture was incubated at 30°C, 200 rpm for 8 – 16 hours (depending on growth)
- 100 ml inoculum Hi-medium (see Attachment E for details) was inoculated with 0.5 ml LB-culture and incubated for 16 – 18 hours at 30°C (200 rpm)

2.4.9.2 Fermentation conditions

The following procedure was used to set the conditions of 1-litre and 3-litre fermentors

- 0.35 l and 0.75 l start volume respectively of Hf.1 medium (for details see Attachment D) was added to each fermentor and autoclaved for 30 minutes at 120°C. Thereafter cooled to 15°C overnight. The temperature was then set to 30°C
- pH electrode was calibrated in buffers with pH 7 and 4 before setting the set point to 6.8. For 3-litre fermentors 12.5 % NH_3 (sterile) was used for pH control. When bacteria grow with the access to glucose (like for this study), acids are normally produced and pH decreases. NH_3 will adjust this pH to the set point of 6.8.
- The oxygen electrodes 0 % level was checked with nitrogen gas. Stirring speed was adjusted to 500 rpm on the 1-litre fermentors and to 1000 rpm on the 3-litre fermentors and the reported dissolved oxygen (DO)-value was adjusted to 100 %. DO is the concentration of dissolved oxygen in the medium and is measured continuously during the fermentation. It is controlled by the stirring speed. Set-value for oxygen concentration in the medium was 20 %.Stirring speed was set to minimum set point at 200 and 350 rpm for respectively 1-litre and 3-litre fermentors.
- Air flow was adjusted to 0.25 vvm (litre air/litre medium, minute). Flow measurements of air into the fermentor is measured by a mass-flow-meter and should be between 0.25 – 1.0 vvm.
- Additions were made to the Hf.1 medium following Attachment E
- 0 % value of CO_2 analyzer was calibrated with N_2 gas. The set value of CO_2 is thereafter set to 5 % calibration gas. CO_2 concentration from the air leaving the fermentor and is reported as percentage of total air flow

Materials and methods

- Inoculation of the fermentors was done. OD_{600} samples of the precultures were taken to calculate the volume necessary for giving a start OD_{600} value of 0.05 in the fermentors

- Exponential feeding was started with initial rate of 5.6 g/fermentor·hour (1l-fermentors) or 12 g/fermentor·hour (3l-fermentors) and exponentially increased with $\mu = 0.20\text{-}0.22$ h^{-1} until reaching a feed rate of 12 g/fermentor·hour (1l-fermentors) or 25 g/fermentor·hour (3l-fermentors). When this stage was reached, feeding was constant thorough out the fermantation

- Samples for glucose measurements were taken regularly to control the that there was not rest of glucose in the medium

- Cell density was measured regularly before reaching induction time ($OD_{600} \approx 80$ - 90) for strains harbouring expression plasmid constructed in this study (Chapter 2.2). Inducer was added to a final concentration of 1 mM.

The different parameters are measured and logged by a computer.

All fermentations were executed in collaboration with SINTEF Materials and Chemistry, department of Biotechnology.

2.4.10   Detection and quantification of recombinant protein

Sodium dodecyl sulphate (SDS) is a commonly used detergent for separating proteins based on their mass and shape during SDS polyacrylamide gel electrophoresis (SDS-PAGE). When SDS is mixed with a protein sample it binds and partially unfolds them. During electrophoresis, where an electric field is applied, the negative charge of SDS will contribute to separating the proteins depending on mass and shape, where smaller polypeptides migrate more rapidly. After separation the proteins can be visualized with using a suitable stain (Nelson and Cox, 2008) or specific proteins can be detected with a method called Western Blot. Here the proteins are transferred from the gel to a nitrocellulose membrane using an electrical current. A specific protein can be detected using antibodies to elements of the proteins, for example c-myc or His6. Method used for detection depends on how the secondary antibody is marked, but one example is conjugation with a enzyme which facilitates detection using its substrate (Snustad and Simmons, 2010).

2.4.10.1   Sample preparation for SDS-PAGE

Recombinant proteins in prokaryotes can be produced as soluble or insoluble fractions (previously referred to as IB). To determine the different amount of these fractions, lysing protocols have to be used. The following protocols were developed for *E. coli* but since *P. putida* also is a gram negative bacterium, it was assumed that the protocols would have the same outcome on both *E. coli* and *P. putida*. Neither of the protocols will separate proteins secreted to the periplasm form proteins inside the cytoplasm.

- 1 ml sample from shake flask or HCDC experiments were centrifuged in 1.5 ml tube (Eppendorf) for 10 minutes as 13 – 14 000 rpm (Heraeus)
- The supernatant was transferred to a 1.5 ml tube (Eppendorf), while the remaining pellet was used for lysis either using protocol 1 or 2. The main difference between these two are the enzyme used to eliminate DNA, which in protocol 1 is DNaseI and in 2 is Benzonase:

Cell lysis protocol 1
- 250 μl 0.5 M Tris-HCl, pH 8.0 containing 2 mg/ml lysozyme (Fluka) was added to the cells and resuspended. Lysozyme is a enzyme that breaks down the cell wall of the bacteria. The enzyme is active in pH range 6.0 – 9.0 created by Tris-HCl.
- 250 μl Sucrose stock solution containing 1 mg/ml DNaseI (Sigma) was added. The mixture was incubated for 1 hour at room temperature shaking. The mixture will eliminate DNA and prevent protease activity
- The lysate was centrifuged at 13000 rpm for 8 minutes (Herasus)
- The supernatant (soluble fraction) was transferred to a clean 1.5 ml tube
- The pellet was resuspended in 500 μl 1 x Running Buffer (ClearPAGE)
- If no dilution was necessary, 20 μl of the samples was added 10 μl 3x Sample Buffer and boiled at 95°C for 5 minutes. Possible dilution was made in 1 x Running Buffer (Clear PAGE)

Cell lysis protocol 2
- The pellet (P1) was resuspended completely in 500 μl 50 mM Tris-HCl, pH 8.0
- 500 μl sucrose stock solution was added and mixed carefully to prevent protease activity

Materials and methods

- 10 μl 100 mg/ml lysozyme (Sigma) and there after 10 μl 10 mg/ml Benzonase (Sigma) was added
- The mixtures were incubated while rotating, for 1 hour at room temperature.
- The samples were then centrifuged at 14 000 rpm (Eppendorf) for 5 minutes at 4°C
- The supernatant (soluble fraction) and the pellet (insoluble fraction) were separated and the pellet resuspended in 500 μl 1 x Running Buffer (ClearPAGE)
- The samples were at – 20°C
- Before using the samples in gel electrophoresis, they were boiled at 95°C in a 2:1 relationship with 3 x Sample Buffer

2.4.10.2   SDS-PAGE

- 15 μl of the boiled samples and 10 μl Precision Plus Protein Dual Color Standard (Figure C- 3) was transferred to a 10 % or 12 % TEO-CI SDS gel (ClearPAGE) and run inside Dual Cool Mini-Vertical PAGE (CBS Scientific) at 150 V (BioRad Power Pac 200) for 1 hour in 1 x Running Buffer (ClearPAGE). For this study, the standard was always placed in the well furthest to the left.

2.4.10.3   Western Blot

Using anti-c-myc as primary antibody

- Gel, filter paper and blotting sponges was incubated in Blotting Buffer while the membrane (Immobilon Millipore Transfer membrane) was incubated in methanol for 10 minutes
- Afterwards this was placed in the following order in Mini Protean blotting system (BioRad): Clear plate, sponge, filter paper, membrane, gel, filter paper, sponge, black plate. This was placed in the blotting chamber containing a block of ice. The blotting chamber was filled with Blotting Buffer before butting the whole system in a box filled with ice to keep the temperature down
- The blotting was run at 70 V for 1 hour
- The membrane was washed 3 x 5 minutes with TBS before blocking the membrane for unspecific binding with the help of Blotto for 1 hour at room temperature
- The proteins were detected with 2 μl of the primary antibody anti-c-myc (Invitrogen) in 10 ml Blotto. The membrane was kept in this solution shaking overnight at 4°C

Materials and methods

- The membrane was washed 3 x 20 minutes in TBST before 10 µl Rabbit Anti-Mouse (Dako) in 10 ml Blotto for detection of the primary antibody was added. This was kept at shaking for 1 hour
- The membrane was washed 3 x 5 minutes in TBST and then 1 x 5 minutes in TBS before the substrates Tetrametylbenzidine and Hydrogen Peroxide (TMB Substrate Reagent Set, BD Biosciences) was mixed 1:1 and added to the membrane
- When the colour reaction was complete, the membrane was transferred to distilled water

Using Tetra His Antibody as primary antibody

- Gel, filter paper and blotting sponges were incubated in Blotting Buffer for 30 minutes. The membrane (Immobilon Millipore Transfer membrane) was incubated in methanol for 15 seconds, 2 minutes in water and 10 minutes in Blotting Buffer
- Afterwards this was placed in the following order in a BioRad blotting system: Clear plate, sponge, filter paper, membrane, gel, filter paper, sponge, black plate. This was placed in the blotting chamber containing a block of ice. The blotting chamber was filled with Blotting Buffer before butting the whole system in a box filled with ice to keep the temperature down
- The blotting was run at 100 V for 1 hour
- The membrane was washed one time with TBS
- The membrane was blocked by incubating it in Blotto (made from 1 g Skim milk Powder in 100 g TBST) for 1 hour at room temperature
- Primary antibody Tetra His Antibody (Qiagen) was diluted 1:7500 in Blotto and added to the membrane. This was incubated overnight, shaking at 4 °C.
- The membrane was washed three times with TBST, each time for 10 minutes
- Secondary antibody Polyclonal Rabbit Anti-Mouse Ig HRP() was diluted 1: 1500 in Blotto was added and incubated for 1 hour at room temperature
- The membrane was washed three times with TBST and one time with TBS, each time for 10 minutes
- The antibodies attached to the proteins of interest were detected by using 3 ml 3,3'5,5' Tetramethylbenzidine (TMB) (Sigma). After incubating until bands were visible, the membrane was transferred to distilled water before scanning the result

3   Results and discussion

The work in this study can be divided into two main parts. The first part was the genetic construction of expression vectors. In total, 7 different vectors were constructed, where the main differences were gene of interest, copy number, and codon optimized genes and signal sequence. The second part was cultivation analysis of possible expression platforms and evaluation of their production of recombinant proteins. *E. coli*, *P. fluorescens* and *P. putida* were evaluated under different conditions with respect to growth and protein production. The cultivations were performed in deep well plates, shake flasks and fed batch fermentations.

3.1   Construction and cloning of wild type copy number plasmids pIFNpelB and pGMpelB

The goal for the first construction strategy (Chapter 1.4, Figure 9, Step 1) was to make two expression plasmids with wild type copy number. pIFNpelB and pGMpelB harboured the *ifn-α2b* and *gm-csf* gene respectively. By using this as starting point, there were many possibilities for later genetic modifications.

All of the three initial plasmids, pDI-8_MV_ifna2, pAT64 and pMV11 (Chapter 2.3), were present in *E. coli* DH5α strains. In the first step of this study, the original *ifn-α2* of pDI-8_MV_ifna2 was replaced gene with the genes *ifn-α2b* and *gm-csf* to form pIFNpelB and pGMpelB respectively.

The three DH5α strains were grown overnight (37°C, 200 rpm) in 3 ml LB with selection before isolating the plasmids (Chapter 2.4.3). Restriction mixtures were made following Chapter 2.4.4 with the enzymes *MunI* (also called *MfeI*) and *NotI*. pAT64 and pMV11 were both expected to give a 6.9 kb fragment in addition to one smaller fragment with size 2.0 and 1.9 kb respectively. These two smaller fragments were the genes *ifn-α2b* and *gm-csf*, and therefore the inserts. pDI-8_MV_ifna2 was expected to be cut in two fragments, one at 7.5 kb and one at 2.0 kb, the largest being the vector. The result from the digestion is shown in Figure 14.
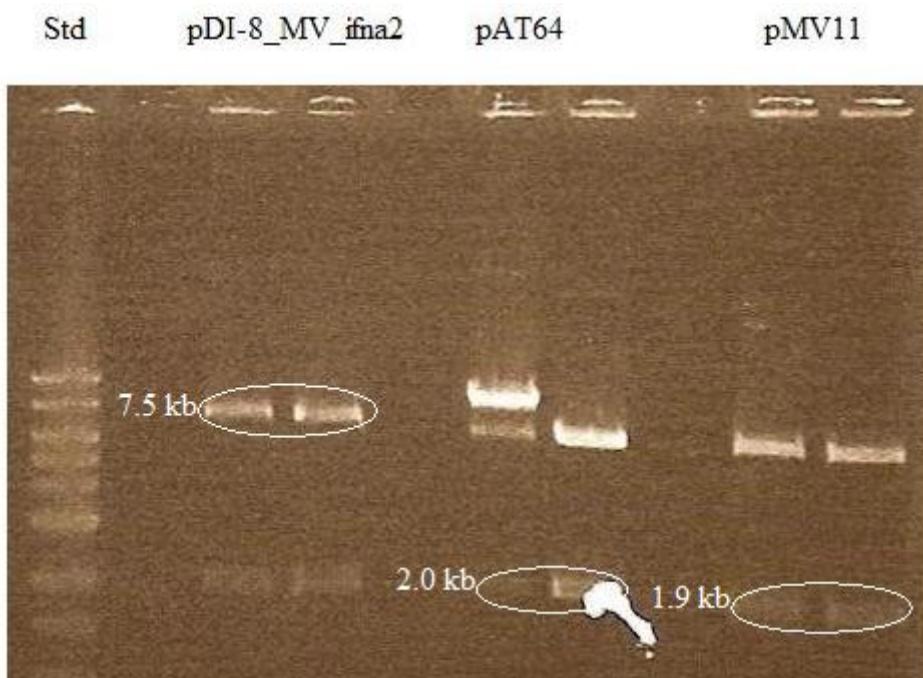
Figure 14: Restriction digestion results of pDI-8_MV_ifna2, pAT64 and pMV11 digested with *NotI* and *MunI*. The marked DNA fragments were extracted from the gel; 7.5 kb fragment from pDI-8_MV_ifna2, 2.0 kb fragment from pAT64 and 1.9 kb fragment from pMV11. The sizes were identified with 1 kb DNA ladder (NEB) standard (left) (Attachment C, Figure C- 1).

Fragments with the expected size on the gel (Figure 14) were identified and isolated as described in Chapter 2.4.6 (QIAquick protocol). Ligation mixtures were made (Chapter 2.4.7) in addition to one mixture with vector DNA only. This was a negative control. Ligation mixtures were transformed into competent *E. coli* DH5α cells (Chapter 2.4.8.1) and plated out on LA with selection. After overnight incubation at 37 °C the plates were inspected. As the negative control had no growth, the transformation was interpreted as successful, but still had to be evaluated through restriction digestion. Single colonies were transferred to 3 ml selective LB medium and incubated overnight at the same temperature (37°C). Plasmids were isolated and restriction mixtures were made (Chapter 2.4.3), where pIFNpelB transformants were digested with *PstI*, while *BsrGI* and *XhoI* were used on pGMpelB. The correct constructed plasmids were expected to give fragments of 4.0, 3.3, 1.2 and 0.9 kb, and 6.2 and 3.2 kb respectively. Transformants that had the expected fragment sizes, shown in Figure 15, were used for further work in this study.

Results and discussion
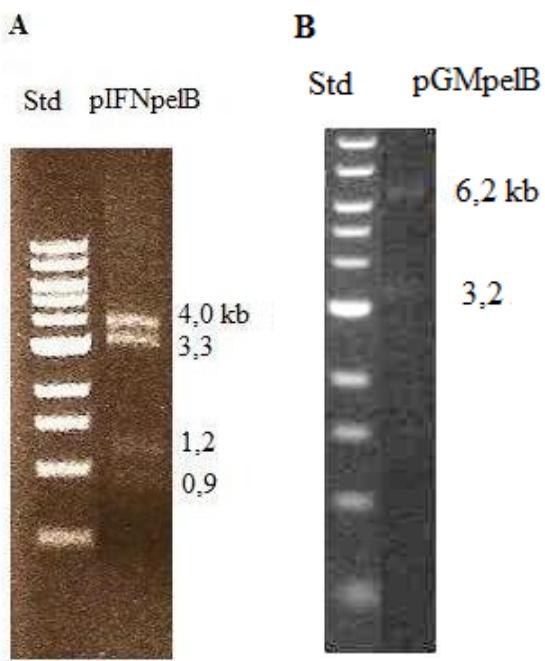
Figure 15: Restriction analysis of two transformants after cloning of target genes (*ifn-α2b* and *gm-csf*) into vector. A) Plasmid expected to be pIFNpelB digested with *PstI*. B) Plasmid expected to be pGMpelB digested with *BsrGI* and *XhoI*. The sizes were identified with 1 kb DNA ladder (NEB) standard (left) (Attachment C, Figure C- 1).

## *3.2 Evaluation of E. coli RV308 strain as expression host for wild type expression vectors*

The plasmids were now present in *E. coli* DH5α cells, which are general cloning hosts. For further experiments and evaluation of ability to produce the recombinant proteins, the plasmids were transferred to a production strain of *E. coli*. These *E. coli* strains would be used for comparisons in the investigation of the *Pseudomonas* species properties as a protein producer. As explained in Chapter 1.3, IFN-α2b and GM-CSF have already been produced in *E. coli* RV308. This strain was therefore chosen to be reference in this study.

The pIFNpelB, pGMpelB, pAT64 and pMV11 were transferred to *E. coli* strain RV308 by transformation (Chapter 2.4.8.1) and different dilutions were placed on selective LA plates. After incubating at 37°C overnight, one colony from each strain was transferred to 3ml LB with selection and grown overnight before storing the culture in glycerol stocks at − 80°C (Chapter 2.4.1).

3.2.1 Cultivation in deep well plates

Precultures of RV308 (pIFNpelB), RV308 (pGMpelB), RV308 (pMV11) and RV308 (pAT64) were grown in 50 ml selective LB medium in 250 ml shake flasks with baffles overnight at 37°C (200 rpm). 10 ml of each pre-culture were spun down and cells were washed with 10 ml 0.9 % sterile NaCl. The cells were resuspended in 1 ml 0.9 % sterile NaCl before transferring 500 µl of the cell suspensions to 50 ml Reduced Hi+YE medium (see Attachment D for details). Parallels of 600 µl inoculated Reduced Hi+YE medium were transferred to deep well plates (Greiner Bio One). Cultures were incubated at 16 and 25°C, 800 rpm. OD_{600} measurements taken regularly and after reaching a value 5, parallels of each culture were induced to a final concentration of 0.5 mM m-toluic acid. After induction the plates were incubated overnight, before freezing at − 20°C.

Figure 16 shows the growth of RV308 strains during the deep well experiment.
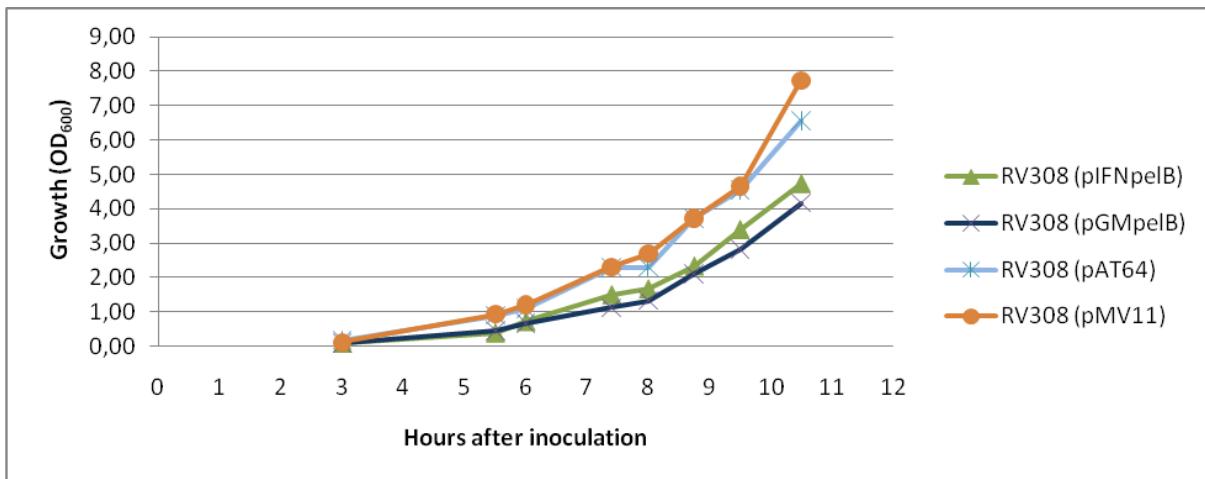
Figure 16: Growth (OD$_{600}$) of *E. coli* RV308 harbouring pIFNpelB, pGMpelB, pAT64 or pMV11, all grown in reduced HiYe medium with selection at 25 °. The densities were measured by OD$_{600}$. For detailed information see Attachment F Table F- 2

E. coli RV308 strains containing pIFNpelB and pGMpelB grew with lower rate compared to the strains with pAT64 and pMV11 (Figure 16). The growth curves are almost identical for these pairs. One reason might be the different selection markers within the plasmids. pIFNpelB and pGMpelB have kanamycin resistance while pAT64 and pMV11 are resistant against ampicillin (Chapter 2.2). The dosage and effect of these antibiotics are not the same and these curves can therefore not be directly compared.

None of the cultures showed growth at 16°C (results not shown).

3.2.1.1 Protein production

The samples induced at OD$_{600}$ ≈5 to a final concentration of 0.5 mM m-toluic acid were used for SDS-PAGE (Chapter 2.4.10.2) after boiling a quantity of the culture diluted with 3x sample buffer. In addition, a 20 mg/l Phox reference sample was prepared like the culture samples. Western Blots protocols using anti-c-myc as primary antibody was followed (Chapter 2.4.10.3). From pAT64 and pIFNpelB it was expected to detect IFN-α2b protein with molecular weight 24.7 kDa, and from pMV11 and pGMpelB the protein GM-CSF with molecular weight 19.7 kDa. The results are shown in Figure 17.

Results and discussion
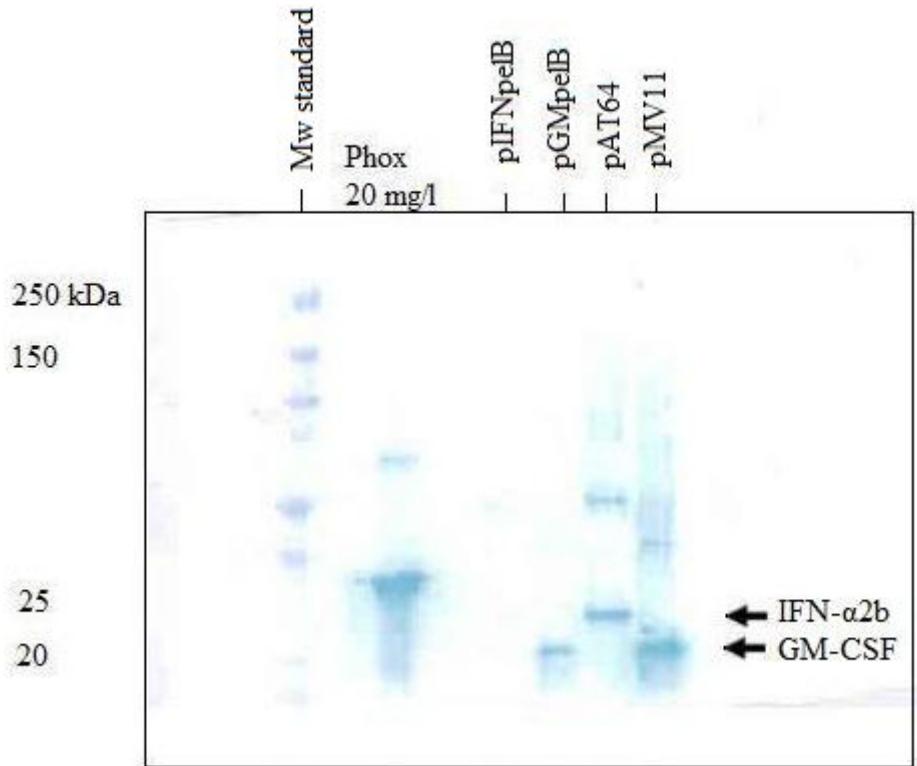
Figure 17: Western Blot results of RV308 strains with plasmids pIFNpelB, pGMpelB, pAT64 and pMV11 (indicated with the top vertical lines). The cultures were grown in deep well plates at 25°C and induced to a final concentration of 0.5 mM m-toluic acid at $OD_{600} \approx 5$. Numbers on the left side represents molecule sizes in kilo Daltons

Figure 17 shows the detected proteins in the RV308 culture samples from the deep well experiment and give an indication on the functionality of the constructed plasmids. Both pAT64 and pMV11 are expression vectors with increased copy numbers (see Chapter 2.2), and can therefore in theory produce more of the recombinant proteins IFN-α2b and GM-CSF respectively. Direct comparison of the amount protein produced from these strains with RV308 (pIFNpelB) and RV308 (pGMpelB) can therefore not be performed. Still, comparison of the molecular weights of produced recombinant proteins is an assurance that the plasmids are successfully constructed. pIFNpelB and pAT64 both harbours the *ifn-α2b* gene (23.7 kDa), but the protein is only detected from RV308 (pAT64). This can indicate that RV308 has problems with producing the protein and should be investigated further.

Both pMV11 and pGMpelB harbours the *gm-csf* gene, and produced GM-CSF protein (19.7 kDa) can detected from the strains harbouring these plasmids (Figure 17). The pGMpelB plasmid is therefore considered to function in RV308 cells.

The concentration of Phox reference (20 mg/l) is too high to make comparisons with the detected bands and should be diluted for further use.

3.2.2 Growth and production experiment of RV308 (pIFNpelB)in shake flasks

Since production of IFN-α2b from the pIFNpelB in RV308 was negative in the deep well experiment (Figure 17), it was decided to run a new experiment with this strain to validate the result. It was chosen to increase the culture volume and perform the experiment in shake flasks. In addition, the culture medium was changed from reduced HiYe medium to another version called HiYe reduced broth and the additional induction solution (see Attachment D for details).

RV308 (pIFNpelB) was grown in 50 ml selective LB overnight at 37°C (200 rpm). 2 % (v/v) from precultures (Attachment F Table F- 1) were used to inoculate two parallels of 35 ml HiYe reduced broth. The strains were grown for 16 hours before 15 ml of the induction solution was added to each culture. One of the parallels was induced to a final concentration of 0.5 mM m-toluic acid. After 5 hours incubation, samples for Western Blot analysis were collected.

3.2.2.1 Protein production

1.8 ml culture samples were prepared following the protocol "Cell lysis protocol 1" (Chapter 2.4.10.1). Western Blot was performed with anti-c-myc as primary antibody (Chapter 2.4.10.2 and 2.4.10.3).

Results from Western Blot showed no bands at expected sizes (data not shown). This might indicate that RV308 has trouble producing the protein. As stated in Chapter 1.3, there has previously been detected production of IFN-α2b from this strain, but the amounts detected have been lower than for GM-CSF. Since the goal for this study is not to evaluate *E. coli* as producer, but to find an alternative expression platform, there is no requirement that *E. coli* has produced the proteins from the constructed plasmids.

3.3 Evaluation of P. fluorescens SBW25 strain as expression platform for pIFNpelB and pGMpelB

For this study, the production potential of two different *Pseudomonas* species was evaluated. Because *P. fluorescens* SBW25 has proven to function as a recombinant protein producer (Chapter 1.2.3.2), this was one of strains evaluated.

Wild type copy number plasmids (pIFNpelB and pGMpelB) were transferred to competent cells of the *E. coli* strain S17.1 (Chapter 2.4.8.1). Conjugation between SBW25 and S17.1 (pIFNpelB) and S17.1 (pGMpelB), were performed according to the conjugation procedure described in Chapter 2.4.8.3 to construct SBW25 (pIFNpelB) and SBW25 (pGMpelB). Dilutions were plated out on selective PIA-agar to isolate *Pseudomonas* species only. One colony of each strain was transferred to 10 ml selective LB medium and grown overnight (30°C, 200 rpm) and frozen in glycerol stocks according to the protocols (Chapter 2.4.1).

3.3.1 Cultivation in deep well plates

It was chosen to evaluate SBW25 (pIFNpelB) and SBW25 (pGMpelB) under the same conditions as for RV308 in Chapter 3.2.1 to compare their production of recombinant proteins.

Precultures of SBW25 (pIFNpelB) and SBW25 (pGMpelB) were grown in 50 ml LB medium in 250 ml shake flasks with baffles overnight at 25˚C (200 rpm). The reason for choosing this temperature (and not 30˚C) was previously protocols used for cultivations of *Pseudomonas* species as SINTEF Department of Biotechnology. Precultures were treated as RV308-strains in Chapter 3.2.1.

Growth measurements (Attachment F Table F- 2) indicated that none of the SBW25 strains grew enough to reach the desired OD_{600}-values for induction ($OD_{600} \approx 5$). The reason for this lack in growth had to be tested further, but because the strains earlier had shown a relatively low OD_{600} values in the precultures (Attachment F Table F- 1), it was suspected that the strain was not able to maintain the plasmid. If the strain loses its plasmid, the antibiotic kanamycin will kill the bacteria.

3.3.2 Plasmid stability evaluation of *P. fluorescens* strains SBW25 and NCIMB10525

Because of the low growth rate of SBW25 (data not shown), plasmid instability was suspected. To examine this, the plasmid stability of two *P. fluorescens* strains, NCIMB10525 and SBW25 were tested. NCIMB10525 was chosen as a possible alternative to SBW25.

1 % (v/v) from overnight precultures (50 ml LB in 250 ml shake flask with baffles, 25 °C, 200 rpm) of the two strains SBW25 (pHH100) and NCIMB (pHH100) (both borrowed from H. Jørgensen, Post doc. NTNU) were used to inoculating 20 ml of three different medias; LB, PIA (for details see Chapter 2.1) and Hi-10YE-10GLY-2GLU (for details see Attachment D) (all with selection). 600 μl parallels were transferred to deep well plates (Greiner Bio One) and incubated at 25 °C overnight (900 rpm).

The cultures were plated out on LA without selection and incubated at 30 °C for 2 days. 75 colonies were transferred to LA with selection and incubated at the same conditions. For both strains, $0 - 13.3$ % of the colonies was able to grown on the selective media (Attachment F Table F- 3). This result shows low plasmid stability indicating that they would not be suitable for recombinant protein production without any genetic modifications of stability elements on the expression plasmids. It also reveals that the lack of growth of SBW25 strains in the deep well experiment was due to low plasmid stability. Since the focus of this study was expression of proteins, and not increasing plasmid stability, it was chosen test other *Pseudomonas* strains. *P. fluorescens* was therefore not used further in this study.

Results and discussion

3.4 Evaluation of P. putida KT2440 as expression platform for wild type copy number vectors

Based on the poor plasmid stability of *P. fluorescens* strains (Chapter 3.3.2) it was decided to evaluate an alternative host form the *Pseudomonas* family. The *P. putida* strain KT2440 was chosen to be the new candidate.

pIFNpelB and pGMpelB were transferred to KT2440 through electro transformation according to the protocol (Chapter 2.4.8.2).

3.4.1 Plasmid stability evaluation of KT2440

SBW25 and NCIMB10525 plasmid instability (Chapter 3.3.2), made it necessary to evaluate the plasmid stability in KT2440 before any analysis of production of recombinant proteins.

1 % (v/v) from overnight precultures of the two strains KT2440 (pHH100) (borrowed from H. Jørgensen, Post doc. NTNU) and KT2440 (pIFNpelB) were used for inoculating 20 ml of the same three media as in Chapter 3.3.2; LB, PIA and Hi-10YE-10GLY-2GLU (all with selection). 600 μl parallels were transferred to deep well plates (Greiner Bio One) and incubated at $25\degree$C overnight (900 rpm). KT2440 (pIFNpelB) was induced with 0.5 mM m-toluic acid at $OD_{600} \approx 1$. Plasmid stability was testes using the same approach as for SBW25 and NCIMB10525 (Chapter 3.3.2).

The plasmid stability in KT2440 (pHH100) was calculated to 70 – 100 % (Attachment F Table F- 3). Since an essential feature of the production strain is to be able maintain the plasmids constructed for this paper, the induced culture of KT2440 (pIFNpelB) were tested for plasmid stability as well. The results here were 100 % stability (Attachment F Table F- 3). This indicated that the *P. putida* strain KT2440 was a good candidate for functioning as expression platform.

3.4.2 Plasmid stability of KT2440 over 20 generations

To ensure that the stability of plasmid in KT2440 was high over several generations, plasmid stability was tested during growth for 20 generations in PIA and M9 media (for details see Chapter 2.1). From this experiment and forward, the incubation temperature was changed to 30°C.

Results and discussion

Precultures of KT2440 (pIFNpelB) was grown in 50 ml selective LB medium overnight at 30°C, 200 rpm. 50 ml of PIA- and M9 medium were inoculated to an OD_{600} value of 0.01 and 0.1 respectively. After reaching OD_{600} values above 1, plasmid stability samples were taken and the cultures were used to inoculate a new shake flask with the same medium to the same start cell density. This was repeated until at least 20 generations (one doubling in OD_{600} equals one generation) had been grown in each media. Plasmid stability tests were performed as before (see Chapter 3.3.2). The results are shown in Table 3 and Table 4.

Table 3: Stability of pIFNpelB in KT2440 over 20 generations (grown in LB medium at 30°C)

Plasmid stability in PIA medium	Inoculated to OD_{600}	OD_{600} after incubation	Number of generations	Plasmid stability[1]
Shake flask 1	0,01	2	8	76 %
Shake flask 2	0,01	2,1	8	81 %
Shake flask 3	0,01	2,1	8	77 %
Total number of generations		24		

[1]**Calculated as % of colonies able to grow in a selective medium after cultivation in unselective medium**

Table 4: Stability of pIFNpelB in KT2440 over 20 generations (grown in M9 medium at 30°C)

Plasmid stability in M9 medium	Induced to OD_{600}	OD after incubation	Number of generations	Plasmid stability[1]
Shake flask 1	0,10	1,80	5	81 %
Shake flask 2	0,10	7,00	7	80 %
Shake flask 3	0,10	5,90	6	81 %
Shake flask 4	0,10	7,60	7	84 %
Total number of generations		25		

[1]**Calculated as % of colonies able to grow in a selective medium after cultivation in unselective medium**

The plasmid stability over more than 20 generations is relatively high ranging from 76 – 81 % in LB medium (Table 3) and 80 – 84 % in M9 medium (Table 4). The reason for this constant plasmid stability over several generations (in both media) is not known. One possible reason may be the strains viability when transferring colonies. Since the stability did not decrease, the strain was interpreted as stable, both in rich (LB) and minimal medium (M9).

3.4.3 Growth and production experiment of KT2440 (pGMpelB) and KT2440 in shake flasks

Plasmid stability test had shown that the *P. putida* strain KT2440 was able to maintain the plasmid in rich and minimal medium for over 20 generations (Chapter 3.4.2, Table 3 and Table 4). This made interesting to evaluate the production of the model proteins from the genes inserted in the vector.

At the time this experiment was planned, electro transformation of pIFNpelB to KT2440 had proven to be difficult to complete. It was therefore chosen to test the production of KT2440 (pGMpelB) in the following experiment. Although the results from this experiment would not be directly transferable to KT2440 (pIFNpelB), it could be an indication. The purpose of the test was to examine the growth preferences of this strain. This would give valuable information about preferable conditions. KT2440 was included for comparisons.

Precultures of KT2440 and KT2440 (pGMpelB) were grown in 50 ml LB (250 ml shake flasks with baffles, selection for KT2440 (pGMpelB)) at 30°C for 16 hours. 50 ml of five different media (PIA-glucose, M9 and Hi-10Ye-10X* with glucose, fructose or glycerol as carbon source (see Chapter 2.1 and Attachment D for details)) was inoculated with 2 % (v/v) from precultures (Attachment F Table F- 4). After 4 hours of incubation at 30°C 200 rpm, all four cultures containing KT2440 (pGMpelB) were split into two bottles. 20 ml was transferred to a new 250 ml sterile shake flask, while the rest (approximately 30 ml) remained in the original bottle. The 20 ml cultures were induced with m-toluic acid to a final concentration of 0.5 mM. Growth and pH measurements were taken regularly throughout the experiment.

3.4.3.1 Growth measurements

The growth (OD_{660}) measurements of KT2440 are shown in Figure 18 and KT2440 (pGMpelB) in Figure 19.

Results and discussion
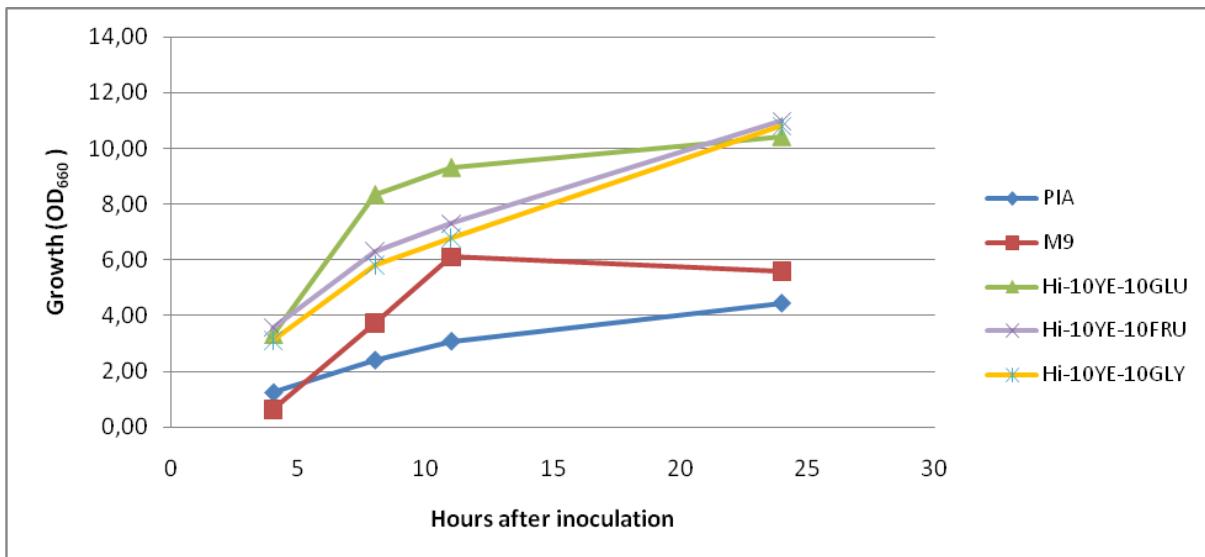
Figure 18: Growth (OD$_{660}$) measurements of KT2440 cultures in different media (indicated on the right side of the graphs) during shake flask growth experiment at 30°C, 200 rpm. For details see Attachment F Table F- 5.

The wild type strain of KT2440 displayed the best growth in the different versions of Hi-10YE-10X medium (Figure 18). All three types of Hi-10YE-10X versions, gave approximately the same cell yield after 24 hours incubation. Compared with each other, the culture grown in Hi-10YE-10GLU showed higher growth rate in the initial phase of the experiment, indicating that glucose may be the preferred carbon source.

pH measurements (Attachment F Table F- 6) showed that cultures grown in M9 and Hi-10YE-10FRU decreased in pH eight hour after inoculation and stayed low compared with the three other media. PIA, Hi-10YE-10GLU and Hi-10YE-10GLY media had pH values of approximately 7.5 at the end of the experiment indicating that substrate had run out. Since pH was only measured for possible evaluation of trends in the different media, no adjustments were done.

Results and discussion
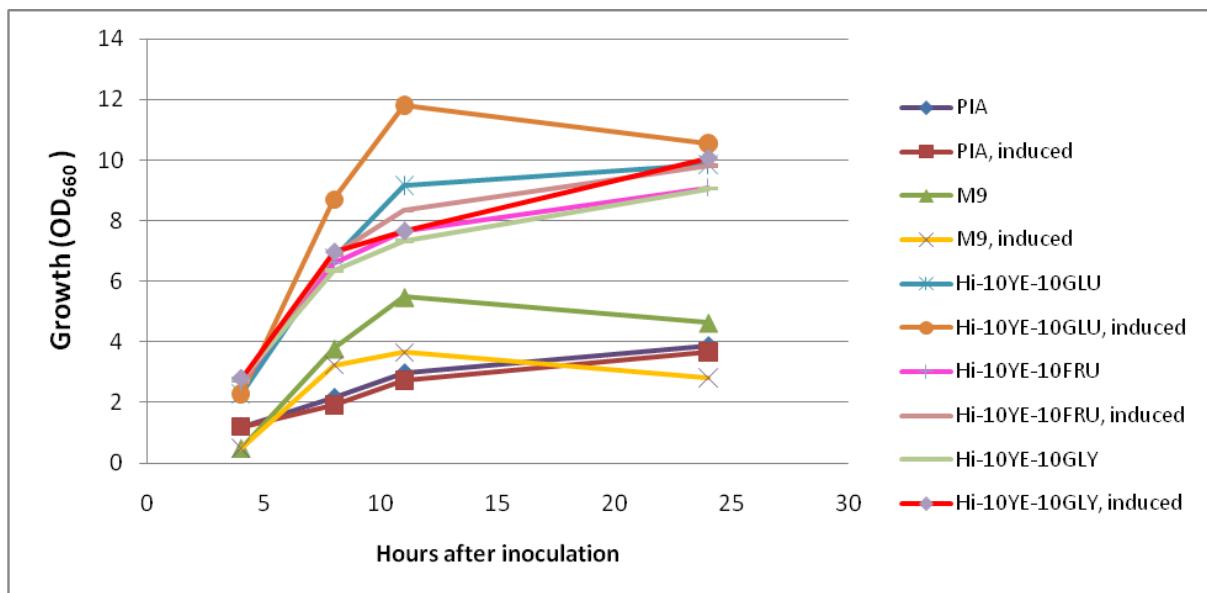
Figure 19: Growth (OD$_{660}$) measurements from KT2440 (pGMpelB) cultures in different media (indicated on the right side of the graphs) during shake flask growth experiment at 30°C, 200 rpm. Parallels were induced with m-toluic acid to a final concentration of 0.5 mM four hours after inoculation. For details see Attachment F Table F- 7

In the growth measurement of KT2440 (pGmpelB) shown in Figure 19, the two PIA cultures (induced and non-induced) show no difference in growth. During most of the experiment, these had the lowest growth rate and were not able to reach the same cell yield after 24 hours. This was experienced with KT2440 as well (Figure 18) indicating that PIA not is the optimal medium for growth.

KT2440 (pGMpelB) grown in M9 medium, induced and non-induced, grew until 11 hours (Figure 19) where medium seemed to be outgrown. The strain grown in Hi-10YE-10GLU had, compared to the non-induced strain, a high growth rate. Also here, growth stopped after 11 hours, probably from outgrowing the medium. HiYe-cultures with the two other carbon sources (fructose or glycerol) showed no significance difference from each other. Based on these observations, Hi-10YE-10GLU seems like the best cultivation medium for optimal growth rate and cell yield.

Both of the PIA cultures had low pH (under 5.5) (Attachment F Table F- 8) after 11 hours. After 24 hours the non-induced PIA culture had a relative high value (over 7). The M9 – cultures had pH values under 7, while all of the HiYe cultures staid at approximately 7.5. Since the optimal pH value for maximal effect of the inducer has been shown to be 6.8

Results and discussion

(Chapter 1.2.4.1.1), it is possible that the inducer did not exploited its potential in Hi-10YE-10X cultures.

3.4.3.2 Plasmid stability of KT2440 (pGMpelB)

To ensure that KT2440 also was able to maintain pGMpelB, this plasmids stability was tested. It was expected that the stability was high based on previous results obtained with pHH100 and pIFNpelB (Chapter 3.4.1 and 3.4.2).

Induced cultures of KT2440 (pGMpelB) grown in PIA, M9 and HiYe was plated out on LA plates without selection and grown at 30°C overnight. 100 colonies were transferred to LA with selection and incubated under the same conditions. Plasmid stability was calculated as the % of colonies able to grow on selective LA after cultivation on LA without selection. The results are shown in Table 5.

Table 5: Results of plasmid stability examination from KT2440 (pGMpelB) grown in PIA, M9 and Hi-10YE-10GLU. The cultures were plated out on LA plates without selection before single colonies were transferred to selective LA plates.

Strain	Medium (with selection)	Plasmid stability[1]
KT2440 (pGMpelB)	PIA	100 %
	M9	100 %
	Hi-10YE-10GLU	100 %

[1]**Calculated as % of colonies able to grow in a selective medium after cultivation in unselective medium**

The plasmid stability was high (Table 5), as expected from the result in Chapter 3.3.2 where KT2440 (pIFNpelB) were examined. The strain KT2440 was therefore regarded as able to stably maintain the relevant plasmids.

3.4.3.3 Protein production

1.8 ml culture samples were prepared for SDS-PAGE following the protocol "Cell lysis protocol 1" (Chapter 2.4.10.1). Western Blot was performed with anti-c-myc as primary antibody (Chapter 2.4.10.3), but no recombinant proteins could be detected (results not shown). Since plasmid stability is high (Table 5), there is no obvious reason why production is absent. The plasmid pGMpelB have been proven to function in RV308, but no recombinant proteins have been detected in *P. putida* or *P. fluorescens* strains.

## 3.4.4 Growth and production experiment of KT2440, KT2440 (pIFNpelB) and KT2440 (pGMpelB) in shake flask

The lack of detected protein production during the shake flask experiment with KT2440 (pGMpelB) and KT2440 in Chapter 3.4.3 was not expected. To ensure that no technical errors were the reason, an additional growth and production experiment was performed. This time, two different media were included in the experiment; HiYe-8GLY-2GLU (for details see Attachment D) and M9-10GLU (for details se Chapter 2.1). Glucose was selected as carbon source because of better growth compared to the two other (fructose and glycerol) in the shake flask experiment in Chapter 3.4.3. PIA medium was eliminated because of previously obtained low growth rate and cell yields in the same experiment (Chapter 3.4.3.1, Figure 18 and Figure 19).

Precultures of KT2440, KT2440 (pIFNpelB) and KT2440 (pGMpelB) were grown in 50 ml selective LB (250 ml shake flask with baffles) at 30°C overnight (16 hours). 100 ml medium (HiYe-8GLY-2GLU and M9-10GLU) in 500 ml shake flasks with baffles was inoculated 2 % (v/v) from precultures (Attachment F, Table F- 9) and incubated at 30°C and 200 rpm. Samples for growth (OD_{600}) and pH measurements were taken throughout the experiment. Cultures were induced 8.5 hours after inoculation by adding 100 µl 500 mM m-toluic acid (final concentration 0.5 mM). pH was adjusted if measured above 7.00 to ensure acidic conditions for the inducer. All of the cultures were grown with a non-induced parallel for later comparison of production.

3.4.4.1 Growth measurements

The results for growth in M9-10GLU and HiYe-8GLY-2GLU are shown in Figure 20 and Figure 21 respectively.

Results and discussion
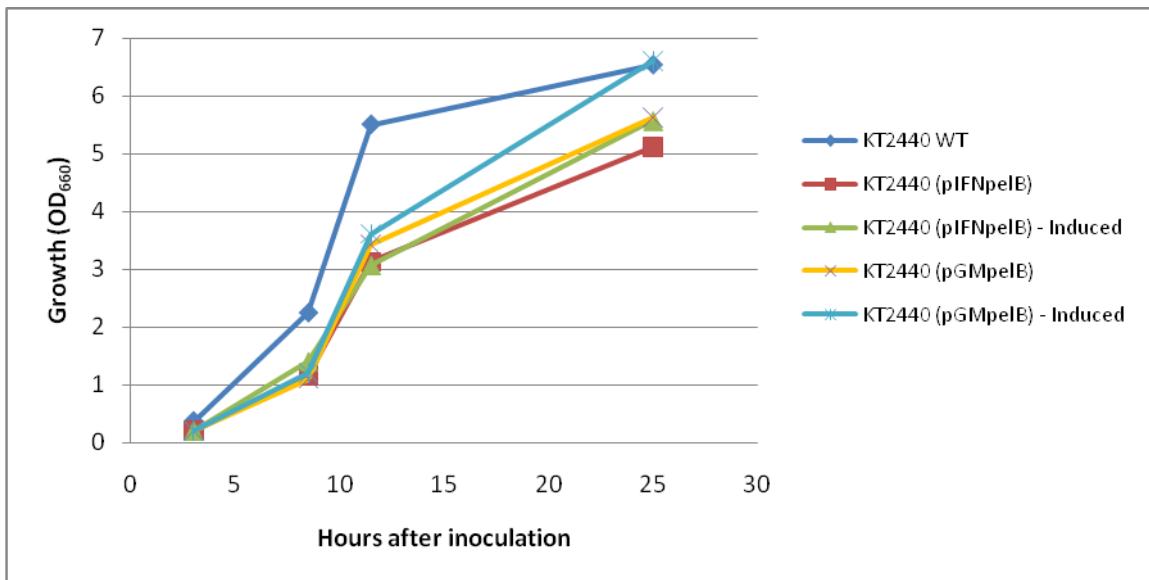
Figure 20: Growth (OD$_{660}$) measurements from KT2440 strains (indicated in the right column) grown in M9-10GLU medium at 30°C. Induction of the strains marked "induced" was performed 8.5 hours after inoculation to a final concentration of 0.5 mM m-toluic acid. For details see Attachment F Table F- 10.
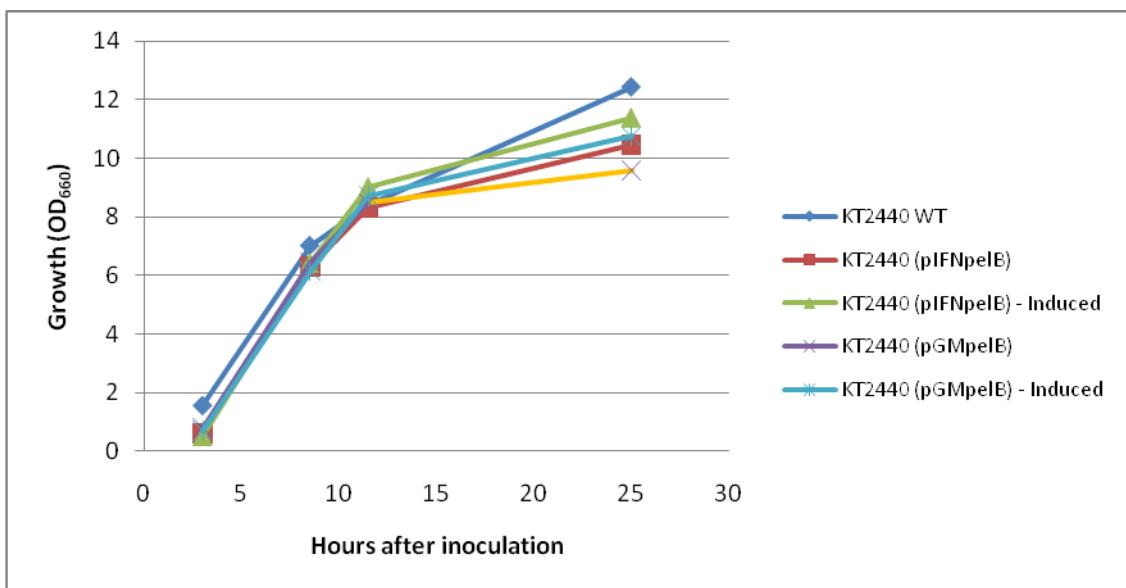
Figure 21: Growth (OD$_{660}$) measurements from strains (indicated in right column) grown in HiYe-8GLY-2GLU medium at 30°C. Induction of the strains marked "induced" was done 8.5 hours after inoculation to a final concentration of 0.5 mM m-toluic acid. For details see Attachment F Table F- 12

In M9-10GLU, KT2440 had the highest initial growth rate (Figure 20), but gave approximately the same cell yield at the end of the experiment. None of the other strains stood out during the growth experiment.

pH values decreased throughout the experiments for cultures in M9-10GLU and was therefore not adjusted (Attachment F, Table F- 11). One adjustment for cultures in HiYe-8GLY-2GLU had to be made 8.5 hours after inoculation (right before induction) to ensure optimal conditions for the inducer (Attachment F, Table F- 13).

3.4.4.2 Protein production

50 ml of cultures grown for 25 hours (Chapter 3.4.4) were centrifuged at 4000 rpm for 10 minutes (Eppendorf). 100 mg wet weight cells were transferred to 1.5 ml tubes (Eppendorf) by diluting them in 0.9 % NaCl. NaCl was then removed by centrifuging for 8 minutes at 12 000 rpm. Samples were kept at $-20°C$. This preparation would enable direct comparison of the different samples as they all contained the same amount of cells (100 mg). 100 mg cells were lysed using "Cell lysis protocol 1" (Chapter 2.4.10.1). Both of the Western blot protocols were performed (Chapter 2.4.10.2 and 2.4.10.3), but only detection with Tetra His as primary antibody gave results. The results are given in Figure 22 and Figure 23.
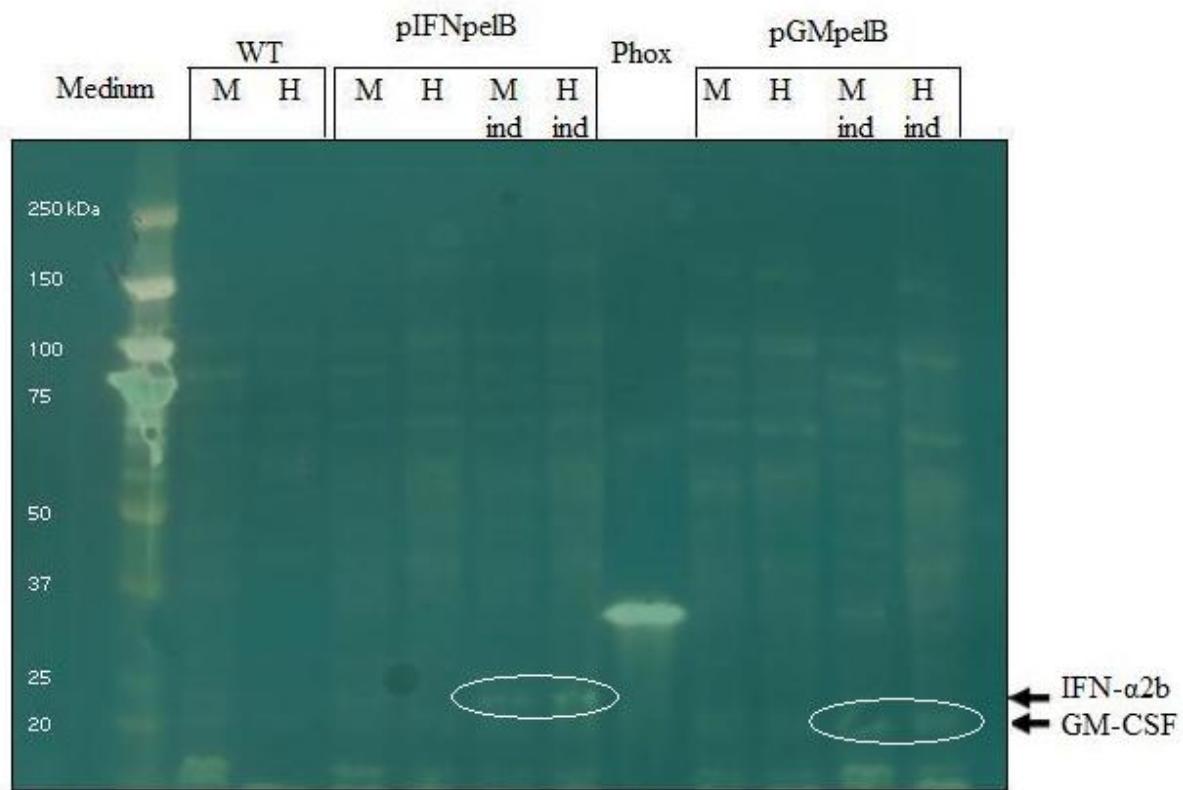
Figure 22: Western Blot results of soluble fractions from KT2440 strains (plasmids indicated in top row). The cultures were grown in 100 ml M9-10GLU (indicated with "M") or HiYe-2GLU-8GLY (indicated with "H") medium at 30°C. "Ind" marks the cultures which were induced with m-toluic acid to a final concentration of 0.5 mM. The concentration of the Phox protein is 4 mg/l. Numbers on the left side represents molecule sizes in kilo Daltons

By comparing KT2440 and non-induced cultures with induced strains, the effect of induction can be observed. It was expected to find the IFN-α2b protein, which KT2440 (pIFNpelB) is designed to produce, at 23.7 kDa. In the soluble fractions at this size (Figure 22), the wild type strain and non-induced strains have no bands. The induced KT2440 (pIFNpelB) strain on the other hand, has one band estimated to be this size. This is observed in both media, and implies that the induction of the *Pm*-promoter has been successful. The bands are significant stronger than what is seen for non-induced cultures and for the wild type.

Results and discussion
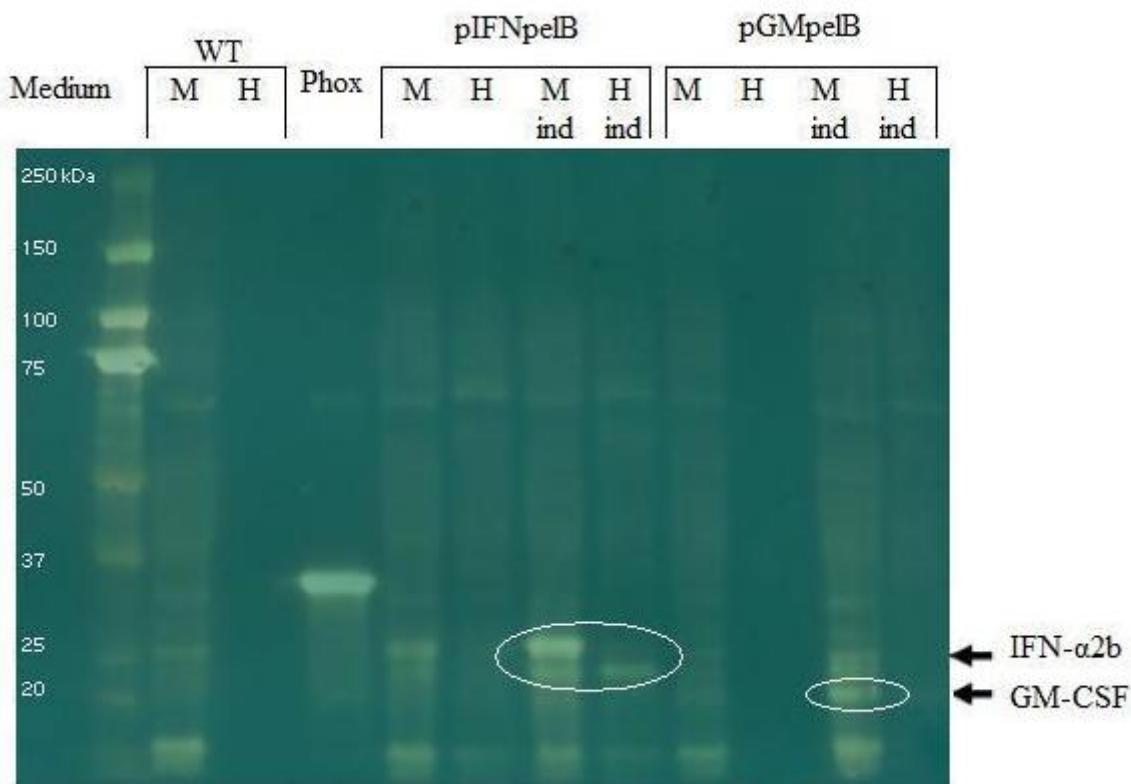
Figure 23: Western Blot results of insoluble fractions from KT2440 strains (plasmids indicated in top row). The cultures were grown in 100 ml M9-10GLU (indicated with "M") or HiYe-2GLU-8GLY (indicated with "H") medium at 30 ̊C. "Ind" marks the cultures which were induced with m-toluic acid to a final concentration of 0.5 mM. Wells marked "WT H", "pGMpelB H" and "pGMpelB H ind" are empty (transferring of samples were impossible due to high viscosity). The concentration of the Phox protein is 4 mg/l. Numbers on the left side represents molecule sizes in kilo Daltons

Only one of the wild type insoluble fractions was possible to apply to the gel. The same problem was experienced with insoluble fractions of KT2440 (pGMpelB) grown in HiYe-2GLU-8GLY. The viscosity of these samples is possibly an indication that DNA has not been completely digested with DNaseI from "Cell lysis protocol 1" (Chapter 2.4.10.1).

The insoluble fractions of induced KT2440 (pIFNpelB) grown in M9-10GLU have two bands at approximately 25 kDa (Figure 23). This is probably due to cleavage of pelB-polypeptide as explained in Chapter 1.2.4. IFN-α2b proteins with and without pelB-sequences will both be detected causing the formation of two bands. Comparison of the size of IFN-α2b without pelB (21.5 kDa) agrees with the smaller band observed. Later detection of these bands will be assumed to have the same possible cause. KT2440 and non-induced strain have also detectable band at the largest size, but these bands are weaker compared to the induced.

Results and discussion

Comparison of induced KT2440 (pIFNpelB) grown in HiYe-2GLU-8GLY with non-induced and KT2440 (Figure 23), leads to detection of one IFN-α2b band. The size of this protein also agrees with the size of IFN-α2b without pelB-polypeptide. Comparison of product yield in the two growth media points towards M9-10GLU being the best suited for production by KT2440 (pIFNpelB).

As explained, viscous insoluble fractions of KT2440 (pGMpelB) grown in HiYe-2GLU-8GLY caused them not possible to analyze because of high viscosity. The results from M9-10GLU are therefore the only once discussed for this strain. GM-CSF protein detected from induced culture is found stronger than KT2440 and non-induced KT2440 (pGMpelB) (Figure 23). This shows that induction has enabled expression of GM-CSF. In summary, production of both proteins has been successful, but the quantities are low when compared to band strength of the reference 4μg/ml Phox.

3.4.5 HCD growth and production experiment (fed batch fermentation) of KT2440, KT2440 (pJB861), KT2440 (pIFNpelB) and KT2440 (pGMpelB)

Production of recombinant proteins from pIFNpelB and pGMpelB had been detected in low cell density experiments (Chapter 3.4.4.2, Figure 22 and Figure 23). For evaluation of KT2440s potential as expression host in high cell density cultivations, fed batch fermentations were performed.

Fed batch fermentations of KT2440, KT2440 (pJB861), KT2440 (pIFNpelB) and KT2440 (pGMpelB) was performed following "Production of proteins in HCDC (fermentation)", here in 1-l fermentors (Chapter 2.4.9). pJB861 was used as a reference based on the fact that it is an expression vector without any of the genes of interest (*ifn-α2b* or *gm-csf*), a empty vector. KT2440 was grown in media with no antibiotics.

3.4.5.1 Growth and respiration measurements

Figure 24 shows the growth for KT2440, KT2440 (pJB861), KT2440 (pIFNpelB) and KT2440 (pGMpelB) after inoculation from precultures in LB medium (Attachment F, Table F- 14).

Results and discussion
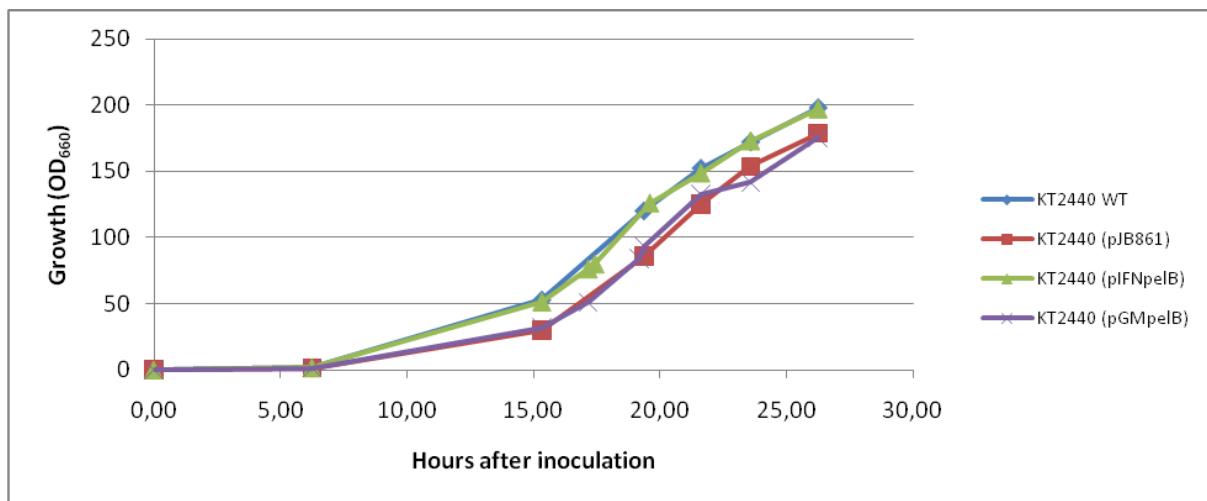
Figure 24: Growth (OD$_{660}$) during fed batch fermentation of KT2440 WT, KT2440 (pJB861), KT2440 (pIFNpelB) and KT2440 (pGMpelB) in Hf.1-medium. Cultures were induced at OD$_{660}$≈ 80-90 to a final concentration of 1 mM m-toluic acid. For details see Attachment F, Table F- 15

The growth curves for KT2440 and KT2440 (pIFNpelB) are somewhat delayed compared to KT2440 (pJB861) and KT2440 (pGMpelB) throughout the experiment (Figure 24). The highest cell densities were obtained with KT2440 and KT2440 (pIFNpelB) reaching OD$_{660}$ of 197.8 and 196.7 respectively 26.25 hours after inoculation. These are similar to what was obtained for RV308-strains in Sletta et al, 2007. The feature of reaching high cell yields in relatively short time is essential when developing a new expression platform. If the production system is successful this will enable higher yield of the recombinant proteins compared to expression platforms only able to reach low cell densities in the same time period.

The specific growth rate (μ) and doubling time for *P. putida* KT2440 can be calculated using equation (1) in Chapter 1.2.5. By using measurements from the phase of free growth, μ was calculated to be 0.53 h^{-1} and 0.58 h^{-1}witch corresponds to a doubling time of 1.20 - 1.31 h (Attachment F Table F- 16). Under the same conditions, the growth rate and doubling time for *E. coli* has been reported in Sletta et al. 2004 to be approximately the same as found here for KT2440.

Respiration curves from the fermentation are shown in Figure 25.

Results and discussion
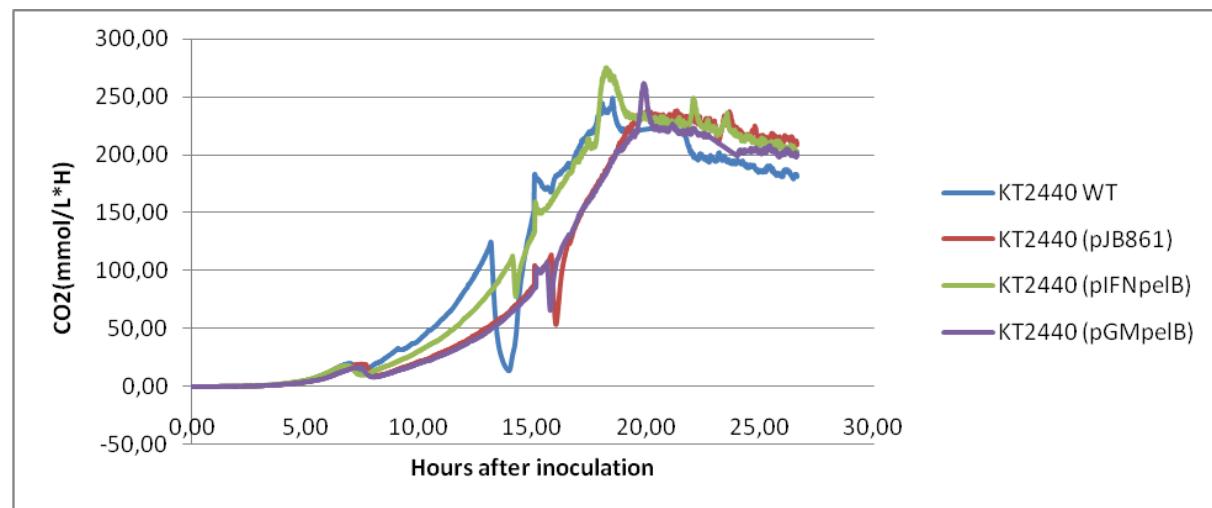
Figure 25: Respiration curve (mmol/L ·H CO_2) from fermentation of KT2440 WT, KT2440 (pJB861), KT2440 (pIFNpelB) and KT2440 (pGMpelB) in Hf.1-medium with selection at 30°C and pH 6.5.

For KT2440 and KT2440 (pIFNpelB), the glucose added form start was utilized after approximately 13 hours, seen as a drop in CO_2 generation (Figure 25). Feeding was started manually leading to the following rapid increase in respiration. For KT2440 (pJB861) and KT2440 (pGMpelB), the glucose metabolism was slower. Feeding was for these two started after 15 hours. Respiration increases evenly for all strains until the induction time.

KT2440 (pIFNpelB) and KT2440 (pGMpelB) was induced 17.4 and 19.4 hours after inoculation respectively. For both strains, it is observed an increase in CO_2 generation directly after induction. This indicates that KT2440 is able to consume the inducer, something that could be expected from the knowledge of this strains origin, toluene degrading *P. putida* mt-2 (Chapter 1.2.3.2.1). To determine if this was the case, the amount of inducer consumed or diffused into the cells was tried quantified in HPLC analysis performed by SINTEF Department of Biotechnology after completed fermentation. It turned out that the setup for this method was not suited for detection of m-toluic acid, resulting in unsuccessful analysis (data not shown). It can therefore only be assumed that metabolism of the inducer the cause in respiration at induction.

3.4.5.2 Plasmid stability

KT2440 have been shown to have high plasmid stability during low cell density cultivations in shake flasks (Attachment F Table F- 3, Chapter 3.4.2 Table 3 and Table 4 and Chapter

3.4.3.2 Table 5). To validate that this ability is intact through high cell densities, plasmid stability was evaluated from samples taken late in the fed batch fermentation (after induction).

0.16 ml sterile glycerol (Sigma) was added to 0.8 ml culture samples before keeping the mixture at − 80°C. The mixture was plated out on LA without selection and incubated overnight (30°C) before transferring 100 colonies to LA with selection. After incubating overnight at the same temperature, plasmid stability was calculated. The result is shown in Table 6.

Table 6: Plasmid stability evaluation of KT2440 (pJB861), KT2440 (pIFNpelB) and KT2440 (pGMpelB) during HCD fed batch fermentation.

Strain	Number of colonies picked	Plasmid Stability[1]
KT2440 (pJB861)	100	92 %
KT2440 (pIFNpelB)	100	100 %
KT2440 (pGMpelB)	100	100 %

[1]Calculated as % of colonies able to grow in a selective medium after cultivation in unselective medium

Plasmid stability results (Table 6) indicate that KT2440 is able to carry plasmids also during HCDC.

3.4.5.3 Protein production

Samples were harvested for SDS-PAGE at induction and the following two, four, six and eight hours. The samples were named P0, P1, P2, P3 and P4 respectively and prepared following "Detection and quantification of recombinant protein" (Chapter 2.4.10). Also samples from induced KT2440 (pIFNpelB) and KT2440 (pGMpelB) grown in M9-10GLU and HiYe-2GLU-8GLY from shake flask growth experiment in Chapter 3.4.4, were prepared for comparison.

The pellet fraction was prepared by the procedure "Cell lysis protocol 2" because of viscous insoluble fractions when using protocol 1 (Chapter 3.4.4.2). Both soluble and insoluble fractions were analyzed for protein content by both of the Western Blot protocols (Chapter 2.4.10.3). As before, the expected band is 23.7 or 21.5 kDa for IFNα2b (with and without pelB polypeptide) and 19.7 kDa for GM-CSF.

Results and discussion

Western Blot with Anti-myc as primary antibody

Figure 26 shows Western blot results from soluble fraction samples using procedure with anti-c-myc as primary antibody. No bands were detected at the expected sizes, but all of the samples had a band at approximately 50 kDa. Since this band also is detected in the reference cultures KT2440 and KT2440 (pJB861), this is probably a protein produced by the KT2440 strain in general. Later detected proteins with these sizes will not be discussed.
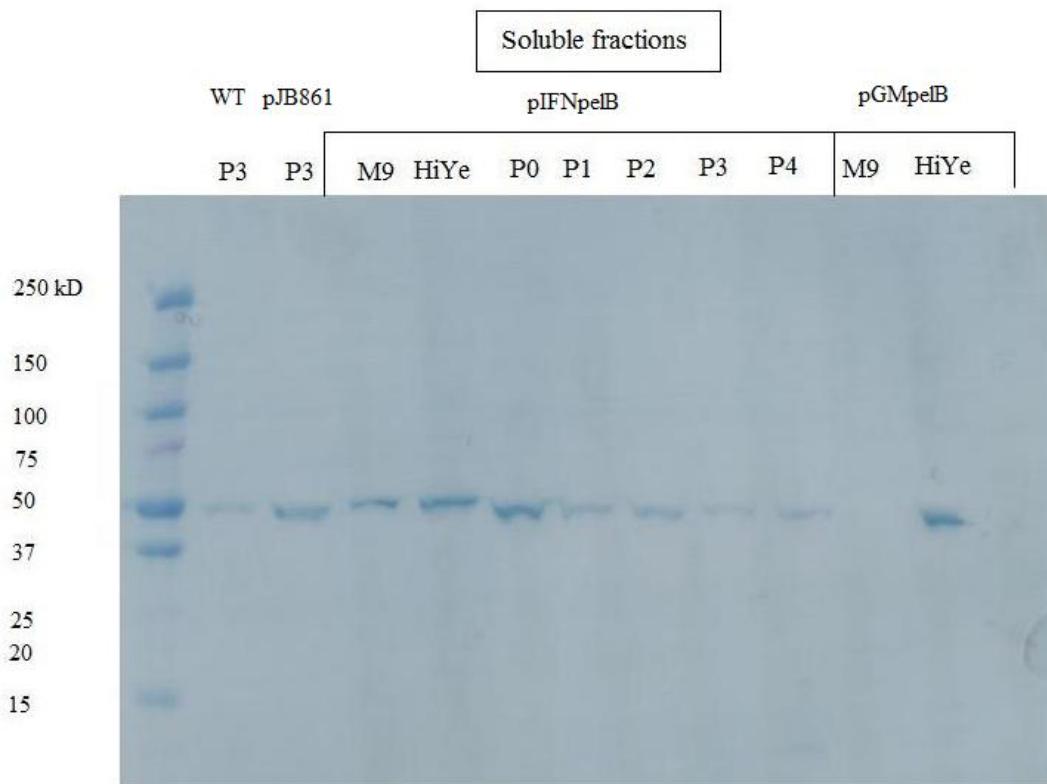
Figure 26: Western Blot results for soluble fractions from 1-l fed batch fermentation of KT2440 strains (second top row) in Hf.1 medium. Samples from induced HiYe-2GLU-8GLY ("HiYe") and M9-10GLU ("M9") cultures from the shake flask experiment in Chapter 3.4.4 were also included. P0-P4 samples from the fed batch fermentation were taken 0, 2, 4, 6 and 8 hours after induction respectively. The numbers on the left side represents molecule sizes in kilo Daltons

Figure 27 shows soluble fractions from KT2440 (pGMpelB) strain and insoluble fractions from reference and pIFNpelB strains.

Results and discussion
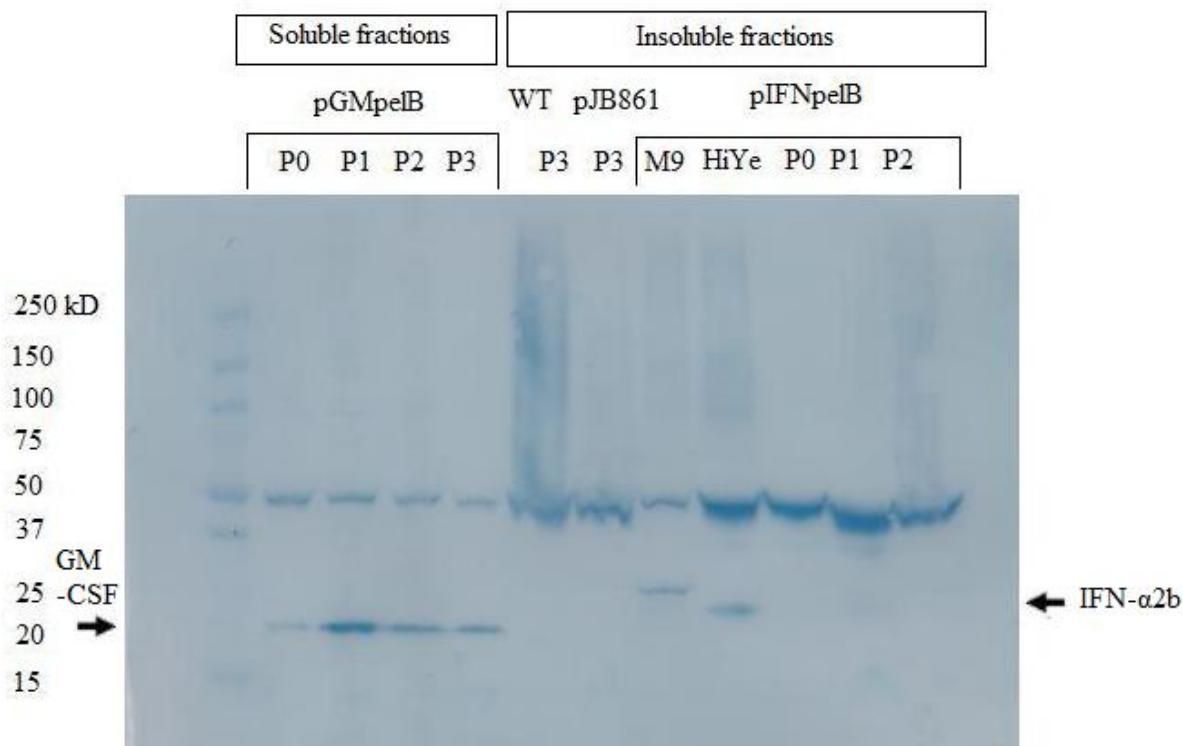
Figure 27: Western Blot results of soluble and insoluble fractions from 1-l fed batch fermentation of KT2440 strains (second top row) in Hf.1 medium. Samples from induced HiYe-2GLU-8GLY ("HiYe") and M9-10GLU ("M9") cultures from the shake flask experiment in Chapter 3.4.4 were also included. P0-P4 samples from the fed batch fermentation were taken 0, 2, 4, 6 and 8 hours after induction respectively. The numbers on the left side represents molecule sizes in kilo Daltons

Recombinant GM-CSF protein is detected at the expected size (19.7 kDa) from KT2440 (pGMpelB) culture (Figure 27). The sample P1 (taken two hours after induction) has the strongest detected amount of the recombinant protein GM-CSF. Since all fermentation samples used for SDS-PAGE preparation had the same initial volume (1 ml), P0 to P4 has an increasing amount of cells. If the quantity of produced protein is proportional to the amount of cells, P2 and P3 are expected to give bands stronger than P1s band. This is not observed. A possible explanation for the decrease in detectable GM-CSF-protein in these samples is instability of protein or leakage into the culture medium. Leakage of protein can be tested by running Western Blot analysis on the supernatant from the culture (Chapter 2.4.10.1). This was performed later in the study (Chapter 3.4.6.2) but gave no detection of recombinant proteins (results not shown). Instability of GM-CSF is therefore thought to be the reason.

Figure 27 show no detected proteins from KT2440 (pIFNpelB) fermentation samples (P0-P2) at the expected size (23.7 kDa). The samples from shake flask experiment reported in Chapter

Results and discussion

3.4.4, on the other hand, have detected protein at different sizes. During the first detection of recombinant protein in these samples (Chapter 3.4.4.2), anti-c-myc protocols (Chapter 2.4.10.3) gave no results (not shown). This may indicate that "Cell lysis protocol 2" enable better protein detection than "Cell lysis protocol 1" (Chapter 2.4.10.1) since the same samples were used in both protocols.

Figure 28 shows recombinant protein detected in insoluble fractions from KT2440 (pIFNpelB) (P0-P2 were shown in Figure 27) and KT2440 (pGMpelB).
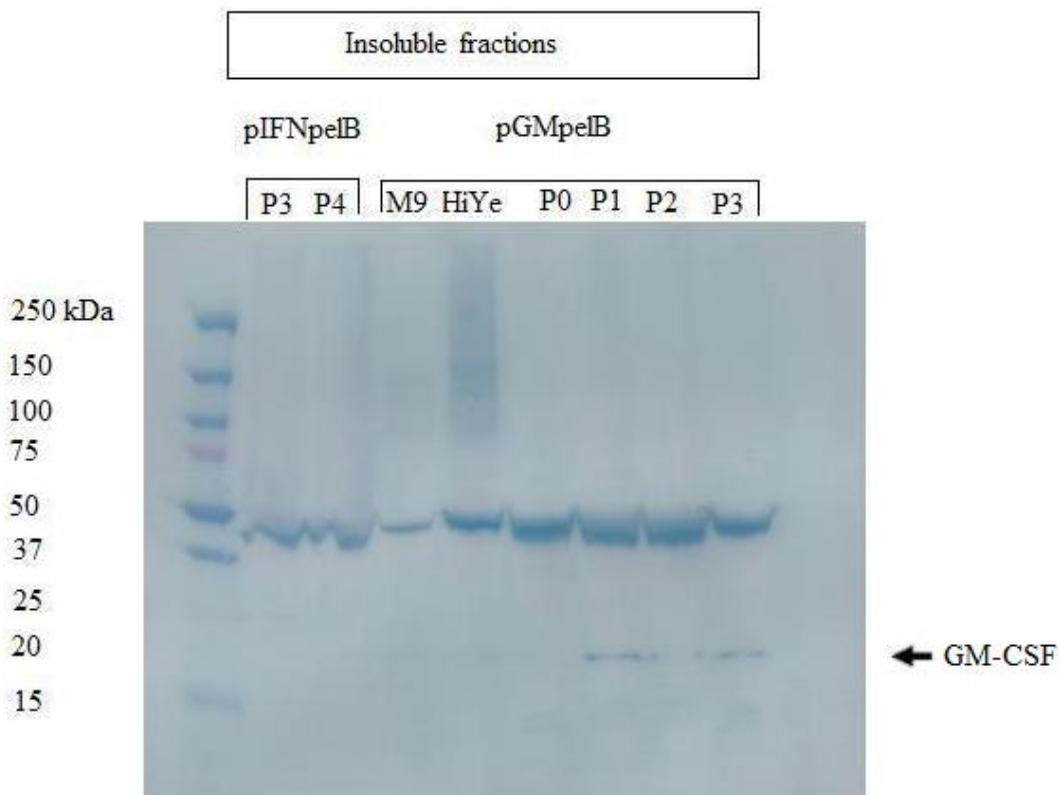
Figure 28: Western Blot results of insoluble fractions from 1-l fed batch fermentation KT2440 strains (second top row) in Hf.1 medium. Samples from induced HiYe-2GLU-8GLY ("HiYe") and M9-10GLU ("M9") cultures from the shake flask experiment in Chapter 3.4.4 were also included. P0-P4 samples from the fed batch fermentation were taken 0, 2, 4, 6 and 8 hours after induction respectively. The numbers on the left side represents molecule sizes in kilo Daltons

KT2440 (pIFNpelB) sample P3 and P4 have no detected protein with expected size (24.7 kDa) (Figure 28). Nor have KT2440 (pGMpelB) samples from experiment described in Chapter 3.4.4 at GM-CSF size (19.7 kDa). Fermentation samples of KT2440 (pGMpelB), on

the other hand, have weak but visible bands. Form this culture, the sample taken 2 hours after induction (P1) has the highest concentration of GM-CSF.

Even though it desirable to only compare samples on the same membrane, one cannot avoid notice the difference in band strength between soluble (Figure 27) and insoluble fractions (Figure 28) from KT2440 (pGMpelB) fermentation samples. As the SDS-PAGE preparation procedure describes (Chapter 2.4.10.1), the insoluble fraction added to the gel is twice as concentrated as the soluble fraction. This shows that KT2440 has a larger quantity of the produced GM-CSF as soluble fraction under the conditions tested.

<u>Western Blot with Tetra His as primary antibody</u>

It was chosen to analyse KT2440 (pIFNpelB) samples from this fed batch fermentations using Tetra His antibody even though anti-c-myc had already been used (previous section). The reason for this was that Tetra His antibody previously had been shown to detect recombinant protein in samples where anti-c-myc did not (Chapter 3.4.4.2). For comparison of detection methods (anti-c-myc versus Tetra-His), three KT2440 (pGMpelB) samples from this fed batch fermentation were also tested. A sample containing Phox protein with a known concentration of 5 mg/l was applied to have the opportunity to estimate the quantity of produced protein. This will also function as an internal standard when comparing membranes. The results from detection with Tetra-His are shown in Figure 29.

Results and discussion
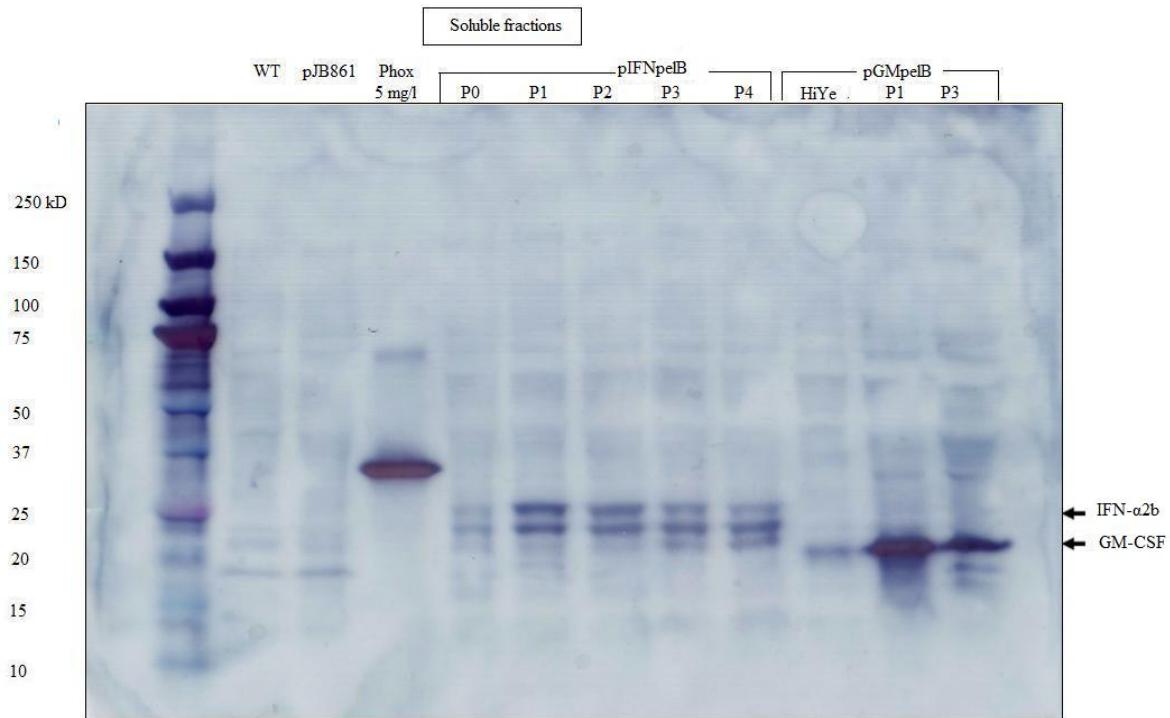
Figure 29: Western Blot results of soluble fractions from 1-l fed batch fermentation of KT2440 strains (second top row) in Hf.1 medium. Samples from induced HiYe-2GLU-8GLY ("HiYe") cultures from the shake flask experiment in Chapter 3.4.4 were also included. P0-P4 samples were taken 0, 2, 4, 6 and 8 hours after induction respectively. Arrows indicated the recombinant proteins detected by Tetra His antibody, and the numbers on the left side represents molecule sizes in kilo Daltons

Figure 29 shows that the soluble fractions from KT2440 (pIFNpelB) has two bands at IFN-α2b's size (21.5- 23.7 kDa). The sample taken at induction (P0) has, compared to the samples taken later, the weakest band. This shows that the induction has effected IFN-α2b production. As for GM-CSF detected in Figure 27, there is a reduced quantity of IFN-α2b later than four hours after induction, indicating protein instability.

As explained, only three of the KT2440 (pGMpelB) samples from the shake flask experiment in Chapter 3.4.4 were part of this run. GM-CSF-production had already been detected from these samples (Figure 27 and Figure 28) and was also detected here (Figure 29). Comparing band strengths from this cultures soluble fractions using anti-c-myc (Figure 27) versus Tetra-His detection (Figure 29) shows big difference, where Tetra-His clearly gives stronger bands. Still, Tetra-His also gives more background noise (more unspecific bands), and detection with anti-c-myc is therefore preferred in this study.

Results and discussion

Comparison between the strength of GM-CSF bands and the strength of Phox (5 mg/l) band (Figure 29) enables estimation of the concentration. The soluble fraction of GM-CSF in the sample taken 2 hours after induction seems to have approximately the strength as the Phox reference. Since 1 ml sample was used to prepare 1 ml soluble fraction (see Protocol in Chapter 2.4.10), the concentration of soluble GM-CSF in the culture is estimated to be 5 mg/l. To estimate this further, both reference and samples need to be diluted, but since the amount detected by Sletta et al. 2007 in *E. coli* was higher (0.39 $\pm$0.03 g/l), this was not performed.

Figure 30 shows insoluble fractions of the same samples shown in Figure 29.
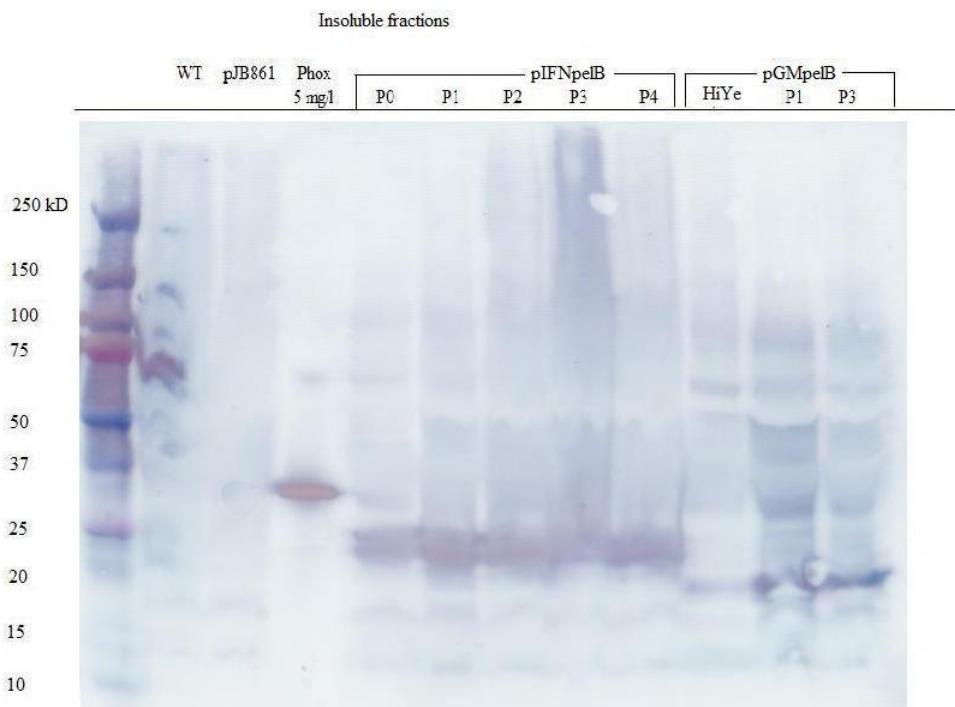
Figure 30: Western Blot results of soluble fractions from 1-l fed batch fermentation KT2440 strains (second top row) in Hf.1 medium. Samples from induced HiYe-2GLU-8GLY ("HiYe") cultures from the shake flask experiment in Chapter 3.4.4 were also included. P0-P4 samples were taken 0, 2, 4, 6 and 8 hours after induction respectively. The numbers on the left side represents molecule sizes in kilo Daltons

The signals from the Western blot were a bit smeared and thus difficult to discuss. Still, there seemed to the same relationships as for soluble fractions.

3.4.6 HCD growth and production experiment (fed batch fermentation) of KT2440 (pGMpelB) with different inducers

The induction effect obtained with m-toluic acid was probably reduced based on the signs that KT2440 has the ability to consume it. By using different inducers of the *Pm/XylS* system, which the strain is reported to not consume, protein production may be increased. The two inducers o-toluic acid and 2-chlorobenzoic acid are chosen to be tested as alternatives to m-toluic acid (Chapter 1.2.4.1.1). Because of more successful production of recombinant proteins from KT2440 (pGMpelB) than KT2440 (pIFNpelB), it was chosen to only examine the effect of the different inducers on KT2440 (pGMpelB). If a positive effect is discovered, one can expect the same effect on the IFNα2b-strain, and then perform the same test to reveal this.

Fed batch fermentations of KT2440 (pGMpelB) was executed as before (Chapter 3.4.5). Four cultures of KT2440 (pGMpelB) were run in total, one induced continually with m-toluic acid by adding this to the feeding solution. The three remaining strains were induced once with m-toluic acid, o-toluic acid or 2-cholorbenzoic acid to a final concentration of 1 mM. The fermentation culture induced with m-toluic acid once will be a repetition of KT2440 (pGMpelB) from the fed batch fermentation in Chapter 3.4.5 and therefore function as a reference to the previous fermentation result.

Induction was performed following the protocol (Chapter 2.4.9.2). Continuous induction was enabled by inducing following the protocol before switch to a 150 ml feeding solution (Attachment E) with m-toluic acid to give a final concentration of 2 mM in the fermentation culture. Samples for Western blot analysis were taken at the time of induction and after two, four and five hours (P0, P1, P2 and P3).

3.4.6.1 Growth and respiration measurements

Growth measurements are shown in Figure 31.

Results and discussion
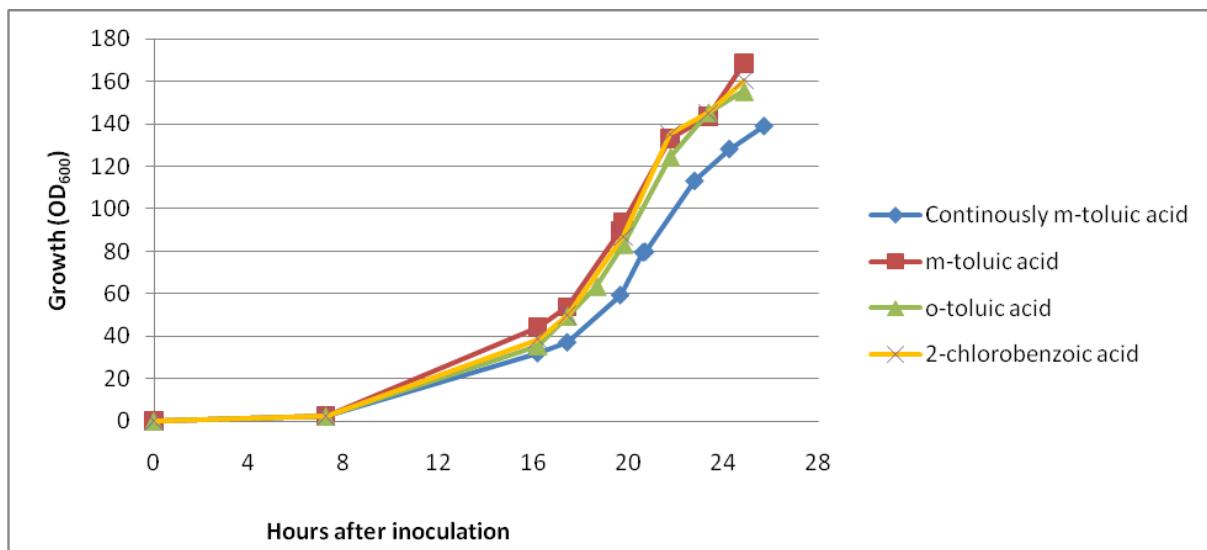
Figure 31: Growth (OD$_{600}$) measurements from 1-l fed batch fermentation of KT2440 (pGMpelB) in Hf.1-medium. Cultures were induced at OD$_{600}\approx$ 80-90. The names to the right indicate which inducer the corresponding graphs had. For details see Attachment F, Table F- 17

In Figure 31, KT2440 (pGMpelB) continuously feed with the inducer shows deviation from the remaining strains with a lower growth rate. Since all cultures consisted of KT2440 (pGMpelB) it was expected to experience similar growth in all cultures. The difference in growth from this strain might therefore be a result of the continuous feeding of inducer.
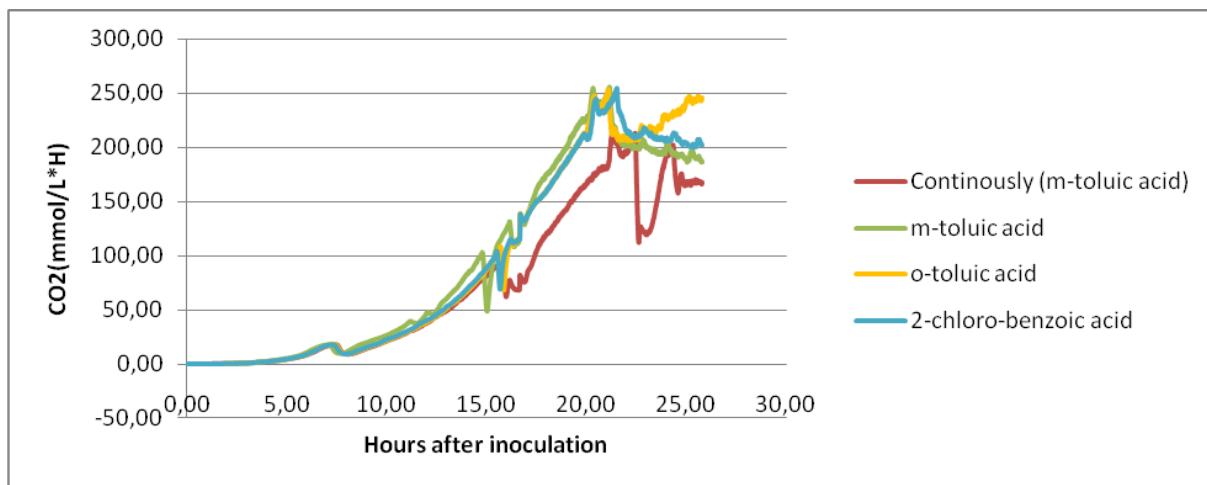
Figure 32: Respiration curve (mmol/L ·H CO$_2$) from fermentation of KT2440 (pGMpelB) in Hf.1-medium with selection at 30°C and pH 6.5. The names to the right indicate which inducer the corresponding graphs had.

KT2440 (pGMpelB) continuously induced has after the feeding start (15.8 hours after inoculation) a lower respiration curve compared to the others (Figure 32). Since respiration

depends on the amount of cells and their consummation of nutrients, the lower cell densities of this strain (Figure 31, 16 – 28 hours after inoculation) may be the explanation.

The remaining cultures of KT2440 (pGMpelB) were induced at the same time (20 hours after inoculation) but with different inducers. For all induced strains there is an observed by an increased CO_2 generation. Since the alternative inducers were not thought to be metabolized by KT2440, it was not expected to see this increase. The result might indicate that KT2440 is able to metabolise both o-toluic acid and 2-chloro-benzoic acid.

3.4.6.2 Protein production

1 ml culture samples were harvested at induction and the following two, four and six hours. The samples were named P0, P1, P2 and P3 respectively and prepared following "Detection and quantification of recombinant protein" (Chapter 2.4.10). The pellet fraction was prepared by the procedure "Cell lysis protocol 2". Supernatant from the cultures (see Chapter 2.4.10.1), soluble and insoluble fractions were analyzed by Western Blot protocols with anti-c-myc as primary antibody (Chapter 2.4.10.3).

The results for recombinant protein production of the two culture of KT2440 (pGMpelB) induced with m-toluic acid are shown in Figure 33. As before, the expected band for GM-CSF is 19.7 kDa.

Results and discussion
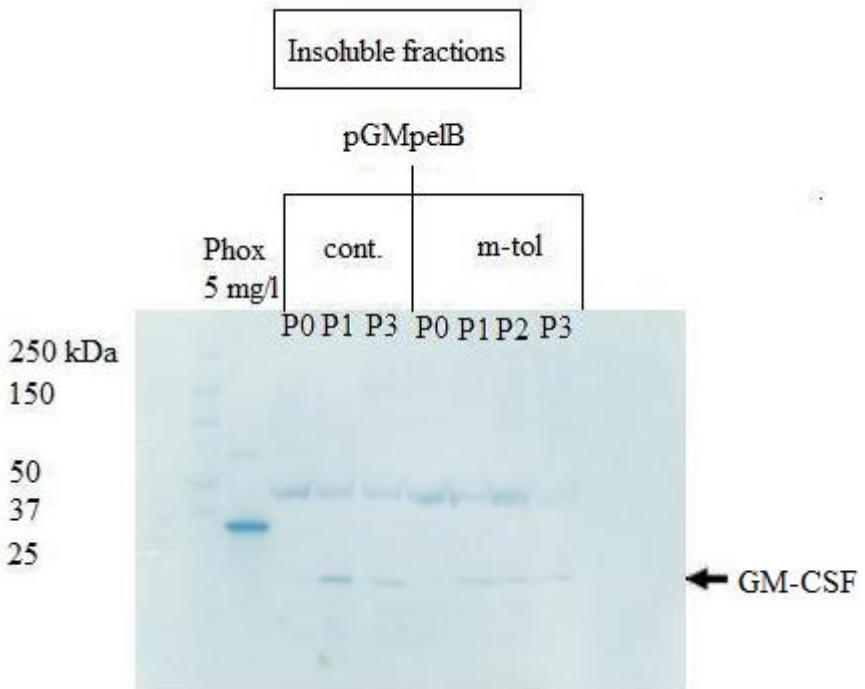
Figure 33: Western Blot results from insoluble fractions from 1-l fed fermentation of KT2440 (pGMpelB) in Hf.1 medium. Samples marked "Cont" were induced continuous while "m-tol" were induced once with m-toluic acid. P0-P3 samples were taken 0, 2, 4 and 6 hours after induction respectively. Arrows indicated the recombinant proteins detected by anti-c-myc antibody, and the numbers on the left side represents molecule sizes in kilo Daltons

No proteins were detected in the supernatant samples (result not shown) indicating that protein does not leak out into the cultivation medium.

KT2440 (pGMpelB) induced with o-toluic acid or 2-chloro benzoic acid had no detectable recombinant proteins in neither soluble nor insoluble fractions (results not shown). In the insoluble fractions of KT2440 (pGMpelB) induced with m-toluic acid (continuously or once) (Figure 33), recombinant GM-CSF protein is detected, but in small quantities compared to the 5 mg/l standard Phox.

KT2440 (pGMpelB) induced with m-toluic acid once is a repetition of the fed batch fermentation of KT2440 (pGMpelB) described in Chapter 3.4.5. Comparison of detected recombinant protein from these runs (insoluble fractions in Figure 28 and Figure 33) show approximately the same amounts of GM-CSF.

Results and discussion

There was not detected production of GM-CSF when using o-toluic acid or 2-choloro benzoic acid. This indicates that these alternative inducers did not function as desired. Based on their induction ratio (m-toluic acid; 18, o-toluic acid; 17 and 2-chloro benzoic acid; 14) (Ramos et al., 1990), a slightly lower induction effect was expected. Still, the observed increase in CO_2 generation at induction may indicate that also these inducers were consumed. o-Toluic acid and 2-chloro benzoic acid was therefore not be further evaluated as alternatives to m-toluic acid in this study.

3.5 Construction of copy number mutant plasmids pIFN271pelB and pGM271pelB

It had been shown that KT2440 (pIFNpelB) and KT2440 (pGMpelB) produced the wanted model proteins (Figure 23 and Figure 33). The amount of production was low. It was therefore tried to increase the production by different strategies (For complete strategy see Chapter 1.4). The first approach was increasing the copy number of the plasmids. The gene *trfA* of the plasmids was exchanged with *trfAcop271C* from pAT64, which also was the source of *ifnα2b* gene. As mentioned in Chapter 1.2.4, it has been shown that this increases the copy number from approximately 4-6 to 17-19 (3.5 fold increase) in *E. coli.*

The plasmid pIFNpelB, pGMpelB and pAT64 were first transferred to competent ER2925 cells (Chapter 2.4.8.1). ER2925 cells were chosen due to the later use of Dcm methylation-sensitive enzyme (*SexAI*). After growing the cells in 3 ml selective LB overnight and isolating plasmids following the protocols (Chapter 2.4.3), they were digested with *SexAI* and *AhdI* (Chapter 2.4.4). DNA fragments were separated by gel electrophoresis (Chapter 2.4.5). It was expected that all plasmids should yield a 2.4 kb fragment (*trfA* and *trfAcop271C* gene) and that the larger fragments of pIFNpelB, pGMpelB and pAT64 should be 7.1, 7.0 and 6.5 kb respectively. The results from gel electrophoresis are shown in Figure 34 and Figure 35.
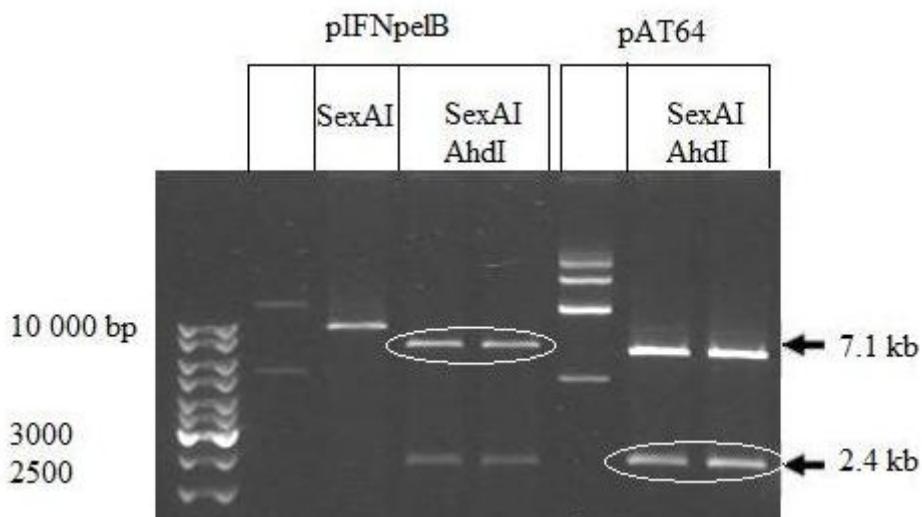
Figure 34: Restriction digestion of the isolated plasmids pIFNpelB and pAT64 with *SexAI* and *SexAI* + *AhdI*. Blank column indicated undigested plasmids. The trfAcop271C fragment (2.4 kb) from pAT64 were to be exchanged with trfA fragment (2.4 kb) from pIFNpelB
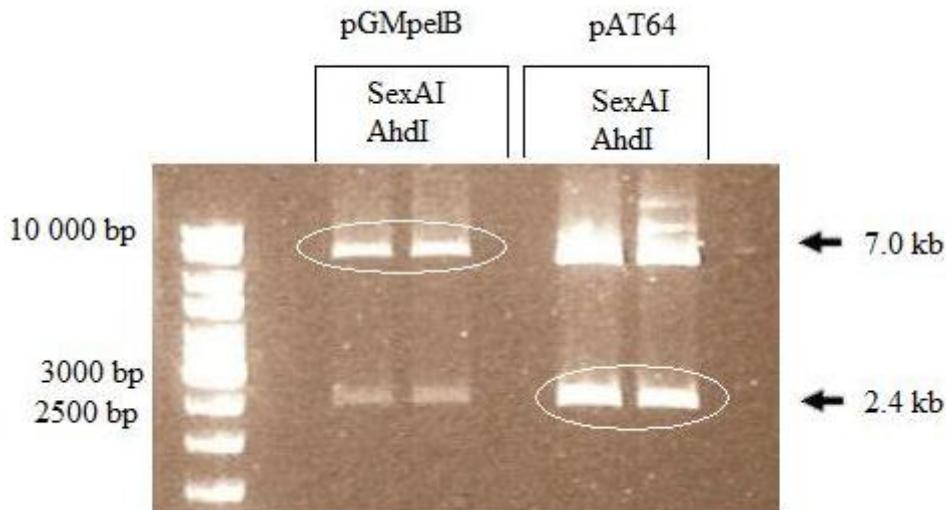
Figure 35: Restriction digestion of the isolated plasmids pGMpelB and pAT64 with *SexAI* + *AhdI*. The trfAcop271C fragment (2.4 kb) from pAT64 were to be exchanged with trfA fragment (2.4 kb) from pGMpelB

Marked DNA fragments (Figure 34 and Figure 35) had the expected sizes and were extracted using QIAEXII Agarose Gel Extraction Protocol (Chapter 2.4.6). The 2.4 kb fragments from pAT64 were assumed to be *trfAcop271C* and were therefore ligated with 7.1 kb fragment from pIFNpelB (Figure 34) and 7.0 kb fragment from pGMpelB (Figure 35) as following protocols described earlier. Ligation mixtures were transformed to competent DH5α cells (Chapter 2.4.8.1).

To control that *trfA* had been exchanged with *trfAcop271C*, plasmids were isolated from the DH5α-transformants and sequenced by Eurofins MWG Operon. Sequence results were compared to trfA-sequence and conclusion was that *trfA* in pIFNpelB and pGMpelB had been replaced by *trfAcop271C* yielding pIFN271pelB and pGM271pelB (Attachment G).

3.5.1 Transferring copy number mutant plasmids to KT2440

pIFN271pelB and pGM271pelB were transferred to KT2440 through electro transformation (Chapter 2.4.8.2). Colonies were transferred to 3ml selective LB and grown overnight. Plasmids were isolated as before (Chapter 3.1) and digested with *NotI* and *NdeI* (Chapter 2.4.4) to control that the up grown strains harboured the correct plasmid. pIFN271pelB and pGM271pelB were expected to give a large DNA fragment of 8.9 kb and a smaller of 5.7 and

4.5 kb respectively. DNA fragments in the digestions were separated by gel electrophoresis (Chapter 2.4.5). The results are shown in Figure 36.
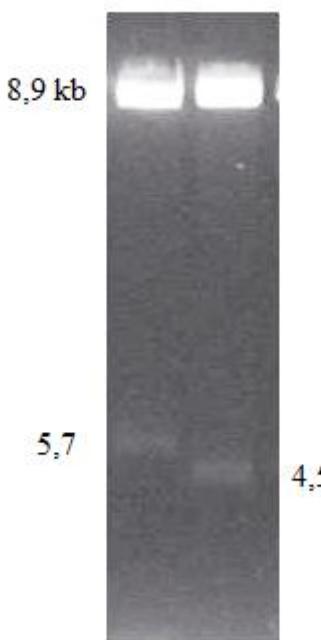
Figure 36: Restriction digestion of pIFN271pelB and pGM271pelB with *NotI* and *NdeI* to control successful electro transformation of plasmids to KT2440. Expected bands from pIFN271pelB (8894 bp + 566 bp) and pGM271pelB (8894 bp + 452 bp) were observed.

Figure 36 shows the expected sizes from digested pIFN271pelB and pGM271pelB. Electro transformation of the plasmids to KT2440 was therefore concluded to be successful.

3.5.2 Control of plasmid copy number in KT2440

To test if the exchange of *trfA* with *trfAcop271C* had the same effect for *P. putida* KT2440 as for *E coli* (Chapter 1.2.4), 3 ml selective overnight LB cultures (30°C, 200 rpm) of KT2440 (pIFNpelB) and KT2440 (pIFN271pelB) were diluted with sterile H_2O to the same OD_{600} value. Plasmids were then isolated as before (Chapter 2.4.3). The culture dilution ensured that plasmids were isolated from the same amount of cells. Restriction digestions with *NotI* were made following the protocol (Chapter 2.4.4), but with plasmid preparation volumes ranging from 1 – 8 μl. *NotI* was expected to cut the plasmids once and since pIFNpelB and pIFN271pelB both have the same size (9460 bp), easy comparison of the band strength (equals the number of plasmids) could be done on the gel. Digested plasmids were separated by gel electrophoresis (Chapter 2.4.5).

The results are shown in Figure 37.
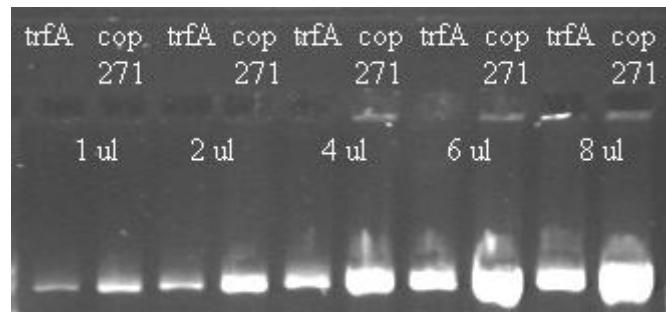
Figure 37: Different versions of replication protein gene, trfA and cop271 (trfacop271C), cut with *NotI*. The volume indicates the added plasmid to the digestion mixture.

Figure 37 shows that the same amount of KT2440 cells have given different concentration of plasmids. This can be observed by comparing the strength of bands form digestion mixtures with the same volume. For example, of the 1 µl digestion mixtures of pIFNpelB and pIFN271pelB (two left columns), pIFN271pelB (marked cop271 in Figure 36) band is stronger than the band from pIFNpelB (marked trfA). By comparing bands, 2 µl of cop271 (lane 4) seems to have approximately the same strength as 6 – 8 µl trfA (lane 7 and 9). This indicates a 3-4 fold increase which is comparable to what was observed in *E. coli* (3.5-fold) (Durland et al., 1990). It is therefore expected that the plasmid copy number in KT2440 has increased from 2-3 to approximately 7 – 11.

3.6 Construction of codon optimized plasmids pIFNs271pelB, pIFNs271pelBs and pGMs271pelBs

The second strategy for increasing the production of recombinant proteins was codon optimization of gene targets and signal sequence with regard to *P. putida* KT2440 (Chapter 1.4). This was performed by Eurofins MWG Operon which synthetically produced these new versions of the genes including the *pelB* sequence. Gene sequence results are shown in Attachment G. The received DNA material (pCR2.1-IFNspelBs and pCR2.1-GMspelBs) was spun down before dissolving in 10 μl TE-buffer. 1 μl of this dissolved gene material was transformed to competent *E. coli* DH5α cells (Chapter 2.4.8.1) and plated out (10x diluted, undiluted, and pellet) on selective LA plates. After incubating at 37°C overnight, one colony from each transformants (DH5α (pCR2.1-IFNspelBs) and DH5α (pCR2.1-GMspelBs)) was transferred to 3 ml selective LB and grown overnight (37°C). The plasmids were isolated following the protocol in Chapter 2.4.3.

To replace the recombinant genes with the codon optimized versions, two different digestions mixtures were made of pIFN271pelB, pCR2.1-IFNspelBs and pCR2.1-GMspelBs. Digestion with *NcoI + NotI* will cut upstream the *pelB* sequence while *NdeI + NotI* will cut downstream. This would, from all plasmids, yield recombinant gene fragments with and without *pelB-*sequence. The different sizes of these fragments are; *ifn-α2b*: 504 bp, *ifn-α2b+pelB*: 566 bp, *gm-csf*: 390 bp and *gm-csf+pelB*: 452 bp. Codon optimized fragments will have the same sizes.

Since the gene will be replaced, digestion of pIFN271pelB, and not pGM271pelB, were made. The fragments were separated by gel electrophoresis (Chapter 2.4.5 and 2.4.5.2). The results from digestion with *NcoI* and *NotI* are shown in Figure 38 and digestion with *NdeI* and *NotI* in
Figure 39.
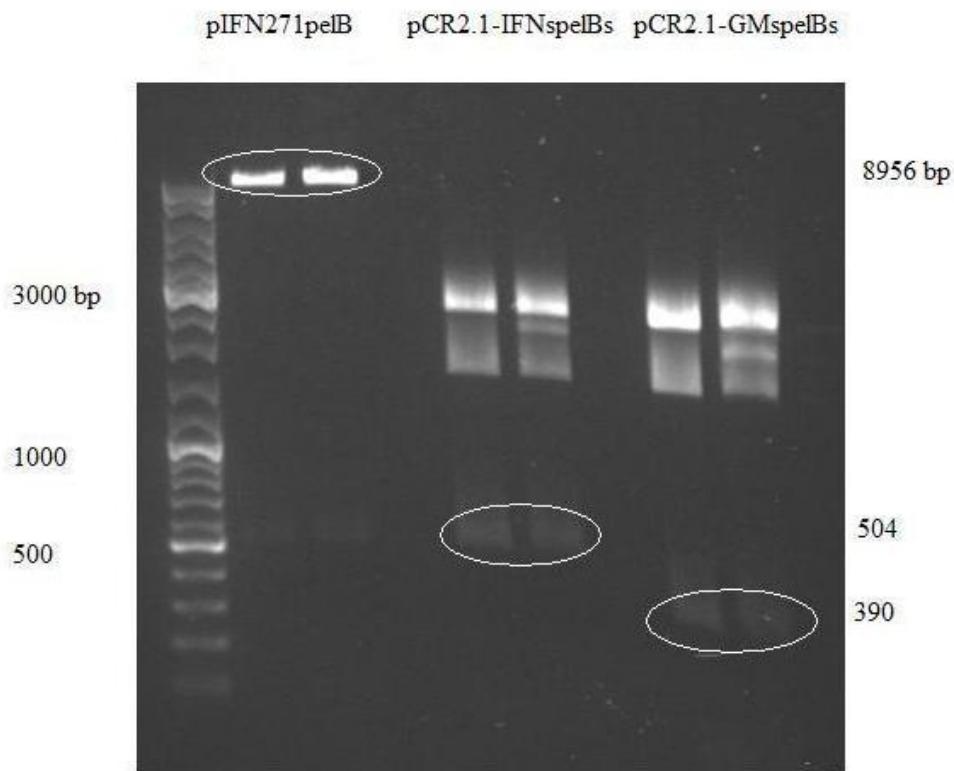
Figure 38: Restriction digestion of pIFN271pelB, pCR2.1-IFNspelBs and pCR2.1-GMspelBs (two parallels of each) with *NcoI* and *NotI*.
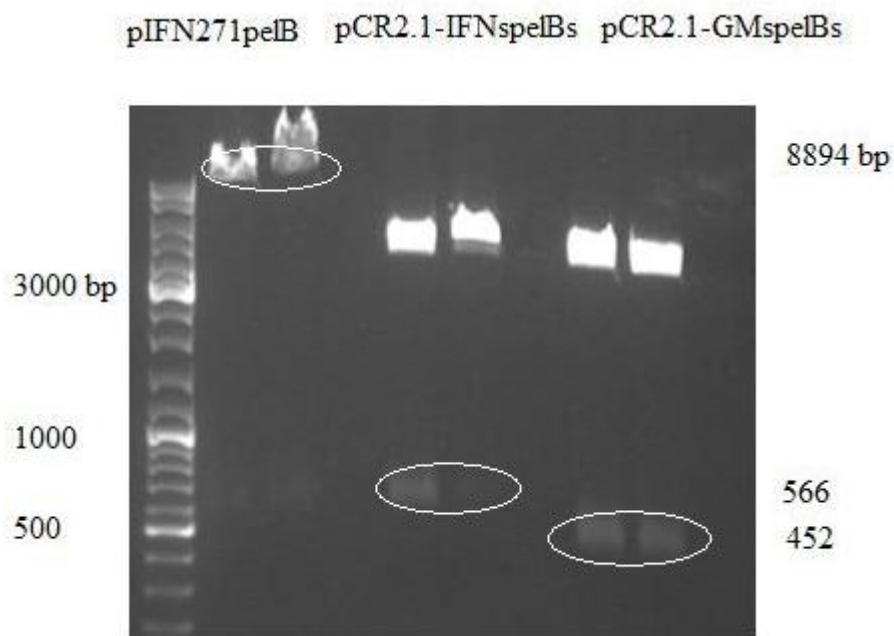
Figure 39: Restriction digestion of pIFN271pelB, pCR2.1-IFNspelBs and pCR2.1-GMspelBs (two parallels of each) with *NdeI* and *NotI*.

Results and discussion

The marked DNA fragments in Figure 38 and Figure 39 were isolated by QIAEX II Agarose Gel Extraction Protocols (Chapter2.4.6). DNA fragments cut with the same enzymes were ligated following the protocols in Chapter 2.4.7 and transferred to DH5α cells (Chapter 2.4.8.1).

To control the transformants DH5α (pIFNs271pelB), DH5α (pGMs271pelB), DH5α (pIFNs271pelBs) and DH5α (pGMs271pelBs), it was chosen to sequence the genes by the use of Eurofins MWG GmbH. The results are shown in Attachment G. The sequences were compared with sequence information of codon adapted genes from the same company in Clone Manager Professional Suite (Sci Ed Central). This revealed that pIFN271pelBs, pIFNs271pelB and pGMs271pelBs were successfully constructed, but that pGM271pelBs had an additional restriction site for *NcoI* inside the codon optimized *GM-CSF* gene. This led to an additional DNA fragment when pCR2.1GMspelBs was digested with *NcoI* and *NotI*. It caused loss of fragments of the gene when DNA fragments were separated by gel electrophoresis. It was tried to get a new version of codon adapted *gm-csfs+pelBs* without this restriction site, but trouble from the company's side led to too short amount of time left to be able to construct pGMs271pelB.

3.6.1 Transferring pIFNs271pelB, pIFNs271pelBs and pGMs271pelBs with codon optimized genes to KT2440

The plasmids (pIFNs271pelB, pIFNs271pelBs, and pGMs271pelBs) were transformed into KT2440 cells by electro transformation (Chapter 2.4.8.2). The only exception from this protocol was that 6 µl DNA was used instead of 2. Single colonies were picked and plated on separate selective LA plates before incubating overnight (30°C). 10 ml selective LB was then inoculated with one colony, incubated overnight (30°C, 200 rpm) before making glycerol cell stocks (Chapter 2.4.1).

3.7 Evaluation of P. putida KT2440 as expression platform for plasmids with increased copy number and codon optimized genes and signal sequence

P. putida KT2440 had been proven to have high plasmid stability in both shake flask cultivations (Attachment F Table F- 3, Chapter 3.4.2 Table 3 and Table 4 and Chapter 3.4.3.2 Table 5) and in HCDC cultivations (Chapter 3.4.5.2 Table 6). Production of both recombinant proteins (IFN-α2b and GM-CSF) had been detected in shake flask experiment (Chapter 3.4.4, Figure 22) and GM-CSF production in HCD fed batch cultivation (Chapter 3.4.5, Figure 29). New expression vectors with copy up mutations and codon optimized genes with and without codon optimized signal sequences had been constructed and transferred to KT2440 (Chapter 3.6). It was now desirable to evaluate the production of recombinant protein in shake flasks and possibly fed batch fermentation. Table 7 shows the strains that were used to evaluate *P. putida* KT2440 as expression platform for plasmids with increased copy number and codon optimized genes.

Table 7: Plasmids used for evaluation of *P. putida* KT2440 as expression platform for copy number mutants and codon optimized genes

Strain	Plasmid name	Gene	Signal sequence	Replication protein
KT2440	pIFNpelB	*ifn-α2b*	*pelB*	TrfA
KT2440	pGMpelB	*gm-csf*	*pelB*	TrfA
KT2440	pIFN271pelB	*ifn-α2b*	*pelB*	TrfAcop271C
KT2440	pGM271pelB	*ifn-α2b*	*pelB*	TrfAcop271C
KT2440	pIFNs271pelB	Codon optimized *ifn-α2b*	*pelB*	TrfAcop271C
KT2440	pIFNs271pelBs	Codon optimized *ifn-α2b*	Codon optimized *pelB*	TrfAcop271C
KT2440	pGMs271pelBs	Codon optimized *gm-csf*	Codon optimized *pelB*	TrfAcop271C

3.7.1 Growth and production experiment in shake flasks

Glycerol cell stocks of the strains shown in Table 7 were plated out on selective LA plates and grown overnight at 30°C. Single colonies were used to inoculate 50 ml selective LB in 250 ml shake flasks with baffles. The precultures were placed in incubators for only 10 hours (in contrast to the usually 16 hours in Chapter 3.4.3 and 3.4.4) to prevent experienced outgrowing of LB medium during overnight incubations in a prestudy (results not shown).

100 ml of HiYe-8GLY-2GLU with selection was inoculated to 4 % (v/v) from precultures (Attachment F,Table F- 18). The reason for this high inoculation volume was the result from a prestudy where a long lag period was experienced (data not shown). Two parallels of each strain were grown to enable comparison of induced and non-induced strain.

The growth and pH were followed throughout the experiment. Cultures were induced at $OD_{600} \approx 3-5$ to a final concentration of 0.5 mM m-toluic acid. pH was adjusted if measured above 7.00. All of the cultures were grown with a parallel which was not induced for later comparison of production.

3.7.1.1 Growth measurements

Figure 40 and Figure 41 shows the growth curves for induced and non-induced cultures. The figures are separated into strains that produce IFN-α2b and GM-CSF proteins respectively.
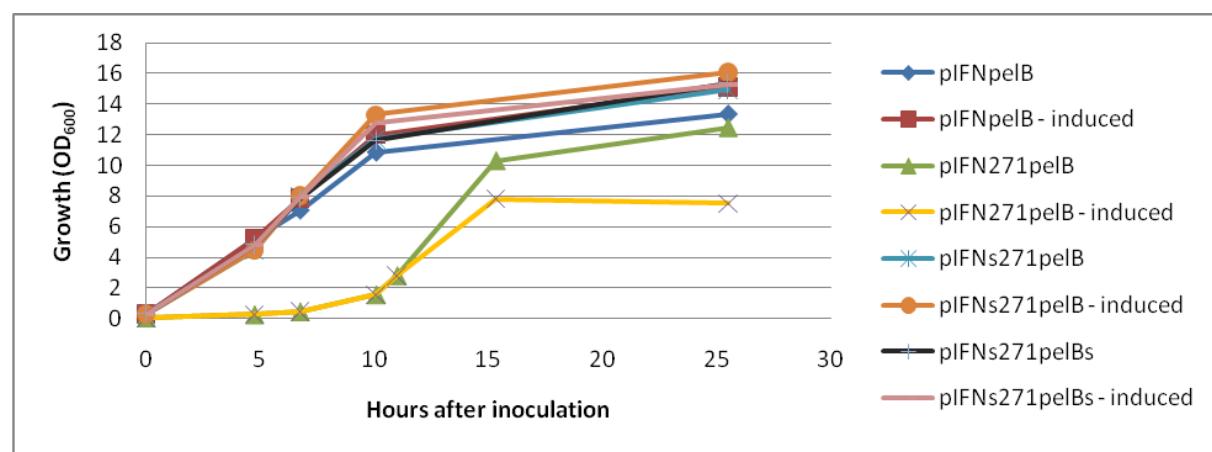
Figure 40: Growth (OD_{600}) measurements from shake flask growth and production experiment with KT2440 containing different plasmids with *ifn-α2b* gene (indicated in the column to the right). The cultures were grown in selective HiYe-2GLU-8GLY medium at 30°C and induced with m-toluic acid when reaching $OD_{600} \approx 3 – 5$. For details see Attachment F, Table F- 19

Results and discussion

Figure 40 shows the growth after inoculation of KT2440 containing different plasmids with *ifn-α2b* gene. Induced KT2440 (pIFN271pelB) did not reach as high cell yield as the remaining strains after overnight incubation. This could indicate that the strain had problem maintaining the plasmid. Still, this effect is not observed in all strains harbouring plasmids with *trfAcop271C*.
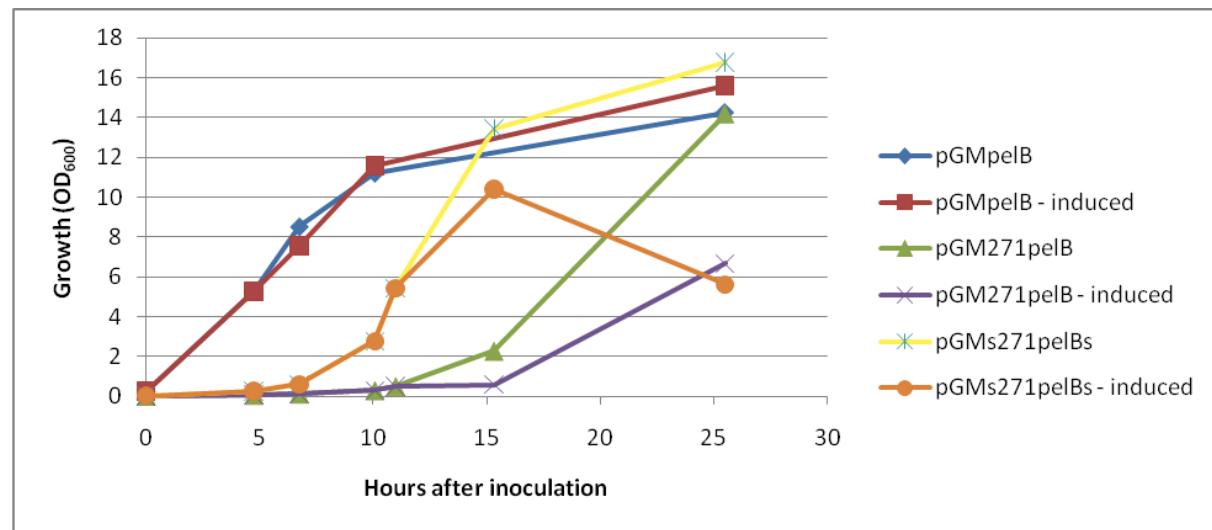
Figure 41: Growth (OD$_{600}$) measurements from shake flask growth and production experiment with KT2440 containing different plasmids with GM-CSF gene (indicated in the column to the right). The cultures were grown in selective HiYe-2GLU-8GLY medium at 30°C and induced with m-toluic acid when reaching OD$_{600}$≈ 3 – 5. For details see Attachment F, Table F- 19

Figure 41 shows the growth after induction of KT2440 containing different plasmids with *gm-csf* gene. KT2440 (pGMpelB) and KT2440 (pGMs271pelBs) were induced at OD$_{600}$ ≈ 3-5, while KT2440 (pGM271pelB) at OD$_{600}$≈ 1 because of slow growth rate. KT2440 (pGMpelB) yielded the same high cell yield after overnight incubation as the majority of the strains (Figure 40 and Figure 41), while KT2440 (pGM271pelB) and KT2440 (pGMs271pelBs) did not. Cultivations of these strains had reached a much lower cell yield after complete experiment. As for KT2440 (pIFN271pelB) (Figure 40) this might indicate plasmid instability.

The genetic modifications of expression vectors (increasing copy number and codon optimization) seemed, for some strains, to have effect on the length of lag phase. This can be observed by comparing the growth in precultures (Attachment F Table F- 20) of this experiment, but it is important to emphasize that the amount cells varies when inoculating from agar plates. KT2440 (pIFNpelB) and KT2440 (pGMpelB) reached OD$_{600}$ values of

approximately 7 in ten hours (which means that they are on the edge on outgrowing the medium). KT2440 (pIFN271pelB), (pGM271pelB) and (pGMs271pelBs) in the same time interval had an OD600 value of 0.5 – 1. If the copy number increase or optimization had been the direct effect for this low initial growth rate, KT2440 (pIFNs271pelB) and KT2440 (pIFNs271pelBs) should also have long lag phases, but this was not the case. They reached OD_{600} 7.0 and 8.3 respectively proving that the high copy number not necessarily affected the growth rate. One reason that might cause this long lag phase was the cells viability on agar plates. It was experienced that the cultures plated out and incubated the day before inoculation of precultures, had shorter lag phases (data not shown).

3.7.1.2 Protein production

5 ml samples were taken at induction and after 2 and 5 hours. The samples were that were centrifuged at 4000 rpm for 10 minutes before separating the supernatant and pellet in different tubes and frozen at - 20˚C until analysis. The pellets were prepared for SDS-PAGE following "Cell lysis protocol 2" (Chapter 2.4.10.1). A sample with known concentration of Phox protein was used as an internal standard. This would enable comparison of membranes and quantification possibilities. Proteins were detected by Western Blot protocols using anti-c-myc as primary antibody (Chapter2.4.10.3).

Wild type copy number strains KT2440 (pIFNpelB) and KT2440 (pGMpelB)

KT2440 harbouring pIFNpelB or pGMpelB had previously been proven to produce the recombinant model proteins, but in low quantities (Chapter 3.4.5.3, Figure 29). Their production in this experiment was therefore used for evaluation of the effects of increasing the copy number and codon optimizing target genes and signal sequences. The detected recombinant proteins from KT2440 (pIFNpelB) and KT2440 (pGMpelB) are shown in Figure 42 and Figure 43 respectively.
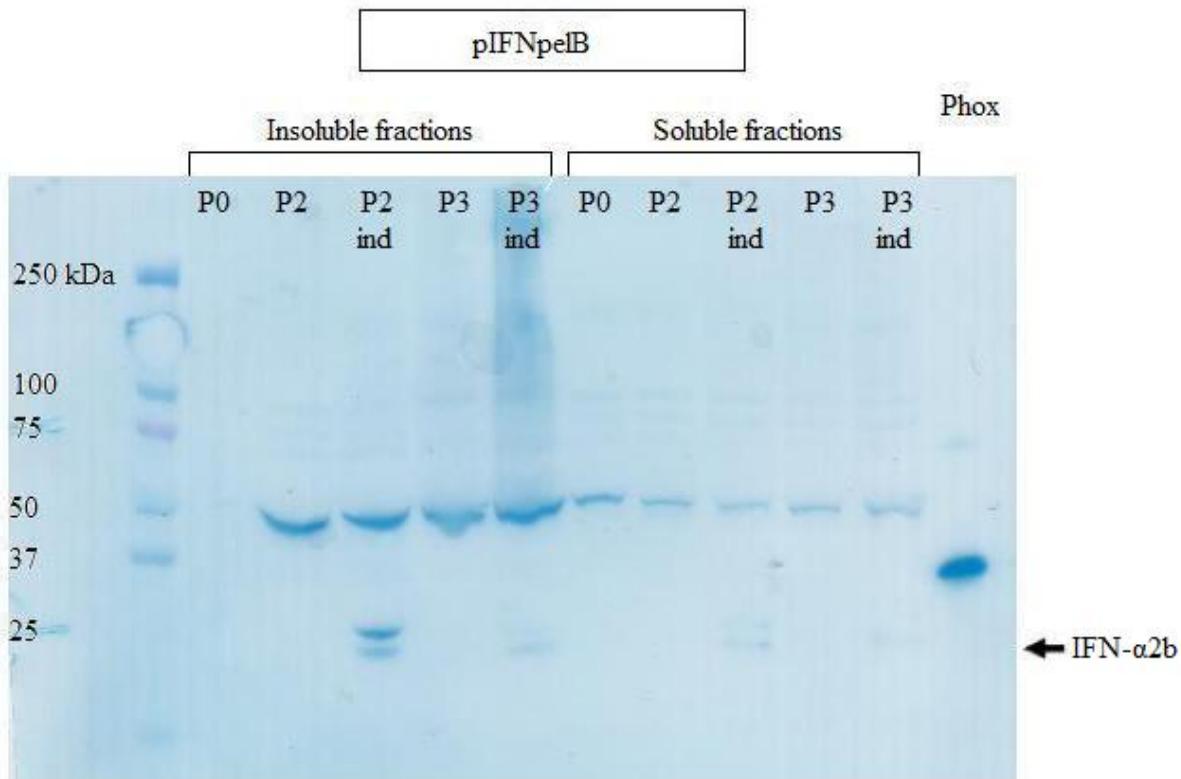
Figure 42: Western Blot results from insoluble and soluble fractions from growth and production experiment in shake flasks. The strains were grown in HiYe-8GLY-2GLU medium at 30°C. The image is the result for KT2440 (pIFNpelB). Induction to a final concentration of 0.5 mM m-toluic acid was performed at OD$_{600}$ ≈ 3-5. P0-P2 samples were taken after 0, 2, 5 hours after induction respectively. P3 was taken after overnight incubation. "ind" marks samples from induced cultures. The arrow indicates IFN-α2b protein detected by anti-c-myc antibody, and the numbers on the left side represents molecule sizes in kilo Daltons. Phox has a concentration of 1 mg/l.

Results and discussion

Figure 43: Western Blot results from insoluble and soluble fractions from growth and production experiment in shake flasks. The strains were grown in HiYe-8GLY-2GLU medium at 30°C. The image is the result for KT2440 (pGMpelB). Induction to a final concentration of 0.5 mM m-toluic acid was performed at $OD_{600} \approx$ 3-5. P0-P2 samples were taken after 0, 2, 5 hours after induction respectively. P3 was taken after overnight incubation. "ind" marks samples from induced cultures. The arrow indicates GM-CSF protein detected by anti-c-myc antibody, and the numbers on the left side represents molecule sizes in kilo Daltons. Phox has a concentration of 1 mg/l.

The highest IFN-α2b yield is measured in the insoluble fraction from sample taken 5 hours after induction (Figure 42, "P2 ind"). Compared to the Phox reference with known concentration (1 mg/l), this is much weaker. Overnight incubation has less detectable protein indicating that the protein may be unstable or leaks into the culture medium. This last hypothesis was tested in Chapter 3.4.6.2. Since the result here was no detectable protein in culture medium, it is assumed that this is not the case here either.

In Figure 43, where the recombinant protein from KT2440 (pGMpelB) is detected, the highest yield of GM-CSF is measured in the sample taken 5 hours after induction ("P2 ind", insoluble and soluble fractions). As for IFN-α2b produced by KT2440 (pIFNpelB), the protein concentration is evaluated to be much lower than 1 mg/l when compared to the Phox

reference. There is less detectable GM-CSF after overnight incubation of the culture. During previous fed batch fermentation, GM-CSF concentrations started to decrease two hours after induction (Chapter 3.4.5.3, Figure 27). It was therefore expected to see the same decline in GM-CSF after overnight incubation in shake flasks. As for KT2440 (pIFNpelB), the reason is probably protein instability.

Copy number mutants KT2440 (pIFN271pelB) and KT2440 (pGM271pelB)

KT2440 (pIFN271pelB) and KT2440 (pGM271pelB) harboured plasmids with a mutation in the gene *trfA* shown to yield 3.5-fold increase in copy number in *E. coli* (Chapter 1.2.4). In Chapter 3.5.2 it was proven that this mutation had the same effect on *P. putida* strain KT2440. Cultivation of KT2440 (pIFN271pelB) and KT2440 (pGM271pelB) would therefore reveal if increased plasmid number would have effect on the production of recombinant proteins. The results from protein detection of IFN-α2b and GM-CSF are shown in Figure 44 and Figure 45.
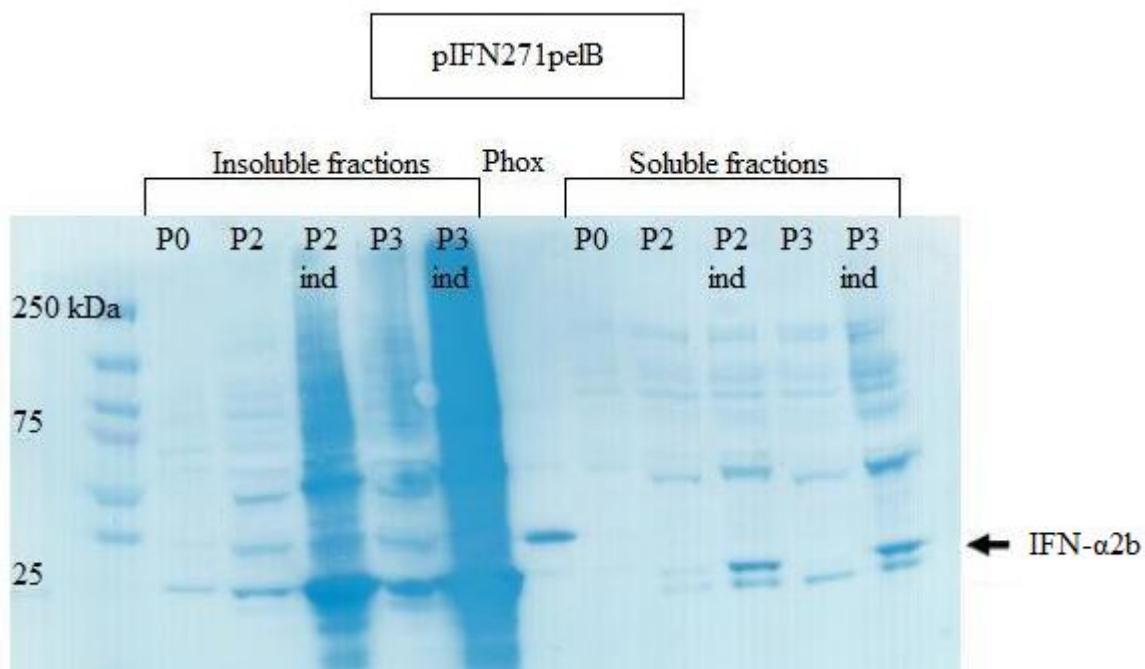
Figure 44: Western Blot results from insoluble and soluble fractions from growth and production experiment in shake flasks. The strains were grown in HiYe-8GLY-2GLU medium at 30°C. The image is the result for KT2440 (pIFN271pelB). Induction to a final concentration of 0.5 mM m-toluic acid was performed at OD$_{600}$ ≈ 3-5. P0-P2 samples were taken after 0, 2, 5 hours after induction respectively. P3 was taken after overnight incubation. "ind" marks samples from induced cultures. The arrow indicates IFN-α2b protein detected by anti-c-myc antibody, and the numbers on the left side represents molecule sizes in kilo Daltons. Phox has a concentration of 1 mg/l.

Results and discussion

Figure 45: Western Blot results from insoluble and soluble fractions from growth and production experiment in shake flasks. The strains were grown in HiYe-8GLY-2GLU medium at 30°C. The image is the result for KT2440 (pGM271pelB). Induction to a final concentration of 0.5 mM m-toluic acid was performed at $OD_{600} \approx 1$. P0-P2 samples were taken after 0, 2, 5 hours after induction respectively. P3 was taken after overnight incubation. "ind" marks samples from induced cultures. The arrow indicates GM-CSF protein detected by anti-c-myc antibody, and the numbers on the left side represents molecule sizes in kilo Daltons. Phox has a concentration of 1 mg/l.

As Figure 44 and Figure 45 shows, the increased plasmid number has had a big effect on the production of both IFNα2b and GM-CSF. Single bands from insoluble fractions in Figure 44 are almost impossible to detect in samples taken later than 5 hours after induction. One wider area at IFN-α2b protein size can still be seen (23.7 kDa). When comparing this with detected IFN-α2b from KT2440 (pIFNpelB) (Figure 42), there is observed at least 5-10-fold increased protein production. This effect on protein production was higher than what was expected from the 3.5-fold increase in plasmid number.

In the soluble fraction from the same strain (KT2440 (pIFN271pelB)), the bands have lower concentrations of IFN-α2b. When compared to the Phox reference, both samples from after 5 hours of induction (P2, P3) have approximately the same strength. Based on judgements done in Attachment H this indicates that the volumetric yield of IFN-α2b is 0.2 mg/l. Previous attempts to produce IFN-α2b from *E. coli* in both shake flasks and fed batch fermentations gave no soluble fraction of this protein (Sletta et al., 2007). KT2440 therefore appears to be a

Results and discussion

better alternative for IFN-α2b production. Non-induced strains have some weak bands at IFN-α2bs size which can be due to leakage from the promoter.

The GM-CSF production has increased compared to KT2440 (pGMpelB) where bands were almost not detectable (Figure 43). In Figure 45, the bands are easily detected and the highest yield of GM-CSF is observed in insoluble fraction after overnight incubation ("P3 ind"). Previously fed batch fermentation with KT2440 (pGMpelB), as well this experiment (Figure 43), indicated that GM-CSF is unstable under longer incubations (Chapter 3.4.5.3, Figure 27). If this is true, the high amount of GM-CSF detected after overnight incubation indicates production is higher than formation of unstable proteins. There also seems to be an increasing amount of soluble GM-CSF through prolonged incubation. Since no band is detected in insoluble fraction after overnight incubation of KT2440 (pGMpelB), P2 samples should be compared. The increased amount of GM-CSF here is estimated to be 3-4 and can therefore be related to increased plasmid number.

Results and discussion

Copy number mutant with codon optimizes gene KT2440 (pIFNs271pelB)
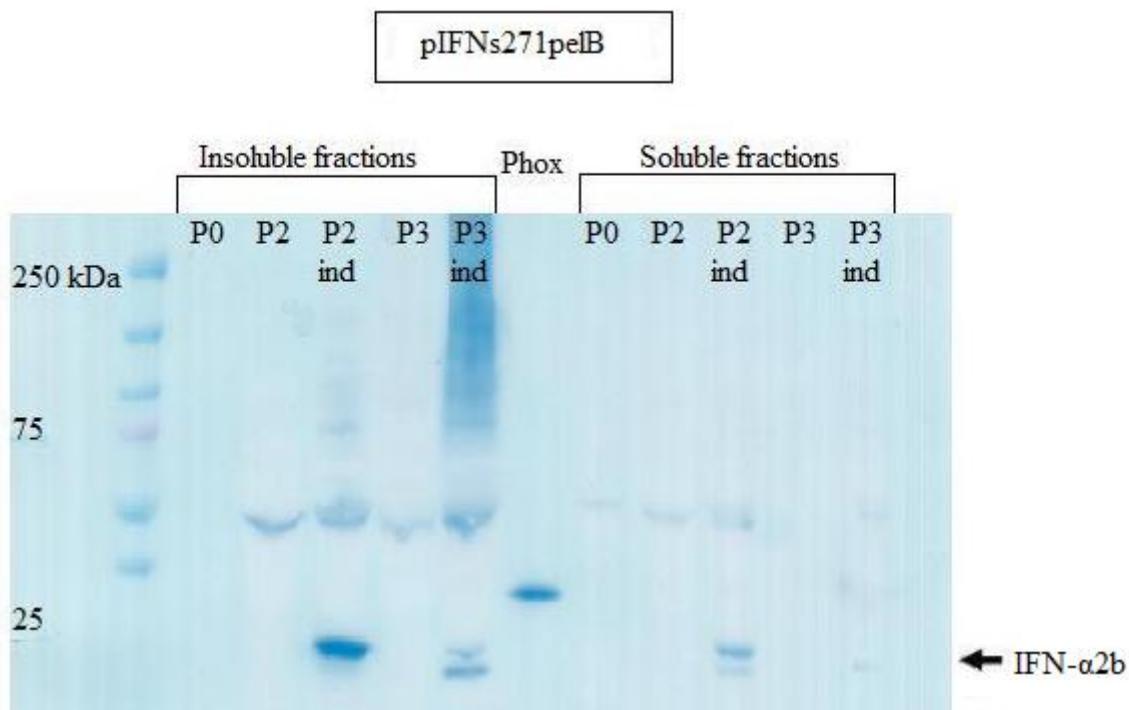
Figure 46: Western Blot results from insoluble and soluble fractions from growth and production experiment in shake flasks. The strains were grown in HiYe-8GLY-2GLU medium at 30°C. The image is the result for KT2440 (pIFNs271pelB). Induction to a final concentration of 0.5 mM m-toluic acid was performed at $OD_{600} \approx$ 3-5.P0-P2 samples were taken after 0, 2, 5 hours after induction respectively. P3 was taken after overnight incubation. "ind" marks samples from induced cultures. The arrow indicates IFN-α2b protein detected by anti-c-myc antibody, and the numbers on the left side represents molecule sizes in kilo Daltons. Phox has a concentration of 1 mg/l.

Figure 46 shows that the highest yield of IFN-α2b is in the insoluble fraction from sample taken 5 hours after induction. When compared to the highest yield from KT2440 (pIFNpelB) (Figure 42), there is obvious that IFN-α2b production has increased. Still, the amount of produced IFN-α2b from KT2440 (pIFNs271pelB) is low compared to KT2440 (pIFN271pelB) production. This suggests that the codon optimization of *ifn-α2b* gene has a negative effect on the production of IFN-α2b protein when compared with copy number mutants. This was quite unexpected since adaptation was done to match KT2440 codon usage.

As described earlier (Chapter 3.6), KT2440 (pGMs271pelB) was not completed for this study.

Results and discussion

Copy number mutants with codon optimized genes and *pelB* sequence
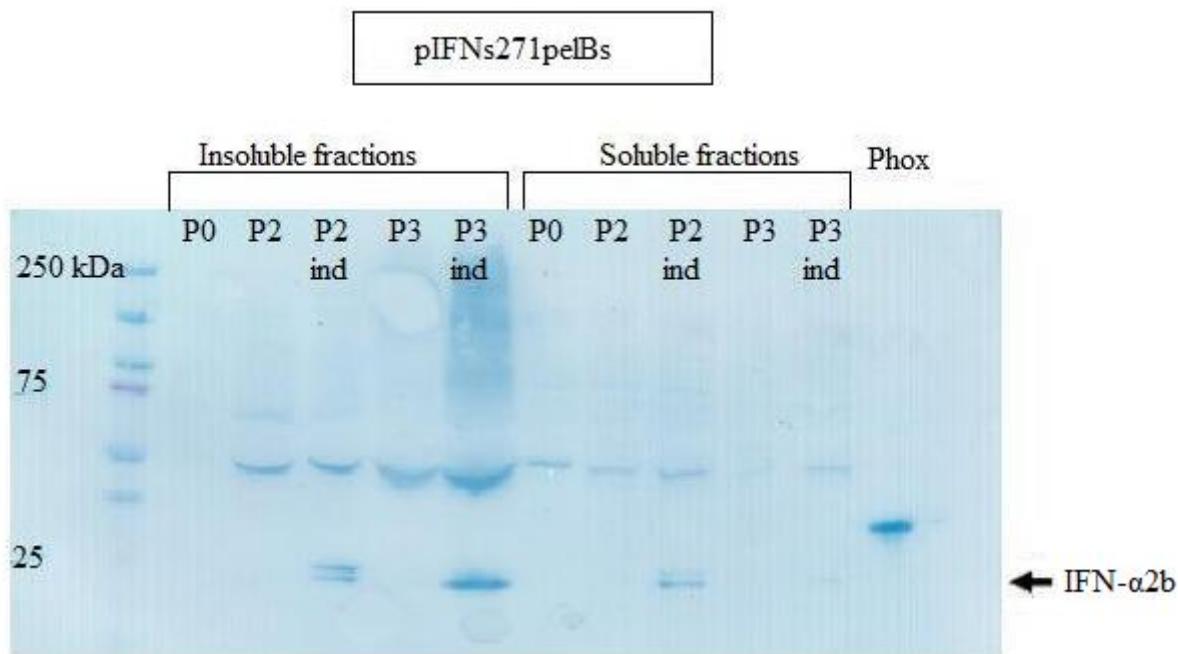
Figure 47: Western Blot results from insoluble and soluble fractions from growth and production experiment in shake flasks. The strains were grown in HiYe-8GLY-2GLU medium at 30°C. The image is the result for KT2440 (pIFNs271pelBs). Induction to a final concentration of 0.5 mM m-toluic acid was performed at $OD_{600} \approx$ 3-5. P0-P2 samples were taken after 0, 2, 5 hours after induction respectively. P3 was taken after overnight incubation. "ind" marks samples from induced cultures. The arrow indicates IFN-α2b protein detected by anti-c-myc antibody, and the numbers on the left side represents molecule sizes in kilo Daltons. Phox has a concentration of 1 mg/l.

Results and discussion
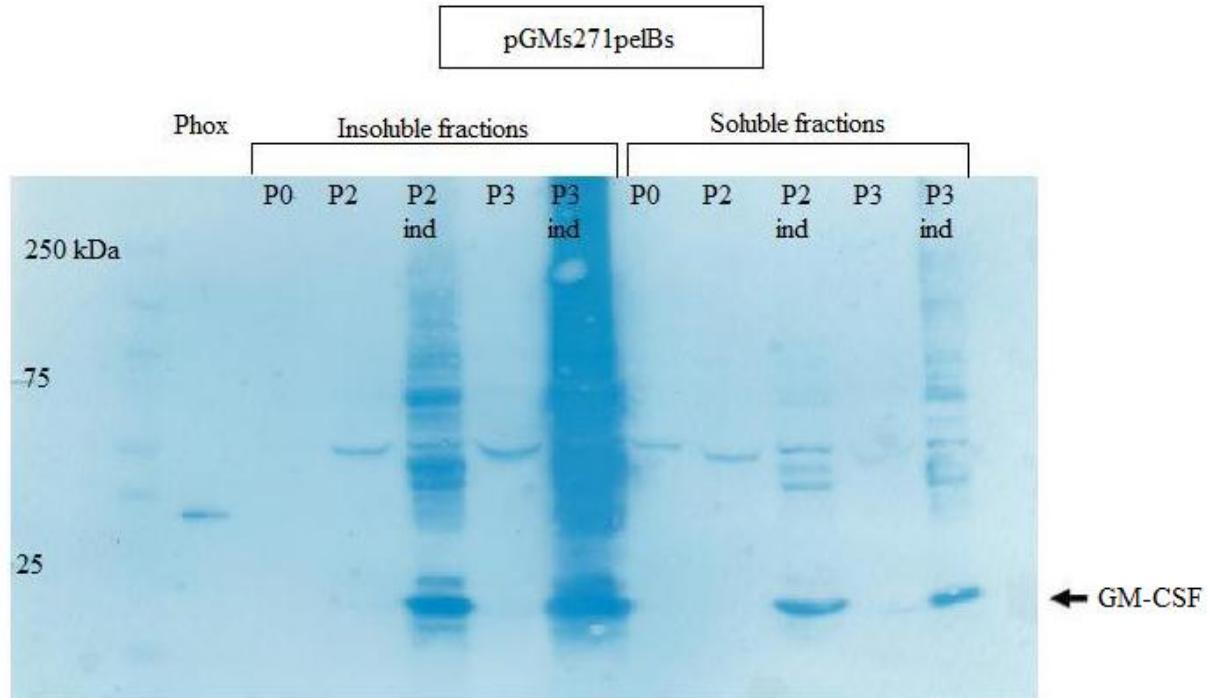
Figure 48: Western Blot results from insoluble and soluble fractions from growth and production experiment in shake flasks. The strains were grown in HiYe-8GLY-2GLU medium at 30°C. The image is the result for KT2440 (pGMs271pelB). Induction to a final concentration of 0.5 mM m-toluic acid was performed at $OD_{600} \approx$ 3-5. P0-P2 samples were taken after 0, 2, 5 hours after induction respectively. P3 was taken after overnight incubation. "ind" marks samples from induced cultures. The arrow indicates GM-CSF protein detected by anti-c-myc antibody, and the numbers on the left side represents molecule sizes in kilo Daltons. Phox has a concentration of 1 mg/l.

The highest yield of IFN-α2b protein in Figure 47 is from overnight incubation (P3 ind). Here, the concentration is evaluated to be lower than 0.1 mg/l when compared to Phox reference. When all IFN-α2b producing KT2440 strains are compared, KT2440 (pIFN271pelB) (Figure 44) has a significant higher production. Codon optimization does therefore not seem to have a positive effect on IFN-α2b production. There are relatively small differences between KT2440 (pIFNs271pelB) and KT2440 (pIFNs271pelB), but codon optimized *pelB* sequence also seems negative. Summarized, the results indicate that increasing the copy number has the best effect on IFN-α2b production. Codon optimization has negative effect in combination with increased plasmid copy number.

As shown in Figure 48, the highest yield of GM-CSF protein from KT2440 (pGMs271pelBs) is found in the insoluble fraction of sample taken after overnight incubation ("P3 ind"). The yield from this strain is significantly higher than what was obtained from KT2440 (pGMpelB) (Figure 43) at the same incubation time. When comparing KT2440 (pGMs271pelBs) with

KT2440 (pGM271pelB) (Figure 45), there is no clear difference. The effect of codon optimization is therefore unknown. When all GM-CSF producing strains are compared, the result indicates that increasing the plasmid number has a positive effect on the production of GM-CSF, while further quantification of protein concentration had to be performed to reveal the effect of codon optimization.

3.7.2 HCD growth and production experiment (fed batch fermentation)

The shake flask experiment (Chapter 3.7.1) showed high production of recombinant proteins from KT2440 (pIFN271pelB) (Figure 44), KT2440 (pGM271pelB) (Figure 45) and KT2440 (pGMs271pelBs) (Figure 48). It was therefore of interest to study the production in a fed batch fermentation to see if the production would increase with the amount of cells.

Two sets of 100 ml inoculums LB precultures were made (Attachment E), one incubated for 16 hours and one for 8 hours to ensure that the cells were in exponential growth when inoculating the inoculums Hi-medium. The fed batch fermentation was performed following the protocol (Chapter 2.4.9), here in 3-litre fermentors. Induction was performed following the protocol (Chapter 2.4.9.2). In addition, the cultures were reinduced two and four hours after the first induction, both times to a final concentration of 0.5 mM.

3.7.2.1 Growth measurements

The growth curves from fed batch fermentation are shown in Figure 49.
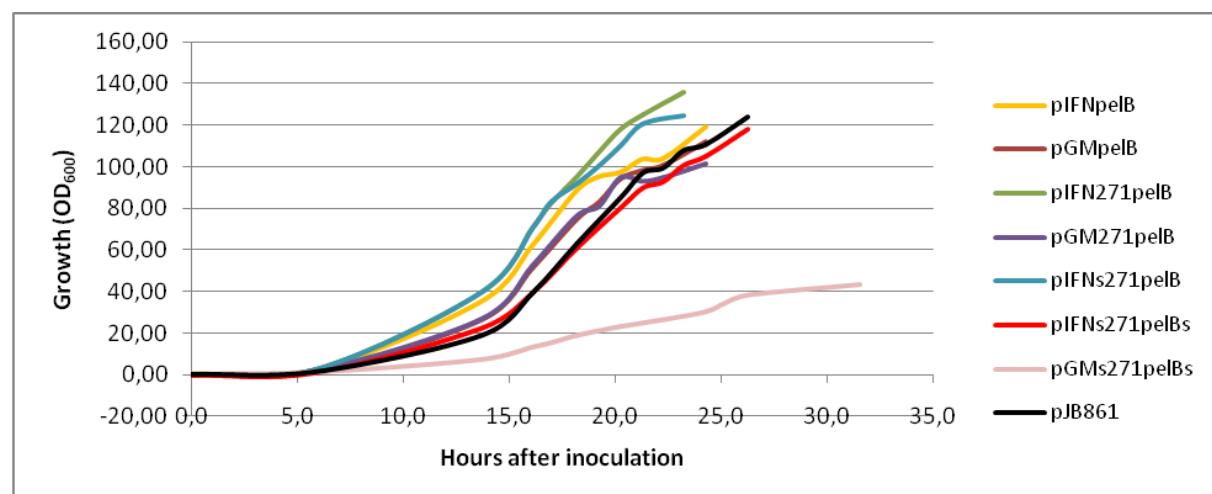
Figure 49: Growth (OD_{600}) measurements of KT2440 strains (harbouring plasmids indicated in the right column) during fed batch fermentation in Hf-1 medium. Cultures were induced at $OD_{600} \approx$ 80-90. For details see Attachment F, Table F- 21

As Figure 49 shows, KT2440 (pGMs271pelBs) had reduced growth rate compared to the rest of strains participating in the fed batch fermentation. As this was not the case for this strain during the shake flask experiment (Chapter 3.7.1.1, Figure 40), it was not expected. KT2440 (pIFN271pelB) was expected to have a long lag phase, but acted as the majority in the fed batch fermentation. KT2440 (pGMs271pelBs) was because of the slow growth rate only induced once, when $OD_{600} \approx 35$.

3.7.2.2 Protein production

Samples were harvested for SDS-PAGE following the protocol (Chapter 2.4.10.1) at induction and the following two, four and six hours. The samples were named P0, P1, P2 and P3 respectively and prepared following "Detection and quantification of recombinant protein" (Chapter 2.4.10).The pellet fraction was prepared by the procedure "Cell lysis protocol 2". Both soluble and insoluble fractions were analyzed for protein content by Western Blot protocol using anti-c-myc as primary antibody (Chapter 2.4.10.3). As before, the expected band is 23.7 kDa for IFNα2b and 19.7 kDa for GM-CSF. The results are shown in Figure 50.
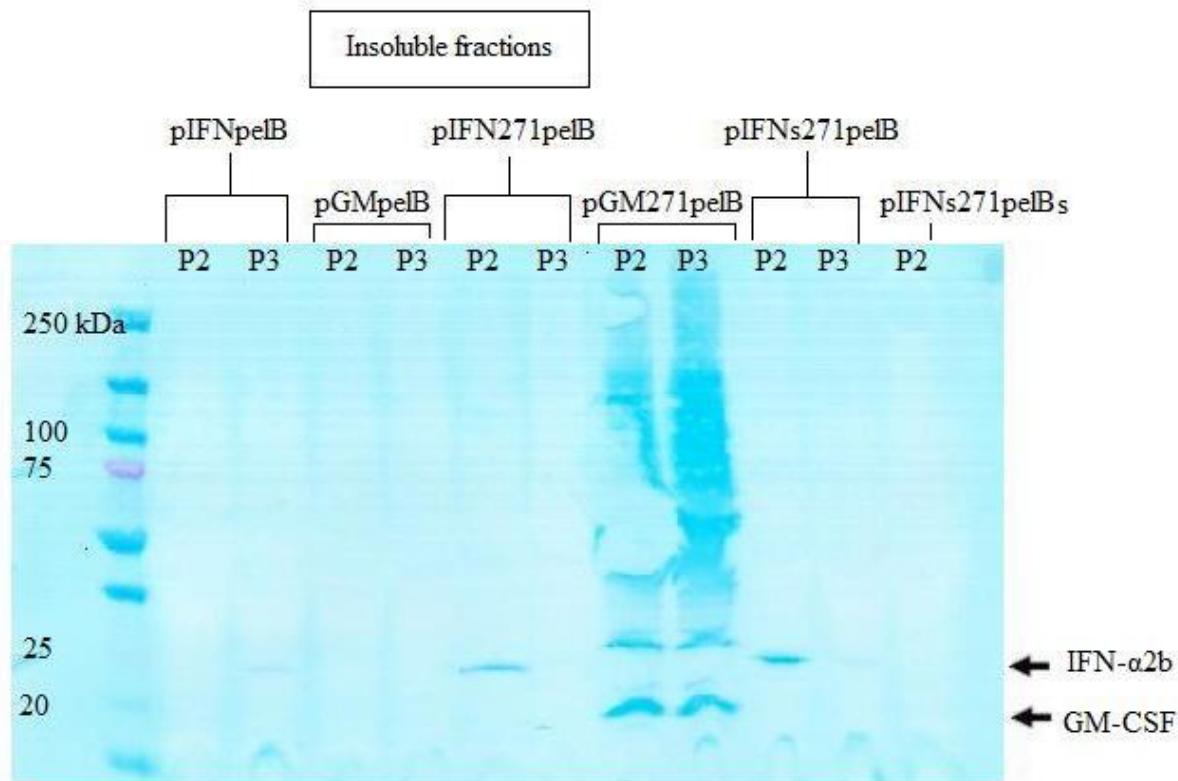
Figure 50: Western Blot results from insoluble fractions from fed batch fermentation of KT2440 strains (plasmid harboured indicated over each well). P2-P3 samples were taken 4 and 6 hours after induction respectively. Arrows indicated the recombinant proteins detected by anti-c-myc antibody, and the numbers on the left side represents molecule sizes in kilo Daltons

No bands were detected in soluble fractions from the fed batch fermentation cultures (result not shown). Nor the insoluble fraction of KT2440 (pGMs271pelBs) gave detection of protein. Because of high production during cultivation of KT2440 (pGMs271pelBs) in shake flasks, this was not expected. The detected recombinant proteins from insoluble fractions of the remaining strains are shown in Figure 50. Two of these strains, KT2440 (pIFN271pelB) and KT2440 (pGM271pelB), were expected to produce high amount of recombinant protein based on their production in shake flask experiment (Figure 44 and Figure 45 respectively). The amount produced in the fed batch fermentation (Figure 50) was therefore much lower than expected. Plasmid stability was also evaluated here (Attachment F Table F- 22), but was found not to be the reason for the low production.

Summarized, the increased protein productions seen in the shake flask experiment were not observed in the fed batch fermentation. Since there is one main difference between the cultivations of these experiments, it is thought that this might be the reason. During the shake flask experiment, induction was performed when the cultures still had glycerol available in abundant quantity. The cultures therefore grew without any limitations. In contrast, induction during the fed batch fermentation was performed when the cultures growth rate was controlled by the exponential feeding of glucose into the fermentor (see Chapter 1.2.5). If the inducer is consumed by KT2440, the fermentation culture will metabolize the inducer shortly after it is added. Shake flask cultures, on the other hand, have glycerol available and will therefore not consume the inducer as fast. The expression of recombinant proteins will thus be different when the same culture of KT2440 is cultivated in shake flasks or fed batch fermentation.

4 Conclusion

Two broad host range expression plasmids with *Pm/XylS* system, *pelB* signal sequence and *trfA* (wild type copy numbers) were constructed for evaluation of recombinant protein production in *P. fluorescens* and *P.putia*. Two human proteins, IFN-α2b and GM-CSF, were chosen as model proteins for the expression system.

During shake flask cultivations, low stability of the expression plasmids in *P. fluorescens* was found. *P. putida* KT2440 showed high plasmid stability under the same conditions and was chosen for further evaluation as expression platform.

Shake flask cultivation conditions for KT2440 that was optimized for growth and protein production revealed production of both model proteins both in rich and minimal media. Further evaluation of T2440 fed batch fermentations performed in minimal medium resulted in the same growth rate reported for *E. coli* cultivated under the same conditions. This indicated potential for KT2440 as industrial protein producer. Possible metabolism of the inducer m-toluic acid was believed to cause low protein production. This led to the evaluation of alternative inducers during fed batch fermentation, but nor o-toluic acid or 2-chloro benzoic acid gave protein production.

Genetic modifications of expression plasmids were performed to evaluate the effects on protein production. The exchange of *trfA* (the gene for replication protein TrfA) with *trfAcop271C* yielded a 3.5-fold increase of plasmid number in KT2440, the same as reported for *E. coli*. A shake flask experiment showed that the change in plasmid number increased the production of IFN-α2b and GM-CSF 3.5-fold or more. Additionally, soluble IFN-α2b was detected, something not reported for *E. coli* under the same conditions. Codon optimization of genes and signal sequence to KT2440 did not have significant effects under the conditions tested.

Fed batch fermentation of KT2440 harbouring genetically modified expression plasmids was performed to further evaluate protein production. Plasmid stability was found as high, but protein production obtained was lower than expected from results in the shake flask experiment. Additional production experiments performed with higher volumes in shake

Conclusion

flasks would reveal if low substrate concentrations during fed batch fermentations caused consummation of the inducer and thus lower production.

P. putida KT2440 have through this study proved as a potential industrial protein producer. It has shown similar growth properties as the widely used *E. coli* during fed batch fermentations. In addition, simple genetic modifications of expression plasmids proved to positively affect the production of model proteins. It is therefore believed that further optimization will increase the volumetric yield of the recombinant proteins.

4.1 Suggestions for further work

Like in most research projects, time is limited and some of the goals are not achieved. Choices have to be made, and there are elements that could be changed or might be eliminated.

The first point that would have strengthened the study is exact quantification of recombinant proteins. For this study, antibodies against C-myc or His6 sequences fused with the target genes were used to detect the model proteins. Standards with known concentrations of Phox-protein was in most cases also part of SDS-PAGE, but with only one dilution. These were compared to the amount of detected protein and gave an indication of the concentration. To quantify the amount of produced protein accurately there could have been executed several dilution of Phox as well as the samples with most amount of detected protein. By comparison of these bands, the concentration of protein produced could be measured and compared with the amount produced in *E. coli*. Detection could also been simplified by choosing model proteins with other detection possibilities. One example is fusion to fluorescent proteins, like mcherry (Clontech). Quantitative imagine directly on the culture would then both shorten detection time and enabled accurate quantification.

Two main genetic modifications were done to the expression vectors in this study. These two were increased copy number and codon optimization. Increased copy number proved to have positive effect on production of both model proteins under the conditions tested, as shown in Chapter 3.7.1.2. It would therefore be interesting to try to increase the copy number further and observe the following effects on protein production. Durland et al. have reported several mutation in *trfA* proven to increase the copy number of a specific plasmid in *E. coli* (Durland et al., 1990). For *trfAcop271C,* the 3.5-fold effect reported was also observed in *P. putida* KT2440 (Chapter 3.5.2). In addition, increased gene dosage had positive effect on expression of recombinant proteins. It would therefore be interesting to see if other mutations reported in Durland et al. 1990 would increase gene dosage and protein production in *P. putida* KT2440 further.

There are also other examples of genetic modifications that could increase production of recombinant proteins. In this study, *pelB* was chosen as signal sequence, but there are alternatives worth exploring. When testing *pelB, ompA* and synthetic signal sequence CSP,

Conclusion

Sletta et al. showed that the choice had high impact on expression of IFN-α2b and GM-CSF in *E. coli (Sletta et al., 2007)*. It would therefore be interesting to see if production would increase by changing *pelB* with one of these alternatives. Other elements proven to effect protein expression levels in *E. Coli* are different sequences in the 5'-untranslated region(UTR) of bacterial mRNA (Berg et al., 2009) and *Pm* promoter mutants (Winther-Larsen et al., 2000a). *Pm* mutants are currently being screened for the effect on protein production in *P. putida*. Positive results here would therefore be worthy of testing.

During this study of *P. putida* KT2440 it was observed an increase in CO_2 generation when inducing with m-toluic acid. Because KT2440 is derivative of the toluene-degrading *P. putida* mt-2 strain, this was assumed to be a sign of consummation. If this was the case, the inducer would be completely removed short time after induction in fed batch fermentations. It was tried to confirm this theory by analysing for m-toluic acid in HCDC samples, but the chosen method was not suited for this purpose. Finding an appropriate method for this analysis could therefore reveal whether KT2440 consume m-toluic acid. If consummation is detected, genetic engineering of the strains genome could remove this ability and increase the effect of induction.

The variation in production of recombinant proteins in this study makes it necessary to perform more studies on the constructed strains. Since there was detected high (but not quantified) amount of recombinant proteins in the shake flask experiment in Chapter 3.7.1, but not in HCDC of the same strains (Chapter 3.7.2), there is obvious that differences in setups effecting recombinant protein production. New experiments could be performed to reveal or if the hypothesis in Chapter 3.7.2.2 that was correct. If the amount of nutrition available at induction was the reason, this could be revealed by running similar shake flask experiment but with larger media volumes.

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Attachments

Attachment A: Abbreviations

Attachment B: Restriction sites nucleotide sequences

Attachment C: Standards: Gel electrophoresis and SDS PAGE

Attachment D: Version of Hi-media

Attachment E: Fermentation solutions

Attachment F: Collection of raw data from experiments

Attachment G: Eurofins sequence data

Attachment H: Attempt to quantify concentration of recombinant proteins in shake flask experiment

Attachment A.　　Abbreviations

A: nitrogen base Adenine

Amp: Ampicillin

bp: base pair

C: nitrogen base Cytosine

DNA: deoxyribonucleic acid

DO: Dissolved oxygen

EDTA: Ethylene Diamine Tetraacetic Acid

ER: endoplasmic reticulum

G: nitrogen base guanine

GOI: gene of interest

IB: inclusion body

Kan: Kanamycin

kb: kilo base pair

kDa: kilo Daltons

LB: Lauria Bertani broth

LA plates: Agar plates made with LB medium

mRNA: messenger RNA

NEB: New England Biolabs

OD(x): Optical density at wave length x

p: promoter region

Pm: Promoter controlling the expression of *meta*-cleavage pathway in pWW0 and recombinant protein in *Pm/XylS* system

rDNA: recombinant DNA

RNA: ribonucleic acid

RNAP: RNA polymerase

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

T: nitrogen base Thymine

t: termination sequence

U: nitrogen base Uracil

Attachment B.   Restriction sites nucleotide sequences

Table B-1: Restriction enzymes used during the clonings of this study. Single letter code N stands for any of the four bases (A, C, G or T), while W codes for A or T. Arrows indicate where the enzyme cuts.

Restriction enzyme	Restriction site (nucleotide sequence)
AdhI	5´...GACNNN▾NNGTC...3´ 3´...CTGNN▴NNNCAG...5´
MunI/MfeI	5´...C▾AATTG...3´ 3´...GTTAA▴C...5´
NcoI	5´...C▾CATGG...3´ 3´...GGTAC▴C...5´
NdeI	5´...CA▾TATG...3´ 3´...GTAT▴AC...5´
NotI	5´...GC▾GGCCGC...3´ 3´...CGCCGG▴CG...5´
SexAI	5´...A▾CCWGGT...3´ 3´...TGGWCC▴A...5´

Attachment C.   Standards: Gel electrophoresis and SDS PAGE

Gel electrophoresis
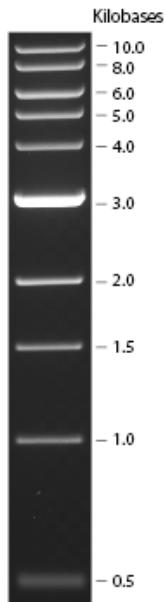
Figure C- 1: 1 kb DNA ladder (NEB) visualized by ethidium staining on a 0.8 % TAE agarose gel. Kilo bases (kb) are indicated in the right column
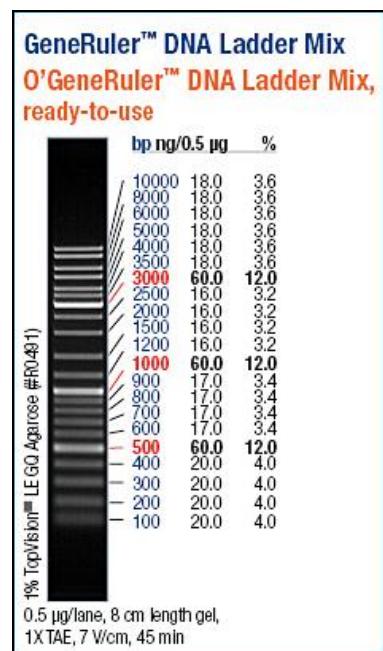
Figure C- 2 GeneRuler^{TM} DNA Ladder Mix 0.5 μg/μl 50 μg. First line to the left indicate base pair sizes (bp) (Fermentas)

SDS-PAGE
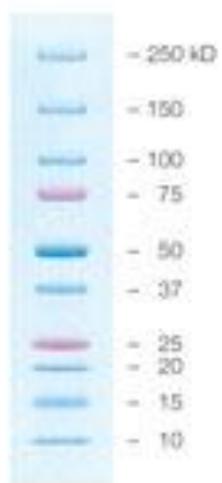
Figure C- 3: Precision Plus Protein^{TM} Standards Dual Color (BioRad). The number to the left indicate the different sizes for poly peptides.

Attachment D.   Version of Hi-media

<u>Stock solution for Hi-and Hf.-medium</u>

6 g/l Fe (III) citrate hydrate (MERCK)

30 g/l H_3BO_3 (Sigma)

10 g/l $MnCl_2 \cdot 4H_2O$ (Riedel-deHäen)

84 g/l EDTA $\cdot 2H_2O$ (titriplex) (Prolabo)

15 g/l $CuCl_2 \cdot 2H_2O$ (MERCK)

25 g/l $Na_2Mo_4O_4 \cdot 2H_2O$ (Riedel-deHäen)

25 g/l $CoCl_2 \cdot 6H_2O$ (Riedel-deHäen)

4 g/l $Zn(CH_3COO)_2 \cdot 2H_2O$ (Fluka)

20 g/l Citrate solution

    20 g/l   Na-Citrat (Sigma)

pH adjusted to 7.0 with 1M NaOH

100 g/l Yeast extract HI

    100 g/l Yeast extract (Oxoid)

Autoclaved at 120°C for 20 minutes

1M $MgSO_4 \cdot 7H_2O$

    246 g/l $MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich)

Autoclaved at 120°C for 20 minutes

250 g/l Glucose $\cdot H_2O$

    250 g Glucose $\cdot H_2O$ (Apotekerproduksjon)

    Ion-free water to total volume 1000 ml

Autoclaved at 120°C for 20 minutes

20 ml/lGlycerol

    590 g/l Glycerol 86-88% (Sigma-Aldrich)

    Ion-free water to total volume 1000 ml

Autoclaved at 120°C for 20 minutes

Attachment D: Version of Hi-media

250 g/l Fructose

     250 g/l Fructose (Sigma)

     Ion-free water to total volume 1000 ml

Autoclaved at 120°C for 20 minutes

250 g/l Glycerol

     250 g/l Glycerol 86-88% (Sigma-Aldrich)

     Ion-free water to total volume 1000 ml

Autoclaved at 120°C for 20 minutes

590 g/l Glycerol

     590 g/l Glycerol 86-88% (Sigma-Aldrich)

     Ion-free water to total volume 1000 ml

Autoclaved at 120°C for 20 minutes

Attachment D: Version of Hi-media

Reduced Hi+YE medium

      8.6 g/l $Na_2HPO_4 \cdot 2H_2O$ (Sigma-Aldrich)

      3 g/l KH_2PO_4 (Riedel-deHaën)

      0.3 g/l NH_4Cl (Riedel-deHaën)

      0.5 g/l $NaCl$ (Apotekerproduksjon)

      3.3 ml stock solution/l Fe (III) citrat hydrate

      0.03 ml stock solution/l H_3BO_3

      0.5 ml stock solution/l $MnCl_2 \cdot 4H_2O$

      0.03 ml stock solution/l EDTA $\cdot 2H_2O$

      0.03 ml stock solution/l $CuCl_2 \cdot 2H_2O$

      0.03 ml stock solution/l $Na_2Mo_4O_4 \cdot 2H_2O$

      0.03 ml stock solution/l $CoCl_2 \cdot 6H_2O$

      0.67 ml stock solution/l $Zn(CH_3COO)_2 \cdot 2H_2O$

      10 g/l MOPS (Sigma)

      817 g/l Ion-free water

pH adjusted to 7.0

After autoclavation the following volume of sterile stock solutions was added:

      50 ml/l 20 g/l Citrate solution

      100 ml/l Yeast extract HI

      0.9 ml/l 1M $MgSO_4 \cdot 7H_2O$

      20 ml/l 590 g/l Glycerol

      8 ml/l 250 g/l Glucose $\cdot H_2O$

Attachment D: Version of Hi-media

Hi-10YE-10GLY-2GLU

     8.6 g/l $Na_2HPO_4 \cdot 2H_2O$ (Sigma-Aldrich)

     3 g/l KH_2PO_4 (Riedel-deHaën)

     1.0 g/l NH_4Cl (Riedel-deHaën)

     10 g/l MOPS (Sigma)

     0.5 g/l NaCl (Apotekerproduksjon)

     10.0 ml stock solution/l Fe(III) citrat hydrate

     0.1 ml stock solution/l H_3BO_3

     1.5 ml stock solution/l $MnCl_2 \cdot 4H_2O$

     0.1 ml stock solution/l $EDTA \cdot 2H_2O$

     0.1 ml stock solution/l $CuCl_2 \cdot 2H_2O$

     0.1 ml stock solution/l $Na_2Mo_4O_4 \cdot 2H_2O$

     0.1 ml stock solution/l $CoCl_2 \cdot 6H_2O$

     2.0 ml stock solution/l $Zn (CH_3COO)_2 \cdot 2H_2O$

     855.5 g/l Ion-free water

pH adjusted to 7.0 before autoclaving at 120°C for 20 minutes

After autoclavation the following volume of sterile stock solutions was added:

     100 ml/l Yeast extract HI

     2.5 ml/l 1M $MgSO_4 \cdot 7H_2O$

     8 ml/l 250 g/l Glucose $\cdot H_2O$

     20 ml/l 85 % Glycerol

Attachment D: Version of Hi-media

HiYe reduced broth

    1. Basis broth

Solution A

       8.6 g/l $Na_2HPO_4 \cdot 2H_2O$ (Sigma-Aldrich)

       3 g/l KH_2PO_4 (Riedel-deHaën)

       0.3 g/l NH_4Cl (Riedel-deHaën)

       0.5 g/l NaCl (Apotekerproduksjon)

       3.3 ml stock solution/l Fe (III) citrat hydrate

       0.03 ml stock solution/l H_3BO_3

       0.5 ml stock solution/l $MnCl_2 \cdot 4H_2O$

       0.03 ml stock solution/l EDTA $\cdot 2H_2O$

       0.03 ml stock solution/l $CuCl_2 \cdot 2H_2O$

       0.03 ml stock solution/l $Na_2Mo_4O_4 \cdot 2H_2O$

       0.03 ml stock solution/l $CoCl_2 \cdot 6H_2O$

       0.67 ml stock solution/l $Zn(CH_3COO)_2 \cdot 2H_2O$

       2 g/l Yeast extract (Oxoid)

       2 g/l Glucose $\cdot H_2O$ (Apotekerproduksjon)

       650 g/l Ion-free water

Solution B:

       20 g/l Citric acid (final concentration 1 g/l)

pH adjusted to 7

Solution C:

       1 M $MgSO_4 \cdot 7H_2O$ stock solution

The basis broth was made by mixing solution A and solution B and adding 0.9 ml of solution C. The mixture was then sterile filtered and eventually added antibiotics.

    2. Induction solution

    25.5 g/l Glycerol (100%) (AnalaR NORMAPUR)

    24.0 g/l Yeast extract (Oxoid)

    To 1000 ml Tap water

Sterile filtered and eventually antibiotics added before use.

Attachment D: Version of Hi-media

Hi-10YE-10X*

Followed the recipe for "Reduced Hi+YE medium" to and including MOPS. Thereafter the following was added:

> 805.4 g/l Ion-free water

pH adjusted to 7.0

After autoclavation the following volume of sterile stock solutions was added:

> 50 ml/l 20 g/l Citrate solution
>
> 100 ml/l Yeast extract HI
>
> 0.9 ml/l 1M $MgSO_4 \cdot 7H_2O$
>
> *40 ml/l 250 g/l C-source:
>
>> if X = GLU: Glucose
>>
>> if X = FRU: Fructose
>>
>> if X = GLY: Glycerol

HiYe-8GLY-2GLU

Followed the recipe for "Reduced Hi+YE medium" to and including MOPS. Thereafter the following was added:

> 820.5 g/l Ion-free water

pH adjusted to 6.5

After autoclavation the following volume of sterile stock solutions was added:

> 50 ml/l 20 g/l Citrate solution
>
> 100 ml/l Yeast extract HI
>
> 0.9 ml/l 1M $MgSO_4 \cdot 7H_2O$
>
> 16 ml/l 590 g/l Glycerol
>
> 8 ml/l 250 g/l Glucose

Attachment E.   Fermentation solutions

Inoculum LB-medium

LB (g)

10 g/l Trypton (Oxoid)

10 g/l Yeast Extract (Oxoid)

5 g/l NaCl (Apotekerproduksjon)

900 g/l Ione free water

90 ml is added to 500-ml-shaking flasks and autoclaved for 20 minutes at 120 °C

Glucose LB – inoculum and Hi

100 g/l Glucose (Apotekerproduksjon) diluted in ion free water in a graduated flask

Autoclaved for 20 minutes at 120 °C

Before inoculation

· 10 ml of "Glucose LB-inoculum and Hi" was added to the 500-ml-shaking flask containing 90 ml LB (g)

· Kanamycin (40 mg/ml) was added to a total concentration of 40 µg/ml

Attachment E: Fermentation solutions

Inoculum Hi-medium

(For stock solutions see Attachment D)

Hi-medium

8.6 g/l Na_2HPO_4 (Sigma-Aldrich)

3.0 g/l KH_2PO_4 (Riedel-deHäen)

1.0 g/l NH_4Cl (Riedel-deHäen)

0.5 g/l NaCl (Apotekerproduksjon)

10 ml stock/l Fe (III) citrate hydrate

0.1 ml stock/l H_3BO_3

1.5 ml stock/l $MnCl_2 \cdot 4H_2O$

0.1 ml stock/l $EDTA \cdot 2H_2O$

0.1 ml stock/l $CuCl_2 \cdot 2H_2O$

0.1 ml stock/l $Na_2Mo_4O_4 \cdot 2H_2O$

0.1 ml stock/l $CoCl_2 \cdot 6H_2O$

2.0 ml stock/l $Zn (CH_3COO)_2 \cdot 2H_2O$

900 g/l ion free water

90 ml is added to 500-ml-shaking flasks and autoclaved for 20 minutes at 120 °C

Before inoculation

- 10 ml of "Glucose LB-inoculum and Hi" is added to the 500-ml-shaking flask containing 90 ml Hi-medium
- 0.234 ml 1M $MgSO_4$-stock was added to each flask
- Kanamycin (40 mg/ml) was added to a total concentration of 40 μg/ml

Attachment E: Fermentation solutions

<u>Glucose 1 stock solution</u>

    96.10 g Glucose · H_2O (Apotekerproduksjon)

    Ion-free water to total volume 200 ml

   Autoclaved at 120°C for 20 minutes

<u>Feeding solution</u>

  Glucose 2/ Mg- feeding solution

    1250 g/l Glucose · H_2O (Apotekerproduksjon)

    Autoclaved separately

    444 g/l $MgSO_4 \cdot 7H_2O$ (Sigma)

    Autoclaved separately

  600 ml autoclaved1250 g/l Glucose · H_2O and 50 ml autoclaved 444 g/l $MgSO_4 \cdot 7H_2O$

  is mixed and connected to the fermentors

  Antifoam

    1+10 diluted Adecanol (Asahi Denka Kogyo)

Autoclaved at 120°C for 20 minutes

Attachment E: Fermentation solutions

Hf.1 medium

16.6 g/l KH_2PO_4 (Riedel-deHaën)

4 g/l $(NH_4)_2HPO_4$ (Sigma)

2.3 g/l Citric acid $\cdot H_2O$ (Sigma)

12.5 ml/l stock Fe (III) citrat hydrate

0.127 ml/l stock H_3BO_3

1.88 ml/l stock $MnCl_2*4H_2O$

0.125 ml/l stock $EDTA*2H_2O$

0.127 ml/l stock $CuCl_2*2H_2O$

0.124 ml/l stock $Na_2Mo_4O_4*2H_2O$

0.124 ml/l stock $CoCl_2*6H_2O$

2.5 ml/l stock Zn $(CH_3COO)_2*2H_2O$

936 g/l Ion-free water

Aliquoted to each fermentor (327.5 ml to 1-L fermentors and 700 ml to 3-L fermentors)before autoclavation.

For 1-L fermentations the following was added to each fermentor

- 20 ml Glucose 1 stock solution
- 2.15 ml 1M $MgSO_4 \cdot 7H_2O$ stock solution
- 0.35 ml 40 mg/ml kanamycin
- 0.2 ml Antifoam

For 3-L fermentations the following was added to each fermentor

- 50 ml Glucose 1 stock solution
- 4.6 ml 1M $MgSO_4 \cdot 7H_2O$ stock solution
- 0.75 ml 40 mg/ml kanamycin
- 0.5 ml Antifoam

Ammonia (25 %) diluted 1:1 (w/w) in ion- free water is used for pH control

Attachment F.   Collection of raw data from experiments

For details concerning measurements of growth and pH, see Chapter 2.4.2

Plasmid stability

To express a recombinant protein from an expression vector it is essential that the host of this vector is able to carry it. Not all bacteria will allow the plasmid inside the cell and therefore eliminate it. To calculate the plasmid stability cultures can be grown on agar plates without selection. After visible colonies have formed, they can be transferred to a selective plate. Now only bacteria harbouring plasmids with resistance will be able to grow.

The plasmid stability is calculated by:

$$\text{Plasmid stability} = \frac{\text{number of upgrown coloines on selection}}{\text{colonies picked from unselected agar}} \times 100\% \qquad \text{Eq. (2)}$$

Attachment F: Collection of raw data from experiments

Calculation of multiplication factor for OD_{600} measurements in deep well plates

The relationship between OD_{600} measurements in cuvettes and 96-wells UV plates (OD-costar) (Costar), used for harvesting samples from deep wall plates, had to be established. This is done by measuring the OD_{600} from the same culture both in cuvette and on a well plate on Spectra Max (BMG Labtech). The multiplication factor is calculated by finding the relationship between OD-cuvette and OD-costar. The calculated factors are shown in Table F-1.

Table F- 1: Growth measurements (OD_{600}) in cuvette and costar plates of precultures for deep-well experiment with *P. fluorescens* SBW25 and *E. coli* RV308 strains (left column). The precultures were grown in 50 ml selective LB medium for at 200 rpm 16 hours. The relationship between OD measurements in cuvette and on costar plates were used to calculate factor needed for OD_{600} measurement during the experiment.

Strain (plasmid)	OD_{600} cuvette	OD_{600} costar plates	Factor (OD_{600} cuvette/ OD_{600} costar)
SBW25 (pIFNpelB)	0.0345	0.00800	4.3
SBW25 (pGMpelB)	0.0265	0.00633	4.2
RV308 (pIFNpelB)	0.3585	0.06866	5.2
RV308 (pGMpelB)	0.3485	0.07033	5.0
RV308 (pAT64)	0.4045	0.07630	5.3
RV308 (pMV11)	0.3965	0.07700	5.2

The measurements showed in Table F- 2 have been multiplied by their dilution factor from Table F- 1.

Table F- 2: Growth measurements (OD$_{600}$) from deep-well experiment of *P. fluorescens* SBW25 and *E. coli* RV308 strains (first and second column from the right). The cultures were grown in reduced HiYe medium at 25°C (800 rpm). Parallels were induced to a final concentration of 0.5 mM m-toluic acid after reaching OD$_{600}$≈ 5.

Strain	Hours after inoculation	Pre culture	3	5,5	6	7,4	8	8,8	9,5	11	22
P. fluorescens	SWB25 (pIFNpelB)	0,35	0,00	0,05	0,09			0,10			0,26
P. fluoresces	SWB25 (pGMpelB)	0,26	0,00	0,06	0,08			0,10			0,34
E. coli	RV308 (pIFNpelB)	3,59	0,10	0,40	0,7	1,5	1,7	2,3	3,4	4,7	
E. coli	RV308 (pGMpelB)	3,49	0,10	0,45	0,7	1,1	1,3	2,1	2,8	4,2	
E. coli	RV308 (pAT64)	4,05	0,18	0,90	1,1	2,3	2,3	3,7	4,6	6,6	
E. coli	RV308 (pMV11)	3,97	0,12	0,94	1,2	2,3	2,7	3,7	4,7	7,7	

Table F- 3: Calculated plasmid stability for *P. fluorescens* and *P. putida* strains (two left columns) grown in 50 ml selective LB, PIA and Hi-10YE-10GLY-2GLU (third column) before plating out on LA without selection. 75 colonies were transferred to LA with selection. KT2440 (pIFNpelB) had been induced with m-toluic acid to a final concentration of 0.5 mM under cultivation in the different media.

Strain		Media (with selection)	Plasmid stability
	SWB25 (pHH100)	LB	13,30 %
		PIA	5,30 %
P. fluorescens		Hi-10YE-10GLY-2GLU	1,30 %
	NCIMB (pHH100)	LB	2,70 %
		PIA	1,30 %
P. fluorescens		Hi-10YE-10GLY-2GLU	0 %
	KT2440(pHH100-synluc)	LB	100 %
		PIA	92 %
P. putida		Hi-10YE-10GLY-2GLU	70 %
	KT2440(pIFNpelB)	LB	100 %
		PIA	100 %
P. putida		Hi-10YE-10GLY-2GLU	100 %
	Induced		

Table F- 4: Growth measurements (OD$_{660}$) of precultures for shake flask growth experiments of KT2440 WT and KT2440 (pGMpelB). The precultures were grown in 50 ml LB (with antibiotics for KT2440 (pGMpelB)) at 30°C, 200 rpm for 16 hours.

Strain: *P. putida*	OD$_{660}$
KT2440 WT	5,6
KT2440 (pGMpelB)	5,66

Table F- 5: Growth measurements (OD$_{660}$) of KT2440 WT grown in 50 ml medium (left column). The cultures were grown at 30°C and 200 rpm.

Strain: KT2440 WT				
Hours after inoculation	**4**	**8**	**11**	**24**
PIA	1,23	2,40	3,08	4,45
M9	0,63	3,72	6,10	5,60
Hi-10YE-10GLU	3,30	8,34	9,32	10,43
Hi-10YE-10FRU	3,57	6,30	7,30	10,98
Hi-10YE-10GLY	3,12	5,82	6,79	10,80

Table F- 6: pH measurements from different media (left column) used in shake flask experiment with KT2440 WT. The cultures were grown at 30°C and 200 rpm

Strain: KT2440 WT				
Hours after inoculation	**4**	**8**	**11**	**24**
PIA	6,3	5,35	5,24	5,75
M9	6,71	6	6,3	6,58
Hi-10YE-10GLU	7,16	7,3	7,38	7,5
Hi-10YE-10FRU	7,25	7,41	7,49	7,47
Hi-10YE-10GLY	7,25	7,46	7,53	7,42

Attachment F: Collection of raw data from experiments

Table F- 7: Growth measurements (OD$_{660}$) of KT2440 (pGMpelB) grown and induced in 50 ml medium (left column). The cultures were grown at 30°C and 200 rpm. Parallels were induced with m-toluic acid to a final concentration of 0.5 mM four hours after inoculation.

Strain: KT2440(pGMpelB)				
Hours after inoculation	**4**	**8**	**11**	**24**
PIA	1,2	2,16	2,97	3,87
PIA, induced		1,92	2,72	3,67
M9	0,5	3,78	5,48	4,63
M9, induced		3,24	3,65	2,81
Hi-10YE-10GLU	2,28	6,78	9,16	9,86
Hi-10YE-10GLU, induced		8,7	11,81	10,55
Hi-10YE-10FRU	2,67	6,6	7,66	9,1
Hi-10YE-10FRU, induced		6,9	8,33	9,8
Hi-10YE-10GLY	2,79	6,36	7,34	9,06
Hi-10YE-10GLY, induced		6,96	7,65	10,06

Table F- 8: pH measurements in the different medium (left column) used to grow KT2440 (pGMpelB). The cultures were grown at 30°C and 200 rpm. Parallels were induced with m-toluic acid to a final concentration of 0.5 mM four hours after inoculation.

Strain: KT2440(pGMpelB)				
Hours after inoculation	**4**	**8**	**11**	**24**
PIA	6,7	5,46	5,43	7,28
PIA, induced		5,34	5,2	4,8
M9	6,8	6,18	6,01	6,56
M9, induced		6,18	5,95	6,12
Hi-10YE-10GLU	7,15	7,36	7,27	7,56
Hi-10YE-10GLU, induced		7,2	7,33	7,52
Hi-10YE-10FRU	7,21	7,36	7,4	7,51
Hi-10YE-10FRU, induced		7,44	7,43	7,48
Hi-10YE-10GLY	7,22	7,45	7,52	7,56
Hi-10YE-10GLY, induced		7,5	7,5	7,48

Table F- 9: Growth measurements (OD$_{660}$) of precultures for shake flask experiment with KT2440 WT, KT2440 (pIFNpelB) and KT2440 (pGMpelB). The precultures were grown in 50 ml selective LB (no antibiotics for KT2440 WT) at 30°C and 200 rpm for 16 hours

Strain	OD$_{660}$
KT2440 WT	6,43
KT2440 (pIFNpelB)	4,43
KT2440 (pGMpelB)	4,72

Table F- 10: Growth measurements (OD$_{660}$) from shake flask experiment with KT2440 WT, KT2440 (pIFNpelB) and KT2440 (pGMpelB) (left column). The cultures were grown in 100 ml M9-10GLU at 30°C and 200 rpm. Induction was performed 8.5 hours after inoculation by adding m-toluic acid to a final concentration of 0.5 mM.

Strain	Hours after inoculation			
	3	8,5	11,5	25
KT2440 WT	0,37	2,24	5,5	6,53
KT2440 (pIFNpelB)	0,2	1,16	3,13	5,12
KT2440 (pIFNpelB) - Induced	0,2	1,4	3,07	5,57
KT2440 (pGMpelB)	0,2	1,1	3,42	5,63
KT2440 (pGMpelB) - Induced	0,2	1,2	3,61	6,6

Table F- 11: pH measurements from shake flask experiment with KT2440 WT, KT2440 (pIFNpelB) and KT2440 (pGMpelB) (left column). The cultures were grown in 100 ml M9-10GLU at 30°C and 200 rpm.

Strain	Hours after inoculation			
	3	8,5	11,5	25
KT2440 WT	6,71	5,86	4,26	3,95
KT2440 (pIFNpelB)	6,82	6,46	4,52	4,86
KT2440 (pIFNpelB) - Induced	6,81	6,47	4,82	5,42
KT2440 (pGMpelB)	6,9	6,41	4,53	5,94
KT2440 (pGMpelB) - Induced	6,8	6,39	4,55	4,07

Table F- 12: Growth measurements (OD$_{660}$) from shake flask experiment with KT2440 WT, KT2440 (pIFNpelB) and KT2440 (pGMpelB) (left column). The cultures were grown in 100 ml HiYe-8GLY-2GLU at 30°C and 200 rpm. Induction was performed 8.5 hours after inoculation by adding m-toluic acid to a final concentration of 0.5 mM.

Strain	Hours after inoculation			
	3	**8,5**	**11,5**	**25**
KT2440 WT	1,55	7,02	8,41	12,44
KT2440 (pIFNpelB)	0,6	6,3	8,32	10,45
KT2440 (pIFNpelB) - Induced	0,5	6,42	9,02	11,39
KT2440 (pGMpelB)	0,8	6,44	8,47	9,58
KT2440 (pGMpelB) - Induced	0,7	6,14	8,71	10,75

Table F- 13: pH measurements from shake flask experiment with KT2440 WT, KT2440 (pIFNpelB) and KT2440 (pGMpelB) (left column). The cultures were grown in 100 ml HiYe-8GLY-2GLU at 30°C and 200 rpm. 8.5 hours after inoculation pH was adjusted from approximately 7.0 to 6.5.

Strain	Hours after inoculation				
	3	**8,5**		**11,5**	**25**
KT2440 WT	7,15	6,99		7,06	7,15
KT2440 (pIFNpelB)	6,94	6,97	6,53	6,61	6,53
KT2440 (pIFNpelB) - Induced	6,84	6,97	6,58	6,62	6,59
KT2440 (pGMpelB)	6,83	6,99	6,53	6,69	6,56
KT2440 (pGMpelB) - Induced	6,98	6,98	6,54	6,59	6,53

Table F- 14: Growth measurements (OD$_{660}$) of precultures for fed batch fermentation with KT2440 WT, KT2440 (pJB861), KT2440 (pIFNpelB) and KT2440 (pGMpelB). The precultures were grown in 100 ml selective Hi-medium (no antibiotics for KT2440 WT) at 30°C and 200 rpm for 16 -18 hours

Strain	OD$_{600}$
KT2440 WT	4,85
KT2440 (pJB861)	8,28
KT2440 (pIFNpelB)	6,89
KT2440 (pGMpelB)	7,08

Table F- 15: Growth measurements (OD$_{600}$) from 1 l-fed batch fermentation with KT2440 WT, KT2440 (pJB861), KT2440 (pIFNpelB) and KT2440 (pGMpelB). The fermentation was executed in Hf.1 medium at 30°C and pH 6.5. Induction was performed by adding m-toluic acid to a final concentration of 0.5 mM when OD$_{600}$ ≈ 80-90.

Strain	Hours after inoculation										
	0,00	6,25	15,33	17,17	17,43	19,18	19,37	19,60	21,62	23,58	26,25
KT2440 WT	0,05	1,68	52,8				120		152,2	172	197,8
KT2440 (pJB861)	0,05	1,34	29,7				85,7		125,1	153,6	178,7
KT2440 (pIFNpelB)	0,05	1,83	51,3	76,2	79,95			125,9	148,5	172,8	196,7
KT2440 (pGMpelB)	0,05	1,38	31,65	51		84	93,2		132,4	141,5	175,6

Table F- 16: Calculated specific growth rate (µ) and doubling time based on OD$_{600}$ measurements during free growth in 1-l fed batch fermentation.

	OD$_{600}$			
HAI	KT2440 WT	KT2440 (pJB861)	KT2440 (pIFNpelB)	KT2440 (pGMpelB)
0,00	0,05	0,05	0,05	0,05
6,25	1,68	1,34	1,83	1,38
	Calculated µ and doubling time for each strain			
µ (h^{-1})	0,56	0,53	0,58	0,53
Doubling time (h)	1,23	1,32	1,20	1,31

Table F- 17: Growth measurements (OD$_{660}$) from 1-l fed batch fermentation with KT2440 (pGMpelB). The fermentation was executed in Hf.1 medium at 30°C and pH 6.5. Induction was performed OD$_{600}$ ≈ 80-90.

Hours after inoculation	*Pseudonomas putida* KT2440 (pDI-gmcsf)			
	Continously m-toluic acid	m-toluic acid	o-toluic acid	2-chloro benzoic acid
0	0,05	0,05	0,05	0,05
7,25	2,21	2,39	2,41	2,37
16,18	32,1	44,1	35,6	38,7
17,43	37,2	53,9	49,5	50,1
18,67			63,6	
19,67	59,4	89,9		
19,8		93,6	83,4	87
20,63	79,53			
20,72	79,8			
21,77		133,2	124,5	135,1
22,8	113,1			
23,37		143,4	145,4	145,02
24,27	128,1			
24,87		168,6	155,5	160,6
25,73	138,9			

Attachment F: Collection of raw data from experiments

Table F- 18: Growth measurements (OD$_{600}$) of precultures (left column) for shake flask experiment. The precultures were grown in 50 ml selective LB medium at 30°C, 200 rpm for 10 hours.

Strain	OD$_{600}$
KT2440 (pIFNpelB)	7,15
KT2440(pGMpelB)	7,10
KT2440(pIFN271pelB)	1,16
KT2440 (pGM271pelB)	0,52
KT2440 (pIFNs271pelB)	6,94
KT2440 (pIFNs271pelBs)	8,28
KT2440 (pGMs271pelBs)	1,00

Table F- 19: Growth measurements (OD$_{600}$) of strains from shake flask experiment in HiYe-8GLY-2GLU at 30°C, 200 rpm. Induction to a final concentration of 0.5 mM m-toluic acid was performed when cultures reached OD$_{600}$ 1-5.

Strain (plasmid)	Hours after inoculation						
	0	4,75	6,75	10,08	11	15,33	25,5
KT2440 (pIFNpelB)	0,29	5,2	7,07	10,85			13,33
KT2440 (pIFNpelB) - induced			7,88	12,01			15,13
KT2440 (pGMpelB)	0,28	5,29	8,53	11,22			14,26
KT2440 (pGMpelB) - induced			7,56	11,58			15,6
KT2440 (pIFN271pelB)	0,04	0,26	0,45	1,56	2,8	10,31	12,47
KT2440 (pIFN271pelB) - induced						7,82	7,51
KT2440 (pGM271pelB)	0,02	0,08	0,14	0,302	0,5	2,28	14,16
KT2440 (pGM271pelB) - induced						0,6	6,67
KT2440 (pIFNs271pelB)	0,28	4,45	8	11,71			14,92
KT2440 (pIFNs271pelB) - induced			8,04	13,31			16,07
KT2440 (pIFNs271pelBs)	0,33	4,82	7,87	11,62			15,36
KT2440 (pIFNs271pelBs) - induced			7,97	12,8			15,26
KT2440 (pGMs271pelBs)	0,04	0,28	0,61	2,77	5,43	13,43	16,78
KT2440 (pGMs271pelBs) - induced						10,42	5,62

Attachment F: Collection of raw data from experiments

Table F- 20: Growth measurements (OD$_{660}$) of precultures for 3-l fed batch fermentation with strains indicated in the left column. The precultures were grown in 100 ml selective Hi-medium at 30°C and 200 rpm for 16 -18 hours

Precultures	OD600
KT2440 (pIFNpelB)	5,94
KT2440 (pGMpelB)	5,92
KT2440 (pIFN271pelB)	4,48
KT2440 (pGM271pelB)	4,04
KT2440 (pIFNs271pelB)	4,44
KT2440 (pIFNs271pelBs)	5,08
KT2440 (pGMs271pelBs)	3,24
KT2440 (pJB861)	6,84

Table F- 21: Growth measurements (OD$_{600}$) from 3 l-fed batch fermentation with strains indicated in the left column. The fermentation was executed in Hf.1 medium at 30°C and pH 6.5. Induction was performed by adding m-toluic acid to a final concentration of 1 mM when OD$_{600}$ ≈ 80-90, thereafter reinduction the following 2 and 4 hours (both times to a final concentration of 0.5 mM).

Strains	Hours after inoculation											
	0,0	5,9	13,7	16,1	16,9	18,2	19,2	20,3	21,3	22,3	24,3	26,3
KT2440 (pIFNpelB)	0,05	2,22	35,30	62,4		89	95	98	104	104	119	
KT2440 (pGMpelB)	0,05	1,60	26,55	51,5		75	83	94	98	101	112	
KT2440 (pIFN271pelB)	0,05	2,31	39,75	71,0	82	95,4	107	118	125			
KT2440 (pGM271pelB)	0,05	2,39	27,00	52,7		76,4	81	94,8	93	94,8	101	
KT2440(pIFNs271pelB)	0,05	2,41	39,82	70,8	83,1	92,1	100,8	110,4	120,9			
KT2440(pIFNs271pelBs)	0,05	1,24	22,72	39,8		61,2		80,4	90	93	105,3	118,2
KT2440(pGMs271pelBs)	0,05	1,07	7,20	13,2	15,15	18,8		23,1		26,4	30,3	38,3
KT2440 (pJB861)	0,05	1,35	18,90	39,5		63,2		85,2	97,2	99,6	111	124,2

Attachment F: Collection of raw data from experiments

Table F- 22: Plasmid stability evaluation of KT2440 (pIFN271pelB), KT2440 (pGM271pelB) and KT2440 (pIFNs271pelB), KT2440 (pIFNs271pelBs) and KT2440 (pGMs271pelBs) during fed batch fermentation. Samples were taken late in the fermentation (after induction)

Strain	Plasmid stability
KT2440 (pIFN271pelB)	99 %
KT2440 (pGM271pelB)	89 %
KT2440 (pIFNs271pelB)	100 %
KT2440 (pIFNs271pelBs)	74 %
KT2440 (pGMs271pelBs)	100 %

Dry weight measurements from KT2440 samples were performed to compare it with known values for *E. coli*.

10 ml ferment cultures were centrifuged at 4000 rpm for 15 minutes. The pellet was washed in 10 ml ion free water before centrifuging again at the same settings. The pellet was washed and placed in a pre weighted aluminium cup. This was left incubating at 50°C overnight before the cup was weighted again. The relationship between OD (600) and dry cell weight is calculated. The results are shown in Table F- 23.

Table F- 23: Calculation of OD_{600}/dried-cell-mass relationship for *P. putida* KT2440.

OD_{600}	Cup (g)	Cup + dried cell mass (g)	Dried cell mass (g/10 ml)	Dried cell mass (g/l)	OD_{600}/dried cell mass
125,10	2,18	2,57	0,40	39,56	3,16
120,90	2,18	2,54	0,36	36,14	3,35

Attachment G.   Eurofins sequence data

Plasmids were isolated by Wizard protocols (see Chapter 2.4.3). 15 µl DNA solutions was transferred to clean 1.5 ml tubes (Eppendorf) and marked with a sequence label from Eurofins MWG Operon. Before sending in for sequencing, a primer was designed.

This was done in Clone Manager Professional Suite. The sequence was chosen to be about 100 bp from the wanted sequence start.

After receiving the sequence results, they were aligned with the template sequence in Clone Manager Professional Suite.

Results from sequencing transformants of pIFN271pelB and pGM271pelB

Primer: trfAseq

Sequence: 5' CCGATCACCTTCACGTTCTAC 3'

trfAcop271C sequence of pIFN271pelB

CTGGTCGATCATGGCCGGTATTACACGAAGGCCGAGGAATGCCTGTCGCGCCTACAGGCGACGGC
GATGGGCTTCACGTCCGACCGCGTTGGGCACCTGGAATCGGTGTCGCTGCTGCACTGCTTCCGCGT
CCTGGACCGTGGCAAGAAAACGTCCCGTTGCCAGGTCCTGATCGACGAGGAAATCGTCGTGCTGTT
TGCTGGCGACCACTACACGAAATTCATCTGGGAGAAGTACCGCAAGCTGTCGCCGACGGCCCGAC
GGATGTTCGACTATTTCAGCTCGCACCGGGAGCCGTACCCGCTCAAGCTGGAAACCTTCCGCCTCA
TGTGCGGATCGGATTCCACCCGCGTGAAGAAGTGGCGCGAGCAGGTCGGCGAAGCCTGCGAAGAG
TTGCGAGGCAGCGGCCTGGTGGAACACGCCTGGGTCAATGATGACCTGGTGCATTGCAAACGCTAG
GGCCTTGTGGGGTCAGTTCCGGCTGGGGGGTTCAGCAGCCACCGGCATGCAAGCTTGAGAGGGTCAG
CTTTATGCTTGTAAACCGTTT

trfAcop271C sequence of pGM271pelB

GGTCGATCATGGCCGGTATTACACGAAGGCCGAGGAATGCCTGTCGCGCCTACAGGCGACGGCGA
TGGGCTTCACGTCCGACCGCGTTGGGCACCTGGAATCGGTGTCGCTGCTGCACTGCTTCCGCGTCCT
GGACCGTGGCAAGAAAACGTCCCGTTGCCAGGTCCTGATCGACGAGGAAATCGTCGTGCTGTTTGC
TGGCGACCACTACACGAAATTCATCTGGGAGAAGTACCGCAAGCTGTCGCCGACGGCCCGACGGA
TGTTCGACTATTTCAGCTCGCACCGGGAGCCGTACCCGCTCAAGCTGGAAACCTTCCGCCTCATGTG
CGGATCGGATTCCACCCGCGTGAAGAAGTGGCGCGAGCAGGTCGGCGAAGCCTGCGAAGAGTTGC
GAGGCAGCGGCCTGGTGGAACACGCCTGGGTCAATGATGACCTGGTGCATTGCAAACGCTAGGGC
CTTGTGGGGTCAGTTCCGGCTGGGGGGTTCAGCAGCCACCGGCATGCAAGCTTGAGAGGGTCAGCTT
TATGCTTGAAACCCGCC

T: Mutations yielding *trfAcop271C*

Attachment G: Eurofins sequence data

Codon optimization results

IFNpelB adapted (572 bp) from pCR.1-IFNspelBs:

CATATGAAGTATCTGCTGCCCACTGCAGCCGCAGGGTTGCTGCTGTTGGCGGCCCAACCAGCCATG
GCTTGCGATTTGCCGCAAACCCACAGCCTGGGTAGCCGTCGCACCCTGATGCTGCTGGCCCAGATG
CGGCGCATTTCGCTGTTTTCCTGCCTGAAAGATCGCCATGACTTCGGCTTTCCGCAGGAGGAGTTCG
GCAACCAGTTCCAGAAAGCCGAAACCATTCCCGTCCTGCACGAAATGATCCAGCAGATCTTCAACC
TCTTCTCGACGAAGGACAGCAGTGCGGCCTGGGACGAAACCCTGCTGGACAAGTTCTACACCGAGC
TGTATCAGCAGCTCAATGACCTGGAAGCGTGCGTGATCCAAGGCGTAGGCGTCACCGAAACGCCG
CTCATGAAGGAGGACTCGATCCTGGCCGTTCGCAAGTACTTCCAGCGGATCACCCTGTACCTGAAG
GAGAAGAAGTACAGCCCTTGTGCGTGGGAAGTGGTGCGTGCCGAAATCATGCGCAGCTTCAGCCT
GTCCACCAACCTGCAGGAGTCGTTGCGCAGCAAAGAGGCGGCCGC

GMpelB adapted (458 bp) from pCR2.1-GMspelBs:

CATATGAAATATCTCCTGCCGACTGCGGCGGCTGGCTTGCTGTTGCTGGCGGCACAACCGGCCATG
GCAGCCCCAGCGCGCAGTCCGAGCCCCAGCACCCAGCCATGGGAGCACGTGAACGCCATTCAGGA
GCCCGTCGCCTGCTGAATCTGTCGCGCGATACCGCCGCCGAGATGAACGAAACGGTTGAGGTGGAT
CAGCGAGATGTTCGACCTGCAGGAACCCACCTGCCTGCAGACCCGCTTGGAGCTGTACAAGCAAG
GCCTGCGGGGTTCGCTGACCAAGCTCAAAGGCCCTCTGACCATGATGGCCTCCCACTACAAGCAGC
ATTGCCCGCCTACCCCGGAAACGAGCTGTGCCACCCAGATCATCACCTTCGAGAGCTTCAAGGAGA
ACCTCAAGGACTTCCTGCTGGTCATCCCGTTTGACTGCTGGGAACCCGTACAGGAAGCGGCCGC

Attachment G: Eurofins sequence data

Sequence and alignment results of pIFNs271pelB, pGMs271pelB, pIFNs271pelBs and pGMs271pelBs

Plasmid isolated from the transformants DH5α (pIFNs271pelB), DH5α (pGMs271pelB), DH5α (pIFNs271pelBs) and DH5α (pGMs271pelBs) were sequenced using the primer Pmfwdseq.

Primer: Pmfwdseq
Sequence: 5' GACAAATCTGGCTCCCCAACTAATGC 3'

Sequence of codon optimized pIFNs271pelB

ACCAGCATAAACTGTCCGGTTTGATAGGGATAAGTCCAGCCTTGCAAGAAGCGGATACAGGAGTG
CAAAAAATGGCTATCTCTAGAAAGGCCTACCCCTTAGGCTTTATGCAACAGAAACAATAATAATGG
AGTCATGAACATATGAAATCCCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCGGCCCAG
CCGGCCATGGCTTGCGATTTGCCGCAAACCCACAGCCTGGGTAGCCGTCGCACCCTGATGCTGCTG
GCCCAGATGCGGCGCATTTCGCTGTTTTCCTGCCTGAAAGATCGCCATGACTTCGGCTTTCCGCAGG
AGGAGTTCGGCAACCAGTTCCAGAAAGCCGAAACCATTCCCGTCCTGCACGAAATGATCCAGCAG
ATCTTCAACCTCTTCTCGACGAAGGACAGCAGTGCGGCCTGGGACGAAACCCTGCTGGACAAGTTC
TACACCGAGCTGTATCAGCAGCTCAATGACCTGGAAGCGTGCGTGATCCAAGGCGTAGGCGTCACC
GAAACGCCGCTCATGAAGGAGGACTCGATCCTGGCCGTTCGCAAGTACTTCCAGCGGATCACCCTG
TACCTGAAGGAGAAGAAGTACAGCCCTTGTGCGTGGGAAGTGGTGCGTGCCGAAATCATGCGCAG
CTTCAGCCTGTCCACCAACCTGCAGGAGTCGTTGCGCAGCAAAGAGGCGGCCGCTGGATCCGAACA
AAAGCTGATCTCAGAAGAAGACCTAAAGGCCTCGGGGGGCCGATCACCATCATCACCATCATTGATA
AGCTTGACCTGTGAAGTGAAAAATGGCGCACATTGTGCGACATTTTTTTTGTCTGCCGTTTACCGCT
ACTGCGTCACGGATCCGGCCGAACAAACTCCGGGAGGCAGCGTGATGCGGCAACAATCACACGGA
TTTCCCGTGAACGGTCTGAATGAGCGGATTATTTTCAGGAAAGT

Sequence of codon optimized pGMs271pelB

CCAGCATAAACTGTCCGGTTTGATAGGGATAAGTCCAGCCTTGCAAGAAGCGGATACAGGAGTGC
AAAAAATGGCTATCTCTAGAAAGGCCTACCCCTTAGGCTTTATGCAACAGAAACAATAATAATGGA
GTCATGAACATATGAAATCCCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCGGCCCAGC
CGGCCATGGGAGCACGTGAACGCCATTCAGGAAGCCCGTCGCCTGCTGAATCTGTCGCGCGATACC
GCCGCCGAGATGAACGAAACGGTTGAGGTGATCAGCGAGATGTTCGACCTGCAGGAACCCACCTG
CCTGCAGACCCGCTTGGAGCTGTACAAGCAAGGCCTGCGGGGTTCGCTGACCAAGCTCAAAGGCCC
TCTGACCATGATGGCCTCCCACTACAAGCAGCATTGCCCGCCTACCCCGGAAACGAGCTGTGCCAC
CCAGATCATCACCTTCGAGAGCTTCAAGGAGAACCTCAAGGACTTCCTGCTGGTCATCCCGTTTGA
CTGCTGGGAACCCGTACAGGAAGCGGCCGCTGGATCCGAACAAAAGCTGATCTCAGAAGAAGACC
TAAAGGCCTCGGGGGGCCGATCACCATCATCACCATCATTGATAAGCTTGACCTGTGAAGTGAAAAA
TGGCGCACATTGTGCGACATTTTTTTTGTCTGCCGTTTACCGCTACTGCGTCACGGATCCGGCCGAA
CAAACTCCGGGAGGCAGCGTGATGCGGCAACAATCACACGGATTTCCCGTGAACGGTCTGAATGA
GCGGATTATTTTCAGGGAAAGTGAGTGTGGTCAGCGTGCAGGTATATGGGCTATGATGTGCCCGGC
GCTTGAGGCTTTCTGCCTCATGACGTGAAGGTGGTTTGTTGCCGTGTTGTGTGGCAGAAAGAAGAT
AGCCCCGTAGTAAGTTTA

Attachment G: Eurofins sequence data

Sequence of codon optimized pIFNs271pelBs
AACCAGCATAAACTGTCCGGTTTGATAGGGATAAGTCCAGCCTTGCAAGAAGCGGATACAGGAGT
GCAAAAAATGGCTATCTCTAGAAAGGCCTACCCCTTAGGCTTTATGCAACAGAAACAATAATAATG
GAGTCATGAACATATGAAGTATCTGCTGCCCACTGCAGCCGCAGGGTTGCTGCTGTTGGCGGCCCA
ACCAGCCATGGCTTGCGATTTGCCGCAAACCCACAGCCTGGGTAGCCGTCGCACCCTGATGCTGCT
GGCCCAGATGCGGCGCATTTCGCTGTTTTCCTGCCTGAAAGATCGCCATGACTTCGGCTTTCCGCAG
GAGGAGTTCGGCAACCAGTTCCAGAAAGCCGAAACCATTCCCGTCCTGCACGAAATGATCCAGCA
GATCTTCAACCTCTTCTCGACGAAGGACAGCAGTGCGGCCTGGGACGAAACCCTGCTGGACAAGTT
CTACACCGAGCTGTATCAGCAGCTCAATGACCTGGAAGCGTGCGTGATCCAAGGCGTAGGCGTCAC
CGAAACGCCGCTCATGAAGGAGGACTCGATCCTGGCCGTTCGCAAGTACTTCCAGCGGATCACCCT
GTACCTGAAGGAGAAGAAGTACAGCCCTTGTGCGTGGGAAGTGGTGCGTGCCGAAATCATGCGCA
GCTTCAGCCTGTCCACCAACCTGCAGGAGTCGTTGCGCAGCAAAGAGGCGGCCGCTGGATCCGAAC
AAAAGCTGATCTCAGAAGAAGACCTAAAGGCCTCGGGGGCCGATCACCATCATCACCATCATTGAT
AAGCTTGACCTGTGAAGTGAAAAATGGCGCACATTGTGCGACATTTTTTTTGTCTGCCGTTTACCGC
TACTGCGTCACGGATCCGGCCGAACAAACTCCGGGAGGCAGCGTGATGCGGCAACAATCACACGG
ATTTCCCGTGAACGGTCTGAATGAGCGGATTATTTTCAGGAAAGTGAGTGTGGTCAGCGTGCAGT

Sequence of codon optimized pGMs271pelBs
ACCAGCATAAACTGTCCGGTTTGATAGGGATAAGTCCAGCCTTGCAAGAAGCGGATACAGGAGTG
CAAAAAATGGCTATCTCTAGAAAGGCCTACCCCTTAGGCTTTATGCAACAGAAACAATAATAATGG
AGTCATGAACATATGAAATATCTCCTGCCGACTGCGGCGGCTGGCTTGCTGTTGCTGGCGGCACAA
CCGGCCATGGCAGCCCCAGCGCGCAGTCCGAGCCCCAGCACCCAGCCATGGGAGCACGTGAACGC
CATTCAGGAAGCCCGTCGCCTGCTGAATCTGTCGCGCGATACCGCCGCCGAGATGAACGAAACGGT
TGAGGTGATCAGCGAGATGTTCGACCTGCAGGAACCCACCTGCCTGCAGACCCGCTTGGAGCTGTA
CAAGCAAGGCCTGCGGGGTTCGCTGACCAAGCTCAAAGGCCCTCTGACCATGATGGCCTCCCACTA
CAAGCAGCATTGCCCGCCTACCCCGGAAACGAGCTGTGCCACCCAGATCATCACCTTCGAGAGCTT
CAAGGAGAACCTCAAGGACTTCCTGCTGGTCATCCCGTTTGACTGCTGGGAACCCGTACAGGAAGC
GGCCGCTGGATCCGAACAAAAGCTGATCTCAGAAGAAGACCTAAAGGCCTCGGGGGCCGATCACC
ATCATCACCATCATTGATAAGCTTGACCTGTGAAGTGAAAAATGGCGCACATTGTGCGACATTTTTT
TTGTCTGCCGTTTACCGCTACTGCGTCACGGATCCGGCCGAACAAACTCCGGGAGGCAGCGTGATG
CGGCAACAATCACACGGATTTCCCGTGAACGGTCTGAATGAGCGGATTATTTTCAGGGAAAGTGAG
TGTGGTCAGCGTGCAGGTATA

The sequences from Eurofins were compared with previously received adapted genes. Alignment was performed in Clone Manager professional Suite by using the restriction enzymes that was used to insert the synthetically genes on both sequenced sequence and the optimized sequence. Alignment had the following results:

Alignment of sequence of codon optimized pIFNs271pelB with IFNpelB adapted

Percent match: 100 %

An alignment score of 100 % homology between the blue segments (504 bp) was found indicating that the plasmid pIFNs271pelB have been successfully constructed.

Attachment G: Eurofins sequence data

<u>Alignment of sequence of codon optimized pGMs271pelB and GMpelB adapted</u>
When the codon optimized sequence of *gm-csfs+pelBs* was prepared for alignment, it was revealed that an additional restriction site for *NcoI* had become part of the gene. This led to an additional DNA fragment when pCR2.1GMspelBs was digested with *NcoI* and *NotI*. It then caused loss of fragments of the gene when DNA fragments were separated by gel electrophoresis. It was tried to get a new version of codon adapted gene and signal sequence without this restriction site, but trouble executing this lead to too short amount of time to construct pGMs271pelB.

<u>Alignment of sequence of codon optimized pIFNs271pelBs and IFNpelB adapted</u>
Percent match: 100 %
The result indicate that the construction of pIFNs271pelBs was successful

<u>Alignment of sequence of codon optimized pGMs271pelBs and GMpelB adapted</u>
Percent match: 100 %
The result indicate that the construction of pGMs271pelBs was successful

Attachment H. Attempt to quantify concentration of recombinant proteins in shake flask experiment

The following is an attempt to quantify concentration of recombinant proteins in the shake flask experiment reported in Chapter 3.7.1. It is important to emphasize that further analysis would have to be done before the accurate concentrations had been determined.

5 ml culture sample were taken 0, 2, 5 hours after induction and after overnight incubation. The samples were centrifuged and cells lysed following "Cell lysis protocol 2" before Western blot analysis. During lysis, 0.5 ml insoluble fraction and 1 ml soluble fraction are formed. For insoluble fraction this means that the concentration of insoluble proteins found in 5 ml culture now is present in 0.5 ml. This equals a 10-fold concentration increase. For soluble fractions, the same relationship yields a 5-fold concentration increase. The concentration found in these fractions therefore has to be divided by the found relationship to determine the concentration in the culture:

$$\text{Insoluble protein, culture (mg/l)} = \frac{\text{Insoluble protein, Western Blot (mg/l)}}{10} \qquad \text{Eq. (3)}$$

$$\text{Soluble protein, culture (mg/l)} = \frac{\text{Soluble protein, Western Blot (mg/l)}}{5} \qquad \text{Eq. (4)}$$

The amount of protein produced is dependent on number of cells present. OD_{600} measurements therefore have to be taken into account when comparing yields of recombinant protein from different samples.

Calculations here are only based on bands comparable to the 1 mg/l reference (Phox). Several cultures have therefore produced more than what is calculated.

Wild type copy number strains KT2440 (pIFNpelB) and KT2440 (pGMpelB)

In Figure 42, the sample with highest IFN-α2b yield from KT2440 (pIFNpelB) is the insoluble fraction "P2 ind" (5 hours after induction) (OD_{600} = 12.01). Concentration of protein here is lower than the reference Phox and can therefore only be quantified as lower than 0.1 mg/l.

Attachment H: Attempt to quantify concentration of recombinant proteins in shake flask experiment

In Figure 43, the samples with highest GM-CSF yield from KT2440 (pGMpelB) are both the insoluble and soluble fractions from "P2 ind" (5 hours after induction) (OD_{600} = 11.58). Also here, concentration is lower than for the reference Phox and can therefore only be quantified as lower than 0.1 and 0.2 mg/l respectively.

Copy number mutants KT2440 (pIFN271pelB) and KT2440 (pGM271pelB)

Concentration of IFN-α2b from KT2440 (pIFN271pelB) is too high to quantify in insoluble fractions from P2 (OD_{600} = 7.82) and P3 (OD_{600} = 7.5) (Figure 44). The soluble fractions of these samples are comparable with Phox in band strength. Using equation (4) gives an estimated 0.2 mg/l IFN-α2b in the culture. If one estimates that there is 10-fold more detected protein in the insoluble fraction sample "P3 ind" than in the reference (Phox), the calculated concentration is 1 mg/l.

KT2440 (pGM271pelB) has the highest yield of GM-CSF in the insoluble fractions from overnight incubation ("P3 ind") (Figure 45). The concentration of GM-CSF here is too high to be quantified. Insoluble fraction "P2 ind" (OD_{600}=0.6) and soluble fraction "P3 ind" (OD_{600}=6.67) have GM-CSF bands comparable to Phox in strength. Based on this information, and equation (3) and (4), the following concentrations were quantified:
-   Insoluble, "P2 ind": 0.1 mg/l GM-CSF in the culture.
-   Soluble "P3 ind": 0.2 mg/l GM-CSF in the culture

Because of the low amount of cells in P2 samples, there is obviously a potential to produce more if induction had been performed later.

Copy number mutant with codon optimized gene: KT2440 (pIFNs271pelB)

The highest yield of IFN-α2b is detected in insoluble fraction "P2 ind" (OD_{600} = 13.31) (Figure 46). This band is evaluated to be stronger than Phox and therefore, based on equation (3) and (4) correspond to more than 0.1 mg/l GM-CSF in the culture.

Copy number mutants wit codon optimized genes and pelB sequence: KT2440 (pIFNs271pelBs) and KT2440 (pGMs271pelBs)

The highest yield of IFN-α2b from KT2440 (pIFNs271pelBs) is detected in the insoluble fraction "P3 ind" (OD_{600} =15.26). The strength of this band is somewhat weaker than for Phox. Concentration of IFN-α2b in the culture is therefore estimated to be lower than 0.1 mg/l

Attachment H: Attempt to quantify concentration of recombinant proteins in shake flask experiment

The highest yield of GM-CSF from KT2440 (pGMs271pelBs) is detected in the insoluble fraction of "P3 ind", but this band is too strong for comparison with Phox. In addition, Phox reference strength compared with its strengths on other membranes indicate weaker blotting. Based on these observations, it was chosen not to quantify GM-CSF concentration, but note it as high compared with KT2440 (pGMpelB).