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Evaluation of *Pseudomonas putida* and *Pseudoalteromonas haloplanktis* as Alternative Hosts for Recombinant Gene Expression

by Evaluating the xylS/*Pm* Expression System
with *mCherry* as Reporter Gene

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Preface

This Master's thesis conclude my degree in Master of Science (M.Sc.) in Chemical Engineering and Biotechnology at the Norwegian University of Science and Technology (NTNU) in Trondheim. This thesis was written at the Department of Biotechnology in collaboration with the biotechnology company *Vectron Biosolutions* AS, and is a continuation of the specialization project carried out in the spring of 2016. All of the experimental work presented here was conducted by me during the fall of 2016 at the Department of Biotechnology's laboratories.

Acknowledgements

I would like to thank my supervisor at NTNU, Professor Trygve Brautaset, for the opportunity to work on this project. Thanks to Vectron Biosolutions AS by Dr. Trond Erik Aune and Dr. Anne Krog for the chance to collaborate with their company and contribute to their work with this thesis. Mainly thanks to my co-supervisor Dr. Anne Krog for the enthusiasm and dedication to the laboratory work and providing me with valuable guidance all the way. Also, I would like to thank the other employees in Vectron Biosolutions, both for good advice and company during long days in the laboratory.

Declaration of Compliance

I declare that this is an independent work according to the exam regulations of the Norwegian University of Science and Technology (NTNU).

Trondheim, January 22, 2017

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Abstract

Different species of bacteria have different intracellular environments. *Escherichia coli* (*E. coli*) is widely used as an expression host for microbial production of recombinant proteins and therapeutics. However, its intracellular environment is not necessarily the best to obtain correct folding of all proteins expressed. Changing the expression host to a species with a more suitable intracellular environment can cause benefits like proper folding for some proteins. Thus, downstream processing after expression to achieve correct folding is no longer needed, resulting in simplified and more inexpensive production. In this thesis, two alternative expression hosts proved to be favorable for recombinant gene expression, *Pseudomonas putida* (*P. putida*) and *Pseudoalteromonas haloplanktis* (*P. haloplanktis*) were compared to *E. coli* for production of recombinant proteins.

The expression vectors used in this thesis includes the xylS/*Pm* expression cassette from the TOL plasmid and is expected to function well in *P. putida* since it originates from this bacterium. The different expression vectors used were shown to function well in *E. coli* by high expression of the reporter gene *mCherry*. The expression vectors pVB-1B0B1-mCh, and pVB-1M0B1-mCh resulted in highest expression of the reporter gene for both soluble and insoluble protein fractions. These vectors carried the mutated 5'-UTR region H39 in combination with wild type (pVB-1M0B1-mCh) and mutated *Pm* promoter ML2-5 (pVB-1B0B1-mCh).

However, in *P. putida* these plasmid vectors resulted in no expression of mCherry. This might be caused by the medium copy number caused by the *cop271*-mutation of *trfA* embedded in the RK2 vectors. The evaluation of *P. haloplanktis* resulted in the establishment of favorable growth conditions, as well as a functioning protocol for conjugation and detection of positive conjugants. However, the preservation of these must be optimized further, so the strains are easier to revive after freezing and evaluation of the expression can be performed more thoroughly and compared to *E. coli*.

Sammendrag

Ulike bakteriearter vil ha ulikt intracellulært miljø. *Escherichia coli* (*E. coli*) er en mye brukt ekspresjonsvert for mikrobiologisk produksjon av rekombinante proteiner og legemidler til tross for at det intracellulære miljøet ikke nødvendigvis er det beste for å oppnå korrekt folding av proteiner. Ved å benytte seg av en annen mikrobiologisk vert, som har et mer passende intracellulært miljø, kan fordeler som korrekt folding av noen proteiner oppnås. På denne måten unngår man etterfølgende prosesseringer av de uttrykte proteinene, dermed en enklere og billigere produksjon. I dette arbeidet vil to alternative ekspresjonsverter undersøkes og sammenlignes opp mot *E. coli*. De alternative vertene er *Pseudomonas putida* (*P. putida*) og *Pseudoalteromonas haloplanktis* (*P. haloplanktis*) og begge disse har tidligere vist lovende egenskaper som sikre bakteriestammer for rekombinant produksjon av proteiner.

Ekspresjonsvektorer brukt i dette arbeidet inneholder en xylS/*Pm* ekspresjonskassett, opprinnelig fra TOL plasmidet isolert fra *P. putida*. På bakgrunn av dette, burde ekspresjonsvektorene kunne replikere i *P. putida*. De ulike ekspresjonsvektorene undersøkt i dette arbeidet viste seg å fungere godt i *E. coli*, hvor det ble oppnådd høyest produksjon av løselig og uløselig mCherry for plasmidvektorene pVB-1B0B1-mCh og pVB-1M0B1-mCh. Hos disse vektorene er den muterte 5'-UTR regionen H39 kombinert med henholdsvis villtype (pVB-1M0B1-mCh) og mutert *Pm* promotor ML2-5 (pVB-1B0B1-mCh).

Produksjonen av proteiner for de samme vektorene i *P. putida* var ikke vellykket. Dette kan komme av *cop271*-mutasjonen for *trfA* genet, som fører til et medium høyt kopitall for RK2-vektoren. Evalueringen av *P. haloplanktis* som en potensiell vert resulterte i etableringen av gunstige vekstbetingelser og en fungerende protokoll for konjugasjon og identifisering av positive konjugerte celler. Det ble dessverre ikke evaluert noen ekspresjon av mCherry da det oppstod problemer med å gjenopplive kulturene som hadde blitt fryst ved -80 °C. Videre arbeid gjenstår for å evaluere produksjonen og sammenligne den med *E. coli*.

Abbreviations

aa	Amino acid
Amp	Ampicillin
Amp^r	Ampicillin resistance
bp	Base pair
DNA	Deoxyribonucleic acid
ds	Double stranded
ins	Insulin
Kan	Kanamycin
Kan^r	Kanamycin resistance
kb	kilo base pair
kD	kilo Dalton
mCh	mCherry
mRNA	messenger RNA
OD_{600}	Optical density at 600 nm
Oligo	Oligonucleotide
p	Plasmid
pVB	Plasmid Vectron Biosolutions
RNA	Ribonucleic acid
rRNA	ribosomal RNA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TOL	Toluene-degradative
tRNA	transfer RNA
wt	Wild type
5'-UTR	5'-untranslated region

Contents

1 Introduction

1.1 Recombinant Protein Expression

Recombinant protein expression is the production of proteins from expression vectors constructed *in vitro* by cloning, joining all or parts of different DNA molecules resulting in a specific DNA sequence [51]. There are different types of expression systems, and they vary in terms of what kind of organism that hosts the expression. The hosts can be animals and plants which are transgenic, or different mammalian cell lines as well as insect cell lines. For microorganisms, *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Escherichia coli* (*E. coli*) are the two most common expression systems for recombinant proteins. These are the favourable systems since they are easy to grow due to simple growth requirements, known genome sequences, biochemical processes, and they are easy to handle. Mammalian expression systems are not as favourable when producing biopharmaceuticals since more complex and expensive growth medias and other production requirements are needed because of the post expressional modification systems. These post translational modifications do not occur in *E. coli*. However, mammalian cell lines stand for the largest production of recombinant proteins, whereas *E.coli* and *S. cerevisiae* together account for only 37% of the total production of recombinant proteins [2]. Plasmid expression vector systems will be used in this work. In general, plasmid vectors need an origin of replication, a dominant selectable marker gene and must harbor at least one unique restriction enzyme site to be a valid expression system. Since plasmids are small in size, the foreign DNA insert can not be larger than 10 - 15 kb [51]. To achieve good recombinant protein expression one needs a suitable host and expression vector, together working as an expression system. This expression system needs tight control of the expression both on a transcriptional and translational level, low-cost inducers as well as stability during scale-up [10].

1.1.1 *E. coli* as a Host

E. coli is relatively simple to work with as a host for recombinant protein expression. It requires inexpensive medias and inducers for its relatively rapid growth. The genetics are well-studied and known, which provides several tools to use in biotechnology and

especially as an expression host. However, the expression may encounter some difficulties when it comes to expressing eukaryotic membrane proteins, for example. This is associated with post-translational modifications, proteolysis and aggregation [58]. Many alterations have been done to the expression vectors and engineering of the strains, resulting in an improved expression for some of these proteins, by avoiding the issues mentioned above [27], [38], [41]. In general the proteins expressed in *E. coli* needs many modifications before they can be used. Therefore the investigation of alternative hosts with expression systems are of interest, exploring the potential other bacteria species have for recombinant protein expression.

1.2 Bacterial Gene Expression

Genes are expressed through transcription of DNA into mRNA followed by translation of mRNA into proteins. To regulate the gene expression the different components within both transcription and translation can be altered, giving rise to an expression system with preferred qualities and characteristics [51].

Both transcription and translation can be divided into three parts; initiation, elongation and termination. For the transcriptional initiation, RNA polymerase will recognize and bind to the promoter sequence which marks the starting point of the transcription. Then the RNA polymerase will synthesize a complementary mRNA strand (5' - 3') from the DNA template. Elongation of the mRNA will continue until a termination signal is reached. The mRNA strand consists of a sequence of codon triplets that each specifies insertion of a single amino acid (aa). The aas are linked together by polypeptide bonds and the elongation of the polypeptide continues until a termination codon is reached and translation is terminated [44].

1.2.1 Transcription

Transcription can be explained as the production of a single strand (ss) RNA copy (mRNA) made from a double helix DNA molecule. It starts at the promoter, a specific

DNA sequence. This initiation occurs at the 3' side of the promoter. Then elongation follows and the process is ended by termination when a specific site downstream of the coding sequence of the gene is reached [44]. The large enzyme RNA polymerase binds to the promoter in the double strand (ds) DNA and start the transcription of the genes. The enzyme moves along the DNA template, synthesising mRNA by recruitment and binding of nucleotides complementary to the bases in the DNA until it reaches a terminator sequence. Then the polymerase stops adding nucleotides to the growing mRNA chain, releases the product and dissociates from the DNA strand. There are two types of termination for prokaryote cells; ρ-dependent and ρ-independent termination. ρ-dependent termination is when the protein ρ binds to the RNA chain, causing the polymerase to pause at a certain DNA sequence. For ρ-independent termination, an intrinsic termination sequence may cause hair-pin loops in the RNA which promote dissociation of the polymerase. This is the most common form of termination in *E. coli* since no additional factors are required [44].

In the absence of other factors, RNA polymerase will recognize and bind to the promoter and produce ss RNA from ds DNA in prokaryotes, which makes most of the protein coding genes transcriptionally active by default. As a transcriptional control, repressor proteins can bind to the DNA sequence adjacent to the RNA polymerase binding site and compromise the initiation. Transcription is a highly regulated process where timing and level of gene expression are important [44].

1.2.2 Translation

Translation is the process where RNAs and their associated proteins decode the linear mRNA sequence obtained from transcription into linear chains of aas called polypeptides or proteins. Translation is also divided into initiation, elongation and termination and take place on the ribosomes. Ribosomes are RNA-rich cytoplasmic granules where protein synthesis take place and all proteins needed to perform the chemical reactions are located. In *E. coli*, a small (30S) and large subunit (50S) come together as 70S ribosome where translation take place in bacteria.

Initiation is when the ribosomal subunits assembles on the mRNA at the purine-rich Shine-Dalgarno sequence (5'-AGGAGGU-3'). It is located 4 - 8 bases upstream of the initiation codon, encoding the aa methionine. This positions the ribosome before translation is initiated [48]. The nucleotide sequence of mRNA is read as a series of triplets known as codons, where each codon specifies the insertion of a single aa. Translation starts at initiation codon AUG and ends with termination codons UAA, UGA or UAG. tRNAs transport a specific aa to the mRNA codon it encodes. The ribosome moves along the 5'-3' direction of mRNA recruiting tRNA with anticodon corresponding to the codons and binding them together in a chain with polypeptide bonds, and continues until a termination codon is reached. The aa is coupled with the corresponding anticodon on tRNA by aminoacyl-tRNA synthetase. This pairing of codon-anticodon directs the addition of the correct aa to the growing polypeptide chain with polypeptide bonds holding the aas together in the chain. Polypeptide bonds are formed when the aa attached to the tRNA in the P site is transferred to the aminoacyl-tRNA in the A site. This uncharged tRNA will leave the complex and the ribosome moves along to the next codon. Since there are no corresponding tRNAs for the termination codons, RF (release factor) proteins will bind instead and cause the ribosome to dissociate. Soluble cytosolic proteins are released from the ribosome after the polypeptide synthesis is complete.

1.3 Gene of Interest - *mCherry*

Reporter genes are used in molecular biology to check for correct integration of a certain gene and proper expression in cells. *mCherry* is used in this study as a reporter gene. It will indicate successful uptake and expression of plasmid by the bacteria. mCherry is a fluorescent protein (fluorophore) and shows *in vitro* expression, which is a much used and an important tool in biotechnology [29], [57], [63]. The expression is visualized by a pink color of the recombinant bacterial culture since mCherry is a part of the red fluorescent proteins derived from a protein isolated from *Discosoma sp.* mCherry is a monomeric fluorescent construct with a maximum absorption peak at 587 nm and an emission maximum peak at 610 nm. Its biological half-life is around 15 minutes [46].

1.4 Expression Vector System

The expression vector system used in this work include the inducible xylS/*Pm* promoter system inserted into the minimal replicons of RK2 [7]. It contains several DNA control elements like the *Pm* promoter, *xylS* gene, 5'-UTR region and *trfA* gene. The *xylS* gene encodes the positive regulator of *Pm* which is activated by an inducer (benzoic acid derivates). 5'-UTR is a 5'-untranslated region at the RNA level. Whereas the *trfA* gene encode initiation of plasmid replication and the copy number.

1.4.1 The RK2 Plasmid

The RK2 plasmid can replicate in many different bacterial species and is therefore denoted as a broad-host-range plasmid [54]. RK2 replicates in many gram-negative bacteria, including *E. coli* and *Pseudomonas putida* (*P. putida*), which are used as hosts in this work [10], [45]. It requires two regions for replication; *trfA* and *ori*V. The *trfA* gene encode initiation of replication and *ori*V acts as origin for vegetative DNA replication [40]. *trfA* also affects the plasmid copy number. The copy number is regulated in a process called hand-cutting by interactions between the origin and TrfA protein [55]. A mutation in *trfA* can have a negative effect on replication from *ori*V, however, copy-up mutations for the *trfA* gene will cause an elevated plasmid copy number [17], [24]. For some plasmids, copy-up mutations have shown to increase the copy number up to 24-fold [59]. The *cop-271* mutation has previously shown to result in a medium copy number around 20 and to work well with the expression vector system used in this work [25], [49].

1.4.2 The xylS/*Pm* Expression System

Toluene-degradative (TOL) plasmid pWWO originates from *P. putida* mt-2 and function in many different bacterial species [7], [15], [19]. It encodes the degradation pathway of toluene and xylene [62]. Expression of the upper pathway genes for the plasmid is controlled by XylR/*Pu* [10]. The transcription factors XylR and XylS positively regulate the upper and meta pathway operons, respectively. The *Pm* promoter is activated by XylS which is controlled by the two promoters *Ps1* and *Ps2* [43]. XylR can also activate transcription from *Ps1*, but *Ps2* is constitutive and low. This ensures an expression level

of XylS which is well controlled [10]. If XylS is overexpressed, it can activate *Pm* without activation of an inducer by binding to the operator *Om* [34]. However, when inducer is present, like benzoic acid derivates, *Pm* will be activated even at low protein concentrations [43]. Figure 1.1 shows a simplified example of how this activation works in the cells. The inducer molecules passively enter the cell and activate xylS by binding to it, forming a complex. This complex activates transcription from *Pm* and causes expression of the gene of interest downstream of the promoter [10].

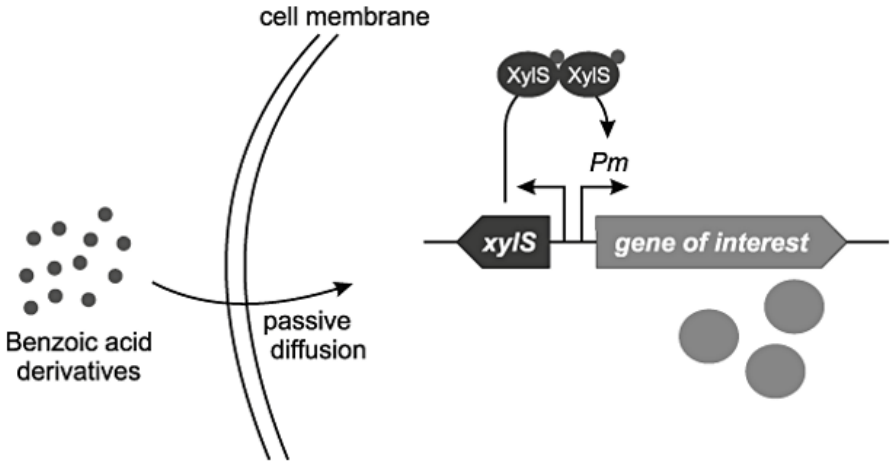


Figure 1.1: The xylS/*Pm* expression system where inducer molecules (benzoic acid derivatives) passively enters the cells and activates the xylS by binding to it. The activated transcription factor XylS will then activate transcription from *Pm* and the gene of interest downstream of *Pm* will be expressed [10].

1.4.3 Expression Vectors Based on pJB658

Expression vector pJB658 constructed by Blatny and colleagues in 1997 [8], based on the expression vector pJB653 [7], is the basis for the expression vectors used in this work. With RK2 mini replicon and expression cassette from the TOL plasmid, a broad-host-range expression vector with an adaptable expression system is formed [7], [8]. The physical map of the pJB658 plasmid is given in Figure 1.2.

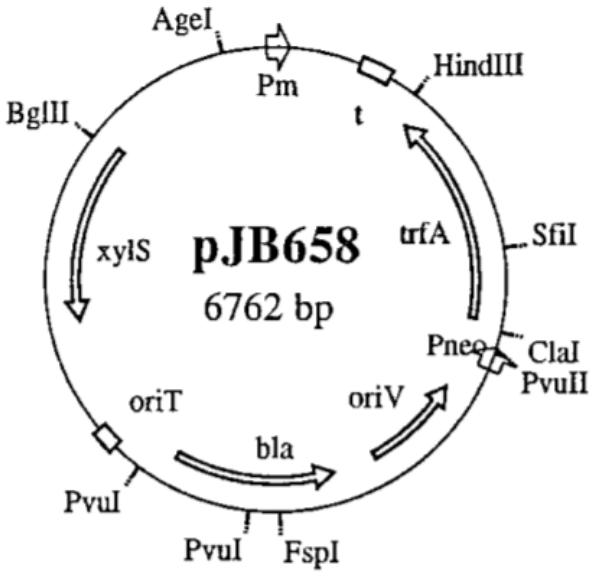


Figure 1.2: Physical map of the expression plasmid pJB658 [8]. The plasmid is based on expression vector pJB653 [7], and harbors the xylS/*Pm* promoter system and RK2 mini replicon.

pJB658 is an expression system suitable for expression in Gram-negative bacteria and contain the *bla* gene which encodes and produces β-lactamase which causes resistance to the antibiotic Ampicillin for the bacteria [8], [31]. pJB658 is by Vectron Biosolutions referred to as pVB-1, and will be called so from here.

More alterations have been done to pVB-1 to improve the vector (Vectron Biosolutions unpublished) [50]. The gene of interest, signal sequence and vector copy number can be changed by one-step cloning procedures for the plasmid [49], [50]. Expression vector pJBphOx shows an example of this. It is a variant of pVB-1, shown in Figure 1.3, and harbors the xylS/*Pm* promoter system and *pelB*-scFv-*phOx-c-myc*-his6 fusion gene downstream of *Pm* [49].

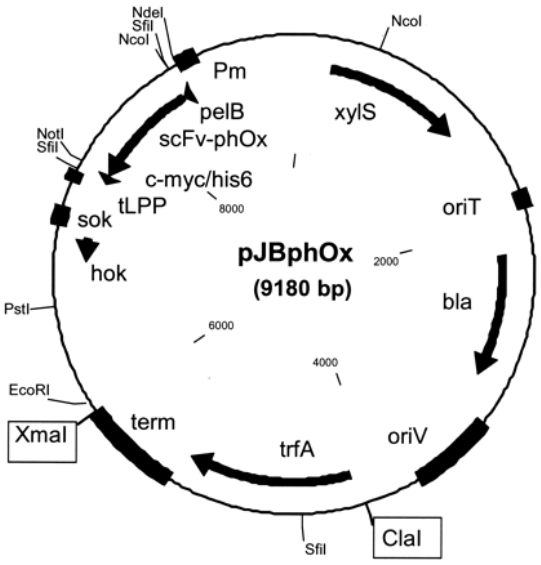


Figure 1.3: Physical map of the expression plasmid pJBphOx [49]. The plasmid is based on pJB658 from Blatny 1997 and harbors the xylS/*Pm* promoter system and *pelB*-scFv-*phOx*-*c*-*myc*-his6 fusion gene downstream of *Pm*.

1.4.4 Important Elements of the Expression Vectors

Important elements of the expression system for the vectors used in this work are the *Pm* promoter, 5'-UTR region and signal peptide *pelB*. Figure 1.4 gives a schematic overview of the nomenclature of the different expression vectors based on these elements.

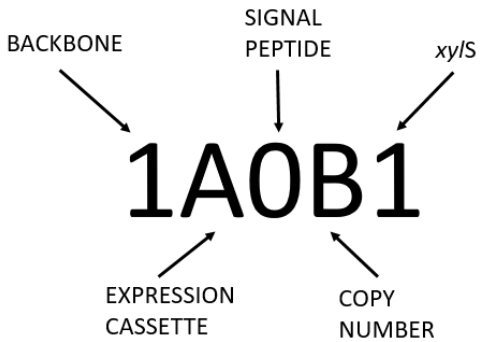


Figure 1.4: Explanation of the nomenclature for the expression vectors used in this work.

The *Pm* promoters used in this study were wild type (wt) and ML2-5 [3]. The mutated promoter ML2-5 was developed by random mutagenesis of a 24-bp region of wt *Pm*. It stimulated expression of reporter genes *luc* and *cel*B, as well as improving the high-level recombinant production of granulocyte-macrophage [3]. For the 5'-UTR region, an alternative mutated region, H39, is used as well as wt. This has shown a contribution to gene expression by stimulation at transcription and protein product level [4]. These two

mutations have also been combined (denoted comPU), resulting in increased expression and production of reporter gene *bla* in *E. coli* [64]. In general, these mutations have shown positive affect on the level of expression. The location of the promoter and 5'-UTR region can be seen upstream of the gene of interest in Figure 1.5. The corresponding restriction sites useful for potential cleaving are marked by the arrows on top [19], [28].

The strong promoter system xylS/*Pm* have shown to give high volumetric yields for human proteins granulocyte-machrophage colony-stimulating factor (GM-CSF) and single-chain antibody variable fragment (scFv-phOx) when the genes were fused together with the signal peptide *pelB* for a high-cell-density cultivation of *E. coli* [49], [50].

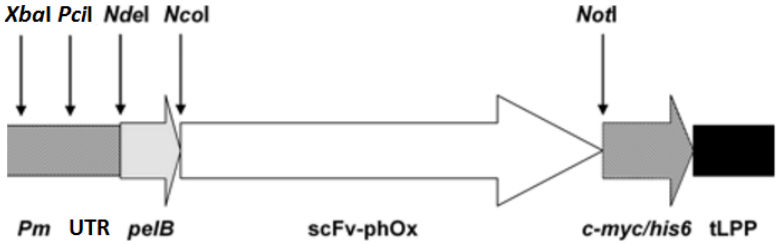


Figure 1.5: Physical map of a section of the expression vector pJBphOx-271d [50]. Signal peptide *pelB* is followed by the gene of interest, here scFv-phOx. Arrows indicate restriction sites useful for potential cleaving of signal peptide, *Pm* promoter and 5'-UTR region.

Another feature of the expression vectors is that upstream of the gene of interest there is a signal peptide, *pelB*, seen in Figure 1.5. This is a translocational signal sequence which translocates the protein from cytoplasm to periplasm. This is favourable for soluble expression with disulfide bonds, since the periplasm is a more suitable environment for forming of disulfide bonds. Downstream of the gene of interest is a *c-myc* tag which antibodies will bind to and enable visualization of the expressed gene of interest on Western blots.

Modifications of the expression vector pVB-1 in this work include *cop-271* mutation of *trfA*, different combinations of wt and mutated *Pm* promoter and 5'-UTR region, and signal peptide *pel*B.

1.5 *Pseudomonas putida*

Pseudomonas putida (*P. putida*) is a rod-shaped gram-negative bacterium with polar flagella which are easily isolated from soil and water related habitats where oxygen is present [23]. It can degrade organic solutions like toluene and growth is optimal around 25 - 30 °C [18].

None of the known strains of *P. putida* are pathogenic towards animals or plants, and the species is considered environmental friendly. For this reason, *P. putida* was declared as a promising candidate for development of a secure strain for recombinant DNA-experiments. *P. putida* works well for this due to its catabolic versatility and suitability for environmental use without causing hazard [42]. Other species of *Pseudomonas*, *P. aeruginosa* [7], [8] and *P. fluorescense* [21], have been proven to work well with the xylS/*Pm* expression system. The transcription regulator XylS originates from the TOL plasmid of *P. putida* pWWO and therefore *P. putida* is considered a suitable host for this expression system [19].

P. putida has previously shown promising properties as a secure strain for recombinant expression with the use of similar expression vectors as in this work, and is therefore evaluated as an alternative host for recombinant expression of proteins (Vectron/Sintef, unpublished results), [26]. Other broad-host-range vectors have been introduced to *P. putida*, producing p-Coumaric acid [11]. Another study has shown positive expression of lipolytic enzymes with similar promoter system as used in this work [56]. In general, *P. putida* is regarded as a versatile host for production of natural products [32], and well-suited for downstream applications of synthetic biology and harsh biochemical reactions [36], [37].

1.6 *Pseudoalteromonas haloplanktis*

Pseudoalteromonas haloplanktis (*P. haloplanktis*) is a rod-shaped gram-negative bacterium belonging to the Antarctica psychrophile bacterium. The size is 0.2 - 1.5 by 1.8 - 3 μm and the curved bacterium is motile by polar flagella [20]. It is isolated from sea samples from the Arctic coast and grows in marine like environments below 20°C [13]. It is also none-spore forming and none-luminescent [20].

The genome of the bacterium was determined in 2005 and given as a circular representation in Figure 1.6 [35]. As seen from the figure there are two chromosomes, 3214944 bp and 635328 bp. There are a large number of tRNA and rRNA genes (marked in colors) which may confirm the fast growth at low temperatures, even though the speed of transcription and translation usually is reduced when the temperature is lowered.

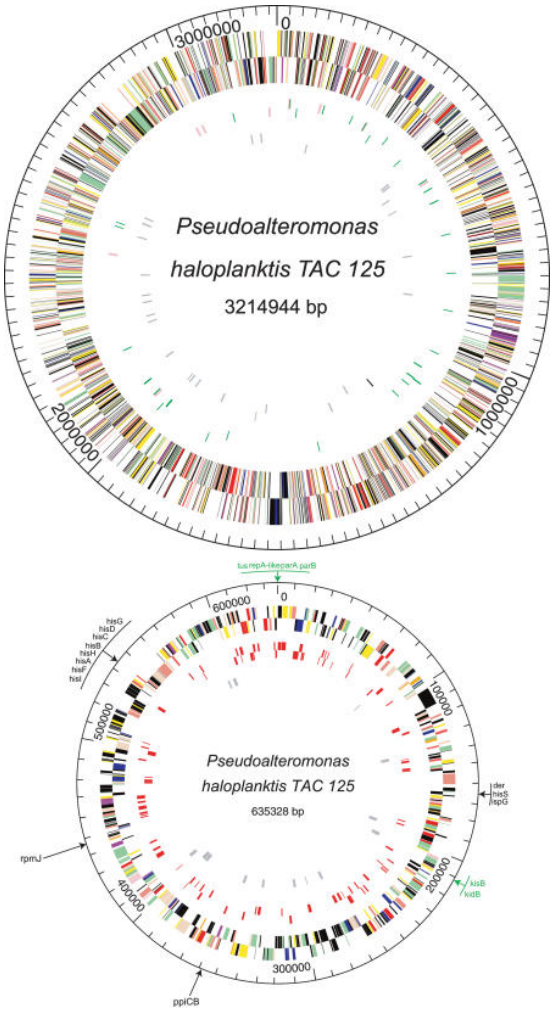


Figure 1.6: Circular representation of the *Pseudoaleteromonas haloplanktis* TAC 125 genome from Medigue et. al., 2005 [35].

P. haloplanktis is considered a good candidate as an expression host for certain kind of proteins due to its relative rapid growth at low temperatures. It can produce many heterologous enzymes in soluble and active state, and include a cold-adapted expression system for recombinant expression of genes and protein production [61]. In addition, the bacteria is not classified as a human pathogen. This means that it is safe to both use and work with in terms of as an expression host for recombinant expression of proteins [35]. Results have shown successful expression of active recombinant proteins, for example hGLA (lysosomal enzyme alpha-galactosidase A), in *P. haloplanktis* TAC125 where expression failed in *E. coli* [58], [61].

In terms of producing psychrophilic enzymes, development of new psychrophilic hosts is considered a good strategy, as well as adapting existing expression systems to low-temperature production. *P. haloplanktis* TAC125 is one of the psychrophilic hosts investigated for this kind of expression and have shown promising results [6]. In the same study, several proteins were expressed in *P. haloplanktis* and also the xylS/*Pm* promoter system was implemented. The Arctic-sourced chitinase was expressed by a similar expression vector used in this thesis, yielding higher levels than the same system in *E. coli*.

1.7 The Aim of This Study

The aim of this study was to establish functional expression vectors based on the broad-host-range plasmid RK2 with the strong xylS/*Pm* promoter system to be used in *E. coli*, *P. putida* and *P. haloplanktis* and evaluate the hosts expression when *mCherry* was used as a reporter gene. The expression in *E. coli* would work as a reference which the two alternative hosts would be compared against.

2 Materials and Methods

2.1 Bacterial Strains and Growth Conditions

Bacterial strains and vectors used in this thesis are given in Table 2.1. All medias and solutions made and used in this work are listed in Section 2.10. Physical maps over the expression plasmids are given in Appendix A.2.

Escherichia coli (*E. coli*) DH5α was used as cloning host for construction of expression vectors [22], *E. coli* BL21(DE3) was used as expression host and *E. coli* S17-1 was used as conjugation host [53]. All culturing of *E. coli* was conducted in LB-medium at 37 °C, both when grown in liquid culture (225 rpm) and on agar plates. Ampicillin (100 μg/mL Amp) and Kanamycin (50 μg/mL Kan) were used as selective antibiotics, determined by the plasmids antibiotic resistance due to the *bla* gene (Amp^r) and *kan* gene (Kan^r), respectively.

Pseudomonas putida (*P. putida*) was one alternative expression host investigated in this work. *P. putida* was cultured in LB-medium at 30 °C both for liquid culture (225 rpm) and LB agar plates. Amp (100 μg/mL) and Kan (50 μg/mL) were used as selective antibiotics.

Pseudoalteromonas haloplanktis (*P. haloplanktis*) TAC125 was the second alternative expression host investigated in this work [35]. *P. haloplanktis* was cultured in TYP-medium at 15 °C, both for liquid culture (225 rpm) and TYP agar plates. Amp (100 μg/mL) was used as selective antibiotics.

Table 2.1: Bacterial strains and plasmids used in this thesis.

Bacterial strain or plasmid	**Proterties**	**Reference**
Escherichia coli		
DH5α	Cloning host	BRL
BL21(DE3)	Production strain	NEB
S17-1	Conjugation host	
Pseudomonas putida	Production strain	
Pseudoalteromonas haloplanktis		
TAC125	Production strain	[39]
pAT64	pIFN30SpelB	[50]
pVB-1	pJB658: RK2-based expression vector harboring *Pm/xylS* regulatory promoter system for expression of cloned genes Amp^r	[8]
pVB-1A0B1	back bone based on pVB-1 and pAT64, *Pm/xylS* regulatory promoter system, wild type *Pm* promoter and 5'-UTR, *trfA* with *cop-271* mutation, Ps1 and Ps2 Amp^r	Vectron Biosolutions unpublished
pVB-1A0B1-mCh	pVB-1A0B1 with fusion gene mCherry-*c-myc*-his6	Vectron Biosolutions unpublished
pVB-1A0B1-mCh_kan	pVB-1A0B1-mCh carrying Kan^r	This work
pVB-1A1B1-mCh	pVB-1A0B1 with fusion gene *pelB*-mCherry-*c-myc*-his6	Vectron Biosolutions unpublished
pVB-1A1B1-mCh_kan	pVB-1A1B1-mCh carrying Kan^r	This work
pVB-1B0B1-mCh	pVB-1A0B1-mCh with mutated *Pm* promoter and 5'-UTR*(comPU)	Vectron Biosolutions unpublished
pVB-1B0B1-mCh_kan	pVB-1B0B1-mCh carrying Kan^r	This work
pVB-1B1B1-mCh	pVB-1A1B1-mCh with mutated *Pm* promoter and 5'-UTR*(comPU)	Vectron Biosolutions unpublished
pVB-1B1B1-mCh_kan	pVB-1B1B1-mCh carrying Kan^r	This work
pVB-1B0B1-IL-1RA	pVB-1A0B1 with IL-1RA-*c-myc*-his6 fusion gene mutated *Pm* promoter and 5'-UTR*(comPU)	Vectron Biosolutions unpublished
pVB-1B1B1-ins	pVB-1A0B1 with fusion gene *pel*B-insulin-*c-myc*-his6 downstream of mutated*Pm* promoter and 5'-UTR*(comPU)	Vectron Biosolutions unpublished
pVB-1L0B1-mCh	pVB-1A0B1-mCh with mutated *Pm* promoter and wt 5'-UTR**(comP)	This work
pVB-1L1B1-mCh	pVB-1L0B1-mCh with with fusion gene *pelB*-mCherry-*c-myc*-his6	This work
pVB-1M0B1-mCh	pVB-1A0B1-mCh with wt *Pm* promoter and mutated 5'-UTR region***(comU)	This work
pVB-5	pVB-1 carrying Kan^r	Vectron Biosolutions unpublished

*comPU: *Pm* variant=ML2-5 and 5'-UTR=H39
**comP: *Pm* variant=ML2-5
***comU: 5'-UTR=H39

2.2 Construction of Plasmid Vectors

2.2.1 Transformation of *E.coli* DH5α

Transformation is the genetic alteration of a cell due to direct up-take of exogenous DNA from the environment through the cell membrane, which then becomes a part of the cells genome. This process can occur natural in many bacterial species, however, it is mostly conducted through artificial means and properties. Only competent cells can take up foreign DNA through transformation. Competent cells have gone through a chemical or physical treatment and the effect is transient. Transformation is one of three methods where exogenous genetic material can be introduced in new bacteria. Conjugation and transduction are the two other methods, where genetic material is transferred from one cell to another by direct contact (Section 2.7) or foreign DNA are injected into a new cell by bacteriophage virus, respectively [1], [51].

Super Competent E.coli DH5α

Preparation of Super Competent (RbCl-competent) *E.coli* DH5α cells were prepared by inoculating *E.coli* DH5α in 10 mL LB medium without antibiotics over night in a shaking incubator at 37 °C. The next day 1 mL of the culture was re-inoculated in 100 mL Psi medium. The optical density measured at 600 nm (OD_{600}) was followed closely, and the cells were harvested after reaching $OD_{600} = 0.4$ by centrifugation at 4 °C, 4500 rpm for 5 minutes. The cells were re-suspended in cold TFB1 and incubated on ice for 5 - 15 minutes. The cells were harvested with the same conditions as when they were washed and re-suspended in cold TFB2. The cell solution was aliquoted in micro centrifuge tubes (100 μL/tube) and frozen immediately in liquid nitrogen and stored at -80 °C. The competent cells were evaluated by transformation with pLit28 and diluted in a dilution series. The competent cells were able to take up the free plasmid since it grew on LB agar plates with Amp.

Transformation of E.coli DH5α

A tube of super competent cells was thawed on ice and added 10 μL ligation mixture and incubated on ice for 30 minutes. The cells were placed in a 42 °C water bath for 35 seconds (heat-shock), and then incubated on ice for 2 minutes. 1 mL of preheated SOC medium (37 °C) was added to the cells and the cells were incubated at 37 °C in a water bath for 60 minutes. After incubation, 100 μL cell solution was plated out on LA-plates added selective antibiotic determined by the plasmids antibiotic resistance. The rest of the solution was centrifueged, cells harvested and resuspended before plated on LA-agar plate with antibiotics. The plates were incubated at 37 °C over night. After incubation colonies were picked, transferred to a new plate and into liquid LB-medium for further incubation at 37 °C over night. Plasmids from the different cultures, originating from the individual colonies were isolated and investigated by restriction digestion. Colonies harboring plasmids that gave correct digestion patterns with chosen restriction enzymes were grown and stored at -80 °C.

2.2.2 Plasmid Isolation

Isolation of plasmid DNA for purification of DNA from bacteria was performed using Promega's Wizard R Plus SV Miniprep DNA Purification System, based on the alkaline lysis method was used in this work. It is a rapid, small-scale isolation which is used for purification of plasmid DNA needed for cloning and verification. First bacteria with the desired plasmid are grown in culture and centrifuged to concentrate all of the cell material into a pellet. The pellet is re-suspended in a buffer with EDTA, which contain cations necessary for the function of DNA degrading enzymes and to stabilize the phosphate backbone of DNA. Then alkaline solution mixed with a strong base is added to the mixture and incubated. This will cause the cell membrane to disrupt and the alkali will impact the chromosomal and plasmid DNA causing it to denaturate, due to the increase in pH. Potassium acetate is then added to acidify the solution and plasmid DNA will stay in the solution, while chromosomal DNA precipitates [5]. After centrifugation, plasmid DNA will be located in the supernatant and chromosomal DNA and other unwanted components are located in the pellet. For isolation of plasmid DNA from the supernatant, the solution is transferred to a column which DNA will binds to when centrifuged. Finally the column is washed and DNA is eluted out by ion free water without nucleases (Wizard®

Plus SV Minipreps DNA Purification System Protocol). Purified DNA was stored at -20 °C.

Concentration Determination

The concentration of plasmids was measured using a Nanodrop Spectrophotometer ND-1000 (Saveen & Werner). The absorbance was measured at 260 nm and 280 nm, and concentration was calculated from Lambert-Beers law. The calculations were conducted automatically by the software associated with the instrument. The ratio between 260:280 was expected to be between 1.8 - 2.0 for samples of plasmid DNA [9].

2.2.3 Restriction Site Digestion of DNA

Construction and verification of the applicable plasmids were obtained by digestion with restriction enzymes. Restriction enzymes binds to specific nucleotide sequences, restriction sites, in the DNA and cleave the double strand (ds) at these sites. In Figure 2.1 digestion of ds DNA with NotI is shown, yielding cohesive ends [51].

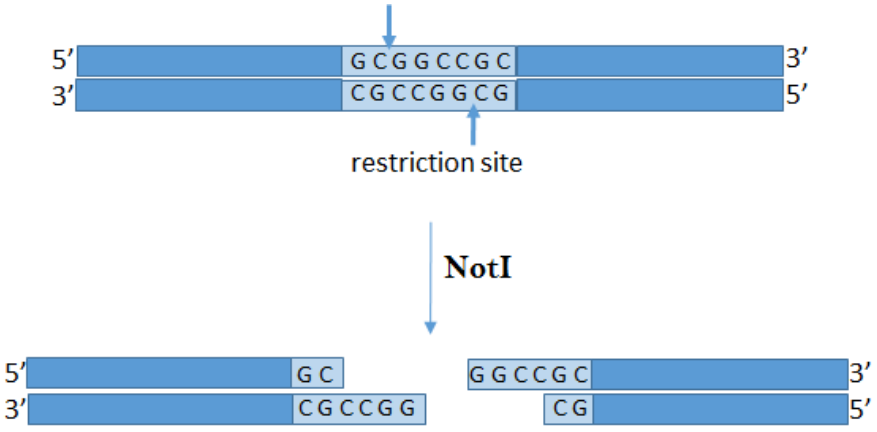


Figure 2.1: Cleavage of DNA strand by restriction enzyme NotI yielding cohesive ends. Figure adapted from Addison Wesley Longman, Inc. (AWLInc).

In Appendix B in Table B.1, an example of the components needed for digestion is given as a sample digestion mixture. The individual restriction enzymes require different buffers to cleave the DNA. Appropriate buffer is found by double digest calculator (NEB).

The digestion mixture is incubated for around 1 hour at 37 °C depending on the enzymes used. 6x loading dye (10 μL) is added to the solution before run on a 0.8 % agarose gel (Section 2.2.4) at 90 volts for 30 minutes. When used for cloning, the desired fragments are cut out from the gel and purified as described in Section 2.2.5. However, when evaluation of bands are in order, the fragments in the gel are only observed by ChemiDoc (Bio Rad).

2.2.4 Gel Electrophoresis

Gel electrophoresis is the process where suspended particles migrate in an electrical field and are thus separate according to size [51]. Small particles will migrate fastest and give rise to bands further down on the gel, whereas larger particles will not migrate as far and leave bands higher up on the gel. Simultaneously as the samples a ladder is also run to work as a reference for the other samples since each bands actual size is known. Ladders used for reference in this study are given in Appendix D in Figure D.1.

Procedure

First the agarose gel is prepared with wells for the DNA samples. Then the samples are dissolved in loading buffer with a dye. This buffer has a density greater than the electrophoresis buffer so the samples will settle at the bottom of the wells instead of diffusing into the electrophoresis buffer. The dye makes it easier to detect the samples after migration. When the DNA solutions are prepared, they are loaded into individual wells of the gel. The power is switched on and the electrophoresis started. The voltage and duration of the electrophoresis is altered according to the size of the gel and the fragments which are going to be separated. When the gel is done, it is photographed using ChemiDoc (Bio Rad).

2.2.5 Gel Extraction of DNA

When gel electrophoresis is completed (Section 2.2.4), the bands on the gel are investigated by ChemiDoc (Bio Rad). The desired bands are cut out from the gel and the DNA is extracted from the gel fragments according to the user manual of Zymoclean Gel DNA recovery Kit (Zymo Research). In short, agarose dissolving buffer (ADB) is added to

the gel fragments and incubated at 55 °C until completely dissolved, transferred to a Zymo-Spin column in a collection tube, centrifuged, washed with wash buffer and eluated directly from the column by DNA eluation buffer. The DNA obtained is stored at -20 °C.

2.2.6 Preparation of Oligonucleotides

Phosphorylation and annealing of oligonucleotides (oligos) were conducted prior to ligation when constructing expression vectors with alternative *Pm* promoter and 5'-UTR region according to the following protocol:

1. Add 7 μL oligo forward primer (100 pmol/μL), 7 μL oligo reverse primer (100 pmol/μL), 1.5 μL 10x T4 DNA ligase buffer and 0.8 μL PNK (T4 polynucleotide kinase) in PCR tubes.
2. Incubate at 37 °C for 30 minutes and 65 °C for 30 minutes in a PCR machine.
3. Add 4 μL 1M NaOH and run the following PCR program for annealing:
   (a) Lid: 105 °C, 95 °C (t = 10 min), 80 °C (t = 2 min), 75 °C (t = 2 min), 70 °C (t = 3 min), 65 °C (t = 5 min), 60 °C (t = 10 min), 55 °C (t = 10 min), 50 °C (t = 5 min), 45 °C (t = 3 min), 40 °C (t = 2 min), 35 °C (t = 1 min), 30 °C (t = 1 min), 25 °C (t = 1 min), 20 °C (t = 1 min), and hold 4 °C.
4. Dilute the reaction 500x (2 μL sample and 998 μL dH_2O). The concentration should be around 5 - 7.5 ng/μL.

The temperature at 95 °C for 10 minutes is to ensure elimination of any existing secondary structures in the oligos, and 60-55 °C is around 15 °C lower than the melting temperature of the oligos which are annealed. The rest of the temperatures should be altered accordingly in terms of this temperature.

Oligos used in this work were *Pm* promoter ML2-5 [3] and 5'-UTR region H39 (LIII-3) [4], both given in Table 2.2.

Table 2.2: Sequences of the different variants of the *Pm* promoter and 5'-UTR region used in this work are given. The sequence for the wild type (wt) and mutated variants are compared. The restriction sites are underlined and the respective restriction enzymes are given above the sequences in italic. For the mutated variants only the differences in the sequence compared to wt are shown, where dotted lines indicate identical sequence.

Element	**Variant**	**Sequence ('5 - 3')**
		XbaI *PciI*
Pm promoter	wt	TCTAGAAAGGCCTACCCCTTAGGCTTTATGCAACATGT
	ML2-5	. . . . . C . . . . . A . . . . . A . . . . .
		PciI *NdeI*
5'-UTR region	wt	ACATGTACAATAATAATGGAGTCATGAACATATG
	H39	. . . . . C . . . . . T . . . . .

The annealing of the forward and reverse primers for the two oligos ML2-5 and H39 will result in cohesive ends, shown in Figure 2.2.

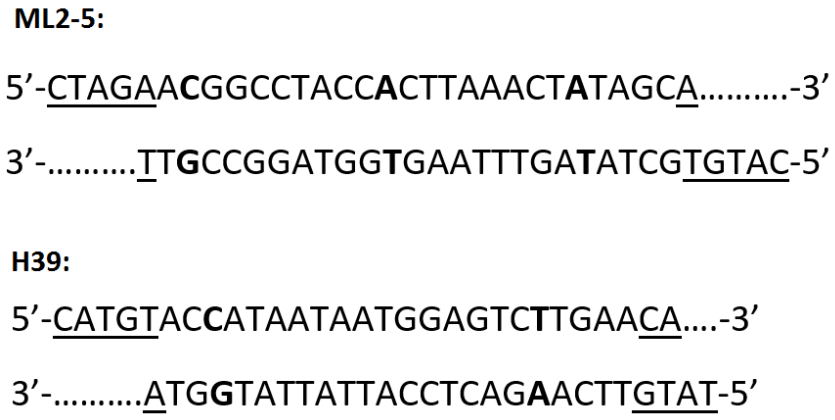


Figure 2.2: Annealing of forward and reverse primers for oligonucleotides ML2-5 and H39 will result in cohesive ends, indicated by the dots in the figure. The underlined regions are the restriction sites for restriction enzymes XbaI and PciI for ML2-5 and PciI and NdeI for H39. The nucleotides in bold text indicates where the mutated versions of the *Pm* promoter and 5'-UTR region differ from wt.

2.2.7 Ligation

Ligation was the last step of the construction process. The ligation reaction was catalyzed by T4 DNA ligase (New England Biolabs). The vector-insert ratio was 1:3 and the amounts needed for the ligation was calculated based on both vector and insert size and concentration of the samples by using the *Ligation Calculator* (http://www.insilico.uni-duesseldorf.de/Lig_Input.htmL). The total ligation mixture was 10 μL, given an example in Appendix B in Table B.2. The mixtures was incubated at 16 °C over night or at room temperature for 1 hour.

2.3 Construction of Expression Vectors

All expression vectors constructed in this work are given in Table 2.1 (Section 2.1), which include expression vectors with alternative *Pm* and 5'-UTR regions and Kan^r. Maps for the different plasmid vectors are given in Appendix A.2.

2.3.1 Expression Vectors with Alternative *Pm* Promoter or 5'-UTR Region

pVB-1L0B1-mCh, pVB-1L1B1-mCh and pVB-1M0B1-mCh are expression vectors with mutated *Pm* promoter (pVB-1L0B1-mCh/pVB-1L1B1-mCh) or 5'-UTR region (pVB-1M0B1-mCh), and constructed as part of this thesis.

pVB-1L0B1-mCh and pVB-1L1B1-mCh

pVB-1L0B1-mCh and pVB-1L1B1-mCh were constructed from pVB-1A0B1-mCh, pVB-1A1B1-mCh and oligo ML2-5 [3], following methods described in Section 2.2. *E.coli* DH5α harboring pVB-1A0B1-mCh and pVB-1A1B1-mCh were cultured in LB-medium. Plasmids were isolated from the cultures (Section 2.2.2) and digested (Section 2.2.3) with restriction enzymes XbaI and PciI, yielding large backbone-XbaI/PciI-fragments (9032 and 9101 bp) and wt *Pm* promoter (32 bp). The digested samples were run on gel electrophoresis (Section 2.2.4). The desired backbone-XbaI/PciI-fragments were then cut out of the gel and plasmid DNA was extracted (Section 2.2.5). Oligo ML2-5 (mutated *Pm* promoter) was phosphorylated and annealed (Section 2.2.6). The plasmid DNA obtained for the backbone-fragments and the prepared oligo ML2-5 were ligated (Section 2.2.7) to finalize the plasmid vectors pVB-1L0B1-mCh and pVB-1L1B1-mCh. *E. coli* DH5α cells were transformed with the ligation mixture (Section 2.2.1). The colonies were verified by a control digestion with XbaI and NotI, to ensure the introduction of the alternative *Pm* promoter ML2-5 and the *mCherry* gene (773 and 8289 bp for pVB-1L0B1-mCh, 842 and 8289 bp for pVB-1L1B1-mCh). A sketch of the construction is given in Figure 2.3.

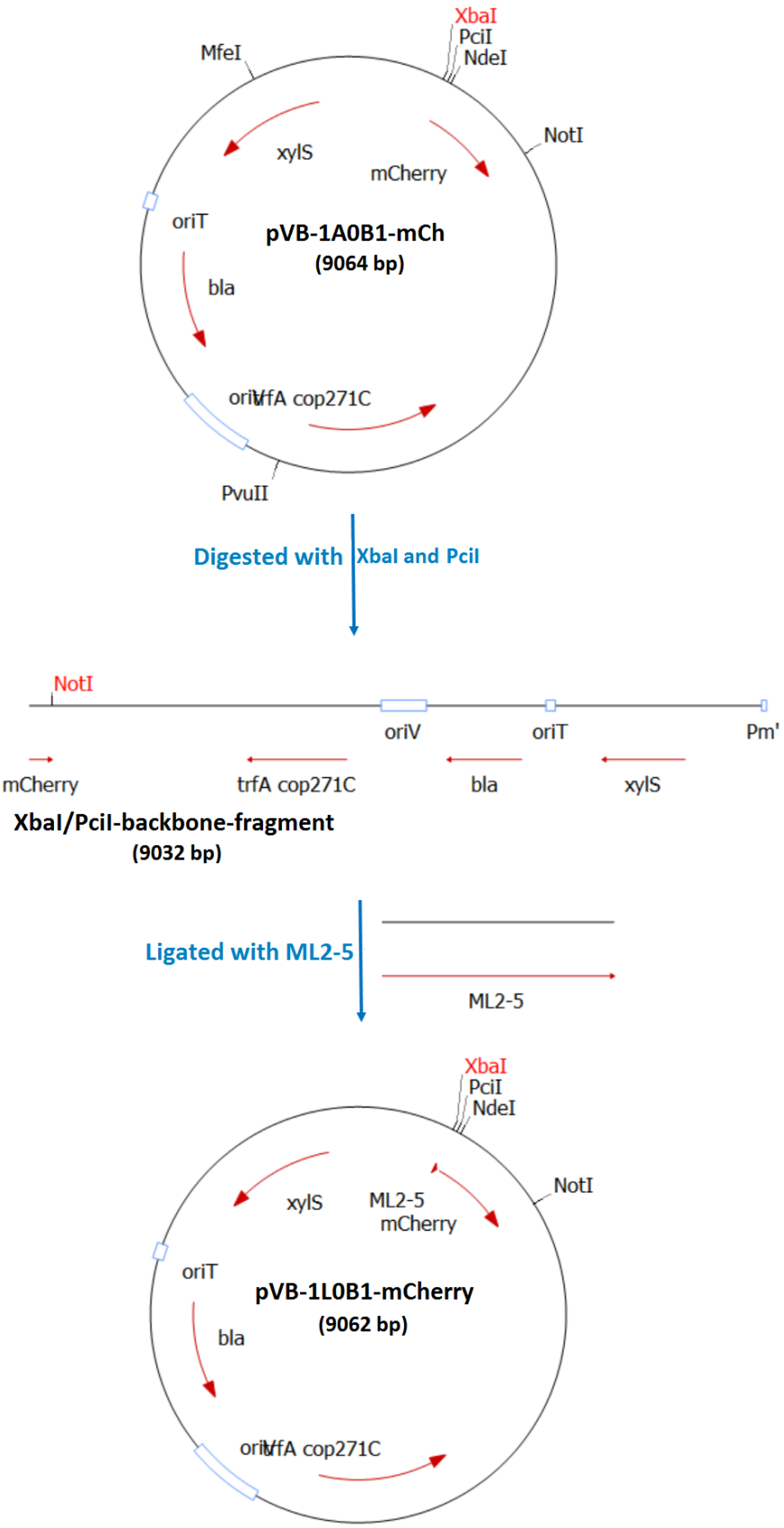


Figure 2.3: Construction of plasmid vector pVB-1L0B1-mCh. pVB-1A0B1-mCh is digested with restriction enzymes XbaI and PciI. The large XbaI/PciI-backbone-fragment obtained after digestion is ligated together with phosphorylated and annealed oligonucleotide ML2-5, yielding expression vector pVB-1L0B1-mCh.

pVB-1M0B1-mCh

pVB-1M0B1-mCh was constructed from pVB-1A0B1-mCh and oligo H39 [4], following methods described in Section 2.2. *E.coli* DH5α harboring pVB-1A0B1-mCh was cultured in LB-medium. Plasmids were isolated from the culture (Section 2.2.2) and digested (Section 2.2.3) with restriction enzymes PciI and NdeI yielding large backbone-PciI/NdeI-fragment (9035 bp) and wt 5'-UTR region (29 bp). The digested samples were run on gel electrophoresis (Section 2.2.4). The desired backbone-PciI/NdeI-fragment (9035 bp) was cut out of the gel and plasmid DNA was extracted (2.2.5). Oligo H39 (mutated 5'-UTR region) was phosphorylated and annealed (Section 2.2.6). The plasmid DNA obtained for the backbone and the prepared oligo H39 were ligated (Section 2.2.7) to finalize the vector pVB-1M0B1-mCh. *E. coli* DH5α was transformed with the ligation mixture (Section 2.2.1). The colonies were verified by a control digestion with PciI and NotI, to ensure the introduction of the alternative 5'-UTR region and the *mCherry* gene (743 and 8321 bp). A sketch of the construction is given in Figure 2.4.

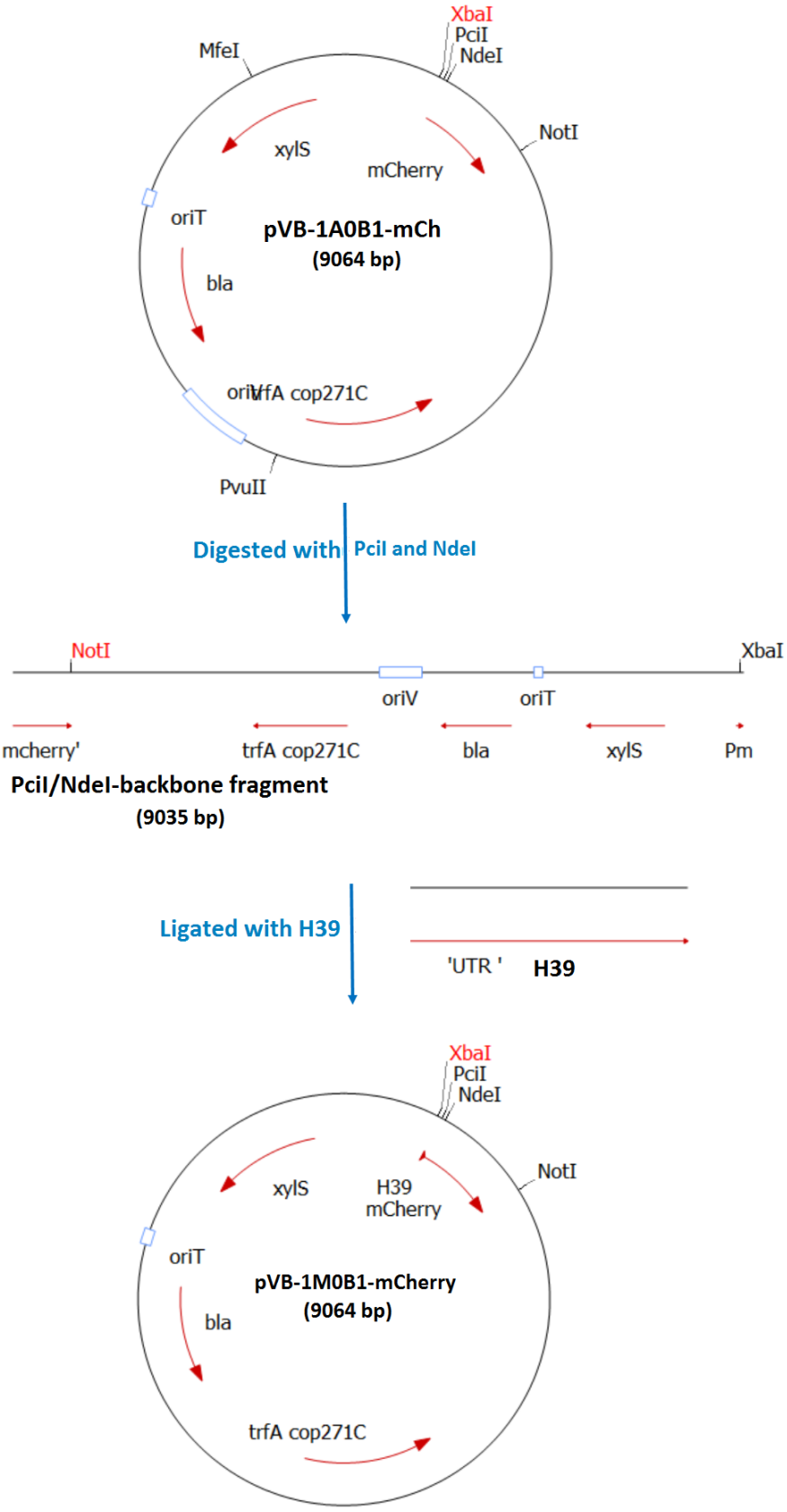


Figure 2.4: Construction of plasmid vector pVB-1M0B1-mCh. pVB-1A0B1-mCh is digested with restriction enzymes PciI and NdeI. The obtained PciI/NdeI-backbone-fragment and phosphorylated and annealed oligonucleotide H39 are ligated together yielding expression vector pVB-1M0B1-mCh.

2.3.2 Expression Vectors with Kanamycin Resistance

pVB-1A0B1-mCh_kan, pVB-1A1B1-mCh_kan, pVB-1B0B1-mCh_kan and pVB-1B1B1-mCh_kan were constructed from corresponding vectors harboring Amp^r (pVB-1A0B1-mCh, pVB-1A1B1-mCh, pVB-1B0B1-mCh and pVB-1B1B1-mCh) and pVB-5 carrying Kan^r, following methods described in Section 2.2. Only the construction of one of the vectors (pVB-1A0B1-mCh_kan) is explained in detail, since the construction of all four expression vectors are the same. *E.coli* DH5α containing pVB-1A0B1-mCh and pVB-5_kan were cultured in LB-medium with appropriate antibiotics. The plasmids were isolated (Section 2.2.2) and digested (Section 2.2.3) with the restriction enzymes PvuII and MfeI. Digestion yielding vector fragments including the *mCherry* gene (5703 bp) and *bla* gene (3361 bp) for pVB-1A0B1-mCh, and *kan* carrying fragment (3906 bp) and other fragment (3337 bp) for pVB-5. The digested samples were run on gel electrophoresis (Section 2.2.4) and the desired fragments were cut out of the gel and DNA was extracted (2.2.5). The DNA obtained was ligated (Section 2.2.7) to finalize the vector pVB-1A0B1-mCh_kan. *E. coli* DH5α was transformed with the ligation mixture (Section 2.2.1). Correct colonies were verified by a control digestion of purified plasmid with XhoI and NdeI to verify the presence of the *mCherry* and *kan* gene when Kan was used as antibiotic selection (6685 and 2924 bp). A sketch of the construction of pVB-1A0B1-mCh_kan by sub-cloning is given in Figure 2.5 [12].

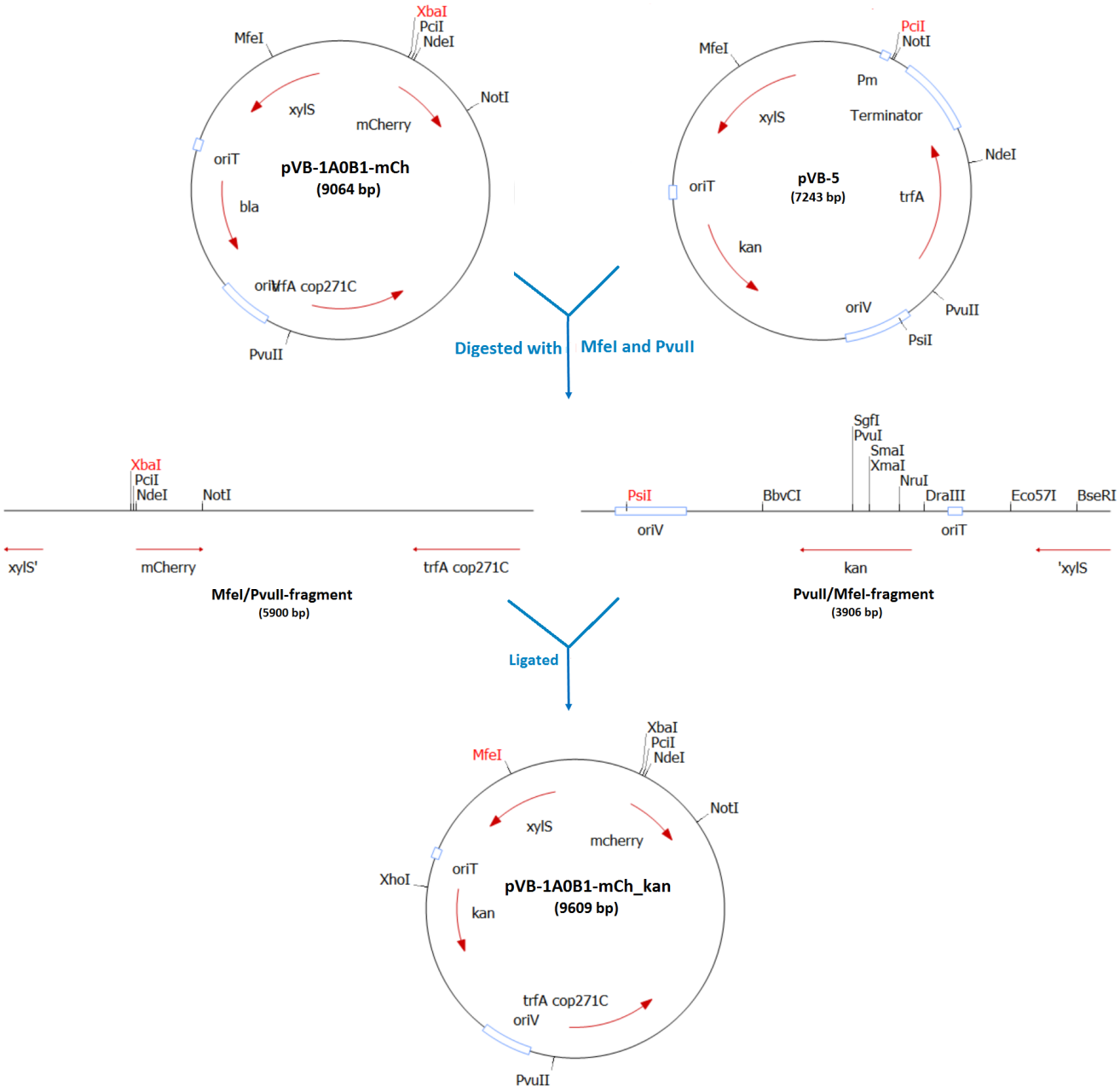


Figure 2.5: Construction of plasmid vector pVB-1A0B1-mCh_kan by sub-cloning. pVB-1A0B1-mCh and pVB-5 are digested with restriction enzymes MfeI and PvuII. Vector fragment MfeI/PvuII (5703 bp) carrying *mCherry* and PvuII/MfeI-fragment (3906 bp) harboring *kan* are ligated together, yielding the expression vector pVB-1A0B1-mCh_kan.

2.4 Transformation of *E. coli* BL21(DE3)

E. coli BL21(DE3) was transformed with different expression vectors to evaluate the expression. It was used as a reference compared to the alternative host for expression. Transformation of *E. coli* BL21(DE3) follow the same procedure as *E. coli* DH5α, described in Section 2.2.1.

2.5 Transformation of *E. coli* S17-1

E. coli S17-1 was used for conjugation with *P. haloplanktis* TAC125. The procedure for TSS-competent cells (Section 2.7.1) was used when transforming *E. coli* S17-1.

2.6 Transformation of *P. putida*

P.putida was transformed with several vectors by electroporation. Large and charged molecules can not passively diffuse across the hydrophobic bi-layer of the cell membrane, but when an electrical field is applied to cells it will cause the permeability of the cell membrane to increase. Chemicals and DNA can then enter and be integrated in the cell. Electrocompetent cells are therefore added plasmid DNA and gently mixed before applying a voltage across a very small distance which is a huge stress on the cells. After this pulse the cells are handled with care and supplied with nutrients and other necessary growth factors so they can start dividing and give rise to many copies of the cells with the newly introduced plasmid DNA [52].

2.6.1 Preparation of Electrocompetent *P. putida* Cells

Electrocompetent *P.putida* cells were prepared, for use in electroporation, by the following procedure.

1. Inoculate *P. putida* cells in 10 mL LB medium. Incubate over night at 30 °C and 225 rpm.
2. Inoculate 100 mL preheated LB with 1 mL on-culture.
3. Incubate at 30 °C and 225 rpm until OD_{540}=0.3-0.5 (about 2.5-3 hours).

4. Transfer the culture to cold 50 mL tubes. Incubate on ice for 30 minutes.
5. Harvest the cells by centrifuging (4 000 xg, 15 minutes, 4 °C).
6. Wash the cells three times with sterile 10 % glycerol and centrifuge in-between each wash (4 000xg, 15 minutes, 4 °C):
   (a) 50 mL in each tube
   (b) 25 mL in each tube
   (c) 5 mL in each tube
7. Resuspend the cells in 0.5 mL 10 % glycerol.
8. Aliquot the cells in 1.5 micro-centrifuge tubes (40 μL/tube), and freeze immediately.
9. Store the cells at -80 °C.

2.6.2 Electroporation of *P. putida*

Electroporation of *P. putida* was done to integrate the desired expression vectors into the alternative host. Electrocompetent *P. putida* cells (Section 2.6.1) was added plasmid DNA and then electroporation carried out with use of Gene Pulser Electroporation System (Bio-Rad) and 2 mm cuvettes (VWR), yielding *P. putida* with wanted integrated genetic material, here in the form of expression vectors. The electroporation was performed according to the following procedure:

1. For each electroporation:
   (a) 40 μL electro-competent cells
   (b) 4 μL chilled DNA
2. Gently mix and incubate on ice for 30 minutes.
3. Electroporation is conducted in a 2 mm cuvette (VWR) and a Gene pulser Electroporation system is used (Bio-Rad) with the following settings:
   (a) 2.5 kV
   (b) 25 μF

(c) 200 Ω

4. Straigth after the pulse the sample is added preheated SOC medium (37 °C) and incubated at 30 °C for 2 hours.
5. Plate out 100 μL culture on LA+Amp100 agar plate.
6. Spin down and plate out the resuspended cell pellet.
7. Incubate the plates for 2 days at 30 °C.

2.7 Conjugation of *P. haloplanktis* TAC125

P. haloplanktis TAC125 cells were conjugated with *E. coli* S17-1 harboring expression vectors carrying the *mCherry* gene [16]. Conjugation is a type of sexual reproduction mechanism where vegetative cells lay adjacent to each other and genetic material can be exchanged and cause recombination. The process was discovered in 1946 by Lederberg and Tatum and is still very useful in biotechnology [30]. One cell is F^+ and contain a F factor which makes it the donor cell. The F^- cell, is the recipient cell, and receives genetic material from the donor cell through a cytoplasmic bridge which forms between the two cells. This is formed by the two cytoplasms fusing together; connecting the two cells and making exchange of DNA possible. The bridge is also called pilus [51].

2.7.1 TSS-competent *E.coli* S17-1 Cells

TSS competent *E. coli* S17-1 cells were prepared, for use in conjugation of *P. haloplanktis* TAC125, by the following procedure.

1. 10 mL starter culture of *E. coli* S17-1 in LB-medium is inoculated over night at 37 °C and 225 rpm.
2. Transfer of 0.1 mL overnight culture to 10 mL fresh LB-medium (1% inoculum).
3. Incubate at 37 °C and 225 rpm until OD_{600nm}= 0.4.
4. Put cell cultures on ice for 5 minutes and centrifuge in Eppendorf tubes (1 mL/tube), 3000 rpm, 10 min.
5. Remove the supernatant and resuspend in 1 mL TSS.

6. Quickly freeze cells in liquid nitrogen and store at -80 °C.

The transformation procedure is similar as heat-shock method described in Section 2.2.1 except preheated LB-medium is used instead of SOC.

2.7.2 Conjugation Procedure

Conjugation of *P. haloplanktis* TAC125 was done to integrate the desired expression vectors from *E. coli* S17-1 into the alternative host. F^+ *E. coli* S17-1 cells (Section 2.7.1) were grown to OD_{600nm} around 1, mixed with *P. haloplanktis* TAC125 with similar OD-value. The mixture was dropped on a LB agar plate and incubated overnight at 30 °C. Next day the cell mass on the plate was collected and diluted in TYP-medium. This culture was further diluted and plated on TYP agar plates with correct antibiotic selection according to the expression vectors integrated and incubated at 15°C for two days. This allow only *P. haloplanktis* which has received the plasmid carrying the antibiotic resistance gene to grow. The growth on the plates are selected by both temperature and antibiotic resistance. This yielded *P. haloplanktis* TAC125 with integrated genetic material, here in the form of expression vectors. This protocol was adapted from Wang et al 2015, which is based on Dominguez et al. 2013 [14], [60].

1. Prepare over night (on) cultures of *E. coli* S17-1 harboring expression vectors and wt *P. haloplanktis* TAC125:
   (a) *E. coli* S17-1: LB medium (added Amp, 100 μg/mL), 37 °C, 225 rpm
   (b) *P. haloplanktis* TAC125: TYP medium, 15 °C, 225 rpm
2. Inoculate 150 μL on-culture in 15 mL medium (1 % inoculum), and incubate until OD_{600nm} is around 0.8-1.0
3. Harvest 2 mL donor cells (*E.coli* S17-1) and 1 mL recipient cells (*P. haloplanktis TAC125)* by centrifugation (4000 rpm, 3 min) and wash with LB-medium.
4. Mix donor and recipient cells briefly before dropping on a LB-agar plate, and incubate over night at 30 °C.
5. Scrape cells and resuspend in TYP-medium and make a serial dilution. Plate out on TYP-agar plates with 100 μg/mL Amp, and incubate at 15 °C for two days.

2.8 Expression of mCherry

mCherry expression was investigated by growing *E. coli* BL21(DE3), *P. putida* and *P. haloplanktis* harboring expression vectors over night in LB and TYP medium added Amp. A small amount of the cultures were added to the distinct media (Hi+Ye Basis medium) yielding a start OD_{600}-value of 0.05. When OD-values reached 2 - 2.5 the cultures were induced with 2 mM m-toluate and incubated over night. The next day the cell pellets were harvested and prepared for analysis by SDS-PAGE and Western blot to visualize the proteins expressed by the different expression vectors for the two hosts. Also the activity of mCherry was measured as described in Section 2.8.4.

2.8.1 Growth of Bacteria and Expression of Recombinant Protein

The same procedure was followed when expressing mCherry in BL21(DE3) and *P. putida*, except the growth temperatures which were 37 °C and 30 °C, respectively. *P. haloplanktis* TAC125 followed a similar procedure except TYP-medium (added Amp) was used instead of Hi + Ye Basis medium and the culture was grown at 15 °C. Bacterial cultures were grown at 37 °C, 30 °C and 15 °C before induction, and after induction with m-toluate the temperatures where reduced to 16°C and 25 °C for *E.coli* BL21(DE3) and *P. putida*. The temperature for *P. haloplanktis* remained at 15 °C. The cultures were grown over night and harvested the next day. The procedure was as follows:

1. Inoculate the main culture with a volume of on-culture yielding a start OD_{600}=0.05 to 30 mL Hi + Ye Basis medium (added 2.5 mL 1 M $MgSO_4$*$7H_2O$, 600 μL Glycerol, 240 μL glucose and 30 μL Amp).
2. Incubate for 3 - 4 hours at 37/30/15 °C, 225 rpm.
3. Follow OD_{600} and induce when values are about 2 - 2.5.
4. Induce samples with 120 μL 0.5 M m-toluate stock (2 mM m-toluate).
5. Incubate over night at 16/25/15 °C, 225 rpm.
6. Transfer 30 mL culture to a 50 mL tube and centrifuge for 10 minuts at 4 °C, 10 000 rpm.

7. Vacuum the supernatant and weigh the pellett.
8. On ice: Resuspend the pellett in 0.9 % NaCl.
9. Centrifuge (11 000 rpm, 5 min, 4 °C) and vacuum the supernatant.
10. Freeze the samples or prepare for SDS-PAGE right away.

2.8.2 Protein Isolation and SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used technique to separate proteins according to their size due to electrophoretic mobility. SDS binds to the polypeptide chain which leads to an even charge per unit mass so molecules will only differ in size. Smaller proteins will migrate the furthest on a membrane and larger once will not migrate as far [47]. In Appendix C in Figure C.1 the ladder used for SDS-PAGE and Western is given. The pre-stained version Dual Color Ladder (Precision Plus Protein Standards, Bio Rad) was used for the experiments in this study.

Protein Isolation

This procedure split up the soluble and insoluble fractions of the proteins obtained from the growth experiment described in Section 2.8.1.

1. Resusupend the pellet in 500 μL Cell Lytic M (Sigma) and incubate for 1 hour on ice.
2. Centrifuge for 8 minutes at 13 000 rpm at room temperature.
3. Transfer 500 μL of the supernatant to a sterile 1.5 micro centrifuge tube (soluble fraction).
4. Resuspend the pellet in 500 μL 1xSDS running buffer and dilute the insoluble fraction 1/5 (200 μl resuspended sample and 800 μL 1xSDS running buffer).
5. Add 15 μL 3x loading buffer to 30 μL sample for both soluble and diluted insoluble and boil the samples at 99 °C for 5 minutes.

SDS-PAGE

The denaturated samples and ladder are loaded into wells of the precast SDS-PAGE gel (Bio-Rad). The gel was run at 130 V for 90 minutes. After completion, the gel was rinsed in distilled water and stained with Instant blue (Expedeon) for 1 hour or over night. The stain was poured off and the gel rinsed with water making it ready for scanning.

2.8.3 Western Blot

Western blot is a procedure used for visualising proteins on a membrane by allowing antibodies to bind tags on the protein. This is a very useful tool for detection of specific proteins. The Trans-Blot Turbo Transfer System from Bio Rad was used for transfer of the proteins from the SDS-PAGE to the blotting membrane. The set-up for the blotting sandwich is given in Appendix C in Figure C.2.

Detection of proteins on the Western blot follow the indirect method where a primary antibody is added first followed by a secondary antibody. The primary antibody binds to the antigen specific for the gene of interest and the secondary antibody contain a label which is directs against the primary antibody. In this work the blotting membranes were incubated with primary antibody Anti-Myc Antibody (Invitrogen), which will bind itself to the *c-myc* downstream of the gene of interest (mCherry) in our expression vectors, seen in Figure 1.5, and be visualized by secondary antibody Polyclonal Rabbit Anti-mouse Immunoglobulins/HRP (Dako Denmark).

Procedure

1. Run SDS-PAGE with wanted samples and ladder.
2. Assemble the blotting sandwich as shown in Figure C.2.
3. Run program mixed MW (molecular weight), duration 7 minutes.
4. Retrieve the blotting membrane and wash it with TBS for 1 minute.
5. Block membrane in cold, freshly made Blocking solution (30 mL) for an hour at room temperature and 100 rpm.

6. Add 30 mL Blocking solution added 3 μL primary antibody. Incubate over night at 4 °C and 100 rpm.
7. Wash the membrane with TBST for 10 minutes, three times.
8. Add 30 mL Blocking solution added 15 μL secondary antibody. Incubate for 1 hour at room temperature and 100 rpm.
9. Wash membrane with TBST for 10 minutes, three times. Rinse with TBS for 10 minutes.
10. Place the blotting membrane in a clean container and add 3 mL substrate, TMB (Thermo Scientific), and incubate in room temperature for 1-5 minutes.
11. Rinse the membrane with MQ-water to stop the reaction and scan the membrane.

2.8.4 Activity of mCherry

Activity of mCherry was determined by use of a micro-plate reader fluorometer (Infinite 200 Pro, Tecan). It measures the amount of relative fluorescent units (RFU) in an sample and generated the data via computer software. The samples were loaded (100μL) in individual wells in an Infinite 200 Pro multifunctional microplate. The samples were measured by setting the system to measure excitation wavelength 584 nm (9 nm bandwidth) and emission wavelength 620 nm (20 nm bandwidth).

2.9 Identification of Oxidase Positive Bacteria by Kovacs Test

Oxidase positive bacteria can be identified by the presence of cytochrome c-oxidase forming a colour complex with the Kovacs oxidase reagent (1g N-tetrametyl-p-fenylendiamin mixed in 100g dH_2O) giving a dark blue color. Figure 2.6 shows a photograph after a conducted Kovacs test where *P. putida* and *P. haloplanktis* are oxidase positive seen by the dark blue color, and *E. coli* is oxidase negative as no color-change occurred after the Kovacs oxidase reagent was added to the culture [33].

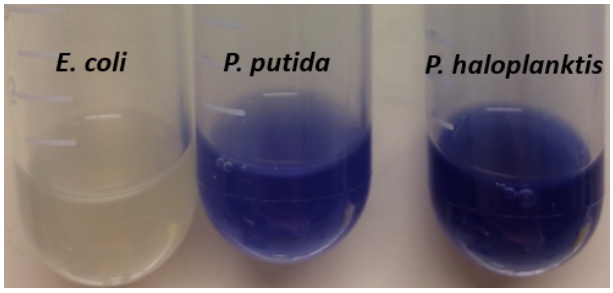


Figure 2.6: Picture of different bacterial cultures after addition of Kovacs oxidase reagent. From the left: *E. coli*, *P. putida* and *P. haloplanktis*

Protocol

1. Grow a fresh culture of bacteria in appropriate media
2. Add 20 μL Kovacs oxidase reagent to 1 mL of cell culture in a test tube.
3. Shake the tube to ensure mixing and thorough oxygenation of the reaction.
4. Observe the color-change within 3 minutes.

Microorganisms are oxidase positive if the color changes within 15-30 seconds. Alternatively, microorganisms can be regarded delayed oxidase positive if the color changes within 2-3 minutes. However, if the color does not change to blue after 3 minutes it is oxidase negative.

2.10 Mediums and Solutions

Mediums and solutions prepared for use in this thesis are given in the following tables.

Lauria-Bertani medium		
Solution	**Amount [g/l]**	**Producer**
Tryptone	10	Oxoid
Yeast extract	5	Oxoid
NaCl	10	Acros organics

Lauria-Bertani agar		
Solution	**Amount [g/l]**	**Producer**
Tryptone	10	Oxoid
Yeast extract	5	Oxoid
NaCl	10	Acros organics
Agar	15	Oxoid

TYP medium*		
Solution	**Amount [mL/l]**	**Producer**
Yeast extract	16	Oxoid
Tryptone	16	Oxoid
Sea salt	10	Sigma

*pH-adjusted to 7.5

TYP agar*		
Solution	**Amount [g/l]**	**Producer**
Yeast extract	16	Oxoid
Tryptone	16	Oxoid
Sea salt	10	Sigma
Agar	15	Oxoid

*pH-adjusted to 7.5

Glycerol 50 %		
Solution	**Amount [mL/l]**	**Producer**
Glycerol 100%	1000	VWR

0.9 % NaCl		
Solution	**Amount [g/l]**	**Producer**
NaCl	9	Acros organics

EDTA		
Solution	**Amount [g/l]**	**Producer**
EDTA	232.5	Oxoid

TrisHCl		
Solution	**Amount [g/l]**	**Producer**
Tris base	6	Fischer Scientific

TFB2		
Solution	**Amount [g/l]**	**Producer**
MOPS	2.1	Fischer Scientific
RbCl	1.21	Acros organics
$CaCl*2H_2O$	11.0	Sigma Aldrich
Glycerol 100%	150 mL/l	VWR prolabs chemicals

TFB1		
Solution	**Amount [g/l]**	**Producer**
KAc	2.94	Merck
RbCl	12.1	Acros organics
$CaCl*2H_2O$	1.945	Sigma Aldrich
$MnCl_2*4H_2O$	15.73	J.T. Baker
Glycerol 100%	150 mL/l	VWR prolab chemicals

Psi media		
Solution	**Amount [g/l]**	**Producer**
Yeast extract	5.0	Oxoid
Tryptone	20	Oxoid
$MgSO_4$	5.0	

TBS		
Solution	**Amount [g/l]**	**Producer**
2 M Tris base	20 mL/l	Fischer Scientific
NaCl	17.6	Acros organics

TBST		
Solution	**Amount [mL/l]**	**Producer**
TBS	1	-
Tween20	0.002	Acros organics

Blocking solution		
Solution	**Amount [g/l]**	**Producer**
Skim milk powder	30	Oxoid

1 M $MgSO_4$*7H_2O		
Solution	**Amount**	**Producer**
$MgSO_4$*7H_2O	12.3 g	Oxoid
Tap water	To 50 mL	Oxoid

Yeast extract Hi		
Solution	**Amount**	**Producer**
Yeast extract	10 g	oxoid
Tap water	To 100 mL	-

Glucose solution		
Solution	**Amount**	**Producer**
Glucose	11.35 g	VWR
Tap water	To 50 mL	

Glycerol solution		
Solution	**Amount**	**Producer**
Glycerol (100%)	50.15 g	VWR
Tap water	To 100 mL	

Fe(III) citrate hydrate stock solution		
Solution	**Amount**	**Producer**
Fe(III) citrate hydrate	6 g	
Distilled, ion free water	1000 mL	

H_3BO_3 stock solution		
Solution	**Amount**	**Producer**
H_3BO_3	30 g	
Distilled, ion free water	1000 mL	

$MnCl_2$*4H_2O stock solution		
Solution	**Amount**	**Producer**
$MnCl_2$*4H_2O	10 g	
Distilled, ion free water	1000 mL	

EDTA*2H_2O stock solution		
Solution	**Amount**	**Producer**
EDTA*2H_2O	84 g	
Distilled, ion free water	1000 mL	

$CoCl_2$*6H_2O stock solution		
Solution	**Amount**	**Producer**
$CoCl_2$*6H_2O	25 g	
Distilled, ion free water	1000 mL	

$Zn(CH_3COO)_2$ *2H_2O stock solution		
Solution	**Amount**	**Producer**
$Zn(CH_3COO)_2$ *2H_2O	4 g	
Distilled, ion free water	1000 mL	

$CuCl_2$*2H_2O stock solution		
Solution	**Amount**	**Producer**
$CuCl_2$*2H_2O	15 g	
Distilled, ion free water	1000 mL	

$Na_2Mo_4O_4$ * 2H_2O stock solution		
Solution	**Amount**	**Producer**
$Na_2Mo_4O_4$ * 2H_2O	25	
Distilled, ion free water	1000 mL	

Basis medium 1			
Solution	**Amount [g/l]**	**Amount [mL/l]**	**Producer**
Na_2HPO_4*2H_2O	8.6		Oxoid
KH_2PO_4	3		Acros organics
NH_4Cl	1		Acros organics
NaCl	0.5		Acros organics
Fe(———) citrate hydrate		10	
H_3BO_3		0.1	
$MnCl_2$*4H_2O		1.5	
EDTA*2H_2O		0.1	
$CuCl_2$*2H_2O		0.1	
$Na_2Mo_4O_4$ * 2H_2O		0.1	
$CoCl_2$*6H_2O		0.1	
$Zn(CH_3COO)_2$ *2H_2O		2	
Distilled, ion free water	To 900 mL		

TSS solution			
Solution	**Amount [g/l]**	**Amount [mL/l]**	**Producer**
10 % PEG8000	100		
5 % DMSO		50	Merck
1M $MgCl_2$		50	Sigma Aldrich
LB medium	To 10 mL		

3 Results

The aim for this thesis was to evaluate different expression vectors in three different hosts, using *mCherry* as a reporter gene. The different expression hosts evaluated were *E. coli* BL21(DE3), *Pseudomonas putida* (*P. putida*) and *Pseudoalteromonas haloplanktis* (*P. haloplanktis*) TAC125. The expression vectors tested in this study are based on the broad-host-range plasmid RK2 with the strong xylS/*Pm* promoter system. The expression of mCherry was evaluated by SDS-PAGE, Western blot, color of cell pellet, and fluorescent measurements for both soluble and insoluble proteins fractions.

Plasmid vectors used in this study are given in Section 2.1, Table 2.1 as well as in a quick guide in Appendix A, Table A.1. Expression vectors both previously constructed (Vectron Biosolutions unpublished) and constructed as apart of this thesis were evaluated and introduced into the individual hosts by transformation, electroporation and conjugation. All of the expression vectors were first evaluated using *E. coli* BL21(DE3) as host and were proven to functioned well in BL21(DE3) resulting in expression of the reporter gene in both soluble and insoluble protein fractions. The expression vectors were then introduced to the alternative hosts.

The electroporation of *P. putida* cells with the same expression vectors as evaluated in *E. coli* BL21(DE3) were not successfully verified for all expression vectors and results obtained for the expression indicate no presence of mCherry.

The introduction of the RK2 plasmid vectors into *P. haloplanktis* TAC125 by conjugation was successful. However, an evaluation of the expression of mCherry was not achieved due to challenges in reviving frozen cultures.

3.1 *Escherichia coli*

In this work, three new expression vectors all carrying *mCherry* as reporter gene were constructed. These expression vectors, together with four previously constructed expression vectors (Vectron Biosolutions unpublished), were introduced into three different *E. coli* strains; DH5α, BL21(DE3) and S17-1. An overview of the expression vectors are given in Appendix A.1, Table A.1 and explained more thoroughly in Section 2.1, Table 2.1. The expression of mCherry was evaluated by SDS-PAGE, Western blot, color of cell pellet and fluorescent measurements for soluble and insoluble protein fractions.

3.1.1 Construction and Introduction of Expression Vectors

Expression vectors pVB-1M0B1-mCh, pVB-1L0B1-mCh and pVB-1L1B1-mCh, all harboring *mCherry* (mCh) as reporter gene, were constructed as described in Section 2.3.1 and introduced into the different *E. coli* strains by heat-shock transformation (Section 2.2.1).

Construction of Expression Vectors with Alternative Pm and 5'-UTR

The expression vectors pVB-1M0B1-mCh, pVB-1L0B1-mCh and pVB-1L1B1-mCh were constructed from pVB-1A0B1-mCh and pVB-1A1B1-mCh by replacing the wt *Pm* promoter and 5'-UTR region with mutated *Pm* promoter ML2-5 [3] and 5'-UTR region H39 [4], all shown in Table 3.1.

Table 3.1: Overview of the differences between the expression vectors pVB-1M0B1-mCh, pVB-1L0B1-mCh and pVB-1L1B1-mCh for their type of *Pm* promoter, 5'-UTR region, signal peptide and antibiotic selection.

Vector	***Pm***	**5'UTR**	**Signal peptide**	**Selection**
pVB-1M0B1-mCh	wt	H39 (LIII-3) [4]	-	Amp
pVB-1L0B1-mCh	ML2-5 [3]	wt	-	Amp
pVB-1L1B1-mCh	ML2-5 [3]	wt	*pelB*	Amp

The expression vector pVB-1M0B1-mCh is pVB-1A0B1-mCh with a mutated 5'-UTR region H39. It was constructed by PciI and NdeI digestion of pVB-1A0B1-mCh to replace the existing wt 5'-UTR region with H39. The large PciI/NdeI backbone fragment was

then ligated with phosphorylated and annealed H39 oligos, yielding the new expression vector. The ligation mixture was transferred by heat shock transformation of *E. coli* DH5α and colonies obtained were evaluated by digestion with PciI and NotI. The results are given in Figure 3.1 and correlates well with the expected bands of 743 and 8321 bp (lane 2 and 3).

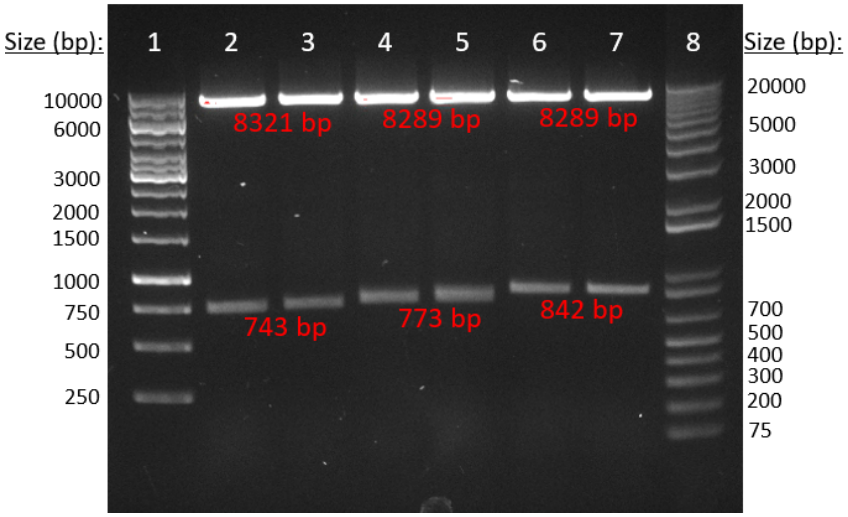


Figure 3.1: Picture of the gel after gel electrophoresis of constructed plasmid expression vectors pVB-1M0B1-mCh digested with PciI and NotI (lane 2-3), pVB-1L0B1-mCh digested with XbaI and NotI (lane 4-5) and pVB-1L1B1-mCh digested with XbaI and NotI (lane 6-7). Lane 1: 1 kb DNA Ladder (GeneRuler). Lane 8: 1 kb Plus DNA Ladder (GeneRuler).

The expression vectors pVB-1L0B1-mCh and pVB-1L1B1-mCh are, respectively, pVB-1A0B1-mCh and pVB-1A1B1-mCh with a mutated *Pm* promoter, ML2-5. They were constructed by XbaI and PciI digestion of pVB-1A0B1-mCh and pVB-1A1B1-mCh to replace the existing wt *Pm* promoter. The large XbaI/PciI backbone fragment were then ligated with phosphorylated and annealed ML2-5 oligos, yielding the new expression vectors. *E. coli* DH5α were transformed by heat-shock with the ligation mixtures and the bacterial cultures were grown on agar plates with antibiotic selection, resulting in colonies. Plasmid DNA was isolated from positive colonies and digested with XbaI and NotI. The results of this digestion mixture after gel electrophoresis are given in Figure 3.1, and correlates well with the expected bands of 773 and 8289 bp for pVB-1L0B1-mCh (lane 4 and 5) and for pVB-1L1B1-mCh 842 and 8289 bp (lane 6 and 7).

Samples of plasmid DNA for the new constructs were sent for sequencing, to fully verify the correct constructions of the plasmid vectors. In Figure E.1, E.2 and E.3 in Appendix E the results are given and show correct integration of the mutated 5'-UTR region H39 and *Pm* promoter ML2-5, respectively, since the DNA sequences of the samples are identical to the references. The references for the constructed vectors were made with computer software *CloneManager*, and were compared in *CloneManager* with the sequencing results obtained for the different plasmid DNA samples.

Introduction of Constructed Expression Vectors into E. coli

Introduction of the expression vectors into *E. coli* DH5α, BL21(DE3) and S17-1 as described in Section 2.2.1 were conducted by heat-shock transformation. The cultures obtained after this transformation were cultivated and selected by its antibiotic resistance on agar plates with antibiotic selection and gave rise to several single colonies. Transformation of DH5α and S17-1 harboring expression vectors pVB-1A0B1-mCh, pVB-1A1B1-mCh, pVB-1B0B1-mCh, pVB-1B1B1-mCh, pVB-1M0B1-mCh, pVB-1L0B1-mCh and pVB-1L1B1-mCh resulted in small white colonies. Some of the colonies with the mutated expression cassette had a light pink color which became more pink within the next day in the fridge. The colonies obtained for BL21(DE3) were a bit larger in size compared to DH5α and S17-1. Negative controls, where no plasmid was added to the competent cells, were transformed simultaneously as the other expression vectors, yielding few or none colonies. To verify the introduction of the new plasmid vectors, plasmid DNA was isolated from single colonies by miniprep and digested with NotI and NdeI. Gel electrophoresis of the digested DNA samples gave rise to bands with similar size as the NdeI/NotI-*mCherry* fragment (714 bp) and NdeI/NotI-backbone fragment (8350 bp), which verifies successful transformation. Pictures of these gels are given in Appendix D, Figure D.2 and D.3 for *E.coli* BL21(DE3) and S17-1, respectively.

3.1.2 Expression of mCherry in *E. coli* BL21(DE3)

E. coli BL21(DE3) cells were transformed with different expression vectors, all carrying the *mCherry* gene. The vectors are divided into, vectors that have been constructed previously (unpublished) and vectors constructed in this study, as seen in Table 3.2. Some

of the expression vectors used in this study have been constructed previously by Vectron Biosolutions (unpublished) and by Nygård T. in previous project during spring 2016.

Table 3.2: Overview of expression vectors previously constructed and constructed in this study with *mCherry* as reporter gene.

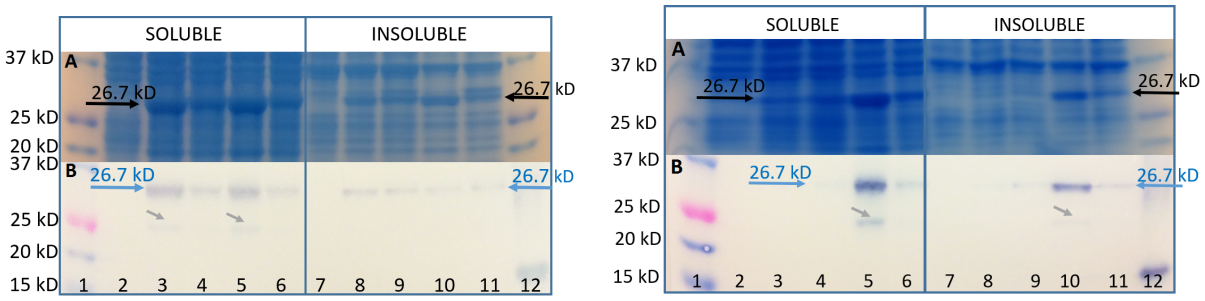
Expression Vectors Previously Constructed *Vectron Biosolutions unpublished*	**Expression Vectors Constructed** *This work*
pVB-1A0B1-mCh	pVB-1M0B1-mCh
pVB-1A1B1-mCh	pVB-1L0B1-mCh
pVB-1B0B1-mCh	pVB-1L1B1-mCh
pVB-1B1B1-mCh	

The expression of mCherry was investigated as described in Section 2.8. Cells were grown in Hi + Ye Basis medium (added $MgSO_4$, Glycerol, Glucose and Ampicillin) at 37 °C until OD_{600} reached 2 - 2.5. There were grown two parallels of each strain to evaluated the effect of inducing and un-inducing the cultures. When the appropriate OD-value was achieved, one culture for each strain were induced by 2 mM m-toluate, and all cultures were grown over night at 16 °C. Next day, cells were harvested from the culture and expressed proteins were separated into soluble and insoluble fractions and visualized by SDS-PAGE and Western blot analysis. Also the color of the supernatant and cell pellets for the different expression vectors were investigated. In Section 3.1.3 the results for the activity of mCherry is presented.

Expression Vectors Previously Constructed

E.coli BL21(DE3) cells were transformed with previously constructed expression vectors (Vectron Biosolutions unpublished) (Project Nygård T.), all harboring the *mCherry* gene, described further in Table 2.1 (Section 2.1) and Table A.1 (Appendix A). Vectors pVB-1A1B1-mCh and pVB-1A0B1-mCh both contain the wt *Pm* promoter and 5'-UTR sequence. pVB-1A1B1-mCh in addition harbor the sequence coding for the signal peptide *pelB* upstream of the gene coding for mCherry. The vectors pVB-1B1B1-mCh and pVB-1B0B1-mCh contain mutated *Pm* promoter ML2-5 and 5'-UTR sequence H39 and pVB-1B1B1-mCh also harbors the coding sequence for signal peptide *pelB*, upstream of the gene coding for mCherry.

In Figure 3.2 (a) and (b) the expression of mCherry in *E.coli* BL21(DE3) for the four expression vectors previously constructed are visualized by SDS-PAGE and Western blot for induced and un-induced cultures, respectively. Both the soluble and insoluble protein fractions were investigated. pVB-1 was used as a negative SDS-PAGE and Western control, and a sample of soluble protein fraction from *E. coli* BL21 harboring pVB-1B1B1-ins (insulin) expression vector (Project Nygård T.) was used as a positive Western control, because it carries the same *c-myc* tag as the expression vectors evaluated in this work.



(a) Induced

(b) Un-induced

Figure 3.2: SDS-PAGE showing the soluble and insoluble protein fractions of induced (a) and un-induced (b) mCherry (26.7 kD) expressed from different expression vectors harboring the *mCherry* gene when *E.coli* BL21(DE3) was used as expression host. Same set-up and order for both gels and membranes evaluated were used for induced (a) and un-induced (b) samples. **A:** SDS-PAGE of soluble (lanes 3-6) and insoluble protein fractions (lanes 8-11) for four different vectors. Lane 1: Ladder (Precision Plus Protein Standards, Dual Color (Bio Rad)), lane 2 and 7: negative SDS-PAGE and Western control (pVB-1), lane 3 and 8: pVB-1A0B1-mCh, lane 4 and 9: pVB-1A1B1-mCh, lane 5 and 10: pVB-1B0B1-mCh and lane 6 and 11: pVB-1B1B1-mCh. Bands representing mCherry are indicated by black arrows. **B:** Western blot of the same vectors and in the same order as for the SDS-PAGE analysis in A. Lane 12: positive Western control (pVB-1B1B1-insulin). Bands of mCherry are indicated by blue arrows.

From the SDS-PAGEs seen in Figure 3.2A, soluble and insoluble expression occurred for both induced (a) and un-induced (b) samples, where bands corresponding to mCherry (26.7 kD) are indicated by black arrows. The Western blots, in Figure 3.2B, distinguishes mCherry from other proteins, due to its *c-myc* tag, and showed expression of both soluble and insoluble protein fractions of mCherry. The highest expression of mCherry were obtained from vectors without signal peptide *pelB* for both induced and un-induced samples. The strongest bands also had one lighter band beneath them (gray arrows). The size of the bands in the Western blots, indicated by blue arrows, correlates well with the

actual size of the mCherry protein, mCherry-*c-myc*-his6 (26.7 kD). Overall the expression for the induced samples are greater than the un-induced. Samples of the insoluble protein fraction are diluted 1/5 and twice as much sample is applied on the SDS-PAGE and Western blot compared to the soluble samples.

In Figure 3.3, images of the supernatant and cell pellets of *E. coli* BL21(DE3) carrying the previously constructed expression vectors all harboring the *mCherry* gene are shown with pVB-1 as a negative control.

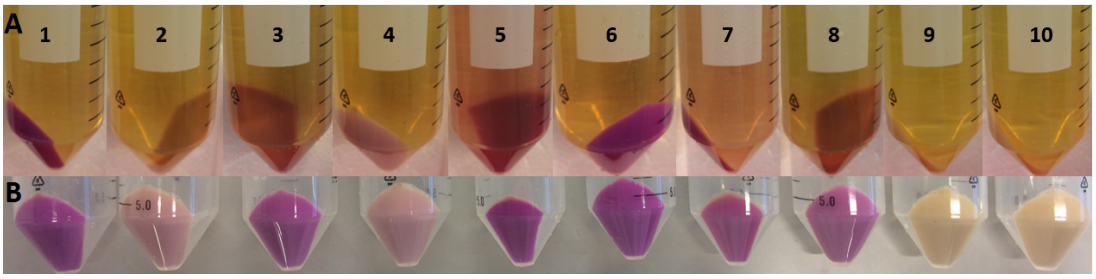


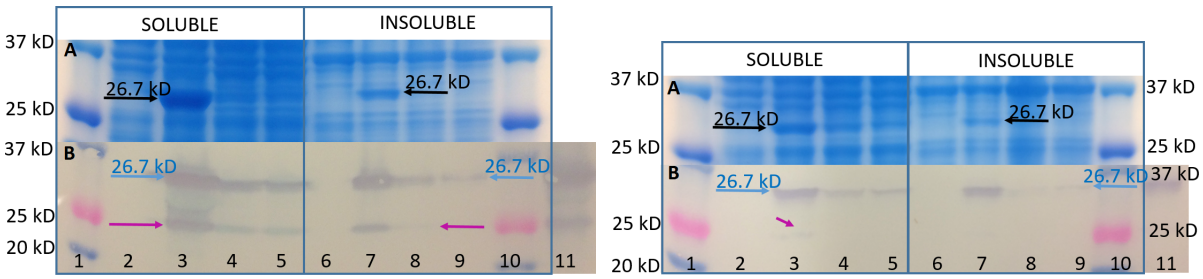
Figure 3.3: Image of supernatant and cell pellett of *E- coli* BL21(DE3) cultures with 4 different expression vectors all harboring the *mCherry* gene. Strains where grown in Hi and Ye medium at 37 °and half of the samples where induced with m-toluate. Induced samples are in odd and un-induced are in even numbers. **A:** Supernatant of induced (odd numbers) and un-induced (even numbers) cultures with mCherry. Number 1 and 2: pVB-1A0B1-mCh, number 3 and 4: pVB-1A1B1-mCh, number 5 and 6: pVB-1B0B1-mCh, number 7 and 8: pVB-1B1B1-mCh and number 9 and 10: pVB-1 (negative control). **B:** Cell pellet for the same cultures and in same order as in A.

Seen from the images in Figure 3.3A the color for the supernatant of the different cultures are quite similar to the light yellow color of the negative control, pVB-1 (number 9 and 10), except for the induced samples of the two expression vectors with mutated *Pm* promoter and 5'-UTR region (pVB-1B0B1-mCh and pVB-1B0B1-mCh, number 5 and 7), which are light red compared to the others. The colors of the cell pellet for all the induced samples of the different expression vectors, shown in Figure 3.3B, are dark purple. For the un-induced samples of the expression vectors with wt *Pm* promoter and 5'-UTR region (pVB-1A0B1-mCh and pVB-1A1B1-mCh, number 2 and 4) the pellets are light pink. The un-induced samples of the expression vectors with mutated *Pm* promoter (ML2-5) and 5'-UTR region (H39) have purple pellets, similar to the induced samples of the same expression vectors (number 6 and 8).

Expression Vectors Constructed in this Study

E.coli BL21(DE3) cells were transformed with the expression vectors constructed in this study, described in Section 3.1.1, Table 3.1. All the vectors harbor the *mCherry* gene and are described further in Section 2.1, Table 2.1 and given in a quick guide in Appendix A.1, Table A.1. Expression vector pVB-1M0B1-mCh contain the wt *Pm* promoter and mutated 5'-UTR sequence H39 (compU). pVB-1L0B1-mCh and pVB-1L1B1-mCh both contain the mutated *Pm* promoter ML2-5 and wt 5'-UTR sequence (comP), respectively, without and with signal peptide *pelB* upstream of the gene coding for mCherry. The expression for the vectors constructed in this study are expected to yield more mCherry than the previously constructed expression vectors with wt *Pm* and 5'-UTR region. Also, the introduction of one mutated element will be compared with the combination of both mutated *Pm* and 5'-UTR (comPU).

In Figure 3.4 the expression of mCherry in *E.coli* BL21(DE3) for these different expression vectors are visualized by SDS-PAGE and Western blot for induced (a) and un-induced (b) samples. Both the soluble and insoluble protein fractions were investigated. pVB-1 was used as a negative control for SDS-PAGE and Western, and soluble protein fraction from *E. coli* BL21(DE3) harboring pVB-1A0B1-mCh as a positive Western-control with its *c-myc* tag.



(a) Induced

(b) Un-induced

Figure 3.4: SDS-PAGE showing the soluble and insoluble protein fractions of induced (a) and un-induced (b) mCherry (26.7 kD) expressed from different expression vectors harboring the *mCherry* gene when *E.coli* BL21(DE3) was used as expression host. Same set-up and order for both gels and membranes evaluated were used for induced (a) and un-induced (b) samples. **A:** SDS-PAGE of soluble (lanes 3-6) and insoluble protein fractions (lanes 8-11) for four different vectors. Lane 1: Ladder (Precision Plus Protein Standards, Dual Color (Bio Rad)), lane 2 and 7: negative SDS-PAGE and Western control (pVB-1), lane 3 and 8: pVB-1A0B1-mCh, lane 4 and 9: pVB-1A1B1-mCh, lane 5 and 10: pVB-1B0B1-mCh and lane 6 and 11: pVB-1B1B1-mCh. Bands representing mCherry are indicated by black arrows. **B:** Western blot of the same vectors and in the same order as for the SDS-PAGE analysis in A. Lane 12: positive Western control (pVB-1B1B1-insulin). Bands of mCherry are indicated by blue arrows.

The SDS-PAGEs seen in Figure 3.4A, shows the soluble and insoluble protein fractions expressed. The bands corresponding to mCherry (26.7 kD) are indicated by black arrows. The highest expression of soluble and insoluble mCherry was detected for vector pVB-1M0B1-mCh (lane 3 and 7) for both induced and un-induced samples. The Western blots, Figure 3.4B, shows bands representing mCherry in both soluble and insoluble protein fractions for all three expression vectors evaluated, indicated by blue arrows, and correlates well with the actual size of the mCherry protein, mCherry-*c-myc*-his6 (26.7 kD). The expression of both soluble and insoluble mCherry were highest for vector with mutated 5'-UTR region H39 (pVB-1M0B1-mCh) for both induced and un-induced samples. For all three vectors there also occurred an additional band just beneath the one representing mCherry indicated by pink arrows. The expression for induced and un-induced samples seem quite similar from the SDS-PAGEs and Western blots. The samples of the insoluble protein fraction are diluted 1/5 and twice as much sample is applied on the SDS-PAGE and Western blot compared to the soluble samples.

In Figure 3.5, images of the supernatants and cell pellets of *E. coli* BL21(DE3) with the three different expression vectors all harboring the *mCherry* gene are shown with pVB-1 as a negative control.

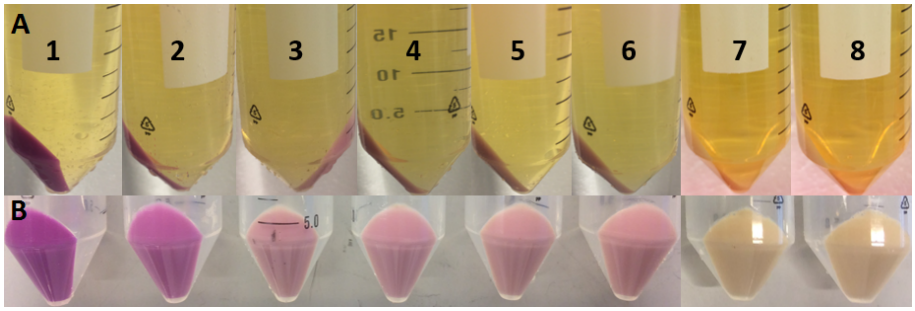


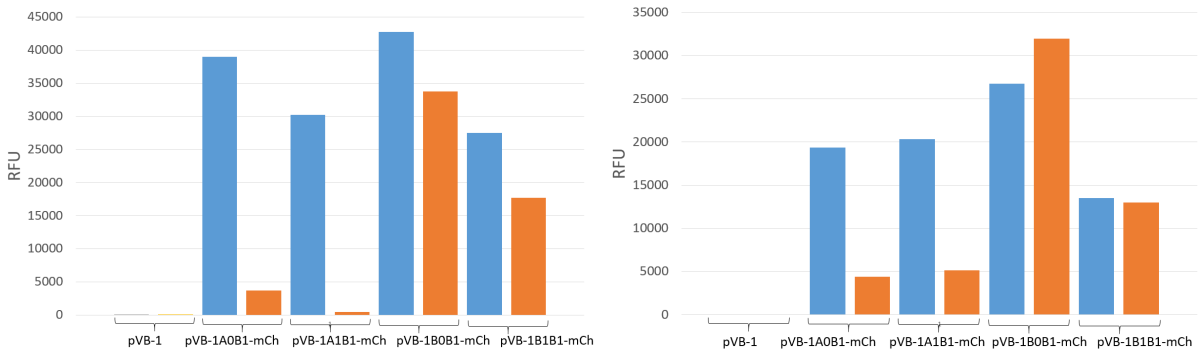
Figure 3.5: Image of supernatant and cell pellet of *E- coli* BL21(DE3) cultures with 3 different expression vectors all harboring the *mCherry* gene. Strains where grown in Hi and Ye medium at 37°and half of the samples where induced with m-toluate. Induced samples are in odd and un-induced are in even numbers. **A:** Supernatant of induced (odd numbers) and un-induced (even numbers) cultures with *mCherry* gene. Number 1 and 2: pVB-1M0B1-mCh, number 3 and 4: pVB-1L0B1-mCh, number 5 and 6: pVB-1L1B1-mCh and number 7 and 8: pVB-1 (negative control). **B:** Cell pellet for the same cultures and in same order as in A.

As seen in Figure 3.5A the color of the supernatant of all the samples are of the similar light yellow color as the negative control. The cell pellets shown in 3.5B are purple for the expression vector with mutated 5'-UTR region H39 (pVB-1M0B1-mCh, number 1 and 2) and light pink for the two other vectors with mutated *Pm* promoter ML2-5 (pVB-1L0B1-mCh and pVB-1L1B1-mCh, number 3-6). The difference between induced and un-induced samples are most visible for the two purple pellets (number 1 and 2), where the induced sample is a bit darker in color than the un-induced sample.

3.1.3 Activity Measurements of mCherry in *E. coli* BL21(DE3)

The activity of mCherry was measured with a spectrophotometer (Tecan), by measuring the relative fluorescence units (RFU) for samples prepared in Section 3.1.2. The samples evaluated were *E. coli* BL21(DE3) carrying expression vectors previously constructed and expression vectors constructed in this study, followed the method described in Section 2.8.4. It is expected higher activity for the induced samples compared to the un-induced.

In Figure 3.6 (a) and (b) the relative fluorescence units (RFU) are given, respectively, for the soluble and insoluble protein fractions of *E. coli* BL21(DE3) for the previously constructed expression vectors: pVB-1A0B1-mCh, pVB-1A1B1-mCh, pVB-1B0B1-mCh and pVB-1B1B1-mCh. pVB-1 was used as a negative control. Induced samples are given in blue and un-induced in orange.



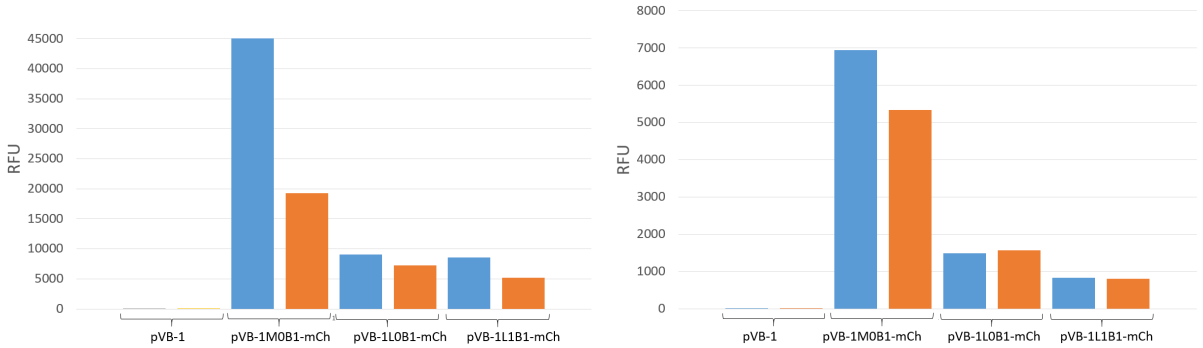
(a) Soluble fraction

(b) Insoluble fraction

Figure 3.6: Relative fluorescence units (RFU) of the soluble (a) and insoluble (b) protein fraction of *E. coli* BL21(DE3) with previously constructed expression vectors (pVB-1A0B1-mCh, pVB-1A1B1-mCh, pVB-1B0B1-mCh, and pVB-1B1B1-mCh), indicated with names under the graphs. Induced samples are in blue and un-induced in orange. pVB-1 was used as a negative control.

Seen in Figure 3.6, the highest values were obtained for the expression vector with both mutated 5'-UTR region H39 and *Pm* promoter ML2-5 (pVB-1B0B1-mCh) for both soluble and insoluble fraction for the induced samples. For the expression vectors with wt 5'-UTR region and *Pm* promoter the difference between induced and un-induced samples are the largest for both the soluble and insoluble fractions. The lowest activity for induced samples in both soluble and insoluble fraction was detected for vector with mutated 5'-UTR H39, *Pm* promoter ML2-5 and signal peptide *pelB* (pVB-1B1B1-mCh). All the induced samples, except insoluble pVB-1B0B1-mCh, have greater activity than their corresponding un-induced samples.

The relative fluorescence units (RFU) are given in Figure 3.7 (a) and (b), respectively, for the soluble and insoluble protein fractions of *E. coli* BL21(DE3) harboring expression vectors constructed in this study: pVB-1M0B1-mCh, pVB-1L0B1-mCh and pVB-1L1B1-mCh. pVB-1 was used as a negative control. Induced samples are given in blue and un-induced in orange.



(a) Soluble fraction

(b) Insoluble fraction

Figure 3.7: Relative fluorescence units (RFU) of the soluble (a) and insoluble (b) protein fraction of *E. coli* BL21(DE3) with different expression vectors (pVB-1M0B1-mCh, pVB-1L0B1-mCh and pVB-1L1B1-mCh), indicated with names under the graphs. Induced samples are in blue and un-induced in orange. pVB-1 was used as negative control.

Seen in Figure 3.7, the highest values were obtained for the expression vector with mutated 5'-UTR region H39 (pVB-1M0B1-mCh), for both soluble and insoluble fraction for the induced samples. The two expression vectors with mutated *Pm* promoter ML2-5 (pVB-1L0B1-mCh and pVB-1L1B1-mCh) obtained significantly lower values for both the soluble and insoluble fractions, and the difference between induced and un-induced samples are not as large as for pVB-1M0B1-mCh. Compared to the expression vectors previously constructed in Figure 3.6, the values are significantly lower for expression vectors pVB-1L0B1-mCh and pVB-1L1B1-mCh, but the activity for pVB-1M0B1-mCh was quite similar to the expression vector with mutated *Pm* ML2-5 and 5'-UTR H39 (pVB-1B1B1-mCh) for both induced and un-induced samples.

3.2 *Pseudomonas putida*

Evaluated *P. putida* as an alternative expression host, by introducing the same expression vectors evaluated in *E. coli* BL21(DE3) (Section 3.1.1), and compared the expression of the reporter gene *mCherry* as described in Section 2.6.

3.2.1 Introduction of Expression Vectors Harboring *mCherry*

P. putida cells were electroporated with the same expression vectors as previously evaluated in *E. coli* (Section 3.1.1), according to the method given in Section 2.6. To check for correct integration of the expression vectors, cultures obtained were grown on LB agar plates (Amp 100 μg/mL) and gave rise to colonies. Plasmid DNA was isolated from the colonies and digested with NdeI and NotI. The digested samples showed poor quality plasmid DNA, and by restriction enzyme digestion it was not possible to verify presence of the correct vectors. Therefore, an alternative method to check for the correct integration of the expression vectors were conducted.

The alternative method was to transform *E. coli* DH5α cells with the poor quality plasmid DNA obtained from electroporated *P. putida* cells. Cultures obtained of electroporated *P. putida* cells were grown on LB agar plates (Amp 100 μg/mL) and gave rise to colonies. Plasmid DNA was isolated from these colonies and used to transform *E. coli* DH5α by heat-shock method (Section 2.2.1). The culture obtained of transformed DH5α cells were grown on same type of agar plates and resulted in very few or none colonies. Some colonies were picked and plasmid DNA was isolated and digested with NdeI and NotI, which gave the expected bands on the gel after gel electrophoresis (Appendix D, Figure D.4). However, most of the digested samples did not give the favourable result after gel electrophoresis this time either.

3.2.2 Expression of mCherry in *P. putida*

Electrocompetent *P. putida* cells were transformed with different expressions vectors harboring the *mCherry* gene multiple times at different dates. Even though the verification of positive introduction of the expression vectors gave ambiguous results, the evaluation

of the expression of mCherry was conducted. Cells were grown in LB (added Ampicillin) at 30°C until OD_{600} was around 2 - 2.5, before induction with 2 mM m-toluate and grown over night at 25°C. Cells were harvested and the supernatants and cell pellets were investigated. The expression vectors previously constructed, pVB-1A0B1-mCh, pVB-1A1B1-mCh, pVB-1B0B1-mCh and pVB-1B1B1-mCh, were evaluated. The vectors are given in Section 2.1, Table 2.1 and a brief overview is given in Appendix A.1, Table A.1.

In Figure 3.8, images shows replicates of the supernatants and cell pellets of *P. putida* electroporated at different dates with the expression vectors previously constructed.

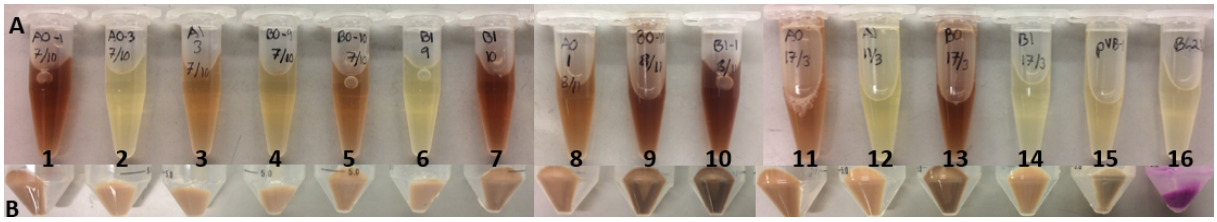


Figure 3.8: Image of supernatant and cell pellet of *P. putida* cultures harboring expression vectors previously constructed. Electroporation of *P. putida* and introduction of the expression vectors were done at different dates, and are divided accordingly. Strains were grown in LB (added 100 μg/mL Amp) medium at 30 °C, and all of the samples where induced with 2 mM m-toluate. **A:** Supernatant of induced samples. Number 1-7: from 2016-10-07, expression vectors pVB-1A0B1-mCh(no 1), pVB-1A0B1-mCh(no 3), pVB-1A1B1-mCh, pVB-1B0B1-mCh(no 9), pVB-1B0B1-mCh(10), pVB-1B1B1-mCh(9) and pVB-1B1B1-mCh(10). Number 8-10: from 2016-11-08, pVB-1A0B1-mCh, pVB-1B0B1-mCh and pVB-1B1B1-mCh. Number 11-14: from 2016-03-17 and 2016-03-11 (project Nygård T.), pVB-1A0B1-mCh, pVB-1A1B1-mCh, pVB-1B0B1-mCh and pVB-1B1B1-mCh Number 15: negative control (pVB-1). Number 16: positive control (BL21(DE3) pVB-1B1B1-mCherry). **B:** Cell pellet for the same cultures and in same order as in A.

Seen in Figure 3.8A there were a lot of variety in terms of color of the supernatant for the different samples. pVB-1 (number 15) is a negative control since we expect no color change of the supernatant. The samples with the darkest red-brown color for each vector are number 1 for pVB-1A0B1-mCh, number 3 for pVB-1A1B1-mCh, number 9 for pVB-1B0B1-mCh and number 10 for pVB-1B1B1-mCh. These samples also have the darkest pellets, seen in 3.8B. None of the cell pellets were purple as the positive control in number 16 (BL21(DE3) pVB-1B1B1-mCh). However, there was a dark red color for several of the supernatants and a darker pellet compared to pVB-1 (number 15).

Because the introduction of the given expression vectors were not completely verified in Section 3.2.1, and the results after a simple growth experiment were deviating, the activity of mCherry was measured before samples were prepared further for SDS-PAGE and Western blot analysis.

3.2.3 Activity Measurements of mCherry in *P. putida*

The activity of mCherry was measured with a spectrophotometer (Tecan), by measuring the relative fluorescence units (RFU) for samples obtained and described in Section 3.2.2. It is expected higher activity for the induced samples compared to the un-induced.

In Figure 3.9 the relative fluorescence units (RFU) are given respectively for the supernatant and insoluble protein fractions of different samples of *P. putida* with expression vectors pVB-1A0B1-mCh, pVB-1A1B1-mCh, pVB-1B0B1-mCh and pVB-1B1B1-mCh. pVB-1 was used as a negative control. Induced samples are in blue and un-induced in orange.

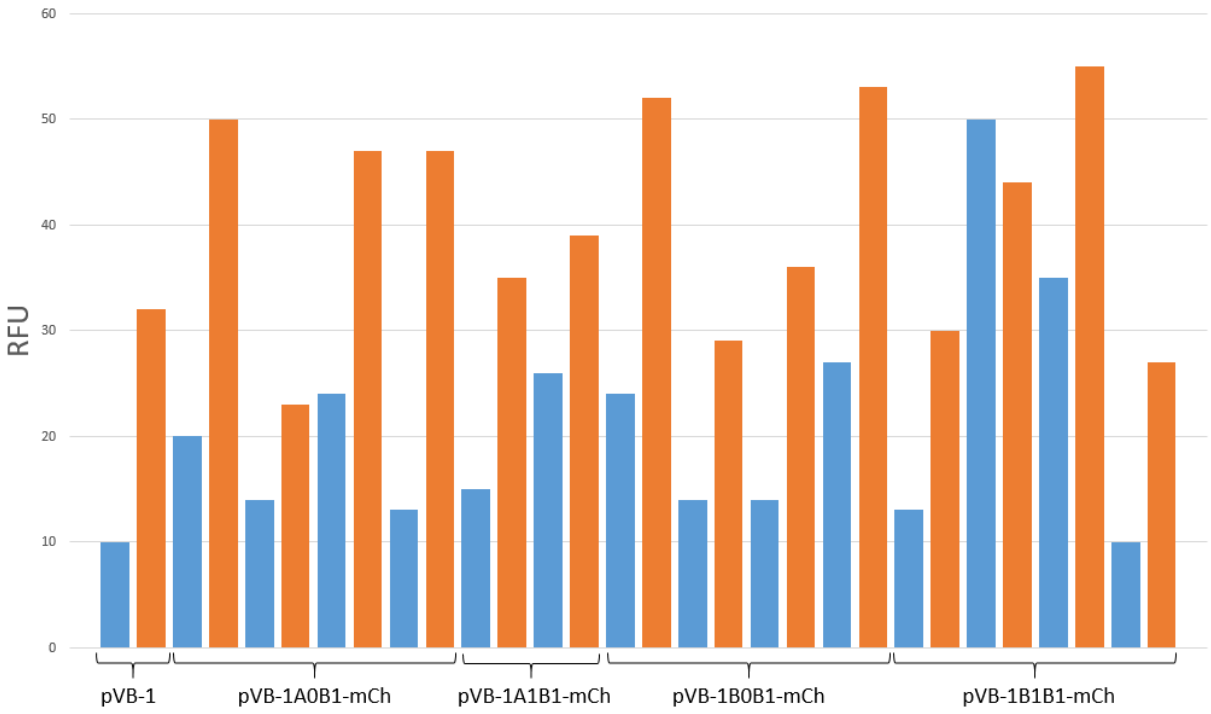


Figure 3.9: Relative fluorescence units (RFU) of the supernatant and insoluble protein fraction of different samples of *P. putida* with different expression vectors as indicated under the graphs. Values for the supernatant are in blue and insoluble in orange.

In general, the values detected showed very low activity for the samples of *P. putida* compared to *E. coli* BL21(DE3) (Figure 3.6 and 3.7) and were similar to the negative control pVB-1. No immediate correlation between the values were detectable, except most of the samples for insoluble fraction had higher activity than the corresponding supernatant samples. Since the results obtained for the activity showed no immediate contribution from mCherry, samples were not prepared further for SDS-PAGE and Western blot analysis.

3.2.4 Identification of Oxidase Positive Bacteria by Kovac's Test

Oxsidase positive bacteria can be identified by the presence of cytochrome c-oxidase as described in Section 2.9 and visualized by a blue color shortly after the Kovac's oxidase reagent is added to a bacterial culture.

To ensure that the strains of *P. putida* harboring expression vectors used in the previous experiments were in fact cultures of *P. putida*, they were checked to be oxidase positive. Cultures for the same strains as used in Section 3.2.2 and 3.2.3 were grown in LB (added Ampicillin) at 30 °C and results after a Kovac's oxidase test are given in Figure 3.10.

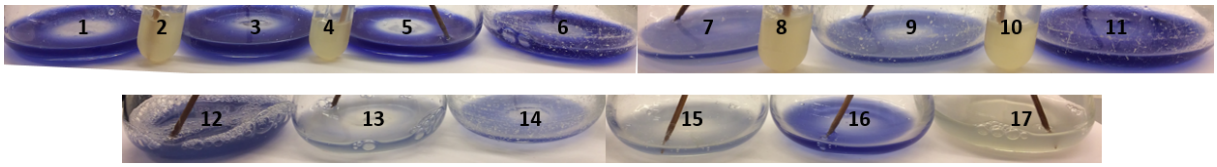


Figure 3.10: Cultures of *P. putida* after addition of Kovac's reagent for strains electroporated at different dates. Number 1-6: 2016-11-07. Number 7-11: 2016-11-08. Number 13-16: 2016-03-17. Number 2, 8 and 17: negative control (*E. coli)* S17-1 with mCherry vector. Number 4 and 10: negative control (*E. coli* BL21(DE3) with mCherry vector). Number 12: Positive control (*P. putida* pVB-1).

Seen in Figure 3.10, all of the different samples of *P. putida* obtained a blue color shortly after adding of the Kovac's reagent, which compared to the negative (*E. coli*) and positive (*P. putida*) control, indicated that all the samples of *P. putida* were oxidase positive.

3.2.5 Antibiotic Resistancy

There was discovered some abnormalities after working with the *P. putida* strain so the antibiotic resistance was investigated for Amp. As seen in Appendix F, Figure F.1 the bacteria could grow on up to 200 μg/mL Amp after incubation of one day at 30 °C. This is unfavorable since the concentration used for antibiotic selection was 100 μg/mL Amp. Comparing the same culture grown on no and 100 μg/mL Amp, the growth was similar after one day of incubation at 30 °C, seen in Figure 3.11.

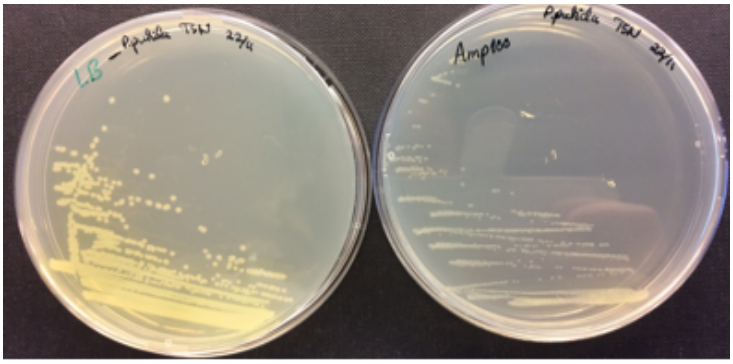


Figure 3.11: Picture of agar plates with bacterial culture of *P. putida* grown on LB agar plate with no (left) and 100 μg/mL Ampicillin (right). Plates were incubated for one day at 30 °C before inspected.

It was investigated weather the bacteria was resistant to the antibiotic Kanamycin (Kan), so the selective antibiotic of the expression vectors could be changed, by replacing the *bla* gene with *kan*, the antibiotic resistance gene for Kan. A culture of *P. putida* was grown in LB medium at 30 °C and plated on LB agar plates with increasing concentrations of Kan, given in Figure 3.12. Inspection of the plates incubated over night shows growth of *P. putida* only on agar plates with concentration less than 5 μg/mL kan.

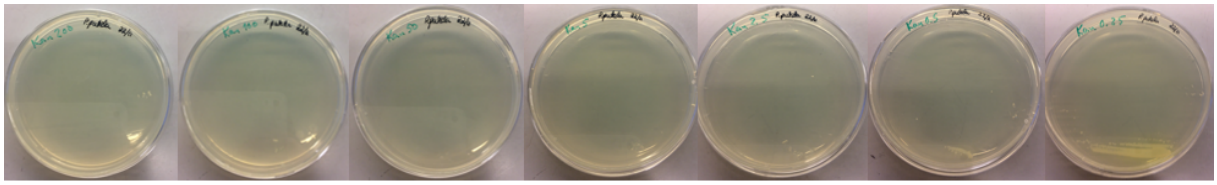


Figure 3.12: Bacterial culture of *P. putida* grown on LB agar plates with different concentrations of Kanamycin (μg/mL). From the left the concentrations are 200, 100, 50, 5, 2.50, 0.50, and 0.25. All plates were incubated for one day at 30 °C before inspected.

3.2.6 Construction of Expression Vectors Harboring *mCherry* and Kanamycin Antibiotic Resistance

P. putida indicated no natural resistance to Kan (Section 3.2.5), so the expression vectors harboring *mCherry* used in Section 3.2.1 were altered to replace the Amp resistance gene (*bla*) with a gene resulting in resistance towards Kan (*kan*).

The vectors pVB-1A0B1-mCh_kan, pVB-1A1B1-mCh_kan, pVB-1B0B1-mCh_kan and pVB-1B1B1-mCh_kan were constructed from the corresponding expression vectors harboring *mCherry* and carrying Amp antibiotic resistance in the form of the *bla* gene by sub-cloning described in Section 2.3.2. Expression vectors pVB-1A0B1-mCh, pVB-1A1B1-mCh, pVB-1B0B1-mCh, pVB-1B1B1-mCh and pVB-5_kan were digested with restriction enzymes MfeI and PvuII. Each digestion of the expression vectors carrying *mCherry* resulted in two MfeI/PvuII-fragments, one harboring the *mCherry* gene (about 5700 bp) and the other harboring the *bla* gene (about 3300 bp). Digestion of pVB-5 resulted in two MfeI/PvuII-fragements, one carrying the *kan* gene (3906 bp) and the other harboring *trf*A and parts of XylS (3337 bp). The digested samples were run on gel electrophoresis and the desired fragments carrying the *mCherry* and the *kan* gene (5700 and 3906 bp) were cut out from the gel and plasmid DNA were extracted. The DNA obtained were ligated and *E. coli* DH5α cells were transformed with the ligation mixture and the culture was plated on LB agar plates with 50 μg/mL Kan. Colonies obtained after incubation over night were picked and plasmid DNA isolated and digested with XhoI and NdeI and examined by gel electropheresis by correct restriction patterns to check the integration of the correct plasmid. The results are given in Figure 3.13. The two bands obtained for most of the parallels correlates well with the expected sizes around 6700 and 3000 bp (6685 and 2924 bp for pVB-1A0B1-mCh_kan).

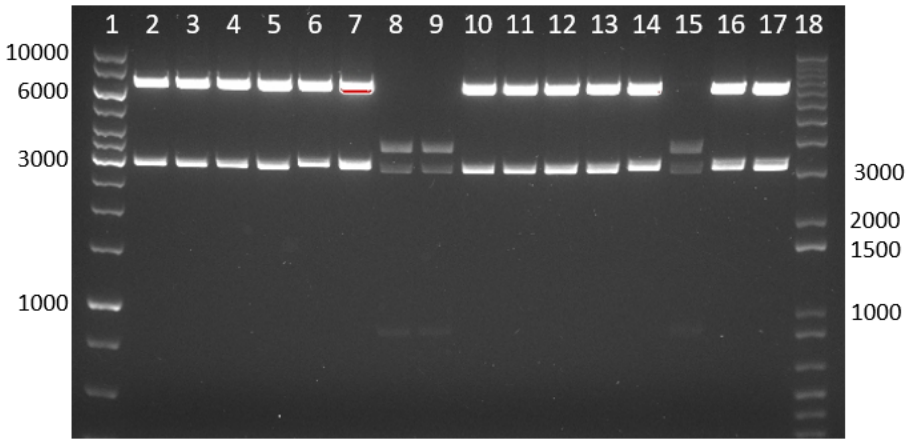


Figure 3.13: Picture of the gel after gel electrophoresis of constructed plasmid expression vectors digested with XhoI and NdeI. Lane 2-5: pVB-1A0B1-mCh_kan, lane 6-9: pVB-1A1B1-mCh_kan, lane 10-13: pVB-1B0B1-mCh_kan and lane 14-17: pVB-1B1B1-mCh_kan. Lane 1: 1 kb DNA Ladder (GeneRuler). Lane 8: 1 kb Plus DNA Ladder (GeneRuler).

Unfortunately, there was not enough time to complete and verify successful electroporation of *P. putida* cells with expression vectors harboring both the *mCherry* and *kan* gene.

3.3 *Pseudoalteromonas haloplanktis*

P. haloplanktis TAC125 was evaluated as an expression host by investigation of growth conditions, generation time and antibiotic resistance. Expression vectors harboring the *mCherry* gene were introduced by conjugation between *P. haloplanktis* cells and *E. coli* S17-1 carrying the desired vectors. Evaluations of the expression were attempted, however, with unfavourable results.

3.3.1 Growth of Expression Host

Growth of *P. haloplanktis* TAC125 was first evaluated in TYP medium with and without shaking at 15 °C. Shaking was evaluated as necessary for growth and 225 rpm was used here on out. Further evaluations of different growth mediums and incubating temperatures were conducted. In Figure 3.14, growth in LB and TYP medium at 15 and 25 °C were evaluated by measuring the optical density (OD_{600nm}) at 600 nm during incubation of *P. haloplanktis*. The values are given as a semi-log plot where OD_{600nm} is plotted against time.

The different growth conditions are indicated by different colors accordingly: TYP medium and 15 °C (blue), TYP medium and 25 °C (gray), LB medium and 15 °C (red) and LB medium and 25 °C (yellow).

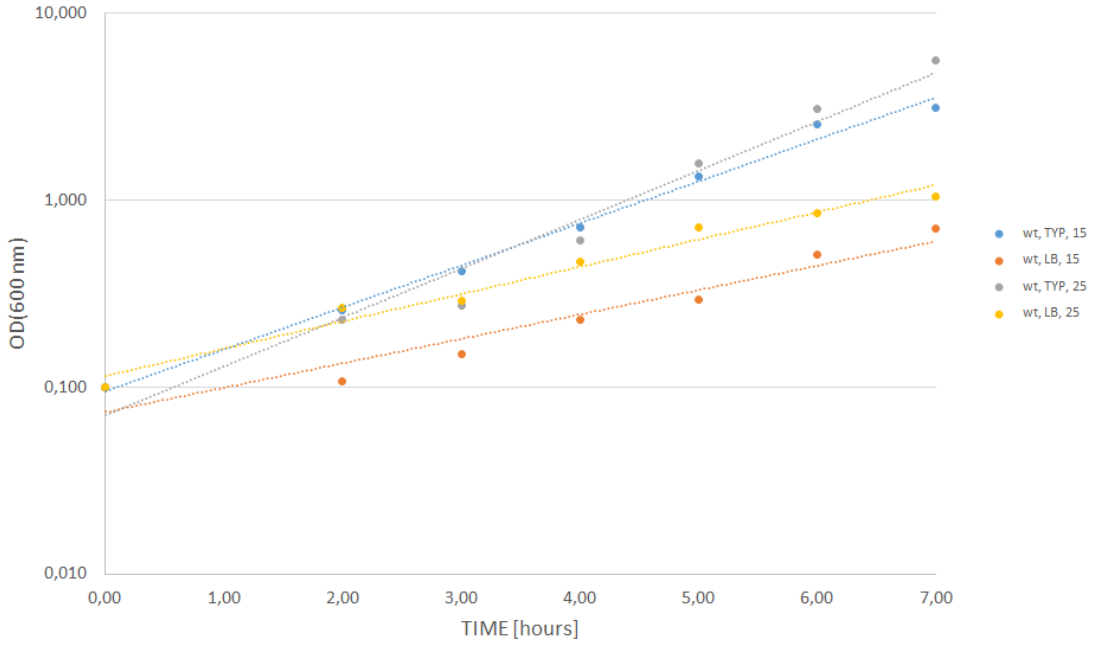


Figure 3.14: The growth rate of wild type (wt) *P. haloplanktis* TAC125 evaluated at different growth conditions (medium and temperature) in four parallels where the optical density measured is plotted against time in a semi-log plot. The parallels are given in different colors with generated trend lines in same color.
Blue: TYP medium, 15 °C, $OD_{600} = 0.095e^{0.517 \cdot t}$, $R^2 = 0.99$
Red: LB medium, 15 °C, $OD_{600} = 0.073e^{0.300 \cdot t}$, $R^2 = 0.93$
Gray: TYP medium, 25 °C, $OD_{600} = 0.071e^{0.603 \cdot t}$, $R^2 = 0.97$
Yellow: LB medium, 25 °C, $OD_{600} = 0.115e^{0.356 \cdot t}$ and $R^2 = 0.98$.

From the generated trend lines obtained for the growth at different conditions in Figure 3.14, the experimental growth rates are given as the slopes for each line and used to calculated the experimental generation times by Equations in Appendix G.1. The calculated generation times are given in Table 3.3. The lowest generation times were obtained for cultures grown in TYP medium and incubated at 25 and 15 °C, respectively.

Table 3.3: Overview of different growth conditions evaluated for *P. haloplanktis* and associated experimental growth rate (μ) and generation time (g) calculated. The equations for the calculations are given in Appendix G.1.

Temperature	***Growth Medium***	μ [h^{-1}]	***g*** [h]
15 °C	LB	0.300	2.31
	TYP	0.517	1.34
25 °C	LB	0.356	1.95
	TYP	0.603	1.15

Even though the generation time calculated above was lower for growth at 25 °C, further growth experiments with *P. haloplanktis* were conducted at 15 °C. This temperature was chosen since it had shown high expression in previous studies [58].

To evaluate the growth of *P. haloplanktis* more thoroughly at the favourable condition determined above, the strain was grown in TYP medium at 15 °C and 225 rpm in a baffled shaking flask. The OD_{600nm} was measured every hour for 13 hours and once more the next day for two cultures with different starting OD-values; 0.1 (blue) and 0.25 (orange). In Figure 3.15, the values are given as a semi-log plot where OD_{600nm} is plotted against time. This is the growth curve of *P. haloplanktis*. The exponential growth phase lasted around 9 hours before the stationary phase occurred.

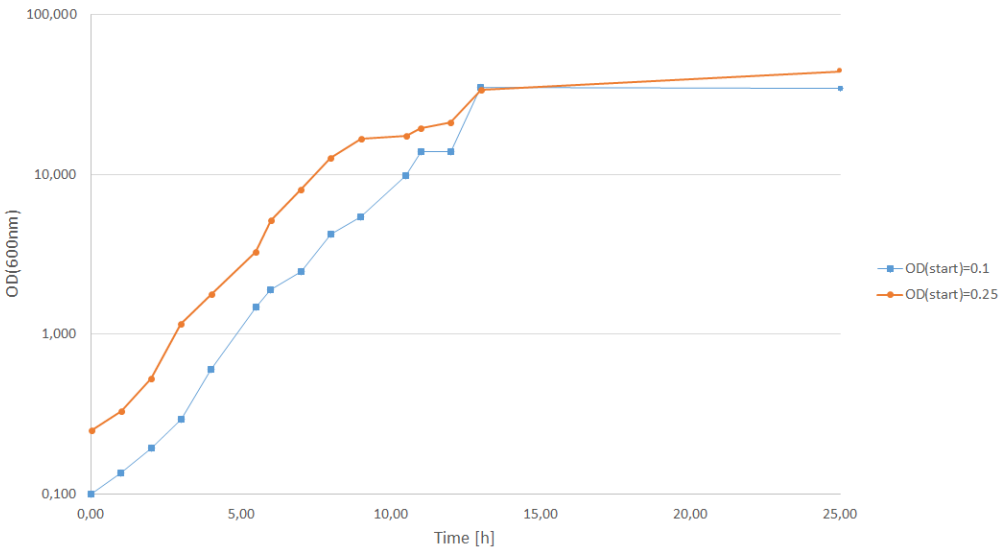


Figure 3.15: The optical density plotted against time for *P. haloplanktis* TAC125 with different optical densities at start; 0.1 (blue) and 0.25 (orange). The bacterial strain was grown in TYP medium at 15 °C and 225 rpm.
Trend lines generated: $OD_{600} = 0.294e^{0.422 \cdot t}$, $R^2 = 0.96$ (orange) and $OD_{600} = 0.093e^{0.460 \cdot t}$, $R^2 = 0.99$ (blue).

From the growth curve, given in Figure 3.15, the experimental growth rate can be found, Figure 3.16, as the slope of the exponential growth phase. The trend lines generated are given as $OD_{600} = 0.093e^{0.460 \cdot t}$ and $OD_{600} = 0.294e^{0.422 \cdot t}$ for the parallels with starting OD-value 0.1 and 0.25, respectively.

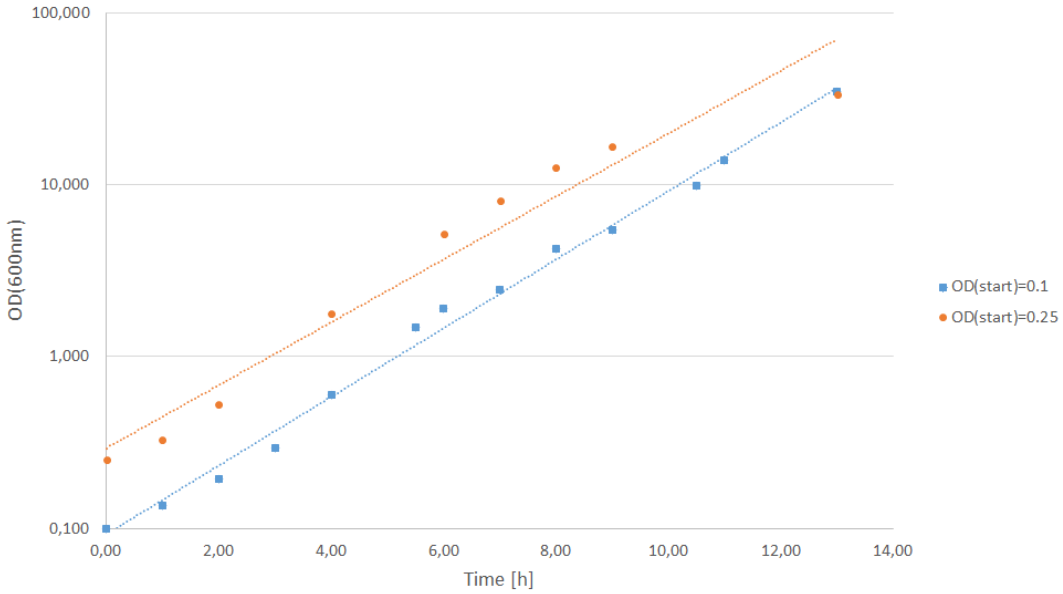


Figure 3.16: The growth rate of *P. haloplanktis* TAC125 is found by evaluating the exponential growth found in Figure 3.15 which gives an experimental growth rate of 0.422 h^{-1} (orange) and 0.460 h^{-1} (blue) found from the trend line generated $OD_{600} = 0.294e^{0.422 \cdot t}$, $R^2 = 0.96$ (orange) and $OD_{600} = 0.0.093e^{0.460 \cdot t}$, $R^2 = 0.99$ (blue).

The generation time can be calculated from the experimental growth rate given in Equation (G.2) in Appendix G.1. The generation times were calculated to be around 1.5 - 1.6 hours.

Samples of *P. haloplanktis* for freezing were taken out during the exponential growth phase. 500 μL sample were mixed with 500 μL 50 % Glycerol before frozen and stored at - 80 °C.

3.3.2 Antibiotic Resistance

Evaluation of the growth on different agar plates for *P. haloplanktis* were conducted as well as evaluating the antibiotic resistance by plating the same culture grown in LB and

TYP on corresponding agar plates with increasing concentrations of Amp or Kan, shown in Figure 3.17, 3.18 and 3.19. The wt bacterium grew fastest on TYP agar plates without antibiotics, and could grow on Amp concentration up to 10 μg/mL and 5 μg/mL for Kan when incubated at 15 °C.

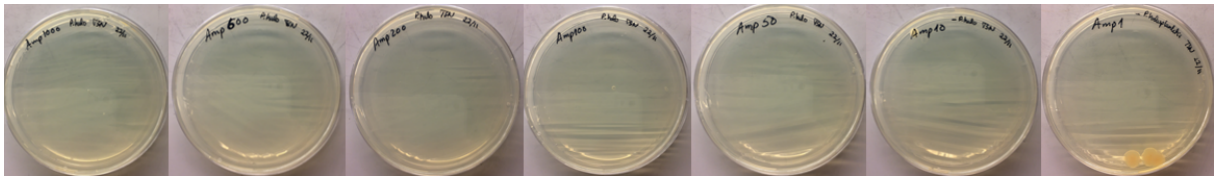


Figure 3.17: Culture of *P. haloplanktis* plated on LB agar plates with increasing Ampicillin concentrations (μg/mL) incubated at 15 °C for two days. Concentrations from the left: 1000, 500, 200, 100, 50, 10, 1.

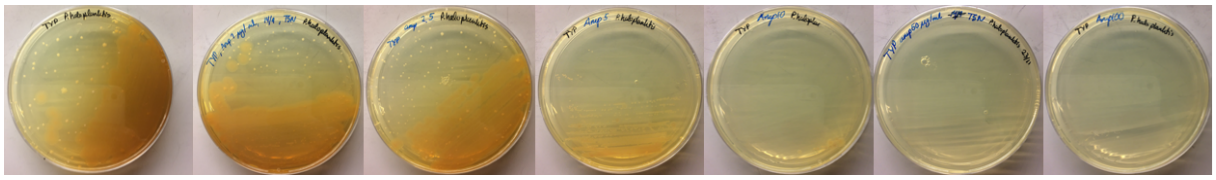


Figure 3.18: Culture of *P. haloplanktis* plated on TYP agar plates with increasing Ampicillin concentrations (μg/mL) incubated at 15 °C for two days. Concentrations from the left: zero, 1, 2.5, 5, 10, 50, 100. Some salt crystals formed in the TYP agar after about three days of incubation.

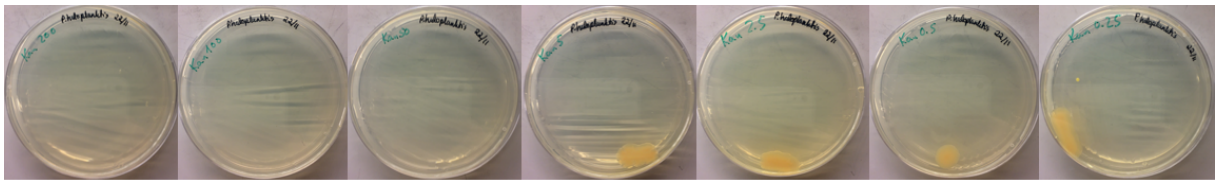


Figure 3.19: Culture of *P. haloplanktis* plates on LB agar plates with increasing Kanamycin concentrations (μg/mL) incubated at 15 °C for two days. Concentrations from the left: 200, 100, 50, 5, 2.5, 0.5, 0.25.

On some of the TYP agar plates with much growth it occurred white, presumably, salt crystals after incubation for more than two days at 15 °C. An example of this can be seen in Figure 3.18 for agar plates with the lowest concentrations of Amp (zero, 1 and 2.5 μg/mL Amp). In Appendix F, Figure F.2 the same TYP agar plate is photographed after incubation at 15 °C for two days and after storage in the fridge (4 °C) as a demonstration of the occurrence of these crystals.

3.3.3 Introduction of Expression Vectors Carrying *mCherry*

P. haloplanktis cells were conjugated with *E. coli* S17-1 harboring the same expression vectors as previously evaluated in *E. coli* (Section 3.1.1). The conjugation procedure, as described in Section 2.7, resulted in growth on TYP agar plates with 100 μg/mL Amp after two days of incubation at 15 °C. This indicates successful conjugation since *E. coli* S17-1 is not able to grow to the same extent at that temperature and that the expression vectors have been integrated due to ability of growth on agar plates with the same antibiotic selection as carried by the expression vectors. However, to fully verify the correct integration of the expression vectors in *P. haloplanktis*, single colonies were picked and grown in TYP medium (added 100 μg/mL Amp). It was experienced difficulties with this inoculation, so several colonies where collected and inoculated together instead. This resulted in growth and further analysis could be conducted. An oxidase test was conducted to verify growth of the correct bacteria (Section 3.3.4). Plasmid DNA were isolated from the cultures and digested with NdeI and NotI. The digested samples showed poor quality plasmid DNA, and restriction enzyme digestion was not possible to verify presence of correct vectors. Therefore an alternative method to check for the correct integration of the expression vectors were conducted, same method as conducted for *P. putida* in Section 3.2.1.

The alternative method was to transform *E. coli* DH5α cells with the poor quality plasmid DNA obtained from *P. haloplanktis* cultures. Plasmid DNA, isolated from these cultures, were used to transform *E. coli* DH5α by heat-shock method (Section 2.2.1). The cultures of transformed DH5α cells obtained were grown on LB agar plates (added 100 μg/mL Amp) and resulted in colonies after over night incubation at 37 °C. Some colonies were picked and plasmid DNA was isolated and digested with NdeI and NotI. The digested samples gave the expected bands after gel electrophoresis for all the different expression vectors, shown in Appendix D, Figure D.5. This verifies a successful integration of the expression vectors harboring *mCherry* into *P. haloplanktis* by conjugation.

3.3.4 Identification of Oxidase Positive Bacteria by Kovac's Test

Verification of bacteria belonging to the group *Pseudomonas* can be done by positive Kovac's oxidase test as described in Section 2.9. Different strains of *P. haloplanktis* harboring the different vectors carrying *mCherry* which had previously shown positive integrations to the bacteria (Section 3.3.3) were grown in TYP media with Amp. The different strains of *P. haloplanktis* originated from conjugation between *E. coli* S17-1 (donor) and *P. haloplanktis* (recipient), and validation of growth of the recipient bacteria were in order. Kovac's oxidase test was conducted and the results are shown in Figure 3.20.

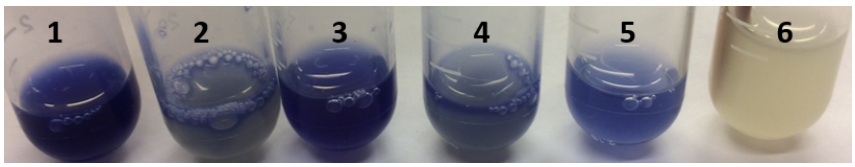


Figure 3.20: Cultures of *P. haloplanktis* after addition of Kovac's reagent for different expression vectors. Number 1: positive control (wt *P. haloplanktis* TAC125), number 2: pVB-1A0B1-mCh, number 3: pVB-1A1B1-mCh, number 4: pVB-1B0B1-mCh, number 5: pVB-1B1B1-mCh and number 6: negative control (*E. coli* S17-1 carrying expression vector harboring *mCherry*).

Seen in Figure 3.20, all of the different samples of *P. haloplanktis* have a blue color, which compared to the negative (*E. coli*) and positive (*P. haloplanktis* wt) control, indicates that all the samples contain cytokrome c-oxidase and are oxidase positive. However, the strength of the color differ somewhat between the samples. The expression vector with wt *Pm* promoter and 5'-UTR region with signal peptide *pelB* (pVB-1A1B1-mCh, number 3) resembles the color of the positive control the most. Whereas sample number 2 (pVB-1A0B1-mCh) and 4 (pVB-1B0B1-mCh) have a blue layer on top of a yellow color due to poor mixing.

3.3.5 Expression of mCherry in *P. haloplanktis*

After introduction of expression vectors harboring the *mCherry* gene were determined as successful (Section 3.3.3), evaluation of the expression of mCherry was conducted. Unfortunately, there was not obtained any presumably positive results for the expression within the time limit of this thesis.

Samples of *P. haloplanktis* which had shown positive integration of expression vectors were frozen with 50 % Glycerol at - 80 °C. These samples were difficult to revive at 15 °C. Also cultivation of single colonies from conjugation-plates after stored in the fridge were problematic to achieve. However, some growth experiments were completed to evaluate the expression of mCherry. This was done by growing the different strains in TYP medium added Amp at 15 °C and inducing the cultures during exponential growth with m-toluate concentrations between 0.5 - 2 mM. Cultures were grown at 15 °C over night and harvested the next day to evaluate the color of the supernatant and pellet. A sample of wt *P. haloplanktis* were grown simultaneously in TYP medium as the other strains to act as a negative control. The resulting colors for the different samples supernatant and pellet were quite similar to wt. The strains were then assumed to not express mCherry and preparation for further analysis were not necessary.

4 Discussion

4.1 *Escherichia coli*

The expression of mCherry from seven individual plasmid vectors was investigated when *E. coli* BL21(DE3) worked as host for the recombinant gene expression. Four of the evaluated vectors had been constructed previously by Vectron Biosolutions, pVB-1A0B1-mCh, pVB-1A1B1-mCh, pVB-1B0B1-mCh, and pVB-1B1B1-mCh (unpublished), while the last three expression vectors to be assessed, pVB-1M0B1-mCh, pVB-1L0B1-mCh and pVB-1L1B1-mCh, were successfully constructed as part of this work. The expression vectors evaluated in this study are chosen because of their different properties. Both wt and a strong expression cassette have been selected to evaluate the impact it will have on expression of the reporter gene used. mCherry was expressed both in the cytoplasm and periplasm of the cells, depending on the presence of signal peptide *pelB*. *pelB* facilitates translocation of the protein from cytoplasm to periplasm. The impact of *pelB* on expression was also evaluated, since the signal peptide was fused together with the *mCherry* gene for some of the expression vectors.

The individual vectors show various levels of expressed mCherry, depending on different promoter strength, 5'-UTR sequence and signal peptide. The expression vectors evaluated in *E. coli* BL21(DE3) were divided into previously constructed and constructed in this study. The results for the expression vectors previously constructed showed that strong expression cassettes, where mutated *Pm* promoter ML2-5 and 5'-UTR region H39 were combined (pVB-1B0B1-mCh), resulted in more expression of mCherry compared to the combination of wt *Pm* and 5'-UTR region (pVB-1A0B1-mCh). For the expression vectors constructed in this thesis, the expression when the mutated versions of these elements were combined with wt versions were evaluated; pVB-1M0B1-mCh (5'-UTR region H39) and pVB-1L0B1-mCh/pVB-1L1B1-mCh (*Pm* promoter ML2-5). The results show the highest expression for the expression vector harboring mutated 5'-UTR region H39 (pVB-1M0B1-mCh). When comparing the expression visualized by SDS-PAGE and Western blot analysis for the expression vectors previously constructed and the expression vectors constructed in this work, expression vectors pVB-1B0B1-mCh and pVB-1M0B1-mCh in-

dicates most expression of both soluble and insoluble mCherry. This is in accordance with previous studies, which showed an elevated level of Amp^r when stronger promoter and improved 5'-UTR sequence were combined (comPU), and also correspond well with previous results obtained for evaluation of one mutated element in combination with wt (comP and comU) [3], [4], [64]. The results obtained by Zwick et al., 2012, where *bla* was used as reporter gene and the resistance levels towards Amp was investigated, showed 5 times as high expression for mutated *Pm* and wt 5'-UTR combined (comP) compared to combination of wt *Pm* and 5'-UTR (wt). For the combination of wt *Pm* and mutated 5'-UTR (comU) the expression was 10 times higher compared to wt. For the combination of both mutated *Pm* and 5'-UTR (comPU), the expression was 25 times greater compared to wt.

The activity measured in this work (Section 3.1.3) for the different expression vectors was (from highest to lowest); pVB-1B0B1-mCh (comPU), pVB-1M0B1-mCh (comU), pVB-1A0B1-mCh (wt), pVB-1A1B1-mCh (wt), pVB-1B1B1-mCh (comPU), pVB-1L0B1-mCh (comP) and pVB-1L1B1-mCh (comP). The results obtained correlates somewhat with the activity measurements of *bla* in Zwick et al., 2012, where the comPU showed highest activity level followed by comU and comP, respectively. The two expression vectors with highest measured activity, are the same obtained in this study and for Zwick et al., 2012, where the values for combination of mutated *Pm* promoter ML2-5 and 5'-UTR region H39 (comPU) are a bit larger than wt *Pm* and mutated 5'-UTR (comU) combined. However, activity measured in this study, for the expression vectors with mutated *Pm* and wt 5'-UTR (comP) are lower than expected since the values obtained in this work are in fact lower than values for both wt *Pm* and 5'-UTR region. Also seen from the activity measurements, as well as indicated by the color of the pellet, expression from vectors with the combination of wt *Pm* and 5'-UTR sequence show the largest increase of activity after induction with m-toulate.

For expression vectors with both mutated *Pm* and 5'-UTR region (comPU), the supernatant of the induced samples had a pink color. This indicates some secretion of expressed proteins to the media, which is not favorable for this gene. However, it may be of interest for expression of other genes as long as the proteins expressed are not toxic for the cells.

The presence of signal peptide *pelB* did not promote the expression of mCherry in *E. coli* seen from the SDS-PAGE and Western blot analysis and demonstrated by the lower values obtained for the activity. Previous research by Sletta et al., 2007, showed increased expression for IFN-α-2b when *pelB* was fused to the gene of interest. This shows that the translocation of mCherry to periplasm does not cause an elevated expression as it does for other proteins.

In this thesis the expression cassette evaluated was shown to be strong for the reporter gene *mCherry*. Previously, the same expression cassette has proven to be strong for *bla* as reporter gene. However, the expression cassette does not always result in high yield of proteins expressed for therapeutic proteins or other reporter genes (Vectron Biosolutions unpublished). The correlation between the strength of the expression cassette and expressed protein depend on the properties of individual proteins as well as the host for the recombinant gene expression [50].

4.2 *Pseudomonas putida*

The electroporation of *P. putida* cells with expression vectors harboring the *mCherry* gene resulted in unsuccessful integration of the plasmid vectors. Previously, the same expression vectors have been successfully integrated to *P. putida* by electroporation and successfully expressed another gene of interest (Project Nygård T., Spring 2016). Perhaps, the integration is successful, but the expression of mCherry is somewhat failing. Evaluation of the expression showed color change for the supernatants and cell pellets for the different strains compared to the negative control, indicating expression of some sort. However, activity measurements conducted showed no expression of mCherry, since the values obtained were around the same values as the negative control. Also the activity measured were substantially lower than the values obtained for the same expression vectors evaluated in *E. coli* BL21(DE3).

Vectors with low copy number will have much less stress on the cell compared to vectors with higher copy numbers since higher copy number usually causes an increased stress on

the cell. Therefore, in working with a new host for expression, a low copy number should be evaluated first. However, in this work a medium copy number was only evaluated for *P. putida* since it had proven to work well with another gene of interest previously (Project Nygård T., Spring 2016). Parallel to this work, electroporation of *P. putida* with expression vectors with different copy numbers was conducted as a small study by Vectron Biosolutions and showed only successful transfer of the expression vectors with the lowest copy numbers (unpublished). Previous work in collaboration with SINTEF have shown difficulties of expression of similar expression vectors with *mCherry* as gene of interest [26],(SINTEF), where the *cop271*-mutation for the *trf*A gene also was used. This may explain the difficulties which occurred in this work since only medium copy number caused by the *cop271*-mutations of the *trf*A gene was used.

4.3 *Pseudoalteromonas haloplanktis*

The conjugation of *P. haloplanktis* TAC125 with expressing vectors harboring the *mCherry* gene was presumably successful since the cultures obtained from conjugation grew on agar plates with correct antibiotic selection at 15 °C and the growth continued when plates were stored in the fridge. The proper integration of the plasmid vectors were verified by an alternative method, resulting in positive integration for all the different expression vectors transferred by conjugation between *E. coli* S17-1 and *P. haloplanktis*. It was shown that these cultures all were oxidase positive by Kovacs oxidase test, ensuring the growth of *P. haloplanktis* and not *E. coli* S17-1. The resistance towards Amp was also examined for the *P. haloplanktis* TAC125 strain and showed no natural resistance. However, the growth and evaluation of mCherry expression for positive conjugants obtained, was not successful due to difficulties in preserving the presumably positive cultures and obtaining samples for freezing, as well as cultivating the frozen samples.

5 Conclusion and Further Work

The aim of this thesis was to establish functional expression vectors to be used in *E. coli*, *P. putida* and *P. haloplanktis* and evaluate the host's expression when *mCherry* was used as a reporter gene. Both *P. putida* and *P. haloplanktis* show promising properties as secure strains for recombinant expression still, more work needs to be done before they can replace *E. coli* as a host.

Expression of reporter gene *mCherry* was successful in *E. coli* and showed highest expression for the expression vectors pVB-1B0B1-mCh and pVB-1M0B1-mCh when expression was evaluated by SDS-PAGE, Western blot, and activity measurements. The evaluation of different control elements in the RK2 plasmid vectors with both *bla* and *mCherry* as reporter genes have found highest expression for the combinations of mutated *Pm* promoter ML2-5 and 5'-UTR region H39 (pVB-1B0B1-mCh, comPU) and wt *Pm* and 5'-UTR region H39 (pVB-1M0B1-mCh, comU). As further work, the evaluation of the expression by SDS-PAGE and Western blot, for the expression vectors proven to have the highest expression should be conducted simultaneously and quantified for further comparison. As well as conducting the experiments several times to verify consistent expression and indicate any deviations.

Expression of mCherry from the expression vectors evaluated here was not successful in *P. putida*. For further experiments, vectors with lower copy number and *kan* as antibiotic resistance marker should be used for better evaluation of the expression of mCherry by the RK2 plasmid vectors in *P. putida* as well as evaluation of other reporter genes.

In this work, there have been successfully established growth conditions and adapted a protocol for conjugation of *P. haloplanktis*, which resulted in successful integration of expression vectors from donor cell *E. coli* S17-1. Tests to verify the correct conjugates have also been established and will be valuable for the further work with this bacterium. However, more work has to be done regarding growth conditions for expression of the

gene of interest and a better functioning procedure for reviving of strains frozen at -80 °C. For freezing of samples, more mediums and variations with glycerol should be investigated. Furthermore, a lower copy number should also be evaluated as well as more reporter genes, to continue the evaluation of *P. haloplanktis* as a host for expression of recombinant proteins.

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A Expression Vectors

A.1 Quick Guide

In Table A.1 there is given an overview over the expression vectors used in this thesis which all carrying the reporter gene *mCherry*. The representation should act as a quick guide to easier distinguish between the different plasmid vectors. The guide assists Table 2.1 (Section 2.1), where the mutations in the *Pm* promoter (ML2-5) and 5'-UTR region (H39) together are denoted as comPU. For some of the vectors constructed in this work the combination of *Pm* and 5'-UTR where both wt and mutated elements together. The combination of wt *Pm* and mutated 5'-UTR are denoted comU and the combination of mutated *Pm* and wt 5'-UTR comP [3], [4], [64].

Table A.1: Overview of differences between expression vectors carrying reporter gene *mCherry* for their type of *Pm* promoter, 5'-UTR, and signal peptide, as well as how the different elements combined are denoted.

	Pm	**5'UTR**	**Signal peptide**	**Combination**
pVB-1A0B1-mCh	wt	wt	-	
pVB-1A1B1-mCh	wt	wt	PelB	
pVB-1B0B1-mCh	ML2-5 [3]	H39 (LIII-3) [4]	-	comPU
pVB-1B1B1-mCh	ML2-5 [3]	H39 (LIII-3) [4]	PelB	comPU
pVB-1L0B1-mCh	ML2-5 [3]	wt	-	comP
pVB-1L1B1-mCh	ML2-5 [3]	wt	PelB	comP
pVB-1M0B1-mCh	wt	H39 (LIII-3) [4]	-	comU

A.2 Overview of Plasmid Vectors

A.2.1 Expression Vectors Previously Constructed

Figures of all the physical maps of the expression vectors harboring the *mCherry* gene are given. In Figure A.1(a) the plasmid vector pVB-1A0B1-mCh is given with the *mCherry-c-myc*-his6 fusion gene. Figure A.1(b) show the plasmid vector pVB-1A1B1-mCh which carry the *pelB-mCherry-c-myc*-his6 fusion gene. Both expression vectors have wt *Pm* promoter and 5'-UTR region, as well as resistance towards ampicillin (Amp^r) by the *bla* gene.

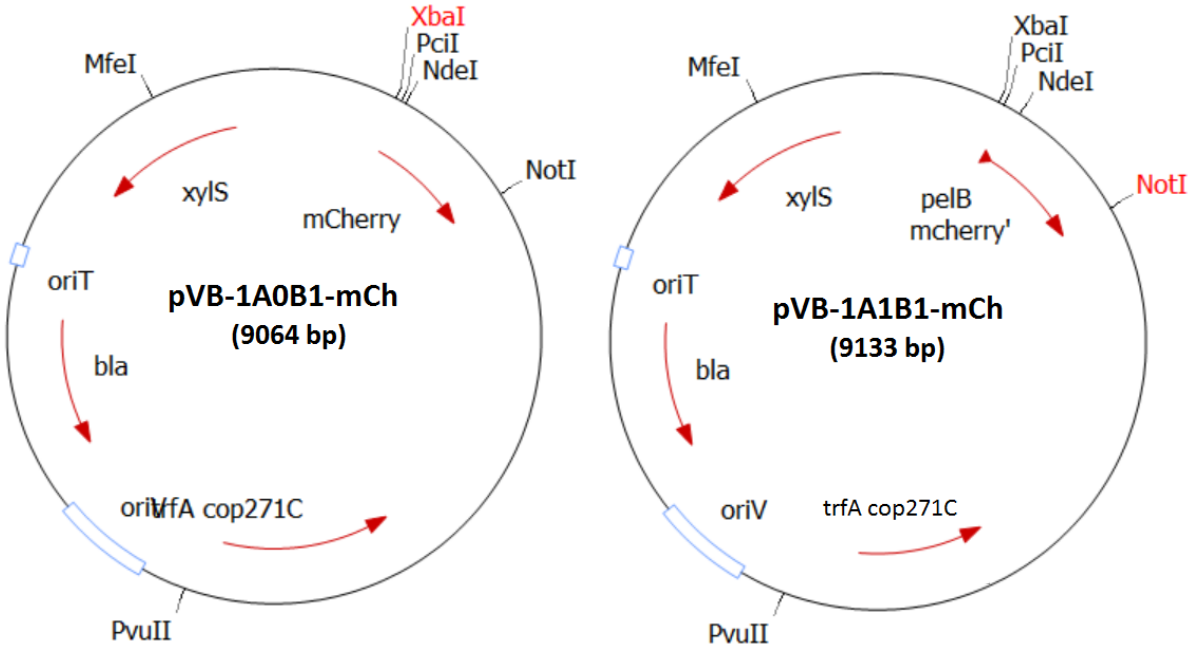


Figure A.1: Physical map for plasmid vectors pVB-1A0B1-mCh and pVB-1A1B1-mCh where restriction sites for the different restriction enzymes useful in this thesis are given. **(a)** Expression vector plasmid pVB-1A0B1-mCh with wt *Pm* promoter and 5'-UTR region, harboring the *mCherry-c-myc*-his6 fusion gene and *bla* gene. **(b)** Expression vector plasmid pVB-1A1B1-mCh with wt *Pm* promoter and 5'-UTR region and harbor the *pel*B-*mCherry-c-myc*-his6 fusion gene and *bla* gene.

In Figure A.2(a) the plasmid vector pVB-1B0B1-mCh is given carrying the *mCherry-c-myc*-his6 fusion gene. Figure A.2(b) show the plasmid vector pVB-1B1B1-mCh which carry the *pel*B-*mCherry-c-myc*-his6 fusion gene. Both expression vectors have mutated *Pm* promoter ML2-5 and 5'-UTR region H39 (comPU) and are Amp^r due to the *bla* gene.

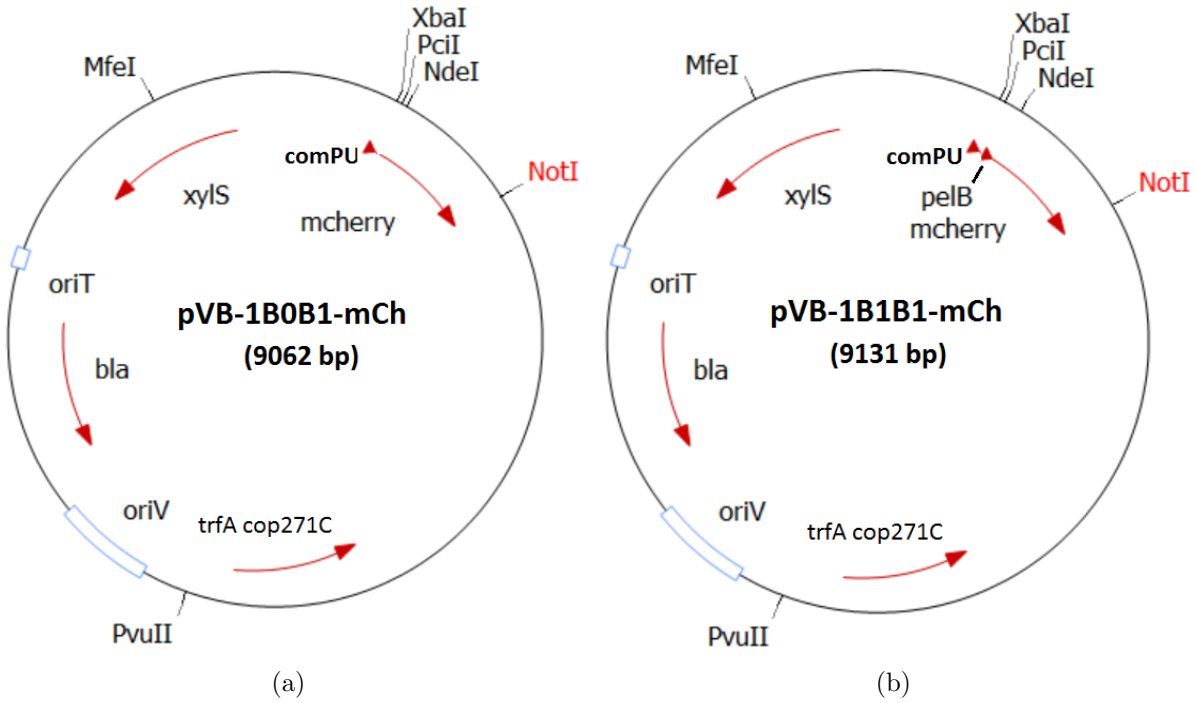
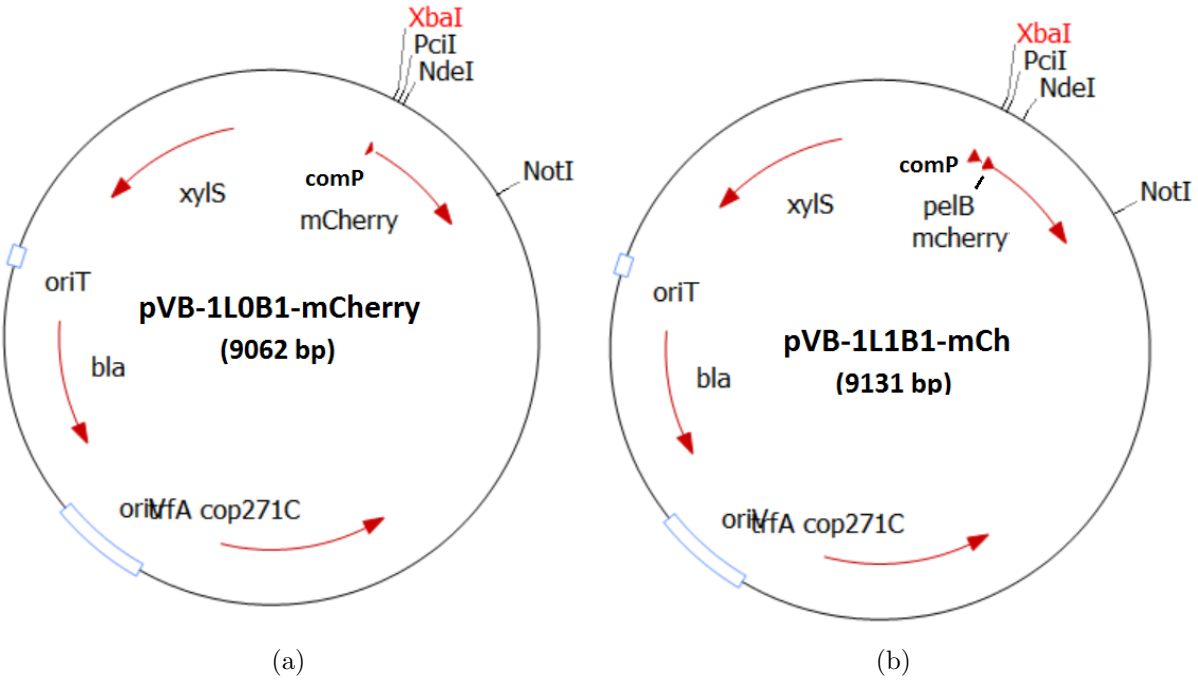


Figure A.2: Physical map for plasmid vectors pVB-1B0B1-mCh and pVB-1B1B1-mCh where restriction sites for the different restriction enzymes useful in this thesis are given. **(a)** Expression vector plasmid pVB-1B0B1-mCh with mutated *Pm* promoter ML2-5 and 5'-UTR region H39 (comPU) and *mCherry-c-myc*-his6 fusion gene and *bla* gene. **(b)** Expression vector plasmid pVB-1B1B1-mCh with mutated *Pm* promoter ML2-5 and 5'-UTR region H39 (comPU), and *pel*B-*mCherry-c-myc*-his6 fusion gene and *bla* gene.

A.2.2 Expression Vectors Constructed In This Work

Expression Vectors with Alternative Pm and 5'-UTR

Expression vectors constructed with alternative *Pm* promoter and 5'-UTR region as part of this work described in Section 2.3.1 are given in Figure A.3 and A.4. In Figure A.3(a) the plasmid vector pVB-1L0B1-mCh is given with the *mCherry-c-myc*-his6 fusion gene. Figure A.3(b) show the plasmid pVB-1L1B1-mCh is given with the *pel*B-*mCherry-c-myc*-his6 fusion gene. Both expression vectors have mutated *Pm* promoter ML2-5 and wt 5'-UTR region (comP) and are Amp^r due to the *bla* gene.



(a) (b)

Figure A.3: Physical map for plasmid vectors pVB-1L0B1-mCh and pVB-1L1B1-mCh where restriction sites for the different restriction enzymes useful in this thesis are given. Both vectors harbor the *bla* gene. **(a)** Expression vector plasmid pVB-1L0B1-mCh with mutated *Pm* promoter ML2-5 and wt 5'-UTR region (comP), and *mCherry-c-myc*-his6 fusion gene. **(b)** Expression vector plasmid pVB-1L1B1-mCh with mutated *Pm* promoter ML2-5 and wt 5'-UTR region (comP), and *pel*B-*mCherry-c-myc*-his6 fusion gene.

In Figure A.4 the plasmid vector pVB-1M0B1-mCh is given with the *mCherry-c-myc*-his6 fusion gene. The expression vector carry wt *Pm* promoter and mutated 5'-UTR region H39 (comU) and are Amp^r due to the *bla* gene.

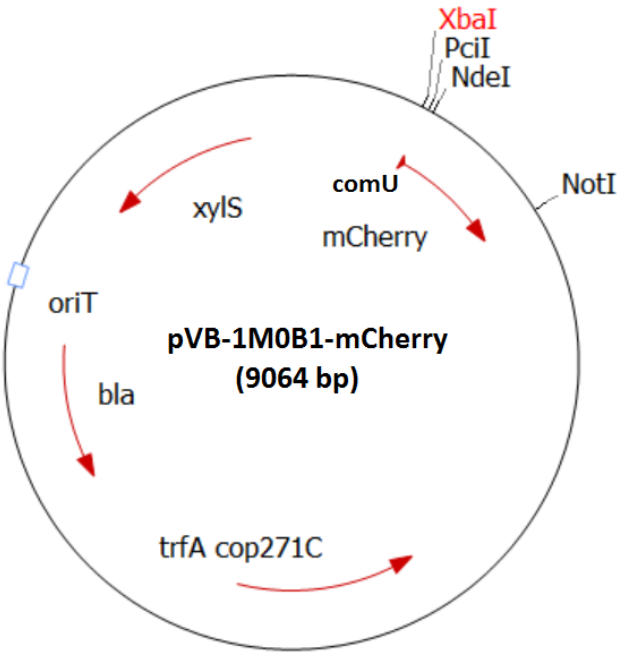


Figure A.4: Physical map of plasmid vector pVB-1M0B1-mCh with wt *Pm* promoter and mutated 5'-UTR region H39 (comU), *mCherry-c-myc*-his6 fusion gene and *bla* gene. The restriction sites for the different restriction enzymes useful in this thesis are given.

Expression Vectors with Kanamycin Resistance

The plasmid vector pVB-5 harboring Kanamycin resistance (Kan^r) due to the *kan* gene is given in Figure A.5 and is used in the construction of expression vectors harboring Kan^r described in Section 2.3.2.

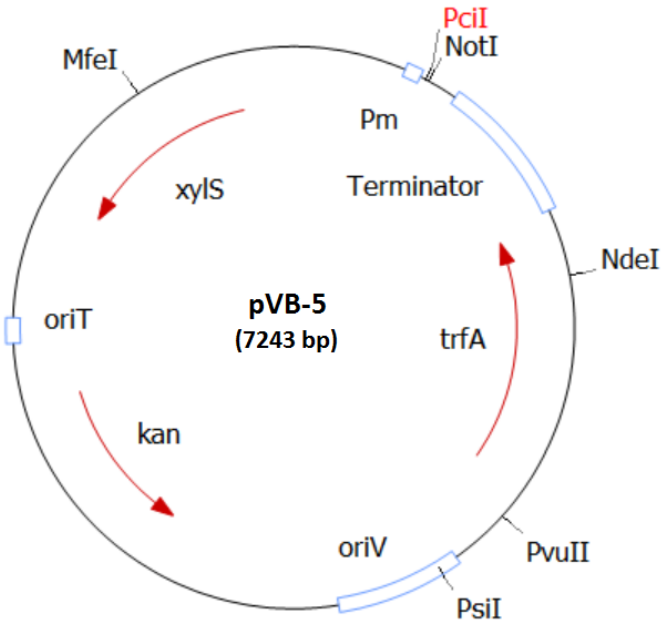


Figure A.5: Expression vector plasmid pVB-5 harboring the xylS/*Pm* expression cassette and wt *trf*A gene. Resistant towards antibiotic Kanamycin by the *kan* gene. Restriction sites for the different restriction enzymes useful in this thesis are given.

Expression vectors constructed with Kan^r are given in Figure A.6 and A.7.

In Figure A.6(a) the plasmid vector pVB-1A0B1-mCh_kan is given with the *mCherry-c-myc*-his6 fusion gene. Figure A.6(b) show the plasmid vector pVB-1A1B1-mCh_kan which carry the *pel*B-*mCherry-c-myc*-his6 fusion gene. Both expression vectors have wt *Pm* promoter and 5'-UTR region and are Kan^r due to the *kan* gene.

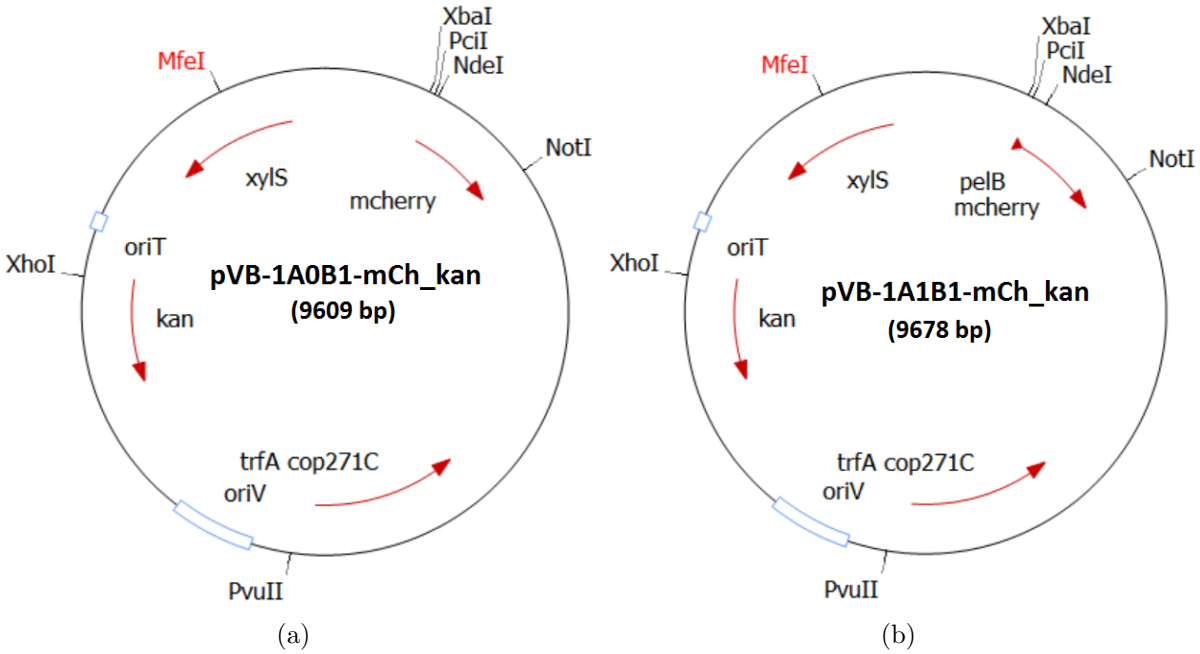


Figure A.6: Physical map for plasmid vectors pVB-1A0B1-mCh_kan and pVB-1A1B1-mCh_kan where both vectors harbor the *kan* gene and are resistant towards kanamycin. **(a)** Expression vector plasmid pVB-1A0B1-mCh_kan with wt *Pm* promoter and 5'-UTR region, *mCherry-c-myc*-his6 fusion gene and resistance towards kanamycin by the *kan* gene. **(b)** Expression vector plasmid pVB-1A1B1-mCh_kan with wt *Pm* promoter and 5'-UTR region, and the *pel*B-*mCherry-c-myc*-his6 fusion gene. The restriction sites for the different restriction enzymes useful in this thesis are given.

In Figure A.7(a) the plasmid vector pVB-1B0B1-mCh_kan is given with the *mCherry-c-myc*-his6 fusion gene. Figure A.7(b) show the plasmid vector pVB-1B1B1-mCh_kan is given with the *pel*B-*mCherry-c-myc*-his6 fusion gene. Both expression vectors have mutated *Pm* promoter ML2-5 and 5'-UTR region H39 (comPU) and are Kan^r due to the *kan* gene.

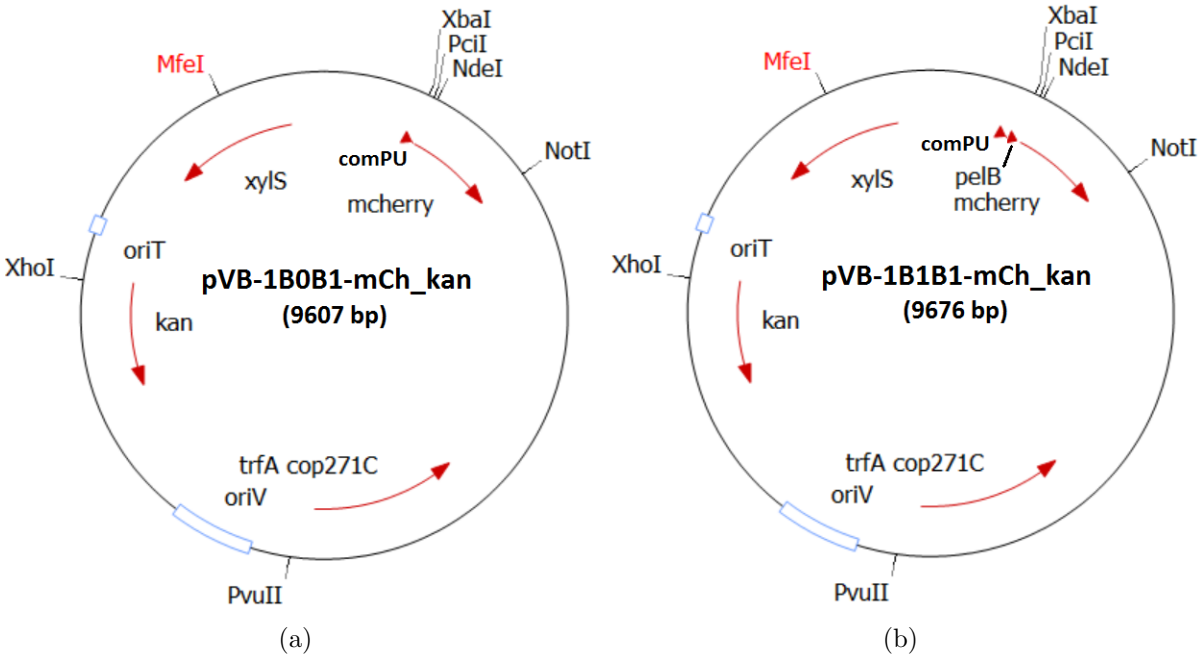


Figure A.7: Physical map for plasmid vectors pVB-1B0B1-mCh_kan and pVB-1B1B1-mCh_kan where restriction sites for the different restriction enzymes useful in this thesis are given. **(a)** Expression vector plasmid pVB-1B0B1-mCh_kan with mutated *Pm* promoter ML2-5 and 5'-UTR region H39 (comPU) and the *mCherry-c-myc*-his6 fusion gene. **(b)** Expression vector plasmid pVB-1B1B1-mCh_kan with mutated *Pm* promoter ML2-5 and 5'-UTR region H39 (comPU) and the *pel*B-*mCherry-c-myc*-his6 fusion gene. Both vectors are resistant towards antibiotic kanamycin due to the *kan* gene.

B Construction of Vectors

When constructing vectors, as described in Section 2.2, digestion and ligation is necessary. In Table B.1, an example of the components needed for digestion (Section 2.2.3) is given as a sample digestion mixture. The individual restriction enzymes require different buffers to cleave the DNA. Appropriate buffer is found by double digest calculator (NEB).

Table B.1: Sample of a digestion mixture for a general digestion.

Solution	**Amount [μL]**
Appropriate Buffer	5
Enzyme 1	1
Enzyme 2	1
H_2O	23
Vector	20
Sum	50

An example of a ligation mixture calculated from the *Ligation Calculator* (http://www.insilico.uni-duesseldorf.de/Lig_Input.htmL) is given in Table B.2. The ligation procedure is further described in Section 2.2.7.

Table B.2: Calculated ligation mixture amounts for a sample ligation with given sizes and concentrations of the DNA fragments by using the *Ligation Calculator* (http://www.insilico.uni-duesseldorf.de/Lig_Input.htmL).

Solution	**Amount [μL]**	**Size [bp]**	**Concentration [ng/μL]**
1xT4 ligase buffer	1	-	-
T4 ligase	1	-	-
Vector	6	8348	9
Insert	2	264	3.2
H_2O	-		
Sum	10		

C Evaluation of Expressed Proteins

In Section 2.8 the expression of mCherry is described where SDS-PAGE and Western blot analysis are to methods evaluated. In Figure C.1 the ladder used for SDS-PAGE (Section 2.8.2) and Western blot (Section 2.8.3) is given. The pre-stained version Dual Color Ladder (Precision Plus Protein Standards, Bio Rad) was used for the experiments in this study.

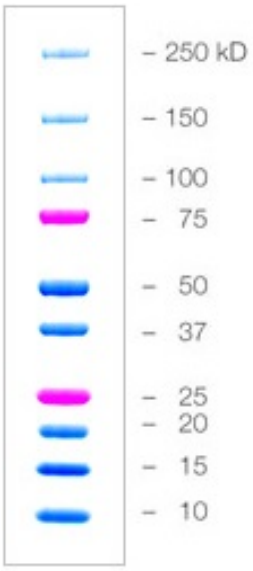


Figure C.1: Precision Plus Protein Standards, Dual Color (Bio Rad).

The set-up for the blotting sandwich used in this study for Western blot analysis (Section 2.8.3) is given in Figure C.2.

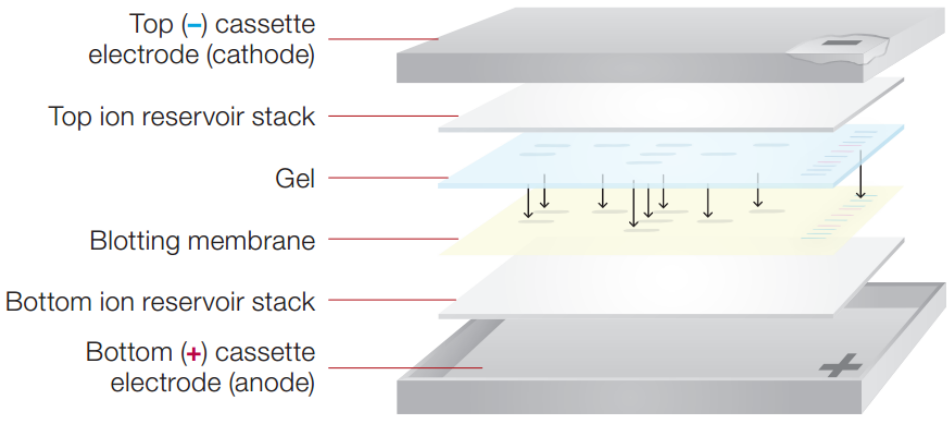


Figure C.2: Assembly of the blotting sandwich for Trans-Blot Turbo Transfer System (Bio Rad).

D Gel Electrophoresis

Ladders 1 kb DNA Ladder and 1 kb DNA Ladder Plus (GeneRuler) are used for reference for gel electrophoresis in this study and are given in Figure D.1.

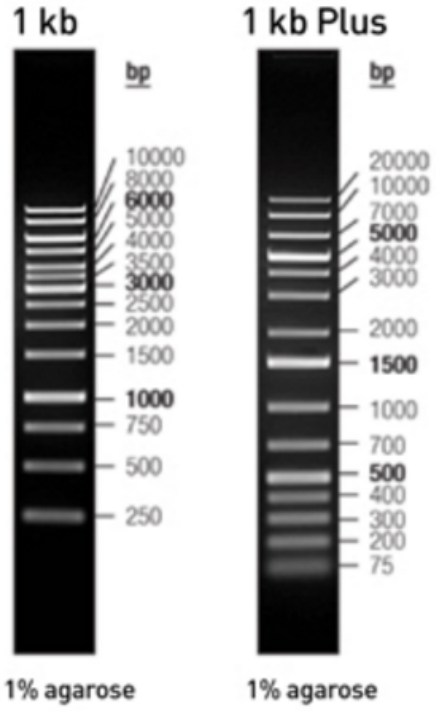


Figure D.1: 1 kb DNA Ladder and 1 kb DNA Ladder Plus (GeneRuler).

Successful integration of the expression vectors previously constructed harboring the *mCherry* gene into *E. coli* BL21 and S17-1 are shown in Figure D.2 and D.3. The expected bands on the gel after gel electrophoresis conducted for samples digested with restriction enzymes NdeI and NotI (around 714 and 8350 bp) are achieved for most of the samples.

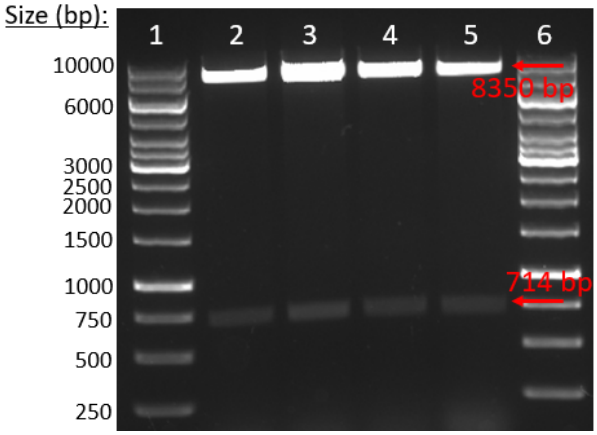


Figure D.2: Positive integration of expression vectors previously constructed into *E. coli* BL21(DE3) shown by a picture of the gel after gel electrophoresis of expression vectors. Samples of pVB-1A0B1-mCh (lane 2), pVB-1A1B1-mCh (lane 3), pVB-1B0B1-mCh (lane 4) and pVB-1B1B1-mCh (lane 5) were digested with restriction enzymes NdeI and NotI and gave rise to the expected bands around 8350 and 714 bp. Lane 1 and 6: 1 kb DNA Ladder (GeneRuler).

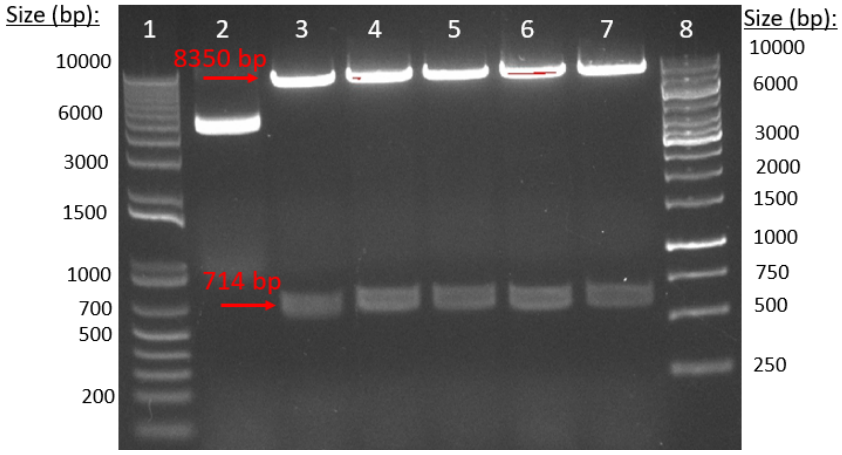


Figure D.3: Positive integration of expression vectors previously constructed into *E. coli* S17-1 shown by a picture of the gel after gel electrophoresis of expression vectors. Samples of pVB-1A1B1-mCh (lane 2-3), pVB-1A0B1-mCh (lane 4-5), pVB-1B1B1-mCh (lane 6) and pVB-1B0B1-mCh (lane 7) were digested with restriction enzymes NdeI and NotI and gave rise to the expected bands around 8350 and 714 bp. Lane 1 and 8: 1 kb Plus and 1 kb DNA Ladder (GeneRuler), respectively.

Successful integration of one expression vector, pVB-1A0B1-mCh, into *P. putida* is shown in Figure D.4. This was the alternative method used to validate the correct integration of plasmid vectors (Section 3.2.1), were *E. coli* DH5α was transformed with plasmid DNA isolated from electroporated *P. putida* cells. The expected bands on the gel after gel electrophoresis conducted for samples digested with restriction enzymes NdeI and NotI (around 714 and 8350 bp) are achieved for one sample and compared to a positiv control in the gel.

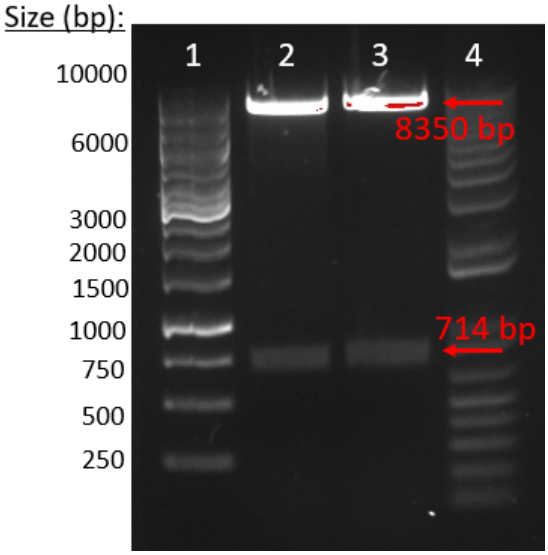


Figure D.4: Positive integration of expression vector pVB-1A0B1-mCh into *P. putida* shown by a picture of the gel after gel electrophoresis of digested samples of the plasmid DNA with restriction enzymes NdeI and NotI, by the alternative method of validating correct integration where *E. coli* DH5α was transformed with the plasmid DNA isolated from electroporated *P. putida* cells. Lane 1 and 4: 1 kb and 1 kb Plus DNA Ladder (GeneRuler), respectively. Lane 2: pVB-1A0B1-mCh. Lane 3: positive control (pVB-1A0B1-mCh in *E. coli* BL21(DE3)).

Successful integration of expression vectors previously constructed into *P. haloplanktis* by conjugation, is shown in Figure D.5. This was the alternative method for verification of positive integration of the plasmid vectors (Section 3.3.3), were *E. coli* DH5α was transformed with plasmid DNA isolated from conjugated *P. haloplanktis* cells. The expected bands on the gel after gel electrophoresis conducted for samples digested with NdeI and NotI (around 714 and 8350 bp) are achieved for all samples evaluated.

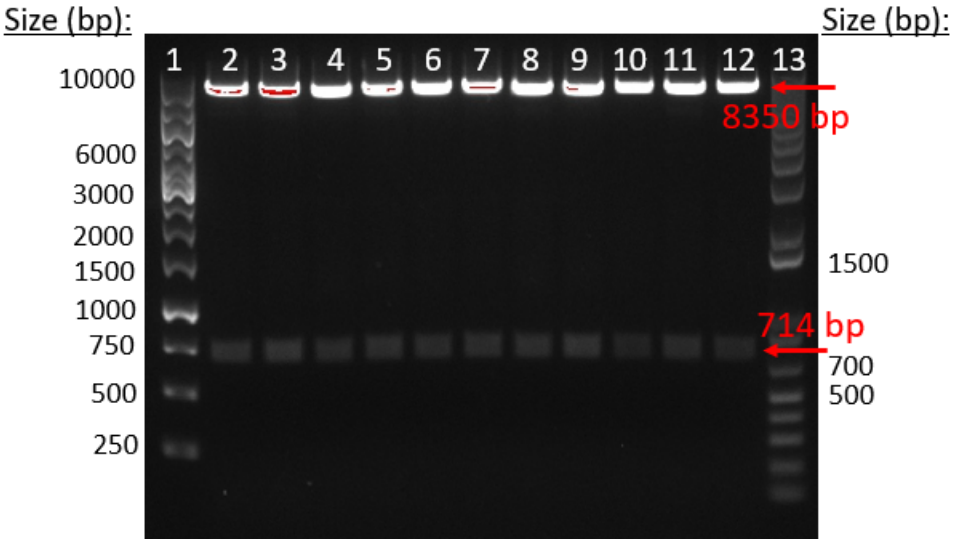


Figure D.5: Positive integration of expression vectors pVB-1A0B1-mCh, pVB-1A1B1-mCh, pVB-1B0B1-mCh and pVB-1B1B1-mCh into *P. haloplanktis* by conjugation, shown by a picture of the gel after gel electrophoresis of digested samples with restriction enzymes NdeI and NotI of the plasmid DNA by the alternative method. *E. coli* DH5α was transformed with plasmid DNA isolated from conjugated *P. haloplanktis* cells and investigated by restriction digestion. Lane 1: 1 kb DNA Ladder (GeneRuler). Lane 2-5: pVB-1A0B1-mCh. Lane 6-8: pVB-1A1B1-mCh. Lane 9-11: pVB-1B0B1-mCh. Lane 12: pVB-1B1B1-mCh. Lane 13: 1 kb Plus DNA Ladder (GeneRuler).

E Sequencing

The newly constructed expression vectors described in Section 2.3.1 and results showed in Section 3.1.1 were sent to sequencing to make sure the small fractions of the mutated *Pm* promoter ML2-5 and 5'-UTR region H39 were correctly integrated with the large XbaI/PciI- and PciI/NdeI-backbone-fragments, respectively. The results are given in Figure E.1, E.2 and E.3, respectively, for expression vectors pVB-1M0B1-mCh, pVB-1L0B1-mCh and pVB-1L1B1-mCh. The sequences for the oligonucleotides ML2-5 and H39 used in this construction are given in Section 2.2.6, Table 2.2 and Figure 2.2.

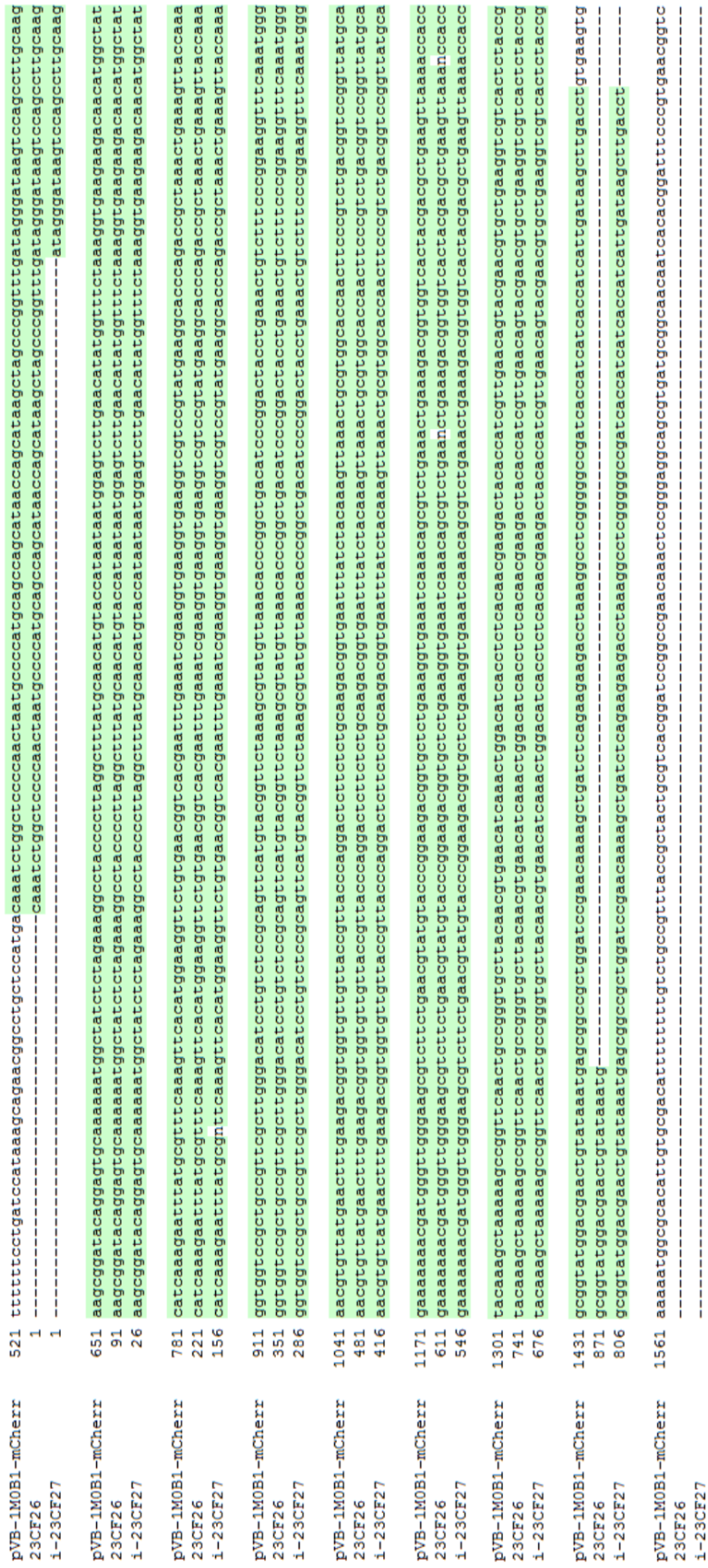


Figure E.1: Sequences of constructed pVB-1M0B1-mCh treated with *Pm*/Xyl*S* forward (second line, 23CF26) and revers primer (third line, i-23CF27) and compared by alignment with the reference expression vector in CloneManager (top line). The areas marked in green are where the sequences are identical.

Figure E.2: Sequences of constructed pVB-1L0B1-mCh treated with *Pm*/Xyl*S* forward and revers primer and compared by alignment with the reference expression vector in CloneManager (top line). The areas marked in green are where the sequences are identical. Unfortunately the reverse sample was of poor quality so it could not be detected.

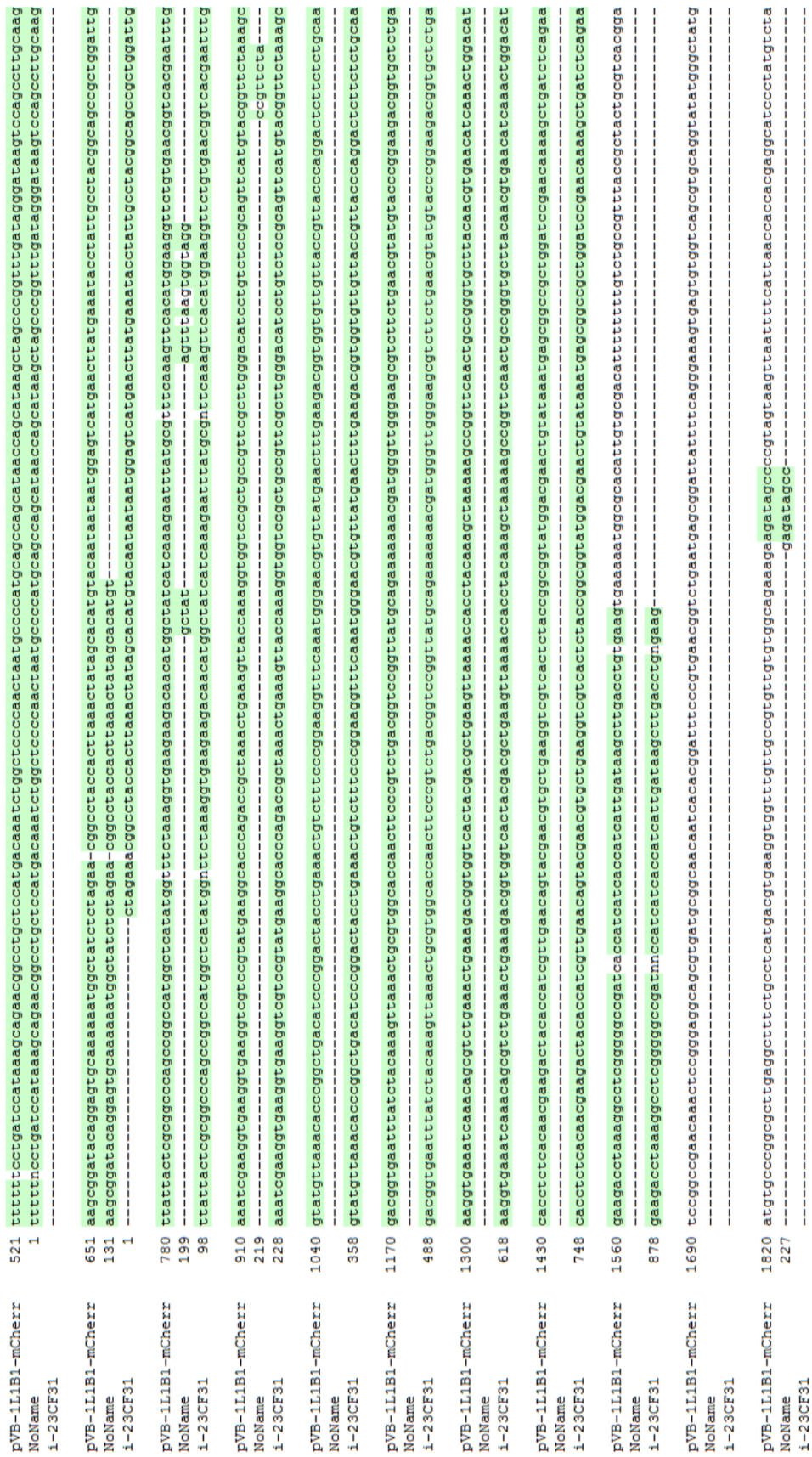


Figure E.3: Sequences of constructed pVB-1L1B1-mCh treated with *Pm*/Xyl*S* forward (second line) and revers primer (third line) and compared by alignment with the reference expression vector in CloneManager (top line). The areas marked in green are where the sequences are identical.

F Growth on Agar Plates

F.1 *Pseudomonas putida*

The same bacterial culture of wt *P. putida* were grown on LB agar plates with increasing concentrations of Ampicillin (Amp). The results after incubation at 30 °C for one day are given in Figure F.1 and show that *P. putida* is able to grow up to 200 μg/ml Amp.

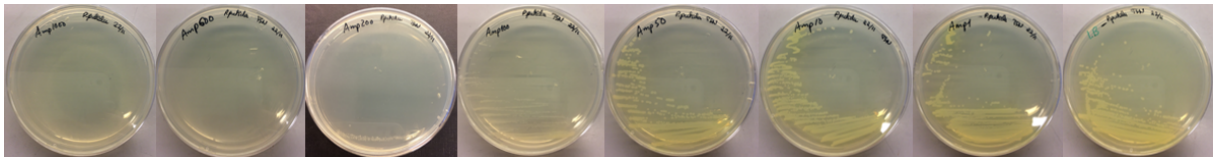


Figure F.1: Bacterial culture of *P. putida* grown on LB agar plates with different concentration of Amp (μg/ml). From the left the concentrations are 1000, 500, 200, 100, 50, 10, 1 and none. All plates are incubated for one day at 30°C.

F.2 *Pseudoalteromonas haloplanktis*

When *P. haloplanktis* was grown on TYP agar plates it occurred some white, presumably salt crystals after incubation for more than two days, as seen in Figure F.2. Here the same TYP agar plate is photographed after incubation at 15 °C for two days and after storage in the fridge (4 °C) as an example of the occurrence of these crystals. It also shows how *P. haloplanktis* continue to grow at this low temperature.

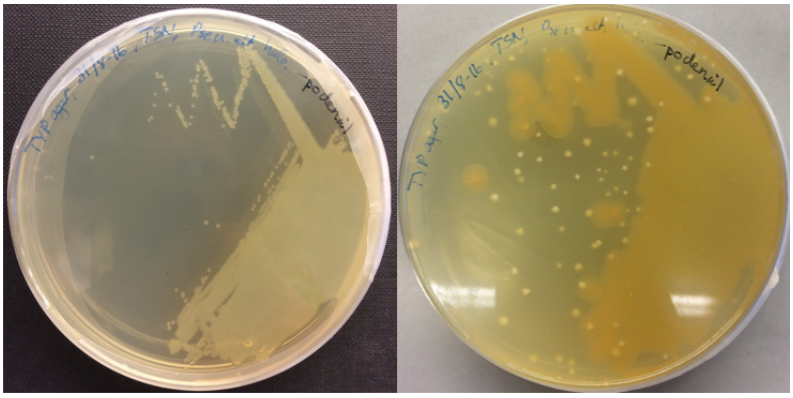


Figure F.2: Culture of *P. haloplanktis* plated on TYP agar plates incubated at 15 °C for two days (left) and then placed in the fridge and taken a picture within a week (right).

G Calculations

G.1 Growth Properties

The growth rate is calculated as the slope of the exponential growth phase. An example of a growth curve is given in Figure 3.15 (Section 3.3), and one example of the growth rate can be seen in Figure 3.16 (Section 3.3). The equation for calculating the growth rate, μ, can be written as:

$$\mu = \frac{lnOD_2 - lnOD_1}{(t_2 - t_1)} \tag{G.1}$$

When the growth rate is known the generation time, g, can be calculated from Equation (G.2).

$$g = \frac{ln2}{\mu} \tag{G.2}$$

G.2 Example Calculations

For example calculations the values found for the slopes in Figure 3.16 are used, μ=0.460 h^{-1} (orange) and μ=0.422 h^{-1} (blue):

$$g_{(orange)} = \frac{ln2}{0.460h^{-1}} = \underline{\underline{1.51h}}$$

$$g_{(blue)} = \frac{ln2}{0.422h^{-1}} = \underline{\underline{1.64h}}$$