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Establishing new XylS/P*m* based Gram-Negative Protein Production Strains

Evaluating *Pseudoalteromonas haloplanktis*
and *Pseudomonas putida*

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Preface

This Master's Thesis concludes my degree Master of Science (M.Sc.) in Biotechnology at the Norwegian University of Science and Technology (NTNU) in Trondheim. The thesis was written at the Department of Biotechnology and Food Science (IBT) in collaboration with Vectron BioSolutions AS under the supervision of Professor Trygve Brautaset (IBT), Anne Krog and Jostein Malmo (Vectron BioSolutions AS).

Acknowledgements

Through the work in this thesis I have got a taste of the worst and best in science.

I would like to thank my supervisor at NTNU, Professor Trygve Brautaset, along with Vectron BioSolutions AS for giving me the opportunity to work on this project.

The employees at Vectron deserves a big thank you for their patience with all my questions. A big thank you to Dr. Anne Krog for guidance and advice along the way, especially towards the end. Also Dr. Jostein Malmo deserves a thank you for invaluable help finalizing this Master's thesis.

Finally, thank you to my fellow students for lunches and late night discussions in the reading room, and to friends and family for coffees, invaluable support and for making me laugh when times are rough.

Declaration of Compliance

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

Trondheim, June 2018

Goro Malene Brennmoen Strypet

Abstract

To date, *Escherichia coli* is the most used bacterial host for recombinant protein production. Some proteins are, however, hard to express in this species due to a reducing intracellular environment and inclusion body formation, among others. The aim of this study was to use the core technology of Vectron BioSolutions AS to establish functional *XylS/Pm* based expression vectors in species alternative to *E. coli*. The candidate species were the Gram-negative strains *Pseudoalteromonas haloplanktis* TAC125, of special interest for production of proteins at low temperatures, and *Pseudomonas putida* KT2440, relevant for high-level production of recombinant protein.

In this study we report successful construction and integration of the pJB658/RK2 minimal replicon based vector pVB-1 mCherry-trfA_Kan with low, medium and high copy number in *P. putida*. High levels of soluble reporter protein (mCherry) was reported from the vector with medium copy number in *P. putida*. Compared to *E. coli* DH5α harboring the same vector, a three-fold increase in expression level was reported for the medium copy number and ~two-fold higher than for the high copy number mutant in *E. coli*. The low and high copy number mutants reported low expression levels of mCherry in *P. putida*. Around 95 % of the protein produced from the medium copy number mutant was soluble, comparable to ~50 % for *E. coli*.

For *P. haloplanktis*, extensive background studies were conducted to map properties important for working with the strain in the lab. Based on these, an expression vector pVB-1 mCherry-wt-ARS_Cm, harboring a Chloramphenicol selection marker and autonomously replicating sequence (ARS) originally from the *P. haloplanktis* plasmid pMtBL was attempted constructed. This was not managed within the time frame of this project. Nonetheless will the acquired insights and knowledge about the nature of the species facilitate further work for establishment of vectors expressing recombinant proteins from the XylS/P*m* expression cassette.

With the integration of an expression vector with a medium copy number and successful production of mCherry, *P. putida* is again regarded as a promising candidate of interest for high-levels protein production in Vectron. No protein production results were obtained for *P. haloplanktis*, but a robust fundament for future work has been made. The species is still highly interesting as a potential expression host, due to its remarkable abilities to grow to high cell densities at temperatures down to 5 °C, also reported in this study.

Sammendrag

Escherichia coli er i dag den mest brukte bakterielle vertsorganismen for rekombinant proteinproduksjon. Noe proteiner er dog vanskelige å uttrykke i denne arten, blant annet på grunn av et reduserende intracellulært miljø og dannelse av uløselige proteinaggregater. Målet med denne studien var å bruke kjerneteknologien til Vectron Biosolutions AS til å etablere funksjonelle XylS/P*m*-baserte ekspresjonsvektorer i alternative arter til *E. coli*. To Gram-negative stammer ble undersøkt. *Psudoalteromonas haloplanktis* TAC125 var av spesiell interesse for produksjon av proteiner ved lave temperaturer, mens *Pseudomonas putida* KT2440 var relevant for høy produksjon av rekombinante proteiner.

Det rapporteres i denne studien om vellykket konstruksjon og integrasjon av den pJB658/RK2 minimalreplikon-baserte vektoren pVB-1 mCherry-trfA_Kan med lavt, middels og høyt kopitall i *P. putida*. Produksjon av store mengder løselig reporterprotein (mCherry) ble rapportert i *P. putida* fra vektoren med medium kopitall. Sammenliknet med produksjonsnivåer i *E. coli* DH5α med den samme vektoren ble det sett en tredobling i mengde protein i *P. putida*, mens sammenliknet med høyt kopitall i *E. coli*, produserte stammen med medium kopitall i *P. putida* dobbelt så mye protein. Stammer med høye og lave kopitall i *P. putida* viste lav proteinproduksjon. Rundt 95 % av proteinet forekom i løselig form i *P. putida*, mens i *E. coli* var forholdet løselig/uløselig protein ~50/50.

For *P. haloplanktis* ble det utført omfattende bakgrunnsstudier for å kartlegge egenskaper som er viktige for videre arbeid med stammen i laboratoriet. Basert på disse forsøkene ble en ekspresjonsvektor, pVB-1 mCherry-wt-ARS_Cm, med en kloramfenikol-seleksjonsmarkør og en autonomt replikerende sekvens (ARS) opprinnelig fra *P. haloplanktis*-plasmidet pMtBL, forsøkt konstruert. Dette arbeidet kom ikke i mål innen tidsrammen for dette prosjektet. Uansett vil økt kunnskap om arten legge til rette for videre arbeid med etablering av vektorer som uttrykker rekombinante proteiner fra XylS / Pm-ekspresjonskasetten.

Med den vellykkede integrasjonen av en ekspresjonsvektor med medium kopitall og vellykket produksjon av mCherry, er *P. putida* nå betraktet som en lovende kandidat av interesse for bruk i proteinproduksjon i Vectron. Ingen proteinproduksjonsresultater ble oppnådd for *P. haloplanktis*, men et solid grunnlag for fremtidig arbeid har blitt gjort. Arten er fortsatt svært interessant som en potensiell ekspresjonsvert, på grunn av sin bemerkelsesverdige evne til å vokse til høye celletettheter og temperaturer rundt 5 ° C, også rapportert i denne studien.

Contents

1 Introduction

1.1 Recombinant DNA Technology

The United Nation defines biotechnology as *"Any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use"*. Mankind was not aware that their beer-brewing in the 12th century would once be called biotechnology. Nor did they know that the reason why the beer became alcoholic was because of millions of small, living cell factories were converting the sugar into alcohol. The discovery of microorganisms and the microscopic world happened in the 17th century. With the discovery of heredity of traits by George Mendel, and later on the structure of DNA in the 1950's, the field of biotechnology started developing fast. The traditional biotechnology lives on today, in the lactic bacteria used to make yoghurt and yeast for baking and beer brewing. With its exponential advances within recombinant DNA technology the last decades, the biotechnology industry has shown a huge potential of economic, scientific, environmental and medical value (1).

The establishment of the first genetically engineered organism was reported in the 1972 by Stanley and Cohen (2). They managed to construct a DNA plasmid *in vitro* by joining endonuclease treated DNA fragments from different plasmids, and subsequently transform this recombinant plasmid into *Escherichia coli.* The replicon showed biological functionality, and had nucleotide sequences from both the parental plasmids (3). This was the beginning of the era of *recombinant DNA technology.* In microbiology, this is based on the principle of inserting the desired piece of DNA into a cloning *vector*, which refers to self-replicating DNA molecules used to carry cloned genes or other pieces of DNA. This vector is transformed into a selected host, and the host grown in culture to obtain numerous clones harboring the recombinant DNA. This is also referred to as gene cloning and can be used to give the host organism itself beneficial properties (the host is the product), or to produce a specific product for harvesting. The recombination of DNA fragments from different DNA sources gives name to the technology, and the technology includes all the techniques required to create self-replicating, biologically active recombinant organisms (1).

Among the many applications of recombinant DNA technology, the oldest application is the production of heterologous proteins in a host organism. Here, the gene of interest is placed behind a closely regulated promoter system, ensuring that protein production happens in a controlled manner (4). Protein production is started by inducing this promoter, making every cell a small fabric for production of protein.

1.2 Bacterial Gene Expression

Two essential processes in a living cell are replication and production of energy. Bacteria grow through cell division (5). Before a cell divides, the genome must be duplicated to give each of the new daughter cells a complete genome set. The genome is copied through *replication*. Furthermore, the cell needs to produce energy to stay alive. To do this, the genetic information in its genome needs to be utilized by expressing the genes. *The Central Dogma of Molecular Biology* states that the information stored in nucleic acids can flow from nucleic acid to nucleic acid, and from nucleic acids to proteins, – but once the information is stored in proteins, it cannot go back (6). Most commonly when expressing genes, the information flow goes from DNA to ribonucleic acid (RNA) to protein. Exceptions exist, however, like RNA replication in viruses with RNA genome, and flow from RNA to DNA caused by the activity of reverse transcriptase. Sometimes the RNA molecule is the end product (7).

Genes are expressed through the processes transcription and translation (7). An overview of these processes is presented in Figure 1. A more thorough explanation will follow in the next sections. The processes differ somewhat in eukaryotes and prokaryotes, and the focus will here be on prokaryotes.

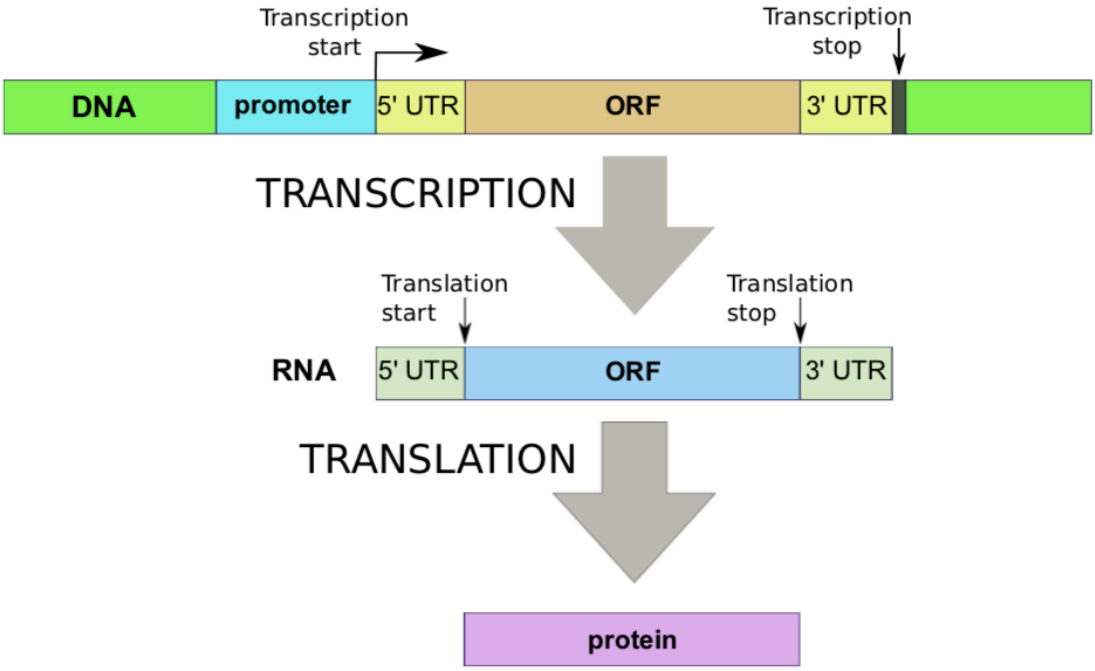

Figure 1: The flow of genetic information for growth and cell division in a living cell. ORF: Open reading frame; UTR: Untranslated region. Figure obtained from (8), adapted from (9).

The system presented in Figure 1 allows regulation at transcriptional, translational and post-translational level. This makes control of production of heterologous protein production possible (7).

1.2.1 Transcription

Transcription can be defined as the production of a single stranded (ss) RNA copy of a specific sequence of double stranded (ds) DNA. It can be divided into three steps: Initiation, elongation and termination. To initiate transcription, RNA polymerase needs to recognize the gne to be transcribed and bind to the DNA sequence. Bacterial RNA polymerase consists of two major components, - the core enzyme and the sigma subunit. The sigma unit is mainly responsible for recognizing the promoter sequence. A piece of regulatory DNA called the *promoter* sequence is located in front of the gene, and this is where the RNA polymerase binds. More specific, the parts of the promoter that is recognized are the -10 and -35 sequences, denoting the number of bases upstream of the transcriptional start site they are located. The consensus sequences for -10 and -35 are TATAAT and TTGACA, respectively. In practice, the sequence will not be identical to these, but differ in up to three bases. It has been found that the level of similarity between the sequences of -10 and -35 and consensus

can affect the strength of the promoter (7). Modification of promoters can thus be utilized in heterologous protein production, to adjust gene expression to an appropriate level in a specific host. The promoter sequence will however not be transcribed (7).

In the elongation step, the core enzyme of RNA polymerase ensure synthesis of RNA in 5'-3'-direction using the DNA strand as a template. The sigma factors stay behind at the promoter sequence, while the core enzyme moves along the DNA strand, adding free ribonucleotides to the 3'-end. When the RNA polymerase reaches a terminator sequence, transcription will be terminated by various mechanisms (7).

Regulation of Promoter Systems

The constitutive "housekeeping genes" in an organism are constantly transcribed to maintain essential processes in the cell. The -10 and -35 sequences of these cells tend to have major similarities with the consensus sequences (7). Other genes, are only needed under specific circumstances (7). Promoter activity can be regulated by modulation of environmental signals like pH, ligands and temperature. These are coupled with transcriptional regulators, which can either facilitate (activators) or repress (repressors) transcription. The regulator proteins bind to specific sites proximal to the binding site of RNA polymerase (4). Positively regulated bacterial expression systems are activated by an inducer-activator complex binding to the DNA sequence upstream of the gene, interfering with the transcription initiation machinery in such a way that transcription is facilitated. A mode of action is to facilitate the recognition and binding of RNA polymerase to the sequence (4).

1.2.2 Translation

After transcription, the mRNA molecules are transported to ribosomes where the mRNA is translated into amino acid chains. In bacteria, translation occurs directly after transcription. This process can also be divided into the three major steps: initiation, elongation and termination. Between the transcriptional start site and the translational start site, there is a short sequence called 5'-untranslated region (5'-UTR). This region is located after the promoter, as shown in Figure 1, and is not translated into amino acids (7). This region contains a sequence approx. Eight bases upstream of the translational start site in the 5'-end of mRNA called the Shine-Dalgarno (SD) sequence where the 3'-end of a small ribosomal subunit binds

by base pairing (1). The bacterial (70S) ribosome consist of a small subunit (30S) and a large subunit (50S). Upon binding to the ribosome, translation is initiated (9).

In the elongation step, the bases in mRNA are read off in groups of three denoted *codons*, each in which represent a new amino acid in the growing amino acid chain. Translation starts at a *start codon* in the mRNA sequence, most commonly with the bases ”AUG” which encode the amino acid methionine. The location of the start codon defines the reading frame of the sequence. Every piece of DNA starting with a start codon that can possibly be translated into a protein is called an open reading frame (ORF) (9). Thus can three different proteins be transcribed from the same DNA sequence if they have different reading frames.

From each codon in the RNA sequence, one new amino acid is added to the amino acid chain by transfer RNA (tRNA). The rate of translation depends on the availability of tRNAs, as one tRNA with anticodon is commonly needed per codon. Some exceptions exist however, where tRNA can read more than one codon. The translational process ends when the ribosome reaches one of three possible stop codons, UGA, UAG or UAA. No tRNAs exist for reading these codons, and hence the elongation of the amino acid chain stops. Instead, release factors recognize these areas, and release the amino acid chain from the last tRNA (7).

The amino acid chain can then fold into a three dimensional structure, and potentially bind to other amino acid chains to make a complete protein (9). A common problem in recombinant protein production is found here, in the transformation of the amino acid chain to a functional three dimensional protein. This can be due to that the intracellular environment of the host organism is different from the environment of the original host of the protein (10).

1.3 Cloning vectors

Bacteria and some eukaryotes contain extrachromosomal genetic units called plasmids. These double stranded, self-replicating DNA units harbor non-essential genes for the organism, but genes that can rather give the cell an advantage under certain environmental conditions. An example is resistance to antibiotics. Plasmids can be represented by up to 100 copies in the cell, and can range in size from < 1 kb to 500 kb (1). Multicopy bacterial plasmids are the most commonly used vectors in molecular cloning and heterologous protein production today. This is due to several properties convenient for cloning experiments (7).

A cloning vector should contain certain genetic elements to make manipulation possible and easy. First, the cloning vector must be autonomous and self-replicating. The plasmid's *replicon* consists of an origin of replication (*ori*), with associated cis-acting regulatory elements (1, 10). Within the replicon is also the incompatibility group of the plasmid determined. *ori* is the start point of DNA replication of the plasmid, (10). In plasmids, this origin is denoted *oriV* ('V' for vegetative replication). The conjugational origin of rolling circle replication is denoted *oriT* ('T' for transfer) (11).

A selective antibiotic marker is often added to the cloning vector. This allows for direct selection for transformants harboring the plasmid, because only the cells containing the vector are able to grow on the selected antibiotic. In addition to confirmation of successful uptake of plasmid, successful insertion of the desired gene has to be detected in some way. This can be confirmed by restriction cutting of the plasmid, where the insert can be detected by changed sizes of resulting DNA fragments or by changed number of fragments (7). Another way to detect insertion is by inserting the gene of interest in a gene, disrupting the transcription of it and hence the activity of the protein it encodes. A widely used method for this is blue/white color screening. The gene of interest is inserted in the *lacZ* gene encoding the enzyme β-galactosidase, which normally cleaves lactose into galactose and glucose. It can also cleave an artificial galactoside called X-gal (5-bromo-4-chloro-3-indonyl β-D-galactoside), resulting in galactose and an insoluble blue dye. This dye will stain colonies upon plating of transformants on an agar plate, unless the insertion of the gene in this gene is successful – resulting in white colonies (7). This is called *screening*, and is not the same as *selection* via an antibiotic marker (1).

To simplify the insertion of the desired DNA into the vector, cloning vectors contain multiple cloning sites (MCS) containing multiple endonuclease restriction cutting sites. Endonucleases cut the dsDNA in specific sites called restriction sites. By treating vector and insert with the same restriction enzymes, complementary ends are made and joining of fragments by ligation easier. Ligation is the joining of two DNA fragments by the enzyme ligase.

Finally, the elimination of all unnecessary plasmid DNA to minimize the size of the vector makes uptake in a host easier (7).

1.4 Bacterial Growth in a Batch

The Growth Cycle

When an appropriate host is transformed with the desired cloning/expression vector, this host needs to be grown in culture to amplify the gene of interest and/or produce the protein. *Growth* in microbiology is defined as an increase in cell number. The growth rate of bacteria is usually expressed as the *generation time*, which is the time it takes for a cell population to double. A batch of bacteria cultivated under optimal conditions in the lab will follow a typical growth curve with defined growth phases. These phases include the lag phase, exponential phase, stationary phase and death phase (5) as shown in **Figure 2**.

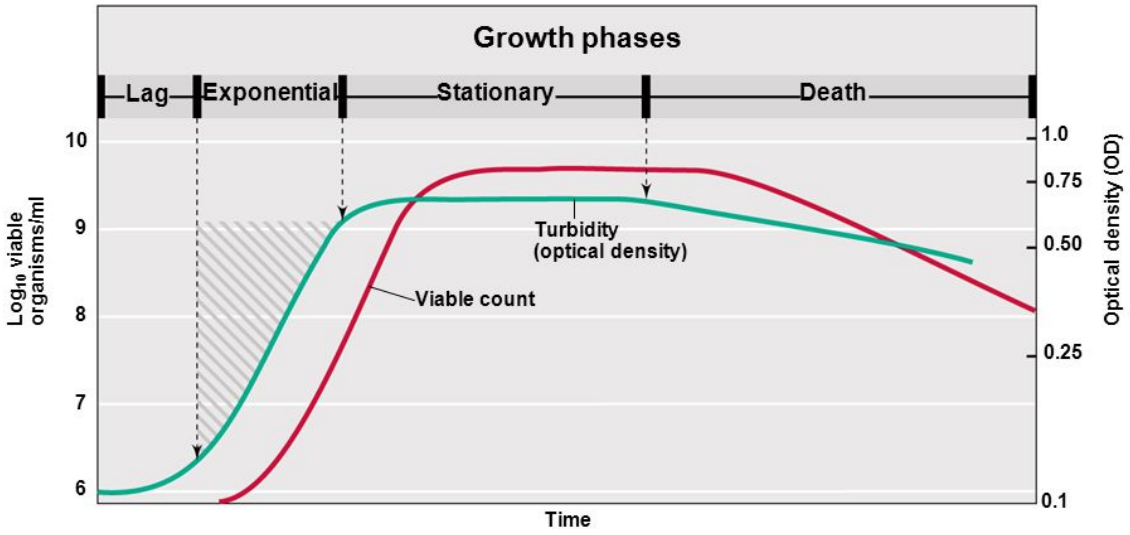

Figure 2: Typical growth curve for a bacterial population. Figure obtained from (5).

After inoculation of bacterial culture into fresh media, the bacteria will need some time to adjust to the new environment and initiate biosynthesis of required proteins before they start growing. This phase with little growth is called the lag phase. The length of the lag phase depends on several factors, e.g. the state of the preculture used for reinoculation (5).

When the bacteria start to grow, they enter the exponential growth phase. This phase is characterized by an exponential growth of the cell population resulting in a constant generation time. This is also the phase where bacteria are regarded healthiest, and is the preferred state for the bacteria to be in when used in the lab. As the growth in this phase is constant, this is a good basis for calculating the growth rate. The growth rate for a specific

bacterial strain is not constant regardless of growth conditions, it will highly depend on e.g. temperature, media and aeration (5).

Bacteria in a batch cannot maintain exponential growth indefinitely. Depletion of essential nutrients and/or accumulation of waste products from the bacterial metabolism will eventually decrease growth, and make the bacteria enter the stationary phase. In this phase the net cell growth is zero. Some bacteria grow and some die, resulting in a constant number of bacteria in the batch. At some point, the bacteria will enter the death phase. The net growth here is negative, as more cells die than there is growth (5).

1.5 *Escherichia coli* as a Host for Protein Production

The most used bacterium in molecular labs is the gram negative, rod shaped *Escherichia coli* isolated from the intestinal tract of mammals. It is about 1-2.5 microns in size, and has flagella attached to its outer membrane ensuring motility (9). *E. coli* has a high growth rate (~20 min generation time), can grow on a cheap medium with easily available components, and it is easy to handle and manipulate genetically. Its genetics are very well characterized (10). All this makes *E. coli* a well suited organism for use in the lab, both as a model organism and as a host for protein expression (7). For the latter purpose, many expression systems have been established, enabling effective production of recombinant proteins.

Some challenges are, however, met when trying to produce recombinant proteins in *E. coli.* Not all recombinant proteins are easily expressed in the intracellular environment of *E. coli* which is known to be reducing. *E. coli* is neither a suited host for proteins that require post-translational modifications, since these pathways are absent in prokaryotes. Psychrophilic enzymes that require lower temperatures for correct folding is another example, since the mesophilic *E. coli* is incapable of growing at low temperatures (<15 °C). Formation of inclusion bodies is also a problem, which decreases the available amount of soluble and active protein. Due to all these problems with *E.coli*, new bacterial species with potential for serving as a protein production host are being investigated (12) (13).

1.6 The XylS/*Pm* Expression System

It is beneficial to use a tightly regulated promoter systems in high-level heterologous protein production, because it makes it possible to decide when and how much protein that should be

produced at all times. One such regulated system is the XylS/P*m* promoter system. This used system has proven to work in a wide range of Gram-negative bacterial species.

The XylS/P*m* system origins from the TOL plasmid pWW0 isolated from *P. putida* (14). The TOL plasmid harbors genes that degrade toluene and other aromatic hydrocarbons. These catabolic genes are organized in two operons, conveniently called the upper and lower pathway (15). The genes in the lower pathway are set under control of a promoter called P*m*. This positively regulated promoter is activated by the protein XylS. XylS belongs to the AraC family of positive transcriptional factors, with a size of 321 amino acids and 36 kDa (16). *xylS* is set under control of two individually regulated tandem promoters called P*s1* and P*s2*. P*s1* is induced by the protein XylR and is σ^{54}-dependent, while P*s2* is σ^{70}-dependent with low, constitutive transcription (17).

To induce the system, benzoic acid derivatives, like *m*-Toluate, are added to cell culture. *m*-Toluate will diffuse through the membrane by passive diffusion. Inside the cell, these effector molecules bind to XylS, and facilitates dimerization of XylS with subsequent binding to P*m* (14). This is presented in Figure 3.

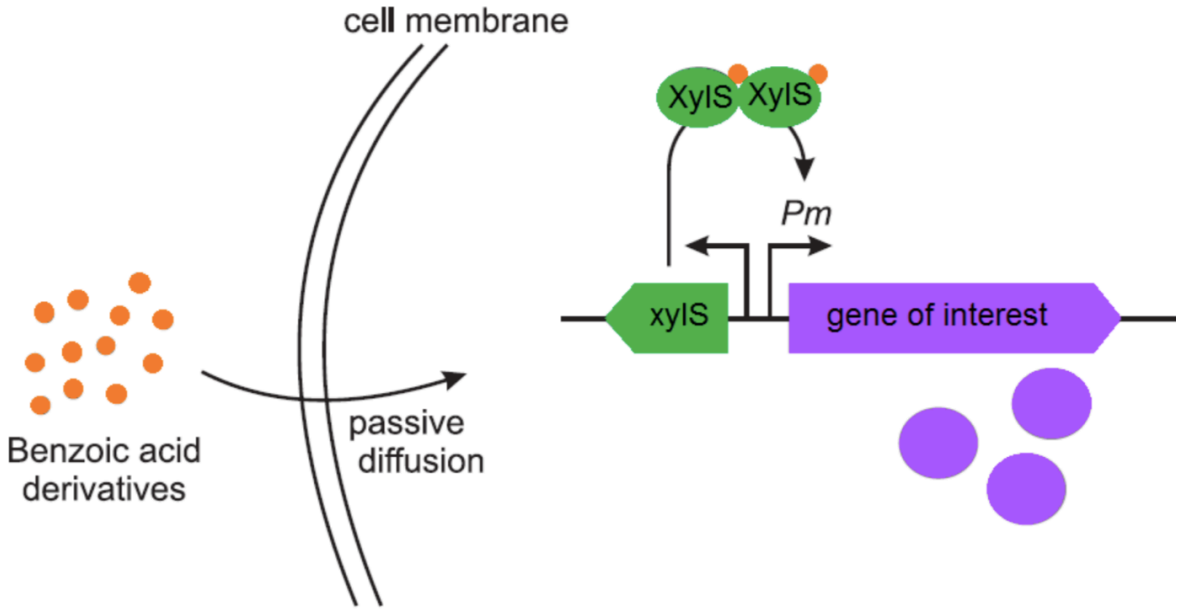

Figure 3: XylS/P*m* expression system. Benzoic acid derivatives diffuse passively through the cell membrane and bind to the activator XylS. This facilitates dimerization of XylS, which causes binding of XylS/effector complex to the *Pm* promoter which initiates transcription of the gene of interest placed behind P*m*. Figure obtained from (4).

XylS will dimerize at an increased rate in the presence of effectors. Dimerization has been reported as crucial for binding of XylS to P*m*. The XylS-effector-P*m*-interaction with subsequent initiation of transcription of a gene of interest is shown in Figure 4

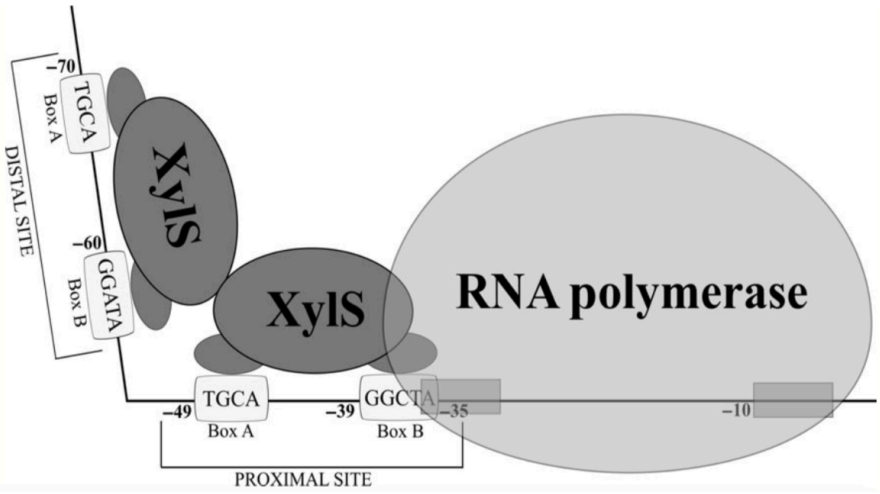

Figure 4: P*m* promoter is activated upon binding of dimerized XylS bound to benzoate derivative effector molecules. This enhances binding of RNA polymerase to the DNA sequence and thus initiation of transcription. Figure obtained from (14).

Modifications of the 5'-UTR and regions of P*m* (including the -10 sequence) and directed evolution of *xylS* have led to increased expression levels from the system (18, 19). Another valuable feature of the system is that inducer molecules can enter the cell by passive diffusion. Thus, no additional systems for uptake of inducer needs to be added. For use of this system in recombinant DNA technology, the degradative proteins encoded for in the two operons exchanged with genes of interest, and thus are the benzoic acid derivatives commonly not metabolized by the cell (4).

1.6.1 RK2 Plasmid

RK2 is a broad-host range plasmid with ability to replicate and maintain in a wide range of Gram negative bacteria (11). Thus, in addition to the simplicity in transferring the plasmid to a new hosts, RK2 has become a valuable tool in genetic engineering. The plasmid is about 60 kb of size, and the copy number in *E. coli* has been found to be 5-7 per chromosome (11). RK2 harbors two essential genes: *oriV,* and the trans-acting replication function (*trfA*) (20). In addition to the vegetative *ori,* RK2 harbors an *oriT* for conjugational replication.

trfA encodes two proteins of 44 and 33 kDa that origin from different translational reading frames within the same gene (21). In *E. coli* and *P. putida*, only one of the proteins are needed for replication, while in other species both TrfA proteins need to be involved. It is believed

that this feature affects the replicons' ability to replicate in many species (22). TrfA controls the frequency of initiation of replication in *oriV*, and hence the copy number of the plasmid in the cell. TrfA is an essential initiator protein that binds to iterons in *oriV* (11). The concentration of TrfA is not the only factor affecting the copy number. Point mutations have been introduced by mutagenesis, and mutants with a copy number up to a 24-fold in *E. coli* have been reported (23). The copy-up mutants in *E. coli* has shown to be copy-up in other species too, and vice versa. The copy number obtained with wild type (wt) *trfA* is low. Mutants of *trfA* giving medium and high copy numbers are denoted *cop271C* (271) and *cop251* (251) respectively. The maximum tolerable copy number of RK2 differs in different hosts and in some species even the wt is too much for the cell to grow (22).

1.6.2 pJB658 Vector

New vectors have been constructed for use in heterologous protein expression based on the RK2 minimal replicon denoting *oriV* and *trfA*. One of these is the pJB658 plasmid, a result from the work of Blatny et al (1997) (24). To construct this vector, the RK2 minimal replicon was spliced with the XylS/P*m* cassette. *oriT* was also included from RK2. The vector was added multiple cloning sites (MCS) downstream of *Pm*, and the Ampicillin (Amp) resistance selection marker *bla* (25). A plasmid map of the pJB658 vector is presented in Figure 5. The vector is also referred to as pVB-1.

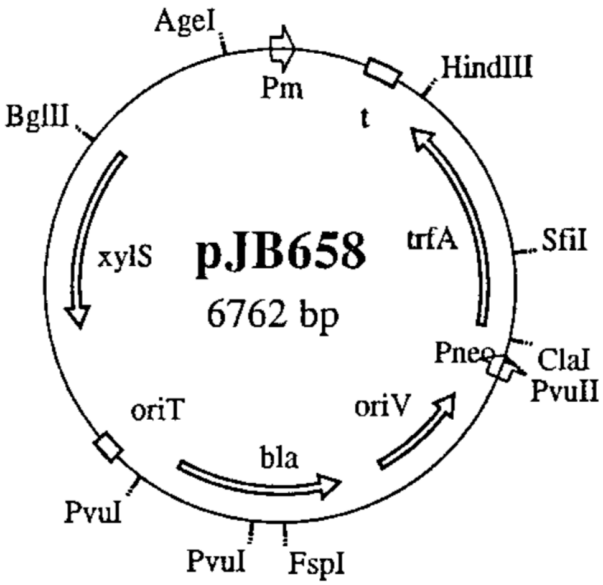

Figure 5: RK2 minimal replicon-based vector pJB658 containing the XylS/P*m* cassette, *oriT*, MCS and the antibiotic selection marker *bla* (Ampicillin^r). Size: 6,762 bp. Figure obtained from Blatny 1997b (24).

1.6.3 pVB-1 Vector

Vectron Biosolutions AS is currently creating a new series of expression vectors based on pJB658, given the name pVB-1 (Vectron Unpublished*).* New core vectors for use in this study were constructed based on pJB658. The following modifications were made:

First, the ampicillin resistance marker in pVB-1 was changed into Kanamycin, using sequence- and ligation-independent cloning (SLIC). *kan* was amplified from the plasmid pVB5 (also referred to as pJB862). This resulted in pVB-1_Kan. Next, the copy number of pVB-1_Kan was changed, using SLIC, resulting in pVB-1-271_Kan and pVB1-251_Kan. *trfAcop271C* and *trfAcop251M* were amplified from the vectors pJB655 cop271 Cluc and pJB655 cop251 Mluc respectively. Finally, a synthetic DNA sequence, containing *bla*, the lpp terminator and the host killing/suppression of killing (*hok/sok*) postsegregational killing system, was introduced. This sequence (generated in CloneManager) is presented in Figure 6.

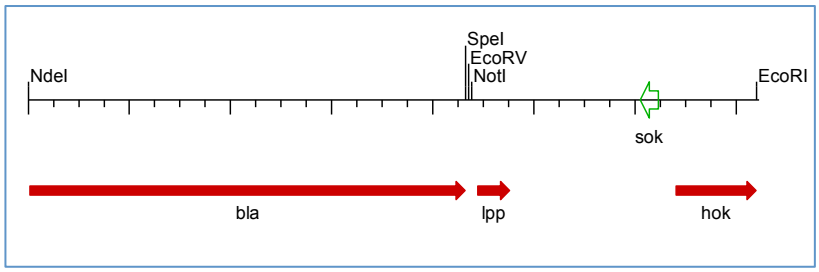

Figure 6: Synthetic sequence inserted in pJB658 to include the Ipp terminator, a hok/sok postsegregational killing system.

The synthetic sequence was cloned into the *Nde*I/*Eco*RI site of pVB1-wt_Kan, pVB-1-271_Kan and pVB-1-251_Kan resulting in pVB1-bla-wt_Kan, pVB-1-bla-271_Kan and pVB-1-bla-251_Kan (Vectron Unpublished). These vectors will throughout this study be referred to as the core vectors. The complete plasmid map of the constructed vectors can be found in **Figure 7**, represented by the wt mutation of *trfA*. The two other core vectors with *trfA* mutations 271 and 251 only differ in individual point mutations in the *trfA* gene, giving medium and high copy number.

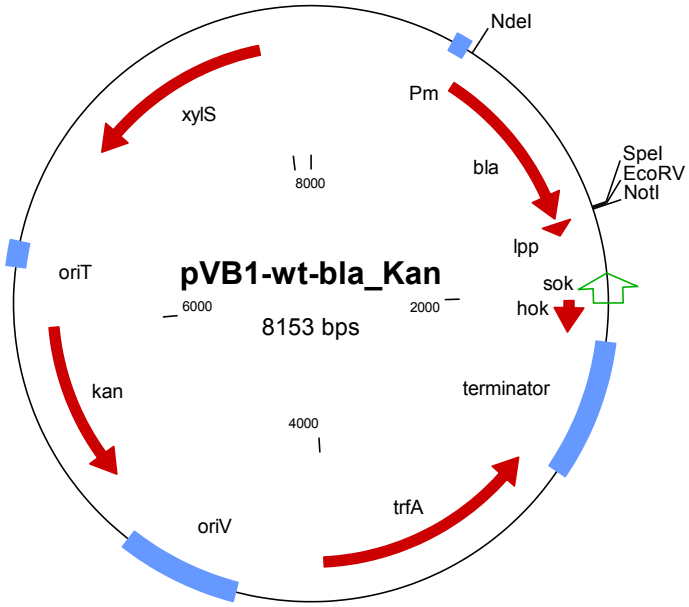

Figure 7: One of the three core vector used as starting points for all constructs in this work. The vector with wt version of the *trfA* gene is shown, the two others were identical except from in *trfA*, and harbored 271 mutation (medium copy number) and 251 mutation (high copy number) respectively.

The *hok/sok* postsegregational killing system origins from plasmid R1. It ensures plasmid stability in a bacterial population by killing plasmid-free segregants directly after cell division, – hence the name. The *hok/sok* locus encodes two RNAs. The Hok mRNA is very stable, while Sok antisense RNA is quickly degraded. The Hok mRNA encodes the protein

Hok, which has the ability to kill the cell. Hok is regulated at a post-transcriptional level by Sok antisense RNA. After cell division, each cell get their share of Hok mRNA and Sok antisense RNA. Sok will quickly degrade while Hok stays present in the cell, and unless more antisense RNA is transcribed from a strong promoter in the plasmid locus, Hok will be translated and kill the cell. Thus must the plasmid harbor the plasmid containing *hok/sok* to survive cell division (26).

Summarized, the three constructed pVB-1 plasmids are smaller in size, and they harbor two antibiotic resistance markers, *bla* and *kan*. In addition, they contain the *hok/sok* system, and an Ipp terminator. The presence of three different mutants of the *trfA* gene resulted in three vectors only differing in the copy number, obtaining vectors with low (wt), medium (c271) and high (251) copy numbers.

1.7 mCherry as a Reporter Protein

An easy way to demonstrate the functionality of an expression system is to use a fluorescent reporter protein. In this study, a red fluorescent protein (RFP) called mCherry was used. This is an improved variant of Discosoma red (DsRed), with faster maturation time and improved photostability. mCherry is exited at wavelengths longer that 600 nm. It has not shown any interference with physiological activity in previous studies performed on *E. coli* and *Bacillus subtilis*, even at high levels (27). These properties altogether make the reporter protein suitable for both *in vivo* studies of cells (28) and *in vitro* studies. mCherry has an excitation maximum of 587 nm, an emission maximum at 610 nm, and the maturation time is 15 minutes (29).

High level expression of mCherry will result in pink bacterial cultures and colonies, and successful expression of the plasmid can be detected by visual inspection. More sensitive analyses of exact amount of protein produced can be measured in a fluorometer.

1.8 Psychrophilic Species for Recombinant Protein Production

Today, most heterologous proteins are produced in protein production systems in well-established mesophilic hosts, like *E. coli* and *B. subtilis*. These systems do however have certain limitations, e.g. when it comes to expressing psychrophilic enzymes. Psychrophilic enzymes refer to proteins with optimal activity at low temperatures, and they originate from

psychrophilic hosts with growth optimum lower than 15 °C. These enzymes have a low intrinsic stability due to high structural flexibility, and can be difficult or impossible to express at mesophile temperatures (30).

Psychrophilic bacteria have shown promising properties of economical and scientific value the recent decade (13, 30, 31). An example of an industrial application of these enzymes is in laundry detergents. By introduction of lipases, amylases and cellulases to a laundry detergent, the need for water heating is lowered due to the activity of the enzyme at low temperatures, and thus less energy is used. This is related to the high structural flexibility of these enzymes Also, the need for chemical additives and the concentration of enzyme in detergents can be lowered (30). Lower enzyme concentrations in reactions are needed because of high reaction rates of the enzyme due to lowered activation energy of the reactions (31). Due to their thermoinstability, they can be inactivated by increased temperature, a feature with potential applications in industry (30).

It is a concern that unless strategies for expressing proteins in cold temperatures are advanced, research will be biased towards the proteins possible to express in mesophiles. Thus, the discovery of potential industrial enzymes with economic potential can be lost (30). When including terrestrial areas, 80 % of the world is permanently cold (13), and the cellular adaptations to low temperatures are only partly understood. In addition to the economical value of finding new resources here, there is a considerable value in investigating the organisms living here to understand how life adapts to cold environments.

The need is therefore seen for recombinant protein production systems that work at adequately low temperatures to ensure correct folding of the enzyme and maintenance of its structural integrity. One solution to this problem may be to adapt already existing mesophile expression systems to cold conditions, or the development of new psychrophilic hosts. The latter is under investigation by ArcticZymes AS and Vectron Biosolutions AS, as a part of a collaboration project. This project aims to broaden the set of hosts used in protein production, with focus on investigating hosts with unique properties that makes them suited for protein production at industrial levels. A candidate for recombinant production of proteins at low temperatures is the psychrophilic species *Pseudoalteromonas haloplanktis*.

1.9 *Pseudoalteromonas haloplanktis* TAC125

Pseudoalteromonas haloplanktis TAC125 is a fast growing bacterium isolated from Antarctic surface seawater (32). The genus *Pseudoalteromonas* is ubiquitous in marine environments and belongs to the class Gammaproteobacteria. *P. haloplanktis* TAC125 are rod-shaped, flagellar, Gram negative and oxidase positive bactera well adapted to saline environments (optimum between 1.5 and 3.5 % NaCl). Its optimum growth temperature is at around 20 °C, but it is able to grow to high cell densities at lower temperatures in optimal media and aeration in the lab (33). The fast growth is thought to be related to the high number of tRNA and rRNA genes, possibly ensuring fast translation of proteins (32). No strong synthesis of biofilms has been reported for the species, but a dense layer of compact cells rapidly formed on the air-water interface has been observed. This suggests that this might be the normal way for *P. haloplanktis* to occupy a biotope (32).

The whole genome of *P. haloplanktis* TAC125 has been sequenced, and the properties of its genome has started to be decoded. Its genome is distributed on two chromosomes, with chromosome I being ~3,215 kb and chromosome II ~635 kb (34). In addition, a self-replicating multicopy plasmid called pMtBL has been identified (4,081 bp) (35). The autonomously replicating sequence (ARS) containing an *ori* has been identified and isolated from pMtBL. The cloning of pMtBL ARS into mesophilic expression systems has been reported. Here, the vector was able to replicate and stably maintain in, among others, *P. haloplanktis* (35). Successful production of soluble recombinant proteins has also been reported (36).

Gaining knowledge about the genome, the remarkable ability of *P. haloplanktis* to adapt to cold environments has been investigated at a molecular level. Lower temperatures means higher solubility of oxygen in water, which can cause challenges for the cells because of reactive oxygen species (ROS). It has been found that *P. haloplanktis* has deleted whole pathways producing ROS, e.g. the molybdopterin-dependent metabolism. Another cold-adapting feature is the presence of dioxygen consuming lipid desaturases, which also contribute to the maintenance of fluidity of the membrane in cold temperatures. Furthermore, its proteome composition shows that it provides a way to avoid deamidation and cyclization of asparagine residues, which often is a problem for recombinant proteins (37). This process is reported to be temperature dependent, making *P. haloplanktis* growing at low temperatures beneficial. Additionally, *P. haloplanktis* has shown promising results for producing

recombinant proteins that tend to form inclusion bodies when produced in mesophilic bacteria (38).

P. haloplanktis can grow on a simple and cheap growth broth (TYP medium), and is not pathogenic to humans. The fast growth, high density cultures, resistance to ROS and the strategies to avoid deamidation of asparagine all makes *P. haloplanktis* is a promising host for expression of foreign protein in low temperatures (34).

Previous Work on Establishing *P. haloplanktis* as a Host for Protein Production

In Vectron, this bacteria has been attempted conjugated with the RK2 minimal replicon-based vectors pVB-1M0B1-mCh, pVB-1L0B1-mCh and pVB-1L1B1-mCh by T. Nygård (39). All these vectors harbored the 271 mutation of *trfA* (medium copy number), and wt P*m* promoter. The size of the plasmids was 9064 bp, and a schematic plasmid map of the vector is presented in **Figure 8**. Even though successful integration of the vectors in *P. haloplanktis* was detected, there were troubles reviving the bacteria from freezer and verify production of mCherry (39).

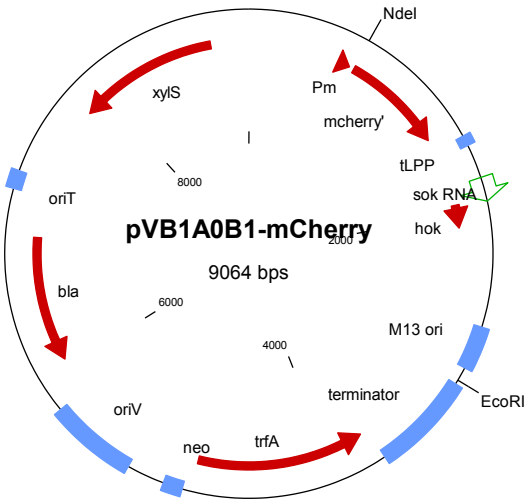

Figure 8: pBJ658/RK2 mininal replicon based vector harboring the Xyls/P*m* expression system and 271 mutant of *trfA*. Conjugation of *P. haloplanktis* TAC125 has previously been conjugated with this vector, with partial success (39).

1.10 *Pseudomonas putida* KT2440

Pseudomonas putida is a Gram negative, aerobic, saprophytic bacteria isolated from soil (40, 41). It has the ability to grow on a versatile set of carbon compounds, including various aromatic compounds. This ability is ensured by the TOL plasmid described in section 1.6. It is not pathogenic to humans and is certified as a biosafety host for cloning of foreign genes (42). The strain has shown ability to express high levels of foreign protein from a broad spectrum. Also, tools for genetic modification and manipulation are available for this strain (41).

P. putida has shown a resistance to ampicilin (Amp), and can grow on agar plates with Amp concentrations up to 200 µg/mL (43). The bacteria are commonly grown at 30 °C in LB medium in the lab. By electron microscopy it was found that P. putida in general had between five and seven polar flagella that ensures motility in the bacteria. An electron micrograph of *P. putida* can be seen in Figure 9 (44).

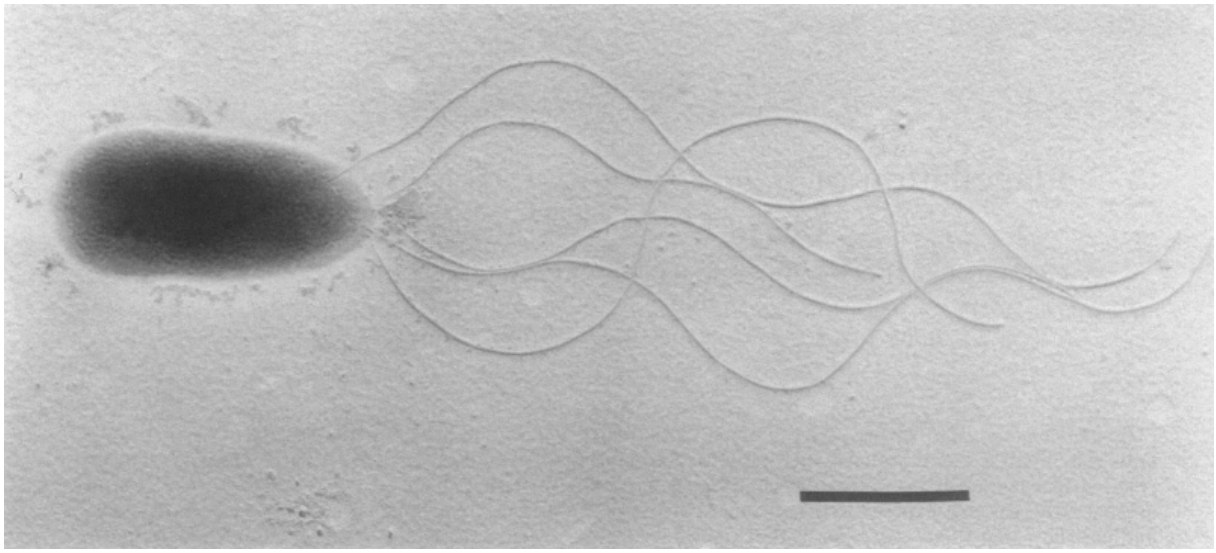

Figure 9: Electron micrograph of Pseudomonas putida PRS2000. The bar is 1µL. Figure obtained from (44).

Previous Work on Establishing *P. putida* as a Host for Protein Production

In Vectron, attempts have been made to tranform *P. putida* with pVB-1 derived vectors harboring the wt and 271 mutant of *trfA* and different mutations in the P*m* promoter. The size of the plasmid was 9,064 bp. The wt *trfA* mutant plasmids were successfully integrated in *P. putida,* but neither of the 271 *trfA* mutants (Vectron Unpublished) (39). Regarding protein production, in the work by Haukås (2011), high level production (~3.5-fold of obtained

amounts in *E. coli*) of IFN-α2b and GM-CSF from *P. putida* KT2440 has been reported from the XylS/P*m* cassette in a plasmid with the 271 mutation of *trfA* (43).

1.11 Aim of Study

The aim of this study was to establish functional XylS/P*m* based expression vectors in species alternative to *E. coli*. The candidate species were the Gram-negative strains *P. haloplanktis* TAC125 and *P. putida* KT2440. The starting point for construction of expression vectors were the pVB-1 bla-trfA_Kan plasmids, with low, medium and high copy number.

To establish *P. haloplanktis* as a production host, background studies to map basic properties important for work with the strain in the laboratory were conducted. Properties made subject for investigation were cultivation conditions, methods for reviving from frozen state and antibiotic sensitivity. Furthermore, a pVB-1 vector with low copy number and an appropriate selection marker, harboring the reporter gene *mCherry* was constructed. Next, the vector was introduced to the bacteria via conjugation to ensure stable maintenance of the plasmid, and finally produce mCherry as a reporter protein.

For *P. putida,* the pVB-1 vectors with low, medium and high copy number were to be introduced to the strain via electroporation, aiming to achieve stable maintenance and high-level production of mCherry.

Finally, the expression levels and solubility of mCherry in these alternative hosts were compared to the levels achieved in *E. coli* DH5α.

2 Materials and Methods

2.1 Media and Solutions

Media and solutions used for the work in this thesis are presented in Appendix A.

2.2 Bacterial Strains and Cultivation Conditions

An overview of bacterial strains used for the work in this thesis is presented in Table 1.

Table 1: Bacterial strains used for the work in this thesis.

Strain	Properties	Source/reference
E. coli DH5α	Cloning host	BRL
E. coli S17-1	Conjugation host (donor of plasmid), F^+	(45)
P. haloplanktis TAC125	Production strain	Institut Pasteur
P. putida KT2440	Production strain	NTNU

E. coli DH5α was used as a general cloning host. *E. coli* S17-1 was used as a host for conjugation of DNA into *P. haloplanktis*. The general cultivation conditions for *E. coli* was in LB medium at 37 °C and 225 rotations per minute (rpm) (tubes or flasks), or on LA agar at 37 °C and no shaking (plates). Selective antibiotics were added to LB medium and agar based on the resistance gene of the harboring plasmid in the bacteria.

For bacteria harboring plasmids that contained the *kan, bla* or *cat* gene encoding Kanamycin (Kan), Ampicillin (Amp) or Chloramphenicol (Cm) resistance, LB with 50 µg/mL Kan (LB+Kan50), 100 µg/mL Amp (LB+Amp100) or 20 µg/mL Cm (LB+Cm20) respectively, were used for cultivation.

P. putida and *P. haloplanktis* were candidates for protein production strains. *P. putida* was generally grown in LB medium at 30 °C and 225 rpm for tubes and shake flasks, and at 30 °C and no shaking on LA agar plates. For cultivation of the wild type (wt) *P. putida*, no

antibiotics were added to the media. Transformants of *P. putida* harboring plasmids with *kan* were cultivated in LB+Kan50. *P. haloplanktis* TAC125 wt was cultivated in TYP medium and 225 rpm, (tubes and flasks) and TYP agar with no shaking (plates) without antibiotics. As a part of this study, *P. haloplanktis* TAC125 wt was cultivated at different temperatures to find the optimal growth temperature for the strain. The most commonly used temperature for ON cultures and reviving from freezer was however 25 °C.

2.3 Overview Vectors

An overview of the bacterial strains and vectors used and constructed in this study are given in Table 2. Plasmid maps of relevance are presented in Appendix B.

Table 2: Overview of strains and vectors used and constructed in this study.

Plasmid	Properties[a]	Source/reference
pVB1-bla-wt_Kan	pJB658 based vector; promoter: *Pm; Ipp* terminator, hok/sok region, *oriT*. Regulator gene: *xylS*; *trfA* mutation: wt; Amp^r and Kan^r; 8,153 bp.	Vectron unpublished
pVB1-bla-271_Kan	pJB658/RK2 minimal replicon based vector; promoter: *Pm;* Regulator gene: *xylS*; *trfA* mutation: *cop271*; Amp^r and Kan^r; 8,153 bp.	Vectron unpublished
pHH100_optmCherry	Source of *mCherry*. Kan^r, 7,819 bp	Jørgensen (NTNU)
pBBR1 MSC	Source of *cat;* Broad-host-range expression vector; Cm^r; 4,700 kb	Antoine et al., 1992 (46)
pMtBL	Plasmid isolated from wt *P. haloplanktis,* source of ARS element; 4,086 bp	Tutino, 2001 (35)
pJB655 cop251 Mluc	Source of mutated trfA gene cop251	Blatny, 1997 (24)
pGEM®-T	Linearized commercial vector with single thymidine (T)-overhangs on 3'-terminals. Multiple cloning region with T7 and SP6 RNA polymerase promoters. Reporter protein: *lacZ*, allows for blue/white screening. Amp^r; 3,000 kb.	Promega (47)
pGEM-ARS	pGEM®-T based vector with ARS insert.	This work
pVB-1-mCh-wt_Kan	pVB-1-bla-wt_Kan based plasmid with *mCherry* inserted in *bla*. Kan^r, 8,003 bp.	This work
pVB-1-mCh-271_Kan	pVB-1-bla-271_Kan based plasmid with *mCherry* inserted in *bla*. Kan^r, 8,003 bp.	This work
pVB-1-mCh-251_Kan	pVB-1-mCh-271_Kan based plasmid with *cop271* exchanged with *cop251*. Kan^r, 8,003 bp.	This work
pVB-1-mCh-wt_Cm	pVB-1-mCh-wt_Kan based plasmid, *kan* exchanged with *cat*. Cm^r, 7,877 bp.	This work
pVB-1-mCh-271_Cm	pVB-1-mCh-271_Kan based plasmid, *kan* exchanged with *cat*. Cm^r, 7,877 bp.	This work
pVB-1-mCh- wt-ARS_Cm	pVB-1-mCh-wt_Cm based plasmid with insertion of ARS element. Cm^r, 8,725 bp.	This work
pVB-1-mCh-271-ARS_Cm	pVB-1-mCh-271_Cm based plasmid with insertion of ARS element. Cm^r, 8,725 bp.	This work
pVB-1 bla-wt_Cm	pVB1-bla-wt_Kan based vector, *kan* exchanged with *cat*. Cm^r, 8027 bp.	This work
pVB-1 bla-271_Cm	pVB1-bla-271_Kan, *kan* exchanged with *cat*. Cm^r, 8027 bp.	This work

[a] Amp^r: Ampicillin resistance; Kan^r: Kanamycin resistance; Cm^r: Chloramphenicol resistance;

2.4 Background Studies of *P. haloplanktis*

A better understanding of the characteristics of *P. haloplanktis* was needed to evaluate it as a host for protein production. Backround knowledge was also needed to effectively design appropriate expression vector for use in the species, with respect to which antibiotic resistance marker to use for instance. Therefore, more in-depth background studies were conducted on the species.

2.4.1 Growth Experiment *P. haloplanktis*

A growth experiment on *P. haloplanktis* TAC125 was conducted to characterize the growth of *P. haloplanktis* TAC125 with respect to cell densities, growth rates and generation times obtainable at different temperatures.

First, a pilot growth study of *P. haloplanktis* was performed at 15 °C, 25 °C, 30 °C and 37 °C with one replicate per temperature. Because *P. haloplanktis* grew well on lower temperature, 15 °C, the full scale growth experiment was performed at 4 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C and 37 °C. All shake flasks in the growth experiment were 500 mL baffled shake flasks containing 50 mL TYP medium without antibiotics, with 225 rpm shaking. The medium was inoculated from ON cultures of *P. haloplanktis* with OD_{600} ≈6 to obtain OD_{600} ≈ 0.2 at time 0. OD measurements were conducted using a Spectramax 384 Plus Spectrophotometer at 600 nanometers (nm) for 14 hours, with one last measurement after 25 hours from reinoculating point.

2.4.2 Generation Time *P. haloplanktis*

The generation time was found by calculating the doubling time of the bacteria under exponential growth. To identify the time points at which the bacteria were in the exponential phase, the ln(OD600) values were plotted against time on a semi-logarithmic plot in Excel. Growth rate and generation times were found for 15 °C, 20 °C, 25 °C and 30 °C The equations used and an example of calculation can be found in Appendix D.

2.4.3 Test of Antibiotic Sensitivity in *P. haloplanktis*

Another part of the background study on *P. haloplanktis* was to map the bacteria's sensitivity to different antibiotics. As antibiotic resistance is a commonly used molecular technique for

selecting for transformants containing a specific plasmid, identification of any natural resistance to an antibiotic is crucial. Therefore, growth of *P. haloplanktis* was tested in five different antibiotics commonly used in a genetics laboratory. TYP medium (5 mL) with the antibiotics presented in Table 3 was reinoculated in tubes (13 mL) from ON culture of *P. haloplanktis* to OD_{600} = 0.02 and incubated for two days (25 °C, 225 rpm). One replicate was included per antibiotic, and OD measurements were performed in cuvettes using a Spectramax 384 Plus Spectrophotometer every 24 h.
Antibiotics used with their respective concentrations are presented Table 3.

Table 3: Concentrations of antibiotics used in the test of antibiotic sensitivity in *P. haloplanktis*.

Antibiotic	Concentration (mg/mL)
Apr	100
Kan	50
Amp	100
Cm	25
Ery	50

After the general test of antibiotic sensitivity in *P. haloplanktis*, the Kan resistance was made subject for further investigation. The aim was to find out which Kan concentration *P. haloplanktis* wt would fail to grow. First, an experiment was performed on growth in TYP with Kan concentrations from 0 to 200 µg/mL, with one replicate per concentration. Then a more sensitive test was performed with Kan concentrations 37.5, 50 and 75 µg/mL, with three replicates per concentration and TYP without Kan as control (one replicate).

2.4.4 Revive *P. haloplanktis* From Freezer

Bacterial glycerol stocks (25 % used in this study) can be stored in the freezer (-80 °C) for years, and revived in media or agar whenever needed. As *P. haloplanktis* was a subject for investigation throughout the work in this thesis, there was a need for making a standardized procedure for reviving *P. haloplanktis* from the freezer. Three growth methods were tested (tubes, shake flasks, plates), two temperatures (15 °C and 20 °C) and three different glycerol stocks (OD_{600} of bacterial cultures used to make 25 % glycerol stocks of ~1, ~5 and ~13).

Three replicates were included per test unit, and to obtain approximately the same amount of cells for each unit, the glycerol stocks were thawed and 10 µL was added to either tube, plate or shake flask before incubation. Growth was measured every 12th hour for 48 hours.

For flasks and tubes, the inoculation volume was transferred to TYP medium by pipetting. For plates, the cells were transferred to the TYP agar plate by pipetting, and the culture was then streaked out using a sterile loop to spread the bacteria, as shown schematically in Figure 10.

Figure 10: Plating of bacterial culture from glycerol stock by application with a pipette and streaking with a sterile loop.

Test of Crystals

Crystals observed on TYP agar plates with *P. haloplanktis* incubated for more than 3 days were tested by picking, streaking on TYP plates and incubation at 15 °C for 3 weeks.

2.5 Inducer Diffusion Study *P. putida* and *E. coli* DH5α

The pVB-1 vectors aimed to use as expression vectors in this study harbor the XylS/P*m* expression cassette, which can be induced by benzoic acid derivatives, e.g. *m*-Toluate. This compound is known to be dose-dependent up to a certain concentration (14). In this experiment, the effect of increased concentrations of *m*-Toluate on growth of *P. putida* wt and *E. coli* DH5α was investigated. The protocol is adapted from (48).

LB was reinoculated with ON cultures of *E. coli* DH5α and *P. putida* wt to OD = 0.1 and aliquoted in individual sterile 96 deep well plates (Masterblock 96w 2mL, VWR). Diluted *m*-

Toluate solutions (20 µL) with different concentrations were added to bacterial culture (1.2 mL) to obtain the final concentrations of *m*-Toluate indicated in Table 4. The composition of these diluted inducer solutions are given under ”Media and Solutions” in Appendix A.

Table 4: Setup in the 96 well deep well plate inducer diffusion study

	1	**2**	**3**	**4**
A	No EtOH *	1	6	15
B	No EtOH	2	6	15
C	No EtOH	2	8	15
D	0**	2	8	20
E	0	4	8	20
F	0	4	10	20
G	1***	4	10	
H	1	6	10	

* Sterile dH_2O water (20 µL) was added instead of inducer.

** Absolute ethanol (20 µL) was added.

*** The number given corresponds to the final concentration of *m*-Toluate in that well.

One deep well plate was used per strain. The deep well plates were incubated at 800 rpm for 25 hours. OD_{540} (*P. putida*) and OD_{600} (*E. coli*) was measured every second hour for 10 hours, with one final measurement after 25 hours in a Tecan Infinite M2000 PRO in a sterile 96 well plate (Costar ® UV plate 96 Well) with LB as blank.

2.5.1 Test of pH Change at Different Concentrations of m-Toluate

m-Toluic acid is a weak organic acid, and has the ability to donate one hydrogen atom from its carboxyl group in a solution and hence lower the pH (49). Therefore, pH was measured in 3 mL LB medium with the following concentrations of *m*-Toluate: 0 mM, 1 mM, 10 mM, 15 mM and 20 mM. The pH meter used was PHM 92 LAB pH METER, NIGEN.

2.6 Molecular Methods for Vector Construction

The molecular methods applied to make the relevant expression vectors are presented in this section, followed by a specific section describing the protocol for constructing each of the vectors.

2.6.1 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a rapid way to amplify DNA by repeated cycles of strand separation and DNA replication. It has revolutionized the area of recombinant DNA technology because it has been made possible to generate large amounts of DNA *in vitro* from very small start amounts of genetic material (7).

A PCR involves three reaction steps: denaturation, annealing and elongation (7):
Denaturation: The temperature is raised (conventionally to 95-98 °C) to make the two DNA strands in the DNA helix separate by breaking the hydrogen bonds between the bases. This makes the DNA strands single stranded. This will also activate the thermostable Taq polymerase.
Annealing: Short single stranded (ss) oligonucleotide sequences called primers bind to ssDNA. The primers are designed to be complementary to a specific bases in the template strands, in each end of the target sequence. This ensures amplification of a specific area of the DNA, not the whole sequence. Primers are normally designed to have a melting point (T_m) around 60-70 °C, and the annealing temperature is set about 10 °C lower than the primers' melting point, depending on the desired specificity of the reaction.
Elongation: DNA polymerase uses the 3'-end of the primers as a starting point, and elongated the nucleotide sequence by adding free deoxyribonucleic acids (dNTPs) to the 3'-end of the sequence. The elongation temperature is normally 72 °C, as this is the optimum temperature for Taq polymerase.

These steps are repeated in cycles in a thermocycler, ensuring an exponential increase in number of amplicons.

Conventional PCR

An example of a PCR reaction is described Table 5. The thermocycler used was C1000 Touch TM Thermal Cycler (Bio-Rad) for all PCR reactions.

Table 5: Example of a conventional PCR reaction performed in a C1000 Touch TM Thermal Cycler (Bio-Rad) with Taq polymerase. The elongation time at 72 °C was 1 min for inserts and 5 min for backbones. For backbones there was also an extra elongation step (marked with *) of 5 min before storage at 4 °C.

Reagent	Amount (µL)			
5x PCR buffer (Q5)	10	**Thermocycler programme**		
Enhancer (Q5)	10	**°C**	**Time**	
Polymerase (Q5)	1	**95**	2 min	
Primer fw 10 pmol/µl	2.5	**95**	30 sec	30x
Primer rev 10 pmol/µl	2.5	**Annealing temp.**	30 sec	
dNTP 10 mM (VWR)	1	**72**	1 min/ 5 min	
Template	1	**72***	5 min	
H_2O	Up to 50	**4**	∞	

CloneAmp HiFi PCR Premix

In addition to the conventional PCR, a commercial High Fidelity PCR Premix (Takara Bio Company) was used. This is a 2x PCR mastermix that contains dNTPs and a high fidelity DNA polymerase with an associated optimized buffer. The premix is aliquoted in PCR tubes, and only primers and template was added as described in Table 6.

Table 6: Recommended mastermix for PCR reactions using HiFi PCR Premix (Takara Biolabs)

Reagent	Amount (µL)	Final concentration
CloneAmp HiFi PCR Premix	12.5	1X
Forward primer (10 pmol/µL)	0.5	0.2 µM
Reverse primer (10 pmol/µL)	0.5	0.2 µM
Template	1	
dH_2O	Up to 25 µL	

No initial denaturation step was included for this PCR, neither was a final elongation step. The elongation step for PCR reactions was 5 seconds for inserts (size ~0.7 kb) and 40 seconds for backbones (size ~8 kb). The thermocycler program used for all Takara PCRs is presented in Table 7.

Table 7: PCR program template for PCR reactions run using HiFi PCR Premix (Takara).

Temperature (°C)	Time	
98	10 sec	
55	15 sec	30x
72	5 sec/kb	
4	∞	

2.6.2 Plasmid Isolation

To start molecular cloning, plasmid DNA needed to be extracted from the cell and purified. A commercial plasmid isolation kit could be used for this purpose. The one applied in this work was ZR MiniPrep™ -Classic (Zymo Research). The method is based on an alkaline lysis protocol combined with a *Fast Spin* column (50).

Zymo Research MiniPrep™ Quick Protocol

Adapted from protocol supplied by manufacturer.

Cells from ON bacterial cultures (5 mL) were harvested by centrifugation (Eppendorf Centrifuge 5804 R, 10,000 g, 5 min). The supernatant was discarded, cells resuspended in Buffer P1 (200 µL) by pipetting and transferred to an 1.5 mL Eppendorf tube. Buffer P2 (200 µL) was added to lyse the cells and denature DNA, and the tubes were mixed by inverting (not vortexed). After incubation in room temperature (2 min), Buffer P3 (400 µL) was added to neutralize the lysis buffer and renature plasmid DNA. The solutions were mixed carefully but thoroughly by inverting the tubes and incubated at room temperature (2 min).

Cell debris was spun down in an Eppendorf MiniSpin centrifuge (13,400 rpm, 3 min). One Zymo-Spin™ IIN column (spin column) per sample was placed in a Collection tube, and the supernatant from the Eppendorf tubes was transferred to the spin column without disturbing the cell pellet. Here, plasmid DNA bound to the column. The tubes were centrifuged (30 sec, 13,400 rpm). The flowthrough was discarded and the columns put back into the same Collection tubes. Cell Wash buffer (200 µL) was added, the spin tubes centrifuged (30 sec, 13,400 rpm), and columns put back into the same Collection tubes. In the final wash step, Plasmid Wash Buffer (400 µL) was added to the column, and the tubes were again centrifuged (1 min, 13,400 rpm).

For elution of DNA, the spin columns were placed in a new, sterile Eppendorf tubes. Plasmid DNA was eluted with Elution Buffer (30 µL) by adding the buffer directly to the column and centrifuging the tubes (30 sec, 13,400 rpm). The isolated plasmids were stored at -20 °C.

2.6.3 Determination of Plasmid DNA Concentration

The concentration of plasmid DNA was determined by measuring absorbance in 1 µL sample at 260 nm in Nanodrop Spectrophotometer ND-1000 (Saveen and Werner) or a Thermo Scientific NanoDrop One. Protein contamination can also be detected by measuring absorption at 280 nm.

2.6.4 Endonuclease Digestion

An invaluable tool in genetic engineering is the application of restriction endonucleases found in bacteria. The type II restriction endonucleases recognize and bind specific recognition sites in non-methylated double stranded (ds) DNA and cleave the DNA by cutting next to a specific nucleotide in each strand. Depending on the nature of the recognition site, the

cleaving of DNA result in either *blunt* or *sticky* ends (7). An example of creation of sticky ends with single stranded (ss) overhangs is shown in Figure 11, for *Nde*I and *Spe*I which were the restriction enzymes used in this work.

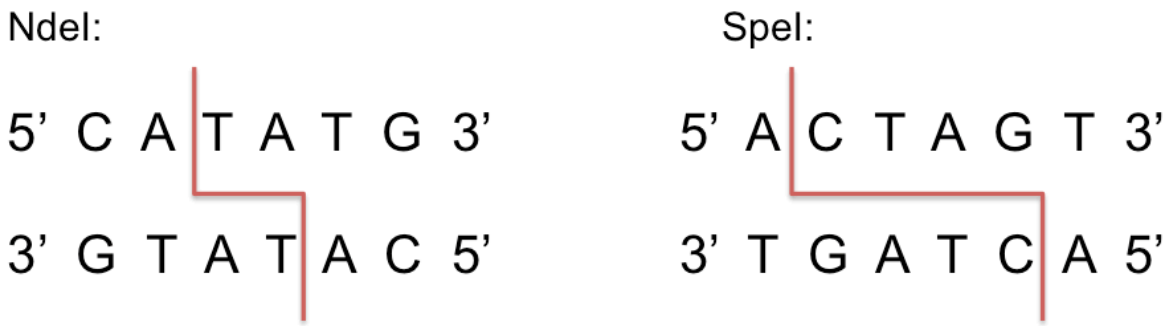

Figure 11: Recognition sites in a nucleotide sequence for the restriction enzymes *Nde*I and *Spe*I respectively. The red line represents where the enzymes will cleave DNA and make sticky ends. Figure adapted from (51, 52).

The benefit of making sticky ends is that two independent pieces of DNA that are cut with the same restriction enzyme can be ligated relatively easily because of the increased specificity. To clone a gene into a plasmid vector, both backbone and insert can be cut with the same restriction enzymes, and then ligated. Temporary hydrogen bonds are formed between corresponding nucleotides in the sticky ends and stabilize the molecule long enough to facilitate covalent sealing by DNA ligase (7).

In this work, the High Fidelity (HF) version of *Spe*I was used due to its beneficial properties, like the minimization of off-target products. Different restriction enzymes have optimal activity in different buffers and temperatures. Both *Nde*I and *Spe*I-HF have 100 % activity in CutSmart® Buffer (NEB) at 37 °C.

A typical restriction cutting reaction is presented in Table 8. For test cut reactions after ligation, a smaller reaction volume was used (10 µL), and all reagents were reduced accordingly.

Table 8: Typical restriction enzyme cutting reaction for use in molecular cloning.

Reagent	Backbone	Insert
10x CutSmart (NEB)	2.5 µL	2.5 µL
***Nde*I (NEB)**	0.5 µL	0.5 µL
***Spe*I-HF (NEB)**	0.5 µL	0.5 µL
Plasmid	10 µL	15 µL
dH_2O	Up to 25 µL	Up to 25 µL

The cutting mix incubated for 1 h (37 °C), before Alkaline Phosphatase, Calf Intestinal (CIP) (NEB) was added to the backbones when to be used for ligation. CIP dephosphorylates nucleic acids, and prevents religation of the linearized plasmid (7).

2.6.5 Agarose Gel Electrophoresis

Gel electrophoresis is a widely used technique in molecular biology for purification and separation of DNA and RNA fragments together with proteins. In this study, DNA fragments from PCR and restriction enzyme cutting reactions were separated by agarose gel electrophoresis. The principle of the method is to separate fragments based on their intrinsic electrical charge. The agarose gels used in this study were made from agarose powder and 1x TAE buffer to a concentration of 0.8 % agarose. This gel is placed in a chamber filled with buffer (1x TAE), and connected to an electrical field (Bio-Rad PowerPac Basic). Other buffers can be used, but the same buffer must be used in gel and chamber. As the power is turned on, DNA molecules applied to the gel will move towards the positive electrode. Small molecules will migrate fast, while big will take longer due to the difference in resistance in the agarose gel (7).

Loading dye is added to the samples to simplify application to the gel. The blue color makes the samples visible, and the glycerol in the loading dye makes the sample sink down to the well. To visualize the DNA, however, a stain must be used (7). GelRed and GelGreen™ Nucleic Acid Gel Stain, 10,000X in water (Biotium) were the stains used in this study, and made it possible to visualize DNA fragments under UV light in a Bio-Rad Molecular Imager ® ChemiDoc ™ XRS+ with Image Lab ™ Software.

By adding a commercial DNA ladder containing DNA fragments with known sizes, the size of the fragments in the relevant sample can be estimated from these. The molecular ladders used in this work are presented in Appendix E.

2.6.6 Extraction of DNA from Agarose Gel

When DNA fragments are successfully separated on an agarose gel, the nucleic acids need to be extracted from the DNA and purified. This can be done by use of Zymoclean™ Gel DNA Recovery Kit (Zymo Research).

Zymoclean™ Gel DNA Recovery Kit Protocol

Adapted from protocol supplied by the manufacturer.

DNA fragments with correct sizes were sliced out from the gel and put in an 1.5 mL Eppendorf tube. The weights of the gel pieces were determined and added 3 parts ADB Buffer per 1 part gel weight. (E.g. 750 μL ADB per 250 mg gel.) Gel and buffer was heated in a water bath (42 °C, 5-10 min) until the gel slice was completely dissolved. The melted agar was transferred to a Zymo-Spin™ IIN column placed in a Collection tube, and centrifuged in a Eppendorf MiniSpin centrifuge (45 sec, 13,400 rpm). The flowthough was discarded, and the columns put back into the same Collection tube. DNA Wash Buffer (200 μL) was added to the column and the tubes centrifuged (30 sec, 13,400 rpm). The flowthrough was again discarded, and the wash step repeated. Before elution of DNA, the columns were placed in a sterile Eppendorf tube. Elution Buffer (12 μL) was added directly to the column, and the tubes were centrifuged (30 sec, 13,400 rpm) to elute DNA. The extracted DNA fragments were stored at -20 °C.

2.6.7 DNA Ligase

Ligation in molecular biology is the joining of free DNA ends by the enzyme DNA ligase. The enzyme seals nicks in the backbone of DNA by using the energy from hydrolyzation of ATP to create a covalent sugar-phosphate bonds between adjacent free DNA ends. T4 ligase is the one most commonly used DNA ligase in genetic engineering, and origins from the bacteriophage T4. This enzyme can ligate both sticky and blunt ends, although at a slower rate for blunt ends due to the lower specificity of blunt ends (7). In this study, only sticky ends were used.

A common ligation reaction is presented in Table 9. A religation reaction without insert is included as control. Ligation reactions were incubated ON (approx. 16 hours) at 16 °C.

Table 9: Typical ligation reaction composition. The molecular ratio between insert and vector is typically between 3:1 and 5:1.

	Ligation (µL)	**Religation (µL)**
T4 Ligase Buffer (NEB)	1	1
T4 Ligase (NEB)	0.5	0.5
Vector	x	x
Insert	x	0
dH_2O	Up to 10 µL	Up to 10 µL

T4 ligase buffer contains ATP, and was used to optimize the conditions for T4 ligase, which is the enzyme used for all ligation reactions in this work. The molecular ratio of insert/backbone is adjustable, but a molar ratio of insert 5:1 backbone is used here. The Ligation Calculator by NEBio was used to calculate this ratio (53).

2.6.8 Transformation of *E. coli*

Transformation is the transfer of "naked" or "pure" DNA from external medium into the cell (7). "Naked" means that the DNA molecule is not associated with any macromolecules, like proteins. Bacterial cells are, under special circumstances, able to take up such naked DNA, and incorporate the genetic information in the cell. Cells capable of this are called *competent*. Some bacteria are naturally competent, while other must be treated chemically to obtain this feature. This can be done by chilling bacteria in the presence of metal ions, e.g. Rb^+ or Ca^{2+}. This loosens the structure of the cell walls, which allows uptake of naked DNA. This is followed by a heat shock with subsequent incubation in rich media without antibiotics which makes the bacteria repair the cell wall (7).

Preparation of E. coli *DH5α RbCl Supercompetent Cells*

LB (100 mL) was reinoculated to 1 % from ON culture *E. coli* DH5α in in a 1 L shake flask and incubated for ~2.5 hours (37 °C, 225 rpm) until OD_{600} = 0.4. The shake flask was then put on ice for 20 min to stop growth. The culture was aliquoted in 3 sterile 50 mL tubes and

cells harvested by centrifuging (Eppendorf Centrifuge 5804 R, 4 °C, 5,000 g and 5 min). The supernatant was discarded, and cells were resuspended in cold TFB1 Buffer (13.3 mL in each tube) by carefully pipetting up and down. After incubation on ice (15 min), the cells were harvested again (4 °C, 5,000 g and 5 min). The cells were carefully resuspended in cold TFB2 Buffer (1 mL per tube), and the resuspension aliquoted in 1.5 mL Eppendorf tubes (100 µL per tube). This step was performed in a sterile bench on ice. Tubes were immediately after aliquotation frozen with liquid nitrogen and stored at -80 °C.

Transformation of E. coli *DH5α*

Chemically competent *E. coli* DH5α cells (100 µL) were thawed on ice for ~10 min. One tube was needed per transformation. For ligation reactions, one tube for religation is commonly added per ligation as control. Up to 10 µL DNA (plasmid or ligation mix) was added to the competent cells and incubated on ice (15-30 min). Next, the cells were heat-shocked in a water bath (35 sec, 42 °C), and then immediately put on ice (2 min). Prewarmed SOC-medium (900 µL) was added to the tubes before incubation in a water bath (37 °C, 1 h).

100 µL of the cell culture was plated on LB agar containing a selective antibiotic. Additionally was 100 µL concentrated cells plated. The remaining cell culture (900 µL) was concentrated by centrifugation (Eppendorf MiniSpin, 4 min, 13,400 rpm), the supernatant decanted and the cells resuspended in the remaining volume before plating.

Transformation of E. coli S17-1

Transformation of plasmids into *E. coli* S17-1 was conducted the same way as for DH5α, described in section 0.

2.6.9 Sequence- and Ligation- Independent Cloning

Sequence- and ligation-independent cloning (SLIC) is a cloning method that does not require endonuclease digestion of DNA or use of DNA ligase. The method is based on the 3'-to-5'-exonuclease activity of T4 DNA polymerase. An optimized SLIC protocol from Jeong et al. was used in this study (54).

Plasmids (backbone and insert) were isolated from ON bacterial cultures using Zymo Research Miniprep™ Quick Protocol. Backbones and inserts were amplified by PCR using primers with a ≥ 15 bp extension homologous to the desired ends of the backbones. The PCR fragments were separated by agarose gel electrophoresis, and the fragments with correct size were sliced out and purified by Zymoclean™ Gel DNA Recovery Kit Protocol.

Backbone and insert were mixed in a molar ratio of 1:2. NEB Buffer 2.1 was added for optimal activity of T4 polymerase. The reaction setup is shown in Table 10.

Table 10: Setup SLIC T4 polymerase reaction to create 5'-end overhangs. x and y are determined from the DNA concentrations of the purified fragments

	Sample	**Control**
Backbone (PCR product)	x µL	x µL
Insert (PCR product)	y µL	0 µL
10X NEB Buffer 2.1 (NEB)	1 µL	1 µL
dH_2O	Up to 10 µL	Up to 10 µL

After mixing and spinning down the tubes, T4 polymerase (0.5 µL) was added and the tubes and incubated in room temperature (2.5 min). 5'-overhangs were created here by T4 polymerase exonuclease activity. The tubes were put on ice immediately after the 2.5 min incubation period to stop the reaction.

Tubes were incubated on ice for 10 min to allow the single stranded 5'-end of insert and backbone to anneal. Next, supercompetent *E. coli* DH5α cells were transformed with the annealed complex directly by the procedure described in section 0. The nicks in DNA are then repaired by the natural reparation machinery of the cell *in vivo*, and the recombinant plasmid is thus able to replicate and function as normal (54). The primers used for SLICs in this study are presented in Appendix C.

2.7 Sequencing

The vectors constructed in this work were examined by sequencing for detailed knowldge about the exact sequence of the plasmids. Primers specifying which area of the vector desired

to sequence were mixed with isolated plasmids after orders from the company, and sent by mail to GCTA, a company offering sequencing services in Germany.

2.8 Construction of New Vectors

2.8.1 Construction of pVB-1 mCherry-wt_Kan and pVB-1 mCherry-271_Kan

The *mCherry* gene was cloned into the core pVB-1 vectors because it was desired to use mCherry as the reporter protein in further use of the constructs as expression vectors in *P. haloplanktis* and *P. putida*. The core vectors harbored the *bla* reporter gene encoding β-lactamase and hence Amp^r. One of the species to be worked with in this study was *P. putida*, which is able to grow on Amp concentrations up to 200 μg/mL (39). Therefore, β-lactamase would not be a suited reporter protein for *P. putida*. Another intention of cloning *mCherry* into these vectors was to compare mCherry and β-lactamase as reporter proteins. Advances and challenges could be evaluated by expressing the reporter proteins from the same vector.

mCherry was cloned into the *Nde*I/*Spe*I restriction sites in the *bla* gene in the core vectors. Insert (*mCherry*) was amplified from the commercial plasmid pHH100_optmCherry using conventional PCR. The primer sequences (1, 2) are listed Appendix C. The 728 bp fragment was isolated by gel electrophoresis, and extracted from gel. Plasmids were isolated from cultures of *E. coli* DH5α harboring pVB-1 mCherry-trfAwt_Kan and pVB-1 mCherry-trfA271_Kan. These plasmids and the *mCherry* PCR product were digested with *Nde*I and *Spe*I-HF. Backbones were treated with CIP before separation of fragments by agarose gel electrophoresis. Backbones (7290 bp) and insert (713 bp) were extracted from gel, and a ligation reaction with T4 ligase was set up with a molecular ratio of 5:1 (insert: backbone). Supercompetent DH5α cells were transformed with the ligation mix by heat shock and plated on LA+Kan50. To test whether the cloning was successful, a test cutting with subsequent el electrophoresis, a test for expression of mCherry and sequencing was conducted.

2.8.2 Construction of pVB1-bla-wt_Cm, pVB1-bla-271_ Cm, pVB1-mCherry-wt_Cm and pVB1-mCherry-271_ Cm

As *P. haloplanktis* showed some natural resistance to Kan, it was decided to make a set of new pVB-1 mCherry vectors based on pVB-1 mCherry-wt/271/251_Kan where *kan* was exchanged with *cat* for use in this species. This was conducted using SLIC.

Plasmids were isolated from ON cultures of *E. coli* DH5α containing the following plasmids: pVB1-bla-wt_Kan, pVB1-bla-271_ Kan, pVB1-mCherry-wt_Kan, pVB1-mCherry-271_ Kan,(backbones) and pBBR-1-MCS-Cm (insert). Successful amplification of pVB-1 backbones and Cm insert using HiFi PCR Premix (Takara) was confirmed by gel electrophoresis, giving bands in the gel with the correct sizes (7187 bp and 741 bp for backbones and *cat*, respectively). Primers (number 3-6) are listed in Appendix C. The fragments were purified and ligated using the T4 polymerase protocol with subsequent transformation of *E. coli* DH5α with ligation mix. This yielded pVB1-bla-wt_Cm, pVB1-bla-271_ Cm, pVB1-mCherry-wt_Cm and pVB1-mCherry-271_ Cm. Successful cloning was checked by test cutting, test for expression of mCherry and sequencing.

2.8.3 Construction of pVB-1 mCherry-251_Kan and pVB-1 mCherry-251_Cm

The intention from the beginning and throughout this work was to use expression vectors with low, medium and high copy numbers. Due to the cloning trouble that will be described in section 3.3.1, it was discovered that there was no core vector containing the 251 mutant of *trfA*. Thus, this gene had to be cloned in from another vector.

Plasmids were isolated from ON cultures of *E. coli* DH5α harboring the plasmids pVB1 mCherry-271_ Kan, mCherry-271_ Cm (backbones), and pJB655 cop251 Mluc (251 insert). SLIC protocol was used, with HiFi PCR Premix (Takara) used for amplification to obtain fragments with complementary ends. The primer sequences (number 7-10) are listed in Appendix C. The PCR fragments were separated by agarose gel electrophoresis, purified and ligated using the T4 polymerase protocol with subsequent transformation of *E. coli* DH5α with ligation mix. This yielded pVB1-mCherry-251_Kan and pVB1-mCherry-251_ Cm. Successful cloning was checked by sequencing.

2.8.4 Construction of pGEM®-T-ARS

pGEM®-T is a commercial, linearized broad host cloning vector from Promega with 3'-end T-overhangs that simplifies insertion of PCR products. The vector allows for blue/white screening (47). As pVB-1 mCherry-wt_Kan did not successfully integrate in *P. haloplanktis,* new strategies for stable maintenance of the plasmid were tried. Thus, it was decided to clone the autonomously replicating sequence (ARS) originally from the pMtBL plasmid of

P. haloplanktis into the pVB-1_Cm vectors. pGEM®-T was used for a simple method of cloning the ARS element into a high-copy number vector able to maintain in *E. coli*.

pMtBL (4081 bp) was isolated from ON *P. haloplanktis* TAC125 culture. The ARS element was amplified via conventional PCR, using primers giving 5'-end adenine(A)-overhangs on the insert. Primers (11, 12) are presented in Appendix C. The ARS element was isolated by agarose gel electrophoresis, the correct band (842 bp) was cut out, and the ARS element extracted from the gel.

The ligation reaction was set up as presented in Table 11 (47). Reactions were mixed by pipetting, and incubated 1 h in room temperature.

Table 11: Ligation reaction of ARS element into the commercial vector pGEM (Promega)

Reaction Component	**Standard Reaction (µL)**	**Positive Control (µL)**
2X Rapid Ligation Buffer, T4 DNA Ligase	5	5
pGEM®-T Vector (50ng)	1	1
PCR Product	1	-
Control Insert DNA	-	2
T4 DNA Ligase (3 Weiss units/µl)	1	1
dH_2O	Up to 10 µL	Up to 10 µL

The ligation mix (10 µL) was heat shocked into *E. coli* DH5α, and plated on LA+Amp100+IPTG+X-Gal* (undiluted and concentrated). White colonies were picked the following day, inoculated in LB+Amp100 and grown ON. Successful cloning was checked by test cutting, test for expression of mCherry and sequencing.

* LB+Amp100 plates were equilibrated to room temperature before IPTG (100 µL, 0.1 M) and X-Gal (50 µL, 20 mg/mL) were spread out on the plate. It was allowed to absorb for 1 h (37 °C) before use.

2.8.5 Attempt to Construct pVB-1 mCherry-ARS-wt_Cm and pVB-1 mCherry-ARS-271_Cm (SLIC)

As pVB-1 mCherry-wt_Kan could not stably maintain in *P. haloplanktis*, new strategies were tried. The aim of constructing the pGEM®-T-ARS plasmid was to further clone the ARS element into the pVB-1_Cm vectors, and then conjugate these into *P. haloplanktis* to see if the plasmid could stably maintain in the species.

Plasmids were isolated from ON cultures of *E. coli* DH5α containing pVB-1 mCherry-wt_Cm, mCherry-271_Cm (backbones) and pGEM®-T-ARS (insert). SLIC protocol was used, aiming to yield pVB-1 mCherry-ARS-wt_Cm and mCherry-ARS-271_Cm. For amplification of ARS from pGEM®-T, Q5 PCR was used. The primer sequences (number 13 and 14) are listed in Appendix C. The PCR product (879 bp) was isolated using agarose gel electrophoresis and purfied. For amplification of the backbones, several attempts were made with both Q5 PCR and HiFi PCR Premix (Takara) without result. The primers (number 15 and 16) are listed in Appendix C. A gradient PCR with both HiFi PCR (Takara) and Q5 PCR was conducted, with annealing temperatures from 51.7-64.4 °C. This was done to increase the specificity of the reaction. The PCR products were separated using agarose gel electrophoresis, which resulted in several bands on the gel. A fragment with a size 7,877 bp (from HiFi PCR, annealing temperature 62.7 °C) was cut out and purified, and a T4 polymerase ligation reaction (from the SLIC protocol) was set up to insert the purified ARS element previously isolated from gel into isolated backbone. DH5α supercompetent cells were transformed with the ligation mix. As this gave no colonies, the T4 polymerase procedure was conducted again with the purified fragment with a size ~8.5 kb (from HiFi PCR, annealing temperature 64.4 °C).

No colonies were obtained, but due to time constraints, no further attempts to clone ARS into pVB-1 were made.

2.9 Conjugation of *P. haloplanktis*

Conjugation is a way of transferring DNA from one cell to another. This transfer method requires cell-cell contact. The donor cell contains a fertility plasmid (F+) containing genes encoding sex pili that holds the cells together. Subsequent formation of a conjugational bridge

that connects the cytoplasm's of the two cells allows DNA to be transferred (55). The plasmid to be transferred starts rolling circle-replication from *oriT*. Single stranded DNA enters the recipient cell, where the plasmid is made circular double stranded (55).

pVB-1 mCherry-wt_Kan and pVB-1 bla-wt_Kan were transferred into *P. haloplanktis* via conjugation, aiming to establish a functional protein expression vector in the species. Originally, the intention was to try conjugation with pVB-1 mCherry-wt_Kan, mCherry-271_Kan, bla-wt_Kan and bla-271_Kan. Since the cloned plasmids with the 271 mutant of *trfA* turned out to actually harbor the wt mutation instead (this was discovered after the conjugation had been conducted), this resulted in attempts of conjugation with wt only.

The plasmids were first isolated from *E. coli* DH5α and transformed to chemically competent E. coli S17-1 (F^+). Cultures of *P. haloplanktis* and *E. coli* S17-1 harboring pVB-1 mCherry-wt_Kan and pVB-1 bla-wt_Kan were grown to $OD_{600} \approx 1$. Donor cells (2 mL) and recipient cells (1 mL) were harvested and washed with LB before carefully mixed and dropped on a LA agar plate. The cell mix was incubated ON (30 °C), and then scraped off and resuspended in TYP. Resuspension (100 µL) from undiluted to 10^{-2} dilution was plated on TYP + Kan50 agar and incubated for seven days (15 °C). As potential conjugants (pVB-1 bla-wt_Kan) did not grow, only the work with potential conjugants (pVB-1-mCherry-wt_Kan) was proceeded. Potential conjugants were picked and reinoculated in TYP+Kan200. By switching between growth in TYP+Kan200 medium and plating on TYP+Kan200 agar (15 °C) to obtain single colonies, clean streak glycerol stocks were made from one single colony.

Since conjugation requires cell-cell contact, both *E. coli* and *P. haloplanktis* would be present in a mixture after conjugation. Thus, there was a need to ensure that the bacteria being picked up and used for further experiments were actually *P. haloplanktis* conjugants, not *E. coli*. Therefore, the potential *P. haloplanktis* conjugants were tested for growth at 15 °C on TYP+Kan200 agar plates (for 2 days) side by side with *E.coli* S17-1 mCherry-wt_Kan. Next, *E.coli* S17-1 mCherry-wt_Kan and potential conjugants were grown in TYP medium (5 mL), where one tube per strain was induced with *m*-Toluate (2 mM) and one uninduced.

2.10 Transformation of *P. putida*

Transformation of DNA into a cell can be done by giving the cell an electric shock. Electrocompetent cells are placed in an "electroporater", and by shocking the cells, the cell wall is opened, allowing DNA to enter the cell (7).

2.10.1 Preparation of *P. putida* electrocompetent cells

Electrocompetent *P. putida* cells were prepared after the following procedure.

Preheated LB (100 mL) was inoculated with ON preculture of *P. putida* and grown until OD_{540}= 0.456 (~1.5 h). The bacterial culture was aliquoted in four cold 50 mL tubes (25 mL in each) and incubated on ice (30 min). Subsequently, the cells were harvested by centrifugation (4,000 g, 4 °C, 15 min, Sorwall Lynx 6000 Centrifuge Thermo Scientific). The cells were then washed three times with cold glycerol (10 %) (25 mL, 12.5 mL and 2.5 mL per tube per time respectively). Tubes were centrifuged (4 000xg, 15 min, 4 °C) between every step to harvest the cells.

Finally, the cell pellet was resuspended in glycerol (10 %, 200 µL), and aliquoted (40 µL per tube). Immediately after aliquotation, the tubes were frozen with liquid nitrogen, and stored at -80 °C. Four tubes were used directly for electroporation.

2.10.2 Electroporation of *P. putida*

pVB1-mCherry-wt_Kan, pVB1-mCherry-271_Kan and pVB1-mCherry-251_Kan plasmids were isolated from ON DH5α cultures. Plasmid (4 µL) and electrocompetent cells (40 µL) were carefully mixed together and incubated on ice (30 min). One tube of electrocompetent *P.* putida cells, where no plasmid was added was included as a negative control. Electroporation was carried out using a Gene Pulser Electroporation System (Bio-Rad) in cold 2 mm cuvettes (VWR) with the following settings: 2.5 kV, 25 µF, 200 Ω. Potential condense was wiped off the cuvette prior to shocking the cells. Immediately after shocking the cells, preheated SOC medium (950 µL) was added, before incubation at 30 °C for 2 h. The last hour of incubation was with shaking.

Bacterial cultures (undiluted and concentrated, 100 µL) were plated on LB+Kan50 and incubated for two days (30 °C). LB+Kan50 (5 mL) in a 13 mL tube was inoculated with

transformants by picking up colonies from plate, and grown ON (30 °C). Successful insertion of plasmid was checked by sequencing, Kovacs test and test for expression of mCherry.

2.11 Kovacs Test

Several methods are developed to characterize microbes. One way is to test for presence of the enzyme cytochrome *c* oxidase, which is present in some bacteria and absent in others. Cytochrome *c* oxidase in bacteria will form a blue color complex when added Kovacs reagent (N',N',N',N'-Tetramethyl-p-phenyl-enediamine dihydrochloride, Sigma-Aldrich). As a result, cultures of oxidase positive bacteria can be distinguished from oxidase negative bacteria by this change in color (5). Both *P. putida* and *P. haloplanktis* are oxidase positive bacteria, whereas *E. coli* is oxidase negative. Thus, an oxidase assay is a suitable way to distinguish these bacteria from *E. coli* (5).

Kovacs reagent (N',N',N',N'-Tetramethyl-p-phenyl-enediamine dihydrochloride) (Sigma-Aldrich) was prepared as described in Appendix A, and protected from light exposure. To start the reaction, Kovacs reagent (20 µL) was added to fresh bacterial culture (1 mL). The tubes were shaken throughout the reaction time to ensure aeration. Color change was to be observed after 15-30 seconds, and within 3 minutes.

2.11.1 Kovacs Test on *P. haloplanktis*

Potential conjugants of *P. haloplanktis* mCherry-wt_Kan were tested with Kovacs test with *P. haloplanktis* TAC125 and *E. coli* DH5α wt as controls.

2.11.2 Kovacs test on *P. putida*

Potential transformants of *P. putida* pVB-1 mCherry-wt_Kan, mCherry-271_Kan and mCherry-251_Kan were tested with Kovacs test with *P. putida* and *E. coli* DH5α wt as controls.

2.12 Test of Potential Conjugants *P. haloplanktis* for Growth on 37 °C

As the Kovacs test on potential conjugants of *P. haloplanktis* mCherry-wt_Kan showed inconclusive results, the cells were tested further. The growth experiment of *P. haloplanktis* wt presented in Section 2.4.1 showed that the species could not grow on 37 °C. Therefore,

ON cultures of potential conjugants were reinoculated in TYP+Kan200 in shake flasks and incubated ON at 37 °C to test for growth. *P. haloplanktis* wt was included as control.

2.13 Expression of mCherry in *E. coli* DH5α

An expression study of mCherry was performed in *E. coli* DH5α harboring the three constructed pVB-1 vectors with wt, 271 and 251 mutations of *trfA*. The aim was to quantify the mCherry production levels obtainable in *E. coli*, and later possibly compare these levels to other species harboring the same vectors. As a side study, fluorescence was measured in production cultures directly and also in cells resuspended in PBS in addition to in the soluble and insoluble fraction of lysed cells.

LB (30 mL) was reinoculated with ON cultures of *E. coli* DH5α pVB-1 mCherry-wt_Kan, mCherry-271_Kan and mCherry-251_Kan and grown to $OD_{600} \approx 1$ (30 °C). *E. coli* DH5α wt was included as control. Two flasks were included per vector, one induced and one uninduced. *m*-Toluate (120 µL, 0.5 M) was added to the flaks to induce expression, giving a final concentration of 2 mM. The production cultures were incubated for 16.5 hours ON (25 °C and 225 rpm). The following day, OD_{600} was measured in the production cultures. Fluorescence was measured in production culture directly and in cells taken out and resuspended in PBS. LB and PBS were included as negative controls. Next, cells were harvested by centrifugation of production cultures in 50 mL tubes and resuspended in PBS according to the wet weight of the pellet (1 mL/100 mg wet weight). Cells were harvested by centrifugation and resuspended in cold lysis buffer (CellLyticB (Sigma-Aldrich) added Benzonase Nuclease (Sigma), 0.2 µL/mL) (500 µL per 100 mg pellet). After incubation in room temperature (1 h), cell debris was harvested by centrifugation, and fluorescence was measured in supernatant and insoluble phase (resuspended in PBS (250 µL)) in a Tecan Infinite M200 PRO. Settings and details for the fluorescence measurements can be fond in Appendix G.

2.14 Expression of mCherry in *P. putida*

Successful electroporation of *P. putida* with pVB-1 mCherry-trfA_Kan vectors (wt, 271 and 251 mutants of *trfA*) was confirmed by Kovacs test and sequencing of plasmid DNA. The next step was then to quantify the amount of reporter protein possible to produce from this

expression cassette in this species. Therefore, an expression study similar to the one performed in *E. coli* DH5α was conducted.

3 Results

3.1 Background studies on *P. haloplanktis*

3.1.1 Optimal Growth Temperature of *P. haloplanktis*

Growth experiments on *P. haloplanktis* wild type (wt) was conducted to characterize the growth of the strain with respect to cell densities, growth rates and generation times obtainable at different temperatures.

TYP medium (50 mL) in baffled shake flasks (500 mL) was inoculated from an over-night (ON) culture grown at 15 °C. The flasks were incubated at 4 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C and 37 °C. For the temperatures 15-37 °C, three replicates of each sample were included, and for 5 °C and 10 °C one replicate was included. The measurements at 20 °C were performed in an individual experiment, under the same conditions as in the other growth experimens with flasks grown at 25 °C as control. The growth curves for 20 °C and 25 °C are presented in Appendix H. OD_{600} was measured in cuvettes every hour (15-30 °C) and every second hour (5 °C, 10 °C and 37 °C) for 14 hours. One last measurment was made after 25 hours from the reinoculation point. OD_{600} as a function of time (days) are presented in Figure 12.

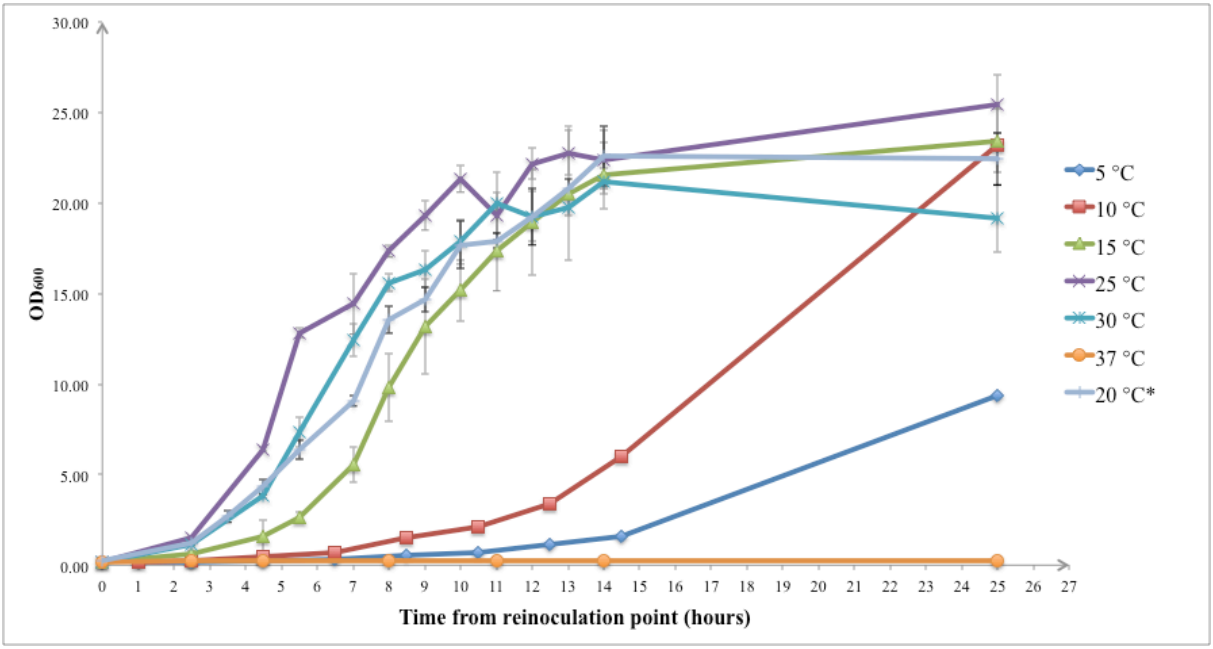

Figure 12: Mean OD_{600} of *P. haloplanktis* grown at 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C and 37 °C for 25 hours in baffles shake flasks (225 rpm). For 15-37 °C: three replicates, for 5 °C and 10 °C: one replicate. Error bars represent standard deviations. *Growth at 20 °C was conducted in a separate experiment, but under the same conditions.

For cultures grown at 25 °C, 30 °C and 37 °C, a culture sample was plated on a TYP agar plate and incubated at 37 °C ON. None of the plates had growth the following day, indicating no contamination of *E. coli.*

To determine the generation time of *P. haloplanktis* grown at different temperatures, a semi-log plot was made using $\ln(OD_{600})$ values as a function of time. The linear areas of the curves in the semi-log plot were used to calculate generation times for *P. haloplanktis* at 15 °C, 20 °C, 25 °C and 30 °C. Growth rate and generation times are presented in Table 12. The calculations can be found in Appendix D.

Table 12: Growth rate and generation time of *P. haloplanktis* in exponential phase grown in baffled shake flasks at 225 rpm at different temperatures.

Temperature (°C)	Growth rate (h^{-1})	Generation time (min)
15	0.51	80.9
20	0.72	57.9
25	0.79	52.9
30	0.70	59.8

The shortest generation time of *P. haloplanktis* was obtained at 25 °C, followed by 20 °C, 30 °C and 15 °C respectively. The bacteria were able to grow at 5 °C and 10 °C, and *P. haloplanktis* grown at 10 °C reached OD_{600} = 23.2 after 25 hours, while *P. haloplanktis* grown at 5 °C reached OD_{600} = 9.4 within the same time span. No growth was detected at 37 °C.

3.1.2 Test of Antibiotic Sensitivity in *P. haloplanktis*

To test whether *P. haloplanktis* had a natural resistance to antibiotics commonly used in molecular biology, a small scale screening experiment was performed growing *P. haloplanktis* in tubes added different antibiotics. The results are presented in Table 13 using a "+/-" system, where "+" represents $OD_{600} \geq 0.1$.

Table 13: Antibiotic sensitivity test of *P. haloplanktis*. "+": $OD_{600} \geq 0.1$. Numbers associated with the individual antibiotics represent their final concentration in growth media (µg/mL). Apr: Apramycin; Amp: Ampicilin; Kan: Kanamycin; Ery: Erythromycin; Cm: Chloramphenicol

Hours	TYP	Apr100	Amp100	Kan50	Ery50	Cm20
24	+	-	-	-	-	-
48	+	-	-	+	-	-

Growth of *P. haloplanktis* was detected in the control (TYP) with no antibiotic after 24 hours, while growth was detected in TYP+Kan50 and after 48 hours.

After the general test of antibiotic sensitivity in *P. haloplanktis*, the Kanamycin (Kan) resistance was made subject for further investigation. The reason was that the core vecors (pVB-1 bla-wt_Kan, bla-271_Kan and bla-251_Kan) used as a starting point for constructing expression vectors for use in *P. haloplanktis* and *P. putida* contained a Kan resistance selection marker. It was therefore crucial to find out whether these could be used in *P. haloplanktis*, or if the selection marker in the plasmids had to be exchanged.

First, a growth experiment was performed at a broad range of Kan concentrations, with one replicate per concentration as presented in Table 14. "Growth" was defined as $OD_{600} \geq 0.1$.

Table 14: Days before growth was detected in *P. haloplanktis* grown in 25 mL TYP medium with different concentrations of Kanamycin (µg/mL) in shake flasks. ”Growth” is defined as $OD_{600} \geq 0.1$. ”-” represents cultures were no growth was detected within 6 days.

Kanamycin concentration (µg/mL)	Time before growth detected (days)
0	1
10	2
25	2
50	-
100	-
150	-
200	-

Growth was detected after 1 day in the control tube without antibiotics, and after 2 days in tubes grown with 10 and 25 µg/mL Kan. No growth was detected in tubes with higher Kan concentrations.

With contradictory results from the two previous experiments concerning growth of *P. haloplanktis* at Kan50, a more sensitive test was performed. The same experimental setup was used, but with Kan concentrations 37.5, 50 and 75 µg/mL and three replicates per concentration. The result is presented in **Figure 13**.

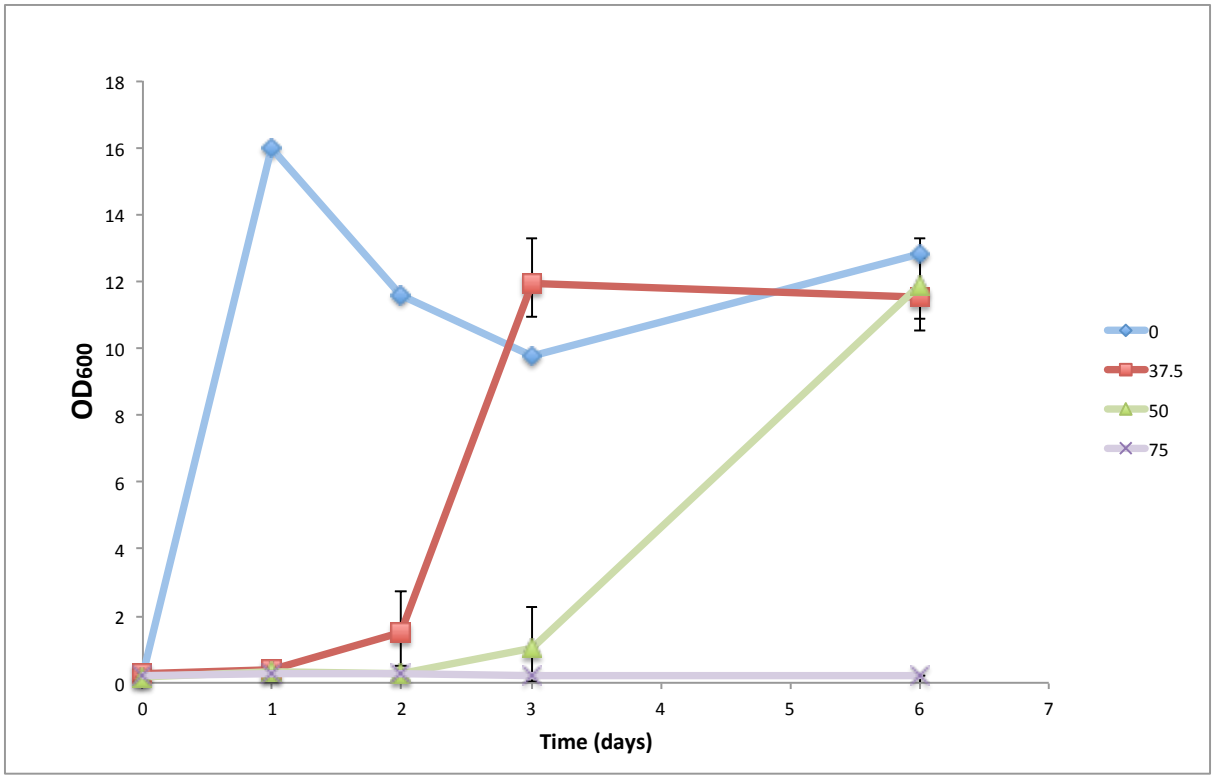

Figure 13: Average OD_{600} from *P. haloplanktis* cultures in TYP+Kan plotted as a function of time (days). The label of each data series represents the concentration of Kan (µg/mL) in TYP. Three replicates were included per concentration of Kan, and the positive control had one replicate. Error bars represent the standard deviation.

For flasks with 37.5 and 50 µg/mL Kan, growth was detected after 2 and 3 days respectively. No growth was detected in 75 µL/mL Kan. These results indicate that *P. haloplanktis* is able to obtain growth in 50 µg/mL Kan, but not in 75 µg/mL Kan in liquid medium. This means that the Kan concentration in growth medium had to be > 75 µg/mL to select for cells harboring the plasmids encoding Kan^r, or the *kan* gene had to be exchanged with a new selection marker.

3.1.3 Revive *P. haloplanktis* From Freezer

As *P. haloplanktis* was a subject for investigation throughout the work in this thesis, the need for standardizing a procedure for reviving *P. haloplanktis* from freezer was seen. Reviving using different growth methods, temperatures and ODs of cells when harvested for making glycerol stocks were investigated. An overview of the variables tested for is given below:

Growth methods:

- TYP agar plate (no shaking)
- 13 mL tube with 5 mL TYP medium (225 rpm)
- 250 mL shake flask with 25 mL TYP medium (225 rpm)

OD_{600} of cells in glycerol stock:

- 1.14
- 4.92
- 13.4

Temperatures:

- 15 °C
- 25 °C

Growth was measured every 12th hour for 48 hours.

Volume of a toothpick:

To make sure every replicate contained approximately the same amount of cells, the glycerol stocks were thawed, and the same volume was transferred to all plates/tubes/flasks. A rough estimate of the volume of a toothpick with frozen cells was found by scraping cells off a frozen glycerol stock with a toothpick and transferring the toothpick to an 1.5 mL Eppendorf tube. Here, the culture was melted off, the toothpick discarded, and the cells were centrifuged down into the tube before the volume was measured with a pipet.

Reviving on plates:

To simplify the characterization of growth on the plates, a ”+/- system”, representing growth increasing from ”-” (no growth) to ”++++” (mature/thick, yellow growth and larger, single colonies). The exact meaning of each representation is given beneath Table 15. OD=1, OD=5 and OD=13 refer to the OD_{600} of the bacterial cultures when harvested for making a glycerol stock for storage in the freezer (-80 °C). The mean growth on plates is presented in Table 15. No replicates diverged from the mean, meaning that the three replicates could be placed in the same category in the “+/-” system.

Table 15: Mean growth of *P. haloplanktis* on TYP agar plates observed every 12th hour for 48 hours. OD=1, OD=5 and OD=13 refer to the OD_{600} of the bacterial cultures when harvested for making a the glycerol stocks used in the experiment. Characterization of growth is based on the "+/- system" described below.

	OD = 1		OD = 5		OD = 13	
Hours	**15 °C**	**25 °C**	**15 °C**	**25 °C**	**15 °C**	**25 °C**
12	-	+	++	++	++	++
24	+	+	++	+++	++	+++
36	++	+++	+++	++++	+++	++++
48	++++	++++	++++	++++	++++	++++

"–"= no growth;

"+"= detectable growth where bacterial culture was applied to the plate;

"++"= growth where bacterial culture was applied to the plate;

"+++"= thick, yellow growth and also small single colonies;

"++++" = mature/thick, yellow growth and larger, single colonies.

All plates reached mature, yellow growth after 48 hours. The amount of bacteria on the plates differed, – the plates from the glycerol stock with OD=1 had less colonies than the plates from OD=5 and OD=13. OD=5 had growth spread out over the whole plate, while growth of the other two ODs was limited to where the glycerol stock was applied to the plate. The bacteria grew in general faster on 25 °C than on 15 °C. There was little or no variation between the replicates, all plates of the same test unit could be put in the same category in the "+/- system".

Reviving in tubes:

Growth from reviving in tubes was characterized OD_{600} measurements. The mean OD_{600} values from the three replicates per test unit are presented in Table 16.

Table 16: Mean OD_{600} values of *P. haloplanktis* revived in tubes in triplicates from different glycerol stocks, grown at 15 and 25 °C at 225 rpm for 48 hours. OD=1, OD=5 and OD=13 refer to the OD_{600} of the bacterial cultures when harvested for making the glycerol stocks used in the experiment.

	OD = 1		OD = 5		OD = 13	
Hours	**15 °C**	**25 °C**	**15 °C**	**25 °C**	**15 °C**	**25 °C**
12	0.06	0.75	0.31	0.83	0.34	0.77
24	1.68	1.45	1.26	1.45	1.28	1.08
36	2.33	1.62	1.96	1.68	1.88	1.49
48	2.82	2.34	2.38	2.78	2.68	2.10

The bacterial cultures had a white/bright yellow color throughout the whole incubation time. The ODs were increasing through the whole incubation time, and after 48 hours all tubes had an OD of ~2.5.

Reviving in flasks:

The results from reviving *P. haloplanktis* in baffled shake flasks at 15 and 25 °C from different ODs of glycerol stocks are presented in Table 17.

Table 17: Mean ODs of three replicate baffled shake flasks with 25 mL TYP medium grown at 15 and 25 °C at 225 rpm for 48 hours. OD=1, OD=5 and OD=13 refer to the OD_{600} of the bacterial cultures when harvested for making a the glycerol stocks used in the experiment.

	OD = 1		OD =5		OD = 13	
Hours	**15 °C**	**25 °C**	**15 °C**	**25 °C**	**15 °C**	**25 °C**
12	0	0	0.15	23.00	0.043	0
24	0.039	8.65*	10.20	**	6.74	0.020
36	18.50	**	**		**	15.3
48	**					**

* One of the replicates did not have any growth at all. The mean is calculated from the two other flasks.

** Cultures were brown/yellow of color, with little or no production of foam. Cultures were discarded at this point.

For the flasks inoculated from the glycerol stock with OD_{600} = 1 on 25°C, one of the replicates had no growth throughout the whole incubation period. The variations between the replicates were larger here than for reviving in tubes (standard deviations not shown).

Reinoculation:

After 12 hours, one of the shake flasks with detected growth (from glycerol stock with OD=5, grown at 25 °C) was used to reinoculate fresh media with 10 µL bacterial culture (*P. haloplanktis*). Also, one colony from TYP agar with detected growth (from glycerol stock with OD=5, grown at 25 °C) was reinoculated in fresh media. Three replicates per temperature per growth method were incubated at 15 °C and 25 °C. The mean ODs at 12, 24 and 36 h is presented in Table 18.

Table 18: Mean ODs of *P. haloplanktis* reinoculated in baffled shake flasks with 25 mL TYP grown at 15 and 25 °C at 225 rpm for 48 hours. Culture from flask used for reinoculation was grown ON at 25 °C for 12 hours and had OD = 15. The colony used for reinoculation was grown on TYP agar at 25 °C ON. **indicate bacterial cultures with high OD that are dark yellow/brown in color, with no production of foam. These cultures were checked by visual inspection and discarded.

	Reinoculated from flask		Inoculated from plate	
Hours	**15 °C**	**25 °C**	**15 °C**	**25 °C**
12	0.38	13.53	0.042	1.77
24	**	**	3.77	**
36			**	

All shake flasks obtained growth faster when reinoculated from plate or flasks than they did when inoculated with bacterial culture from freezer. Growth was faster at 25 °C than at 15 °C. All cultures reached high ODs and a color change.

3.1.4 Crystal on plates with TYP agar + *P. haloplanktis*

A crystal-like structure was observed in plates incubated with *P. haloplanktis* for more than three days on 15 °C. Incubation at 15 °C was continued for these plates for four weeks. The crystal-like structure presented in **Figure 14** is ~3 weeks old. The crystals became larger over time. An experiment was performed where the structures were picked and streaked again on new TYP agar plates with subsequent incubation on 15 °C. This resulted in neither growth-like structures nor new crystal-like structures.

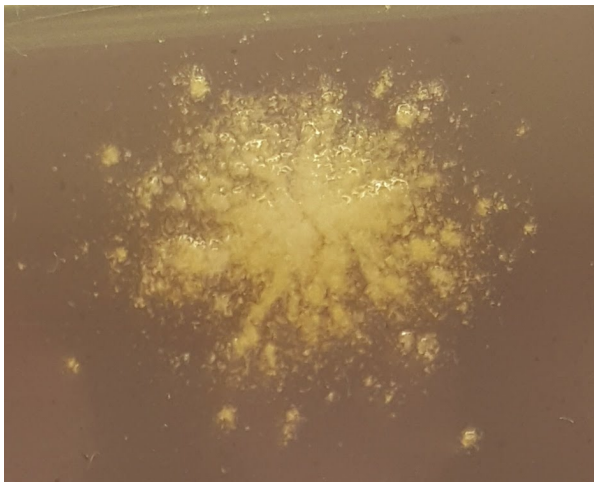

Figure 14: Crystal-like structure observed on TYP plates with growing *P. haloplanktis* wt after incubation for 3 weeks at 15 °C.

3.2 Inducer Diffusion Study

The inducer diffusion study was conducted to investigate how increased concentrations of *m*-Toluate would affect growth of *P. putida* wt and *E. coli* DH5α. The experiment is relevant when evaluating potential hosts for recombinant protein production because it is desired to use different concentrations of *m*-Toluate to "tune" the expression. Tuning of expression is possible because activation of the P*m* promoter and hence transcription, is known to be dose-dependent up to a certain point (48). It is already known that *m*-Toluate is able to enter the cells of *E. coli*, hence *E. coli* DH5α was included in the experiment as a positive control.

OD of *P. putida* was measured at 540 nm, and OD of *E. coli* at 600 nm. The mean ODs at every measuring point with associated standards deviations are presented Figure 15 and Figure 16 for *P. putida* and *E. coli,* respectively.

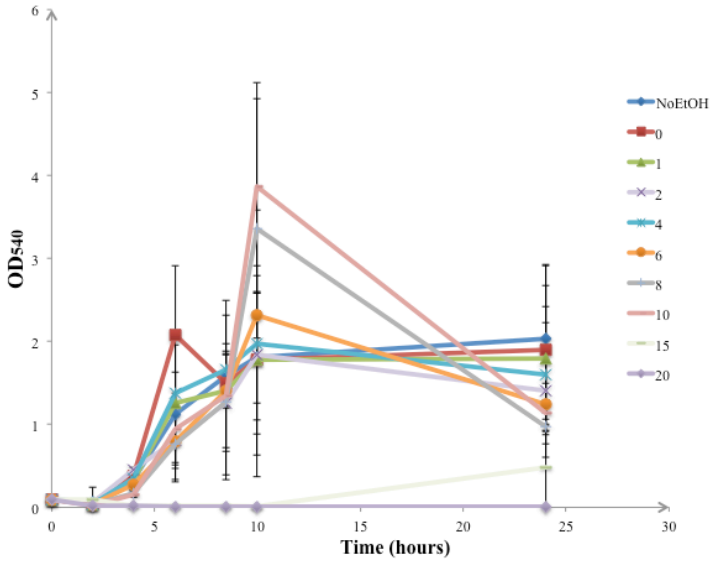

Figure 15: Mean OD_{540} of *P. putida* grown in LB plotted as a function of time with error bars representing standard deviations. 1.2 mL bacterial culture was grown at 30 °C in deep well plate with triplicates of each concentration of inducer. The label of each data series represents the final concentration of m-Toluate (dissolved in absolute ethanol) in the well. NoEtOH =20 µL RO water; 0 = 20 µL EtOH.

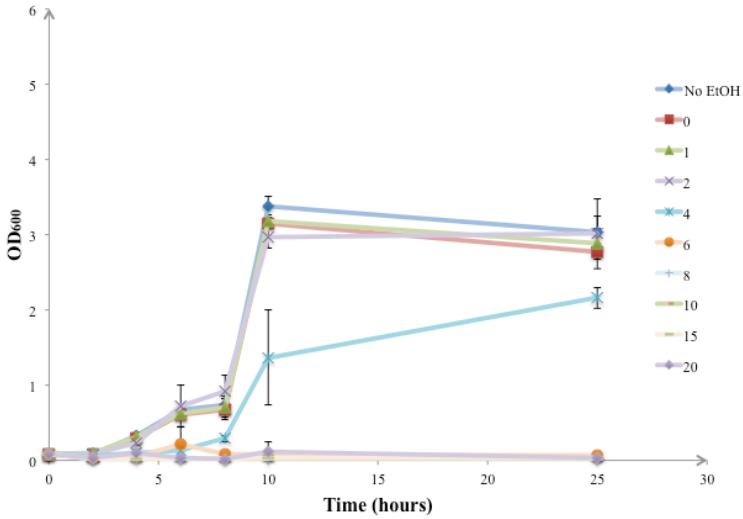

Figure 16: Mean OD_{600} of *E. coli* DH5α grown in LB plotted as a function of time with error bars representing standard deviations. 1.2 mL bacterial culture was grown at 37 °C in a deep well plate with triplicates of each concentration of inducer. The label of each data series represents the final concentration of m-Toluate (dissolved in absolute ethanol) in the well. NoEtOH = 20 µL RO water; 0 = 20 µL EtOH.

P. putida showed normal growth in concentrations of *m*-Toluate up to 10 mM. A small increase in OD up to ~0.5 was also observed at 15 mM *m*-Toluate in the time span from 10 h to 24 h. The final OD values measured at 24 h were all ~1-2 for *P. putida* growth in *m*-Toluate concentrations from 0-10 mM, including NoEtOH. No growth was detected for 20 mM m-Toluate. The standard deviations were large and overlapping.

E. coli showed normal growth up to 4 mM *m*-Toluate. The final OD values of cultures grown in *m*-Toluate concentrations from 0-4 mM were all between 2 and 3, including NoEtOH. No growth was detected for concentrations 6-20 mM *m*-Toluate. The standard deviations here were smaller than for *P. putida*.

3.2.1 pH of LB + Inducer

pH was measured in LB medium with different concentrations of *m*-Toluate to check the effect of the inducer used in this study. This was done because *m*-Toluate, also referred to as *m*-Toluic acid is an organic acid with a potential to change the pH of the medium.

Table 19 shows the pH in LB medium after adding 0-20 mM *m*-Toluate.

Table 19: pH of LB with different concentrations of m-Toluate.

***m*-Toluate concentration (mM)**	**0**	**1**	**10**	**15**	**20**
pH	7.00	6.78	5.60	4.92	4.70

As expected, the pH is decreasing when increasing the concentration of m-Toluate. A small acidification could be seen at 1 mM m-Toluate with increasing drop in pH as the concentration of *m*-Toluate gets higher.

3.3 Cloning Results

A description of the plasmids used and constructed in this work is found in section 2.3. The work to construct new plasmids is described in section 2.8. The aim of constructing new vectors was to create expression vectors that could stably maintain and express protein at certain levels in the bacterial species *P. haloplanktis* and *P. putida*. Plasmids were

continuously tested in the intended species after construction. A new cloning/modification of vectors would start immediately if it was found that the tested vectors were not useful.

3.3.1 Cloning Trouble

A lot of time was spent on working with incorrect plasmids containing for instance the wrong mutation of *trfA* through this study. This was not discovered before after some time because the sequencing was specified to the parts of the plasmid that were changed in the cloning and its transitions. This extended the time used on cloning by moths. Hence, the time left for experiments with the constructs was limited. An explanation of what happened can be found in Appendix F.

3.3.2 Construction of pVB-1 mCherry-wt_Kan and pVB-1 mCherry-271_Kan

The *mCherry* gene was cloned into the core pVB-1 vectors because it was desired to use mCherry as the reporter protein instead of β-lactamase. The intention of constructing these vectors was to use them as expression vectors in *P. haloplanktis* and *P. putida*. The core vectors pVB-1 bla-wt_Kan and bla-271_Kan (Vectron) were used as a base, and *bla* was exchanged with *mCherry* in the *Nde*I/*Spe*I restriction sites.

mCherry was successfully amplified, purified via gel electrophoresis, digested with *Nde*I and *Spe*I, and purified again via gel electrophoresis, obtaining a 713 bp fragment with *Nde*I/*Spe*I sticky ends. pVB-1 bla-wt_Kan and bla-271_Kan were isolated from ON cultures of E.coli, digested with *Nde*I/*Spe*I and sucessfully separated via gel electrophoresis, giving 7,290 bp fragments. Successful ligation of insert and plasmid with subsequent heat shock into supercompetent DH5α cells was confirmed by detection of colonies on LA+Kan50 agar plates incubated ON (37 °C). Correct insertion of mCherry was confirmed by test cutting with *Nde*I/*Spe*I giving the correct sizes (7290 + 713 bp), sequencing and ON cultures of the strains with inducer, obtaining pink cultures.

3.3.3 Construction of pVB1-bla-wt_Cm, pVB1-bla-271_ Cm, pVB1-mCherry-wt_Cm and pVB1-mCherry-271_ Cm

As *P. haloplanktis* showed some natural resistance to Kan in the antibiotic sensitivity test, it was decided to make a set of new pVB-1 vectors based on pVB-1 mCherry-wt/271_Kan and pVB-1 bla-wt/271_Kan. Here, *kan* was exchanged with *cat* encoding Cm^r for use in this

species. The 251 mutants were not included because it was not believed that a plasmid with high copy number could stably integrate in *P. haloplanktis,* based on previous studies.

SLIC protocol was used to exchange *kan* with *cat* in pVB1-bla-wt_Kan, pVB1-bla-271_ Kan, pVB1-mCherry-wt_Kan, pVB1-mCherry-271_ Kan, yielding pVB1-bla-wt_Cm, pVB1-bla-271_ Cm, pVB1-mCherry-wt_Cm and pVB1-mCherry-271_ Cm. pBBR-1-MCS-Cm was used as source of the *cat* gene. HiFi PCR Premix (Takara) was used for amplification. The primers sequences (3-6) are listed in Appendix C. Successful cloning was confirmed by test cutting and sequencing.

3.3.4 Construction of pVB-1-mCherry-251_Kan and pVB-1-mCherry-251_Cm

The intention from the beginning and throughout this work was to use expression vectors with low, medium and high copy numbers. Due to the cloning trouble described in section 3.3.1, it was discovered that there was no core vector containing the 251 mutant of *trfA*. Thus, this gene had to be cloned in from another vector: pJB655 cop251 Mluc, to obtain all three core vectors. pVB1-mCherry-271_ Kan, pVB1-mCherry-271_ Cm were used as backbones, and the *trfA* gene was exchanged using SLIC. Primer sequences (number 7-10) are listed in Appendix C. Successful cloning was confirmed by sequencing.

3.3.5 Construction of pGEM-ARS

As pVB-1 mCherry-wt_Kan did not successfully integrate in *P. haloplanktis,* new strategies for integration of the plasmid was tried. It was decided to clone the autonomously replicating sequence (ARS) originally from the pMtBL plasmid of *P. haloplanktis* into the pVB-1_Cm vectors via the commercial vector pGEM®-T. The latter was used as a simple method of cloning the ARS element into a high-copy number vector able to maintain in *E. coli* for further use. Moreover, pGEM®-T allowes for blue/white screening, which simplifies detection of cells harboring vectors with a successful integration of the ARS element.

The pMTBL (4081 bp) plasmid was isolated from *P.haloplanktis* wt via Miniprep, the ARS element amplified via PCR giving 5'-end A-overhangs, and the 842 bp fragment was successfully separated on agarose gel electrophoresis. Next, the element was ligated into pGEM®-T (with 3'-end T-overhangs). Ligation mix was transformed into E.coli DH5α via

heat shock. A positive control with a commercial insert and a negative religation control were included. The plates after ON incubation at 37 °C can be seen in Figure 17.

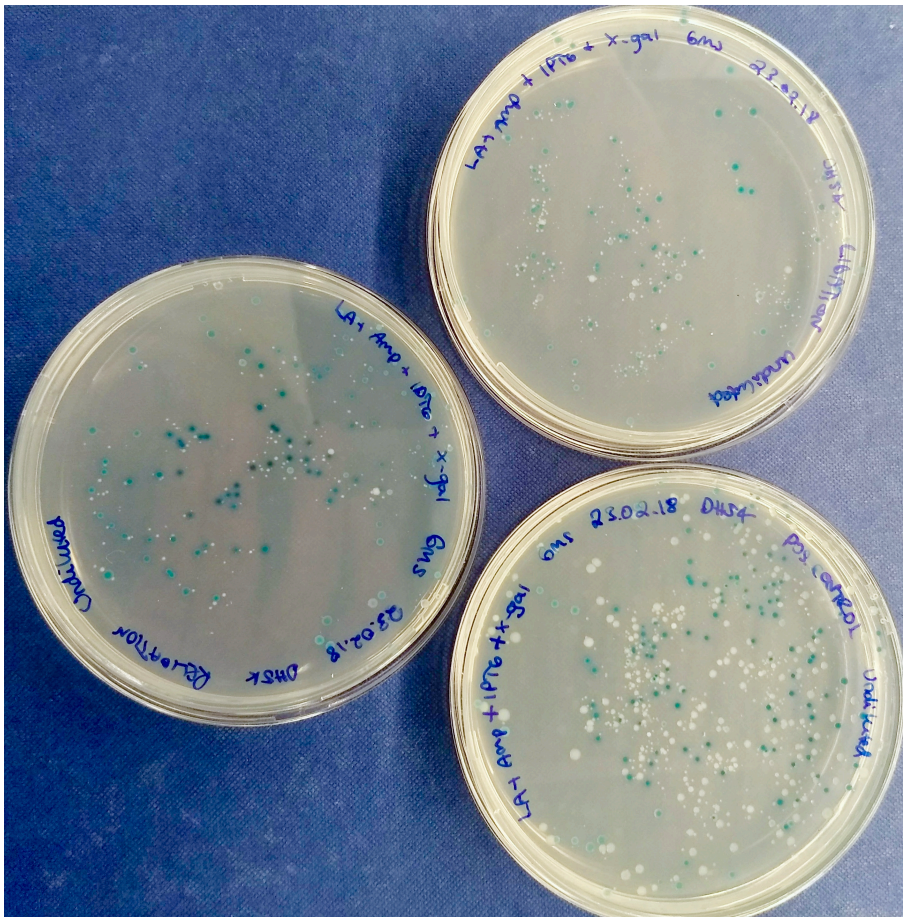

Figure 17: Colonies of *E. coli* DH5α transformed with ligation mix of ARS and pGEM®-T and controls on LA+Amp100+IPTG+X-Gal plates. Left: religation; up to the right: ligation pGEM®-T-ARS; down to the right: positive control. The colours of the picture are slightly modified to better show the blue/white colors.

White colonies indicated a disruption of expression of β-galactosidase, due to the missing ability to degrade X-Gal into an insoluble blue compound – and hence a successful insertion of DNA in the gene encoding β-galactosidase. White colonies were picked from the ligation plate, cells grown ON, plamids isolated and sent for sequencing. The sequencing results showed insert of ARS in pGEM in the right orientation.

3.3.6 Attempt to Construct pVB-1 mCherry-wt-ARS_Cm and mCherry-271-ARS_Cm

The final aim of cloning ARS into pGEM was to clone the element further into pVB-1 mCherry-wt_Cm and mCherry-271_Cm for use in *P .haloplanktis.* The ARS element origin

from *P. haloplanktis*, and therefore was the insertion of it in the vectors believed to enable stable maintenance of the plasmid in this species.

The ARS element in pGEM®-T-ARS was successfully amplified via Q5 PCR and the fragment (879 bp) was isolated using agarose gel electrophoresis. The primer sequences (number 13 and 14) are listed in Appendix C. The Q5 PCR of pVB-1 mCherry-wt_Cm and mCherry-271_Cm (backbones) with subsequent separation using agarose gel electrophoresis resulted in a smear, indicating no specific product. The primers (number 15 and 16) are listed in Appendix C. Gradient PCRs were then conducted with annealing temperatures from 51.7-64.4 °C, using both Q5 PCR and HiFi PCR Premix (Takara). This resulted once more in a smear for all annealing temperatures for the Q5 PCR. For the Takara PCR, several bands were observed on the gel. The lanes with fragments obtained from annealing temperatures 62.7 °C and 64.4 °C had several bands for both backbones, but one with a size 7,877 bp. These were cut out, purified and used in the T4 polymerase ligation reaction. Both the fragment from 62.7 °C and 64.4 °C were tried in two individual ligation reactions for both mutants (wt and 271), but after transformation of DH5α with the ligation mixes, no colonies were obtained. Thus, the ligation was considered unsuccessful. Due to time limitations, no further attempts to clone ARS into pVB-1 was made in this work.

3.4 Conjugation of *P.haloplanktis* with pVB-1 mCherry-wt_Kan and pVB-1 bla-wt_Kan

pVB-1 mCherry-wt_Kan was the first vector constructed, and an early attempt to conjugate this and the core vector pVB-1 bla-wt_Kan into *P. haloplanktis* was made, with the aim of integrating a functional protein expression vector in the species.

The donor strain was *E. coli* S17-1 and the full conjugation procedure is described in section 2.9.

3.4.1 Test of Potential *P. haloplanktis* Conjugants for Growth at 15 °C

Since conjugation requires cell-cell contact, both *E. coli* and *P. haloplanktis* would be present in a mixture after conjugation. Thus, there was a need to ensure that the bacteria being picked up and used for further experiments are actually *P. haloplanktis* conjugants, not *E. coli*.

Several tests were performed to compare and distinguish the potential conjugants from *E. coli* S17-1.

ON cultures of *E. coli* S17-1 bla-wt_Kan and potential conjugants were streaked on TYP+Kan100 and incubated for 2 days (15 °C) to check for difference in growth. These plates after 2 days of incubation are shown in **Figure 18**.

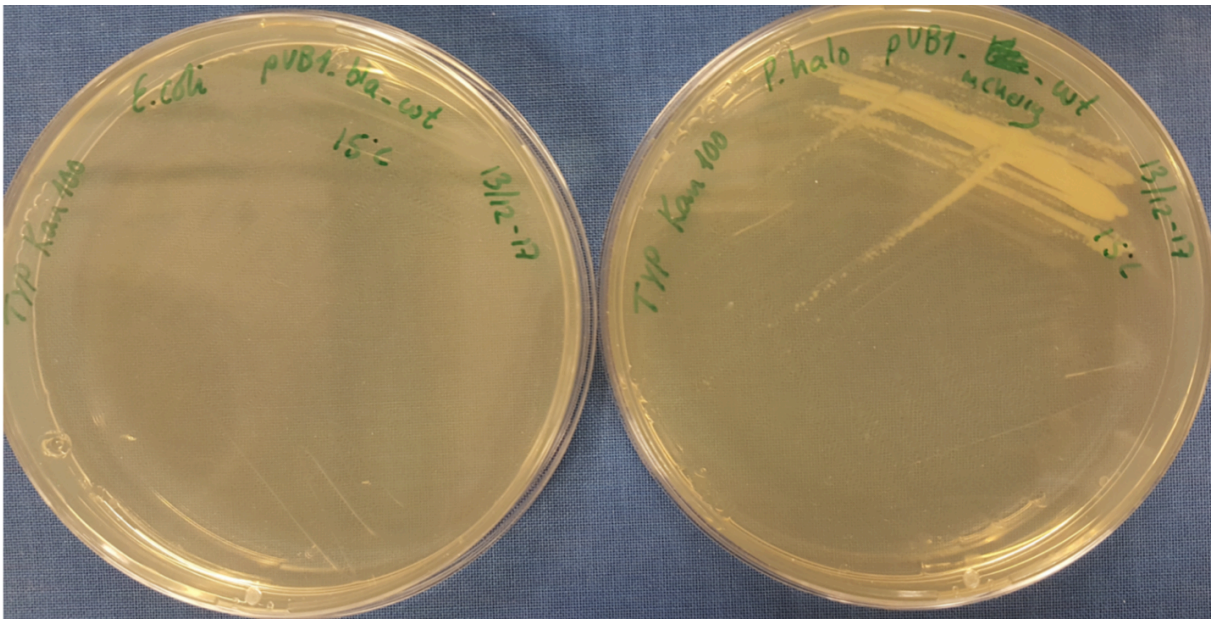

Figure 18: TYP+Kan100 plates streaked with *E. coli* pVB-1 bla-wt_Kan (left) and *P. haloplanktis* potential conjugant grown at 15 °C for 2 days.

E. coli S17-1 showed less growth than the potential conjugants after incubation at 15 °C for 2 days.

3.4.2 Test of Potential *P. haloplanktis* Conjugants for Production of mCherry

Reinoculum of potential conjugants and *E. coli* S17-1 mCherry-wt_Kan were grown in TYP+Kan200 (15 °C, 2 days). For each species, one induced and one uninduced tube was included. Cells from these cultures were spun down, and the results are presented in Figure 19.

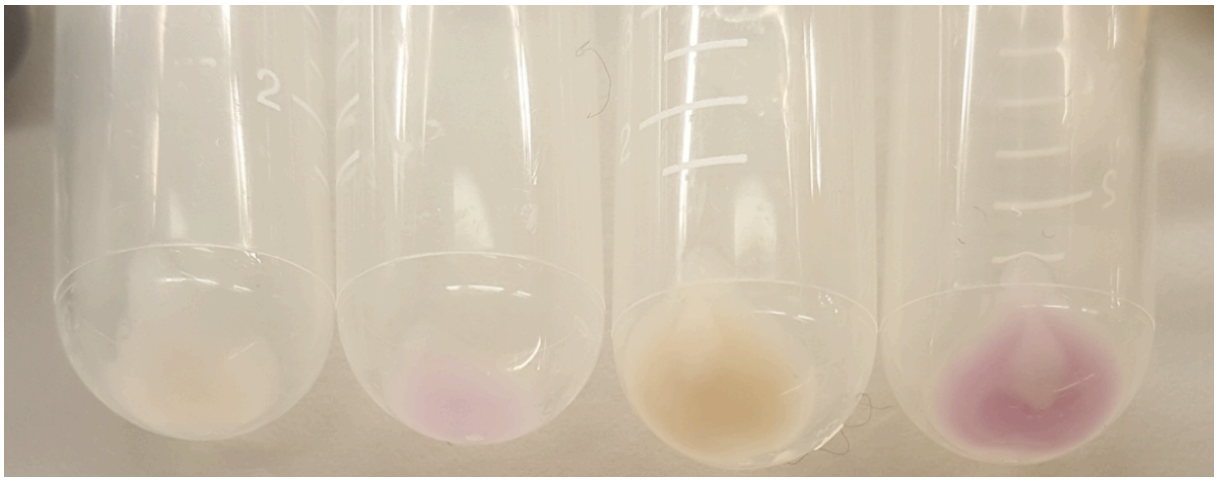

Figure 19: Test of mCherry expression in potential conjugants of *P. haloplanktis* grown for 2 days at 15 °C. Inducer: *m*-Toluate (2 mM). From the left: *E. coli* S17-1 mCherry-wt_Kan (uninduced); *E. coli* S17-1 mCherry-wt_Kan (induced); potential conjugants (uninduced); potential conjugants (induced).

The potential conjugants had more cell mass, and the cells had a stronger pink color, inducating more growth and more mCherry production in the potential conjugants.

3.4.3 Kovacs Test of Potential *P. haloplanktis* Conjugants

Since conjugation requires cell-cell contact, there is a chance that the baceria picked can be *E. coli* and not *P. haloplanktis*. A Kovacs test was conducted on potential *P. haloplanktis* conjugants to verify that the bacteria were oxidase positive. This is contradictory to *E. coli* which is oxidase negative. A color change would indicate oxidative positive bacteria in cell culture. Fresh cultures of *E.coli* DH5α, *P. haloplanktis* wt and potential conjugants were prepared. The OD of the three cell cultures were not measured, but by visual inspection it was observed that the culture of *P. haloplanktis* wt was more dense than the other two. The results are shown in Figure 20.

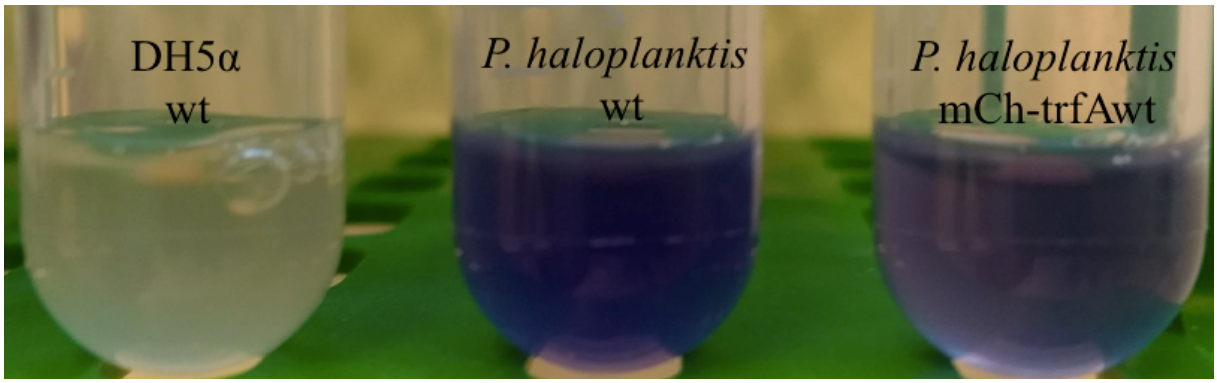

Figure 20: Kovacs test on *P. haloplanktis* potential conjugants (marked *P. haloplanktis* mCh-trfAwt) with *E. coli* DH5α and *P. haloplanktis* wt as controls. Picture taken after approx. 4 min reaction time.

Color change was observed after 1 min in *P. haloplanktis* wt, while *E. coli* DH5α wt showed no color change. Color change was observed after 3-4 min in potential conjugants. The upper time limit for color change for a true positive test is 3 minutes. *P. haloplanktis* potential conjugants changed thus color at, or above the upper limit of what is approved as a positive result. The color of potential conjugants was not as strong as in *P. haloplanktis* wt, but its cell culture did also have a lower OD. The color change was regarded inconclusive due to differences in OD600 and too long reaction time (> 3 min).

3.4.4 Test of Potential *P. haloplanktis* Conjugants for Growth at 37 °C

As the Kovacs test on potential conjugants of *P. haloplanktis* mCherry-wt_Kan showed inconclusive results, the cells were tested further. The growth experiment of *P. haloplanktis* wt presented in section 3.1.1 showed that the species could not grow on 37 °C. Therefore, ON cultures of potential conjugants were reinoculated in TYP+Kan200 in shake flasks and incubated ON at 37 °C to test if they could grow on 37 °C. *P. haloplanktis* wt was included as control. The result is presented in Figure 21.

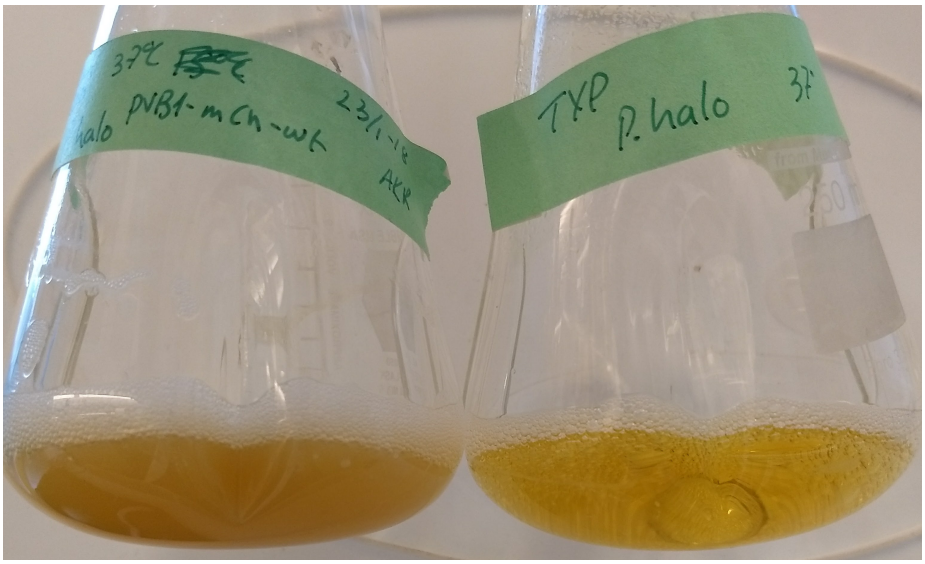

Figure 21: Potential conjugants of *P. haloplantkis* pVB-1 mCherry-wt_Kan (left) and *P. haloplantkis* wt (right) grown ON in TYP+Kan200 at 37 °C. Left: Kan200. Dense growth was observed to left, no growth (clear medium) was observed the right.

This experiment was performed twice, both times with the same results. What previously most likely was believed to be potential conjugants were able to grow on 37 °C, thus this could not this be *P. haloplanktis*. No further experiments were conducted on this strain.

3.5 Electroporation of *P. putida*

Electrocompentent *P. putida* cells were transformed with pVB-1 mCherry-wt_Kan, mCherry-271_Kan and mCherry-251_Kan via electroporation. The transformants + negative control were plated on LA+Kan50 after incubation with SOC (2 h, 30 °C), and after incubation ON (30 °C) there were many colonies on the transformant plate, and no colonies on the control plate. Colonies were picked, the cells grown in LB+Kan50 before plasmids were isolated and sent for sequencing. The sequencing results showed a successful integration of plasmids harboring all three mutants of *trfA*. The cultures of *P. putida* also had a different smell than the ones of *E. coli*.

3.5.1 Test for Production of mCherry

A small scale check for expression of mCherry was done for all three mutants, where *m*-Toluate (2 mM final concentration) was added to cultures with OD_{540} = 1 incubated ON (25 °C). This resulted in pink cultures for wt and 271 (results not shown). For 251, fluorescence was measured with LB and induced *P. putida* wt culture (no plasmid) as controls. The 251

mutant emitted a hundred-fold more fluorescence than the controls (results not shown). Thus, it was confirmed that *P. putida* with all three mutants of pVB-1 mCherry_Kan produced mCherry.

3.5.2 Kovacs Test

After confirmation of introduction of correct plasmid by sequencing and verified production of mCherry, a Kovacs test was performed to verify that the bacteria were *P. putida* (oxidase positive) and not for instance a contamination of *E. coli* (oxidase negative). Fresh cultures of *E. coli* DH5α wt, *P. putida* wt and *P. putida trfA*wt, *trfA*271 and *trfA*251 were grown to equal ODs and added Kovac's reagent. The result is presented in Figure 22.

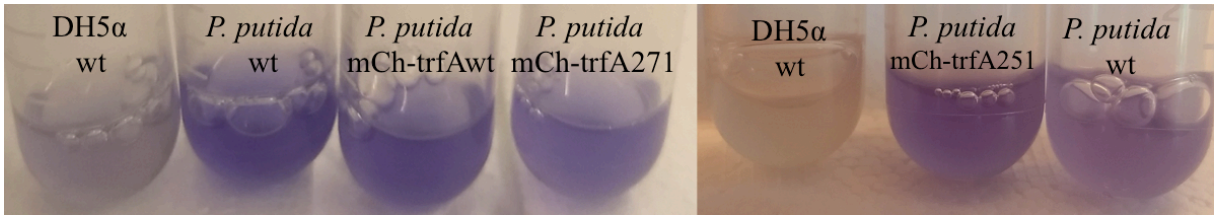

Figure 22: Kovacs test of *P. putida* transformants with *E. coli* DH5α wt and *P. putida* wt as controls. Picture is taken after 2 min (left picture) and 1 min (right picture) reaction time, with ODs of 0.4 and 1.0 respectively. As the *trfA*251 mutation was tested at a later time point, the ODs diverged from each other at the test point, and therefore are the controls included also in picture 2.

The cultures of *P. putida* wt and the potential transformants of *P. putida* turned blue within 1 minute. The DH5α wt culture in the test with *P. putida* wt and 271 showed a very weak colour change after ~2 min. This indicated that all *P. putida* transformants were oxidase positive.

3.6 Expression Study of mCherry in *E. coli* DH5α and *P. putida*

After successful integration of pVB-1 mCherry-trfA_Kan, mCherry-271_Kan and mCherry-251_Kan into *E. coli* DH5α and *P. putida* by heat shock and electroporation respectively, the expression levels of mCherry were investigated by performing an expression study. The aim was to quantify the mCherry production levels obtainable in both species for subsequent comparison of the species.

Production cultures were grown to OD ≈ 1 in 250 mL shake flasks (30 mL LB+Kan50, 30 °C) before induced to a final concentration of 2 mM m-Toluate and incubated ON (16.5 h, 25 °C). OD measurements were made at 600 nm for *E. coli* DH5α and at 540 nm for *P. putida*. For each species, the wt strains without plasmid were included as negative controls (induced and uninduced). The following day, the cells were harvested and lysed. The amount of lysis buffer used was doubled compared to the protocol (Vectron) to get enough supernatant for the measurements. The insoluble phase was resuspended in PBS and fluorescence was measured in soluble and insolube phase.

Fluorescence measured in E.coli and P.putida wt without plasmid was substracted from the measured values of fluorescence in wt, 271 and 251 (induced from induced, within every group respectively) to account for background fluorescence in medium, inducer and cells. The final, relative fluorescence units (rfu) for each mutant, induced and uninduced, with their respective uncertainties are presented in Figure 23 for *E.coli* and Figure 24 for *P. putida.*

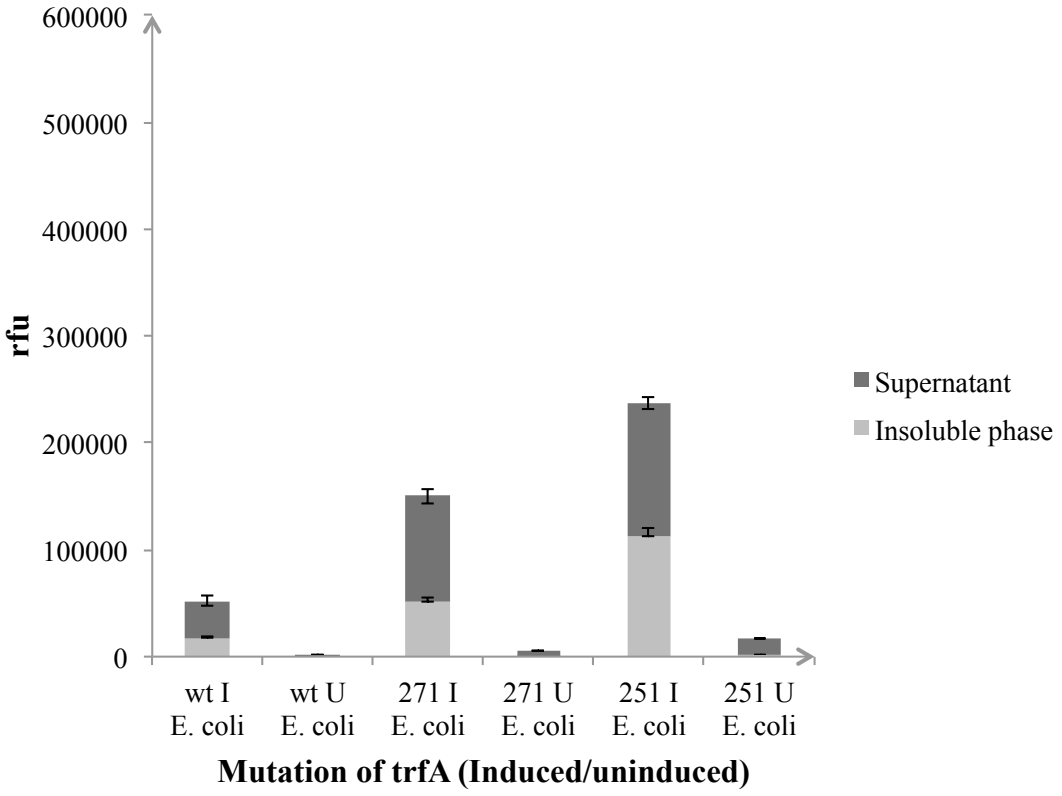

Figure 23: Relative fluorescence units (rfu) of mCherry measured in insoluble/insoluble phase of lysed cells of *E. coli* pVB-1 mCherry-wt_Kan, mCherry-271_Kan and mCherry-251_Kan (induced (I) and uninduced (U)) after 16 h incubation at 25 °C after induction. Error bars represent standard deviations.

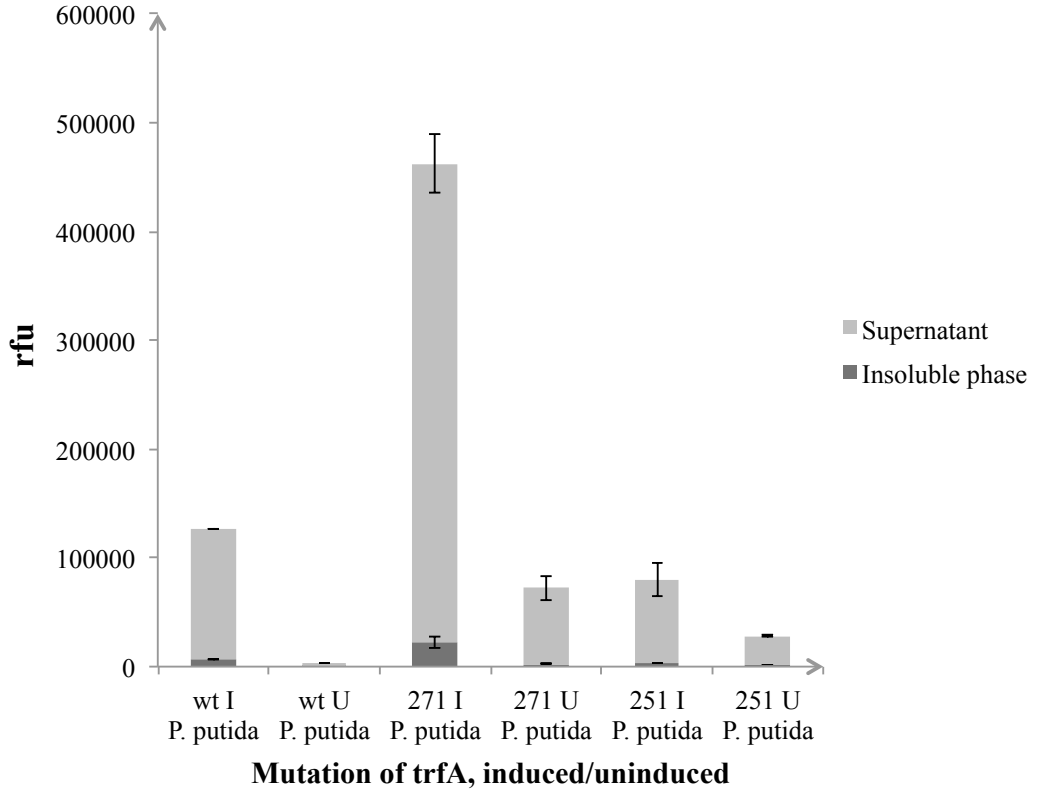

Figure 24: Relative fluorescence units (rfu) of mCherry measured in lysed cells of *P. putida* KT2440 pVB-1 mCherry-wt_Kan, mCherry-271_Kan and mCherry-251_Kan (induced (I) and uninduced (U)) after 16.5 h incubation at 25 °C after induction. Error bars represent standard deviations.

In *E. coli*, the expression levels increased with increasing copy numbers. This was not the case in *P. putida*. The wt mutant had a ~2.5 times higher expression level in *P. putida* than the in *E. coli*. The 271 mutant had a ~2 times higher expression level in *P. putida* than in *E. coli*. The 251 mutant on the other hand, had a ~3.5 times higher expression level in *E. coli* than in *P. putida*. The expression levels in the uninduced production cultures seemed to be higher in *P. putida*, compared to their respective induced production cultures. This indicates that the expression vector is more ”leaky” in *P. putida,* and has a higher background expression of mCherry than *E. coli*. The amount of protein in soluble phase compared to in insoluble phase is higher in *P. putida,* where a large overweight of the protein is found in the soluble phase.

3.6.1 Comparison of Fluorescence Measurements in Different Sample Preparations

For *E. coli,* fluorescence was also measured directly in bacterial culture and in cells resuspended in PBS. Lysing of cells is time consuming, and therefore it was desired to investigate if measuring fluorescent directly in production culture or on cells resuspended in

PBS could give realistic results with respect to the relative fluorescence in the different strains with different copy numbers.

The relative fluorescence units for each strain are presented in Figure 25.

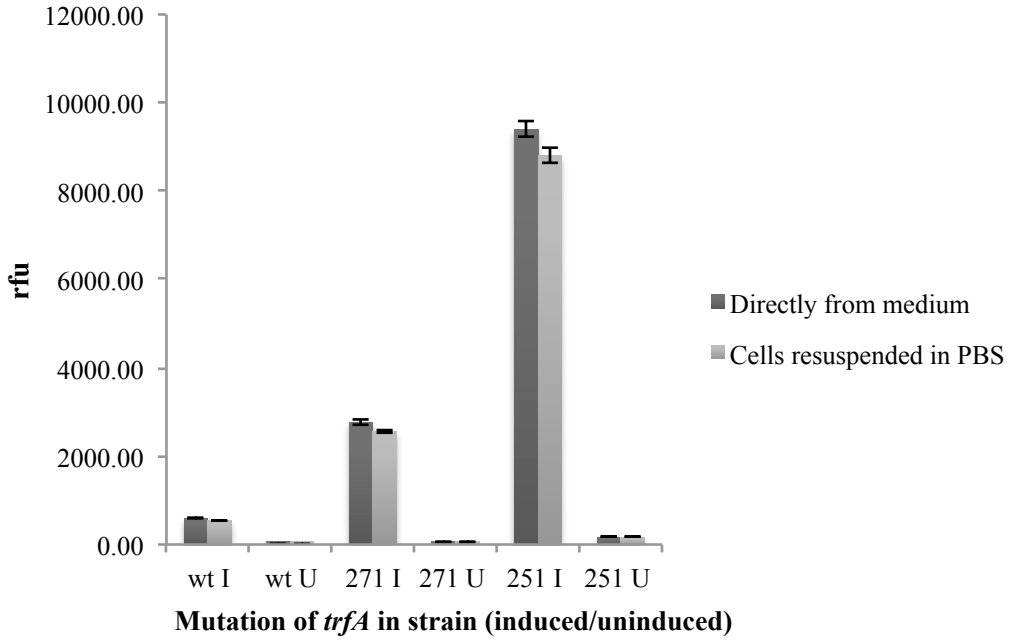

Figure 25: Relative fluorescence units (rfu) of mCherry measured in cells resuspended in PBS/ bacterial cultures of *E. coli* pVB-1 mCherry-wt_Kan, mCherry-271_Kan and mCherry-251_Kan (induced and uninduced) after 16 h incubation at 25 °C after induction. Fluorescence was measured in Tecan Infinite M200 PRO with exitation wavelength 580 nm and emission measured at 615 nm. Error bars represent standard deviations.

When adding up the fluorescence measured in the soluble and insoluble phase of lysed cells, this represents a "true" value for the relative amount of protein present in the cell. These values also say something about how much protein that is produced in cells harboring each of the three pVB-1 mutants with different copy numbers. It is of interest how much they produce compared to each other, and this can be calculated. The intensity of the fluorescence measured in cells harboring the 271 mutant divided by the intensity of the fluorescence measured in cells harboring the wt mutant, gives a measure on how much protein that is produced in the 271 strain compared to in the wt strain. Such ratios were calculated for 251/wt and 271/wt for the total fluorescence in the lysed cells, the fluorescence measured directly on cell cultures, and the fluorescence measured in cells resuspended in PBS for comparison. The ratios are presented in **Table 20**. Thus it could be seen whether measuring

fluorecence in unlysed cells could give a realistic measure on how much protein the strains produced compared to each other.

Table 20: Ratios between fluorescence measured in the different strains used in the expression study, for induced and uninduced cultures.

	Ratio	LB (bacterial culture)	PBS (resuspension of cells)	Lysed cells (total)
I	**271/wt**	5.0	4.7	2.9
U	**271/wt**	5.0	4.9	5.4
I	**251/wt**	12.5	16.1	4.6
U	**251/wt**	2.8	14.7	18.8

The ratios of fluorescence measured in the different strains in LB and PBS resuspension are quite similar for all strains. The ratios of the lysed cells do differ from the ratios found for unlysed cells, however. Based on the 271/wt ratio found for lysed cells, which is assumed to be the "true" value, almost three times more protein is produced in the 271 strain (induced) than in the wt strain (induced). According to the ratios found for unlysed cells, the 271 strain (induced) produce almost 5 times more protein than the wt strain (induced). Similarly, the 251/wt ratio indicates that the 251 strain (induced) produce 12-16 times more proteins than the wt strain (induced), while according to the ratios found for unlysed cells, the 251 strain (induced) produce 4.6 times more protein than the wt strain (induced). There is ergo a difference in ratios found in lysed an unlysed cells for induced cultures. For uninduced cultures, the ratios did not diverge as much for 271/wt, but for 251/wt they did.

3.6.2 Stain of Bacterial Cultures with *P. putida*, LB and *m*-Toluate

In the expression study, a brown/coppery colour was observed after ON incubation (25 °C) in all the shake flasks containing *P. putida*, LB and *m*-Toluate, including the control *P. putida* wt with no plasmid. No stain was observed in the uninduced shake flasks. The ON production cultures transferred to 50 mL tubes are shown in Figure 22 (cells were not spun down in the 251 cultures when the picture was taken).

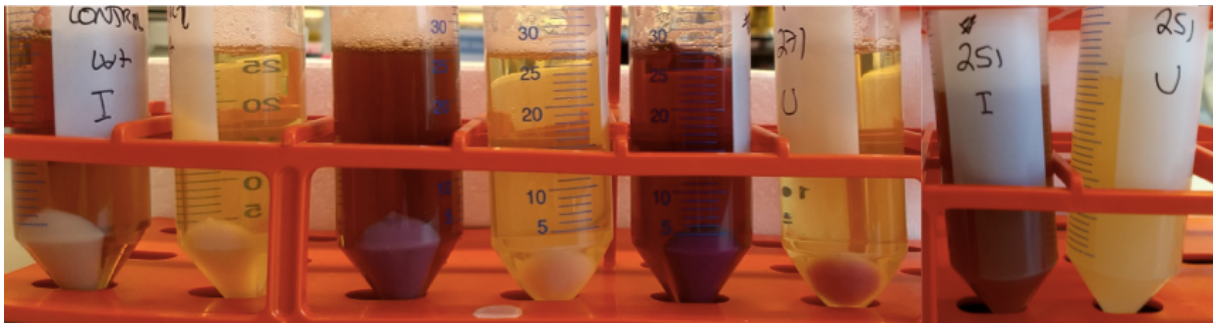

Figure 26: Cultures of P. putida grown ON in LB+Kan50. For each mutant, one induced (I) and one uninduced (U) culture is included. Inducer: *m*-Toluate (2mM). From the left: wt control (induced and uninduced); pVB-1 mCherry-wt_Kan (I and U); pVB-1mCherry-271_Kan (I and U); mCherry-251 (I and U – cells not spun down).

In the tube containing *P. putida* pVB-1 mCherry-271_Kan and inducer, a darker layer of precipitate with the same color as the stain in medium can be seen on top of the cells spun down. The same observation was made for all induced cultures. Fluorescence values measured in induced cultures of *P. putida* wt control (stained) and *E. coli* wt control (not stained) were both low compared to the fluorescence measured in the cultures of strains harboring the plasmid (induced and uninduced). This indicated that the stain did not affect the fluorescence measurements of mCherry.

4 Discussion

4.1 Cell Death caused by Toxic Concentrations of *m*-Toluate or Lowered pH in *E. coli* and *P. putida*

All vectors constructed in this study harbor the XylS/P*m* expression system which can be induced by *m*-Toluate. As the XylS/P*m* promoter system is dose-dependent up to a certain level (14), the expression level can be ”tuned” by using different concentrations of inducer. Therefore, it was of interest to see how the potential hosts for protein expression behaved in increasing concentrations of inducer. High concentrations of *m*-Toluate inside the cells have been reported to be toxic and cause cell death (48).

As *m*-Toluate was dissolved in absolute ethanol, a control with water and one with absolute ethanol were included to exclude that the ethanol would have an impact on the growth. High levels of ehanol are known to be toxic for the cells. The results showed that the growth was similar in wells with water and ethanol, isolating *m*-Toluate as the only variable potentially causing decreased growth in the experiment.

The graph presented Figure 16 shows that *E. coli* grew normal (compared to the two controls) up to 4 mM *m*-Toluate. At 4 mM, decreased growth rates were seen. From 6 mM and up to 20 mM *m*-Toluate, no growth of *E. coli* was detected. For *P. putida*, the results indicate that the cells are able to grow on concentrations of *m*-Toluate up to, and including 10 mM. This indicated that for *E. coli*, 4 mM of *m*-Toluate represent toxic levels for the cells leading to decreased cell growth, and that the inducer indeed is capable of permeating the cells. For *P. putida*, toxic levels of *m*-Toluate were reached at 15 mM according to the decreased growth, indicating either that the species is more tolerant for high levels of inducer, or that the inducer is not able to permeate the cells to a similar extent as in *E. coli*. The XylS/P*m* system origins from *P. putida,* and therefore it is assumed that the inducer is able to enter the cells. Hence is the increased tolerance for *m*-Toluate of *P. putida* a more likely explanation.

As *m*-Toluate is an organic acid with pKa = 4.27 possible effect on pH were also investigated. *P. putida* was no longer able to grow at 15 mM *m*-Toluate. Here, the pH was 4.92. Thus, it

cannot be know whether it is the decreased pH or toxic levels of *m*-Tuloic acid that affected growth of *P. putida*. For *E. coli*, the cells started dying at 6 mM *m*-Toluate. Here, the pH was somewhere between 6.58 and 5.60, which is a less dramatic drop in pH. Therefore, it could be assumed that pH is not the reason for decreased growth of *E. coli*.

The cultivation of bacteria in a deep well plate allows for a large-scale experiment, however, with many replicates, but some challenges were met using 96 well plates for measuring the OD. The variation among the three replicates were large, as could be seen from the standard deviations. This could have affected the results.

Due to time constraints, the experiment was not conducted for *P. haloplanktis*, but should be performed in future work to supplement the background studies on the species.

4.2 Successful Electroporation of *P. putida* with Low, Medium and High Copy Number Plasmids

There have been problems introducing pVB-1 derived vectors harboring the 271 mutant of *trfA* (medium copy number) to *P. putida* in Vectron. Large-scale attempts have been made, using different promoters and inserts, but without successful integration and/or stable maintenance of the plasmid (Vectron Unpublished). One case of successful integration has been reported, however, resulting in functional production (43). To our knowledge, no attempts to integrate a plasmid with the 251 *trfA* mutant has been reported. It has also been attempted to establish minimal RK2 replicons with copy up mutants in *Pseodomonas aeruginosa*, but without success (22).

Haugan 1994 tested the tolerance for copy-up mutants of RK2in several different species, and it was reported that for some species, even the wt copy number of RK2 caused a to big burden for the cells to grow. It was postulated that there is a specific upper tolerance level for copy number of the plasmid in every species before cell death is initiated. According to their experience, this was believed to apply for all Gram negative bacteria (22). A high copy number plasmid is reported to cause a metabolic burden for the cell, which can lead to plasmid instability, and hence decreased growth or lead to cell death (10).

In this work, *P. putida* was successfully electroporated with the pVB-1 mCherry-wt_Kan, mCherry-271_Kan and mCherry-251_Kan. Successful integration was confirmed by sequencing of plasmids, verified production of mCherry and positive and conclusive results of the Kovacs test. Several factors could have contributed to the successful integration of all three mutations. One factor is the size. The pVB-1 plasmids in this study were almost 1 kb smaller than the ones previously attempted to transfer into *P. putida* by Vectron. This could have made uptake of the vector into the cell easier. The smaller size also constitute a smaller metabolic burden for the cells, because less resources are needed to make new plasmids (7). Furthermore, the vectors used in this study encoded Kan^r, contradictoty to the vectors used previously in Vectron which encoded Amp^r. As *P. putida* is naturally resistant to Amp (39), very high concentrations of Amp were used to distinguish the transformants from wt *P. putida*, and this might have constituted a burden for the cells, resulting in poor growth of transformants (Vectron Unpublished).

Uptake and stable maintenance of vectors can also be insert-dependent. If the vector harbors a protein of interest that toxic for the cell, the cells will not be able to grow. Since the plasmids were successfully integrated, the *mCherry* insert is believed not to affect the cells negatively. This is consistent with literature reporting that no interference with biological activities have been reported for mCherry in *E. coli* and *B. subtilis*, even for high concentrations of the protein (56).

The motivation for introducing a copy-up mutant in this strain was to investigate whether the increased copy number could lead to higher expression levels of recombinant proteins. This relationship has been observed in *E. coli* (10). *trfA* wt mutants of the pVB-1 plasmid have previously been introduced to *P. putida* with success, but the levels of protein produced from the low-copy plasmid were lower than in *E. coli* with higher copy number. The establishment a vector with medium copy number able to integrate and maintain stably in P.putida did therefore ensure continued interest in the species as a potential host for recombinant protein production in Vectron, more specific in the BIA project.

4.3 Evaluation of *P. putida* as a Host for Protein Production

In the expression study performed on *E. coli* and *P. putida* harboring pVB-1 mCherry-wt/271/251_Kan, three aspects were investigated. First, the difference in expression levels

caused by copy number of the plasmid. Next, the effect of different hosts on expression levels was investigated and finally, the solubility of mCherry was compared between the species.

In *E. coli*, previous studies have reported a correlation between copy number and levels of protein expression (10). This was consistent with the findings in *E.coli* DH5α this study, as the measured fluorescence increased from pVB-1 harboring wt via 271 to 251 mutation of *trfA*. For *P.putida*, this pattern was seen from wt to 271, but not for the 251 mutant of *trfA*. A plausible reason for the decreased expression from this mutant is that the high copy number of the plasmid made up a considerable metabolic burden for the cell, and hence few extra resources for protein production were available (7). Some protein production was detected, however, confirming the functionality of the gene and protein.

The total amount of mCherry produced in the two species differed. Measurements based on the total mCherry content in the cells showed that the 271 mutant in *P. putida* produced 3-fold more protein than the 271 mutant in *E. coli*, and a 2-fold more protein than from the 251 mutant in *E. coli*. Regarding the solubility of the protein, it was found that most of the protein in *P. putida* was found in the soluble phase. This was not the case in *E. coli*, where the amount of soluble/insoluble protein was almost 50/50. When producing proteins for industrial purposes, it is crucial to have a high fraction of the proteins in the soluble phase to increase the availability of the protein and ensure the proteins are active (10). Increased solubility of proteins is one of the reasons for the ongoing research to find alternative hosts to *E. coli* (57). mCherry is in general a protein with high solubility (28), but the results indicate nonetheless that the produced protein is more soluble in *P. putida* than in *E. coli* under the conditions tested. The increased solubility properties could potentially be transferred to other proteins when exchanging *mCherry* with e.g. the gene encoding proinsulin, but this will have to be investigated.

The high expression levels of soluble protein obtained from the medium copy number muntant, combined with the versatility of this bacteria makes *P. putida* a promising potential production strain for heterologous proteins.

4.4 Comparison of Methods for Measuring Fluorescence

The fluorescence of induced *E. coli* cells harboring pVB-1 mCherry-wt_Kan, mCherry-271_Kan and mCherry-251_Kan was measured using different methods. One way of finding relative fluorescent units is to harvest and lyse the cells, before the total fluorescnce is measured in both supernatant and insoluble phase (cell debris). This procedure is time consuming, and therefore it was investigated if measurements of fluorescence could be made directly on production culture or on harvested cells resuspended in buffer, and still give the same reliability on the measurements. Measuring fluorescence in unlysed cells also make measurements of protein levels *in vivo* possible.

Firstly, the results showed that the fluorescence measured in bacterial culture and cells resuspended in PBS was fairly similar. This indicated that a very small fraction of mCherry was secreted to the medium in *E. coli*. Also, it indicated that the LB did not affect the measurements on unlysed cells. Next, when comparing the measured fluorescence in unlysed and lysed cells, the relationship between the different mutants is not consistent. ”Lysed cells” correspond to the total amount of protein measured in the soluble and insoluble phase together.

The measurements on unlysed cells did, however, say something about which of the strains that produced more protein than the others. The usefulness of the method will depend on how sensitive the measurements should be. In this specific experiment, it was desired to measure the accurate expression levels of mCherry for use in a comparison between species. It was also of interest to see how much of the protein that was found in the soluble phase versus the insoluble phase of the cell, since this is of importance in the evaluation of the strains as protein production hosts. Therefore was the direct measurements not suited for use in this experiment, but can be useful for a quick check of relative expression levels in other experiments.

4.5 Color Change of Bacterial Cultures of *P. putida* grown in LB and added *m*-Toluate

The production cultures of *P. putida* in LB added *m*-Toluate aquired a copper/brown color during the experiment. It was a concern that this color could affect the measurements of mCherry due to the similar in color, and hence give non-conclusive results about amount of

protein produced in the cultures. Stain was only observed in *P. putida* induced cultures, while *E. coli* DH5α remained unaffected. The color change occured also in the *P. putida* wt control production culture. The measured fluorescence values in induced cultures of *E .coli* wt (no plasmid) and *P. putida* wt (no plasmid) were however of the same quantity, and therefore it was concluded that the stain did not emit fluorescence light that disturbed the measurements of mCherry in the expression study.

Little is known about the nature of the color. When harvesting cells by centrifugation the medium was still stained, thus it was assumed that a part of the color compound was soluble. A layer of copper/brown precipitate was also observed as a layer on top of the cell pellet, indicating that some of the stain was present in an insoluble form. As the color change occured also in the *P. putida* wt control production culture, the stain was unrelated to mCherry production, or to presence of the pVB-1 plasmid. Derived from the inducer diffusion study, the only difference between the induced and uniduced cultures were the presence of 2 mM *m*-Toluate and a lowered pH in the medium. Therefore, the stain must derive from the presence of inducer, lowered pH or both. No journals reporting similar observations for *P. putida* and *m*-Toluate were found, so only speculations can be made about the nature of the formation of this stain. As the TOL plasmid origins from *P. putida*, there is a chance that *m*-Toluate could serve additional functions in the cell, and might induce genes in the genomic DNA of *P.putida* causing this color change.

4.6 Evaluation of Growth and Cultivation of *P. haloplanktis*

Growth characteristics of *P. haloplanktis* were investigated in detail in this study, with respect to temperature, antibiotic sensitivity and methods for reviving from freezer. Understanding the nature of a potential host organism for protein production is crucial to be able to exploit its full potential, as well as encounter potential challenges.

4.6.1 A Standardized Method for Reviving of *P. haloplanktis* from Freezer was Established

Different bacterial species can have different preferences for long-time storage at -80 °C, and some species are hard to revive from the frozen state. *P. haloplanktis* showed inconsistencies in growth when revived from freezer in ON cultures. This made the species hard to work with, and the need for a systematic study to establish a standardized, predictable way of

reviving it was desired. Therefore, reviving *P. haloplanktis* in flasks, tubes and plates were tested on 15 and 25 °C for glycerol stocks made with cells harvested at OD= 1, 5 and 13. For OD=13, the culture was grown ON and harvested the next day.

Regarding temperature, the bacteria revived faster at 25 °C than at 15 °C in both flasks and on plates. This was consistent with the findings in the growth study presented in section 3.1.1 regarding the general growth rate of *P. haloplanktis*, showing an optimal growth temperature at 25 °C. The cultures in tubes grown at 25 °C grew faster than at 15 °C after 12 hours, but from 24 hours no notable difference in growth between the temperatures could be seen.

Among the conditions tested, the optimal density of cells for making glycerol stock to obtain fast revival turned out to be OD_{600}=5. According to the growth study conducted in this thesis, cultures at OD_{600}=5 are in the exponential phase, and since the cell densities are higher than for OD=1. Cells harvested after ON incubation (OD_{600}=13) are comparably in the stationary phase, and hence the cells would probably need some time to adjust to new medium before returning to the exponential phase (5). This, or that a part of the cells in the ON culture are dead can explain the faster revival from stocks with OD_{600}=5 in flasks and tubes. For plates, the cells from glycerol stocks with OD=5 revived at the same time as OD=13 at 25 °C. All glycerol stocks used in this experiment had, however, been in the freezer only for about a week. It would be interesting to investigate whether there is a difference in reviving cells from stocks stocks that are several years old versus newly made freezer stocks.

Regarding growth methods, the growth differed a lot in tubes and flasks. In tubes, the OD of the cultures did not pass 3, even after 48 hours. This was contradictory to reviving in flasks, where the ODs became ~25 within 12 hours, once the cells entered the exponential phase. This might be explained by the availability of oxygen for the bacteria grown in flaks, as the baffles and shaking allow for a higher oxygen transfer coefficient (kLa) in the medium (58). The ater/air surface is also larger in flasks than in tubes, allowing more air to mix with medium. kLA denotes the liquid phase oxygen concentration at saturation at any time. Reviving in tubes was nonetheless considered reliable, as all 18 tubes showed growth within 24 hours.

Summarized, cell culures to be used for glycerol stocks should be harvested at a higher OD within the range of exponential phase, like OD=5 when fast growth is desired. The preferred

temperature for reviving was in general 25 °C. Throughout this project, *P. haloplanktis* was commonly revived in flasks, but according to this study, tubes can also be a good alternative. This could save media, space in incubators, and the method also proved to be reliable. However, if higher amounts of cell mass are required, or a fast revival is needed, flasks should be used.

4.6.2 Evaluation of Growth of *P. haloplanktis* at Different Temperatures

The growth study of *P. haloplanktis* showed that the shortest generation times were obtained at 25 °C, followed by 20 °C, 30 °C and 15 °C respectively. The optimum temperature of *P. haloplaktis* has been stated to be 20 °C (34), but it is in this study concluded that the optimum temperature is 25 °C for growth in TYP in baffled shake flasks. *P. haloplanktis* is known to be a psychrophilic bacterium, so the fast growth at 30 °C which is considered a mesophilic temperature was not expected. This result is however promising for further work with this species in the lab. The equipment in most labs are directed towards work with mesophilic bacteria, and the versatility of this bacterium to grow at both mesophilic and psychrophilic temperatures increases its applicability.

P. haloplanktis was in addition able to grow at 5 °C and 10 °C, and the cultures reached high ODs after 25 hours. This is consistent with other studies performed on *P. haloplanktis* TAC125 at low temperatures, stating that *P. haloplanktis* can reach high ODs even at 0 °C (33). Due to limitations in equipment in the lab, it was not possible to test for growth at 0 °C in this work. The feature of reaching very high cell densities and relatively fast growth at low temperatures is remarkable. Since decreased temperatures has shown to facilitate correct folding (31), this is promising for *P. haloplanktis* as potential expression host for psychrophilic enzymes and hard-to-express heterologous proteins from mesophilic hosts.

Finally, no growth was detected at 37 °C. This information can be used to distinguish between *P. haloplanktis* and *E. coli*.

4.6.3 *P. haloplanktis* is Resistant to Kanamycin

To test whether *P. haloplanktis* had a natural resistance to antibiotics commonly used in molecular biology, a small scale screening experiment was performed. *P. haloplanktis* was shown to be sensitive to Ery, Amp, Apr and Cm, but not Kan. There was a special interest in

the Kan resistance because the core vecors (pVB-1 bla-wt_Kan, bla-271_Kan and bla-251_Kan) contained a Kan resistance marker. It was therefore crucial to evaluate if these vectors could be used in *P. haloplanktis*, or if the selection marker in the plasmids had to be changed. Therefore, the limit for growth of *P. haloplanktis* on medium containing Kan was investigated closer.

The detected limit for growth of *P. haloplanktis* on Kan was 75 µg/mL in liquid TYP medium. This is consistent with the literature (34), where *P. haloplanktis* showed growth on Kan after 2 days. In Wang (2015), *P.haloplanktis* TAC125 also showed some resistance to Apr, although less than for Kan. This is inconsistent with the findings in this study. However, only one replicate was used in the initial screening, and the experiment should be repeated with more replicates.

4.7 pVB-1 mCherry-wt_Kan Would Not Integrate in *P. haloplanktis*

In an early phase of this work, the first constructs pVB-1 mCherry-wt_Kan and bla-wt_Kan were attempted conjugated into P.haolplanktis via the F^+ *E. coli* S17-1 strain. Later, the work of constructing vectors more optimal for *P. haloplanktis* was started. The diluted cell mass with S17-1 and *P.haloplanktis* was first plated on TYP+Kan50. The antibiotic experiments showed that *P. haloplanktis* to some extent could grow on Kan50 in liquid medium. It is believed that the growth of *P. haloplanktis* wt without the plasmid would be limited, but not eliminated by the Kan concentration in the agar. There was a difference in the sizes of the colonies, however, and the larger colonies were assumed to be transformants harboring the plasmid due to the increased resistance to Kan. When picking conjugants for incubation in medium, TYP+Kan200 was used. Based on the antibiotic study, all wt *P. haloplanktis* cells without the plasmid were expected to die in this concentration of Kan.

From the test of growth of S17-1 and *P. haloplanktis* potential conjugants on TYP agar at 15 °C for 2 days, the potential conjugants grew well while *E. coli* showed poor growth. Thus it was believed that growing the bacteria at 15 °C was a decent way of distinguishing the bacteria. Single colonies were picked plate TYP+Kan200 agar plate grown for two days at 15 °C, and incubated ON in TYP+Kan200 to make glycerol stocks. Single colonies origin from one single cell, and thus is the genetic material in all the cells of this colony the same. For the subsequent tests for expression of mCherry with *E. coli* as control, it was clear that the

amount of growth in the potential conjugants was higher, and it also produced mCherry, verifying that the bacterium contained the plasmid. In shake flasks, *P. haloplanktis* reached highed cell densities than *E. coli*, but as shown in the revive from freezer experiment, this was not the case in tubes. Therefore is the higher growth in potential conjugants not assumed to be an indication of this being *P. haloplanktis*. The subsequent Kovacs test showed somewhat inconclusive reults, since the color change happened gradually around 3 minutes, and also because the ODs of the samples were different. The faster growth on plate at 15 °C and the positive Kovas test indicated that the conjugants were *P. haloplanktis* harboring the desired vector. The final growth test at 37 °C showed that the potential conjugants were able to grow at this temperature, while the wt of *P. haloplanktis* could not. No element in the pVB-1 plasmid has, to our knowledge, the ability to make *P. haloplanktis* grow on such high temperatures. Thus, the potential conjugants were concluded not to be *P. haloplanktis,* but rather *E.coli* S17-1 used as a donor for the pVB-1 plasmid. No categorical conclusions could be drawn about what happened in this experiment, but it is likely that the growth of *E. coli* S17-1 at 15 °C was underestimated. The signs point towards that it was a mix of *E. coli* and *P. haloplanktis,* but since the glycerol stock was made from one single colony from an agar plate, this could not be the case.

Previous attempts to conjugate *P. haloplanktis* with a pJB658/RK2 derived mutant has been done (39). Here, the plasmid used was pVB-1A0B1-mCherry with the 271 mutant of *trfA* giving a medium copy number and *bla* was the anitibiotic resistance marker. This vector was thought to be successfully integrated in *P. haloplanktis* because of positive results of the Kovacs test. No test on 37 °C or test for production of mCherry was conducted on these cells due to trouble with reviving them from freezer. In this study, it can not be excluded that the transformants picked were a mixture of *E. coli* and *P. haloplanktis,* or simply wt *P. haloplanktis.* This is due to the nature of the Amp^r. β-lactamase is secreted to the medium, and by growth of large colonies the cells can produce enough β-lactamase for other bacteria in the immediate proximity to be able to grow (5).

Since the constructed pVB-1 vectors would not integrate in *P. haloplanktis* in our work, a new design of the vector was planned. The Kan resistance marker was decided to be exchanged with a Chloramphenicol resistance marker, based on the antibiotic sensitivity tests in this study, and the work of (34). Based on this work where no *P. haloplanktis* could be verifies, it was decided to clone in the autonomously replicating sequence, ARS, described in

the introduction, because studies showed that mesophilic plasmid systems harboring this element was stably maintained in *P. haloplanktis* (59).

4.8 Evaluation of *P. haloplanktis* as a Host for Protein Production

Unfortunately, there was not time to complete the construction of pVB-1 mCherry-ARS-wt_Cm and subsequently conjugate *P. haloplanktis* with the plasmid. The Cm resistance marker, the ARS element and a modified protocol (growing potential conjugants on 4 °C to exclude growth of the mesophilic *E. coli,* suggested in (35)), were all factors likely to increase the chances of success. A concern could be potential trouble with inculpabilities of the pVB-1 plasmids and the pMtBL plasmid naturally present in *P. haloplanktis*. This needs to be assessed if further trouble with integrating the plasmid into the host are observed.

Summarized, a fundament has been built for further work with *P. haloplanktis* as a potential host for recombinant protein production. Further work needs to be done before psychrophilic enzymes can be produced in high levels in this species, but motivation for continued work is found in the enormous economical potential demonstrated by *P. haloplanktis*' remarkable adaptations to cold environments and fast growth. In other words, there is no reason to terminate the work with this bacterium.

5 Suggestions for Further Work

For *P. putida*, the next natural step would be to exchange the *mCherry* gene with a gene encoding proinsulin, or another commercial protein. This could be done to check whether the plasmids can still be integrated in the species with a different insert, as well as investigate if the solubility properties of the protein and the high-level expression could be maintained for other recombiant proteins. Next, the exact copy numbers of the pVB-1 mCherry-wt/271/251_Cm plasmids could be explored in both *E.coli* and *P.putida* using real time PCR. Further, *E. coli* BL21 be transformed with the pVB-1 plasmids with subsequent expression studies for a better comparison of production levels. This is due to that BL21 is a better protein production strain than of *E. coli* DH5α. A long term goal could be to assess the secretion properties of this species with potential advances to simplify downstream processing of proteins produced.

For *P. haloplanktis* the next step would be to finish construction of pVB-1 mCherry-ARS-wt_Cm and then repeat the conjugation with both the plasmid with and without the ARS element. A small change in the procedure involving growing potential conjugants at 4 °C should be considered, to better distinguish *P. haloplanktis* from *E. coli* S17-1. Also other methods to quickly distinguish the two species should be included, e.g. qPCR of genomic DNA. Immediate growth tests of the potential conjugants on 37 °C should also be conducted. A suggestion is to pick several potential conjugants, make a master plate as when setting up a test cutting for ligation, and ”screen” the colonies by ON culvivation at 37 °C to check for presence of *E. coli*. The inducer diffusion studies should also be performed to supplement the background studies on the species, but this time with adjusted pH. Next, almost all of the measurements used to characterize growth of *P. haloplankits* were OD-measurements. It would strengthen the results from these studies to do a cell count on plates for cell cultures at different ODs, to see how many viable cells different ODs represent. Finally, the growth experiment on 5 °C 10 °C should be repeated with more replicates to get reliable quantifications of the generation times at these temperatures, as these are the most interesting temperatures for future expression of protein in *P. haloplanktis*.

6 Conclusions

In this project, essential elements of Vectron's expression system were used to attempt constructing vectors that could stably maintain and produce high levels of highly soluble protein in *P. putida*. The pBJ658/RK2 minimal replicon based modified pVB-1 vector with low, medium and high copy numbers were successfully integrated in *P. putida*. High levels of soluble mCherry were produced in *P. putida* harboring the pVB-1 mCherry-271_Kan vector (medium copy number). These levels were approximately twofold higher than the highest levels achieved (from the 251 mutant) in *E. coli* DH5α. *P. putida* transformed with pVB-1 mCherry-wt_Kan and mCherry-251_Kan (low and high copy number) did also produce mCherry, but in lower quantities. These results make *P. putida* a promising candidate for future production of other proteins, in higher quantities and in a more soluble form than in *E. coli*.

For *P. haloplanktis*, the species can achieve high cell densities in a remarkable temperature range from 5-30 °C. An attempt to conjugate the bacteria with pVB-1 mCherry-wt_Kan was made, but without success due to the potential conjugants ability to grow on 37 °C, indicating that it was *E. coli* and not *P. haloplanktis*, as the latter is not able to grow at 37 °C. Some difficulties were met regarding distinguishing potential conjugants of *P. haloplanktis* from *E. coli* S17-1 (donor strain).

Construction of a new vector harboring an autonomously replicating sequence, ARS, originally from *P. haloplanktis* was started, in addition to an exchange of the resistance marker to obtain Cm^r instead of Kan^r. Due to time limitations, the construction work was not completed, and no attempts to conjugate *P. haloplanktis* with this vector were made. Despite the lack of successful establishment of a functional expression vector in *P. haloplanktis*, a fundament for further work has been made in this project.

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Appendices

A. Buffers and solutions

Standard procedure for autoclaving is 20 min at 121 °C. Where storing conditions are not specified, buffers and solutions were stored at room temperature.

A.1 Growth Media

Lysogeny Broth (LB), cultivation of *E. coli* and *P. putida*

10.0 g Tryptone (OXOID)

5.0 g Yeast Extract (OXOID)

5.0 g NaCl (VWR)

Up to 1 L dH_2O

Autoclaved.

LB Agar (LA): growth of *E. coli* and *P. putida*

15 g Bacterial Agar (Oxoid) to 1 L LB medium*

Autoclaved, poured in petri dishes (approx. 30 mL per plate) and stored at 4 °C.

*If a selective antibiotic was to be added, the agar was cooled to under 50 °C before mixing in the antibiotic and pouring the plates.

TYP Medium, cultivation of *P. haloplanktis*

16.0 g Tryptone (Oxoid)

16.0 g Yeast extract (Oxoid)

10.0 g Sea salt (Sigma)

Up to 1 L dH_2O

pH adjusted to 7.5 with 1 M NaOH before autoclaving.

TYP Agar, growth of *P. haloplanktis*

15 g Bacterial Agar (Oxoid) to 1 L TYP medium (adjusted to pH = 7.5)*

Autoclaved, plates poured (approx. 30 mL agar per plate) and stored at 4 °C.

*If a selective antibiotic was to be added, the agar was cooled to under 50 °C before mixing in the antibiotic and pouring the plates.

Psi Medium, preparation of supercompetent *E. coli* cells

5.0 g Yeast extract (Oxoid)

20.0 g Tryptone (Oxoid)

10.24 g $MgSO_4 \cdot 7H_2O$ (VWR)

Up to 1 L dH_2O

pH adjusted to 7.6 with KOH before autoclaving.

Super Optimal Broth (SOB) medium, transformation of bacteria

5 g Yeast extract (Oxoid)

20 g Tryptone (Oxoid)

0.584 NaCl (VWR)

0.186 KCl (Merck)

2.92 $MgSO_4$ 7•H_2O (VWR)

Up to 1 L dH_2O

Autoclaved.

Super Optimal Catabolite-repression (SOC) Medium, heat shock of *E. coli*

100 mL SOB medium

2 mL 20 % glucose solution (VWR) (filter sterilized)

Autoclaved, aliquoted in 13 mL tubes and stored at -20 °C.

A.2 Other Solutions

TFB1 Buffer, supercompetent *E. coli* cells

0.588 g KAc (Merck)

2.42 g RbCl (Aros Organics)

0.389 g $CaCl_2 \cdot 2H_2O$ (Merck)

3.146 g $MnCl_2 \cdot 4H_2O$ (J. T. Baker)

30 mL Glycerol (99.5 %) (VWR)

Up to 200 mL with dH_2O

pH adjusted to 5.8 using *diluted* C_2H_5OH (0.1 M). Sterilized by filtration.

TFB2 Buffer, supercompetent *E. coli* cells

0.21 g Morpholinopropanesulfonique (MOPS) (Fisher Bioreagents)

1.1 g $CaCl_2 \cdot 2H_2O$ (Merck)

0.121 g RbCl (Acros Organics)

15 mL Glycerol (99.5 %) (VWR)

Up to 100 mL dH_2O

pH adjusted to 6.5 with diluted NaOH. Sterilized by filtration.

50 % Glycerol, for making bacterial glycerol stocks

50 mL Glycerol absolute (99.9 %) (VWR)

50 mL dH_2O

Autoclaved.

10 % Glycerol, preparation of electrocompetent *P. putida* cells

10 mL sterile Glycerol (50 %) (VWR)

90 mL sterilized dH_2O

Phosphate Buffered Saline (PBS), mCherry expression study

8 g NaCl (VWR)

0.2 g KCl (pro)

1.44 g Na_2HPO_4 (Merck)

0.24 g KH_2PO_4 (Merck)

Autoclaved.

Lysis buffer, mCherry expression study

5 mL Cellytic B (Sigma)

1.0 µL Benzoase Nuclease (Aldrich)

Prepared just before use and kept cold.

1.8 M Stock of *m*-toluate (also referred to as *m*-Toluic acid), inducer

2.451 g m-Toluate (Aldrich)

Up to 10 mL Ethanol absolute (VWR)

Sterile filtered and stored at -20 °C.

Diluted Solutions of m-Toluate, inducer diffusion study

The diluted solutions of *m*-toulate with varying concentrations used in the inducer diffusion study were made using the 1.8 M stock solution of *m*-Toluate. Solutions were made so that 20 µL stock added to 1.2 mL bacterial culture would give a final concentration of 0-20 mM *m*-Toluate. Compositions of the solutions are presented in **Feil! Finner ikke referansekilden.**.

Table 1: Preparation of *m*-Toluate diluted solutions with concentrations from 0-20 used in inducer diffusion study. *Desired final concentration* refers to the final concentration of *m*-Toluate in 1.2 mL bacterial culture used in the study, when 20 µL diluted solution was added.

Stock concentration (M)*	**Desired final concentration (mM)**	**Volume of 1,8 stock (uL)**	**Volume of ethanol (uL)**
0	0	0.0	1000.0
0.06	1	33.3	966.7
0.12	2	66.7	933.3
0.24	4	133.3	866.7
0.36	6	200.0	800.0
0.48	8	266.7	733.3
0.6	10	333.3	666.7
0.9	15	500.0	500.0
1.2	20	666.7	333.3

Stored at -20 °C.

Kovacs Reagent, Kovacs test

0.05 g Kovacs reagent (N',N',N',N'-Tetramethyl-p-phenyl-enediamine dihydrochloride) (Sigma-Aldrich)

Up to 5 mL dH_20

Covered in aluminium foil due to light sensitivity. Prepared just before use.

50xTris-acetate-EDTA (TAE), stock buffer for gel electrophoresis

242 g Tris-base (Tris(hydroxymethyl)aminomethane) (Sigma)

57.1 mL Acetic acic (100 %) (VWR)

100 mL 0.5 M EDTA (pH 8) (VWR)

Up to 1 L dH_2O

Autoclave.

1xTAE, buffer for gel electrophoresis

400 mL 50xTAE

19.6 L dH_2O

0.8 % Agarose, gel electrophoresis

3.2 g Agarose (Lonza)

400 mL 1xTAE

Boiled in microwave until the agarose is dissolved. Added 20 µL GelRed TM Nucleic Acid Gel Stain, 10,000X in water (Biotium) or GelGreen TM Nucleic Acid Gel Stain, 10,000X in water (Biotium). Stored at 60 °C.

X-Gal, blue/white screening

100 mg 5-bromo-4-chloro-3- indolyl-β-d-galactoside

Up to 2 mL N',N'-dimethyl-formamide

Covered with aluminum foil and stored at -20 °C.

IPTG stock solution (0.1 M), blue/white screening

1.2 g IPTG

Up to 50 mL dH2O

Filter sterilize and store at 4 °C.

A.3 Antibiotics

20 mg/mL Chloramphenicol stock

0.2 g Chloramphenicol (Sigma)

Up to 10 mL Ethanol absolute (99.9 %) (VWR)

Sterile filtrated, aliquoted in Eppendorf tubes (1 mL per) and stored at -20 °C.

50 mg/mL Kanamycin stock

0.5 g Kanamycin sulfate (BioChemica PanReac, AppliChem ITW Reagents)

Up to 10 mL dH_2O

Sterile filtrated, aliquoted in Eppendorf tubes (1 mL per) and stored at -20 °C..

100 mg/mL Ampicilin stock

1.0 g Ampicilin sodium salt (BioChemica PanReac, AppliChem ITW Reagents)

Up to 10 mL dH_2O

Sterile filtrated, aliquoted in Eppendorf tubes (1 mL per) and stored at -20 °C..

50 mg/mL Erythromycin stock

0.5 g Erythromycin (Sigma)

Up to 10 mL dH_2O

Sterile filtrated, aliquoted in Eppendorf tubes (1 mL per tube), and stored at -20 °C.

100 mg/mL Apramycin stock

1.0 g Apramycin sulphate salt (Sigma)

Up to 10 mL dH_2O

Sterile filtrated, aliquoted in Eppendorf tubes (1 mL per tube), and stored at -20 °C.

B. Plasmid Maps

Several plasmids were constructed in this study. The plasmid maps are presented here in figure 1-4.

Core vectors:

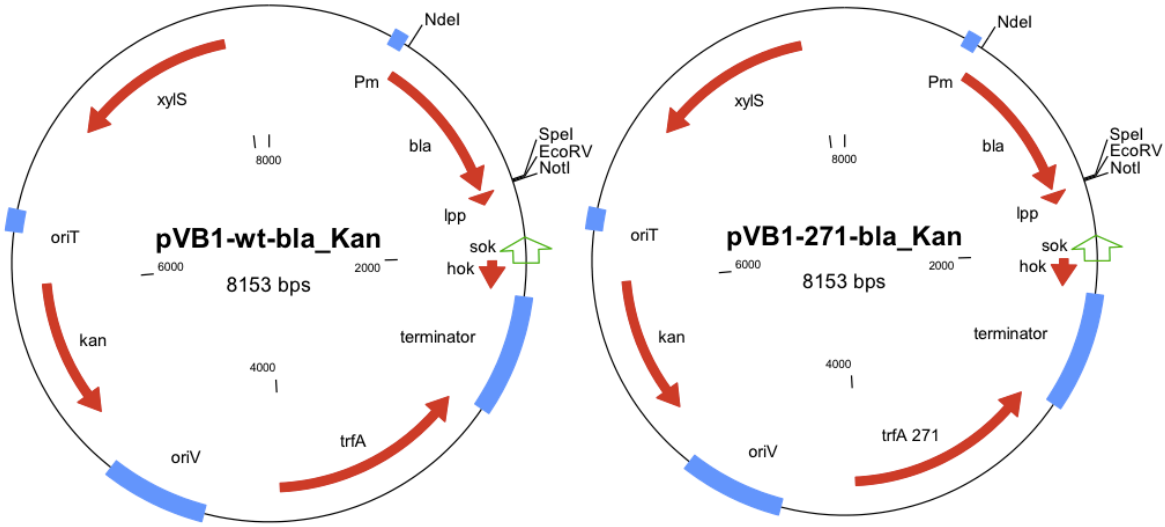

Figure 1: pVB-1 bla-wt_Kan and pVB-1 bla-271_Kan, referred to as ”core vectors” used as a starting point for all plasmids constructed for use as expression vectors in this study.

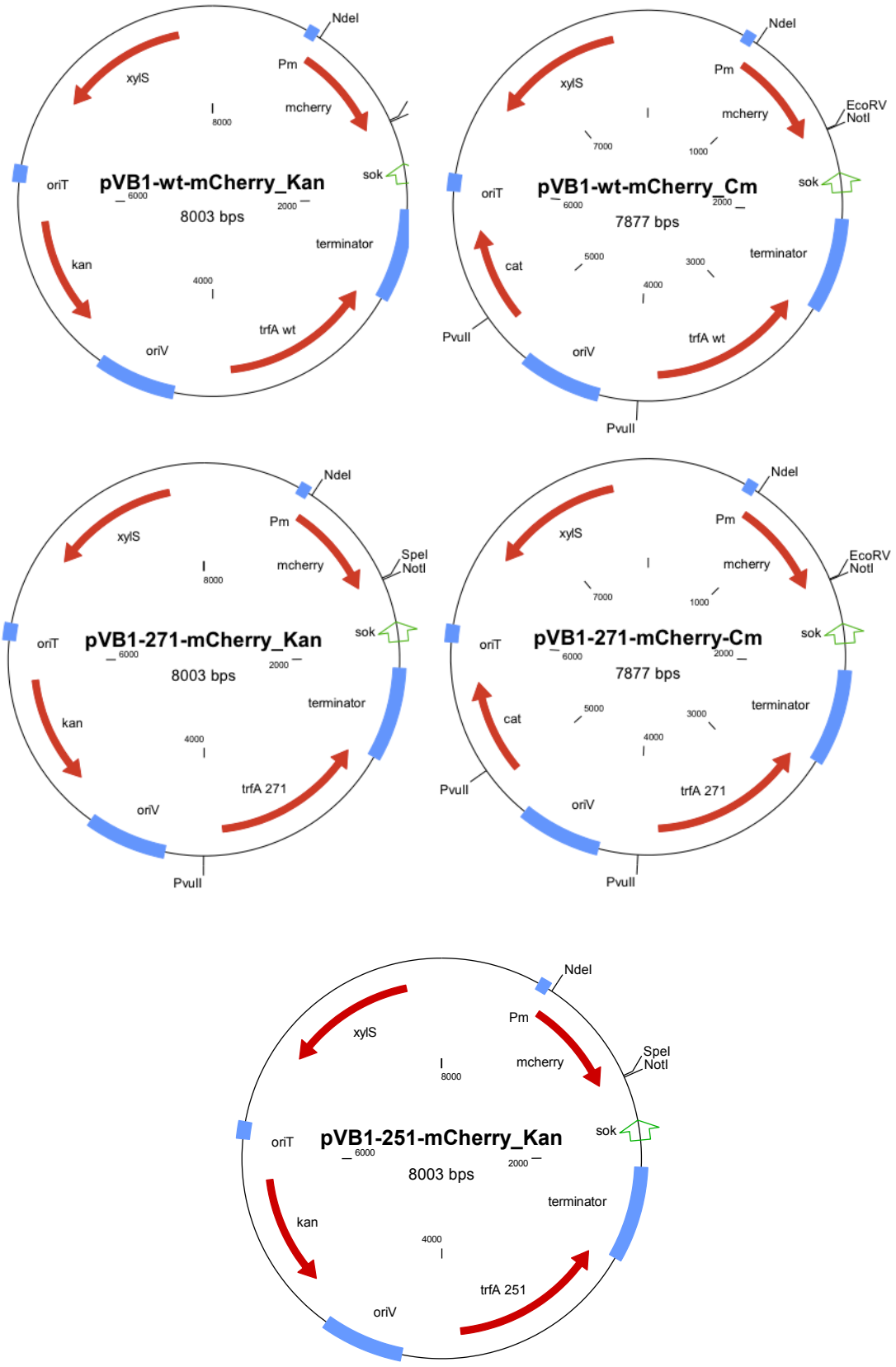

Figure 2: Plasmid maps for pVB-1 vectors harboring the *mCherry* gene and Kan (left and bottom) and Cm (right) resistance markers with different mutations of *trfA*. Size of the plasmid is indicated inside the ring.

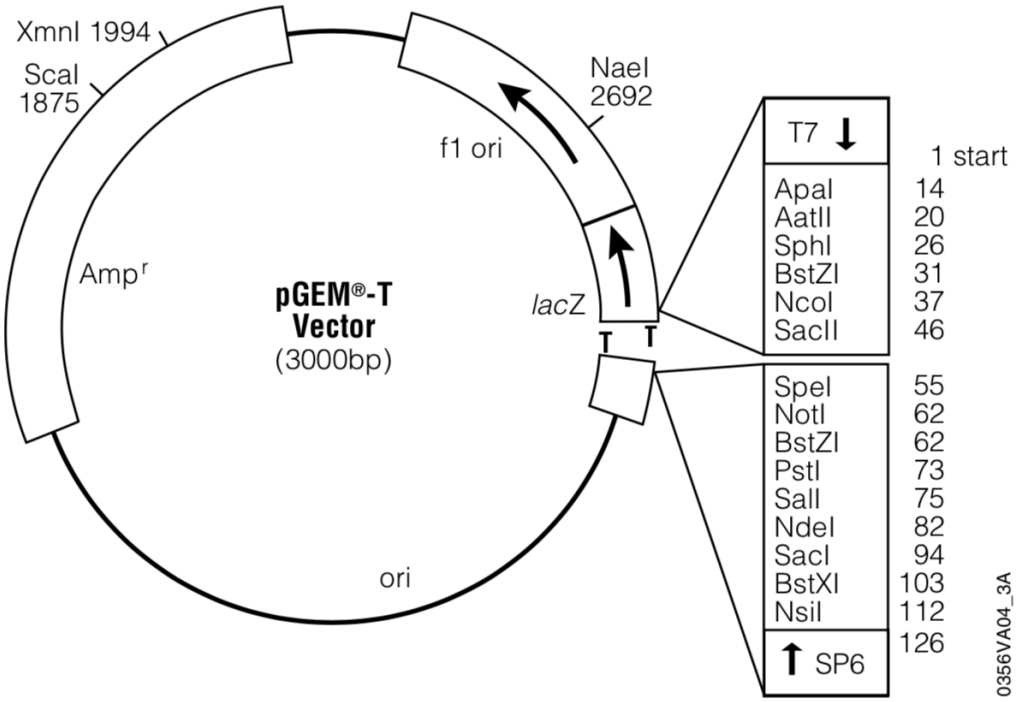

Figure 3: pGEM®-T vector map and sequence reference points. Map obtained from (47).

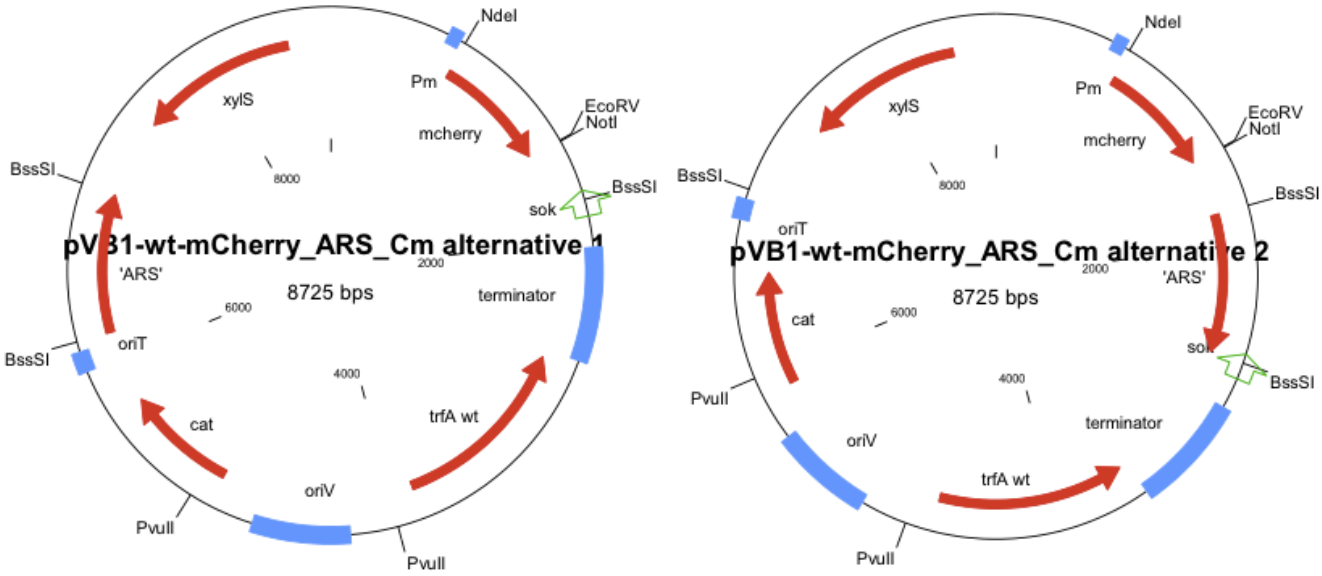

Figure 4: Plasmid maps for pVB-1 mCherry-ARS_Cm showing two possible insertions of ARS in the pVB-1 backbone in separate orientations.

C. Primer Sequences Table

Table 2: Overview of primers used in this study and their purpose.

Number	Primer	Sequence (5'-3')	Purpose
1	mCherry-fw (NdeI)	TTTTCATATGGTTTCT AAAGGTGA	Construct pVB-1-mCh-wt/271_Kan
2	mCherry rev (SpeI)	TTTTACTAGTTCATT TATACAGTTCGTC	
3	Vf-pVB1-mCher	AACTCTTCCTTTTTCAATATTAT TGAAGC	Amplification of backbones to construct pVB-1-mCh-wt/271_Cm
4	Vr-pVB1-mCher	TCTGTCAGACCAAGTTTACTCAT	
5	1f-pBBR1-o(Vec)	TGAGTAAACTTGGTCTGACAGA TTAATGAATCGGCCAACG	Amplification of insert (*cat*) to construct pVB-1-mCh-wt/271_Cm
6	1r-pBBR1-o(Vec)	GCTTCAATAATATTGAAAAAGG AAGAGTTATGGAGAAAAAAATC ACTGGATA	
7	Vf-pVB1-bla-Kan	TCACCCTCCTTGCGGGAT	Amplification of backbones to construct pVB-1 mCherry-251_Kan and mCherry-251_Cm
8	Vr-pVB1-bla-Kan	GGCCTTGTGGGGTCAGTT	
9	1f-pVB1-cop271	CTGACCCCACAAGGCCCTAGCG TTTGCAATGCAC	Amplification of insert (*cop251M*) to construct pVB-1 mCherry-251_Kan and mCherry-251_Cm
10	1r-pVB1-cop271	CCCGCAAGGAGGGTGAATGAAT CGGACGTTTGAC	
11	ARS-f	GCTATAGTTTTAACTGTAAGACT	Amplification of ARS from pMtBL (making adenosine overhangs) to construct pGEM®-T-ARS
12	ARS-r	TTATTTAACATAAAAACCGTTAT CG	

13	Vf-pVB1-mCher	CTGGGAAAACCCTGGCGTTACC	Amplification of backbones to construct pVB-1 ARS-mCherry-trfA_Cm 6225, 6166
14	Vr-pVB1-mCher	TCACGACGTTGTAAAACGACGG	
15	1f-pGEMT-ARS_-o(Vec)	GTCGTTTTACAACGTCGTGAGCTATAGTTTTAACTGTAAGACTC	Amplification of insert (ARS) from pGEM®-T-ARS to construct pVB-1 ARS-mCherry-trfA_Cm
16	1r-pGEMT-ARS_-o(Vec)	CGCCAGGGTTTTCCCAGTTATTTAACATAAAAACCGTTATCG	

D. Calculating the Generation Time of *P. haloplanktis*

The measured OD600 of *P. haloplanktis* was plotted as a function of time in a semi-logaritmic plot in Excel, and the growth rate was found by use of Equation 1:

$$\mu = \frac{lnOD_2 - lnOD_1}{t_1 - t_2}$$

The generation time can then be found by using the growth rate, μ, as described in Equation 2:

$$g = \frac{ln2}{\mu}$$

Example for growth of P. haloplanktis at 25 °C:

$$\mu = \frac{\ln(12.83) - \ln(0.17)}{5.5\ h - 0\ h} = 0.79\ h^{-1}$$

Generation time:

$$g = \frac{ln2}{0.79\ h^{-1}} = 0.88\ h$$

E. Molecular Weight Standards Used in Agarose Gel Electrophoresis

Molecular weight standards (DNA ladders) were used to determine the sizes of DNA fragments separated by agarose gel electrophoresis. These ladders contain nucleic acids with fragments of known molecular sizes, and the sizes of unknown DNA fragments can be found by running the ladders next to the samples on the gel. The ladders used in this study were 1 kb Plus Invitrogen DNA Ladder (Thermofisher) and 1 kb DNA Ladder (NEB) and are presented in **Feil! Finner ikke referansekilden.** and **Feil! Finner ikke referansekilden.** respectively.

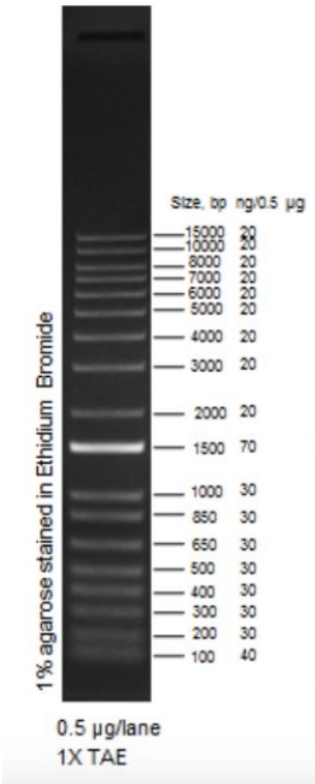

Figure 5: 1 kb Plus DNA Ladder (ThermoFisher) run on 1 % agarose in 1xTAE. Stained with Ethidium Bromide and visualized under UV light.

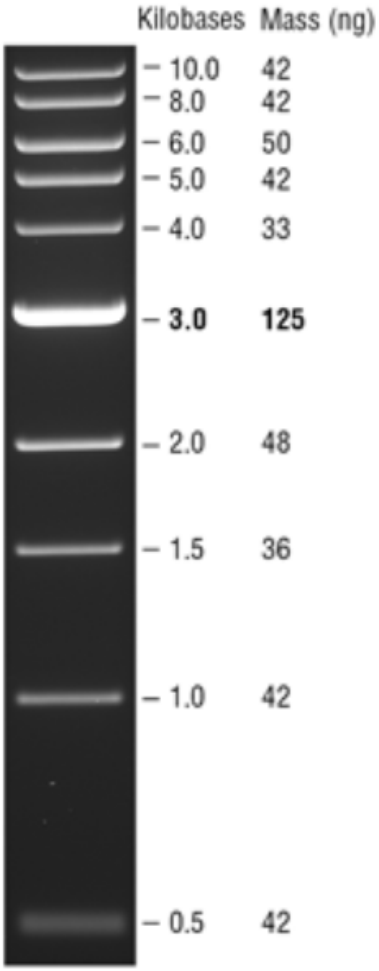

Figure 6: 1 kb DNA Ladder (NEB) run on 0.8 % agarose in 1xTAE. Stained by GelRed and visualized under UV light.

F. Cloning trouble

Some issues with the cloning work were met through the year. The core vectors (referring to pVB-1 bla-wt_Kan, bla-271_Kan and bla-251_Kan) were the starting point for all clonings. In the first attempt to clone mCherry into the core vectors to obtain mCherry-trfA_Kan, pVB-1 trfA_Kan plasmids without the *bla* gene were used instead due to referral to incorrect location in freezer. This was discovered after 4 weeks with several unsuccessful cloning attempts, by test cutting with different restriction enzymes. The next problem was discovered after several months, when the pVB-1 mCherry-wt_Kan, mCherry-271_Kan and mCherry-251_Kan were thought to be successfully costructed. Successful insertion of mCherry was confirmed by sequencing, but only the mCherry gene with transitions to the vector was sequenced. A subsequent expression study was performed in *E. coli*, with results that indicated similar expression levels in wt and 271, and higher levels in 251. This was contradictory to other results with similar vectors in *E. coli*. Thus was the *trfA* gene also sequenced, and it was discovered that wt=wt, 271=wt and 251=271. It was assumed that a mistake was done when picking bacteria from freezer to make ON cultures to start the cloning experiment, and the cloning work started again, in parallel with the construction of the pVB-1 mCherry-trfA_Cm plasmids. After a few more months, overcoming cloning trouble by using different cloning strategies, the 251 mutant was constructed. The restriction test cut showed correct sizes of the fragments. Due to time limitations, an expression study was performed in *E. coli*, the mutants were electroporated into *P. putida*, and a new expression study was performed for this species before the sequencing results came back. Sequencing showed that the *trfA* mutant was still 271, not 251. Thus, there was a mistake not only in my own stocks, but also in Vectrons stocks. Then, the cop251 had to be collected from a different vector to make pVB-1 mCherry-251_Kan, and the cloning work started again to make pVB-1 mCherry-251_Kan and pVB-1 mCherry-251_Cm.

Many of the experiments planned in this thesis required successful contruction of vectors. With several setbacks and a lot of time spent on working on the wrong plasmids, the time left to do other experiments was rather limited. The problems could have been avoided by sequencing the whole plasmid in th every beginning to verify that the start point was correct.

G. Settings of Tecan for Measuring Fluorescence of mCherry

Fluorescence of the protein mCherry produced by *E. coli* and *P. putida* in the expression study conducted in this work was mesured in a fluorometer. The instrument used was a Tecan Infinite M200 PRO. The 96 well plates used were 96F Nunclon Delta Black Microwell SI (Thermo Scientific). The fluorescence was measured with the following settings:

Plate: [NUN96fb] – ThermoFisher Scientific-Nunclon 96 Flat Back
Exitation: 580 nm
Emission: 615 nm
Number of flashes: 25
Mode: Top
Z-Position: Manual, 20000 µm
Gain: Manual, 80
Multiple Reads per well, Type Square (filled), Size 2x2, Border 750 mm
Integration time: 20 µs

H. Growth experiment of *P. haloplanktis* at 20 °C and 25 °C

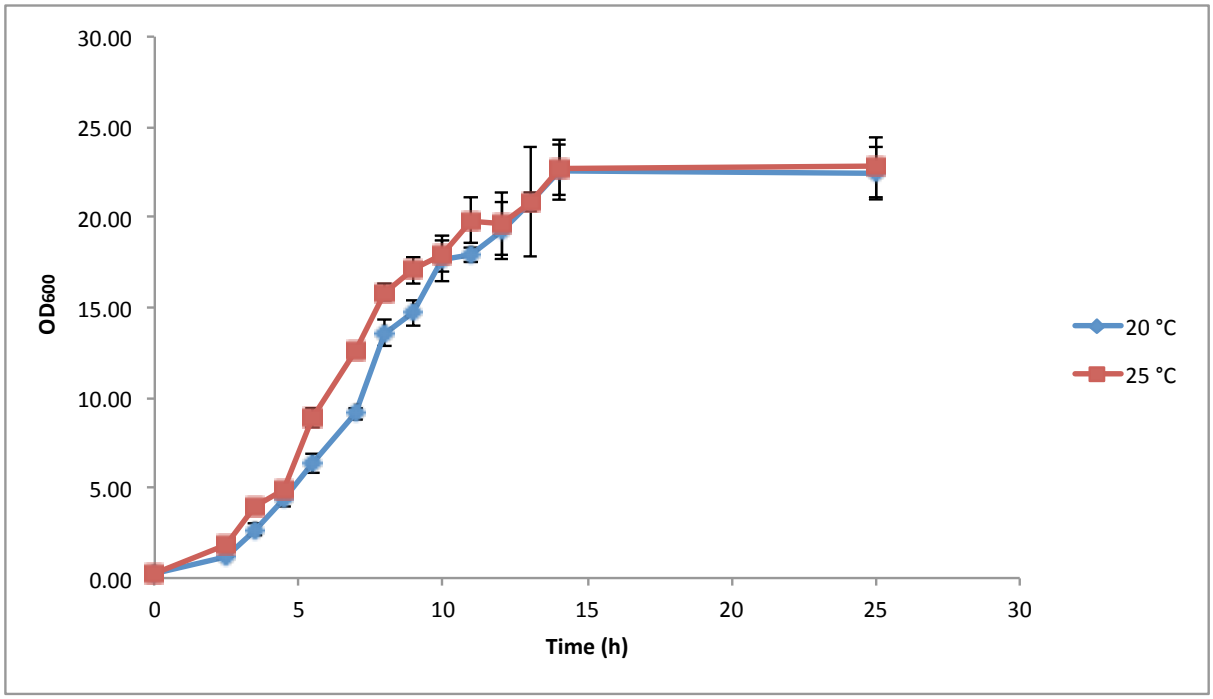

Figure 7: Mean OD_{600} of *P. haloplanktis* grown at 20 °C and 25 °C for 25 hours in baffles shake flasks (225 rpm). n=3. Error bars represent standard deviations.