Anne Sofie Krakeli

Improving expression from the XylS / *Pm* promoter in *Corynebacterium glutamicum*

Master's thesis in Biotechnology (5 year)

Supervisor: Trygve Brautaset, Jostein Malmo, Pieter van Kuilenburg

May 2019

Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

NTNU
Kunnskap for en bedre verden

Anne Sofie Krakeli

Improving expression from the XylS / *Pm* promoter in *Corynebacterium glutamicum*

Master's thesis in Biotechnology (5 year)
Supervisor: Trygve Brautaset, Jostein Malmo, Pieter van Kuilenburg
May 2019

Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

NTNU
Kunnskap for en bedre verden

Preface

This thesis concludes by degree in Master of Science 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), Dr. Jostein Malmo (Vectron Biosolutions AS) and Pieter van Kuilenburg (Vectron Biosolutions AS).

Acknowledgements

I would like to thank Vectron for giving me the opportunity to work on this project and the employees at Vectron for always helping me whenever I needed it and for including me in their social environment. My supervisors at Vectron, Dr. Jostein Malmo and Pieter van Kuilenburg deserve special thanks for helping me both in the lab and in the writing process, your input and support has been much appreciated. I would also like to thank my friends, family and fellow students for their love and support, for sharing my enthusiasm and for cheering me up whenever I needed it. A special thanks to my good friend Camilla for helping me when mCherry was not my friend and for always looking on the bright side of things, and to my roommate Karoline for her endless support and care.

Abstract

Through recombinant DNA technology, genes can be transferred between species and expressed in a non-native host. Recombinant proteins are used for various purposes in industries ranging from food and textile to biotherapeutics. As the number of applications utilizing recombinant proteins is growing, the need for novel and improved expression systems increases. For years, the Gram-negative *Escherichia coli* (*E. coli*) has been the preferred prokaryotic host for heterologous protein expression. However, due to its limited secretion capabilities that complicates and production of endotoxins that can raise safety concerns, the attention has been drawn to the Gram-positive species, such as *Corynebacterium glutamicum* (*C. glutamicum*). In recent years, *C. glutamicum* has been reported as a promising host for heterologous protein expression. However, compared to the well-established host *E. coli*, the number of suitable expression vectors and systems are severely limited. Therefore, the aim of this thesis was to establish use of the well-recognized XylS / *Pm* expression system in *C. glutamicum*.

In this study, the purpose was to identify bottlenecks limiting the expression levels from the XylS / *Pm* expression system. First, *C. glutamicum* codon-optimization of common reporter genes (mCherry and GFP) was compared to *E. coli* codon-optimized variants to determine if use of suboptimal codons limited the expression levels observed. Results showed that *E. coli* codon-optimized mCherry resulted in the highest yield of functional protein. Next, expression levels of the positive regulator XylS was evaluated at protein level. Despite successful, although weak, detection of mCherry from XylS / *Pm* expression vectors, no XylS was detected in *C. glutamicum*. The expression of mCherry from XylS / *Pm* was independent on induction (leak expression), which corroborates with the lack of detecting the positive regulator XylS. Furthermore, when comparing expression from a vector based on XylS / *Pm* with an *E. coli* / *C. glutamicum* shuttle vector, results indicated that the level of transcripts is limiting the protein production levels. Together, the results suggest that transcript levels should be increased by enabling expression of XylS to improve performance of the XylS / *Pm* expression system in *C. glutamicum*. This was attempted by changing the existing *xylS* promoters to *C. glutamicum* native promoters. However, despite several attempts, the cloning work needed to generate the constructs failed and was not finished.

Sammendrag

Ved hjelp av rekombinant DNA-teknologi kan gener overføres mellom arter og uttrykkes i andre arter enn opphavsarten. Rekombinante proteinr brukes til en rekke formål i ulike industrier, fra mat- og tekstilindustri til terapeutisk industri. Nye bruksområder for rekombinante proteiner oppdages stadig, noe som fører til et økt behov for nye og forbedrede ekspresjonssystemer. I de siste årene har den Gram-negative *Escherichia coli* (*E. coli*) vært industriens foretrukne prokaryote vert for heterolog proteinproduksjon. Da *E. coli* har begrenset sekresjonskapasitet, som kompliserer nedstrøms proteinrensing, og produserer endotoksiner, som potensielt kan være en helsemessig utfordring, har fokuset blitt flyttet til de Gram-positive artene, som *Corynebacterium glutamicum* (*C. glutamicum*). I de senere årene har *C. glutamicum* vist seg som en lovende vert for heterolog proteinproduksjon, men til sammenligning med den veletablerte *E. coli*, er antallet egnede ekspresjonsvektorer og systemer svært begrenset. Intensjonen med denne avhandlingen er derfor å etablere det velkjente XylS / *Pm* ekspresjonssystemet i *C. glutamicum.*

Formålet med studien var å identifisere mulige flaskehalser som begrenser ekspresjonsnivået fra XylS */Pm* ekspresjonssystemet. Først ble *C. glutamicum* kodonoptimaliserte versjoner av velkjente reportergener (mCherry og GFP) sammenlignet med varianter kodonoptimalisert for *E. coli* for å undersøke om bruken av suboptimale kodon begrenset de observerte ekspresjonsnivåene. Resultatene viste at den *E. coli*-kodonoptimaliserte versjonen av mCherry ga det høyeste utbytte av funksjonelle proteiner. Deretter ble ekspresjonsnivået av den positive regulatoren XylS evaluert på proteinnivå. Til tross for vellykket, om enn svak, deteksjon av mCherry fra XylS /*Pm* ekpresjonsvektorer, ble XylS ikke detektert i *C. glutamicum*. Ekspresjonen av mCherry fra XylS / *Pm* var uavhengig av induksjon, noe som samsvarer med den manglende deteksjonen av den positive regulatoren XylS. Da ekspresjonen fra den XylS/*Pm*-baserte vektoren ble sammenlignet med en annen skyttelvektor for *E. coli* / *C. glutamicum*, tydet resultatene på at transkripsjonsnivået begrenset produksjonsnivået av proteinet. Samlet tydet resultatene på at transkripsjonsnivået burde øke dersom XylS fikk muligheten til å fremme ekspresjon fra XylS / *Pm* -systemet i *C. glutamicum*. Dette ble forsøkt ved å erstatte de eksisterende promotorene for *xylS* med promotorer fra *C. glutamicum* selv. Til tross for flere forsøk fungerte ikke kloningen og den ble dermed ikke fullført.

Table of contents

1 Introduction

1.1 Systems for heterologous protein production

By using recombinant DNA technology, scientists can transfer DNA from one species into another and express the gene and the encoded recombinant protein in a heterologous host. The market for protein expression is fast growing, with an estimated value of $2,851 million in 2022 compared to $1,654 in 2017 [1]. Recombinant proteins are used in a number of industries, ranging from food and textile to biotherapeutics [2]. The growing market is in constant need for novel and improved expression systems as the applications of recombinant proteins continuous to expand, especially for therapeutic purposes [3-5]. Systems for production of recombinant proteins are mainly divided into two groups, prokaryotic and eukaryotic. For the purpose of industrial protein production, both economical and quality aspects must be considered when choosing a host system. Compared to eukaryotic cells, bacterial cells are easier to genetically manipulate and is generally cheaper to cultivate giving high yields of recombinant proteins. However, prokaryotes are not always able to produce the protein in a functional form as they lack essential mechanisms for post-translational modifications that are found in eukaryotes. The use of prokaryotic systems for heterologous protein production also poses a risk of toxic by-products, this is especially a problem when the protein product is intended for therapeutic purposes [6].

Among the prokaryotic systems, *Escherichia coli* (*E. coli*) is the most common hosts for heterologous protein expression. As a host, the Gram-negative *E. coli* has some intrinsic disadvantages, including production of endotoxins and the formation of inclusion bodies [2, 6]. For this reason, the search for alternative prokaryotic host systems has shifted its focus toward the Gram-positive species as they do not produce endotoxins and are in possession of protein secreting mechanisms, which is a highly-requested trait by the industry [7].

1.2 From DNA to functional protein

Deoxyribonucleic acid (DNA) is a polymer of nucleotides. These nucleotides are made up by the sugar deoxyribose, a phosphate group and nitrogenous base. In total, there are four different nucleotides based on their nitrogenous base; adenine (A), guanine (G), cytosine (C) and thymine (T). These nitrogenous bases are paired with each other in a set combination, A to T and G to C, and held together by hydrogen bonds [8]. For the genetic code of DNA to be

converted into a functional protein, it needs to undergo two essential processes, transcription and translation (**Figure 1-1**).

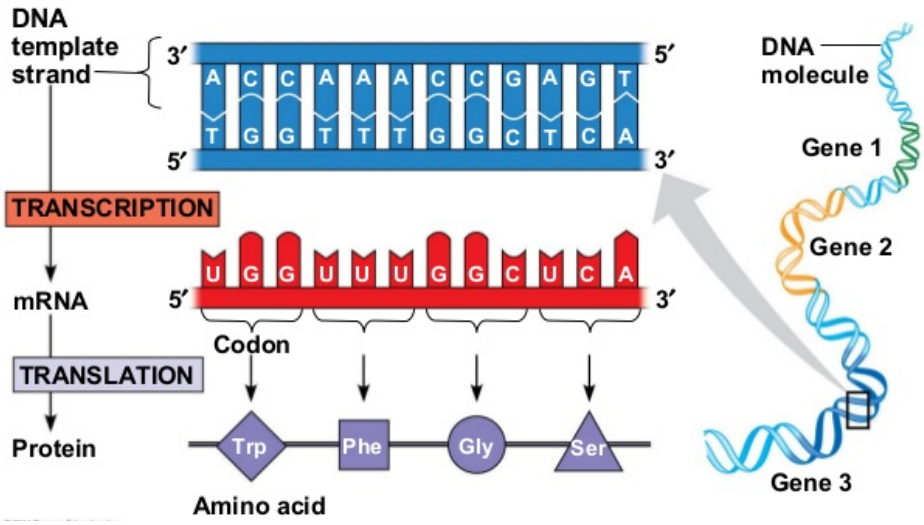


Figure 1-1: The process from gene to protein. Figure obtained from Reece & Campbell [8].

1.1.1 Transcription

Transcription is the process where DNA is converted into messenger RNA (mRNA) by RNA polymerase. RNA has a similar structure to DNA, except RNA is single-stranded and contains ribose instead of deoxyribose and the nitrogenous base thymine is replaced by uracil, which pairs with adenine. The RNA polymerase holoenzyme consists of five subunits, β, β', α, ω and σ (sigma). The sigma factor is responsible for binding of the RNA polymerase to the DNA, while β, β', α_2 and ω make up the core enzyme responsible for the RNA synthesis. The sigma factor recognizes the -10 and -35 regions of the promoter, the transcription initiation site, and facilitates binding of the RNA polymerase holoenzyme to the DNA. After binding to the promoter, the sigma factor dissociates, leaving the core enzyme to perform the synthesis. Using the 3'to 5' DNA template strand, the core enzyme synthesizes a mRNA strand in a 5' to 3' direction, adding approximately 40 nucleotides per second to the growing mRNA strand [9]. **Figure 1-2** shows the structures of a typical prokaryotic gene.

Some genes are transcribed continuously as their product is required for maintaining normal cell functions. These genes are generally referred to as housekeeping genes and they are usually controlled by constitutive promoters, promoters initiating binding of RNA polymerase continuously. Other genes are only transcribed when their product is required by the cell under specific conditions, these genes are controlled by inducible promoters [9].

Transcription is terminated as the RNA core enzyme reaches the terminator sequence downstream of the gene. The terminator sequence consists of two inverted repeats, separated by a non-repeating segment. When the core enzyme transcribes the terminator sequence, the RNA corresponding to the inverted repeats form a stem-loop structure that causes the RNA polymerase to pause. The terminator sequence is followed by a string of A residues in the DNA, which is transcribed and base paired to U residues in the RNA. As U:A base pairs only have two hydrogen bonds holding them together, a longer segment of U:A base pairs will eventually dissociate and thereby terminate transcription [10]. Some terminator sequences require a protein factor known as Rho to terminate transcription. Rho binds to the transcribed Rho termination sequence and causes a conformational change in the RNA polymerase. The RNA polymerase pauses and Rho separates the mRNA from the DNA, causing RNA polymerase to dissociate [9].

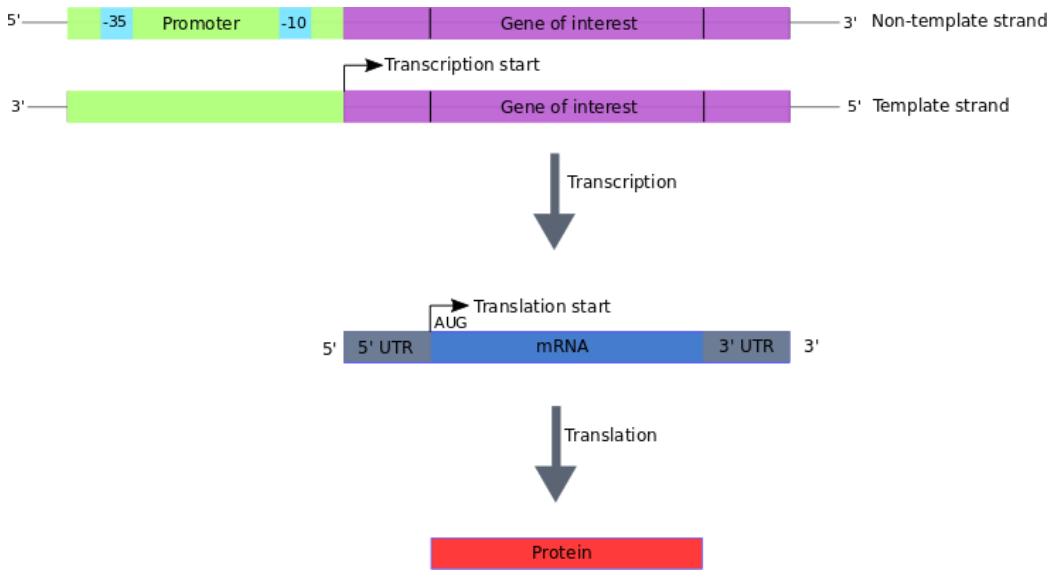


Figure 1-2: The gene of interest is transcribed from the DNA template strand into mRNA, which is translated into a polypeptide chain, the basis of a protein. UTR = untranslated region

1.1.1 Translation

The information stored in mRNA are read in triplet codes known as codons and translated into a polypeptide chain through the process of translation. There are four steps of the translation process: initiation, elongation, termination and ribosome recycling. In prokaryotes, the decoding of the mRNA codons is carried out by the 70S ribosome, consisting of two subunits, 30S and 50S. [11]

The process of translation initiation starts with the formation of the 30S initiation complex (30S IC). Many prokaryotic mRNAs contain a specific translation initiation region known as the Shine-Dalgarno (SD) sequence at their 5' end. The 16S rRNA of the 30S subunit contains an anti-SD sequence (aSD) that is complementary to the SD sequence on the mRNA. Formation of the SD-aSD complex through base pairing docks the mRNA to the 30S subunit. Three initiation factors (IF) are required for the formation of the 30S IC, IF1, IF2 and IF3. IF2 is responsible for recruiting the initiator tRNA to the AUG start codon. As the start codon AUG encodes methionine, the initiator tRNA is charged with methionine. The initiator tRNA is different from the methionine tRNAs used for elongation, as the α-NH_2 group of the methionine is shield by a formyl transferase, the initiator tRNA is therefore often referred to as fMet-tRNA or $tRNA_{fMet}$. This distinction makes IF2 able to recognize and bind fMet-tRNA. With the guidance of IF2, fMet-tRNA binds to the AUG codon with high affinity. IF1 and IF3 "proofreads" the codon / anticodon base pairing and allows for the 50S subunit to dock to the 30S IC, resulting in IF dissociation and complete assembly of the 70S ribosome [12].

Through the process of elongation, the mRNA codons are decoded and translated into an amino acid sequence. A schematic overview showing the structural features of a 70S ribosome is shown in **Figure 1-3**. Charged tRNAs (aa-tRNAs) enter the ribosome at the acceptor site (A-site), the amino acid is then transferred to the to the growing polypeptide at the peptide site (P-site) and the tRNA is released from the exit site (E-site) [11].

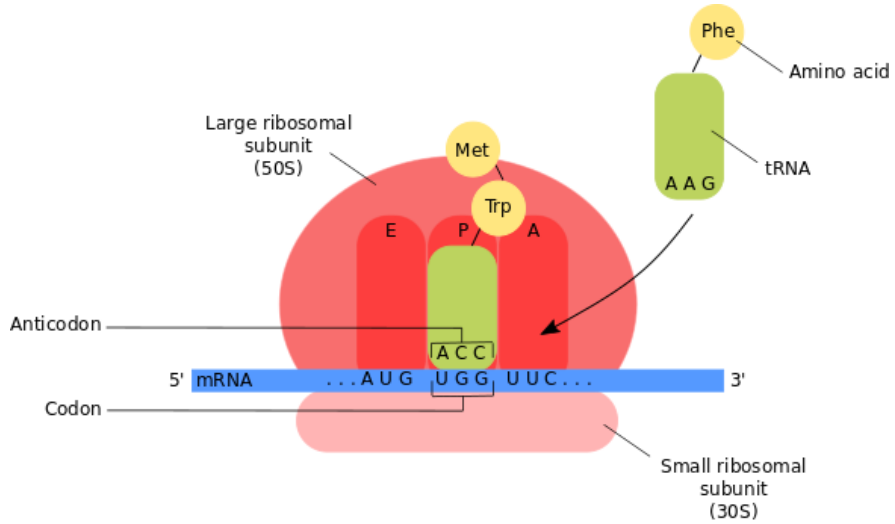


Figure 1-3: Codon translation by the ribosome.

After the binding of the fMet-tRNA at the P-site and the assembly of the 70S ribosome, the mRNA codon following the start codon AUG is exposed at the A-site. The elongation factor (EF) EF-Tu is a general elongation factor that binds to all aa-tRNAs and directs them to a complementary codon at the A-site. As con*tac*t is established between the mRNA codon and the anticodon of the aa-tRNA, EF-Tu hydrolyses guanosine triphosphate (GTP) and the guanosine diphosphate (GDP) bound EF-Tu releases the aa-tRNA at the A-site and dissociates from the ribosome. The ribosome has an intrinsic peptidyl transferase activity that catalyzes the formation of peptide bonds between the amino acid of the tRNA in the A site and the polypeptide of the tRNA in the P-site. With the help of EF-G, the ribosomal subunits move relative to each other and the tRNAs located at the A and P-site is moved to the P and E-site, respectively. When the ribosome reaches a stop codon, which there are no tRNAs for, release factors (RF) recognizes the mRNA stop codons and releases the polypeptide chain from the ribosome. The ribosomal units of the 70S needs to be recycled, a process aided by the ribosome recycling factor (RRF) and EF-G, that removes the 50S subunit, and IF3 that splits up the remaining 30S subunit, tRNAs and mRNA [13].

1.3 *Escherichia coli* as a host for heterologous protein expression

E. coli is the predominantly used prokaryotic host for heterologous protein expression as it is well-characterized, fast-growing and easy to genetically modify. The gram-negative bacterium can grow to high densities and is relatively cheap to cultivate [14].

Even though *E. coli* is a good host for expressing many recombinant proteins, it has some intrinsic disadvantages. As *E. coli* is a Gram-negative bacterium, it lacks efficient pathways for extracellular secretion. Meaning that in order to recover intracellular heterologous proteins, cells need to be lysed. Cell lysis of *E. coli* poses a risk of endotoxin contamination from the lipopolysaccharides in the outer membrane. For recovery of proteins intended for therapeutic use, this is highly undesirable as endotoxins may cause serious side effects such as high fever and death [15]. The formation of inclusion bodies is also a disadvantage for *E. coli*. During high-level protein expression, insoluble protein intermediates tends to aggregate and form inclusion bodies in the cytoplasm. Even though inclusion bodies can make the isolation process easier [16], recovering proteins from inclusion bodies is an extensive process involving solubilization, refolding and purification [17]. Cultivation temperatures are usually lowered after induction to reduce inclusion body formation, however a reduction in

temperature will also reduce the activity of helper proteins (chaperones) facilitating correct protein folding [14].

Many eukaryotic proteins require post-translational modifications that *E. coli* is not capable of performing, like glycosylation [5]. *E. coli* also have trouble forming disulfide bonds as the reducing environment in the cytoplasm hinders the formation of disulfide bonds between two cysteine residues in the polypeptide. The cytoplasm of eukaryotic cells is also reducing, but polypeptides are directed by signal peptides to the endoplasmic reticulum (ER) and its oxidative environment for folding. The environment in the periplasm of Gram-negative bacteria resembles the oxidizing environment of ER. Polypeptides can be transported to the periplasm by fusing it to a periplasm translocation signal peptide, and thereby form the necessary disulfide bonds [18]. Periplasm translocation of proteins does however not exclude the risk of intermediate aggregation, as inclusion body formation may occur in the periplasm as well [16].

1.4 *Corynebacterium glutamicum* as a host for heterologous protein expression

C. glutamicum is a Gram-positive bacterium of the *Actinobacteria* phyla. In an industrial perspective, *C. glutamicum* was first used for production of L-glutamate shortly after it was discovered in 1956. With the advancement of tools for genetic engineering and the publication of the bacterium's genome, *C. glutamicum* expanded its working area and is today used for production of a wide range of bio-based chemicals such as alcohols, organic acids, polymers and other amino acids. [19]. *C. glutamicum* have also been reported as a promising host for heterologous protein expression. One of the most promising intrinsic features of *C. glutamicum* is the presence of native secretion pathways. Secretory production of heterologous proteins is highly desirable as it simplifies down-stream processing and allows for continuous culturing [20]. There are two major secretion pathways in *C. glutamicum*, the Sec pathway and the Tat pathway. The Sec pathway is the main pathway and is responsible for secreting unfolded proteins over the membrane, while the Tat pathway translocate folded proteins [20, 21].

Being a gram-positive species, *C. glutamicum* do not produce endotoxins like *E. coli* and is generally recognized as safe (GRAS) [21]. *C. glutamicum* show low levels of extracellular

protease activity, with only one known gene encoding an extracellular protease [19], making it a suitable candidate for production of protease-sensitive proteins [21]. Like *E. coli*, *C. glutamicum* is fast growing and able to grow to high densities using a broad spectrum of carbon sources [19]. However, the transformation efficiency of *C. glutamicum* is lower than for *E. coli* and the number of suitable expression vectors and expression systems are limited [21].

Genome modifications has been done to improve the ability of *C. glutamicum* as a host strain for heterologous protein production. The presence of prophages in bacterial genomes are quite common, in some species, prophages makes up 10-20% of their entire genome [22]. As the presence of prophages represent a genomic instability, prophage-free strains have been constructed. The native *C. glutamicum* ATCC 13032 contains three prophages; CGP1, CGP2 and CGP3. A prophage-free variant of *C. glutamicum* ATCC 13032 was constructed, *C. glutamicum* MB001 [23]. In this study, *C. glutamicum* MB001 (DE3) was used, a modified variant of *C. glutamicum* MB001. A part of the DE3 region from *E. coli* BL21 (DE3) was integrated in the genome of *C. glutamicum* MB001, making the new *C. glutamicum* MB001 (DE3) strain. The DE3 region contains the T7 RNA polymerase *gene 1*, placed under control of the isopropyl-β-D-1-thiogalactopyranoside (IPTG) inducible *lac*UV5 promoter. Hence establishing a IPTG-inducible T7 expression system in *C. glutamicum* [24].

1.5 The XylS/*Pm* expression system

Inducible expression systems are a useful tool for high-level gene expression. These systems are usually regulated by an environmental factor like temperature, pH or a ligand. The promoters of these systems can be subjected to positive or negative control by transcription factors [25]. Positively regulated promoters require binding of an activator for the RNA polymerase to also bind the promoter and initiate transcription. In negatively regulated promoters, a repressor is bound to the promoter, blocking the ribosomal binding site and preventing RNA polymerase from binding. For transcription to be initiated, the repressor needs to dissociate from the promoter. In both cases, the binding of an inducer to the transcription factor is required for transcription to be initiated [26].

The XylS/*Pm* regulator / promoter system is a positively regulated expression system originating from the pWWO TOL plasmid in *Pseudomonas putida*. In its native state, the

XylS/*Pm* system controls an operon involved in aromatic hydrocarbon degradation, but it is perhaps best known for its use in high-level protein expression. The transcriptional activator XylS, a 36 kDa and 321 amino acid protein of the AraC family, dimerizes upon activation and binds the *Pm* promoter, initiating RNA polymerase binding and thereby transcription of the downstream gene of interest (GOI) [27].

The expression of XylS is regulated by two tandem promoters, the σ^{54}-dependent and inducible *Ps1* promoter and the σ^{70}-dependent and constitutive *Ps2* promoter. XylS is activated by a benzoate-derived inducer. To date, 31 inducer compounds of different induction ratios are identified. The inducer enters the cells through passive diffusion and binds to XylS, activating it and causing it to dimerize with another activated XylS-inducer complex. If overexpressed, XylS may spontaneously dimerize without the presence of an inducer and consequently bind *Pm* and initiate transcription [27]. Transcription from *Pm* can be mediated during all growth phases, as RNA polymerases can bind to *Pm* using different sigma factors [25]. **Figure** 1-3 shows a schematic overview of the XylS/*Pm* expression system and its regulatory elements.

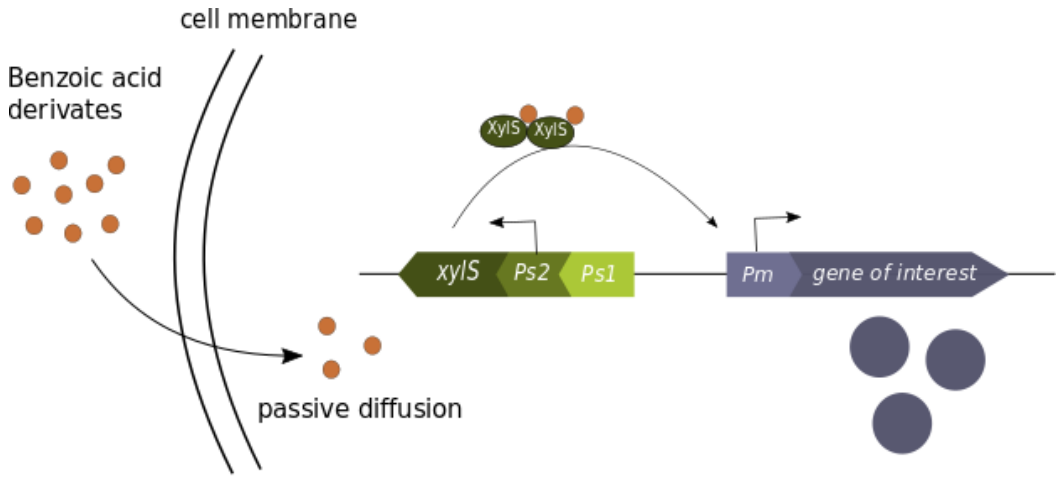


Figure 1-4: Schematic representation of the XylS / Pm expression system. XylS is constitutively expressed from *Ps2*. The inducer diffuses through the cell membrane, binds and activates XylS, causing it to dimerize. Activated XylS binds Pm, promoting RNA polymerase binding and thereby transcription of the gene of interest. Figure adapted from [25].

1.6 Reporters

In molecular biology, genes with easily detectable products are used as reporters [28]. Reporters can for example be used to monitor promoter activity, protein interaction or subcellular protein localization. Ideally the reporter of choice should not be expressed by the host organism itself and provide a sensitive and quantitative assay without harming the host [29]. The *lacZ gene* encoding β-galactosidase is a well-known reporter gene. Naturally β-galactosidase cleaves the disaccharide lactose into the monosaccharaides glucose and galactose. β-galactosidase can also cleave artificial galactose compounds such as ONPG, which splits into galactose and o-nitrophenol. O-nitrophenol is a yellow compound that can easily be detected and quantified. [30]

Another common group of reporters are fluorescent proteins. Unlike many of the other reporters, fluorescent proteins have no need for exogenously added substrates or cofactors, making them ideal for tracking gene expression *in vivo* [31]. When choosing a fluorescent protein as a reporter, there are several factors to consider. The fluorescent protein should be photostable, not pose a toxic effect on the system in use and be bright enough to exceed the autofluorescence emitted by the cells. In addition, minimal environmental sensitivity is important to ensure that only the factors being studied effects the protein [32]. In general, reporters emitting light of a longer wave-length are considered to be a better option as they are less phototoxic for cells and reduces autofluorescence and scattering [33]. **Figure 1-5** shows the excitation and emission specter of some common fluorescent proteins.

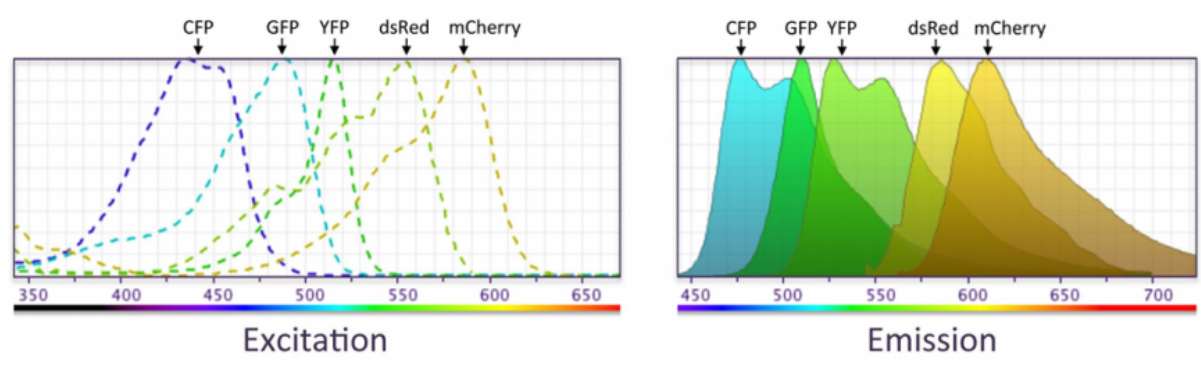


Figure 1-5: Excitation and emission spectra of some common fluorescent factors. Wave-length in nm on the x-axis. Figure obtained from the University of Dundee [34].

1.6.1 Green fluorescent protein (GFP)

Green fluorescent protein (GFP) originates from the jellyfish *Aequorea victoria* and was first discovered by Shimomura et al as an associated protein to aequorin [35], a protein that fluoresces blue when binding to Ca^{2+} (λ_{max} =470 nm). When in excited state, aequorin transfers energy to the associated GFP, which in turn causes GFP to emit green light (λ_{max} =508 nm), giving the *Aequorea victoria* its characteristic blue-green glow [36]. There are no indication of GFP interfering with normal cell functions, and no need for exogenous substrates or cofactors. [31].

1.6.2 mCherry

In 1999 Matz et al cloned six different fluorescent proteins from Anthozoa species with homologous regions to GFP from *Aequorea Victoria* [37], one of these being DsRed from a *Discosom*a species. DsRed is a tetramer with an excitation maximum at 558 nm and an emission maximum at 583 nm [38]. Being a tetramer, DsRed is not ideal for fusion tagging, but through several rounds of directed evolution, a series of monomeric red-shifted fluorescent proteins known as mFruit was constructed [39]. Among the red fluorescent proteins, mCherry is considered to be the better option as it is relatively photostable, bright and shown to function in fusion [33]. mCherry has an excitation maximum of 587 nm and an emission maximum of 610 nm [39].

1.7 Codon optimization

Combining the four possible nucleotides in mRNA into a three-letter code, gives 64 unique codons. Three of these are translation stop signals, leaving 61 codons encoding amino acids. However, there are only 20 amino acids, meaning that several codons encode the same amino acid. **Figure 1-6** gives an overview of all the possible 3-letter codons in the mRNA and the corresponding amino acid. When two or more codons encode the same amino acid, we call them synonym codons. Synonym codons are not necessarily recognized and decoded by the ribosome in the same rate, the codon optimality is dependent on ribosome decoding and the availability of suitable tRNAs. During translation, tRNAs from the cytoplasmic tRNA-pool are recruited by the ribosome A-site and bound to the complementary codon. The speed in which the tRNA is bound to the A-site is dependent on the cytoplasmic level of the tRNA in question, meaning that binding of rare tRNAs take longer and thereby causes a delay in translation. Consequently, codon optimality and its corresponding tRNA level are important

factors in determining translation rates. Genomes tend to show a bias in their codon usage, with optimal codons being overrepresented, especially in highly expressed genes [40].

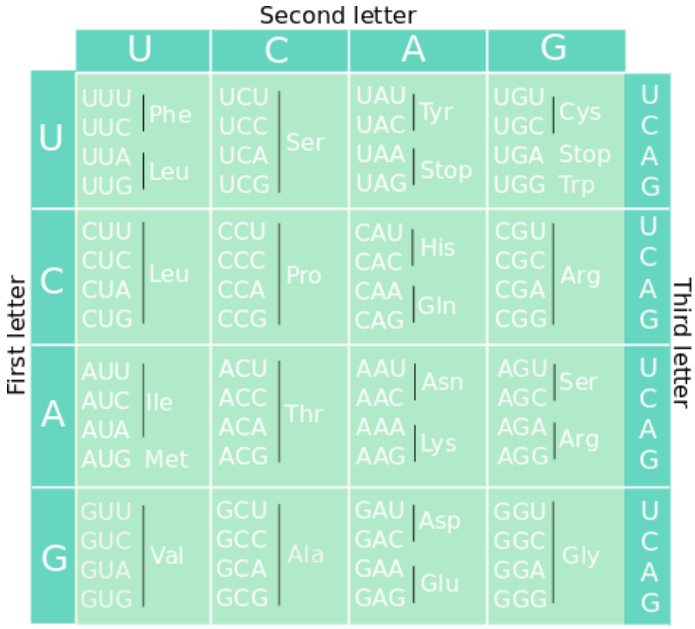


Figure 1-6: The three-letter codons as found in mRNA and their corresponding amino acid.

In heterologous protein expression, codon usage may pose a potential problem when trying to express a gene encoded with codons that are rarely used in the host [41]. For example, the AGA and AGG codons for arginine are rare in *E. coli*, while quite common in eukaryotes. If heterologous genes containing these codons are expressed in *E. coli*, it can affect the stability of the plasmid and the transcribed mRNA, hinder protein accumulation and in some cases, suppress protein synthesis and cell growth. In general, there are two strategies for avoiding codon bias. You can either change rare codons into the bacterium's preferred codon, or expand the bacterium's tRNA repertoire by coexpressing or overexpressing genes of rare tRNAs. However, reports indicate that the results of the latter method are somewhat inconsistent [16, 41]. tRNAs require extensive processing before they can be used in the process of translation. Overexpressing tRNA genes alone might therefore not increase the level of functional and available tRNAs as other factors might be limiting the process. The other alternative, changing the codon composition of the gene to the host's preferred synonym codons, is often a more successful and less time-consuming strategy as synthetic codon-optimized genes are provided by commercial suppliers.

1.7.1 Codon bias in *C. glutamicum*

Being a species with high G + C content, *C. glutamicum* show an expected bias towards G and C codons, with C being the predominant base in the third position of the codon. The C3 bias is particularly prominent in highly-expressed genes [42].

1.8 Choosing a suitable promoter

The level of protein present in a cell is affected by the rate of transcription, translation and degradation, with transcription and translation initiation often being the rate-limiting steps. Transcription initiation is mainly regulated by the promoter elements, and translation initiation is tightly regulated by ribosomal binding site (RBS) strength [43]. With transcription initiation being the first obstacle, optimization of the promoter element is key on the way to successful protein expression.

Several inducible *E. coli* promoters have been used for protein expression in *C. glutamicum*, including the *tac* promoter [44, 45]. The *tac* promoter is a hybrid between the *trp* and *lac* promoters of *E. coli*, with consensus sequences at both the -10 at -35 regions of the promoter. The promoter can be submitted to repression by the lac repressor or induced by isopropyl-β-D-thiogalactoside (IPTG) [46]. However, *C. glutamicum* is less permeable to the IPTG inducer than *E. coli*, which consequently results in lower expression yields. There is also an additional economical aspect of this, as IPTG is a rather expensive compound and therefore not ideal as an inducer for industrial scale production [44].

As established regulatory expression systems have proven to be less efficient in *C. glutamicum*, modifications of these systems are required for *C. glutamicum* to reach its potential as a host for heterologous protein production. A possible solution could be to incorporate *C. glutamicum* native promoters, both inducible and constitutive, in these regulatory systems [47]. By using a strong constitutive promoter, the associated gene is likely to be excessively expressed without the adding of an inducer. However, it is important to emphasize that upregulating transcription of a target gene does not necessarily mean that the yield of target protein will be higher. Transcription initiation is not the only obstacle on the way from gene to protein, and excessive expression could affect cell growth [48].

1.8.1 *C. glutamicum* native promoters

Several native promoters suitable for metabolic engineering have been characterized for *C. glutamicum*. In a study by Shang et al., where the strength of 16 native promoters was tested, the promoter strength covered a span of 31-fold with P_{dapA} being the weakest and P_{pgk} being the strongest promoter [45]. The following *C. glutamicum* native promoters were chosen as alternative *XylS* promoters in this study P_{tuf}, P_{sod}, P_{dapA} and P_{dapB}. **Table 1-1** gives a description of their native application in *C. glutamicum*. P_{tuf} and P_{sod} are considered to be strong constitutive promoters, while P_{dapA} and P_{dapB} are identified as relatively weak [45].

Table 1-1: Native *C. glutamicum* promoters used in this study.

Promoter	Description	Reference
P_{tuf}	Promoter of the *tuf* gene encoding elongation factor TU	[19]
P_{sod}	Promoter of the *sod* gene encoding superoxide dismutase	[19]
P_{dapA}	Promoter of the *dapA* gene encoding dihydrodipicolinate synthase	[49, 50]
P_{dapB}	Promoter of the *dapB* gene encoding dihydrodipicolinate reductasee	[50]

1.9 Shuttle vectors

Shuttle vectors are used to move genes between two or more unrelated host organisms. If the vector contains the necessary machinery to regulate expression of the gene or genes, it is often referred to as an expression vector. The shuttle vector must be able to replicate in both hosts, which often requires multiple origins of replication (ori) [51]. Plasmids used in this study are based on the pXMJ19 *E.coli* / *C. glutamicum* shuttle vector constructed by Jakoby et al in 1999 [52]. pXMJ19 is a based on the high copy number pK18 *E. coli* plasmid and the low copy number pBL1 *C. glutamicum* cryptic plasmid [52], with an estimated copy number of 30 in *C. glutamicum* [53]. The final pXMJ19 plasmid has a promoter-less *cat* gene encoding chloramphenicol acetyltransferase conferring chloramphenicol resistance up to 50 μg/mL, origin of replication in *E. coli, ori* PUC, and in *C. glutamicum*, *ori* BL1, the $lacI^q$ gene, the rrnB terminators T1 and T2 and the IPTG-inducible *tac* promoter [52]. The reporter gene *mCherry* was inserted under the control of the *tac* promoter in the pXMJ19 plasmid, leading to the pXMJ19-mCherry **(Figure 1-7**) constructed by M. Johnsgaard [54]. Using pXMJ19-mCherry as a template, an insertion of the *XylS/Pm* expression cassette, with an

accompanying deletion of the *tac* promoter, the pVB-4A0E1-mCherry plasmid was constructed, placing *mCherry* under *Pm* control [55].

Figure 1-7: pXMJ19-mCherry and pVB-4A0E1-mCherry. Constructed by M. Johnsgaard [54, 55]

1.10 Aim of study

The XylS / Pm expression system has previously been successfully established in several gram-negative species, this study is a part of the ongoing project of establishing the system in alternative gram-positive host species. In previous work, a XylS/ *Pm* expression vector was constructed, but the expression system has not yet been proven to function in *C. glutamicum*. The aim of this study will be to establish a functional and inducible XylS / *Pm* based promoter system in *C. glutamicum* by using *C. glutamicum* codon optimized reporters and native promoters controlling the expression of XylS activator.

2 Materials and Methods

2.1 Materials

2.1.1 Media and solutions

Media and other solutions used for the experimental work of this thesis are listed in Appendix A.

2.1.2 Bacterial strains, plasmids and growth conditions

Two *E. coli* strains and one *C. glutamicum* strain were used in this work, these are presented in **Table 2-1** together with a description of their application and origin. Plasmids used in this work, including plasmids constructed during the project, are listed in **Table 2-2** together with relevant properties and source origin.

All *C. glutamicum* strains were grown in Bran Heart Infusion Supplemented (BHIS) medium and all *E. coli* strains were grown in lysogeny broth (LB). For expression purposes, both *C. glutamicum* and *E. coli* were grown in Hi + Ye medium. If harboring plasmids with antibiotic resistance, the appropriate antibiotic was added to the medium. Liquid cultures were incubated at 37°C and 225 revolutions per minute (RPM), unless other conditions are specified. For bacterial growth on plates, BHIS agar was used for *C. glutamicum* and LB agar was used for *E. coli*, the agar was supplemented with antibiotics if needed and plates were incubated at 37°C unless other conditions are listed. Unless other concentrations are listed, final antibiotic concentrations of media and agar were 25 ng/μL chloramphenicol (Cm), 100 ng/μL ampicillin (Amp) and 50 ng/μL kanamycin (Kan).

Table 2-1: Bacterial strains used in this study, including their application and source.

Strain	Application	Source
E. coli DH5α	Cloning work	Bethesda Research Laboratories (BSL)
E. coli BL21	Heterologous protein expression	NEB
C. glutamicum MB001 (DE3)	Heterologous protein expression	Kortmann, M., et al [24]

Table 2-2: Plasmids used in this study, including relevant properties and source.

Plasmid	Relevant properties [a]	Source
pXMJ19	*E. coli* / *C. glutamicum* shuttle vector, Cm^r	Jakoby et al [52]
pXMJ19-mCherry	pXMJ19 with *mCherry* insert under P_{tac} control, Cm^r	M. Johnsgaard [54]
pMA-T-C_gluta_opt-mCherry	Source vector for *C. glutamicum* codon optimized *mCherry*, Amp^r.	GeneArt (Thermo Fisher Scientific)
pXMJ19-optm_mCherry	pXMJ19 with *C. glutamicum* codon optimized *mCherry* insert from *Pm*A-T-C_gluta_opt-mCherry under P_{tac} control, Cm^r	This study
pMA-GFP	Source vector for *GFP*, Amp^r	GeneArt (Thermo Fisher Scientific)
pXMJ19-GFP	pXMJ19 with *GFP* insert from *Pm*A-GFP under P_{tac} control, Cm^r	This study
pUC57-Kan-GFP_optm_glut	Source vector for *C. glutamicum* codon optimized *GFP*, Kan^r	GenScript
pXMJ19-optm_GFP	pXMJ19 with *C. glutamicum* codon optimized *GFP* insert from pUC57-GFP-optm_glut under P_{tac} control, Cm^r	This study
pVB-4A0E1-mCherry	pXMJ19 backbone with *mCherry* and *XylS /Pm* expression cassette inserts. *mCherry* under *Pm* promoter control. Cm^r	M. Johnsgaard [55]
pASK1-mCherry	pVB-4A0E1-mCherry without the c-myc and 6His protein tag. Removal of *Hind*III recognition site,. Cm^r	This study
pASK2-mCherry	Based on pASK1, NdeI restriction site upstream of *mCherry* changed to *Hind*III with site directed mutagenesis,. Cm^r.	This study
pASK2-optm_mCherry	pASK2 with *C. glutamicum* codon optimized *mCherry* insert from *Pm*A-T-C_gluta_opt-mCherry. Cm^r	This study
pVB-1C0C1-GFP	Source vector for *GFP*. Kan^r	Vectron Biosolutions
pASK1-GFP	pASK1 with *GFP* insert from pVB-1C0C1-GFP. Cm^r	This study
pASK2-optm_GFP	pASK2 with *C. glutamicum* codon optimized *GFP* insert from pUC57-GFP-optm_glut. Cm^r	This study
pVB-4A0E1-mCherry_Ptuf	pVB-4A0E1-mCherry with *XylS* under control of the *C. glutamicum* native P_{tuf} promoter. Cm^r	This study
pVB-4A0E1-mCherry_Psod	pVB-4A0E1-mCherry with *XylS* under the control of the *C. glutamicum* native P_{sod} promoter. Cm^r	This study
pVB-4A0E1-mCherry_PdapA	pVB-4A0E1-mCherry with *XylS* under the control of the *C. glutamicum* native P_{dapA} promoter. Cm^r	This study
pVB-4A0E1-mCherry_PdapB	pVB-4A0E1-mCherry with *XylS* under the control of the *C. glutamicum* native P_{dapB} promoter. Cm^r	This study

[a] Amp^r, ampicillin resistance; Kan^r, kanamycin resistance; Cm^r, chloramphenicol resistance.

2.2 Introduction to methods

In this chapter, a brief theoretical introduction of the methods used in this thesis will be given.

2.2.1 Restriction enzyme cloning

Bacterial cells protect themselves from foreign DNA, often indicating a viral infection, by cutting the foreign DNA into smaller pieces. This process is aided by restriction enzymes (RE), a type of endonucleases that breaks the phosphate bond between adjacent nucleotides on both DNA strands. Restriction enzymes are sequence specific, meaning that they recognize and cut specific DNA sequences, an ability that makes them a useful tool in genetic engineering. **Figure 2-1** show the recognition site of *Eco*RI, the first restriction enzyme isolated from *E. coli*, hence the roman number I and *Eco*. Bacteria protect their own genome by methylating sequences identical to recognition sites, ensuring that the restriction enzymes only cut foreign DNA. Type I restriction enzymes make the cut distal to their recognition site, while Type II restriction enzymes cut within the recognition site itself. Restriction enzymes cutting at the same place on both strands generates blunt ends. By cutting the DNA at different places, complementary overhangs know as sticky ends are generated. Generally, restriction enzymes generating sticky ends are more convenient in genetic engineering as DNA fragments with complementary sticky ends can re-join more precisely. [56]. DNA cloning using restriction enzymes (RE) is a well-established cloning method in molecular biology. Different DNA molecules can be digested with the same RE, generating complementary overhangs, facilitating the combination of DNA from two sources through DNA ligation.

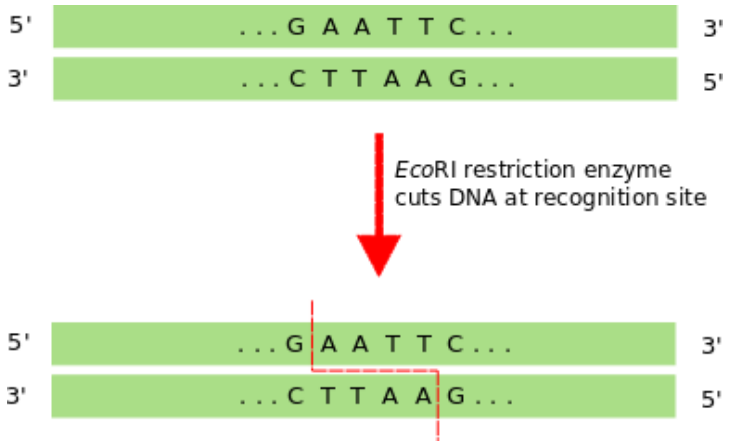


Figure 2-1: The DNA recognition site of the restriction enzyme *Eco*RI. Figure modified from [56].

Agarose gel electrophoresis

Agarose gel electrophoresis is a method that uses an electric filed to separate macromolecules based on their size, charge and physical properties. Fragments with high molecular weight will move slower through the gel, as they are obstructed by the pores in the gel in a greater extent than molecules of a lower molecular weight. The size of the pores is determined by the agarose concentration, which is usually between 0.7% and 3%, with higher concentrations giving a denser agarose network and smaller pores. As DNA is rich in phosphate, it will have a naturally negative charge and when influenced by an electric field, DNA will move toward the positive pole [57]. By using a DNA-binding fluorophore and exposing the gel to UV-light, the DNA fragments in the agarose gel is visualized after terminated electrophoresis. The DNA-binding fluorophore can be included in the gel solution or added after in a staining solution [58].

DNA ligation

DNA ligation is the formation of phosphodiester bonds between the 3'-hydroxyl and 5'-phosphate end groups in adjacent nucleotides. The formation is catalyzed by DNA ligases, which can be divided into two categories based on their required cofactor. DNA ligases from eukaryotic cells, T bacteriophages and archaea require ATP, while ligases of bacteria are dependent on NAD^+ [59].

2.2.2 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a method for amplifying specific DNA sequences. The method utilizes a thermostable DNA polymerase, primers complementary to the flanking regions of the target sequence, nucleotides and a thermocycler. Multiple copies of the target sequence are made by repeating 3 temperature steps in a set number of cycles:

- **Step 1:** Denaturation. High temperature causes the breakage of hydrogen bonds in the DNA and the double stranded DNA is separated into two single stranded molecules. Denaturation temperatures typically lie around 90ºC.
- **Step 2:** Annealing. The temperature is lowered to 50-60 ºC, which facilitates primer binding to complementary sequences on the single stranded template.
- **Step 3**: Elongation. The temperature is increased to the ideal temperature of the DNA polymerase used in the reaction, typically around 70 ºC. The DNA polymerase binds to the 3'end of the primer and elongates the strand in a 5'-3'direction by using the single stranded DNA as a template.

Theoretically the amount of target sequence will be exponentially amplified after the third cycle [60].

2.2.3 Site-directed mutagenesis (SDM)

Site-directed mutagenesis (SDM), a method used to introduce specific alterations in double stranded DNA, either in form of a deletion, insertion or substitution of bases [61]. The method utilizes two primers, where one of them contains the desired mutation. The primers bind on opposite strands in a "back-to-back" positions. A single linear product containing the desired mutation will be generated during the first PCR cycle, subsequent PCR cycles will exponentially amplify the desired product [62].

2.2.4 Sequence and ligation independent cloning (SLIC)

Sequence and ligation independent cloning (SLIC) utilizes the 3' to 5' exonuclease activity of the T4 DNA polymerase to create 5' overhangs. The method allows for multiple fragment assembly, without being dependent on compatible restriction sites between vector and insert and DNA ligases. Vectors are linearized using either restriction enzyme digestion or PCR, and inserts are amplified using PCR and primers with extensions homologous to the ends of the vector. Vector and insert(s) are then mixed and incubated at room temperature with T4 DNA polymerase, which generates complementary 5' overhangs on the vector and insert. When the reaction is put on ice, the complementary overhangs will anneal and the new construct can be used to transform competent cells. During the transformation process, the nicks are sealed through homologous recombination [63]

2.2.5 Gibson assembly

The Gibson assembly method allows for assembling of multiple DNA fragments in a single isothermal reaction. DNA fragments with overlapping terminal sequences are mixed together with an exonuclease with 5' activity, a DNA polymerase and a DNA ligase and the mix is incubated at 50°C for a minimum of 15 minutes. The 5' exonuclease degrades the 5' ends of the dsDNA fragments, creating complementary single-stranded overhangs. These complementary overhangs anneal and the DNA polymerase extends the 3' end and DNA ligase seals the nicks [64].

2.2.6 Fluorometry

Some compounds will absorb light of specific wavelengths when they are illuminated. The absorbed energy result in electron excitation, and as the electrons return to their ground state, energy is emitted as fluorescence. The wavelength of fluorescence is longer than the light originally absorbed as some of energy is lost to other means in the process. By using a fluorescence spectrometer, the wavelength of emitted fluorescence can be measured [65].

2.2.7 SDS-PAGE and Western blot

Nucleic acids have a naturally negative charge and will therefore migrate toward the positive pole by themselves when subjected to an electric field during electrophoresis. The charge of a protein is dependent on the overall charge of its amino acids, which can be positive, neutral or negative. For proteins to be separated based on their molecular weight, these intrinsic charges need to be covered, a process facilitated by the detergent sodium dodecyl sulfate (SDS). SDS is composed of a long hydrophobic hydrocarbon tail and a hydrophilic sulfate group at the end of the tail. By boiling protein samples in a SDS solution, the protein unfolds from its tertiary structure and the hydrophobic tail of SDS binds to the polypeptide chain, covering the charges of the protein and preventing refolding. The hydrophilic sulfate group of SDS gives the unfolded polypeptide chain a net negative charge and prevents protein refolding by repelling other negative sulfate groups. As longer polypeptide chains will bind more SDS molecules with negative sulfate groups, the net negative charge of the unfolded protein is proportional to the molecular weight (size) of the protein. Electrophoresis of DNA and RNA are normally performed with agarose gels, but since proteins generally are smaller than DNA and RNA, they are separated on polyacrylamide gels which have smaller pores, hence the name polyacrylamide gel electrophoresis (PAGE, SDS-PAGE). For protein visualization, the polyacrylamide gel is stained. If specific proteins are to be identified, SDS-PAGE is often combined with a Western blot. Separated proteins are transferred to a nitrocellulose membrane by applying an electric current which will drive the negatively charged proteins from the polyacrylamide gel over to the membrane. A blocking solution, often containing re-hydrated powdered milk, is added for shielding of all non-specific bind sites on the nitrocellulose membrane. A primary antibody solution, with antibodies specific for the target protein, is added and the primary antibody-protein complex is detected by the adding of a secondary antibody solution. For easy detection, secondary antibodies are often tagged with enzymes producing colored or fluorescent compounds [66].

2.2.8 Quantitative PCR (qPCR)

Real-time PCR, or quantitative PCR (qPCR) uses a fluorescent dye to measure DNA amplification during each cycle of the PCR. This eliminates the use of electrophoresis to assay the result [67]. By inverting RNA transcripts into complementary DNA (cDNA), transcript levels can be assayed using qPCR. cDNA synthesis, or reverse transcription, is the process of converting RNA into complementary DNA by using reverse transcriptase. Reverse transcriptase is a RNA dependent DNA polymerase that was originally discovered in retroviruses, where it facilitates the integration of the viral RNA genome into the host's DNA genome [68].

DNA amplification in qPCR can be measured by using either fluorescent probes (TaqMan) or fluorescent dyes that bind the PCR product [69]. The use of TaqMan probes for quantification relies on the 5'- 3' exonuclease activity of the *Thermus aquaticus* (*Taq*) DNA polymerase degrading a labeled probe binding within the target sequence and the consequently release of signal [70]. Alternatively, fluorescent dyes like SYBR Green I (SG) can be utilized. SG interacts with the minor groove in double stranded DNA (dsDNA), causing it to fluoresce [71]. **Figure 2-2** shows a graphic representation of SYBR Green's interacting properties. In this study, qPCR using SYBR Green was used to evaluate the level of *mCherry* transcripts in *C. glutamicum*.

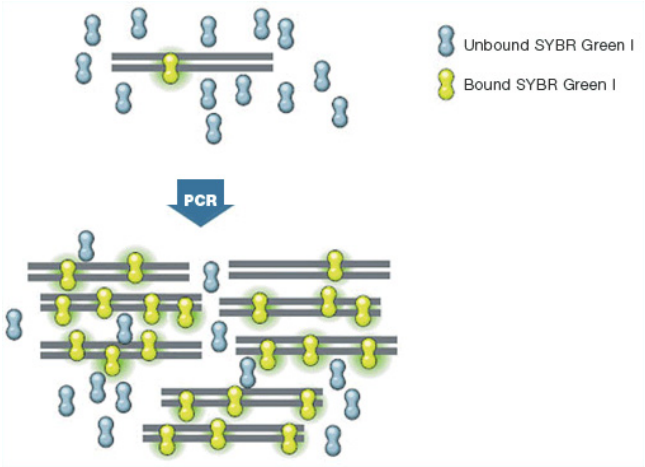


Figure 2-2: SYBR Green binds to double stranded (ds) DNA and fluoresces, when a template is amplified by PCR, more dsDNA is generated, subsequently binding more SYBR Green, causing an increased fluorescence signal. Figure obtained from Bio-Rad [72].

If qPCR is used to evaluate transcript levels, RNA must be isolated from the cells prior to cDNA synthesis and qPCR. The quality of the isolated RNA in this study was analyzed using the Agilent 2100 Bioanalyzer system (Agilent Technologies). The Bioanalyzer provides

automated electrophoresis of DNA, RNA and proteins, in addition to digital data concerning concentration, molecular size, integrity and purity. The software returns electropherograms which lets you track the electrophoresis of RNA as a function of time, whit low molecular weight RNA molecules being detected first. The software also calculates a RNA integrity number (RIN) ranging from 1 to 10, with 10 being fully intact RNA. An example of a electropherogram of an RNA sample with RIN value of 10 is presented in **Figure 2-3**, with 16S and 23S RNA peaks indicated. The peak between 20 and 25 seconds (s) is an intrinsic RNA marker.

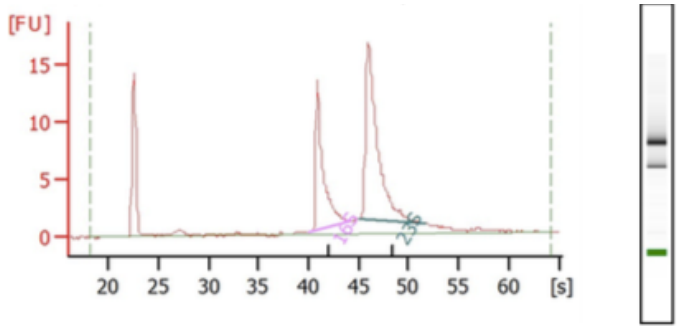


Figure 2-3: Bioanalyzer electropherogram (left) and the gel image (right) of total RNA sample isolated from *E. coli* BL21 WT with RNA integrity number (RIN) of 10. Peaks of 16S and 23S RNA indicated in the figure, marker peak between 20 and 25 seconds (s).

2.2.9 Analyzing qPCR data

Theoretically, the number of target DNA is doubled with every PCR cycle, causing an exponential amplification. As more target DNA is produced, more SYBR Green will bind and cause an increased fluorescence signal, making the amplification possible to track in real time. The background signal detected in the early cycles are referred to as the baseline. To prevent background signal from being detected as "real" amplification, the range of the baseline must be set high enough to exclude the noise, but at the same time allow for early detection of "real" amplification. Amplification is detected when the signal crosses the threshold, the level where the amplification signal is statistically significant higher than the baseline level. The cycle number of which the signal crosses the threshold is known as the threshold cycle (C_t). As such, the C_t value reflects the initial level of target cDNA in the sample, with samples of greater target concentration giving lower C_t values than samples with lower target concentration. The detected fluorescent signal from SYBR Green is normalized against a passive reference dye, giving a Rn value for the PCR reaction. From this Rn value, the Rn value of the baseline signal is subtracted, resulting in a ΔRn value. The fluorescent

signal detected for each sample is presented as ΔRn detected at the end of each cycle. **Figure 2-4** shows an amplification plot with the baseline and threshold level indicated, and the C_t values of four different samples.

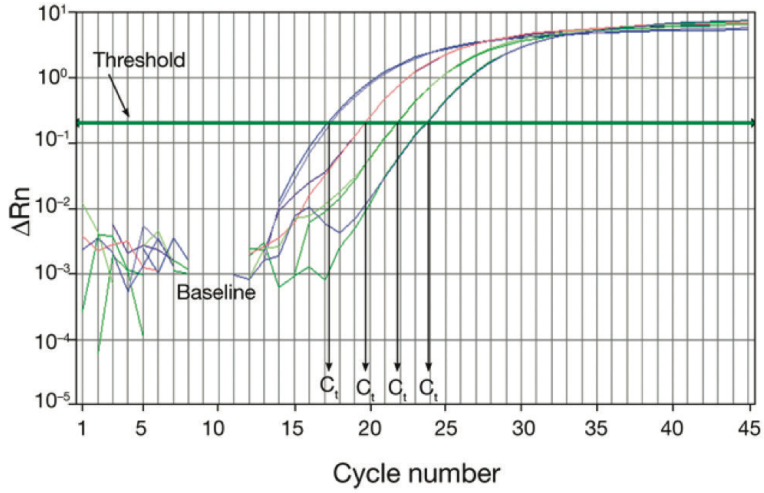


Figure 2-4: Amplification plot with indicated threshold and baseline levels. Threshold cycles (C_t values) for 4 different reactions indicated. Figure obtained from Real-time-PCR handbook (Thermo Fisher Scientific) [73].

For quantitatively comparing amplification between a gene of interest and a calibrator sample, the difference in C_t values (ΔC_t) can be used to calculate the fold difference using **Equation 1**.

$$Fold\ difference = 2^{\Delta C_t} \quad \textbf{(Equation 1)}$$

However, this form of comparison is inadequate as it does not normalize the samples in terms of sample quantity, quality or reaction efficiency. By including both a calibrator sample and an endogenous control, e.g. a housekeeping gene, the C_t value for the gene of interest is normalized in both the test sample and the calibrator sample. The resulting $\Delta\Delta C_t$ value can be used to calculate the relative quantification (RQ) between the test sample and the calibrator sample, while considering internal normalization (**Equation 2**).

$$Calibrator\ sample: C_{t\ GOI} - C_{t\ endo} = \Delta C_{t\ calibrator}$$

$$Sample: C_{t\ GOI} - C_{t\ endo} = \Delta C_{t\ sample}$$

$$\Delta C_{t\ sample} - \Delta C_{t\ calibrator} = \Delta\Delta C_t$$

$$RQ = 2^{-\Delta\Delta C_t} \textbf{ (Equation 2)}$$

From the C_t values, an algorithm in the QuantStudio™ Design and Analysis Software v1.5 (Applied Biosystems) calculates the relative quantification (RQ) between calibrator sample and test sample.

To assess the presence of primer dimers or non-specific amplification, a melting curve analysis was conducted. As the melting temperature of a DNA fragment is affected by various factors such as GC content and fragment length, different fragments can be distinguished from one another by their melting temperature. When the dsDNA product is heated, the strands dissociates into single strands. This dissociation will cause release of SYBR Green and a subsequent decrease in fluorescence signal. A dissociation plot is generated by plotting fluorescence against the temperature. The first derivative (-ΔF / ΔT) of the dissociation plot is plotted against temperature, yielding a graphical visualization of the melting point for the DNA fragment. **Figure 2-5** show an example of a -ΔF / ΔT vs. temperature plot, with fragment specific peaks between 80 and 85ºC and the presence of non-specific products melting at a lower temperature to the left (around 75 ºC).

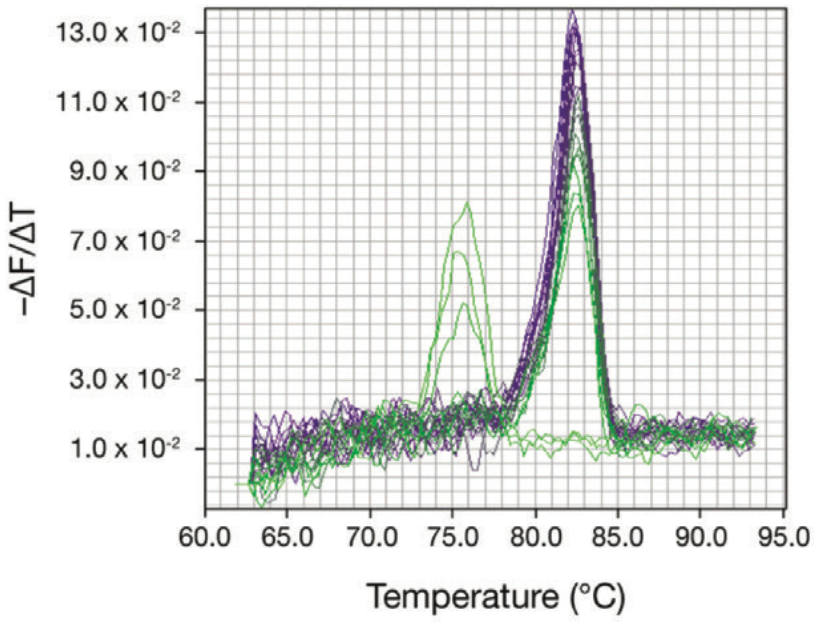


Figure 2-5: Example of -ΔF / ΔT vs. temperature (ºC) plot. Figure obtained from Real-time-PCR handbook (Thermo Fisher Scientific) [73].

2.3 Methods

2.3.1 Plasmid isolation

Cultures of strains containing the desired plasmid were grown overnight at 37°C and 225 RPM and plasmids were isolated using the Monarch Plamid Miniprep Kit (NEB) with accompanying protocol. Manufacturer's protocol is listed in Appendix C.1. The concentration of isolated plasmid was measured using the NanoDrop (NanoDrop One, Thermo Scientific). Plasmids intended for whole genome sequencing by Genscript were isolated using NucleoBond Xtra Midi (MACHEREY-NAGEL), manufacturer's protocol is listed in Appendix C.2.

C. glutamicum proved difficult to lyse with only the Plasmid Lysis Buffer (B2) from the Monarch Plasmid Miniprep Kit (NEB), additional lysozyme pre-treatment was necessary to get a sufficient concentration of isolated DNA. Two different strategies were used, a simpler lysozyme pre-treatment and a more extensive lysozyme treatment including different Tris buffers. The latter method was used in the beginning but later changed to cut down to the simpler method due to findings in the study.

Procedure: Lysozyme pre-treatment
C. glutamicum overnight cultures were pelleted by centrifugation (16 000 x g, 3.5 min) and resuspended in the kit's Plasmid Resuspension Buffer (B1) with 15 mg/ mL Chicken egg white lysozyme (Sigma-Aldrich) added. Samples were incubated at 37°C and 300 RPM in a heating block for two hours. After the lysis pre-treatment, 200 µL of the Plasmid Lysis Buffer (B2) was added and the manufacturer's protocol was followed as described.

Procedure: Tris and lysozyme pre-treatment
C. glutamicum overnight cultures were pelleted by centrifugation (11 000 RPM, 5 min) and washed with 1 mL of 10 mM Tris-HCl. The cell pellet was then frozen at -80°C for a minimum of 60 minutes and thawed at room temperature. The pellet was resuspended in 250 µL 0.5 M sucrose-10 mM Tris and lysozyme was added to 15 mg/mL. Samples were incubated at 37°C for 60 minutes and pelleted by centrifugation (16 000 x g, 3 min). The pellet was resuspended in 300 µL 10 mM Tris - 10 mM EDTA before 35 µL of 10% SDS was added and samples were incubated at room temperature for 15 minutes. Samples were pelleted by centrifugation (16 000 x g, 5 min) and the supernatant was transferred to a new Eppendorf tube and saved for a later step. The protocol of the Monarch Plasmid Miniprep Kit

(NEB) was then followed as described, with the saved supernatant being pooled with the rest of the sample after the neutralization step (between step 4 and 5 in the protocol, see protocol in Appendix C.1.

2.3.2 Restriction enzyme digestion

DNA fragments intended for restriction enzyme digestion were mixed together with the relevant restriction enzyme according to the reagents listed in **Table 2-3**. The restriction enzyme mix was incubated at 37°C for 1 to 18 hours. After complete digestion, fragments were separated using gel electrophoresis.

Table 2-3: Components for typical restriction enzyme digestion mix.

Component	Volume (µL)
10x CutSmart Buffer (NEB) or appropriate buffer	2.5
Restriction enzyme X	1
Restriction enzyme Y	1
DNA sample	10
CIP (NEB) (only added for digestion of backbone vector)	1
dH_2O	up to 25

An overview of the REs used in the construction of expression vectors with alternative reporters are given in **Table 8-4** in Appendix I.

2.3.3 Agarose gel electrophoresis

Prior to gel loading, samples were mixed with Gel Loading Dye Purple (6X, NEB). Samples were loaded on an 0.8% agarose gel with GelGreen or GelRed (Biotium) and run in 1x Tris-Acetate-EDTA (TAE) buffer. The 1 kb DNA Ladder (NEB) or 50 bp DNA Ladder (Invitrogen) was included in the run as a molecular weight size standard, the molecular weight ladders used in this study are included in Appendix E. Current was applied using the Bio-Rad PowerPac Basic Power Supply, voltage and run time were adapted to the fragments being separated. The ChemiDoc XRS+ (Bio-Rad) was used to image the agarose gel after terminated electrophoresis.

2.3.4 Extraction of DNA from agarose gel

DNA fragments were cut out from the gel and extracted using the Zymoclean Gel DNA Recovery Kit (Zymo Research) with accompanying protocol. Manufacturer's protocol is

listed in Appendix C.3. The concentration of extracted DNA was measured using the NanoDrop (NanoDrop One, Thermo Scientific).

2.3.5 DNA ligation

DNA ligation was used to seal the nicks in the constructed recombinant DNA. DNA ligation was performed using T4 DNA ligase from NEB. A molar ratio of 1:3 vector to insert was used when preparing the ligation mix. Components of the DNA ligation mix are shown in **Table 2-4**. The ligation mix was incubated at room temperature for 30-60 minutes and heat inactivated at 65°C for 10 minutes. The mix was then chilled on ice and 1-5 µL of the mix was used to transform a 100 µL of competent *E. coli* DH5α cells.

Table 2-4: Components of typical DNA ligation mix.

Component	20 µL reaction
10x T4 DNA Ligase Buffer (NEB)	2 µL
Vector DNA	50 ng
Insert DNA	1:3 molar vector:insert ratio
dH_2O	to 20 µL
T4 DNA Ligase (NEB)	1 µL

2.3.6 Polymerase chain reaction

In this study, PCR was used to amplify DNA sequences intended for cloning, for introducing restriction sites and for identifying the presence of construct in bacterial colonies. PCR was either conducted with isolated plasmids as DNA templates or with bacterial colonies (colony PCR).

Procedure PCR:

DNA holding the sequence desired for amplification and complementary primers were mixed together with reagents according to **Table 2-5**. The PCR reaction was then conducted in a thermocycler (Eppendorf® Mastercycler). **Table 2-6** show an example of cycling conditions used for a typical PCR reaction. The annealing temperature was altered to fit the optimal annealing temperature of the primer pair used in the reaction.

Table 2-5: Reagents used for Q5 PCR mix and the volume (µL) of each of the components per reaction.

Reagent	Volume (µL)
5x Q5 Reaction Buffer (NEB)	10
5x Q5 High GC Enhancer (NEB)	10
Forward primer (10 *Pm*ol/µL)	2.5
Reverse primer (10 *Pm*ol/µL)	2.5
dNTP 10 mM	1
Template DNA	1
Q5 DNA polymerase (NEB)	0.5
dH_2O	22.5

Table 2-6: Cycling conditions for a typical PCR program.

Number of cycles	Temperature (°C)	Time (minutes)
1	98	5
30	98	1
	55*	1
	72	0,5
1	72	5
1	4	Hold

* The annealing temperature was altered to fit the ideal annealing temperature of the primers.

Procedure colony PCR:

A fresh colony was picked from an agar plate with growing bacteria and resuspended in 20 µL PCR mix made of 12 µL dH_2O, 4 µL 5x Q5 Reaction Buffer (NEB) and 4 µL 5x Q5 High GC Enhancer (NEB). Samples were boiled at 95°C for 15 minutes in a PCR machine and centrifuged for 5 minutes at 2000x g. The top 10 µL of the supernatant was transferred to a new tube and treated as the template DNA. The PCR mix was prepared according to **Table 2-5**. An example of a PCR program used for colony PCR with an annealing temperature of 53°C is shown in **Table 2-7**. The annealing temperature was altered to fit the optimal annealing temperature for the primers used in each PCR reaction.

Table 2-7: Example of PCR program used for colony PCR. * The annealing temperature was altered to fit the ideal annealing temperature of the primers.

Number of cycles	Temperature (°C)	Time (minutes)
1	95	10
1	98	5
30	98	1
	53*	1
	72	0,5
1	72	5
1	4	Hold

* The annealing temperature was altered to fit the ideal annealing temperature of the primers.

2.3.7 Site-directed mutagenesis

The Q5 Site-Directed Mutagenesis Kit (NEB) and the ASK17 / ASK18 primer pair (A complete overview of primers are included in Appendix D) were used to introduce a *Hind*III site upstream of the GOI in pVB-4A0E1(-Not)-mCherry. The reagents used to set up a 25 µL PCR reaction is presented in **Table 2-8**, and the cycling conditions used for the SDM are presented in **Table 2-9**.

Table 2-8: Reagents and volumes of a 25 µL SDM PCR reaction.

Reagents	Volume (µL)
Q5 Hot Start High-Fidelity 2x Master Mix (NEB)	12.5
Forward primer ASK17 (10 *Pm*ol/ µL)	1.25
Revers primer ASK18 (10 *Pm*ol/ µL)	1.25
Template DNA pVB-4A0E1 (-NotI)-mCherry (1-25 ng)	1
Nuclease-free water	9

Table 2-9: Cycling condition for SDM PCR reaction.

Number of cycles	Temperature (ºC)	Time
1	98	30 sec
25	98	10 sec
	56	25 sec
	72	4,5 min
1	72	2 min
1	4	Hold

The PCR product was mixed with the kit's Kinase, Ligase & DpnI (KLD) Treatment according to **Table 2-10** and incubated at room temperature for 5 minutes. 5 µL of the KLD mix was used to transform 100 µL of competent *E. coli* DH5α as described in chapter 2.3.10

Table 2-10: Reagents of the KLD Treatment.

Reagents	Volume (µL)
PCR product	1
2X KLD Reaction Buffer	5
10X KLD Enzyme Mix	1
Nuclease-free water	3

2.3.8 Sequence- and ligation independent cloning (SLIC)

The CloneAmp HiFi PCR Premix (Takara Bio) and the accompanying protocol was used to amplify the pVB-4A0E1-mCherry backbone by PCR without the *Ps1* and *Ps2* promoters intended for SLIC. A master mix was prepared by adding the reagents listed in **Table 2-11**. Samples were cycled in a thermal cycler with the cycling conditions listed in **Table 2-12**. The elongation step in the PCR program is calculated from 60 sec / kb (60 sec* 9 kb = 9 minutes). The PCR product was treated with 1 µL *Dpn*I (NEB) and incubated at room temperature for 40 minutes. Fragments were separated using agarose gel electrophoresis and extracted using the Zymoclean Gel DNA Recovery Kit (Zymo Research). The concentration of extracted DNA was measured using the NanoDrop (NanoDrop One, Thermo Scientific).

Table 2-11: Reagents of the Takara Master Mix.

Reagent	Volume
CloneAmp HiFi PCR Premix (Takara Bio)	12.5 µL
FW primer: pVB4_XylS_SLIC_fw (10 *Pm*ol/µL)	0.75 µL
RV primer: pVB4_XylS_SLIC_rv (10 *Pm*ol/µL)	0.75 µL
Template DNA pVB-4A0E1-mCherry	> 100 ng (X µL)
Sterilized distilled water	up to 25 µL

Table 2-12: Cycling conditions for Takara 2-step PCR reaction.

Number of cycles	Temperature	Time
40	98°C	10 sec
	68°C	9 min (60 sec/kb)

Alternative promoters P_{tuf}, P_{sod}, P_{dapA} and P_{dapB} native to *C. glutamicum* were amplified using colony PCR as described in chapter **2.3.6.** The specific primers and the annealing temperatures used to amplify the four native promoters, P_{tuf}, P_{sod}, P_{dapA} and P_{dapB}, are listed in **Table 2-13**. Fragments of the colony PCR reactions were separated using agarose gel electrophoresis and extracted using the Zymoclean Gel DNA Recovery Kit (Zymo Research). The concentration of extracted DNA was measured using the NanoDrop (NanoDrop One, Thermo Scientific).

Table 2-13: Forward (FW) and reverse (RV) primer and the annealing temperature (TA) used to amplify the C P_{tuf}, P_{sod}, P_{dapA} and P_{dapB} promoters native to *C. glutamicum* by PCR, including the expected fragment size given in base pairs (bp).

Promoter amplified	FW primer	RV primer	TA (°C)	Fragment size (bp)
P_{tuf}	Ptuf-fw-SLIC	Ptuf-rv-SLIC	52	230
P_{sod}	Psod-fw-SLIC	Psod-rv-SLIC	48	222
P_{dapA}	PdapA-fw-SLIC	PdapA-rv-SLIC	45	231
P_{dapB}	PdapB-fw-SLIC	PdapB-rv-SLIC	49	230

For the SLIC reaction, linearized vector and promoter insert was mixed at a molar ratio of 1:2 together with the reagents listed in **Table 2-14**. Samples were mixed and briefly spun down. 0.5 µL of T4 DNA polymerase (NEB) was added to mix and samples were incubated at RT for 2.5 minutes and immediately transferred to ice to stop the reaction. Samples were incubated on ice for 10 minutes before 1.5 µL of the ligation mix was used to transform 100 µL of competent *E. coli* DH5α cells. Transformants were plated on selective LB agar plates with 25 ng/µL CM and incubated overnight at 37°C. Plasmids isolated from random colonies were sent for LightRun sequencing at Eurofins Genomics.

Table 2-14: Reagents of SLIC reaction.

Reagent	Volume
Linearized backbone + insert in a molar ratio of 1:2.	X µL
NEBuffer 2.1 (NEB)	1 µL
Sterilized distilled water	Up to 10 µL

2.3.9 Gibson Assembly

The native promoters P_{tuf}, P_{sod}, P_{dapA} and P_{dapB} were amplified by colony PCR from the genome of *C. glutamicum* MB001 (DE3) as described in chapter **2.3.8**. The pVB-4A0E1-mCherry expression vector was amplified in 3 fragments (A, B, C), excluding the *Ps1* and

Ps2 xylS promoters. PCR mixes were setup using pVB-4A0E1-mCherry as template with the reagents listed in **Table 2-5**. The specific primer pairs used for the 3 different reactions are listed in **Table 2-15** together with the specific annealing temperatures used in the PCR program. Fragments are designated A, B and C according to the information given in **Table 2-15**. The general cycling conditions used for the PCR amplification of fragment A, B and C are shown in **Table 2-16**. The complete sequences of all primers are listed in Appendix D.

Table 2-15: Forward (FW) and reverse (RV) primer used for amplifying the pVB-4A0E1-mCherry expression vector in 3 fragments (A, B, C) without the *Ps1* and *Ps2* promoters. The annealing temperature (TA) and expected fragment size for each of the PCR reactions are included.

Fragment	FW primer	RV primer	TA (°C)	Expected fragment size (bp)
A	pVB4_XylS_SLIC_rv	ASK21	63	3088
B	ASK22	ASK23	70	2960
C	ASK24	pVB4_XylS_SLIC_fw	60	2999

Table 2-16: Cycling conditions used for amplifying the pVB-4A0E1-mCherry expression vector in preparation for Gibson assembly. The annealing temperature (TA) was adjusted to the primer pair used, specific temperatures are listed in Table X.

Number of cycles	Temperature (°C)	Time (minutes)
1	98	5
30	98	1
	TA	1
	72	1,5
1	72	5
1	4	Hold

1 µL DpnI (NEB) were added to the PCR products and incubated at room temperature for 40 minutes. Fragments were separated using agarose gel electrophoresis and extracted using the Zymoclean Gel DNA Recovery Kit (Zymo Research). The concentration of extracted DNA was measured using the NanoDrop (NanoDrop One, Thermo Scientific).

The NEBuilder HiFI DNA Assembly Master Mix (NEB) was used to perform the Gibson assembly of the three pVB-4A0E1-mCherry fragments and the native *C. glutamicum* P_{tuf}, P_{sod}, P_{dapA} and P_{dapB} promoters, yielding four new vector constructs: pVB-4A0E1-mCherry_Ptuf, pVB-4A0E1-mCherry_Psod, pVB-4A0E1-mCherry_dapA and pVB-4A0E1-mCherry_dapB. Assembly reactions were set up on ice with the reagents listed in **Table 2-17**. Samples were incubated at 50°C for 60 minutes in a thermocycler and immediately transferred to ice after

incubation. 2 µL of the assembly mix was used to transform 100 µL of competent *E. coli* DH5α cells as described in chapter **2.3.10.** Transformed cells were plated on selective LA plates with 25 ng/µL CM added and incubated at 37°C overnight. Plasmids isolated from random colonies were sent for LightRun sequencing at Eurofins Genomics.

Table 2-17: Components of the Gibson Assembly mix.

Reagent	4 Fragment Assembly
Backbone fragment A, B, C	0.05 *Pm*ol of each (X µL)
Promoter fragment	0.05 *Pm*ol (Y µL)
NEBuilder HiFI DNA Assembly Master Mix (NEB)	10 µL
Deionized water	10 - X - Y µL
Total volume	20 µL

2.3.10 Transformation of *E. coli*

Preparation of RbCl competent *E. coli* cells

Wild type *E. coli* inoculated and incubated ON at 37°C and 225 RPM. 0.5 mL of the ON culture was inoculated in 50 mL Psi medium and incubated at 37°C and 225 RPM until OD600 reached 0.4. The culture was then incubated on ice for 15 minutes before cells were harvested (4000 RPM, 5 min, 4°C). Cells were carefully resuspended in 20 mL cold TFB1 and incubated in ice for 5-15 minutes. Cells were harvested (4000 RPM, 5 min, 4°C) and resuspended in 1.5 mL cold TFB2. 100 µL aliquots were made and cells were snap-freezed with liquid nitrogen and stored at -80°C.

Heat-shock transformation of competent *E. coli* cells

RbCl competent cells were thawed on ice, when completely thawed, the desired vector was added to the cells and the mix was incubated on ice for 20 minutes. Cells were then heat-shocked in a 42°C water bath for 35 seconds and immediately transferred to ice and incubated for a minimum of 2 minutes. 900 µL of pre-heated (37°C) SOC was added and cells were incubated at 37°C and 225 RPM for 1 hour. Cells were then plated on selective LB agar plates.

2.3.11 Transformation of *C. glutamicum*

Preparation of competent *C. glutamicum* cells:
A pre-culture of *C. glutamicum* MB001 (DE3) WT was incubated overnight in 5 mL BHIS medium at 30°C and 225 RPM. 500 µL of the pre-culture was inoculated in 25 mL BHIS and incubated at 30°C and 225 RPM until OD600 was 1.5. Cells were then harvested by centrifugation (4500 RPM, 5 min, 4°C) and resuspended in 25 mL cold TG buffer. Cells were harvested again at the same conditions and washed in 25 mL cold 10% glycerol solution and then centrifuged using the same conditions as previously. The supernatant was discarded and the pellet was resuspended in the back-flow supernatant.

Transformation of competent *C. glutamicum* cells by electroporation:
To a volume of 100 µL of competent *C. glutamicum* cells, 0.1-10 µg of DNA was added. After carefully mixing cells and DNA, cells were transferred to a cold 0.2 mm electroporation cuvette (BioRad) and electroporated at 2500 V, 25 µF and 200 Ω in a GenePulser Xcell (BioRad). Immediately after electroporation, cells were transferred to 4 mL 46°C pre-heated BHIS medium and incubated at 46°C for 6 minutes. Cells were then regenerated at 37°C and 225 RPM for 1 hour and then at 30°C and 225 RPM for 30 min. After regeneration, cells were centrifuged (4500 RPM, 5 min), resuspended in back-flow and plated on selective BHIS plates.

2.3.12 Expression and detection of reporter protein

For expression, strains of *E. coli* BL21 and *C. glutamicum* MB001(DE3) were used. Strains were inoculated in 4 mL media, BL21 in LB and *C. glutamicum* in BHIS, with appropriate antibiotics added. Pre-cultures were incubated over night at 37°C and 225 RPM. Expression experiment was conducted in 250 mL shake flasks and in 48-well FlowerPlates (m2p-labs) in a BioLector (m2p-labs).

Expression of reporter protein in shake flasks:
Production cultures were made by inoculating pre-culture in 30 mL Hi + Ye media with appropriate antibiotics to a starting OD_{600} of 0.01 in 250 mL baffled flasks. Cultures were incubated at 30°C and 225 RPM until OD600 reached 1 and then induced with the appropriate inducer. pXMJ19 vectors with the IPTG inducible P*tac* promoter were induced with 1 mM IPTG and XylS/*Pm* vectors were induced with 2mM m-toluate. After induction, cultures were

incubated overnight at 25°C and 225 RPM. The next day, OD600 was measured for all production cultures.

Expression of reporter protein in FlowerPlate

Production cultures of 1200 µL were made by inoculating pre-culture in Hi + Ye media with appropriate antibiotics to a starting OD_{600} of 0.2 in a 48-well FlowerPlate without optodes (m2p-labs). Production cultures were incubated in a BioLector (m2p-labs) at 30°C and 1300 RPM until the majority of the cultures reached OD600 1-2. Cultures were then induced with appropriate inducer, 12 µL 0.1M IPTG (final concentration of 1 mM IPTG) for pXMJ19 vectors and 4,8 µL 0.5M m-toluate (final concentration of 2 mM m-toluate) for XylS / *Pm* vectors. After induction, the plate was incubated at 25°C and 1300 RPM overnight. OD_{600} and RFU of the overnight cultures were measured using the Infinite 200pro (Tecan) microplate reader. Measured RFU values were normalized against OD600 values.

2.3.13 Fluorometry

For fluorescence measurements, 20 µL bacterial culture was diluted 1:10 with 180 µL 1x phosphate-buffered saline (1x PBS) in a black bottom plate and relative fluorescence units (RFU) was measured using Infinite 200pro microplate reader (Tecan). An orbital shaking step was added before RFU values were measured, with shaking duration and amplitude set to 15 seconds and 3 mm. Excitation and emission wavelengths used to measure RFU for mCherry were 584 nm and 620 nm, respectively. Excitation and emission wavelengths used to measure RFU for GFP were 485 nm and 515 nm, respectively.

2.3.14 SDS-PAGE

Cultures were harvested by centrifugation (10 000 g, 10 min, 10°C) and wet weight determined. Samples were normalized by resuspending cell pellets in 1 mL 1x PBS / mg wet weight. 1 mL of normalized samples were taken out and transferred to new 1.5 mL Eppendorf tubes, cells were then harvested (10 000 g, 5 min, 4°C) and resuspended in 250 µL CelLytic B Cell Lysis Reagent (Sigma-Aldrich) with 15 mg/mL lysozyme (Sigma-Aldrich) and 0.5 µL benzonase nuclease (Sigma-Aldrich) added. *C. glutamicum* samples were incubated at 37°C and 300 RPM for 2 hours, *E. coli* samples were incubated at the same conditions for 1 hour. The lysate was centrifuged (10 000 g, 5 min, 4°C) and the supernatant, the soluble fraction, was transferred to a new 1.5 mL Eppendorf tube. The remaining cell pellet, the insoluble fraction, was resuspended in 500 µL 1x SDS Running Buffer. Both the soluble and insoluble

fraction was diluted 1:15 with 1x SDS Running Buffer. 20 µL 1:15 diluted sample was mixed with 10 µL 3x loading dye containing SDS and DTT and boiled at 95°C for 5 minutes. 10 µL of each samples was loaded on to a 12% ClearPAGE SDS Gel (C.B.S Scientific), the Precision Plus Protein Dual Colour standards (Bio-Rad) ladder was also included, a figure including the molecular weight markers in ladder is included in Appendix E. Electrophoresis was run for 100 minutes at 130V in 1x SDS Running Buffer. Gels were stained with InstantBlue (Expedeon) for 1 hour in room temperature or overnight in the fridge and imaged with the ChemiDoc XRS+ (Bio-Rad).

2.3.15 Western blot

While mCherry was detected by manually changing the different solutions, XylS was detected by using the iBind Flex Western System (Thermo Fisher Scientific). For detection of XylS, a positive control containing 50 ng purified XylS (GenScript) was included in the SDS-PAGE run. The Trans-Blot Turbo System (Bio-Rad) was used to transfer proteins from the SDS gel to the nitrocellulose membrane.

Solutions used to detect mCherry present are included in Appendix A.5. The membrane was treated with skim milk blocking solution and incubated for a minimum of 1 hour in room temperature. The membrane was then rinsed with 1x PBS and incubated in the primary antibody solution overnight in a cold environment. After rinsing in 30 mL 1x PBST for 3x10 minutes, the membrane was incubated for 1 hour in the secondary antibody solution at room temperature. The membrane was washed in 30 mL 1x PBST for 3x10 minutes and in 30 mL 1x PBS for 10 minutes before it was stained with 2 mL 3,3',5,5'-Tetramethylbenzidine (TMB, Sigma-Aldrich). The staining reaction was stopped after 1-10 minutes with the adding of water.

XylS detection using the iBind Flex Western System:

Solutions used for the iBind Flex Western system are included in Appendix B. The blotted membrane was immerged in 15 mL 1x iBind Flex Solution while the other solutions were prepared (~30 minutes). The iBind Flex Card was placed on the stage and 10 mL 1x iBind Flex Solution was evenly distributed across the flow region, the membrane was placed on top of the card with the protein-side down and the low molecular weight region closest to the stack. Solutions were added to the insert wells according to the information given **Table 8-2** in Appendix B. The insert lid was closed and the membrane was incubated for a minimum of

2.5 hours. After incubation, the membrane was rinsed twice with 20 mL of distilled water for 2 minutes. For staining of the membrane, 2 mL of 3,3',5,5'-Tetramethylbenzidine (Sigma-Aldrich) was added to the membrane. After 1-10 minutes, the reaction was stopped with distilled water.

2.3.16 RNA isolation

The following *C. glutamicum* strains were inoculated in liquid BHIS with appropriate antibiotics added and grown overnight at 37°C: *C. glutamicum* WT, *C. glutamicum* pXMJ19-mCherry and *C. glutamicum* pVB-4A0E1-mCherry. Two production cultures per strain were inoculated in Hi+Ye media with appropriate antibiotics to a starting OD_{600} of 0,05. Cultures were incubated at 30°C until OD_{600} reached about 2 and induced with appropriate inducer, 1mM IPTG for pXMJ19 vectors and 2mM m-toluate for XylS / *Pm* vectors. After induction, cultures were incubated overnight at 25°C. 1 mL samples were taken from the culture for RNA isolation 1 hour and 3 hours after induction, a sample was also taken the next morning. Samples taken for RNA isolation were pelleted (16 000 x g, 2 min) and pellet resuspended in 2 mL RNAlater (Ambion), samples were stored in the fridge overnight and moved to -80°C the next day.

For the RNA isolation, samples were diluted to 1mL with OD_{600} of 1The Quick-RNA Fungal / Bacterial Miniprep Kit (Zymo Research) was used for isolating total RNA from *C. glutamicum*. The associated protocol was followed, but an additional lysis treatment was added. Cells were harvested from the 1 mL samples by centrifugation (16 000g, 3 min) and resuspended in 800 µL RNA Lysis Buffer (Zymo Research). 5.33 µL of the Ready-Lyse Lysozyme Solution (Lucigen) was added to each tube before the samples were transferred to the ZR BashingBead™ Lysis Tube. Samples were incubated at 37°C and 225 RPM for 40 minutes. Samples were then disrupted using a TissueLyser II (QIAGEN) for 3 x 5 minutes at 30 Hz. The manufacturer's protocol was then followed as described from step 3, the complete protocol is included in Appendix C.4.

The 2100 Bioanalyzer instrument (Agilent Technologies) and the associated 2100 Expert Software (Agilent Technologies) was used to assess the RNA quality of isolated samples from *E. coli* and *C. glutamicum*. The system's "Prokaryote Total RNA Nano" program was selected. The chip was prepared according to the manufacturer's protocol.

2.3.17 cDNA synthesis

cDNA synthesis was performed using the SuperScript IV VILO Master Mix with ezDNase enzyme (Invitrogen). The kit combines both genomic DNA (gDNA) digestion and cDNA synthesis. Components for gDNA digestion (**Table 2-18**) was mixed in a tube on ice and samples were incubated at 37°C for 2 minutes. Samples were briefly centrifuged and put back on ice. Components for cDNA synthesis were added to the tube and samples were treated with a series of temperature steps in a thermo cycler, temperature steps are given in **Table 2-19**. The concentration of cDNA was measured using the NanoDrop One (Thermo Scientific) and samples were stored at -80°C.

Table 2-18: Components of the 10 µL gDNA digestion reaction and the added cDNA synthesis components.

Components gDNA digestion	**Volume**
10X ezDNase Buffer	1 µL
ezDNase enzyme	1 µL
Template RNA (1pg - 2.5µg total RNA)	varies
Nuclease-free water	to 10 µL
Components added for cDNA synthesis	
SuperScript IV VILO Master Mix	4 µL
Nuclease-free water	6 µL
Total volume cDNA synthesis:	20 µL

Table 2-19: Temperature steps and incubation time for cDNA synthesis

Step	**Temperature (°C)**	**Time (minutes)**
Primer annealing	25	10
Reverse transcription	50	10
Enzyme inactivation	85	5

2.3.18 qPCR

The PowerUp SYBR Green Master Mix (Applied Biosystems) and QuantStudio 5 Real-Time PCR System (Applied Biosystems) were used for setting up the qPCR. cDNA samples were diluted 1:100 and a series of 10-fold dilutions were made from the 1:100 diluted sample.

Components listed in **Table 2-20** were mixed in a MicroAmp® EnduraPlate™ Optical 96-Well Clar Reaction Plate (Applied Biosystems), primer pairs used are listed in **Table 2-21**.

Oligonucleotide sequences for the primer pairs used for qPCR are included in Appendix D. After mixing, the plate was centrifuged briefly and reactions were run in the QuantStudio 5 Real-Time PCR System (Applied Biosystems). Cycling conditions used are given in **Table 2-22**. The QuantStudio™ Design and Analysis Software v1.5 (Applied Biosystems) was used to analyse the generated data.

Table 2-20: Components of qPCR reaction mix.

Component	Volume
PowerUp SYBR Green Master Mix (2X)	10 µL
Forward primer (10 *Pm*ol/µL)	1 µL
Reverse primer (10 *Pm*ol/µL)	1 µL
cDNA template	2 µL
Nuclease-free water	6 µL
Total volume	20 µL

Table 2-21: Primer pairs used for qPCR.

FW primer	RV primer	Description of use
ASK11	ASK12	Amplification of 16S housekeeping gene in *C. glutamicum*
ASK25	ASK26	Amplification of *mCherry*
E.c-16S-f	E.c-16S-r	Amplification of 16S housekeeping gene in *E. coli*

Table 2-22: Cycling condition for qPCR with subsequent melting curve analysis steps**.**

Number of cycles	Temperature (°C)	Time
1	50	2 min
1	95	10 min
40	95	25 sec
	60	1 min
1	95	1 sec
1	60	20 sec
1	95	1 sec

3 Results

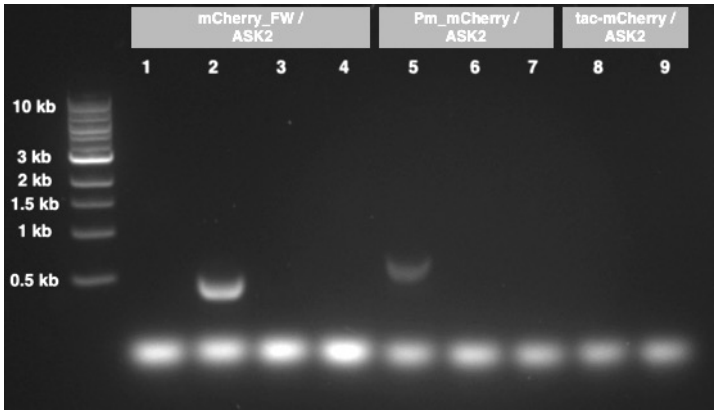
3.1 Verifying the pXMJ19-mCherry and pVB-4A0E1-mCherry constructs in *C. glutamicum*

The work in this thesis is a continuation of the work conducted by a previous master student [55]. During the previous work, there were several problems related to the constructed recombinant *C. glutamicum* strains. This includes insufficient lysis, cloning problems thought to be related to an incorrect pXMJ19 plasmid map and trouble of verifying the presence of constructed expression vectors in *C. glutamicum*. As one of the problems in the previous work had been unexpected fragment patterns on gel after RE digestion and gel electrophoresis of plasmids isolated from *C. glutamicum*, this experiment was repeated with four thought to be single-cut REs, with the intention of linearizing the plasmids. After digestion, none of the plasmids displayed the expected single bands associated with linearization. Instead all samples had the same smear tendency, with some stronger size specific bands, indicating what the previous work proposed, a potential problem related to incorrect plasmid maps. The image of the gel of the linearization digestions are included in **Figure 8-7** in Appendix G.2. To ensure correct plasmid maps, the plasmids pXMJ19-mCherry and pVB-4A0E1-mCherry were sequenced. The generated plasmid maps are presented in **Figure 8-4** and **Figure 8-5** in Appendix F. Compared to the previously used plasmid maps, the generated plasmid maps differed from the previous ones in eight different places, most of them being single base changes. However, according to the new plasmid maps, none of the REs used in the linearization had more than one recognition site in the plasmid, meaning that the linearization should have worked in the previous experiments. This indicated that the plasmids isolated from recombinant *C. glutamicum* were not pXMJ19-mCherry or pVB-4A0E1-mCherry.

3.1.1 Construct verification by colony PCR

In parallel to the plasmid sequencing, colony PCR and subsequent gel electrophoresis were used to verify the presence of pXMJ19-mCherry and pVB-4A0E1-mCherry in *C. glutamicum*. If containing the correct construct, amplification with primer pair FW_mCherry / ASK2 would amplify a region within *mCherry* and give a fragment of 582 bps, FW_Pm_mCherry / ASK2 would amplify *Pm* / UTR plus *mCherry* and give a fragment of 794 bps and FW_tac_mCherry / ASK2 would amplify the *tac* promoter plus *mCherry* and give a fragment of 908 bps. The functionality of the primer pairs was first verified in *E. coli* harbouring pXMJ19-mCherry and pVB-4A0E1-mCherry. An image of the agarose gel showing expected

fragments for *E. coli* are included in **Figure 8-6** in Appendix G.1. Following primer verification, colony PCR were conducted for verification of constructs in *C. glutamicum*, *E. coli* was included as a positive control. An image of the agarose gel after electrophoresis of PCR product is included in **Figure 3-1**. Positive mCherry amplification was only detected for *E. coli* pVB-4A0E1-mCherry amplified with mCherry_FW / ASK2 (lane 2) and Pm_mCherry / ASK2 (lane 5). No amplification indicating presence of pXMJ19-mCherry or pVB-4A0E1-mCherry was detected for *C. glutamicum*.



Lane	Strain	Primer pair
1	*C. glutamicum* pXMJ19	mCherry_FW / ASK2
2	*E. coli* DH5α pVB-4A0E1-mCherry	mCherry_FW / ASK2
3	*C. glutamicum* pXMJ19-mCherry	mCherry_FW / ASK2
4	*C. glutamicum* pVB-4A0E1-mCherry	mCherry_FW / ASK2
5	*E. coli* DH5α pVB-4A0E1-mCherry	Pm_mCherry / ASK2
6	*C. glutamicum* pVB-4A0E1-mCherry	Pm_mCherry / ASK2
7	*C. glutamicum* pXMJ19	Pm_mCherry / ASK2
8	*C. glutamicum* pXMJ19	tac_mCherry / ASK2
9	*C. glutamicum* pXMJ19-mCherry	tac_mCherry / ASK2

Figure 3-1: Agarose gel after colony PCR and gel electrophoresis of *C. glutamicum* MB001 (DE3) harbouring pXMJ19, pXMJ19-mCherry or pVB-4A0E1-mCherry. Lane content given in Table X. Ladder: 1 kb DNA ladder (NEB).

3.1.2 Verifying constructs through mCherry expression

In parallel to plasmid sequencing and colony PCR, mCherry expression from pXMJ19-mCherry and pVB-4A0E1-mCherry in *C. glutamicum* was evaluated in an expression experiment with *E. coli* pXMJ19-mCherry and pVB-4A0E1-mCherry included as positive controls. The induced expression of mCherry measured as relative fluorescence units (RFU) normalized against OD_{600} (cell density) at harvesting is included in **Figure 3-2**. mCherry fluorescence was detected for the induced pXMJ19-mCherry and pVB-4A0E1-mCherry in *E.*

coli, but not for *C. glutamicum*, indicating no presence of functional mCherry protein in *C. glutamicum*. For the non-induced samples, mCherry was detected for *E. coli* pXMJ19-mCherry and pVB-4A0E1-mCherry, but not in any of the *C. glutamicum* samples (data not shown). The protein content of these samples was evaluated using SDS-PAGE and Western blot, images of the SDS-PAGE gel and Western blot for the soluble fraction are included in **Figure 3-3**. Images of the insoluble fraction are included in **Figure 8-8** Appendix G.3. Both the SDS-PAGE and the Western blot show equivalent results to the expression experiment, with mCherry only being detected for *E. coli* pXMJ19-mCherry (lane 13 and 14) and pVB-4A0E1-mCherry (lane 15 and 16).

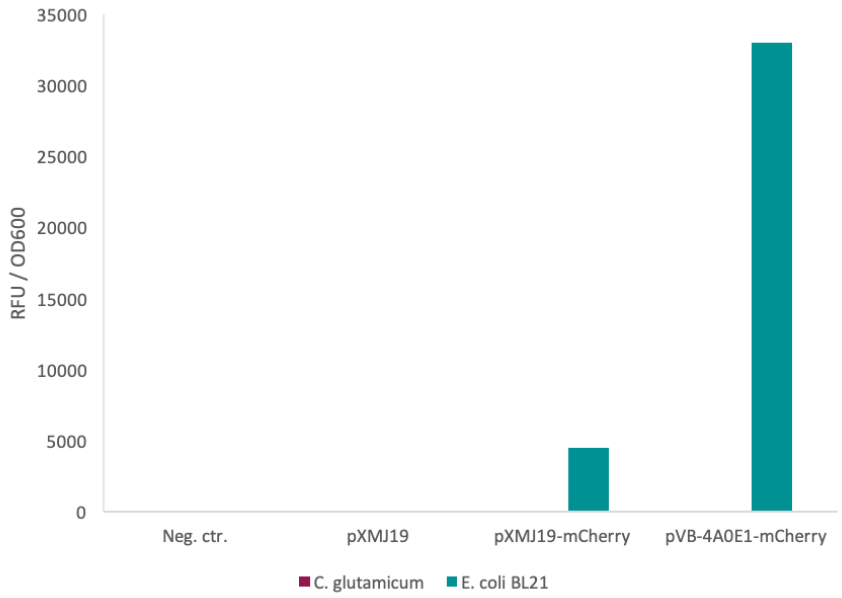
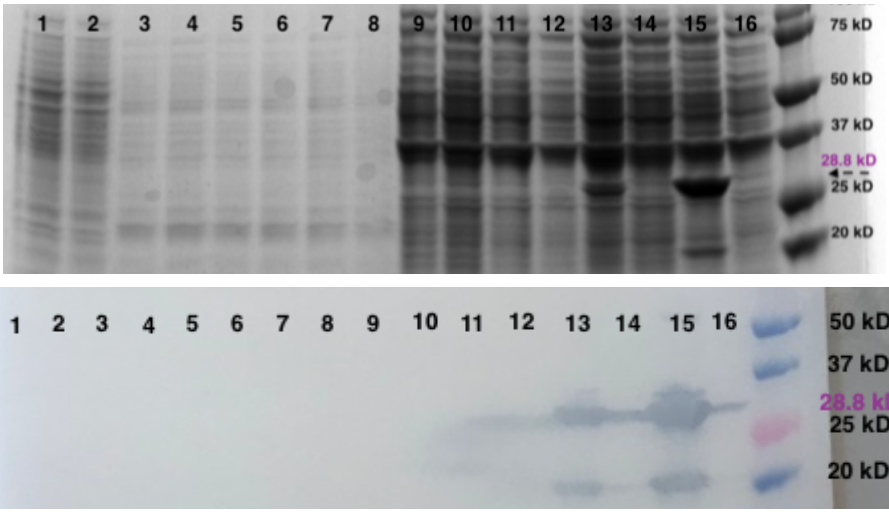


Figure 3-2: mCherry expression in *C. glutamicum* and *E. coli* measured as relative fluorescence units (RFU) / OD_{600} at harvesting. Induced samples only.



Lane	Strain	Lane	Strain
1	*C. glutamicum* (no vector) I	9	*E. coli* BL21 pVB1 I
2	*C. glutamicum* (no vector) NI	10	*E. coli* BL21 pVB1 NI
3	*C. glutamicum* pXMJ19 I	11	*E. coli* BL21 pXMJ19 I
4	*C. glutamicum* pXMJ19 NI	12	*E. coli* BL21 pXMJ19 NI
5	*C. glutamicum* pXMJ19-mCherry I	13	*E. coli* BL21 pXMJ19-mCherry I
6	*C. glutamicum* pXMJ19-mCherry NI	14	*E. coli* BL21 pXMJ19-mCherry NI
7	*C. glutamicum* pVB-4A0E1-mCherry I	15	*E. coli* BL21 pVB-4A0E1-mCherry I
8	*C. glutamicum* pVB-4A0E1-mCherry NI	16	*E. coli* BL21 pVB-4A0E1-mCherry NI

Figure 3-3: SDS-PAGE gel (top image) and Western blot of soluble fraction (bottom image) for *C. glutamicum* MB001 (DE3) and *E. coli* BL21. Lane content given in the table below. Size of mCherry (28.8 kD) indicated in purple. Ladder: Precision Plus Protein Dual Color standards (Bio-Rad).

3.1.3 Antibiotic resistance assay

As none of the previous assays could detect the presence of expression vectors in *C. glutamicum*, an antibiotic resistance assay was conducted to rule out the possibility of *C. glutamicum* being naturally resistant to chloramphenicol (CM). If this was the case, it could be a possible explanation to why the bacterium was able to grow in the selective CM media without harbouring the expression vector. Overnight cultures of *C. glutamicum* pXMJ19, pXMJ19-mCherry, pVB-4A0E1-mCherry and *C. glutamicum* (no vector) were plated on BHIS agar plates with 0, 5, 25 and 50 ng/mL CM added. **Figure 3-4** show the plates after 3 days of incubation at room temperature. Growth of *C. glutamicum* (no vector) (the leftmost column) was only detected on the plate without CM and not on any of the plates with CM added. The recombinant *C. glutamicum* strains were able to grow on all the plates. However, the recombinant strains seemed to have a different colour than the *C. glutamicum* (no vector) strain. While *C. glutamicum* (no vector) have a distinct yellow colour, the vector carrying

strains seemed whiter, indicating a contamination. Based on this, it was decided to redo the transformation of *C. glutamicum* with expression vectors isolated from *E. coli* DH5α.

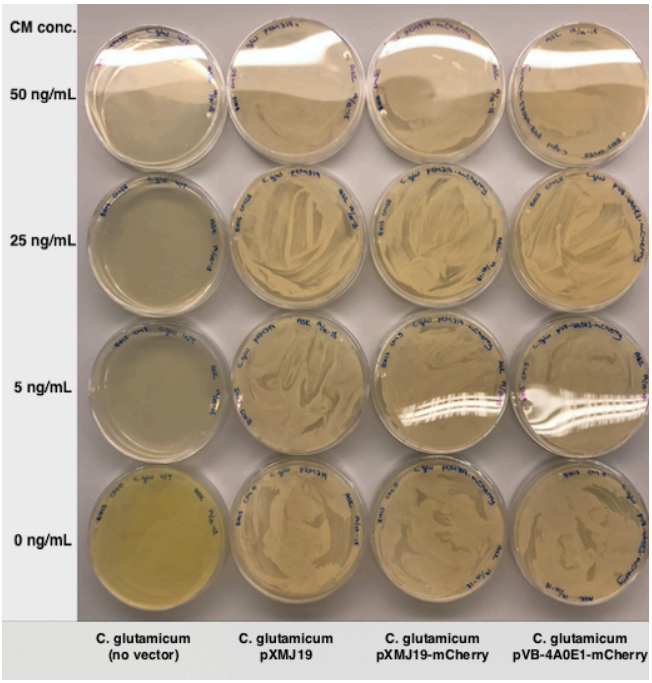


Figure 3-4: Antibiotic resistance assay for *C. glutamicum* MB001 (DE3). From the left: *C. glutamicum* (no vector) (column 1), *C. glutamicum* pXMJ19 (column 2), *C. glutamicum* pXMJ19-mCherry (column 3) and *C. glutamicum* pVB-4A0E1-mCherry (column 4) plated on BHIS plates with chloramphenicol (CM) added in the following concentrations from the top: 50 ng/mL (row 1), 25 ng/mL (row 2), 5 ng/mL (row 3) and 0 ng/mL (row 4).

The same antibiotic resistance assay was conducted with the new transformants. **Figure 8-9** in Appendix G.4 shows the different CM concentration plates with the new *C. glutamicum* pXMJ19, pXMJ19-mCherry and pVB-4A0E1-mCherry transformants after incubation. Like the old ones, the new transformants could grow on all four CM concentrations. In **Figure 3-5** below, the new transformants are aligned with the "old" *C. glutamicum* pVB-4A0E1-mCherry BHIS CM25 plate and the *C. glutamicum* (no vector) BHIS CM0 plate (same as in **Figure 3-4**) for easier comparison. The new transformants seemed to resemble the yellow colouring seen for *C. glutamicum* (no vector). To further investigate if the new transformants were different than the previously used strains, colony PCR and expression experiments were conducted.

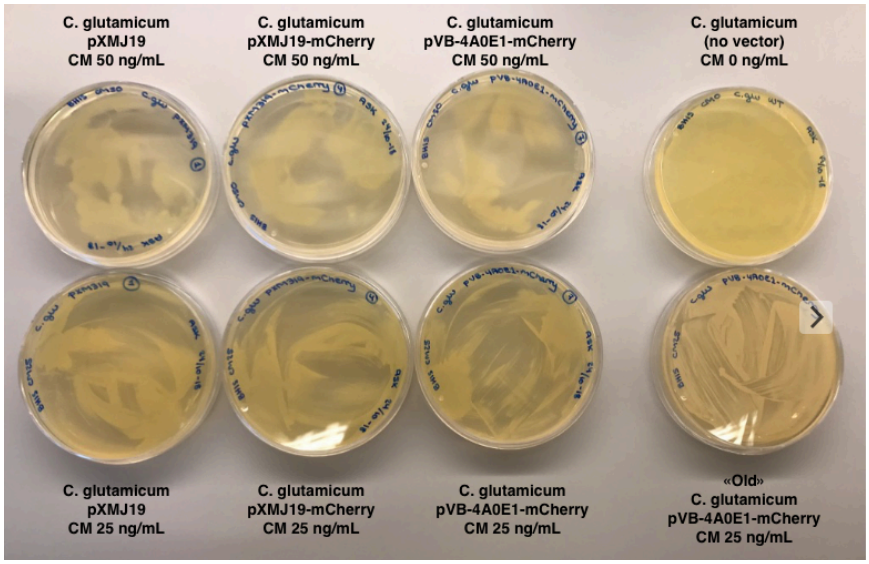
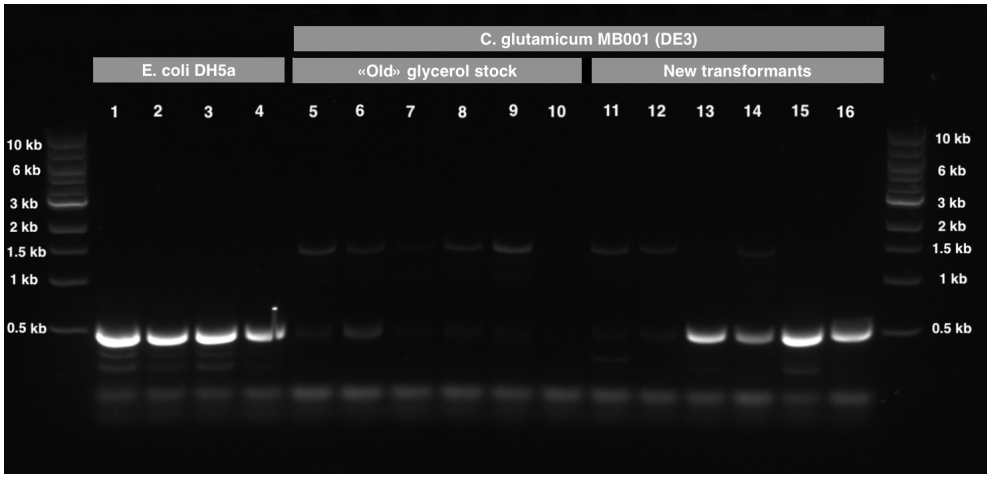


Figure 3-5: Comparing new *C. glutamicum* pXMJ19, pXMJ19-mCherry and pVB-4A0E1-mCherry transformants with *C. glutamicum* (no vector) and the "old" *C. glutamicum* pVB-4A0E1-mCherry.

3.1.4 Verifying presence of constructs in new *C. glutamicum* transformants

Using the primers mCherry_FW and ASK2 primer pair, new *C. glutamicum* transformants were screened for the presence of mCherry constructs. The old *C. glutamicum* glycerol stock and *E. coli* were included as control samples. All samples were run with two different DNA concentrations, 1:1 and 1:10. PCR products were separated using gel electrophoresis, an image of the gel is included in **Figure 3-6**. The expected 582 bps band was present for the positive *E. coli* controls (lane 1-4) and in the new transformants of *C. glutamicum* harbouring pXMJ19-mCherry (lane 13/14) and pVB-4A0E1-mCherry (lane 15/16). There seems to be some unspecific amplification around 1.5 kB for some of the *C. glutamicum* samples, weak bands are also present around the expected mCherry size of 582 bps for some of the *C. glutamicum* samples without mCherry inserts, e.g., in lane 5, 6, 11 and 12.



Lane	Strain	Lane	Strain
1	*E. coli* DH5α pVB-4A0E1-mCherry (1:1)	9	Old *C. glutamicum* pVB-4A0E1-mCherry (1:1)
2	*E. coli* DH5α pVB-4A0E1-mCherry (1:10)	10	Old *C. glutamicum* pVB-4A0E1-mCherry (1:10)
3	*E. coli* DH5α pXMJ19-mCherry (1:1)	11	New *C. glutamicum* pXMJ19 (1:1)
4	*E. coli* DH5α pXMJ19-mCherry (1:10)	12	New *C. glutamicum* pXMJ19 (1:10)
5	Old *C. glutamicum* pXMJ19 (1:1)	13	New *C. glutamicum* pXMJ19-mCherry (1:1)
6	Old *C. glutamicum* pXMJ19 (1:10)	14	New *C. glutamicum* pXMJ19-mCherry (1:10)
7	Old *C. glutamicum* pXMJ19-mCherry (1:1)	15	New *C. glutamicum* pVB-4A0E1-mCherry (1:1)
8	Old *C. glutamicum* pXMJ19-mCherry (1:10)	16	New *C. glutamicum* pVB-4A0E1-mCherry (1:10)

Figure 3-6: Agarose gel after colony PCR and gel electrophoresis of *C. glutamicum*, old and new transformants, and *E. coli* DH5α positive controls. Lane content given in Table X. Ladder: 1 kb DNA ladder (NEB).

To further examine if the new transformants were harbouring vectors capable of expressing mCherry, an expression experiment with induced samples only was conducted (**Figure 3-7**) mCherry fluorescence was detected for the new *C. glutamicum* transformants from both the pXMJ19-mCherry (729 RFU/OD_{600}) and pVB-4A0E1-mCherry mCherry (186 RFU/OD_{600}) expression vectors. Compared to the positive BL21 control, the mCherry fluorescence level was much lower in the new *C. glutamicum* transformants, with 19 609 RFU/OD_{600} detected for *E. coli* BL21 pVB-4A0E1-mCherry.

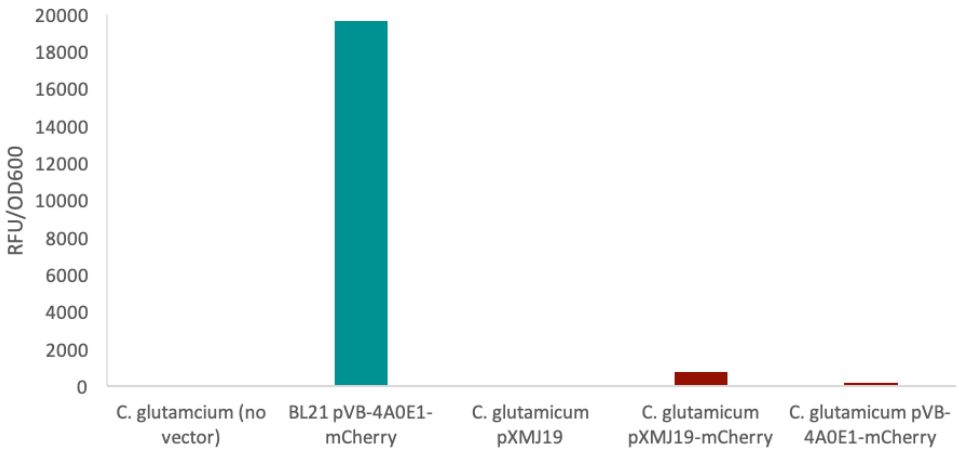
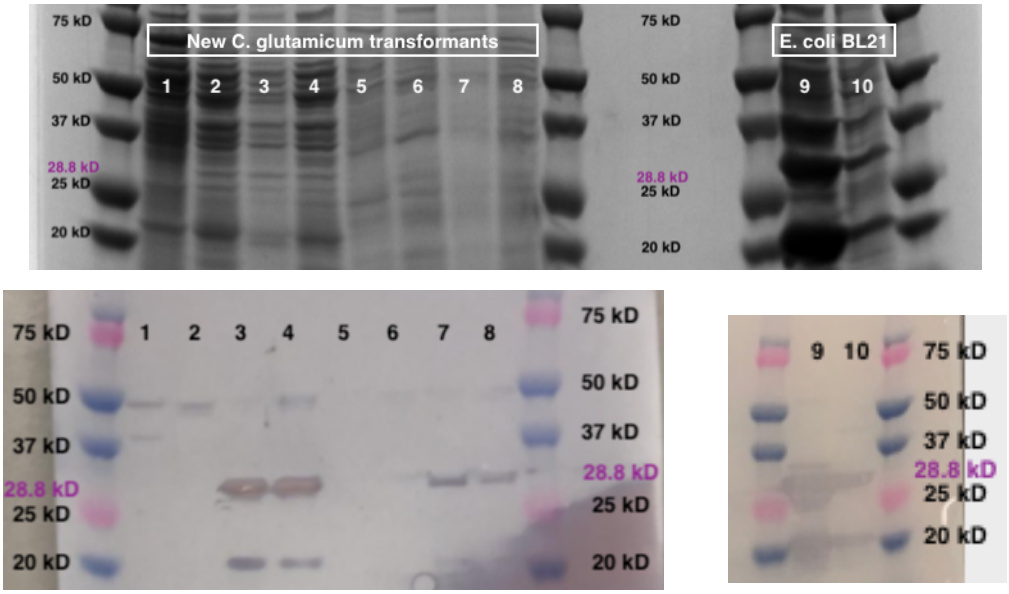


Figure 3-7: mCherry expression in new *C. glutamicum* transformants, *C. glutamicum* (no vector) and *E. coli* BL21 pVB-4A0E1-mCherry included as negative and positive control, respectively. mCherry expression measured as relative fluorescence units (RFU) / OD_{600} at harvesting. Induced samples only.

mCherry presence in the new *C. glutamicum* transformants were assayed using SDS-PAGE and Western blot (**Figure 3-8**). On the SDS-PAGE gel, the positive BL21 pVB-4A0E1-mCherry control had strong bands corresponding to the expected size of mCherry (28.8 kD) in both the soluble (lane 9) and insoluble (lane 10) fraction. For *C. glutamicum*, no strong bands corresponding to mCherry overproduction were visible (lane 1-8). In the Western blot, bands corresponding to mCherry were present for *C. glutamicum* pXMJ19-mCherry (lane 3 and lane 7) and pVB-4A0E1-mCherry (lane 4 and lane 8), as well as in the positive BL21 control (lane 9 and lane 10).



Lane	Strain	Lane	Strain
1	*C. glutamicum* (no vector) (sol)	6	*C. glutamicum* pXMJ19 (insol)
2	*C. glutamicum* pXMJ19 (sol)	7	*C. glutamicum* pXMJ19-mCherry (insol)
3	*C. glutamicum* pXMJ19-mCherry (sol)	8	*C. glutamicum* pVB-4A0E1-mCherry (insol)
4	*C. glutamicum* pVB-4A0E1-mCherry (sol)	9	*E. coli* pVB-4A0E1-mCherry (sol)
5	*C. glutamicum* (no vector) (insol)	10	*E. coli* pVB-4A0E1-mCherry (insol)

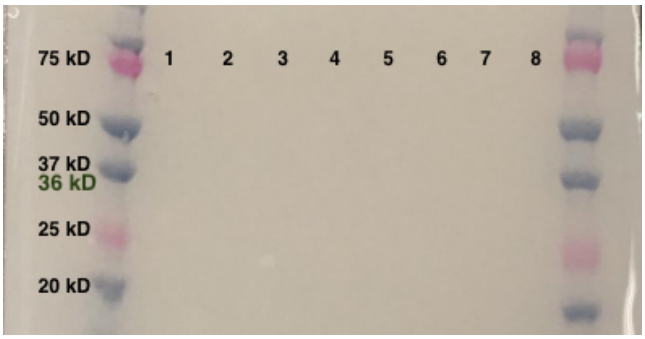
Figure 3-8: Image of SDS-PAGE gel (top image) and Western blot (bottom image) of soluble and insoluble protein fraction of new *C. glutamicum* transformants (left image), *E. coli* BL21 pVB-4A0E1-mCherry included as a positive control (right image). Lane content given in the table below. Ladder: Precision Plus Protein Dual Colour standards (Bio-Rad). The size of mCherry (28.8 kD) indicated in purple writing.

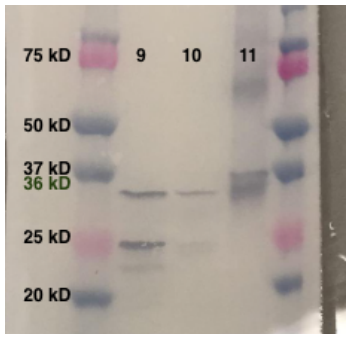
Based on the presence of *mCherry* amplification by colony PCR (**Figure 3-6**), the positive mCherry fluorescence (**Figure 3-7**) and the presence of bands corresponding to mCherry in the Western blot (**Figure 3-8**), the expression vectors pXMJ19-mCherry and pVB-4A0E1-mCherry were presumed to be present in the new set of *C. glutamicum* transformants. At this point in the study, the "old" glycerol stocks were discarded and the new set of *C. glutamicum* transformants will only be referred to as *C. glutamicum* from now on.

3.2 XylS detection

As mCherry expression from *Pm* is dependent on activation by the XylS inducer, the presence of XylS in induced *C. glutamicum* was evaluated using Western blot. *E. coli* pVB-4A0E1-mCherry and purified XylS protein were included in the run as positive controls. An image of the blot is included in **Figure 3-9**. Bands corresponding to the expected 36 kD size of XylS

were only present for *E. coli* pVB-4A0E1-mCherry soluble (lane 9) and insoluble (lane 10) fraction. The bands in *E. coli* corresponded to the band for the purified XylS control (lane 3). Bands corresponding to XylS were not detected for any of the *C. glutamicum* samples, indicting no expression of XylS from the *Ps1* and *Ps2* promoters in *C. glutamicum*.





Lane	Strain	Lane	Strain
1	*C. glutamicum* (no vector) (sol)	6	*C. glutamicum* pXMJ19 (insol)
2	*C. glutamicum* pXMJ19 (sol)	7	*C. glutamicum* pXMJ19-mCherry (insol)
3	*C. glutamicum* pXMJ19-mCherry (sol)	8	*C. glutamicum* pVB-4A0E1-mCherry (insol)
4	*C. glutamicum* pVB-4A0E1-mCherry (sol)	9	*E. coli* pVB-4A0E1-mCherry (sol)
5	*C. glutamicum* (no vector) (insol)	10	*E. coli* pVB-4A0E1-mCherry (insol)
		11	Purified XylS protein (50 ng)

Figure 3-9: Image of a XylS Western blot of soluble (sol) and insoluble (insol) protein fraction of *C. glutamicum* (lane 1-8) and *E. coli* BL21 (lane 9-10). All samples were induced. Lane content indicated in the table. Ladder: Precision Plus Protein Dual Colour standards (Bio-Rad). The size of XylS (36 kD) indicated in dark green writing.

3.3 Constructing vectors with alternative reporters

To evaluate if a *C. glutamicum* codon optimized reporter would yield higher expression of mCherry, the previously used *E. coli* optimized *mCherry* gene was replaced with a *C. glutamicum* optimized version, *optm_mCherry*. Additionally, GFP was tested as an alternative reporter, both a non-optimized and *C. glutamicum* optimized version.

3.3.1 Cloning of alternative reporters under P_{tac} control

The alternative reporter genes *GFP*, *optm_mCherry* and *optm_GFP* were cloned from the following source vectors, pMA-GFP, pMA-T-C_gluta_optm_mCherry and pUC57-Kan-GFP_optm_glut, respectively. The alternative reporters were placed under P_{tac} control in the pXMJ19 vector by replacing the *mCherry* reporter gene in pXMJ19-mCherry. **Figure 3-10** give a schematic overview of the construction of the resulting pXMJ19-GFP, pXMJ19-

optm_mCherry and pXMJ19-optm_GFP expression vectors. The pMA-T-C_gluta_optm_mCherry and pUC57-Kan-GFP_optm_glut source vectors had compatible RE recognition sites with the pXMJ19-mCherry vector, in this case *Hind*III and *Bam*HI recognition sites. The *optm_mCherry* and *optm_GFP* reporters were therefore isolated from the source vectors by RE digestion, fragment separation by gel electrophoresis and gel extraction. The pXMJ19-mCherry vector was digested with the same RE enzymes and combined with the optimized reporters by ligation. The source vector of GFP, pMA-GFP, did not have compatible recognition sites with pXMJ19-mCherry, PCR was therefore used to amplify the reporter with flanking regions containing the recognition sites for *Hind*III and *Bam*HI. Both pXMJ19-mCherry and the GFP PCR amplicon were subjected to RE digestion and fragment separation and combined by ligation, resulting in the pXMJ19-GFP expression vector.

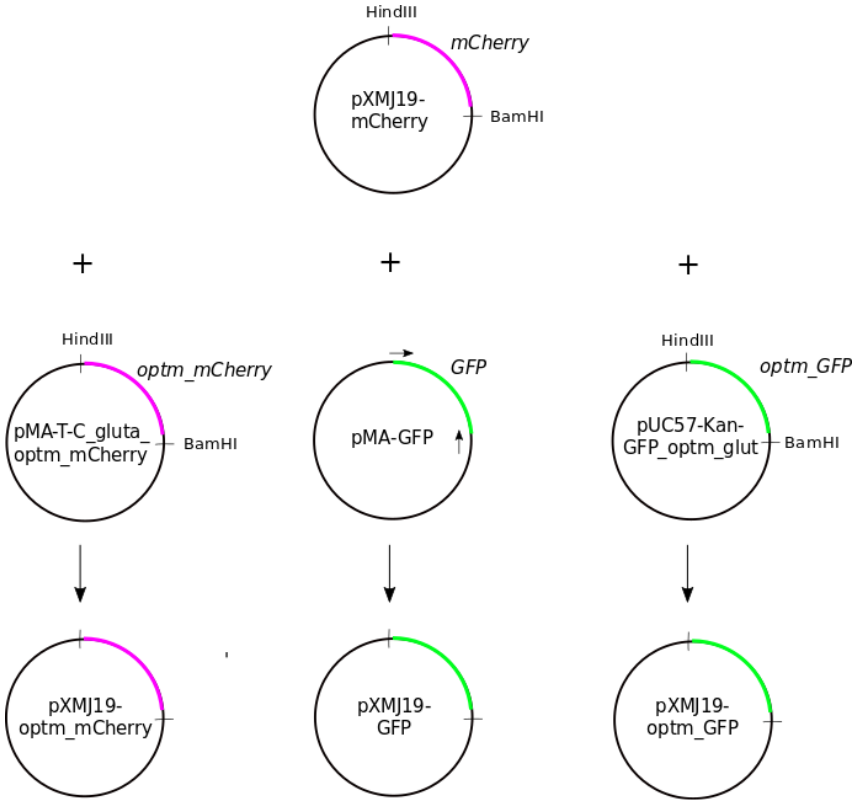


Figure 3-10: Overview of the cloning of pXMJ19-optm_mCherry, pXMJ19-GFP and pXMJ19-optm_GFP.

E. coli DH5α was transformed with the constructed pXMJ19-optm_mCherry, pXMJ19-optm_GFP and pXMJ19-GFP plasmids. To assay if the constructed vectors had the expected fragments, plasmids were isolated from random colonies and subjected to RE digestion and gel electrophoresis (data not shown). Plasmids with expected fragments sizes were sent for sequencing which confirmed correct cloning and used to transform *C. glutamicum*. The

presence of expression vectors in C. glutamicum was verified using either RE digestion or colony PCR (Appendix J).

3.3.2 Modifying the pVB-4A0E1 backbone

In pVB-4A0E1-mCherry there is a *Nde*I site just upstream of the GOI, however this cannot be utilized for RE and ligation cloning as there is also a *Nde*I site in the oriBL1 (origin of replication in *C. glutamicum*). For easier cloning access of the pVB-4A0E1 vector, the vector was modified through RE digestion, relegation and site-directed mutagenesis. **Figure 3-11** gives a schematic overview of the steps taken to modify the vector and the RE recognition sites changed in the process.

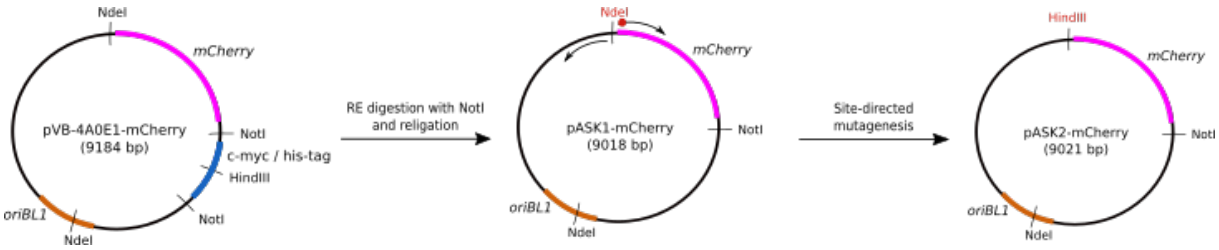


Figure 3-11: Construction of pASK1-mCherry and pASK2-mCherry.

Through RE digestion with *Not*I and the following religation of the vector, the c-myc and 6his-tag region between the two *Not*I sites in the pVB-4A0E1-mCherry vector was excluded, and consequently removing the only *Hind*III site in the vector. The religated vector, was designated pASK1-mCherry. Images of the agarose gel after electrophoresis of the *Not*I digested pVB-4A0E1-mCherry vector and the control digestions of plasmids isolated from random DH5α colonies transformed with the religated pASK1-mCherry vector are included in Appendix H, all samples showed the expected 166 and 9018 bps fragments associated with *Not*I digestion. Plasmids isolated from random colonies of *E. coli* DH5α pASK1-mCherry were sent for sequencing which confirmed correct cloning.

Through site-directed mutagenesis (SDM), the *Nde*I site upstream of the GOI in pASK1-mCherry was changed to a *Hind*III site, resulting in the pASK2-mCherry vector. With pASK2-mCherry, any given reporter with an upstream *Hind*III site and downstream *Not*I site could be cloned under *Pm* control. *E. coli* DH5α were transformed with the constructed pASK2-mCherry, and plasmids were isolated from random colonies and digested with

*Hind*III and *Not*I. The fragments of the digested plasmids were separated by gel electrophoresis for the purpose of assaying the presence of expected fragments of 8303 bps and 718 bps. One of the plasmids seemed to have correct fragments, which was later confirmed by sequencing results. An image of the agarose gel after gel electrophoresis of the control digested pASK2-mCherry are included in Appendix H.

3.3.3 Cloning of alternative reporters under *Pm* control

The *C. glutamicum* codon optimized reporters, *optm_mCherry* and *optm_GFP*, were cloned under *Pm* control in the modified pASK2 vector. A schematic overview of the construction of pASK2-optm_mCherry and pASK2-optm_GFP are given in **Figure 3-12**. *Optm_mCherry* was amplified from the source vector pMA-T-C_gluta_optm_mCherry with primers introducing a downstream *Not*I site. The source vector for *optm_GFP*, pUC57-Kan-GFP_optm_glut had compatible *Hind*III and *Not*I sites, and *optm_GFP* was therefore isolated from the source vector through RE digestion. Through RE digestion of both the pASK2-mCherry backbone and the reporter inserts, fragment separation by gel electrophoresis and gel extraction, the backbone and insert were combined through ligation, resulting in the pASK2-optm_mCherry and pASK2-optm_GFP expression vectors.

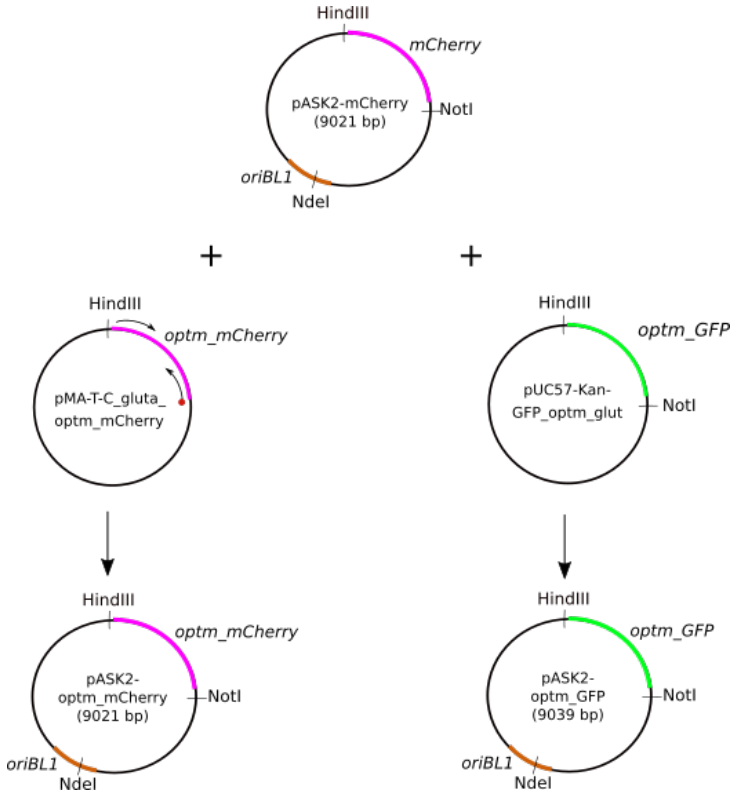


Figure 3-12: Construction of pASK2-optm_mCherry and pASK2-optm_GFP, placing optm_mCherry and optm_GFP under Pm control.

The *C. glutamicum* non-optimized GFP reporter were cloned under *Pm* control through RE digestion and ligation. **Figure 3-13** gives a schematic overview of the construction of pASK1-GFP. The *GFP* reporter gene was amplified from the pVB-1C0C1- GFP source vector through PCR amplification. Both the insert amplicon and the pVB-4A0E1-mCherry backbone vector were digested with *Xba*I and *Not*I and ligated together, resulting in the pASK1-GFP expression vector. Note that the pASK1 backbone in pASK1-GFP is the same backbone as in the intermediate step towards pASK2-mCherry (**Figure 3-11**), they are both missing the c-myc / 6his-tag region between the *Not*I sites in the original pVB-4A0E1-mCherry vector.

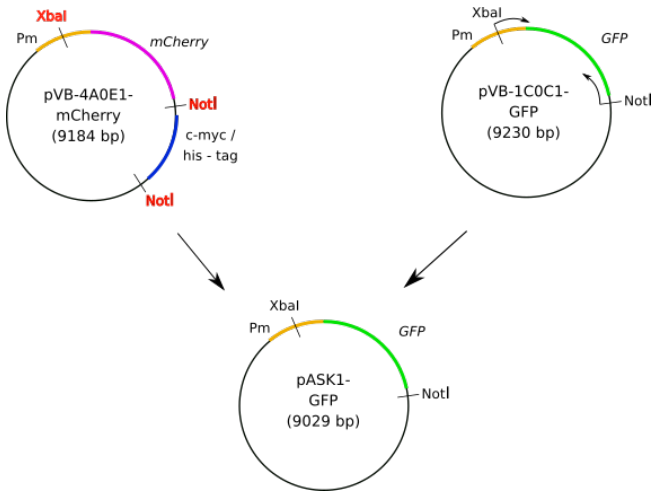


Figure 3-13: Construction of pASK1-GFP, placing *gfp* under Pm control.

E. coli DH5α was transformed with the constructed pASK2-optm_mCherry, pASK2-optm_GFP and pASK1-GFP plasmids. To assay if the constructed vectors had the expected fragments, plasmids were isolated from random colonies and subjected to RE digestion and gel electrophoresis (data not shown). Plasmids with expected fragments sizes were sent for sequencing which confirmed correct cloning and used to transform *C. glutamicum*. The presence of expression vectors in C. glutamicum was verified using either RE digestion or colony PCR (Appendix J).

3.4 Expression from alternative reporters

Expression from the constructed expression vectors with *optm_mCherry, optm_GFP* and *GFP* reporters were compared with the original expression vectors, pXMJ19-mCherry and

pVB-4A0E1-mCherry in both *E. coli* and *C. glutamicum*. Expression was performed in a 48-well FlowerPlate in a BioLector.

3.4.1 Effect of *C. glutamicum* codon optimized *mCherry* as a reporter

Induced and non-induced samples were run in the BioLector, and the measured mCherry expression is presented in **Figure 3-14**. High levels of mCherry expression were detected for *E. coli* Bl21 pXMJ19-mCherry (3190 RFU / OD_{600}) and pVB-4A0E1-mCherry (12 249 RFU / OD_{600}). mCherry was also detected for induced *C. glutamicum* harboring pXMJ19 (389 RFU / OD_{600}) and pVB-4A0E1-mCherry (123 RFU / OD_{600}), but at a lower level than for *E. coli*. mCherry fluorescence from the expression vectors with *optm_mCherry* reporter was not detected in *C. glutamicum* or in *E. coli* BL21. mCherry fluorescence was not detected for the negative controls or for the pXMJ19 shuttle vector control. For the non-induced samples, mCherry expression was detected from *E. coli* BL21 pXMJ19-mCherry (85 RFU / OD_{600}) and pVB-4A0E1-mCherry (773 RFU / OD_{600}) at a considerably lower level than for the induced samples of the same expression vectors. For the non-induced samples of *C. glutamicum*, mCherry expression was only detected from pVB-4A0E1-mCherry (111 RFU / OD_{600}), which is similar to the level detected in the induced sample of the same expression vector (123 RFU / OD_{600}). mCherry levels of the non-induced *C. glutamicum* pXMJ19-mCherry sample were at the same level as for the no vector and shuttle vector controls, indicating no mCherry expression from the non-induced pXMJ19-mCherry. As seen for the induced samples, mCherry was not detected from the *optm_mCherry* reporter in neither pXMJ19-optm_mCherry nor pASK2-optm_mCherry in neither of the species.

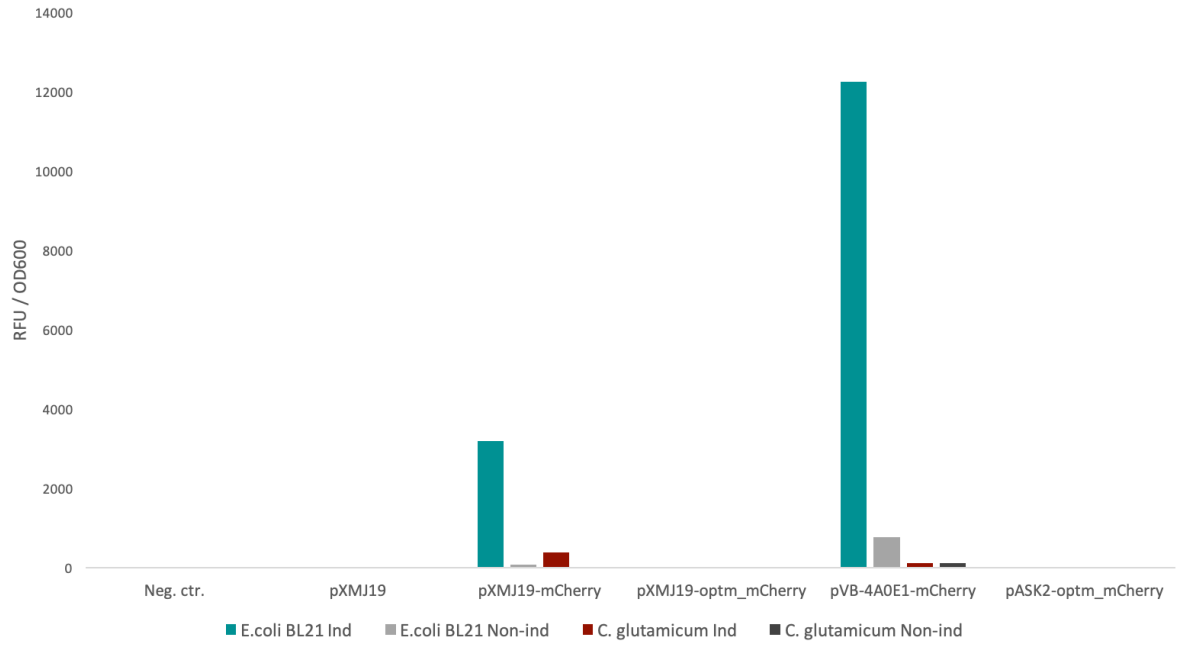


Figure 3-14: Expression of mCherry in *C. glutamicum* and *E. coli* BL21 measured as relative fluorescence units (RFU) / OD_{600} at harvesting. Cultures cultivated 48-well FlowerPlates in the BioLector, induced and non-induced samples. Negative controls: *E. coli* BL21 pVB1 (empty vector) and *C. glutamicum* (no vector).

3.4.2 Effect of *C. glutamicum* codon optimized GFP as a reporter

GFP and *C. glutamicum* codon optimized *GFP* (*optm_GFP*) were tested as alternative reporters to *mCherry*. **Figure 3-15** shows the GFP expression from cultivation in BioLector detected for the samples as RFU / OD_{600}. High levels of GFP was detected from the *E. coli* BL21 pASK1-GFP induced sample (41 960 RFU / OD_{600}). A relative high level of expression was also detected for the non-induced sample of the same expression vector (21 507 RFU / OD_{600}). The induced pXMJ19-GFP in *E. coli* BL21 also expressed some GFP (4730 RFU / OD_{600}). The background fluorescence in the control samples (negative no vector control and pXMJ19 shuttle vector control) were similar to the fluorescence levels detected in the expression vectors harbouring *GFP* and *optm_GFP* inserts in *C. glutamicum*. Indicating that independent of induction, none of the *C. glutamicum* samples could express GFP at detectable levels.

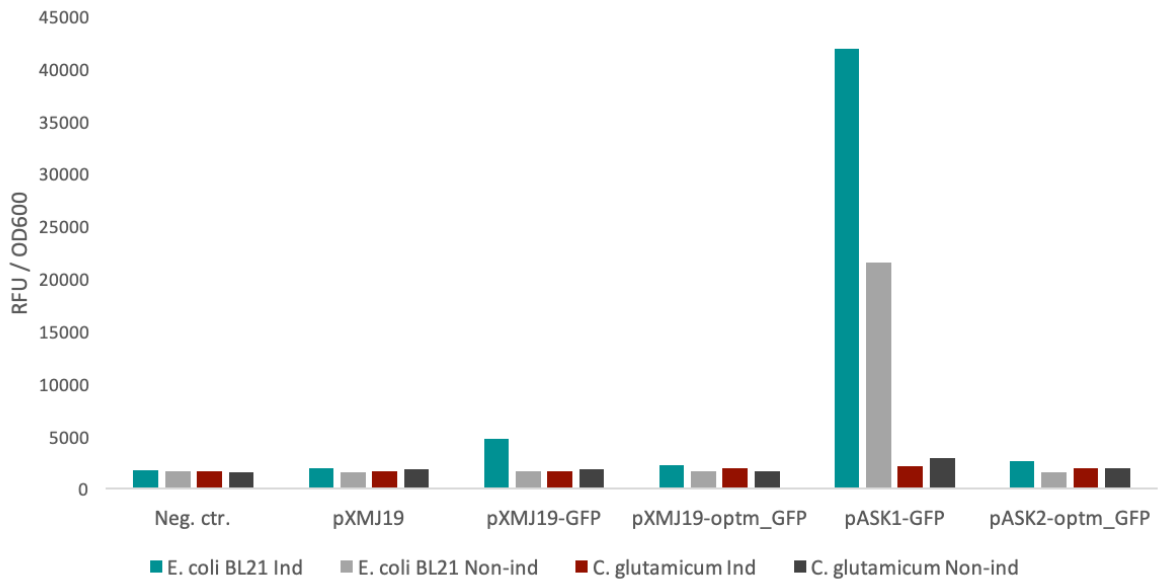


Figure 3-15: Expression of GFP in *C. glutamicum* and *E. coli* BL21 measured as relative fluorescence units (RFU) / OD_{600} at harvesting. Cultures cultivated 48-well FlowerPlates in the BioLector, induced and non-induced samples. Negative controls: *E. coli* BL21 pVB1 (empty vector) and *C. glutamicum* MB001 (DE3).

As neither GFP nor *C. glutamicum* codon-optimized mCherry were detected at higher levels than the previously used mCherry construct codon-optimized for *E. coli*, further work in this study continued using this reporter.

3.5 Optimizing RNA isolation from *C. glutamicum*

The level of mCherry mRNA was evaluated in order to determine if mCherry was limited by transcription or translation in *C. glutamicum*. To evaluate the mRNA levels of *C. glutamicum*, RNA needed to be isolated from the bacterium. This proved to be a difficult process and a total of ten alternative pre-treatments were tested, which are listed in **Table 3-1** below. For further details about the pre-treatments, see Appendix K. A volume of 1 mL samples with OD_{600} = 1 were pelleted and resuspended in the resuspension buffer with alternatively added lysozyme solution. For the enzymatic lysis pre-treatments, two different lysozyme solutions were used, lysozyme from chicken egg white and Ready-Lyse Lysozyme Solution, a recombinant lysozyme solution. For the isolation step, two kits were tested, the RNAqueous Total RNA Isolation Kit and the Quick-RNA Fungal / Bacterial Miniprep Kit. Electropherograms were used to assay the quality of the total RNA isolated. Initially, the automatically calculated RNA Integrity Number (RIN) value given by the software was intended for quality assessment of the isolated RNA. However, samples isolated with the Quick-RNA Fungal / Bacterial Miniprep Kit did not return a RIN value, this was probably

due to an unexpected signal in the 5S region of the electropherogram as the kit from Zymo Research elutes RNA molecules of low molecular weight. Consequently, electropherograms had to be visually evaluated.

Table 3-1: Overview of tested pre-treatments for RNA isolation from *C. glutamicum*.

Nr.	Resuspension buffer	Lysozyme solution	Lysozyme conc.	Incubation time at 37°C	Mechanical disruption	Isolation Kit
1	TE	Chicken egg white (Sigma-Aldrich)	1 mg /mL	2 hours		RNAqueous Total RNA Isolation Kit
2	TE	Chicken egg white (Sigma-Aldrich)	15 mg/mL	2 hours		RNAqueous Total RNA Isolation Kit
3	TE	Chicken egg white (Sigma-Aldrich)	15 mg/mL	1 hour		RNAqueous Total RNA Isolation Kit
4	TE	Chicken egg white (Sigma-Aldrich)	20 mg/mL	30 minutes		RNAqueous Total RNA Isolation Kit
5	50 mM EDTA	Chicken egg white (Sigma-Aldrich)	2 mg/mL	1 hour		RNAqueous Total RNA Isolation Kit
6	TE buffer, 2x DNA /RNA Shield	Chicken egg white (Sigma-Aldrich)	10 mg/mL	30 minutes		Quick-RNA Fungal / Bacterial Miniprep Kit
7	RNA Lysis Buffer				1x5 min at 30 Hz	Quick-RNA Fungal / Bacterial Miniprep Kit
8	RNA Lysis Buffer				3x5 min at 30 Hz	Quick-RNA Fungal / Bacterial Miniprep Kit
9	RNA Lysis Buffer	Chicken egg white (Sigma-Aldrich)	15 mg/mL	40 min	3x5 min at 30 Hz	Quick-RNA Fungal / Bacterial Miniprep Kit
10	RNA Lysis Buffer	Ready-Lyse Lysozyme Solution (Lucigen)	5, 33 µL	40 min	3x5 min at 30 Hz	Quick-RNA Fungal / Bacterial Miniprep Kit

E. coli samples were isolated with the RNAqueous Total RNA Isolation Kit (Thermo Fisher Scientific) with no additional lysis pre-treatment. Unlike RNA isolation from *C. glutamicum*, isolation from *E. coli* were an easier process yielding acceptable RNA quality with almost every isolation. **Figure 3-16** shows the electropherograms of total RNA isolated from three random and representative *E. coli* BL21 samples, where all three have two peaks around 40 and 45 seconds, indicating the presence of intact 16S and 23S RNA, respectively.

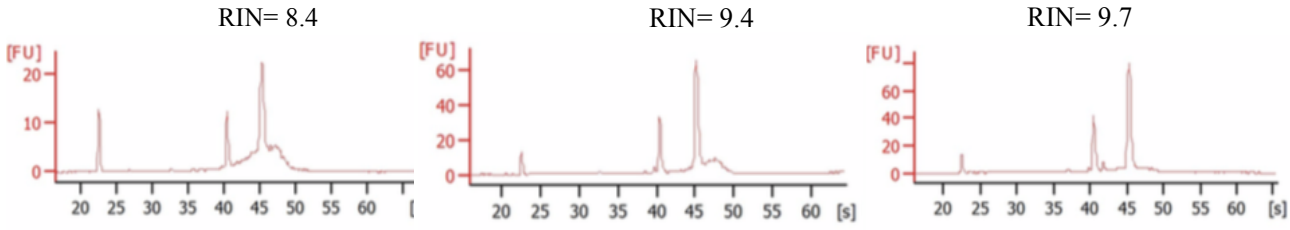


Figure 3-16: Bioanalyzer electropherograms of total RNA isolated from *E. coli* BL21. FU = fluorescence units. RNA integrity number (RIN) of the RNA samples indicated on the top, where 10 is the best, indicating intact RNA and 1 the worst, indicating completely degraded RNA.

RNA samples isolated with the lysozyme from chicken egg white and the RNAqueous Total RNA Isolation Kit (Pre-treatment 1-5) did not show peaks in the 16S and 23S region (**Figure 8-16** in Appendix K.1). As RNA-isolation with the RNAqueous Total RNA Isolation Kit and the chicken egg white lysozyme pre-treatment proved to be unsuccessful, the Quick-RNA Fungal / Bacterial Miniprep Kit was tested as it states to be suitable for Gram-positive species and has the necessary components needed for mechanical lysis.

The Quick-RNA Fungal / Bacterial Miniprep Kit was also tested without mechanical disruption, with a pre-treatment of TE buffer and chicken egg white lysozyme solution, with an additional 2x DNA / RNA Shield (Zymo Research) step (pre-treatment 6). No 16S and 23S peaks were detected for RNA isolated using this pre-treatment (**Figure 8-17** in Appendix K.2). Mechanical disruption for 1x5 min (pre-treatment 7) and 3x5 min (pre-treatment 8) at 30 Hz, with no enzymatic lysis pre-treatment added, was also tested (**Figure 8-17** in Appendix K.2). Neither of these mechanical lysis pre-treatments showed expected 16S and 23S peaks.

A combined 3x5 min at 30 Hz mechanical and enzymatic lysis pre-treatment was tested to further examine if RNA isolation from *C. glutamicum* could be improved. In addition to a 15 mg/mL (~480 000 U) chicken egg white lysozyme pre-treatment (pre-treatment 9), a pre-treatment with ~160 000 U unopened Ready-Lyse Lysozyme Solution (pre-treatment 10) was included in the run, as this was thought to be the most RNase-free lysozyme solution available in the lab. Electropherograms of RNA isolated using the two alternative lysozyme treatments, with the subsequent 3x5 min at 30 Hz mechanical treatment, are included in **Figure 3-17**.

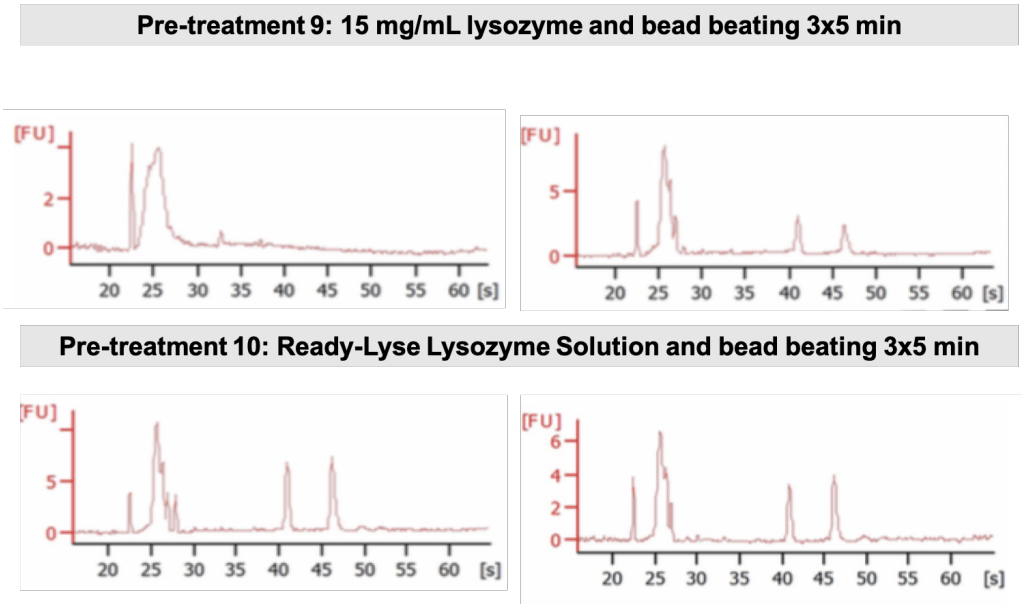


Figure 3-17: Bioanalyzer electropherograms of total RNA isolated from *C. glutamicum* using pre-treatment 9 and 10 and the Quick-RNA Fungal / Bacterial Miniprep Kit (Zymo Research). Two representative electropherograms included for each pre-treatment.

For the *C. glutamicum* samples treated with 15 mg/mL chicken egg white lysozyme and bead beating (top two electropherograms, Figure X), a broad, rounded peak was detected early, at around 25 seconds, for one of the samples (top left electropherogram). As the broad peak indicates the presence of large quantities of small RNA fragments, this sample was thought to be degraded. The other *C. glutamicum* sample treated with the 15 mg/mL chicken egg white lysozyme treatment (top right electropherogram) had peaks in the expected 16S and 23S RNA region. *C. glutamicum* samples treated with the Ready-Lyse Lysozyme Solution (bottom two electropherograms) had sharp peaks in the expected 16S and 23S region, indicating the presence of intact RNA. As the Ready-Lyse Lysozyme Solution combined with mechanical lysis for 3x5 minutes at 30 Hz gave the best results for RNA isolation from *C. glutamicum*, this pre-treatment was used to isolate total RNA from *C. glutamicum* for the purpose of cDNA synthesis and subsequent qPCR. Electropherograms of the RNA samples used for cDNA synthesis are included in Appendix L.

3.6 *mCherry* mRNA quantification

Following successful RNA isolation, RNA was converted to cDNA through reverse transcription. qPCR was used to assess the *mCherry* RNA levels in *C. glutamicum* and *E. coli*. *mCherry* expression was normalized against an endogenous control in form of the 16S housekeeping gene. The generated amplification plot is included in **Figure 3-18**. Initially, the intention was to compare *mCherry* expression in *C. glutamicum* to the expression in *E. coli*, but as the 16S data for *E. coli* was unreliable, due to poor primer efficiency, they could not be used for normalization. The *E. coli* samples were therefor excluded from further assays of relative quantification. Due to time constraints, qPCR was not repeated for 16S in *E. coli.* The detected C_t values for *mCherry* and 16S amplification in *C. glutamicum* are included in **Table 3-2**.

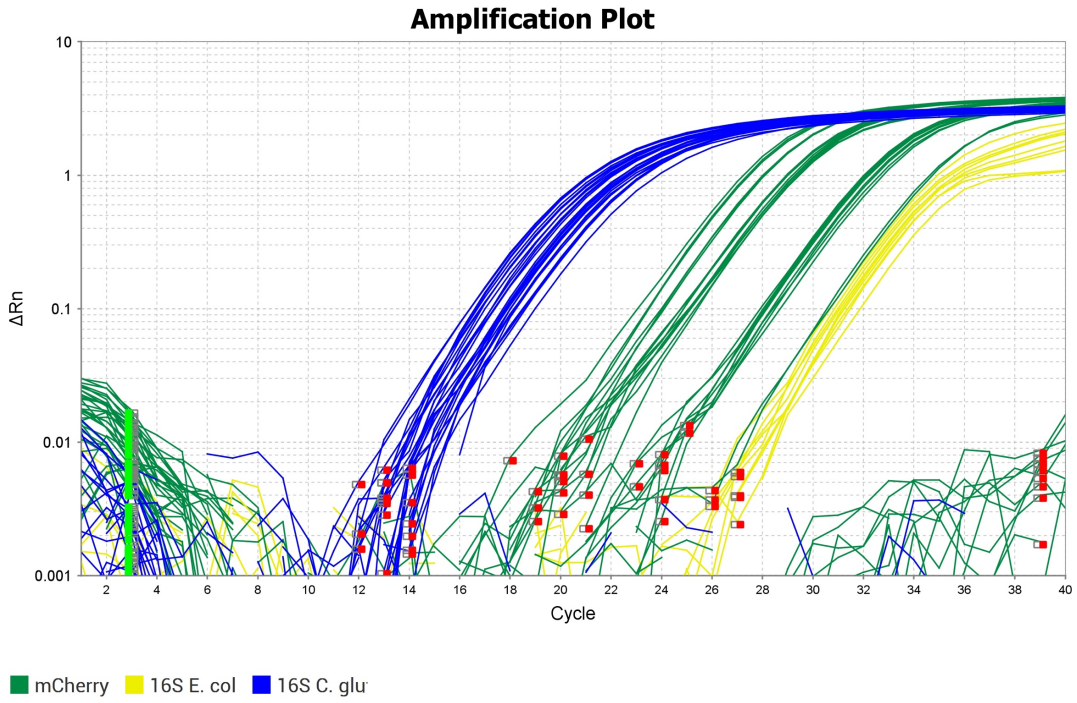


Figure 3-18: Amplification plot for induced and non-induced samples of *C. glutamicum* and *E. coli* pXMJ19-mCherry and pVB-4A0E1-mCherry. Samples were obtained three hours after induction and 16 hours after induction. Level of mCherry transcript indicated by green lines. 16S transcripts in *E. coli* (yellow lines) and *C. glutamicum* (blue lines) included as reference genes.

Table 3-2: Threshold values (C_t) values for the endogenous 16S control and mCherry in *C. glutamicum* samples of different sampling points (SP). C_t values based on the mean value of three technical replicates with standard deviation included (C_t SD). *C_t value detected in one of the three technical replicates.

SP	*C. glutamicum* sample	16S ctr.		mCherry	
		C_t	C_t SD	C_t	C_t SD
3h	Calibrator sample (no vector)	18,253	0,115	-	-
	pVB-4A0E1-mCherry (induced)	17,983	0,201	26,112	0,026
	pVB-4A0E1-mCherry (non-induced)	17,875	0,068	26,459	0,109
	pXMJ19-mCherry (induced)	18,546	0,087	24,575	0,151
	pXMJ19-mCherry (non-induced)	18,749	0,064	29,556	0,134
16h	Calibrator sample (no vector)	19,258	0,130	34,958*	-
	pVB-4A0E1-mCherry (induced)	19,937	0,032	29,283	0,065
	pVB-4A0E1-mCherry (non-induced)	19,305	0,218	29,653	0,334
	pXMJ19-mCherry (induced)	19,844	0,467	26,514	0,057
	pXMJ19-mCherry (non-induced)	19,154	0,138	32,023	0,355

*C_t value detected in one of the three technical replicates.

A melt curve analysis was also conducted to assay the presence of non-specific amplification. The -ΔF / ΔT vs. temperature plot for mCherry and 16S amplification in *C. glutamicum* are included in Appendix M. Melting peaks for mCherry and 16S amplification were detected around 82°C and 81°C, respectively. No other peaks were detected in the plots, indicating absence of non-specific amplification.

The level of mCherry transcripts in the different *C. glutamicum* samples were normalized against the 16h *C. glutamicum* (no vector) calibration sample and the relative quantification were calculated. The results are displayed in **Table 3-3** below. Compared to the calibrator sample with no expression vector, all the *C. glutamicum* samples harboring *mCherry* expression vectors showed upregulated *mCherry* gene expression. The highest upregulation was seen for the induced 3h pXMJ19-mCherry sample (815-fold upregulation) and the induced ON pXMJ19-mCherry sample (522-fold upregulation). An upregulation was also seen for the non-induced samples of these vectors with a 30-fold and 7-fold, respectively.

For *C. glutamicum* harboring pVB-4A0E1-mCherry, mCherry transcript levels were upregulated for both induced and non-induced samples at both sampling points. At the 3h sampling point, the induced pVB-4A0E1-mCherry sample was upregulated by a 190-fold and

the non-induced by a 139-fold. For the overnight sample, the induced pVB-4A0E1-mCherry sample was upregulated by 82-fold and the non-induced by a 41-fold. A general tendency of higher upregulation levels for the 3h samples than for the overnight samples was observed, indicating lower transcripts levels at the overnight sampling point.

Table 3-3: Relative mCherry expression compared to *C. glutamicum* MB001 (DE3) (no vector).

***C. glutamicum* strain**	**Harvested after 3 h**		**Harvested after 16h**	
	Induced	**Non-induced**	**Induced**	**Non-induced**
pXMJ19-mCherry	815	30	522	7
pVB-4A0E1-mCherry	190	139	82	41

3.7 Constructing expression vectors with alternative *xylS* promoters

Several attempts were made to construct expression vectors with the native *C. glutamicum* promoters P_{tuf}, P_{sod}, P_{dapA} and P_{dapB} controlling *xylS* expression, using two different cloning approaches, SLIC and Gibson assembly.

3.7.1 Isolating native promoters from the *C. glutamicum* MB001 (DE3) genome

The native *C. glutamicum* promoters, P_{tuf}, P_{sod}, P_{dapA} and P_{dapB}, were amplified from the genome of *C. glutamicum* MB001 (DE3) using colony PCR. Fragments were separated by agarose gel electrophoresis and extracted from gel. Images of the agarose gels of the PCR products for the different promoters are included in Appendix N. Fragments of correct sizes were extracted from the gel and used for cloning in both the SLIC and Gibson assembly approach.

3.7.2 Cloning of native promoter expression vectors using SLIC

For the SLIC approach, pVB-4A0E1-mCherry was amplified as one fragment using PCR with primers excluding *Ps1* and *Ps2*, the original promoters controlling *xylS* transcription. **Figure 3-19** gives an overview of the construction of pVB-4A0E1-mCherry_Ptuf, pVB-4A0E1-mCherry_Psod, pVB-4A0E1-mCherry_PdapA and pVB-4A0E1-mCherry_PdapB using SLIC. Several attempts were made to amplify the pVB-4A0E1-mCherry backbone, without the expected fragment of 9017 bps being detected with gel electrophoresis After extensive troubleshooting with different cycling conditions, temperatures and PCR mixes, a 2-step PCR

reaction with the PCR Premix from Takara Bio gave the most promising result yielding fragments of ~ 9 kb. Linearized backbone and amplified native promoter were combined using SLIC.

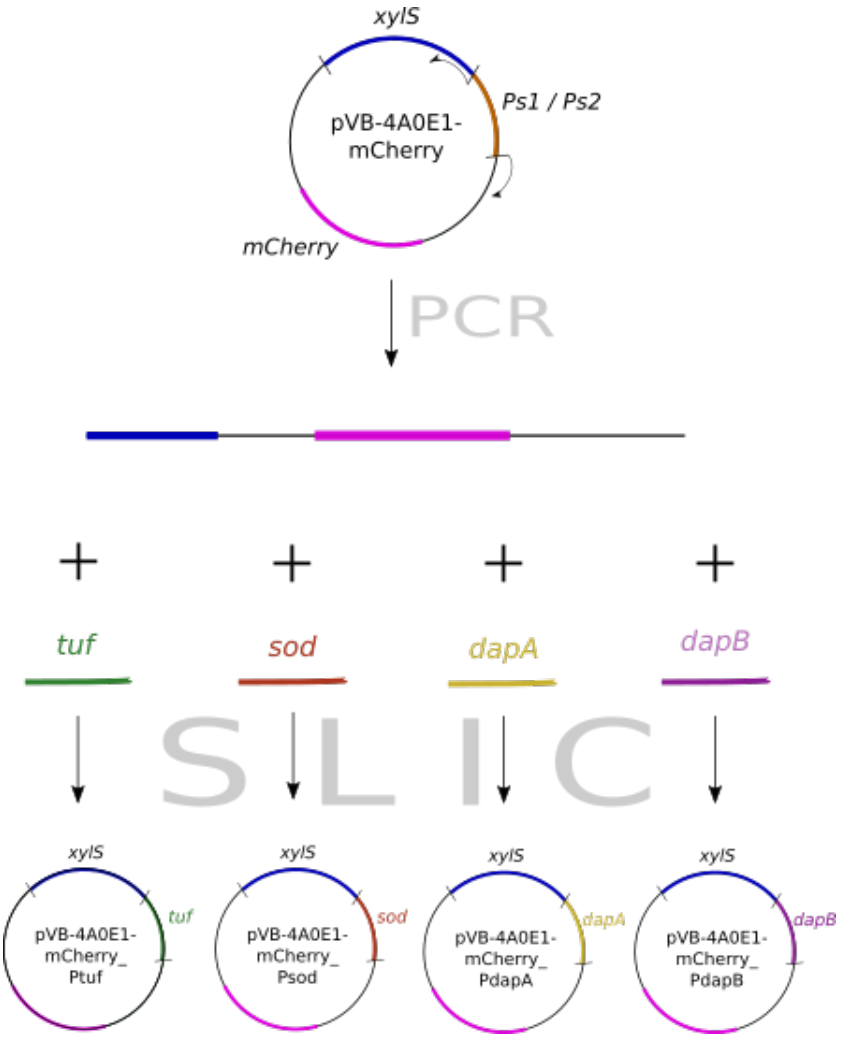


Figure 3-19: Construction overview of pVB-4A0E1-mCherry_Ptuf, pVB-4A0E1-mCherry_Psod, pVB-4A0E1-mCherry_PdapA and pVB-4A0E1-mCherry_PdapB using SLIC. The pVB-4A0E1-mCherry vector was amplified using primer pVB4_XylS_SLIC_fw and pVB4_XylS_SLIC_rv, excluding the original *Ps1* and *Ps2* promoters. Linearized backbone fragment mixed with the four alternative promoters amplified from the *C. glutamicum* MB001 (DE3) genome and combined by SLIC.

E. coli DH5α was transformed with the native promoter expression vectors. An image of the agar plates with DH5α transformants is included in **Figure 3-20**. Interestingly, colonies of different colors were observed, ranging from white to bright pink for all the expression vectors, indicating high levels of mCherry expression in some of the colonies. In addition to sending plasmids for sequencing, plasmids isolated from random colonies of all colors were *Bam*HI linearized and run on gel to evaluate if plasmids had the expected fragments sizes. An

agarose gel with the digested fragments of plasmids isolated from three bright pink, three light pink and three white colonies of pVB-4A0E1-mCherry_Ptuf are presented in **Figure 3-21**, linearized pVB-4A0E1-mCherry was included as a reference (lane 10). Plasmids of bright pink and light pink pVB-4A0E1-mCherry_Ptuf colonies had bands of similar size (expected 9217 bp), corresponding to the pVB-4A0E1-mCherry reference of 9184 bps. The bands of linearized plasmids isolated from white colonies had travelled further in the gel than the fragments from the pink colonies and the reference plasmid, indicating a lower molecular weight, approximately around 5 kb. Sequencing results revealed that all plasmids that were sent still had the *Ps1* and *Ps2* promoter upstream of *xylS*. Meaning that none of the SLIC constructed pVB-4A0E1-mCherry_Ptuf, pVB-4A0E1-mCherry_Psod, pVB-4A0E1-mCherry_PdapA and pVB-4A0E1-mCherry_PdapB plasmids were correct.

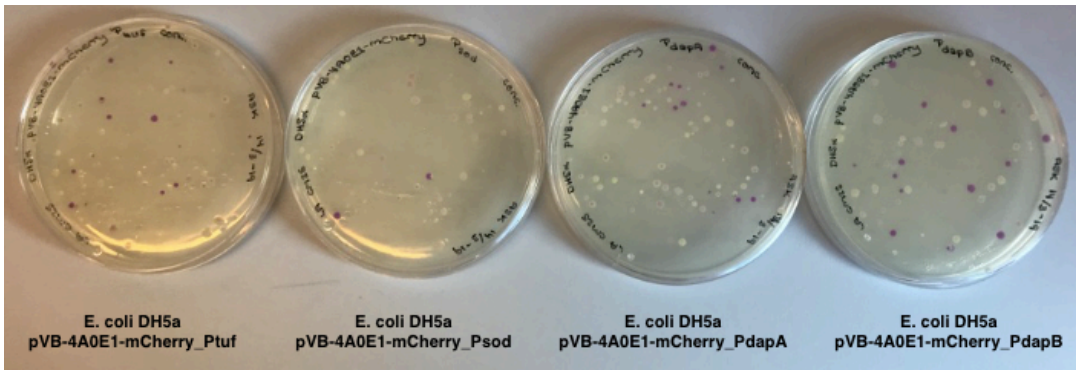


Figure 3-20: *E. coli* DH5α transformed with SLIC constructed pVB-4A0E1-mCherry_Ptuf, pVB-4A0E1-mCherry_Psod, pVB-4A0E1-mCherry_PdapA and pVB-4A0E1-mCherry_PdapB. From the left (1) DH5α pVB-4A0E1-mCherry_Ptuf. (2) DH5α pVB-4A0E1-mCherry_Psod. (3) DH5α pVB-4A0E1-mCherry_PdapA. (4) DH5α pVB-4A0E1-mCherry_PdapB.

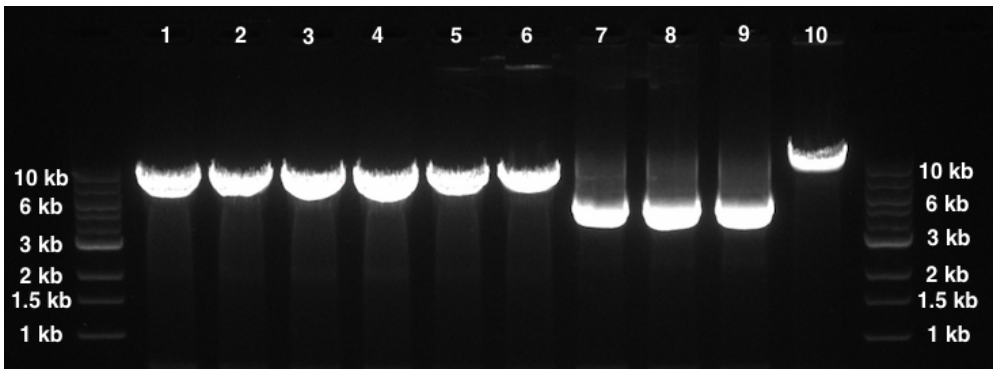


Figure 3-21: Agarose gel after gel electrophoresis of BamHI linearized plasmids isolated from (SLIC) pVB-4A0E1-mCherry_Ptuf colonies of different colors. Lane 1-3: Bright pink colonies. Lane 4-6: Light pink colonies. Lane 7-9: White colonies. Lane:10: BamHI linearized pVB-4A0E1-mCherry (9184 bps). Expected fragment size of BamHI linearized pVB-4A0E1-mCherry_Ptuf: 9217 bps. Ladder: 1 kb DNA ladder (NEB).

3.7.3 Cloning of native promoter expression vectors using Gibson assembly

As an alternative to the unsuccessful SLIC approach, the pVB-4A0E1-mCherry backbone was amplified using PCR in three fragments designated A, B and C. The three backbone fragments were amplified without the *Ps1* and *Ps2* promoters of *xylS*. The three linearized backbone fragments and the native promoter fragment were mixed in equimolar amounts and combined into a circular plasmid using Gibson assembly. An overview of the construction of pVB-4A0E1-mCherry_Ptuf, pVB-4A0E1-mCherry_Psod, pVB-4A0E1-mCherry_PdapA and pVB-4A0E1-mCherry_PdapB using Gibson assembly is included in **Figure 3-22**.

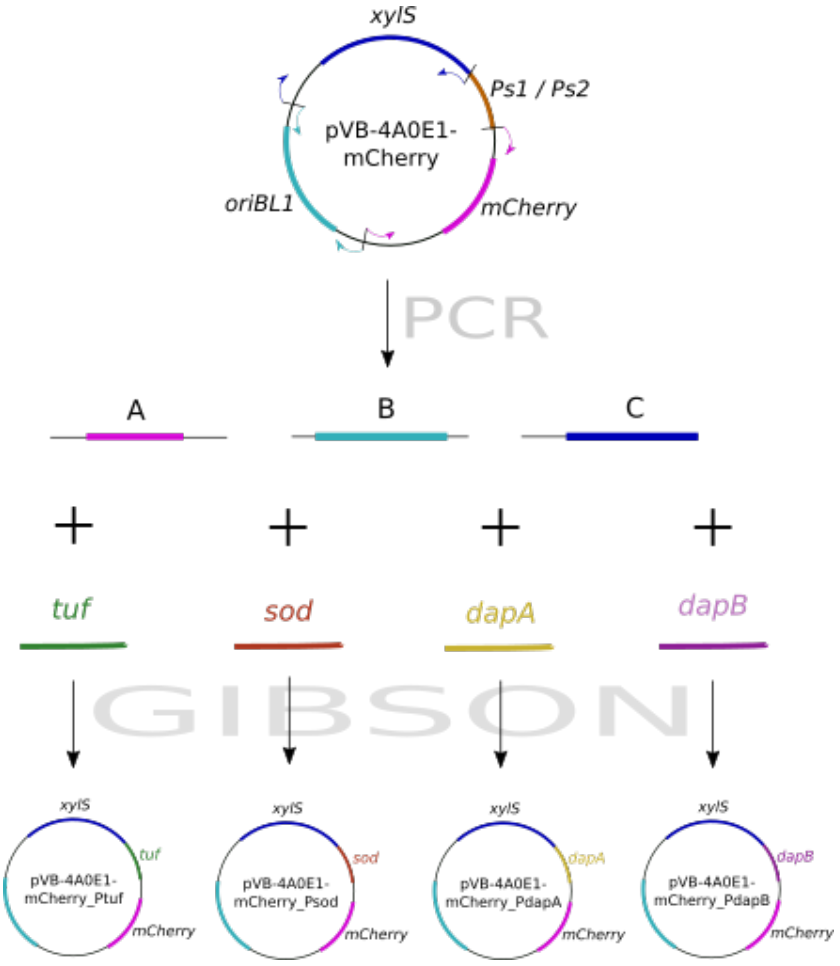


Figure 3-22: Construction overview of pVB-4A0E1-mCherry_Ptuf, pVB-4A0E1-mCherry_Psod, pVB-4A0E1-mCherry_PdapA and pVB-4A0E1-mCherry_PdapB using Gibson assembly. The pVB-4A0E1-mCherry vector was amplified in three fragments, excluding the *Ps1* and *Ps2* promoters, using the following primer pairs: Fragment A: pVB4_XylS_SLIC_rv and ASK21 (pink arrows). Fragment B: ASK22 and ASK 23 (turquoise arrows). Fragment C: ASK24 and pVB4_XylS_SLIC_fw (blue arrows). Linearized backbone fragments were mixed with the 4 alternative promoters, P_{tuf}, P_{sod}, P_{dapA} and P_{dapB}, amplified from the *C. glutamicum* MB001 (DE3) genome and combined by Gibson assembly.

The expected sizes for the three amplified backbone fragments were 3088 bps for A, 2960 bps for B and 2999 bps for C. **Figure 3-23** shows an image of the agarose gel after

electrophoresis of the three backbone fragments. All three fragments had bands of similar sizes, and since expected size for all of them were around ~ 3 kb, the amplification was assumed successful and the bands were extracted from the gel.

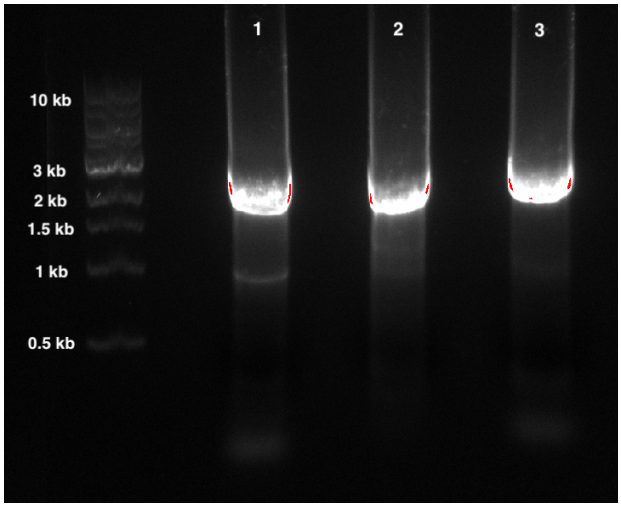
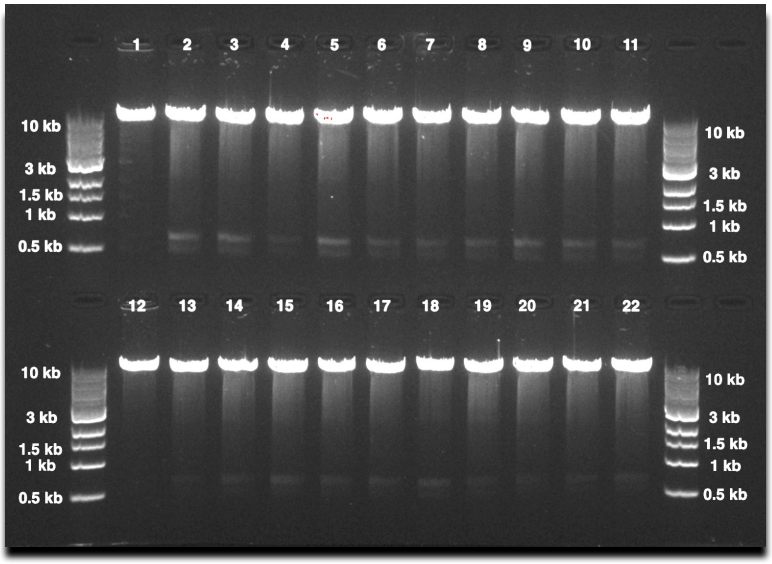


Figure 3-23: Agarose gel after gel electrophoresis of the three backbone pVB-4A0E1-mCherry fragments (A, B and C). Lane 1: Fragment A (3088 bps). Lane 2: Fragment B (2960 bps). Lane 3: Fragment C (2999 bps). Ladder: 1 kb DNA ladder (NEB).

After Gibson assembly, competent *E. coli* DH5α were transformed with the assembly mix and plated on selective LA CM25 plates. Unlike the SLIC transformants, none of the Gibson transformants were pink, they were all white. Plasmids were isolated from random colonies and sent for sequencing.

The same plasmids that were sent for sequencing were also linearized using *Bam*HI for fragment size control. Digestion mixes were subjected to gel electrophoresis and the image of the agarose gel is included in **Figure 3-24**. A *Bam*HI digested pVB-4A0E1-mCherry was included as a reference sample of 9184 bps (lane 1 and 12). Expected bands of ~9 kb, corresponding to the reference sample (9184 bps), were present for all the *Bam*Hi digested pVB-4A0E1-mCherry_Ptuf (lane 2-6), pVB-4A0E1-mCherry_Psod (lane 7-11), pVB-4A0E1-mCherry_PdapA (lane 13-17) and pVB-4A0E1-mCherry_PdapB (lane 18-22) plasmids, indicating successful linearization. However, weak low molecular weight bands were also visible for most of the newly constructed plasmids, but not in the reference sample (lane 1 and 12), potentially indicating multiple *Bam*HI recognition sites in the plasmid. Sequencing results showed that the cloning of alternative *xylS* promoters were unsuccessful and that the *Ps1* and *Ps2* promoters were still present in the constructed plasmids. Due to time constraints,

no further attempts were made to construct expression vectors with native *C. glutamicum* promoters controlling *xylS* transcription.



Lane	Plasmid	Expected fragments size (bps)
1	pVB-4A0E1-mCherry	9184
2-6	pVB-4A0E1-mCherry_Ptuf	9217
7-11	pVB-4A0E1-mCherry_Psod	9209
12	pVB-4A0E1-mCherry	9184
13-17	pVB-4A0E1-mCherry_PdapA	9218
18-22	pVB-4A0E1-mCherry_PdapB	9217

Figure 3-24: Agarose gel after gel electrophoresis of BamHI linearized plasmids isolated from *E. coli*. Lane content given in table below. Ladder: 1 kb DNA ladder (NEB).

4 Discussion

4.1 Difficulties with verifying presence of construct in *C. glutamicum*

The first phase of this study focused on verifying the presence of expression vectors in *C. glutamicum*, as this had proven to be problematic in previous work conducted on the recombinant strain [55]. Despite extensive troubleshooting, mCherry constructs were not detected in the recombinant *C. glutamicum*. As the bacterium could grow in selective CM25 media, and all analysis indicated the absence of expression vectors which would confer CM resistance, an antibiotic resistance assay was conducted to rule out the possibility of *C. glutamicum* being naturally resistant to CM. On plates, a clear colour difference was observed between the thought to be recombinant *C. glutamicum* MB001 (DE3) and the *C. glutamicum* MB001 (DE3) with no vector. While the suspected recombinant *C. glutamicum* appeared white, the control without vector was more yellow and only able to grow on plates without chloramphenicol, thus ruling out the possibility of natural resistance. *C. glutamicum* is known to have a natural yellow pigmentation as it produced the C50 carotenoid decaprenoxanthin [74], which raised the question on whether the white looking recombinant *C. glutamicum* from the previously generated recombinant strains were in fact *C. glutamicum* at all.

4.2 GFP as an alternative reporter

As an alternative to the previously used mCherry reporter, GFP was tested as a possible alternative, both in the form of an optimized and a non-optimized version for *C. glutamicum*. GFP fluorescence was detected for pXMJ19-GFP and pASK1-GFP plasmids in *E. coli*, but not in *C. glutamicum*. The expression vectors with a *C. glutamicum* optimized GFP reporter, pXMJ19-optm_GFP and pASK2-optm_mCherry did not express GFP in *C. glutamicum* either. Several previous studies have successfully used GFP as a reporter in *C. glutamicum* [75, 76], indicating that the bacterium possess the necessary machinery for correct folding of the protein. The absence of GFP expression in *C. glutamicum* was therefore unexpected. However, in the case of pXMJ19-GFP and pXMJ19-optm_GFP, where the reporter is cloned under *tac* control, a previous study using pXMJ19 based expression vectors did not detect GFP fluorescence when GFPuv were cloned under *tac* control either [76]. If the absence of GFP expression was caused by transcriptional, translational or post-translational mechanisms

is unknown and as the *E. coli* optimized mCherry reporter was shown to function in *C. glutamicum*, no further evaluation of GFP as a reporter was conducted.

4.3 *C. glutamicum* codon optimized reporters

Species tend to show a codon bias in their use of synonym codons, with some codons being more common than others. In its simplest form of understanding, codon optimized genes should be expressed at a higher rate as the hosts tRNA composition allows for a more rapid translation of the mRNA [40]. Previous studies have shown codon optimization to cause higher product yields in *C. glutamicum*. In the work of Yang et al [77], codon optimization alone improved the activity of nitrile hydratase from 2,97 U/mg DCW to 7,31 U/mg DCW. When the *E. coli ldcC* gene encoding lysine decarboxylase was replaced with a *C. glutamicum* optimized version, the diaminopentane product yield was increased by 25% in *C. glutamicum* [78]. Even by changing only five codons in the N-terminal coding region of PHA synthase to *C. glutamicum's* preferred synonym codons, production of Poly(-3-Hydroxybutyrate) was improved by 27,1% [79]. On the other hand, if only a single codon was used to express each amino acid, it would increase the risk of repetitive elements in the gene and potentially cause stem-loop formations in the transcribed mRNA, which may hinder ribosome binding and thereby block translation of the codon optimized gene [41]. However, the optimized genes in this study was ordered as synthetic genes from suppliers (Genscript and Thermo Fisher Scientific) using multiparametric optimization algorithms stating to consider mRNA secondary structures as a parameter. It is therefore unlikely that the absence of expression from optimized reporters were due to such stem-loop formations.

C. glutamicum was able to express the *E. coli* codon-optimized version of *mCherry*, both from *tac* and *Pm*, the absence of expression from *optm_mCherry* was therefore somewhat unexpected as the bacterium clearly possess the machinery needed for functional folding of the protein. However, a C to T mutation was identified in the *optm_mCherry* gene in all the pASK2-optm_mCherry plasmids sent for sequencing. The mutation was detected at the 3' end of the gene, and as the pXMJ19-optm_mCherry plasmids were only sequenced using a FW primer, the potential presence of the same mutation was not detected in pXMJ19-optm_mCherry. The mutation caused a CAG glutamine codon to be changed to a TAG codon, which would be transcribed into a UAG stop codon in the mRNA, which probably lead to premature termination of translation and expression of a non-functional mCherry protein. At

the same time, it is suspicious that this mutation occurred in all the pASK2-optm_mCherry plasmids sent for sequencing (6 in total). Either way, the cloning was not repeated as a decision was made to focus on cloning of alternative *xylS* promoters, as the non-optimized mCherry reporter could be used and XylS expression was thought to be a bigger obstacle at that point. If the cloning of *optm_mCherry* was to be repeated at a later stage, the possibility of the gene being incorrectly synthesized by the supplier should be ruled out before new cloning attempts are made.

4.4 Expression limited by transcription or translation?

C. glutamicum was able to express *E. coli* optimized mCherry in a functional form, as mCherry was detected both in the form of transcribed mRNA (qPCR), fluorescence and protein bands on the Western blot. Similar levels of expression were detected for the induced and non-induced samples of pVB-4A0E1-mCherry, indicating leaky expression and a non-functional transcription regulator (XylS). Essentially, there are two possible explanations for why mCherry could be expressed in the non-induced sample, one being leaky expression from *Pm* and the other being XylS activation by m-toluate independent XylS dimerization. However, spontaneous XylS dimerization is generally a feature of high XylS concentrations [27], and since XylS was not detected by Western blot for any of the *C. glutamicum* samples, the latter explanation is highly unlikely. This suggest that the detected mCherry expression from the XylS / Pm system was caused by leaky *Pm* expression. Together, the results indicated that XylS/ *Pm* expression was mainly limited by transcription due to absence of the activator. However, as the mCherry gene used in pXMJ19-mCherry and pVB-4A0E1-mCherry were codon optimized for *E. coli*, it is reasonable to assume that some translational delay in *C. glutamicum* occurred as well.

To overexpress XylS, the original *Ps1* and *Ps2* promoters, originating from *Pseudomonas putida* [27] were intended to be replaced by *C. glutamicum* native, constitutive promoters of different strengths. As native promoters contain familiar binding domains for the hosts sigma factors, it is more likely to be expressed than foreign promoters. The constitutive *Ps2* promoter of the XylS /*Pm* cassette is detected by σ^{70}, facilitating low levels of XylS transcription continuously [27]. All sigma factors found in *C. glutamicum* belongs to the σ^{70} family, with SigA being the primary sigma factor responsible for transcription of housekeeping genes [47, 80]. The 2.4 DNA binding domain of SigA is 90% identical to the

equivalent region in *E. coli* σ^{70}, suggesting a relatively conserved -10 region in *C. glutamicum*. However, the *C. glutamicum* -35 region is less conserved and the 4.2 DNA binding domain responsible for -35 recognition in σ^{70} and SigA is only 66% identical [80]. If SigA had trouble recognizing and binding the -35 region of *Ps2*, this would lead to XylS not being transcribed and possibly explain why XylS was not detected for *C. glutamicum* pVB-4A0E1-mCherry. However, *C. glutamicum* was able to express mCherry from the *E. coli* derived *tac* promoter in pXMJ19-mCherry, which is also recognized by σ^{70} [81], but to a much lower level than detected in *E. coli*. This means that SigA of *C. glutamicum* was able to bind to the *tac* promoter region, despite its less conserved -35 region. If SigA was able to recognize and initiate *xylS* transcription from *Ps2*, XylS expression must have been hindered either at translational or post-translational stages. As the XylS / *Pm* system is new to *C. glutamicum*, it is unknown if the bacterium has the necessary folding machinery to correctly assemble XylS into a functional form. As transcript levels of *xylS* was not evaluated in this study, it is not possible to say if XylS expression was limited by transcription, translation or post-translational mechanisms. However, it is likely that mCherry transcription was mainly limited by transcription due to absence of XylS activator.

4.5 Trouble with cloning of native *C. glutamicum* promoters controlling *xylS*

As SigA is responsible for initiating transcription from housekeeping genes, it should be able to facilitate *xylS* transcription if it was controlled by a housekeeping promoter native to *C. glutamicum*. Despite several attempts, using both SLIC and Gibson assembly, expression vectors with native promoters controlling *xylS* was not successfully constructed. Sequencing results of thought to be successful constructs, showed that all vectors still had the original *Ps1* and *Ps2* promoters upstream of *xylS*. A possible explanation for this could be insufficient *Dpn*I treatment and removal of template DNA, consequently leading to the pVB-4A0E1-mCherry template potentially being extracted together with the amplified *Ps1* and *Ps2* free backbone fragment. This is a more likely scenario in the case of SLIC, as the backbone was amplified in one single fragment with only a 167 bps difference from the pVB-4A0E1-mCherry template, which would be difficult to distinguish on gel. However, this explanation becomes less likely when the Gibson approach returned similar results as SLIC, with *Ps1* and *Ps2* still present in all the expression vectors sent for sequencing. As the backbone for Gibson assembly was amplified in three ~3 kb fragments, it is highly unlikely that the 9 kb pVB-

4A0E1-mCherry template was extracted from the gel together with the ~3 kb fragments. A more likely explanation could be unspecific amplification facilitated by the pVB4-SLIC-fw and pVB4-SLIC-rv primer, the primer pair responsible for excluding *Ps1* and *Ps2*, as this primer pair was used in both the SLIC and Gibson approach. The trouble of getting the 9-kb amplicon for SLIC, with numerous attempts yielding fragments of incorrect sizes, also supports this hypothesis. As none of the constructed expression vectors with native *C. glutamicum* promoters proved to be correct, this remains to be completed to further evaluate the potential of the XylS / *Pm* expression cassette in *C. glutamicum*. For further work, the Gibson assembly method is preferable to SLIC in this case, as the backbone in question is large and amplifying the backbone in several smaller fragments is easier than the one-fragment amplification needed for SLIC. In either case, the *Ps1* and *Ps2* excluding primer pair should be redesigned to rule out any possible problems related to the primer pair used in this study.

5 Conclusion

The previously used recombinant strains were probably contaminated by an unknown source and had to be discarded. When transformation of *C. glutamicum* was repeated, the presence of mCherry constructs was verified. *C. glutamicum* was not able to express any of the GFP reporters, no further investigations were conducted on possible bottlenecks for GFP in *C. glutamicum* as the *E. coli* optimized mCherry reporter was shown to function and therefore used in further work. None of the *C. glutamicum* codon-optimized reporters were detected in *C. glutamicum* in the form of functional protein yielding fluorescence. In the case of *optm_mCherry*, the absence of protein was probably caused by a mutation in the GOI, resulting in premature termination of translation. The presence of XylS activator could not be detected in *C. glutamicum* in the form of functional protein, which probably caused the absence of inducible mCherry expression from the XylS / *Pm* system in *C. glutamicum*. It is therefore reasonable to conclude that mCherry expression was mainly limited by transcriptional activation of XylS. If XylS expression was hindered at a transcriptional, translational or post-translational level could not be determined in this study.

6 Further work

As XylS expression could not be detected in *C. glutamicum*, further work should focus on upregulating XylS levels. To evaluate if XylS expression is limited by transcription or translation, the level of *xylS* transcript in *C. glutamicum* should be determined. Expression vectors were *Ps1* and *Ps2* are replaced with native *C. glutamicum* promoters controlling *xylS* transcription was not successfully constructed during this thesis and remains to be completed. Additionally, to further adapt the XylS / Pm system to *C. glutamicum* and its native sigma factors, the 5' UTR of *Pm* could be replaced by a region native to the bacterium itself. The expression vectors in this study are based on the pXMJ19 shuttle vector, with an estimated plasmid copy number of 10-30 in *C. glutamicum.* However, plasmids with significantly higher copy numbers have shown to be stable and not effect cell growth in *C. glutamicum.* By using a high-copy number plasmid, more templates are present in each cell, which poses as a promising possibility for increased heterologous protein expression. The possibility of establishing the XylS / *Pm* system in a high-copy plasmid should definitively be explored to fully evaluate the potential of the system in *C. glutamicum*

7 References

1. MarketsandMarkets. *Protein Expression Market by Type (E. coli, Mammalian, Yeast, Pichia, Insect, Baculovirus, Cell-free), Products (Competent Cells, Reagents, Instruments, Services), Application (Therapeutic, Research, Industrial) & End User - Global Forecast to 2022*. 2017 [cited 2019 25.04.2019]; Available from: https://www.marketsandmarkets.com/Market-Reports/protein-expression-market-180323924.html.
2. Rai, M. and H. Padh, *Expression systems for production of heterologous proteins*. Vol. 80. 2000.
3. Andersen, D.C. and L. Krummen, *Recombinant protein expression for therapeutic applications.* Current Opinion in Biotechnology, 2002. **13**(2): p. 117-123.
4. Chen, R., *Bacterial expression systems for recombinant protein production: E. coli and beyond.* Biotechnology Advances, 2012. **30**(5): p. 1102-1107.
5. Demain, A.L. and P. Vaishnav, *Production of recombinant proteins by microbes and higher organisms.* Biotechnology Advances, 2009. **27**(3): p. 297-306.
6. Reena Gomes, A., et al., *An Overview of Heterologous Expression Host Systems for the Production of Recombinant Proteins*. Vol. 4. 2016. 346-356.
7. Van Mellaert, L. and J. Anné, *Gram-Positive Bacteria as Host Cells for Heterologous Production of Biopharmaceuticals*, in *Novel Frontiers in the Production of Compounds for Biomedical Use*, A. Van Broekhoven, F. Shapiro, and J. Anné, Editors. 2002, Springer Netherlands: Dordrecht. p. 277-300.
8. Reece, J.B. and N.A. Campbell, *Campbell biology*. 9th ed. 2011, Boston: Benjamin Cummings / Pearson. xlvi, 1263 pages.
9. Clark, D.P. and N.J. Pazdernik, *Chapter 11 - Transcription of Genes*, in *Molecular Biology (Second Edition)*, D.P. Clark and N.J. Pazdernik, Editors. 2013, Academic Press: Boston. p. 309-335.
10. Madigan, M.T., et al., *Chapter 4 - Molecular Microbiology*, in *Brock Biology of Microorganisms (Fourteenth Edition)*. 2015, Pearson: Boston. p. 131-166.
11. Clark, D.P. and N.J. Pazdernik, *Chapter 13 - Protein Synthesis*, in *Molecular Biology (Second Edition)*, D.P. Clark and N.J. Pazdernik, Editors. 2013, Academic Press: Boston. p. 369-416.
12. Gualerzi, C.O. and C.L. Pon, *Initiation of mRNA translation in bacteria: structural and dynamic aspects.* Cell Mol Life Sci, 2015. **72**(22): p. 4341-67.
13. Rodnina, M.V., *Translation in Prokaryotes.* Cold Spring Harb Perspect Biol, 2018. **10**(9).
14. Gopal, G.J. and A. Kumar, *Strategies for the Production of Recombinant Protein in Escherichia coli.* The Protein Journal, 2013. **32**(6): p. 419-425.
15. Srivastava, P. and J.K. Deb, *Gene expression systems in corynebacteria.* Protein Expression and Purification, 2005. **40**(2): p. 221-229.
16. Hannig, G. and S.C. Makrides, *Strategies for optimizing heterologous protein expression in Escherichia coli.* Trends Biotechnol, 1998. **16**(2): p. 54-60.
17. Singh, A., et al., *Protein recovery from inclusion bodies of Escherichia coli using mild solubilization process.* Microbial cell factories, 2015. **14**: p. 41-41.

18. de Marco, A., *Strategies for successful recombinant expression of disulfide bond-dependent proteins in Escherichia coli.* Microb Cell Fact, 2009. **8**: p. 26.
19. Lee, J.Y., et al., *The Actinobacterium Corynebacterium glutamicum, an Industrial Workhorse.* J Microbiol Biotechnol, 2016. **26**(5): p. 807-22.
20. Liu, X., et al., *Protein secretion in Corynebacterium glutamicum.* Critical Reviews in Biotechnology, 2017. **37**(4): p. 541-551.
21. Liu, X., et al., *Expression of recombinant protein using Corynebacterium Glutamicum: progress, challenges and applications.* Critical Reviews in Biotechnology, 2016. **36**(4): p. 652-664.
22. Casjens, S., *Prophages and bacterial genomics: what have we learned so far?* Mol Microbiol, 2003. **49**(2): p. 277-300.
23. Baumgart, M., et al., *Construction of a prophage-free variant of Corynebacterium glutamicum ATCC 13032 for use as a platform strain for basic research and industrial biotechnology.* Appl Environ Microbiol, 2013. **79**(19): p. 6006-15.
24. Kortmann, M., et al., *A chromosomally encoded T7 RNA polymerase-dependent gene expression system for Corynebacterium glutamicum: construction and comparative evaluation at the single-cell level.* Microbial Biotechnology, 2015. **8**(2): p. 253-265.
25. Brautaset, T., R. Lale, and S. Valla, *Positively regulated bacterial expression systems.* Microb Biotechnol, 2009. **2**(1): p. 15-30.
26. Clark, D.P. and N.J. Pazdernik, *Chapter 16 - Regulation of Transcription in Prokaryotes*, in *Molecular Biology (Second Edition)*, D.P. Clark and N.J. Pazdernik, Editors. 2013, Academic Press: Boston. p. 493-525.
27. Gawin, A., S. Valla, and T. Brautaset, *The XylS/Pm regulator/promoter system and its use in fundamental studies of bacterial gene expression, recombinant protein production and metabolic engineering.* Microb Biotechnol, 2017. **10**(4): p. 702-718.
28. Clark, D.P. and N.J. Pazdernik, *Chapter 7 - Cloning Genes for Analysis*, in *Molecular Biology (Second Edition)*, D.P. Clark and N.J. Pazdernik, Editors. 2013, Academic Press: Boston. p. 194-226.
29. Schenborn, E. and D. Groskreutz, *Reporter gene vectors and assays.* Molecular Biotechnology, 1999. **13**(1): p. 29-44.
30. Clark, D.P. and N.J. Pazdernik, *Chapter 19 - Analysis of Gene Expression*, in *Molecular Biology (Second Edition)*, D.P. Clark and N.J. Pazdernik, Editors. 2013, Academic Press: Boston. p. 581-614.
31. Chalfie, M., et al., *Green fluorescent protein as a marker for gene expression.* Science, 1994. **263**(5148): p. 802.
32. Shaner, N.C., P.A. Steinbach, and R.Y. Tsien, *A guide to choosing fluorescent proteins.* Nat Methods, 2005. **2**(12): p. 905-9.
33. Davidson, M.W. and R.E. Campbell, *Engineered fluorescent proteins: innovations and applications.* Nat Methods, 2009. **6**(10): p. 713-17.
34. Dundee, U.o. *Fluorescent reporter protein expression*. [cited 2019 13.05.2019]; Available from: https://www.lifesci.dundee.ac.uk/technologies/flow-cytometry-cell-sorting/techniques/fluorescent-reporter-protein-expression.
35. Tsien, R.Y., *THE GREEN FLUORESCENT PROTEIN.* Annual Review of Biochemistry, 1998. **67**(1): p. 509-544.

36. Inouye, S. and F.I. Tsuji, *Aequorea green fluorescent protein: Expression of the gene and fluorescence characteristics of the recombinant protein.* FEBS Letters, 1994. **341**(2): p. 277-280.
37. Matz, M.V., et al., *Fluorescent proteins from nonbioluminescent Anthozoa species.* Nature Biotechnology, 1999. **17**: p. 969.
38. Baird, G.S., D.A. Zacharias, and R.Y. Tsien, *Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral.* Proceedings of the National Academy of Sciences, 2000. **97**(22): p. 11984.
39. Shaner, N.C., et al., *Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein.* Nature Biotechnology, 2004. **22**: p. 1567.
40. Hanson, G. and J. Coller, *Codon optimality, bias and usage in translation and mRNA decay.* Nat Rev Mol Cell Biol, 2018. **19**(1): p. 20-30.
41. Gustafsson, C., S. Govindarajan, and J. Minshull, *Codon bias and heterologous protein expression.* Trends in Biotechnology, 2004. **22**(7): p. 346-353.
42. Liu, G., et al., *Codon Usage Patterns in Corynebacterium glutamicum: Mutational Bias, Natural Selection and Amino Acid Conservation.* Comparative and Functional Genomics, 2010. **2010**.
43. Shi, F., M. Luan, and Y. Li, *Ribosomal binding site sequences and promoters for expressing glutamate decarboxylase and producing γ-aminobutyrate in Corynebacterium glutamicum.* AMB Express, 2018. **8**(1): p. 61.
44. Yim, S.S., et al., *Isolation of fully synthetic promoters for high-level gene expression in Corynebacterium glutamicum.* Biotechnol Bioeng, 2013. **110**(11): p. 2959-69.
45. Shang, X., et al., *Native promoters of Corynebacterium glutamicum and its application in L-lysine production.* Biotechnol Lett, 2018. **40**(2): p. 383-391.
46. de Boer, H., L.J. Comstock, and M. Vasser, *The Tac Promoter: A Functional Hybrid Derived from the Trp and Lac Promoters*. Vol. 80. 1983. 21-5.
47. Pátek, M., et al., *Promoters of Corynebacterium glutamicum.* Journal of Biotechnology, 2003. **104**(1): p. 311-323.
48. Wei, H.B., et al., *Identification and application of a novel strong constitutive promoter in Corynebacterium glutamicum.* Annals of Microbiology, 2018. **68**(6): p. 375-382.
49. Vasicová, P., et al., *Analysis of the Corynebacterium glutamicum dapA promoter.* Journal of bacteriology, 1999. **181**(19): p. 6188-6191.
50. Cremer, J., L. Eggeling, and H. Sahm, *Cloning the dapA dapB cluster of the lysine-secreting bacterium Corynebacterium glutamicum.* Molecular and General Genetics MGG, 1990. **220**(3): p. 478-480.
51. Madigan, M.T., et al., *Brock biology of microorganisms*. 2014.
52. Jakoby, M., C.E. Ngouoto-Nkili, and A. Burkovski, *Construction and application of new Corynebacterium glutamicum vectors.* Biotechnology Techniques, 1999. **13**(6): p. 437-441.
53. Yang, J., et al., *Biosynthesis of rare ketoses through constructing a recombination pathway in an engineered Corynebacterium glutamicum.* Biotechnology and Bioengineering, 2015. **112**(1): p. 168-180.

54. Johnsgaard, M., *First steps towards implementation of Corynebacterium glutamicum as an alternative production host for Vectron Biosolutions*, in *Department of Biotechnology and Food Science*. 2017, Norwegian University of Science and Technology. p. 58.
55. Johnsgaard, M., *Evaluation of the XylS/Pm Expression Cassette in Corynebacterium glutamicum*, in *Department of Biotechnology and Food Science*. 2018, Norwegian University of Science and Technology. p. 103.
56. Clark, D.P. and N.J. Pazdernik, *Chapter 5 - Manipulation of Nucleic Acids*, in *Molecular Biology (Second Edition)*, D.P. Clark and N.J. Pazdernik, Editors. 2013, Academic Press: Boston. p. 125-161.
57. Mohanty, A.K., M.L. Yadav, and S. Choudhary, *Gel Electrophoresis of Proteins and Nucleic Acids*, in *Protocols in Semen Biology (Comparing Assays)*, N. Srivastava and M. Pande, Editors. 2017, Springer Singapore: Singapore. p. 233-246.
58. Corthell, J.T., *Chapter 3 - Agarose Gel Electrophoresis*, in *Basic Molecular Protocols in Neuroscience: Tips, Tricks, and Pitfalls*, J.T. Corthell, Editor. 2014, Academic Press: San Diego. p. 21-25.
59. Rossi, R., et al., *Functional characterization of the T4 DNA ligase: A new insight into the mechanism of action.* Nucleic Acids Research, 1997. **25**(11): p. 2106-2113.
60. Clark, D.P. and N.J. Pazdernik, *Chapter 6 - Polymerase Chain Reaction*, in *Molecular Biology (Second Edition)*, D.P. Clark and N.J. Pazdernik, Editors. 2013, Academic Press: Boston. p. 163-193.
61. Qi, D. and K.B.G. Scholthof, *A one-step PCR-based method for rapid and efficient site-directed fragment deletion, insertion, and substitution mutagenesis.* Journal of Virological Methods, 2008. **149**(1): p. 85-90.
62. Hemsley, A., et al., *A simple method for site-directed mutagenesis using the polymerase chain reaction.* Nucleic acids research, 1989. **17**(16): p. 6545-6551.
63. Jeong, J.Y., et al., *One-Step Sequence- and Ligation-Independent Cloning as a Rapid and Versatile Cloning Method for Functional Genomics Studies.* Applied and Environmental Microbiology, 2012. **78**(15): p. 5440-5443.
64. Gibson, D.G., et al., *Enzymatic assembly of DNA molecules up to several hundred kilobases.* Nature Methods, 2009. **6**: p. 343.
65. Williams, R.T. and J.W. Bridges, *FLUORESCENCE OF SOLUTIONS: A REVIEW.* Journal of clinical pathology, 1964. **17**(4): p. 371-394.
66. Clark, D.P. and N.J. Pazdernik, *Chapter 15 - Proteomics: The Global Analysis of Proteins*, in *Molecular Biology (Second Edition)*, D.P. Clark and N.J. Pazdernik, Editors. 2013, Academic Press: Boston. p. 459-491.
67. Bustin, S.A., et al., *Quantitative real-time RT-PCR – a perspective.* Journal of Molecular Endocrinology, 2005. **34**(3): p. 597-601.
68. Madigan, M.T., *Brock biology of microorganisms*. 2015, Harlow: Pearson Education.
69. Ponchel, F., et al., *Real-time PCR based on SYBR-Green I fluorescence: an alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions.* BMC biotechnology, 2003. **3**: p. 18-18.
70. Holland, P.M., et al., *Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase.*

Proceedings of the National Academy of Sciences of the United States of America, 1991. **88**(16): p. 7276-7280.
71. Navarro, E., et al., *Real-time PCR detection chemistry.* Clin Chim Acta, 2015. **439**: p. 231-50.
72. Bio-Rad. *SYBR Green qPCR Supermixes*. [cited 2019 13.05]; Available from: http://www.bio-rad.com/featured/en/sybr-green-for-qpcr.html.
73. Scientific, T.F., *Real-time PCR handbook*. 2016, Thermo Fisher Scientific. p. 68.
74. Heider, S.A., P. Peters-Wendisch, and V.F. Wendisch, *Carotenoid biosynthesis and overproduction in Corynebacterium glutamicum.* BMC Microbiol, 2012. **12**: p. 198.
75. Meissner, D., et al., *Comparative analysis of twin-arginine (Tat)-dependent protein secretion of a heterologous model protein (GFP) in three different Gram-positive bacteria.* Applied Microbiology and Biotechnology, 2007. **76**(3): p. 633-642.
76. Lee, J., *Development and characterization of expression vectors for Corynebacterium glutamicum.* J Microbiol Biotechnol, 2014. **24**(1): p. 70-9.
77. Yang, Z., et al., *Efficient inducible expression of nitrile hydratase in Corynebacterium glutamicum.* Process Biochemistry, 2019. **76**: p. 77-84.
78. Kind, S., et al., *Systems-wide metabolic pathway engineering in Corynebacterium glutamicum for bio-based production of diaminopentane.* Metabolic Engineering, 2010. **12**(4): p. 341-351.
79. Jo, S.-J., et al., *Improvement of Poly(3-Hydroxybutyrate) [P(3HB)] Production in Corynebacterium glutamicum by Codon Optimization, Point Mutation and Gene Dosage of P(3HB) Biosynthetic Genes.* Journal of Bioscience and Bioengineering, 2007. **104**(6): p. 457-463.
80. Patek, M. and J. Nesvera, *Sigma factors and promoters in Corynebacterium glutamicum.* J Biotechnol, 2011. **154**(2-3): p. 101-13.
81. Dombroski, A.J., et al., *The sigma subunit of Escherichia coli RNA polymerase senses promoter spacing.* Proc Natl Acad Sci U S A, 1996. **93**(17): p. 8858-62.

8 Appendices

Appendix A: Growth media and solutions

A.1 Growth media

Brain Heart Infusion Supplemented (BHIS) medium

Brain Heart Infusion Broth (Sigma-Aldrich)	18.5 g
Sorbitol (Sigma-Aldrich)	45.5 g
Distilled water	500 mL

Sterilized by autoclaving for 20 minutes at 120°C.

Brain Heart Infusion Supplemented (BHIS) agar

Brain Heart Infusion Broth (Sigma-Aldrich)	18.5 g
Sorbitol (Sigma-Aldrich)	45.5 g
Agar (OXOID)	7.5 g
Distilled water	500 mL

Sterilized by autoclaving for 20 minutes at 120°C.

LB medium

Tryptone (OXOID)	2.5 g
Yeast extract (OXOID)	1.25 g
NaCl (VWR)	2.5 g
Distilled water	250 mL

Sterilized by autoclaving for 20 minutes at 120°C.

LB agar plates

Tryptone (OXOID)	5.0 g
Yeast extract (OXOID)	2.5 g
NaCl (VWR)	5.0 g
Agar (OXOID)	7.5 g
Distilled water	500 mL

Sterilized by autoclaving for 20 minutes at 120°C.

A.2 Media and solutions for preparation of competent *E. coli*

Psi medium

Yeast extract (OXOID)	2.5 g
Tryptone (OXOID)	10 g
$MgSO_4 \bullet 7H_2O$ (VWR)	5.12 g
Distilled water	to 500 mL

pH adjusted 7.6 using KOH. Sterilized by autoclaving for 20 minutes at 120°C.

TFB1

KAc (potassium acetate) (VWR)	0.588 g
RbCl (rubidium chloride) (Alfa Aesar)	2.42 g
$CaCl_2 \bullet 2H_2O$ (Sigma-Aldrich)	0.389 g
$MnCl_2 \bullet 4H_2O$ (VWR)	3.146 g
Glycerol (99.5%) (VWR)	30 mL
Distilled water	to 200 mL

pH adjusted to 5.8 using diluted acetic acid. Sterilized by filtration.

TFB2

MOPS (Fisher Scientific)	0.21 g
$CaCl_2 \bullet 2H_2O$ (Sigma-Aldrich)	1.1 g
RbCl (Alfa Aesar)	0.121 g
Glycerol (99.5%) (VWR)	15 mL
Distilled water	to 100 mL

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

A.3 Media for transformation of competent *E. coli*

Super Optimal Broth (SOB)

Yeast extract (OXOID)	0.5 g
Tryptone (OXOID)	2.0 g
NaCl (VWR)	0.058 g
KCl (Merck)	0.019 g
$MgSO_4$ (VWR)	0.24 g
Distilled water	100 mL

Sterilized by autoclaving for 20 minutes at 120°C

Super Optimal Broth with Catabolite repression (SOC)

SOB	100 mL
Glucose solution 20 Mm, filter sterilized	2 mL

A.4 Media for protein expression

Hi + Ye medium

100 mL Yeast Extract Hi and 900 mL Basis medium 1 (**Table 8-1**) combined to make 1 L Hi + Ye Basis medium. After autoclaving, 2.5 mL 1M $MgSO_4 \bullet 7H_2O$ was added to 1 L Hi + Ye Basis medium. Glucose solution (240 µL / 30 mL medium) and glycerol solution (600 / 30 mL medium) was also added to the medium.

Table 8-1: Components of Basis medium 1 for Hi + Ye medium*.

Component	g/L	mL stock
$Na_2HPO_4 \bullet 2H_2O$ (Honeywell Fluka)	8.6	
KH_2PO_4 (Merck)	3	
NH_4Cl (Sigma-Aldrich)	1	
NaCl (VWR)	0.5	
Fe(III) citrate hydrate		10
H_3BO_3		0.1
$MnCl_2 \bullet 4H_2O$		1.5
EDTA $\bullet 2H_2O$		0.1
$CuCl_2 \bullet 2H_2O$		0.1
$Na_2Mo_4O_4 \bullet 2H_2O$		0.1
$CoCl_2 \bullet 6H_2O$		0.1
$Zn(CH_3COO)_2 \bullet 2H_2O$		2
Distilled water	To 900 mL	

*Sterilized by autoclaving for 20 minutes at 120°C

Yeast extract Hi

Yeast extract (OXOID)	10 g
Tap water	to 100 mL

Sterilized by autoclaving for 20 minutes at 120°C

1M MgSO4 • 7H2O

$MgSO_4 \bullet 7H_2O$ (VWR)	12.3 g
Distilled water	to 50 mL

Sterilized by autoclaving for 20 minutes at 120°C

Glucose solution for Hi + Ye

Glucose (VWR)	11.35 g
Tap water	to 50 mL

Sterilized by autoclaving for 20 minutes at 120°C

Glycerol solution for Hi + Ye

Glycerol (99.5%) (VWR)	50.15 g
Tap water	to 100 mL

Sterilized by autoclaving for 20 minutes at 120°C

Stock solutions

Stock solutions were prepared by Vectron Biosolutions.

Fe(III) citrate hydrate (Sigma-Aldrich)	3 g
Distilled water	500 mL
H_3BO_3 (Merck)	0.75 g
Distilled water	25 mL
$MnCl_2 \bullet 4H_2O$ (VWR)	2.5 g
Distilled water	250 mL
EDTA $\bullet 2H_2O$ (VWR)	2.1 g
Distilled water	25 mL
$CuCl_2 \bullet 2H_2O$ (ACROS)	0.375 g
Distilled water	25 mL

$Na_2Mo_4O_4 \bullet 2H_2O$ (VWR)	0.625 g
Distilled water	25 mL
$CoCl_2 \bullet 6H_2O$ (Merck)	0.625 g
Distilled water	25 mL
$Zn(CH_3COO)_2 \bullet 2H_2O$ (Merck)	1 g
Distilled water	250 mL

A.5 Solutions for SDS-PAGE and Western blot

1x SDS running buffer

20x SDS Running Buffer (C.B.S. Scientific)	50 mL
Distilled water	950 mL

1x Phosphate-Buffered Saline (PBS)

NaCl (VWR)	4 g
KCl (Merck)	0.1 g
Na_2HPO_4 (Honeywell Fluka)	0.72 g
KH_2PO_4 (Merck)	0.12 g
Distilled water	400 mL

pH adjusted to 7.4 with HCl.

Total volume adjusted to 500 mL with distilled water.

Phosphate-Buffered Saline with Tween-20 (PBST)

1x PBS	1L
Tween-20 (VWR)	500 µL

Skim milk blocking solution

1x PBS	30 mL
Skim milk powder (Sigma-Aldrich)	0.9 g

Primary antibody solution

1x PBS	30 mL
Skim milk powder (Sigma-Aldrich)	0.3 g
Rabbit (Rb) pAb to mCherry (abcam)	5 μL

Secondary antibody solution

1x PBS	30 mL
Skim milk powder (Sigma-Aldrich)	0.3 g
Goat anti-Rb antibody (abcam,1:1000)	30 μL

A.6 Inducers

1M *m*-Toluic acid, inducer

m-Toluic acid (Sigma Aldrich)	0.68 g
99.5% ethanol	10 mL

Sterilized by filtration.

1M IPTG, inducer

IPTG (VWR)	2.38 g
Distilled water	10 mL

Sterilized by filtration

A.7 Antibiotics

Ampicillin stock (100 mg/mL)

Ampicillin (PanReac AppliChem)	2.0 g
Distilled water	20 mL

Sterilized by filtration and aliquoted in Eppendorf tubes. Stored at -20°C.

Chloramphenicol stock (25 mg/mL)

Chloramphenicol (Sigma-Aldrich)	0.125 g
Ethanol 99.9%	5 mL

Sterilized by filtration and aliquoted in Eppendorf tubes. Stored at -20°C.

Kanamycin stock (50 mg/mL)

Kanamycin sulfate (PanReac AppliChem)	0.5 g
Distilled water	10 mL

Sterilized by filtration and aliquoted in Eppendorf tubes. Stored at -20°C.

A.8 Other solutions

50 mM Tris-HCl

Tris hydrochloride (Sigma-Aldrich)	0.3 g
Distilled water	to 50 mL

pH adjusted to 8.0 with HCl

Sterilized by autoclaving for 20 minutes at 120°C or sterilized by filtration for RNA isolation purposes.

0.5 M Ethylene Diamine-Tetra-acetic Acid (EDTA)

EDTA • $2H_2O$ (VWR)	93 g
Distilled water	500 mL

pH adjusted to 8.

Sterilized by autoclaving for 20 minutes at 120°C

TE buffer

50 mM Tris-HCl	5 mL
50 mM EDTA	0.5 mL

Prepared and stored in RNase free tube.

0.5 M sucrose - 10 mM Tris - solution

Sucrose	4,28 g
10 mM Tris-HCl	25 mL

10 mM Tris - 10 mM EDTA

0.5 M EDTA	500 µL
10 mM Tris	24.5 mL

10% Sodium dodecyl sulfate (SDS)

SDS	5 g
Distilled water	50 mL

50x Tris-Acetate-EDTA (TAE) buffer

Tris-base (Sigma-Aldrich)	242 g
Acetic acid (VWR)	57.1 mL
0.5 M EDTA	100 mL
Distilled water	up to 1000 mL

Sterilized by autoclaving for 20 minutes at 120°C

0.8% Agarose gel with GelGreen / GelRed

Agarose	3.2 g
GelGreen / GelRed (Biotioum)	20 μL
1x TAE	400 mL

Heated in a microwave for 2 minutes.

Cooled down to 50°C.

Appendix B: Solutions for iBind Flex Western System (Invitrogen)

Solutions were added to the insert wells of the iBind Flex Western Device according to **Table 8-2**.

1x iBind Flex Solution (per membrane)

iBind Flex 5X Buffer	12 mL
100 x Additive	0.6 mL
Distilled water (to 60 mL)	47.4 mL

Primary antibody solution (1:5000)

1x iBind Flex Solution	5 mL
Rabbit Anti XylS polyclonal Ab (GenScript)	5 μL

Secondary antibody solution (1:400)

1x iBind Flex Solution	5 mL
Goat anti-Rabbit IgG Fc HRP (abcam)	12.5μL

Table 8-2: Placement and the volume of the different solutions in the iBind insert wells.

Row	Solution	Volume (mL)
1	Primary antibody solution (1:5000)	4
2	1x iBind Flex Solution	4
3	Secondary antibody solution (1:400)	4
4	1x iBind Flex Solution	12

Appendix C: Manufacturer's protocol

C.1 Monarch Plasmid Miniprep Kit (New England Biolabs)

Before you begin:

- Add 4 volumes of ethanol (≥95%) to one volume of Plasmid Wash Buffer 2.
- All centrifugation steps should be carried out at 16,000 x g (~ 13,000 RPM)
- If precipitate has formed in Lysis Buffer (B2), incubate at 30-37ºC, inverting periodically to dissolve.
- Store Plasmid Neutralization Buffer (B3) at 4ºC after opening.

Protocol steps:

1. Pellet 1-5 mL bacterial culture by centrifugation for 30 seconds. Discard supernatant.
2. Resuspend pellet in 200 µL Plasmid Resuspension Buffer (B1)
3. Add 200 µL Plasmid Lysis Buffer (B2), gently invert tube 5-6 times, and incubate at room temperature for 1 minute.
4. Add 400 µL of Plasmid Neutralization Buffer (B3), gently invert tube until neutralized, and incubate at room temperature for 2 minutes.
5. Centrifuge lysate for 2-5 minutes.
6. Carefully transfer supernatant to the spin column and centrifuge for 1 minute. Discard flow-through.
7. Re-insert column in the collection tube and add 200 µL of Plasmid Wash Buffer 1. Centrifuge for 1 minute.
8. Add 400 µL of Plasmid Wash Buffer 2 and centrifuge for 1 minute.
9. Transfer column to clean 1.5 mL microfuge tube.
10. Add ≥ 30 µL DNA Elution Buffer to the center of the matrix. Wait for 1 minute, then spin for 1 minute to elute DNA.

C.2 NucleoBond® Xtra Midi (MACHEREY-NAGEL)

Plasmid DNA purification (NucleoBond® Xtra Midi / Maxi)

Protocol-at-a-glance (Rev. 14)

		Midi		Maxi	
1–3	**Cultivation and harvest**	4,500–6,000 x *g* 4 °C, 15 min			
4–5	**Cell lysis** *(Important: Check Buffer LYS for precipitated SDS)*	High-copy / low-copy 8 mL / 16 mL Buffer RES 8 mL / 16 mL Buffer LYS RT, 5 min		High-copy / low-copy 12 mL / 24 mL Buffer RES 12 mL / 24 mL Buffer LYS RT, 5 min	
6	**Equilibration of the column and filter**	12 mL Buffer EQU		25 mL Buffer EQU	
7	**Neutralization**	8 mL / 16 mL Buffer NEU Mix thoroughly until colorless		12 mL / 24 mL Buffer NEU Mix thoroughly until colorless	
8	**Clarification and loading of the lysate**	Invert the tube 3 times Load lysate on NucleoBond® Xtra Column Filter			
9	**1st Wash**	5 mL **Buffer EQU** !		15 mL **Buffer EQU** !	
10	**Filter removal**	Discard NucleoBond® Xtra Column Filter		Discard NucleoBond® Xtra Column Filter	
11	**2nd Wash**	8 mL **Buffer WASH** !		25 mL **Buffer WASH** !	
12	**Elution**	5 mL Buffer ELU		15 mL Buffer ELU	
13	**Precipitation**	**NucleoBond® Xtra Midi**	**NucleoBond® Xtra Midi Plus**	**NucleoBond® Xtra Maxi**	**NucleoBond® Xtra Maxi Plus**
		3.5 mL Isopropanol Vortex 4,5–15,000 x *g* 4 °C, 30 min	3.5 mL Isopropanol Vortex RT, 2 min Load NucleoBond® Finalizer	10.5 mL Isopropanol Vortex 4,5–15,000 x *g* 4 °C, 30 min	10.5 mL Isopropanol Vortex RT, 2 min Load NucleoBond® Finalizer Large
14	**Washing and drying**	2 mL 70 % ethanol 4,5–15,000 x *g* RT, 5 min 10–15 min	2 mL 70 % ethanol ≥ 6 x air until dry	4 mL 70 % ethanol 4,5–15,000 x *g* RT, 5 min 15–30 min	4 mL 70 % ethanol ≥ 6 x air until dry
15	**Reconstitution**	Appropriate volume of TE buffer	200–800 µL Buffer TRIS	Appropriate volume of TE buffer	400–1000 µL Buffer TRIS

MACHEREY-NAGEL GmbH & Co. KG · Neumann-Neander-Str. 6–8 · 52355 Düren · Germany
Tel.: +49 24 21 969-270 · Fax: +49 24 21 969-199 · tech-bio@mn-net.com · ***www.mn-net.com***

C.3 Zymoclean Gel DNA Recovery Kit (Zymo Research)

Protocol:

- Buffer Preparation: Add 24 mL 100% ethanol (26 mL 95% ethanol) to the 6 mL DNA Wash Buffer concentrate. Add 96 mL 100% ethanol (104 mL 95% ethanol) to the 24 mL DNA Wash Buffer concentrate.
- Perform all centrifugation at ≥ 10 000 x g

1. Excise the DNA fragment from the agarose using a razor blade, scalpel or other device and transfer it into a 1.5 mL microcentrifuge tube.
2. Add 3 volumes of ADB to each volume of agarose excised from the gel (e.g, for 100 μL (mg) agarose gel slice add 300 μL of ADB).
3. Incubate at 37-55 ºC for 5-10 minutes until the gel slice is completely dissolved.

   For DNA fragments > 8 kb, following the incubation step, add one additional volume (equal to that of the gel slice) of water to the mixture for better DNA recovery (e.g. 100 μL agarose, 300 μL ADB, and 100 μL water).

4. Transfer the melted agarose solution to a Zymo-Spin™ Column in a Collection Tube.
5. Centrifuge for 30-60 seconds. Discard the flow-through.
6. Add 200 μL of DNA Wash Buffer to the column and centrifuge for 30 seconds. Discard the flow-through. Repeat the wash step.
7. Add ≥ 6 μL DNA Elution Buffer or water directly to the column matrix. Place column into a 1.5 mL tube and centrifuge for 30-60 seconds to elute DNA.

C.4 Quick-RNA™ Fungal / Bacterial Miniprep Kit (Zymo Research)

Protocol:

Perform all steps at room temperature and centrifugation at 10,000-16,000 x g for 30 seconds, unless specified.

1. Resuspend a fresh or frozen cell pellet in 800 µl RNA Lysis Buffer1 and transfer
2. the mixture to a ZR BashingBeadTM Lysis Tube.
3. Secure in a bead beater fitted with a 2 ml tube holder assembly and process2.
4. Centrifuge the ZR BashingBeadTM Lysis Tube for 1 minute.
5. Transfer 400 µl supernatant to a Zymo-SpinTM IIICG Column4 in a Collection Tube and centrifuge for 30 seconds. Save the flow-through!
6. Add 1 volume ethanol (95-100%) to the flow-through in the Collection Tube and mix well.
7. Transfer the mixture to a Zymo-SpinTM IIC Column4 in a Collection Tube and centrifuge for 30 seconds5. Discard the flow-through.
8. Add 400 µl RNA Prep Buffer to the column and centrifuge for 30 seconds. Discard the flow-through.
9. Add 700 µl RNA Wash Buffer to the column and centrifuge for 30 seconds. Discard the flow-through.
10. Add 400 µl RNA Wash Buffer to the column and centrifuge for 2 minutes to ensure complete removal of the wash buffer. Transfer the column carefully into an RNase-free tube (not provided).
11. Add 50 µl DNase/RNase-Free Water directly to the column matrix and centrifuge for 30 seconds.

Alternatively, for highly concentrated RNA use ≥25 µl elution.

The eluted RNA can be used immediately or stored at -70°C.

Appendix D: Oligonucleotides overview

Table 8-3 gives an overview of the oligonucleotides used in this study with 5'-3' sequences and a short description of their application.

Table 8-3: Oligonucleotides used in this study. Elongation direction indicated, forward (FW) and reverse (RV).

Primer	Sequence 5'-3'	Description of use
mCherry_FW	AGGTGAAGGTCGTCCGTATG	Colony PCR. Verify *mCherry* presence (FW)
ASK2	ATACCGCCGGTAGAGTGACG	Colony PCR. Verify *mCherry* presence (RV)
Pm_mCherry	TTGCAAGAAGCGGATACAGG	Colony PCR. Verify *Pm*/UTR plus *mCherry*
tac_mCherry	CATCGGAAGCTGTGGTATGG	Colony PCR. Verify *tac* promoter plus *mCherry*
ASK5	GCAAGAAGCGGATACAGGAGTG	Amplify XbaI-UTR-GFP-NotI from pVB-1C0C1-GFP. (FW)
ASK6	TGACGCAGTAGCGGTAAACG	Amplify XbaI-UTR-GFP-NotI from pVB-1C0C1-GFP. (RV)
ASK7	ATGATCATATGATGGTTTCTAAGGGCG	Amplify *optm_mCherry* from pMA, replacing *Hind*III with NdeI
ASK8	CCGGGGATCCTCATGCTCATTTATA	Amplify *optm_mCherry* from pMA, eliminating downstream XbaI site
ASK9	GGTTTCTAAGGGCGAAGAGG	Colony PCR. Verify presence of *optm_mCherry*
ASK10	ACAGCCAAGCTGAATTCG	Colony PCR. Verify presence of *optm_mCherry*
ASK11	GGTGTAGCGGTGAAATG	qPCR primer. mRNA quantification of 16S RNA in *C. glutamicum.* (FW)
ASK12	AGTTACTGCCCAGAGAC	qPCR primer. mRNA quantification of 16S RNA in *C. glutamicum.* (RV)
ASK13	ACAGCCAAGCTGAATTCG	Reverse sequencing pXMJ19
ASK14	CTCGCAAGCTTATGGCGAGCAAA	Cloning non-optimized *GFP* into pXMJ19
ASK15	TTATTCGCCGGCGCCTAGGCATTA	Cloning non-optimized *GFP* into pXMJ19
ASK16	CATCCGCCAAAACAGCCAAG	Sequencing of pVB-4A0E1 vectors (RV)
ASK17	GAGTCATGAAagcttATGGTTTCTAAAGGTG	Site-directed mutagenesis primer, replacing NdeI with *Hind*III cloning site
ASK18	CATTATTATTGTACATGTTGC	Site-directed mutagenesis primer, replacing NdeI with *Hind*III cloning site
ASK19	GACTCACTATAGGGCGAATTGG	Amplify *optm_mCherry*, introducing ds NotI site (FW)
ASK20	GCGGCCGCTCATTTATAGAGCTCGTCCATTCC	Amplify *optm_mCherry*, introducing ds NotI site (RV)

ASK21	TTCTAGTGTAGCCGTAGTTAGGCCACCACTTC	Gibson assembly. Combined with pVB4_XylS_SLIC_rv
ASK22	ACGGCTACACTAGAAGAACAGTATTTGGTATC	Gibson assembly. Combined with ASK23
ASK23	GCGACGCCCAACCTGCCATCACGAGATTTCGATTC	Gibson assembly. Combined with ASK22
ASK24	CAGGTTGGGCGTCGCTTGGTCGGTCATTTC	Gibson assembly. Combined with pVB4_XylS_SLIC_fw
ASK25	ACGGTGGTGTTGTTACCGTT	qPCR primer. mRNA quantification of non-optimized *mCherry.* (FW)
ASK26	ACGTTCAGAAGACGCTTCCC	qPCR primer. mRNA quantification of non-optimized *mCherry.* (RV)
17-6	GTGCACCAATGCTTCTG	Sequencing of pXMJ19 vectors (FW)
E.c-16S-f	CCAGCAGCCGCGGTAAT	qPCR primer. mRNA quantification of 16S RNA in *E. coli.* (FW)
E.c-16S-r	TGCGCTTTACGCCCAGTAAT	qPCR primer. mRNA quantification of 16S RNA in *E. coli.* (RV)
pVB4_XylS_SLIC_fw	ATGGATTTTTGCTTATTGAACG	Amplifying the pVB-4 vector without *Ps1* and *Ps2.* (FW)
pVB4_XylS_SLIC_rv	AGCAGAACGAAGACGTTC	Amplifying the pVB-4 vector without *Ps1* and *Ps2.* (RV)
Ptuf-fw-SLIC	TGGCCGTTACCCTGCGAATGTCAGCAGAACGAAGACG	Amplifying P_{tuf} from the *C. glutamicum* genome. (FW)
Ptuf-rv-SLIC	TAAGCAAAAATCCATTGTATGTCCTCCTGGACTTCGT	Amplifying P_{tuf} from the *C. glutamicum* genome. (RV)
Psod-fw-SLIC	TAGCTGCCAATTATTCCGGGCTAGCAGAACGAAGACG	Amplifying P_{sod} from the *C. glutamicum* genome. (FW)
Psod-rv-SLIC	TAAGCAAAAATCCATGGGTAAAAAATCCTTTCGTAG	Amplifying P_{sod} from the *C. glutamicum* genome (RV)
PdapA-fw-SLIC	TAAGCAAAAATCCATTAGAGTTCAAGGTTACCTT	Amplifying P_{dapA} from the *C. glutamicum* genome. (FW)
PdapA-rv-SLIC	CGCAAAGCTCACACCCACGAAGCAGAACGAAGACG	Amplifying P_{dapA} from the *C. glutamicum* genome. (RV)
PdapB-fw-SLIC	TATGCTCCTTCATTTTCGTGAGCAGAACGAAGACG	Amplifying P_{dapB} from the *C. glutamicum* genome. (FW)
PdapB-rv-SLIC	TAAGCAAAAATCCATAAGGGCAACTTAAGTCTCAT	Amplifying P_{dapB} from the *C. glutamicum* genome. (RV)

Appendix E: Molecular weight standards

Molecular weight standards with reference bands of known sizes were used for molecular weight estimation of fragments separated by agarose gel electrophoresis and SDS-PAGE, and for size estimation of bands on Western blot membranes. For agarose gel electrophoresis, the 1 kb DNA Ladder (NEB) or the 50 bp DNA Ladder (Invitrogen) were added to the agarose gel prior to gel electrophoresis. Figures with indicated fragment sizes for the 1 kb DNA Ladder (NEB) and the 50 bp DNA Ladder (Invitrogen) are included in **Figure 8-1** and **Figure 8-2**, respectively. The Precision Plus Protein™ Dual Color Standard (Bio-Rad) was added to the polyacrylamide gel prior to SDS-PAGE and used for estimation on SDS-PAGE gels and Western blots, an image of the standard with indicated fragment sizes are included in **Figure 8-3**.

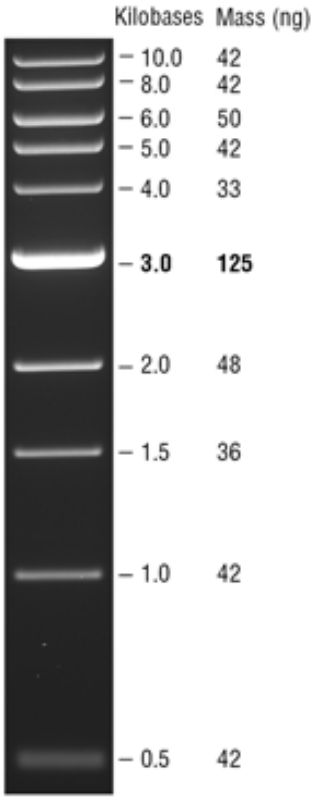


Figure 8-1: Image of 1 kb DNA ladder (NEB) with indicated sizes given in kilobases (kb) and mass (ng). Reference band at 3 kb and 125 ng.

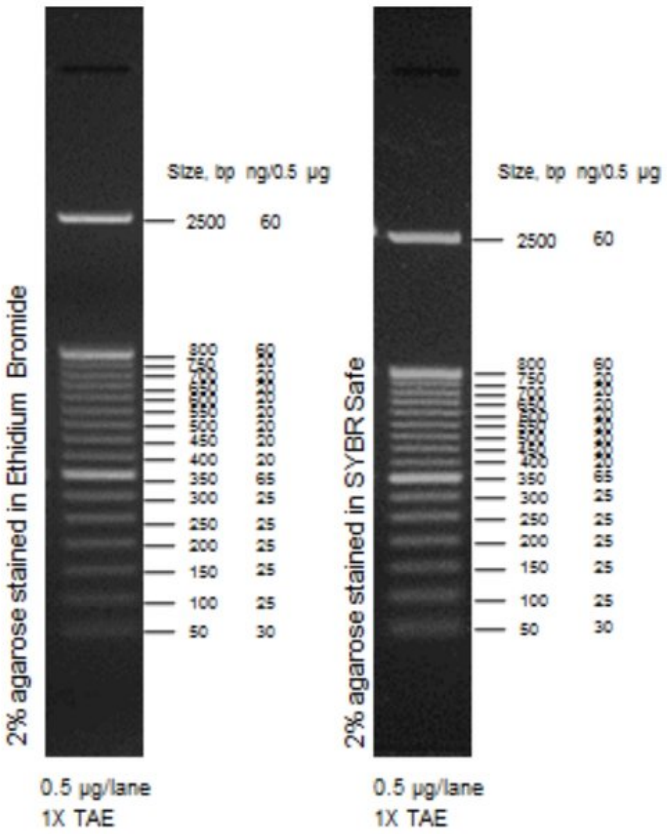


Figure 8-2: Image of 50 bp DNA Ladder (Invitrogen) with indicated fragments sizes given in base pairs (bp) and ng / 0.5 µg. Reference bands at 2500, 800 and 350 bp.

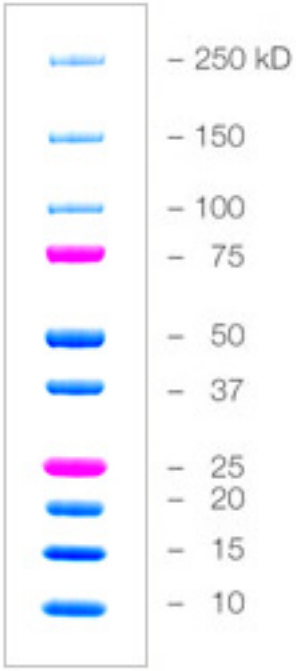


Figure 8-3: Image of Precision Plus Protein™ Dual Color Standard (Bio-Rad) with indicated fragment sizes given in kilo Dalton (kD). 8 blue-stained bands and 2 pink reference bands at 75 and 25 kD.

Appendix F: pXMJ19-mCherry and pVB-4A0E1-mCherry plasmid maps.

Plamid maps of pXMJ19-mCherry (**Figure 8-4**) and pVB-4A0E1-mCherry (**Figure 8-5**) were generated in Benchling using sequencing data obtained from Genscript.

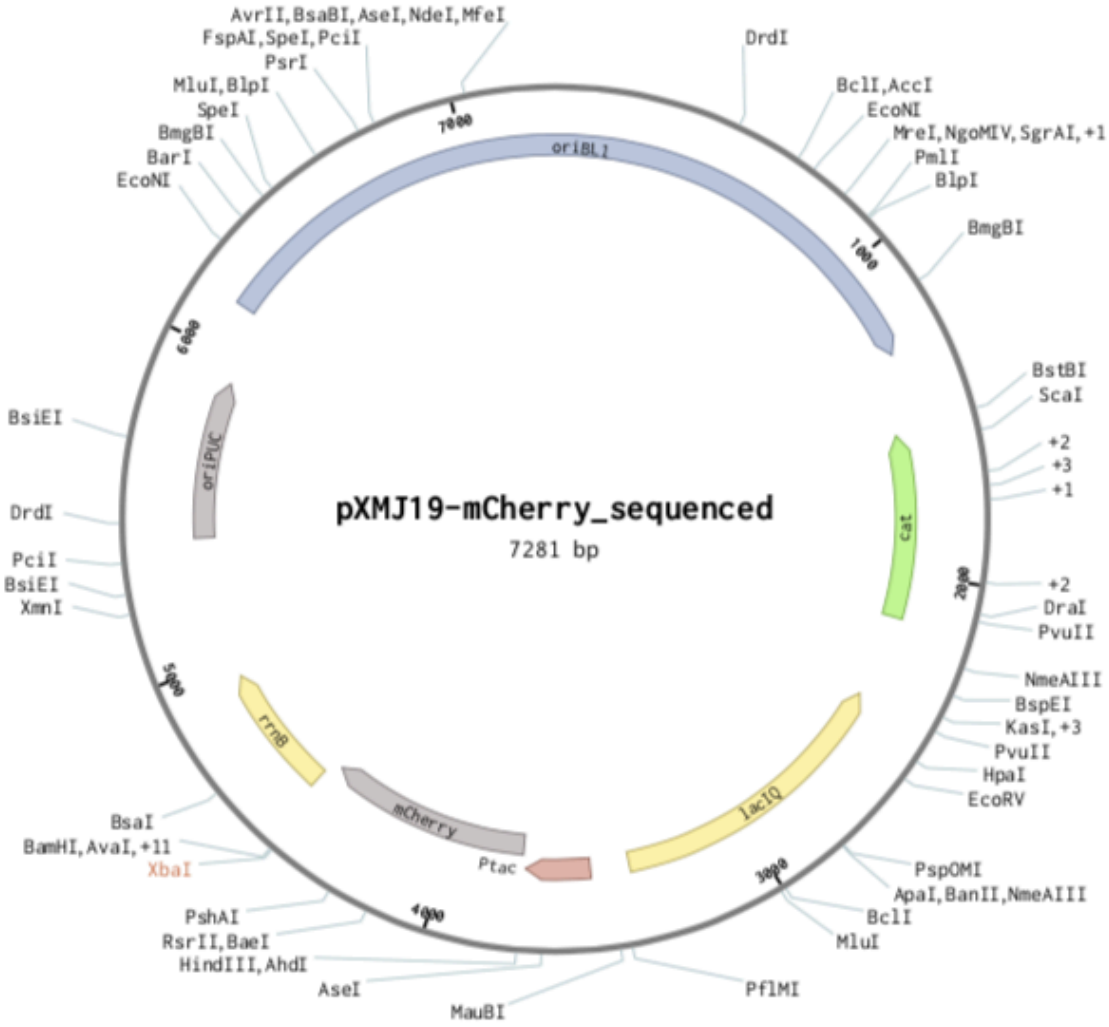


Figure 8-4: Plasmid map of pXMJ19-mCherry (7281 bp). Ori PUC = Origin of replication (ORI) in *E. coli*, Ori BL1 = ORI in *C. glutamicum*, cat = chloramphenicol acetyltransferase gene, $lacI^q$ gene, mCherry gene under P_{tac} promoter control, rrnB = T1 and T2 terminators.

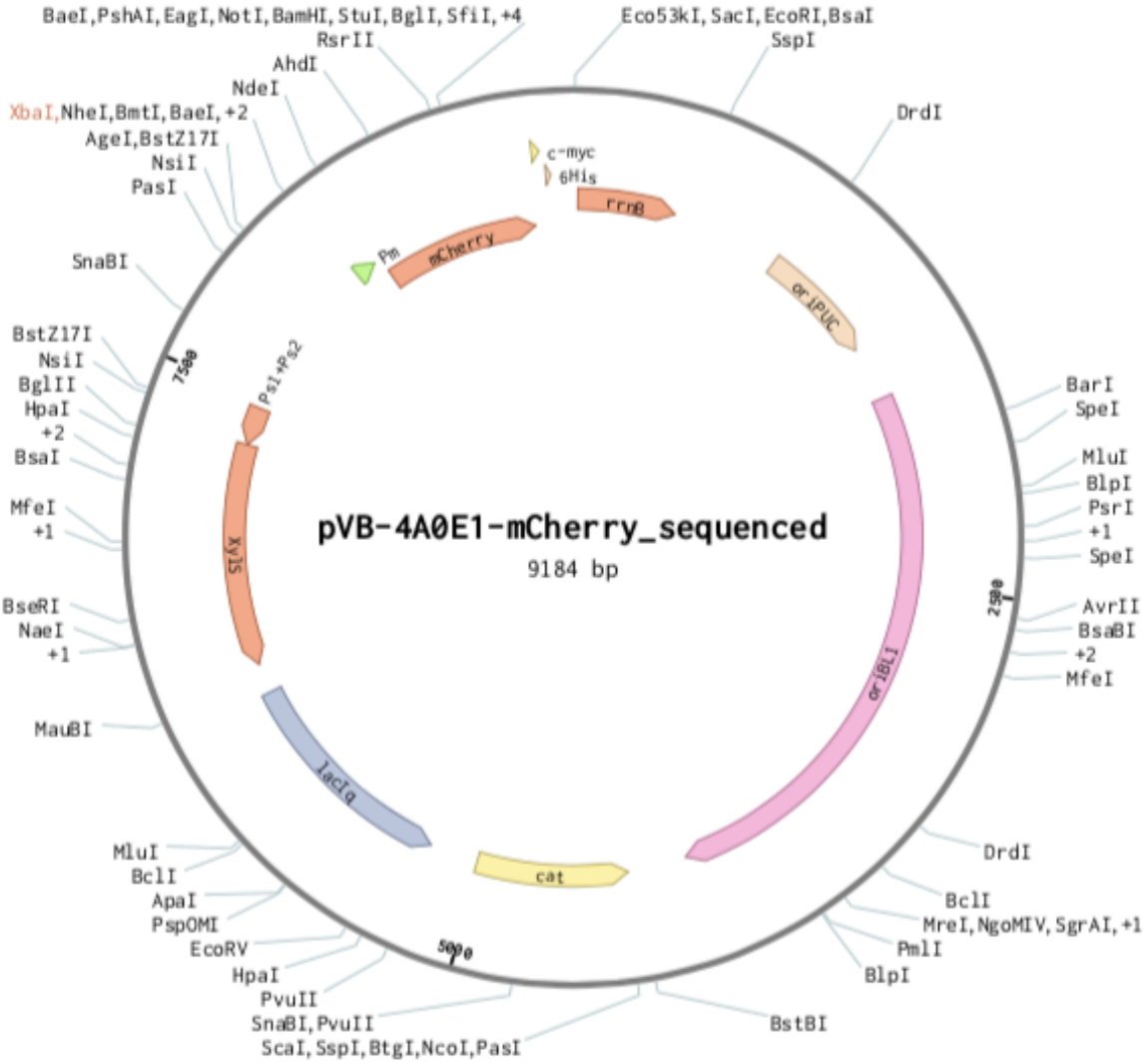
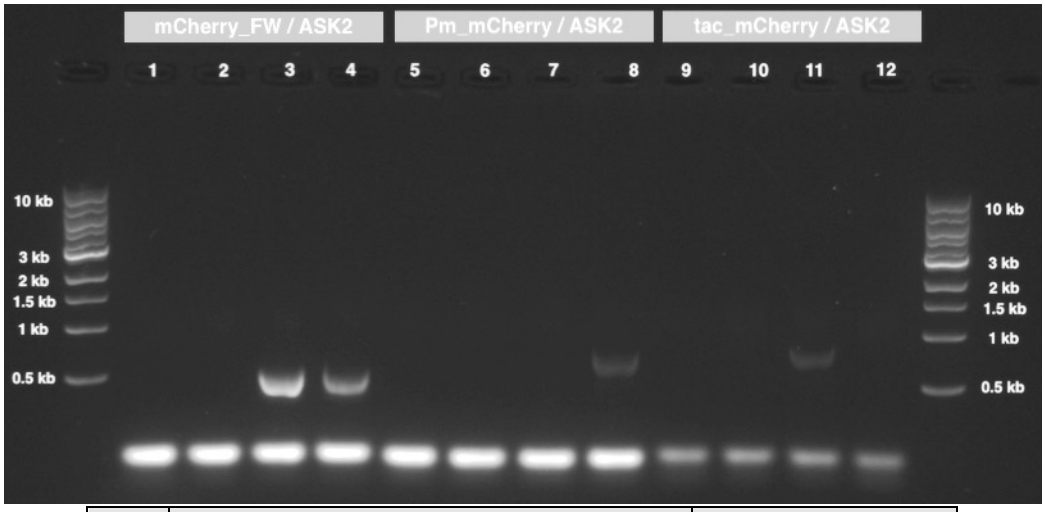


Figure 8-5: Plasmid map of pVB-4A0E1-mCherry (9184 bp). Ori PUC = Origin of replication (ORI) in *E. coli*, Ori BL1 = ORI in *C. glutamicum*, cat = chloramphenicol acetyltransferase gen, $lacI^q$ gene, *xylS* under *Ps1* and *Ps2* control, *mCherry* under *Pm* control, c-myc and 6His protein tag, rrnB = T1 and T2 terminators.

Appendix G: Verifying presence of vectors in "old" *C. glutamicum* transformants

G.1 Verifying primers for colony PCR in *E. coli*

Primers intended for colony PCR were first verified using *E. coli* DH5α colonies harbouring pXMJ19, pXMJ19-mCherry and pVB-4A0E1-mCherry. If colonies contained correct constructs, amplification with primer pair mCherry_FW / ASK2 would amplify a region within *mCherry* and give a fragment of 582 bps, Pm_mCherry / ASK2would amplify *Pm* / UTR plus *mCherry* and give a fragment of 794 bps and tac_mCherry / ASK2 would amplify the *tac* promoter plus *mCherry* and give a fragment of 908 bps. **Figure 8-6** shows a picture of the agarose gel after gel electrophoresis of the PCR products. The low-molecular weight band present in all sample lanes are probably caused by primer dimerization



Lane	Strain	Primer pair
1	ntc	mCherry_FW / ASK2
2	*E. coli* DH5α pXMJ19	mCherry_FW / ASK2
3	*E. coli* DH5α pXMJ19-mCherry	mCherry_FW / ASK2
4	*E. coli* DH5α pVB-4A0E1-mCherry-mCherry	mCherry_FW / ASK2
5	ntc	Pm_mCherry / ASK2
6	*E. coli* DH5α pXMJ19	Pm_mCherry / ASK2
7	*E. coli* DH5α pXMJ19-mCherry	Pm_mCherry / ASK2
8	*E. coli* DH5α pVB-4A0E1-mCherry-mCherry	Pm_mCherry / ASK2
9	ntc	tac_mCherry / ASK2
10	*E. coli* DH5α pXMJ19	tac_mCherry / ASK2
11	*E. coli* DH5α pXMJ19-mCherry	tac_mCherry / ASK2
12	*E. coli* DH5α pVB-4A0E1-mCherry-mCherry	tac_mCherry / ASK2

Figure 8-6: Agarose gel after gel electrophoresis and colony PCR of *E. coli* DH5α harbouring pXMJ19, pXMJ19-mCherry or pVB-4A0E1-mCherry. Lane content given in Table X. Ladder: 1 kb DNA ladder (NEB).

G.2 Linearization of plasmids isolated from *C. glutamicum*

Plasmids were isolated from *C. glutamicum* pXMJ19, pXMJ19-mCherry and pVB-4A0E1-mCherry with the alternative pre-treatment described in section **2.3.1**. Plasmids were submitted to single cut RE digestion with *Xba*I, *Hind*III, *Eco*RI and *Bam*HI, all digestions were run in samples. The expected fragment sizes for successful linearization are 6601 bps for pXMJ19, 7281 bps for pXMJ19-mCherry and 9184 bps for pVB-4A0E1-mCherry. **Figure 8-7** shows a picture of the agarose gel after gel electrophoresis.

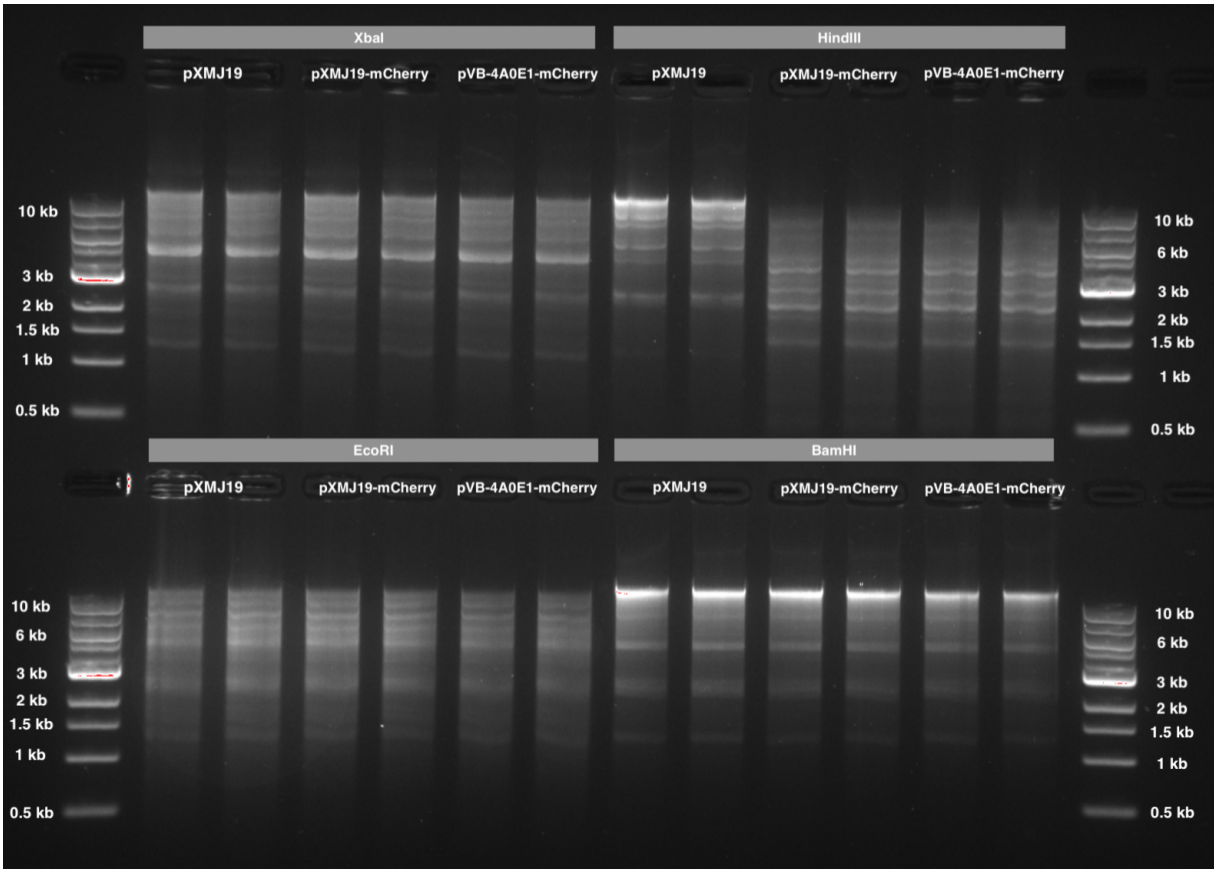
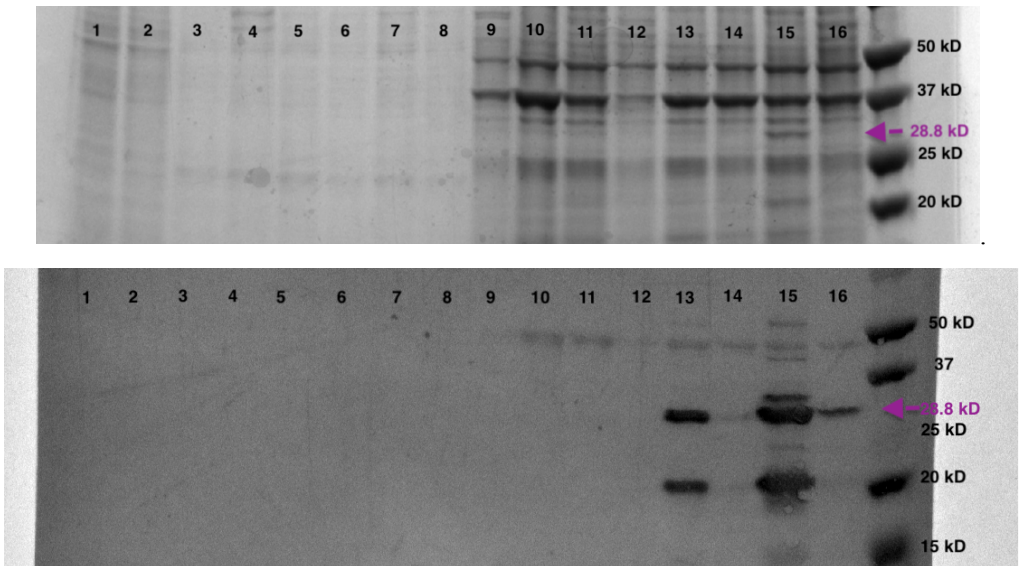


Figure 8-7: Agarose gel after gel electrophoresis of linearized plasmids isolated from *C. glutamicum*. Parallel samples of pXMJ19, pXMJ19-mCherry and pVB-4A0E1-mCherry digested using restriction enzyme XbaI, HindIII, EcoRI and BamHI. Ladder: 1 kb DNA ladder (NEB).

G.3 SDS-PAGE and Western blot of insoluble fraction

The insoluble protein content of *C. glutamicum* and *E. coli* were analysed using SDS-PAGE and Western blot, Images of the SDS-PAGE gel and Western blot are included in **Figure 8-8**. Lane content given in Table X for both figures.



Lane	Strain	Lane	Strain
1	*C. glutamicum* (no vector) I	9	*E. coli* BL21 pVB1 I
2	*C. glutamicum* (no vector) NI	10	*E. coli* BL21 pVB1 NI
3	*C. glutamicum* pXMJ19 I	11	*E. coli* BL21 pXMJ19 I
4	*C. glutamicum* pXMJ19 NI	12	*E. coli* BL21 pXMJ19 NI
5	*C. glutamicum* pXMJ19-mCherry I	13	*E. coli* BL21 pXMJ19-mCherry I
6	*C. glutamicum* pXMJ19-mCherry NI	14	*E. coli* BL21 pXMJ19-mCherry NI
7	*C. glutamicum* pVB-4A0E1-mCherry I	15	*E. coli* BL21 pVB-4A0E1-mCherry I
8	*C. glutamicum* pVB-4A0E1-mCherry NI	16	*E. coli* BL21 pVB-4A0E1-mCherry NI

Figure 8-8: Image of SDS-PAGE-gel (top image) and Western blot (bottom image) of insoluble protein fraction for *C. glutamicum* and *E. coli* BL21. Lane content given in Table below. Size of mCherry (28.8 kD) indicated in purple. Ladder: Precision Plus Protein Dual Color standards (Bio-Rad).

G.4 Antibiotic resistance assay for new *C. glutamicum* transformants

New *C. glutamicum* pXMJ19, pXMJ19-mCherry and pVB-4A0E1-mCherry were plated on BHIS agar with 0, 5, 25 and 50 ng/mL CM added. **Figure 8-9** show the bacterial growth on the plates after incubation.

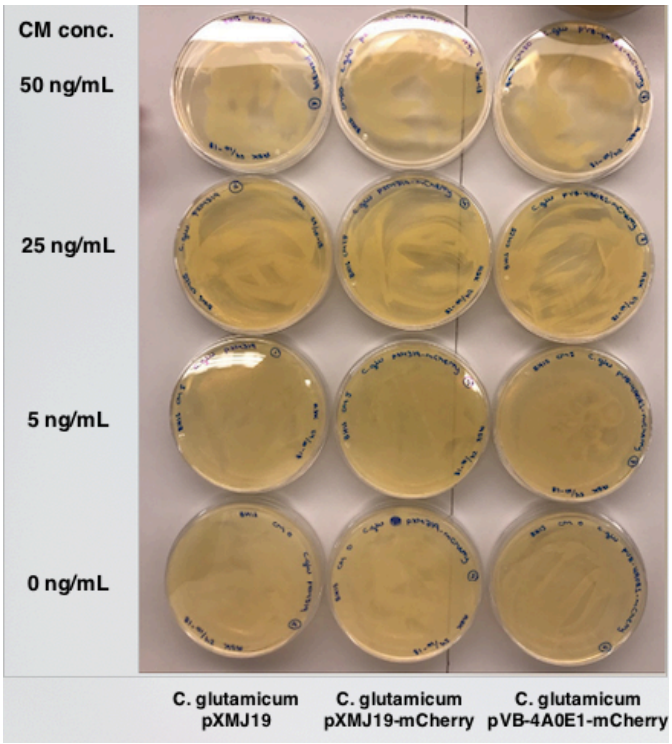


Figure 8-9: Antibiotic resistance assay for new *C. glutamicum* MB001 (DE3) transformants. From the left: *C. glutamicum* pXMJ19 (column 1), *C. glutamicum* pXMJ19-mCherry (column 2) and *C. glutamicum* pVB-4A0E1-mCherry (column 3) plated on BHIS plates with chloramphenicol (CM) added in the following concentrations from the top: 50 ng/mL (row 1), 25 ng/mL (row 2), 5 ng/mL (row 3) and 0 ng/mL (row 4).

Appendix H: Modifying the pVB-4A0E1 backbone

The pVB-4A0E1-mCherry backbone was modified through RE digestion with NotI and site-directed mutagenesis. An of the agarose gel of NotI digested pVB-4A0E1-mCherry are presented in **Figure 8-10**, together with the NotI control digestions of plasmids isolated from four random *E. coli* DH5α transformants. An image of HindIII and NotI control digestions for the site-directed mutagenesis product, pASK2-mCherry, isolated from four random *E. coli* DH5α transformants are presented in **Figure 8-11**.

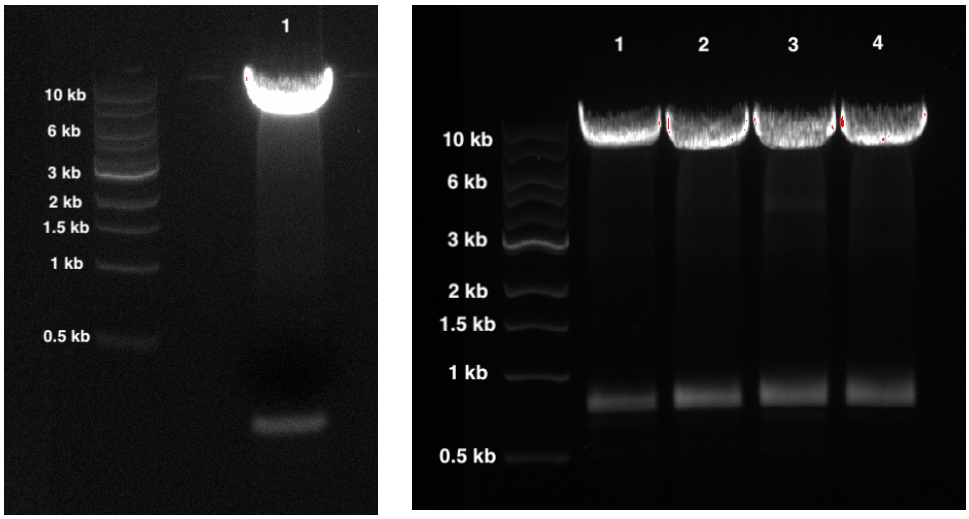


Figure 8-10: Left image: Agarose gel after electrophoresis of NotI digested pVB-4A0E1-mCherry (lane 1). Expected fragments of 9018 bps and 166 bps. Right image: Agarose gel after electrophoresis of XbaI and NotI digested pASK1-mCherry from four random *E. coli* DH5α transformants (lane 1-4). Expected fragments of 8243 bps + 775 bps. Ladder in both images: 1 kb DNA ladder (NEB).

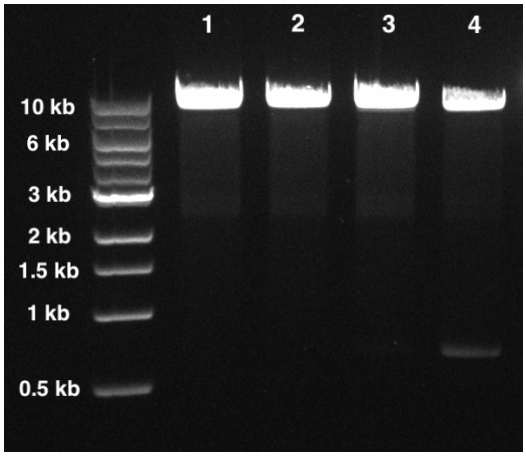


Figure 8-11: Agarose gel after electrophoresis of HindIII and NotI digested pASK2-mCherry from four random *E. coli* DH5α transformants (lane 1-4). Expected fragments of 8303 bps + 718 bps. Ladder:1 kb DNA ladder (NEB).

Appendix I: Primer and RE overview for alternative reporter expression vectors

Table 8-4 gives an overview of the alternative reporter gene vectors constructed using restriction enzyme and DNA ligation cloning. The overview includes template vectors, primers used to introduce compatible recognition sites if needed and restriction enzymes used in the cloning process

Table 8-4: Overview of expression vectors with alterative reporters cloned with restriction enzyme and DNA ligation.

Constructed vector	Template vectors	PCR Primers	RE digestion	Fragment sizes (bp)
pXMJ19-GFP (7295 bp)	pXMJ19-mCherry		*Hind*III *Bam*HI-HF	**6558** + 723
	pMA-GFP	ASK14 ASK15	*Hind*III *Bam*HI-HF	**737** + 10
pXMJ19-optm_mCherry (7281 bp)	pXMJ19-mCherry		*Hind*III *Bam*HI-HF	**6558** + 723
	pMA-T-C_gluta_opt-mCherry		*Hind*III *Bam*HI-HF	2417 + **723**
pXMJ19-optm_GFP (7307 bp)	pXMJ19-mCherry		*Hind*III *Bam*HI-HF	**6558** + 723
	pUC57-GFP-optm_Cgluta		*Hind*III *Bam*HI-HF	2543 + **749** + 29
pASK1-GFP (9029 bp)	pVB-4A0E1-mCherry		*Xba*I *Not*I	**8243** + 775 + 166
	pVB-1C0C1-GFP	ASK5 ASK6	*Bam*HI-HF *Not*I	**786** + 87
pASK2-optm_mCherry (9021 bp)	pASK2-mCherry		*Hind*III *Not*I	**8303** + 718
	pMA-T-C_gluta_opt-mCherry	ASK19 ASK20	*Hind*III *Not*I	**718** + 6
pASK2-optm_GFP	pASK2-mCherry		*Hind*III *Not*I	**8303** + 718
	pUC57-GFP-optm_Cgluta		*Hind*III *Not*I	2256 + **736** + 29

Appendix J: Verifying presence of constructed expression vectors in *C. glutamicum*

The presence of pXMJ19-optm_mCherry and pXMJ19-optm_GFP in recombinant *C. glutamicum* was detected by control digestion with *Hind*III and *Bam*HI and subsequent fragment reparation by agarose gel electrophoresis, an image of the gel is included in **Figure 8-12**. Expected fragment sizes is 6571 bps + 724 bps for pXMJ19-optm_mCherry and 6558 bps + 749 bps for pXMJ19-optm_GFP.

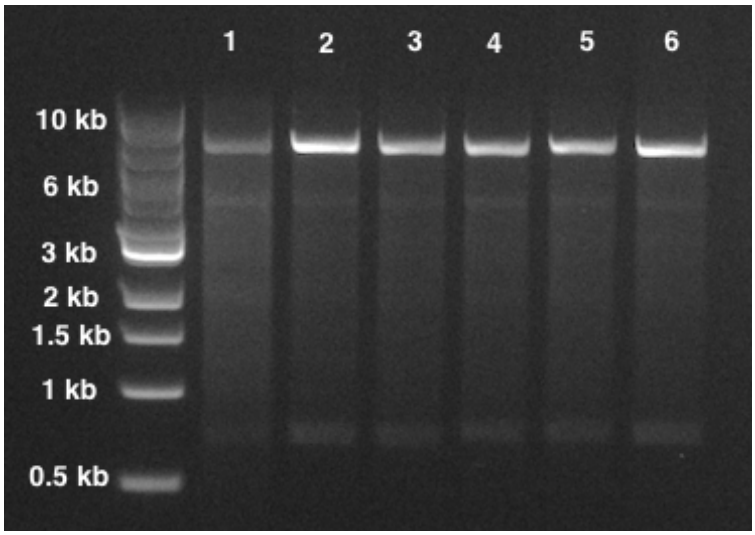


Figure 8-12: Agarose gel of HindIII and BamHI digested pXMJ19-optm_mCherry (lane 1-3) and pXMJ19-optm_GFP (lane 4-6) isolated from recombinant *C. glutamicum*. Ladder: 1 kb DNA ladder (NEB).

The presence of pASK2-optm_mCherry, pASK2-optm_GFP, pASK1-GFP and pXMJ19-GFP in recombinant *C. glutamicum* was verified using colony PCR. The primer pairs used for each reaction and the expected fragment size are included in **Table 8-5**. Images of the agarose gels after gel electrophoresis of PCR products for pASK2-optm_mCherry and pASK2-optm_GFP are included in **Figure 8-13**, pASK1-GFP in **Figure 8-14** and pXMJ19-GFP in **Figure 8-15**.

Table 8-5:Primer pairs and the expected fragment size for verification of recombinant *C. glutamicum* by colony PCR.

***C. glutamicum* strain**	**Primer pair (FW / RV)**	**Expected fragment size (bps)**
pASK2-optm_mCherry	ASK9 / ASK10	743
pASK2-optm_GFP	ASK3 / ASK16	879
pASK1-GFP	ASK3 / ASK16	869
pXMJ19-GFP	ASK4 / ASK13	985

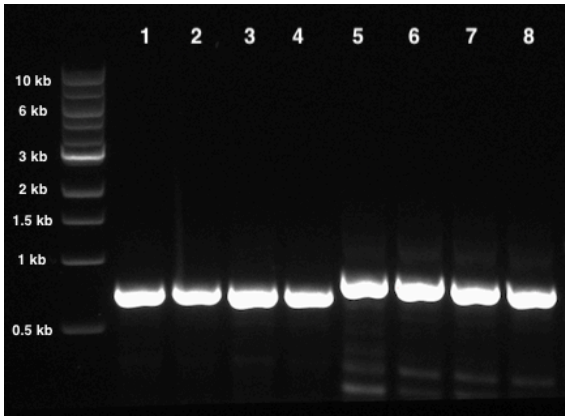


Figure 8-13: Agarose gel after gel electrophoresis and colony PCR of *C. glutamicum* pASK2-optm_mCherry (lane 1-4) and pASK2-optm_GFP (lane 5-8). Ladder: 1 kb DNA ladder (NEB).

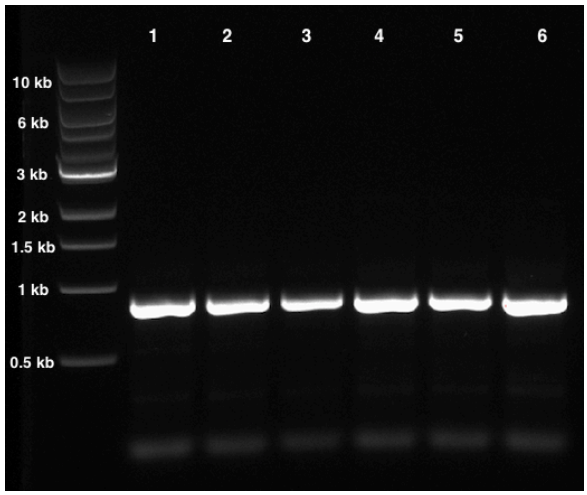


Figure 8-14: Agarose gel after gel electrophoresis and colony PCR of *C. glutamicum* pASK1-GFP (lane 1-6). Ladder: 1 kb DNA ladder (NEB).

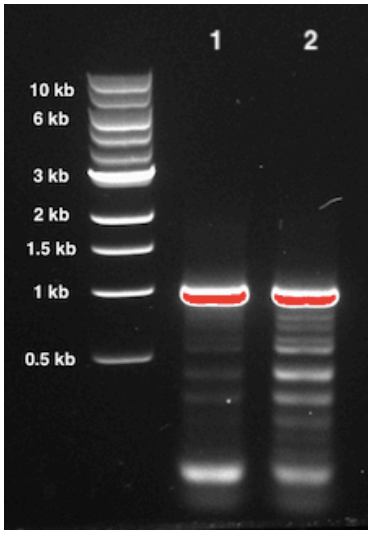


Figure 8-15: Agarose gel after gel electrophoresis and colony PCR of *C. glutamicum* pXMJ19-GFP (lane 1-2). Ladder: 1 kb DNA ladder (NEB).

Appendix K: Optimizing lysis pre-treatment for RNA-isolation

For RNA isolation from *C. glutamicum*, several different lysis-pre-treatments were tested, an overview of the unsuccessful pre-treatments are listed in **Table 8-6**. RNA was isolated using either RNAqueous Total RNA Isolation Kit (Thermo Fisher Scientific) or Quick-RNA Fungal / Bacterial Miniprep Kit (Zymo Research). Representative electropherograms for RNA samples isolated using the RNAqueous Total RNA Isolation Kit (Thermo Fisher Scientific) with pre-treatments 1-5 are presented in **Figure 8-16**. Representative electropherograms for RNA samples isolated using the Quick-RNA Fungal / Bacterial Miniprep Kit (Zymo Research) with pre-treatments 7-8 are presented in **Figure 8-17**, representative electropherograms for pre-treatment 9 and 10 are included in the result section of this thesis.

Table 8-6: Lysis pre-treatments tested for RNA-isolation from *C. glutamicum*

Nr.	Buffer solution	Lysozyme conc.	Incubation time at 37°C	Isolation Kit	Comment
1	100 µL TE buffer	1 mg /mL	2 hours	RNAqueous Total RNA Isolation Kit	Pre-treatment suggested by the kit, with a prolonged incubation time. • Autoclaved buffers used
2	100 µL TE buffer	15 mg/mL	2 hours	RNAqueous Total RNA Isolation Kit	Increased lysozyme concentration.
3	100 µL TE buffer	15 mg/mL	1 hour	RNAqueous Total RNA Isolation Kit	Increased lysozyme concentration, decreased incubation time.
4	100 µL TE buffer	20 mg/mL	30 minutes	RNAqueous Total RNA Isolation Kit	Increased lysozyme concentration, decreased incubation time.
5	400 µL 50 mM EDTA	2 mg/mL	1 hour	RNAqueous Total RNA Isolation Kit	Successful pre-treatment for another gram-positive species in the lab group
6	400 µL TE buffer, 400 µL 2x DNA /RNA Shield (Zymo Research)	10 mg/mL	30 minutes	Quick-RNA Fungal / Bacterial Miniprep Kit	400 µL 2x DNA/RNA Shield added after incubation with TE 10 mg/mL lysosyme. Centrifuged, 800 µL RNA Lysis Buffer added to supernatant and protocol followed from step 4.
7		-	-	Quick-RNA Fungal / Bacterial Miniprep Kit	Manufacturer's protocol followed, with tissue disruption in the TissueLyser II (QIAGEN) for 1x5min at 30 Hz.
8				Quick-RNA Fungal / Bacterial Miniprep Kit	Manufacturer's protocol followed, with tissue disruption in the TissueLyser II (QIAGEN) for 3x5min at 30 Hz.
9	800 µL RNA Lysis Buffer (Zymo Research)	15 mg/mL	40 min	Quick-RNA Fungal / Bacterial Miniprep Kit	15 mg/mL lysozyme added to the RNA Lysis Buffer and incubated for 40 minutes before the protocol was followed as described.
10	800 µL RNA Lysis Buffer (Zymo Research)	5, 33 µL Ready-Lyse Lysozyme Solution (Lucigen)	40 min	Quick-RNA Fungal / Bacterial Miniprep Kit	5, 33 µL Ready-Lyse Lysozyme Solution added to the RNA Lysis Buffer and incubated for 40 minutes before the protocol was followed as described.

K.1: Electropherograms of RNA isolated with the RNAqueous Total RNA Isolation Kit

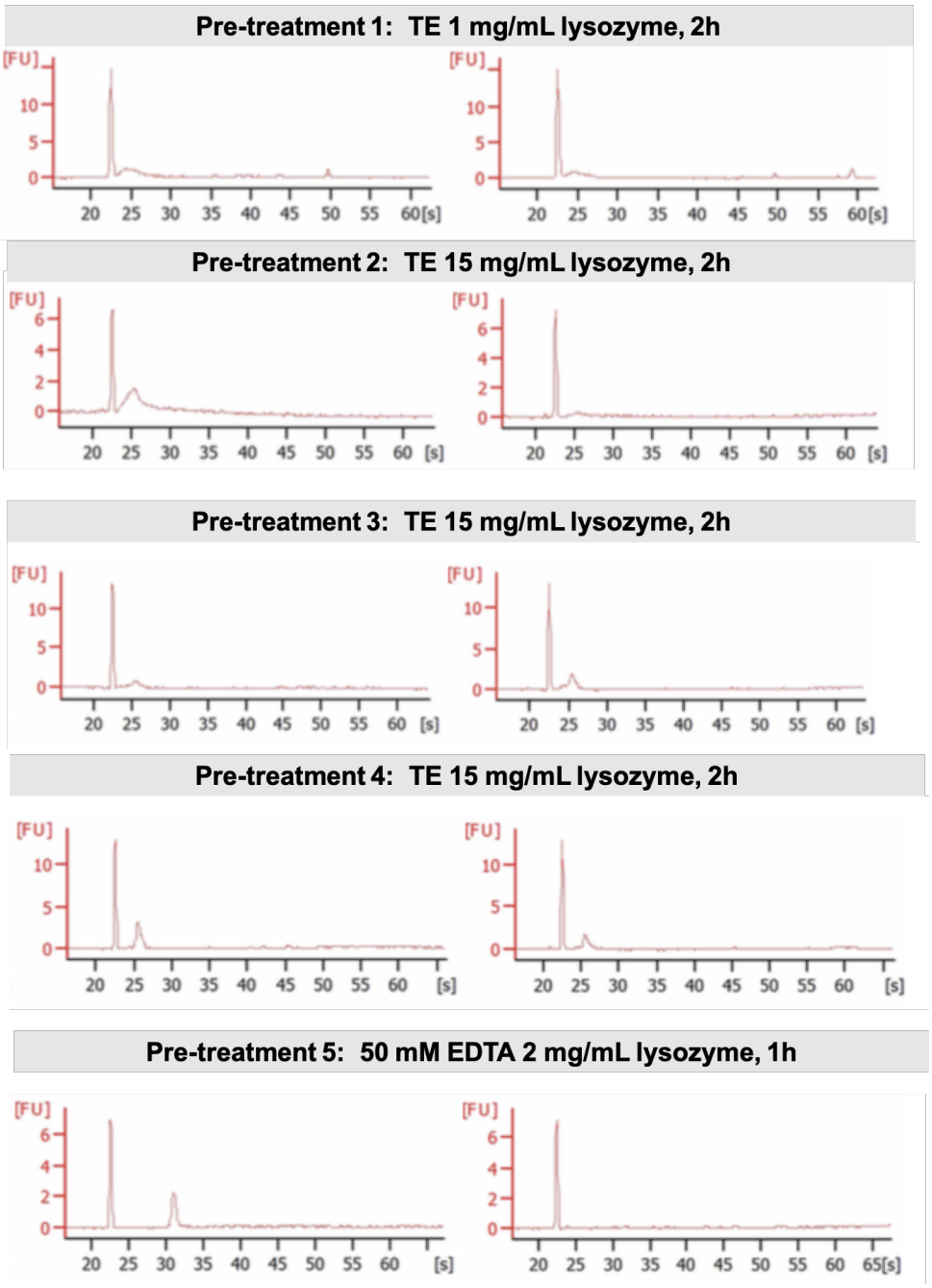


Figure 8-16: Electropherograms of RNA samples isolated from *C. glutamicum* using pre-treatment 1-5 and the RNAqueous Total RNA Isolation Kit (Thermo Fisher Scientific). RNA samples analysed using the Agilent 2100 Bioanalyzer system (Agilent Technologies).

K.2: Electropherograms of RNA isolated with the Quick-RNA Fungal / Bacterial Miniprep Kit

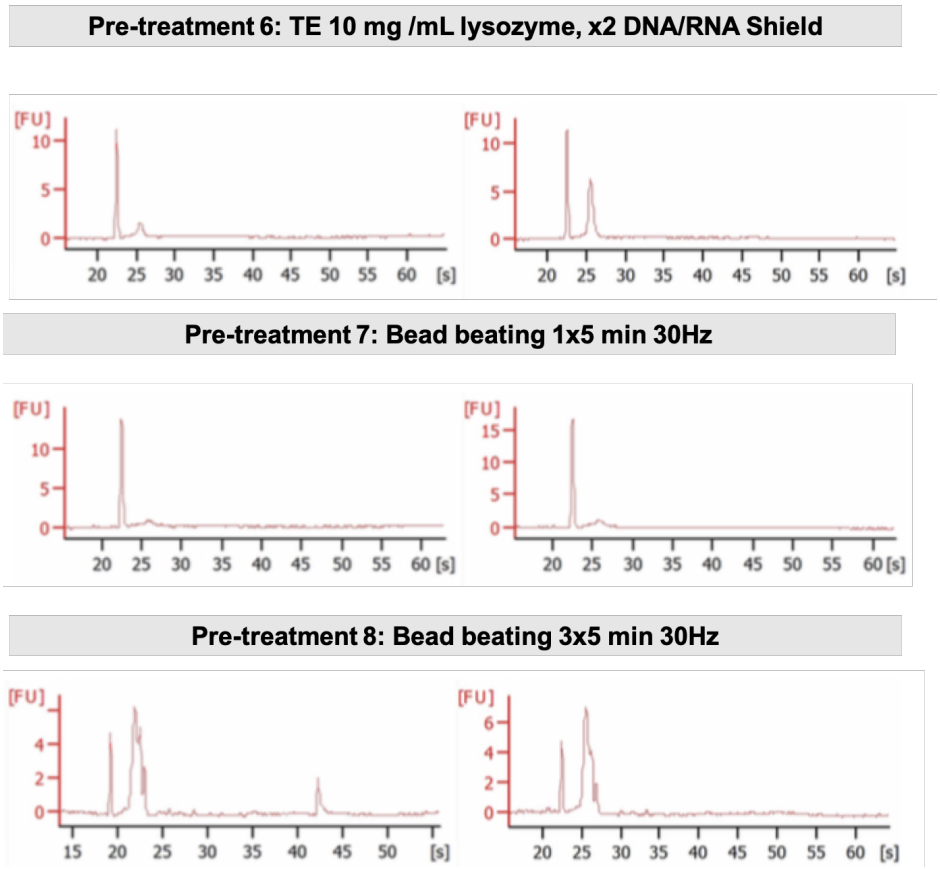


Figure 8-17: Electropherograms of RNA samples isolated from *C. glutamicum* using pre-treatment 6- 8 (presented in Table X) and the Quick-RNA Fungal / Bacterial Miniprep Kit (Zymo Research). RNA samples analysed using the Agilent 2100 Bioanalyzer system (Agilent Technologies).

Appendix L: Electropherograms of RNA samples used for qPCR

Electropherograms for the total RNA samples used for qPCR analysis. *C. glutamicum* samples presented in **Figure 8-18** and *E. coli* in **Figure 8-19**.

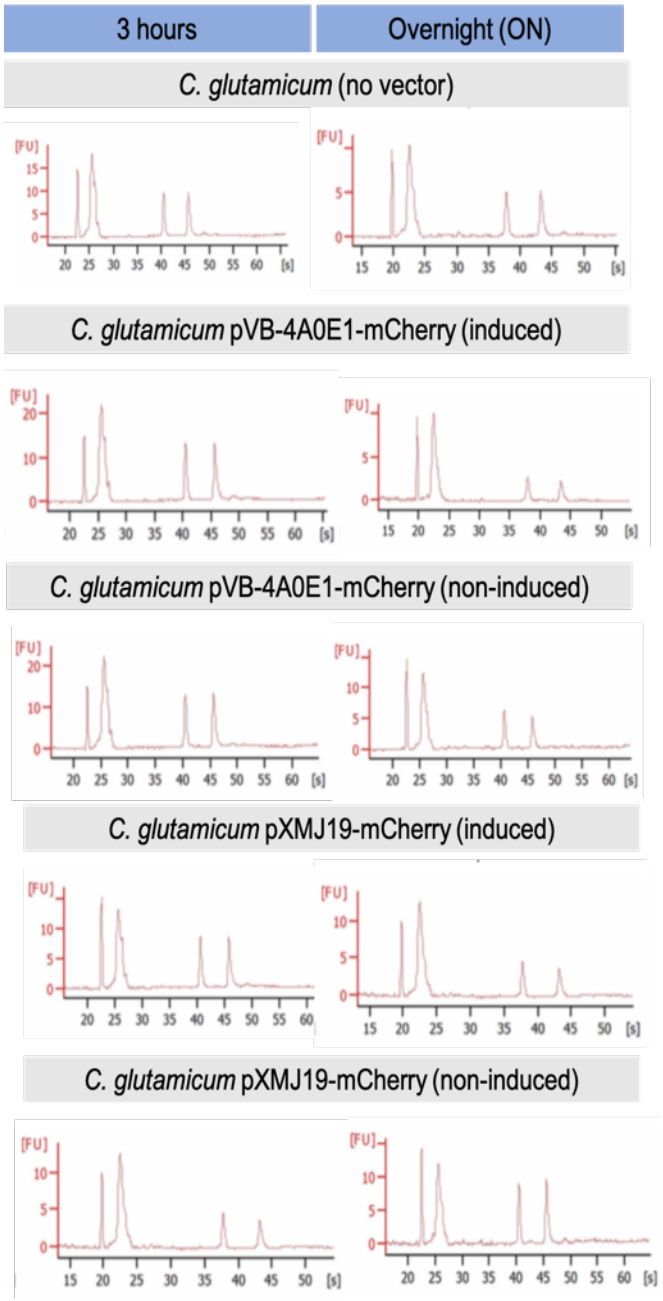


Figure 8-18: Electropherograms for RNA isolated from *C. glutamicum* at sample points 3 hours post-induction and overnight (16 hours post-induction).

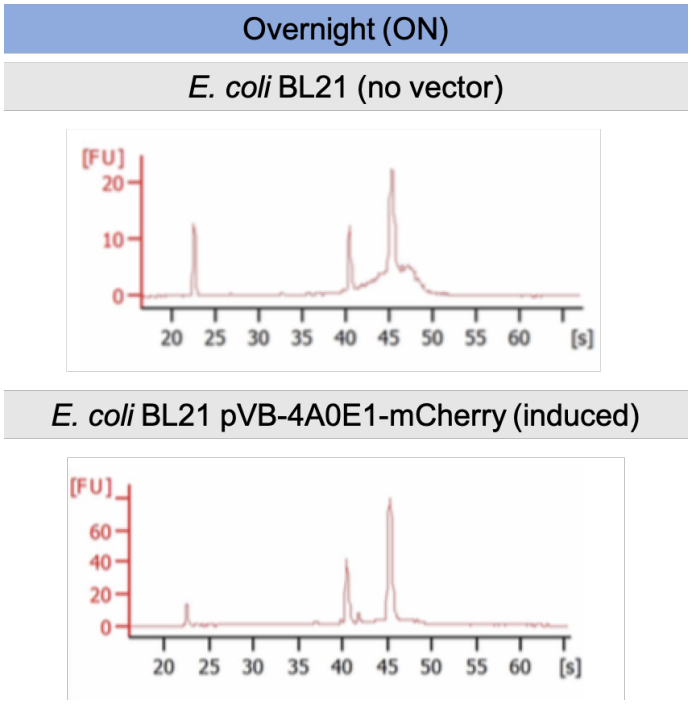


Figure 8-19: Electropherograms for RNA isolated from *E. coli* at sample points 3 hours post-induction and overnight (16 hours post-induction).

Appendix M: Melting curve analysis

The -ΔF / ΔT vs. temperature plot for mCherry and 16S amplification in *C. glutamicum* are included in **Figure 8-20** and **Figure 8-21**, respectively. Melting peaks for mCherry were detected around 82°C and around 81°C for 16S amplification.

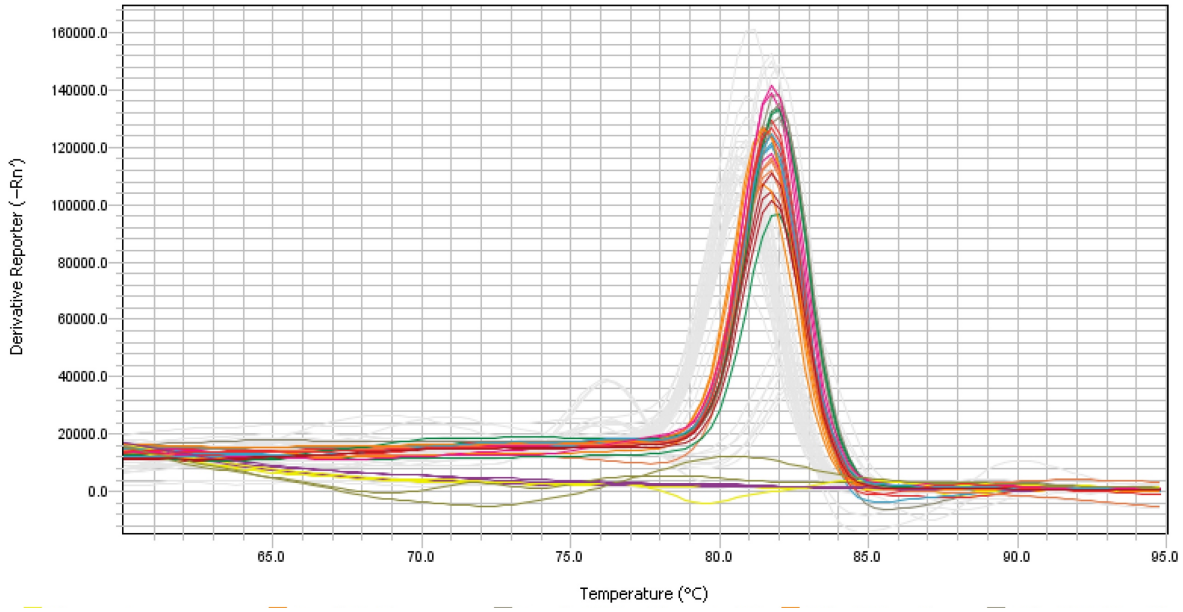


Figure 8-20: -ΔF / ΔT vs. temperature plot for mCherry transcripts in *C. glutamicum*, peaks detected around 82°C. Plot generated by the QuantStudio™ Design and Analysis Software v1.5 (Applied Biosystems)

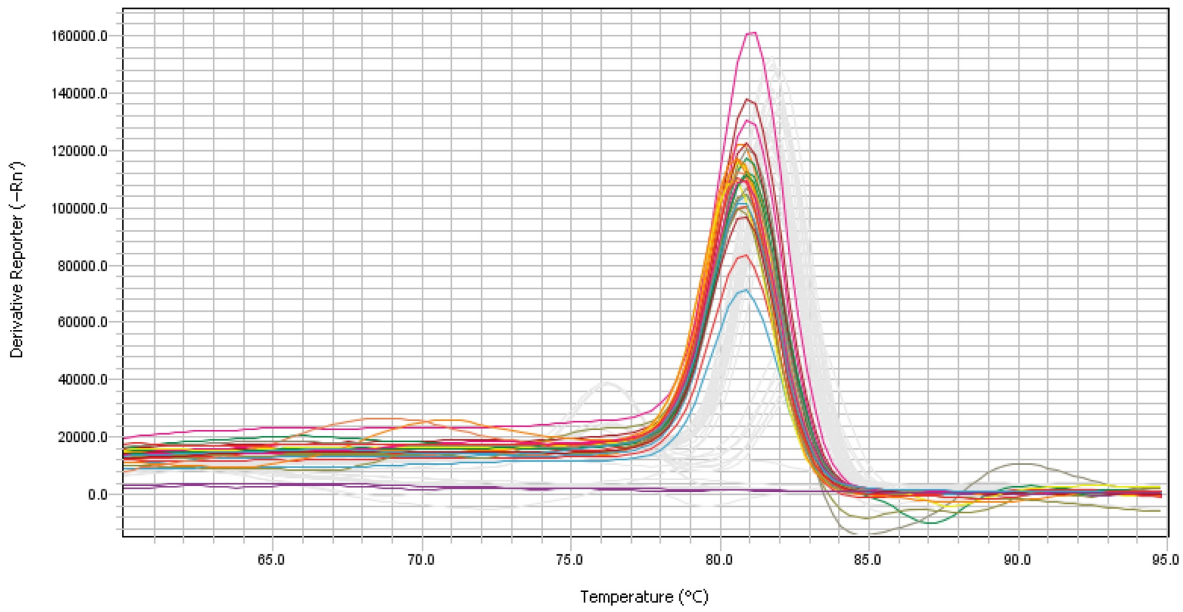


Figure 8-21: -ΔF / ΔT vs. temperature plot for 16S transcripts in *C. glutamicum*, peaks detected around 81°C. Plot generated by the QuantStudio™ Design and Analysis Software v1.5 (Applied Biosystems)

Appendix N: Isolating *C. glutamicum* native promoters

Four *C. glutamicum* native promoters (P_{tuf}, P_{sod}, P_{dapA} and P_{dapB}) were isolated from the genome of *C. glutamicum* MB001 (DE3) using colony PCR. The primer pairs used for amplifying the promoters are presented in **Table 8-7** together with the expected fragment size and the specific annealing temperature used for the PCR reaction. P_{tuf} and P_{sod} were extracted from the agarose gel shown in **Figure 8-22** Some troubleshooting was needed for amplification of P_{dapA} and P_{dapB}, fragments were therefore not extracted from the gel in **Figure 8-22**. With an addition of 5 cycles for both P_{dapA} and P_{dapB}, and an increase in annealing temperature from 48 to 49°C for P_{dapB}, fragments were visible on gel and extracted from the agarose gel shown in **Figure 8-23**.

Table 8-7: Forward (FW) and reverse (RV) primer and the annealing temperature (TA) used to amplify the *C. glutamicum* native P_{tuf}, P_{sod}, P_{dapA} and P_{dapB} promoters by colony PCR, including the expected fragment size given in base pairs (bp).

Promoter amplified	FW primer	RV primer	TA (°C)	Fragment size (bp)
P_{tuf}	Ptuf-fw-SLIC	Ptuf-rv-SLIC	52	230
P_{sod}	Psod-fw-SLIC	Psod-rv-SLIC	48	222
P_{dapA}	PdapA-fw-SLIC	PdapA-rv-SLIC	45	231
P_{dapB}	PdapB-fw-SLIC	PdapB-rv-SLIC	49	230

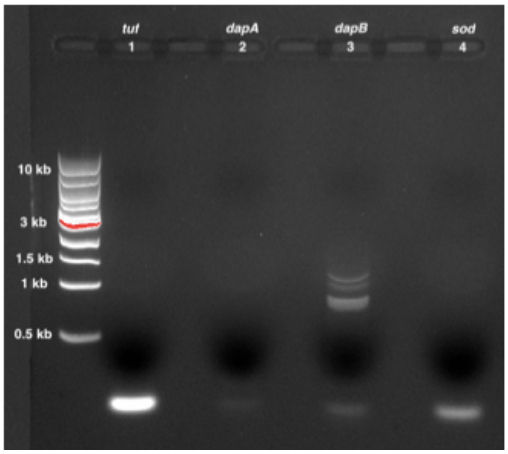


Figure 8-22: Image of agarose gel after gel electrophoresis with the native P_{tuf} (lane 1), P_{dapA} (lane 2), P_{dapB} (lane 3) and P_{sod} (lane 4) amplified by colony PCR from the *C. glutamicum* MB001 (DE3) genome. Ladder:1 kb DNA ladder (NEB).

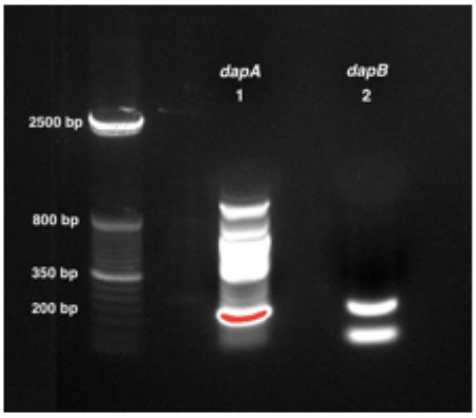


Figure 8-23: Image of agarose gel after gel electrophoresis with the native P_{dapA} (lane 1) and P_{dapB} (lane 2) amplified by colony PCR from the *C. glutamicum* MB001 (DE3) genome. Ladder 50 bp DNA Ladder (Invitrogen).

Anne Sofie Krakeli

Improving expression from the XylS / *Pm* promoter in *Corynebacterium glutamicum*

NTNU
Kunnskap for en bedre verden