NTNU

Norwegian University of
Science and Technology

Evaluation of the XylS/*Pm* Expression Cassette in *Corynebacterium glutamicum*

Maiken Johnsgaard

Chemical Engineering and Biotechnology
Submission date: March 2018
Supervisor: Trygve Brautaset, IBT
Co-supervisor: Maëliss Lemoine, Vectron Biosolutions AS

Norwegian University of Science and Technology
Department of Biotechnology and Food Science

Preface

This Master's Thesis concludes my degree Master of Science (M.Sc.) in Chemical Engineering and Biotechnology at the Norwegian University of Science and Technology (NTNU) in Trondheim. The thesis was written at the Department of Biotechnology and Food Science (IBT) in collaboration with Vectron Biosolutions AS under the supervision of Professor Trygve Brautaset (IBT) and Maëliss Lemoine (Vectron Biosolutions AS).

Acknowledgements

Finishing this thesis proved to be quite the bumpy ride, and I definitely couldn't have done it alone.

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

The employees at Vectron deserves a big thank you for including me, for teaching me and for helping me whenever I needed it.

Both Maëliss Lemoine and Dr. Anne Krog deserves a special thanks. They have both inspired me and helped me in every part of the project, including giving me a push when needed. I am deeply grateful for all the patience and the valuable input along the way.

I would also like to say thank you to all the fellow students for making long days in the lab fun and memorable. And last, but not least, thank you to my dear friends and family for motivation, support, hugs and comforting words. Without you, there is no way I would have done any of this.

Declaration of Compliance

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

Trondheim, March 2018
Maiken Johnsgaard

Abstract

For bacteria, the most common expression system for recombinant protein production is *Escherichia coli* (*E. coli*). However, a large fraction of recombinant proteins produced in *E. coli* are in an insoluble form which sometimes makes them irrelevant for therapeutic applications. There could be several advantages in producing recombinant proteins in other bacteria, such as *Corynebacterium glutamicum* (*C. glutamicum*), especially when it comes to proteins that are difficult to secrete or dependent on effective downstream processes. *C. glutamicum* has an ability to secrete proteins into the growth medium, and has a different intercellular environment which could result in soluble protein expression when *E. coli* fails. The aim of this thesis was, therefore, to test and adapt the expression technology of Vectron Biosolutions, the XylS/*Pm* expression cassette, to the production of recombinant proteins in *C. glutamicum*.

Two *C. glutamicum*/*E. coli* shuttle vectors were constructed. Both of them, pXMJ19-mCherry and pVB-4A0E1-mCherry harboring the XylS/*Pm* expression cassette, were found to be functional in *E. coli*, producing mCherry in high amounts when induced with IPTG and *m*-toluate respectively, yielding pink cultures. For both of them, most of the mCherry protein was found in a soluble state.

Both vectors were successfully transferred into *C. glutamicum*. However, none of them resulted in mCherry production. To further investigate why no mCherry protein was obtained, and to possibly figure out if the bottleneck was at *xylS* or *mCherry* level and also if transcription or translation of these proteins were the main issue, the transcript levels of *mCherry* and *xylS* were evaluted. For *C. glutamicum* harboring pXMJ19-mCherry, *mCherry* transcriptis could be identified and transcript level increased 12 times when the culture was induced with IPTG. However, the amount of mCherry transcript was significantly less than for *E. coli*. For *C. glutamicum* harboring pVB-4A0E1-Cherry, no *mCherry* transcript could be identified. From this, it could not be confirmed whether *mCherry* is a suitable reporter gene in *C. glutamicum*. The results also showed that when compared to *E. coli*, the amount of *xylS* transcript from the XylS/*Pm* expression cassette in *C. glutamicum* was very low.

Even though no functional shuttle vector expressing recombinant proteins from the XylS/*Pm* expression cassette has been verified, *xylS* transcription has been identified as a bottleneck for protein expression from the XylS/*Pm* expression cassette in *C. glutamicum*. This work is a contribution to the ongoing research into developing *C. glutamicum* as an alternative bacterial host.

Samandrag

For rekombinant proteinproduksjon i bakteriar er *Escherichia coli* (*E. coli*) den vanlegaste verten. Ein stor del av dei rekombinante proteina som vert produsert i *E. coli* er ikkje løyselege, og kan dermed ikkje nyttast i terapeutisk høve. Å produsere rekombinante protein i andre bakteriar, til dømes *Corynebacterium glutamicum* (*C. glutamicum*), kan ha derfor fleire fordelar. Dette gjeld særleg protein som er vanskelege å skilja ut eller er avhengig av effektive nedstraumsprosessar. *C. glutamicum* har ei evne til å skilja ut protein til vekstmediet og har eit anna intercellulært miljø som kan resultera i løyselege protein, der same resultatet ikkje hadde vore mogleg å oppnå i *E. coli*. Føremålet med denne masteroppgåva var å teste og tilpasse Vectron Biosolutions sin ekspresjonsteknolgi, XylS/*Pm*-ekspresjonskassetten, til produksjon av rekombinante protein i *C. glutamicum*.

To *C. glutamicum*/*E. coli* skyttelvektorar vart konstruert. Både pXMJ19-mCherry og pVB-4A0E1-mCherry som inneheld XylS/*Pm*-ekspresjonskassetten, var funksjonelle i *E. coli*. Begge produserte store mengder mCherry når dei vart indusert høvesvis med IPTG og *m*-toluate, noko som gav rosa kulturar. Størstedelen av mCherry-proteinet var løyseleg.

Overføringa av båe vektorane til *C. glutamicum* var vellukka, men ingen av dei resulterte i produksjon av mCherry. For å vidare undersøkje kvifor det ikkje var noko produksjon av mCherry, identifisere ein eventuell flaskehals og finne ut om transkripsjon eller translasjon av desse proteina var hovudproblemet, vart transkripsjonsnivåa til *mCherry* og *xylS* evaluert. For *C. glutamicum* med pXMJ19-mCherry, vart det identifisert *mCherry* transkripsjon, og transkripsjonsnivået vart 12 gongar så høgt då kulturen vart indusert med IPTG. Samanlikna med transkripsjonsnivået i *E. coli*, var mengda identifisert i *C. glutamicum* mykje lågare. For *C. glutamicum* med pVB-4A0E1-mCherry vart det ikkje identifisert noko *mCherry* mRNA. Frå dette kunne ein ikkje trekkje ein konklusjon om kor vidt *mCherry* egnar seg som rapportørgen i *C. glutamicum*. Resultata viste òg at om ein samanlikna mengda *xylS* mRNA frå XylS/*Pm*-ekspresjonskassetten i *C. glutamicum* med mengda *xylS* mRNA i *E. coli*, var mengda mykje lågare i *C. glutamicum*.

Sjølv om ingen funksjonell skyttelvektor som uttrykk rekombinante protein frå XylS/*Pm*-ekspresjonskassetten har vorte verifisert, har *xylS* transkripsjonen vorte identifisert som ein flaskehals for proteinekspresjon frå XylS/*Pm* ekspresjonskassetten i *C. glutamicum*. Dette arbeidet er eit bidrag til pågåande forskning innan utvikling av *C. glutamicum* som ein alternativ ekspresjonsvert.

List of Abbreviations

aa	amino acid
Amp	Ampicillin
BHI	Brain Heart Infusion medium
BHIS	Brain Heart Infusion supplemented medium
bp	base pair
cDNA	complementary DNA
Cm	Chloramphenicol
dH_2O	distilled H_2O
ddPCR	droplet digital polymerase chain reaction
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
GTP	guanosine triphosphate
IPTG	isopropyl-β-D-1-thiogalactopyranosid
LA	Luria-Bertani Broth Agar medium
LB	Luria-Bertani Broth medium
mRNA	messenger RNA
OD_{600}	optimal density measured at wavelength of 600 nm
ORF	open reading frame
ori	origin of replication
PCR	polymerase chain reaction
RFU	realtive fluorescent unit
RIN	RNA integrity number
RNA	ribonucleic acid
rRNA	ribosome RNA
SD	Shine-Dalgarno
ssDNA	single-stranded DNA
TOL	toluene-degradative
tRNA	transfer RNA
UTR	untranslate region
wt	wild type
α	alpha
β	beta
σ	sigma

Contents

Chapter 1

Introduction

1.1 Recombinant Protein Production

Biotechnology is defined by the United Nations as any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use. Biotechnology has been around since humans began manipulating the natural environment to improve their food supplies, housing and health [15]. Traditional biotechnology products like bread, cheese, wine and beer which have been made for centuries rely on microorganisms, such as yeast, to modify the original ingredients [15]. Today, biotechnology can be divided into several branches such as blue (marine), green (agricultural), red (medical) and white (industrial) biotechnology. Medical and industrial biotechnology may include molecular biology and genetic engineering, and the work presented in this study would fall into these categories.

Recombinant DNA technology is based on the pioneering work of Stanley Cohen and Herbert Boyer who invented the technique of DNA cloning [17] and presented in 1973 the first genetically engineered organism: *Escherichia coli* (*E. coli*) harboring a plasmid conferring antibiotic resistance [16]. Recombinant DNA technology has since become the basis for almost all biotechnology research [15]. It consists essentially of generating fragments of DNA containing specific sequences and incorporate them into a vector adapted to the host organism. The newly built vector is introduced into a host organism, which is grown in culture to produce numerous clones. Clones containing the relevant DNA fragment are then selected [23]. The association of DNA molecules from different origins is the definition of recombinant DNA, hence the name of the technology. Recombinant DNA technology includes all the techniques used to create recombinant DNA, which can either be used to benefit the host itself or to produce a desired substance for harvesting.

Within the field of recombinant DNA technology, one of the oldest goals is expression of proteins [12]. This is achieved from expression vectors constructed *in vitro* and then introduced into carefully selected host organisms. With an increased understanding of the

fundamentals of DNA, RNA and protein regulation in the host organism, cells can be manipulated to express cloned genes for large-scale production of, among other, therapeutic proteins and industrial enzymes. The expression of cloned genes in host organisms and the resulting protein products are also studied to gain knowledge about protein function and properties et cetera. The first genetically engineered drug approved by the U.S. Food and Drug Administration (FDA) was recombinant human insulin produced in *E. coli*, humulin, in 1982 [24, 38] and by 2015, almost 400 recombinant proteins-based products had been approved as biopharmaceuticals (therapeutic products manufactured using biotechnology [60]) and used as hormones, vaccines, antibodies et cetera [64]. The techniques for production of recombinant proteins has gained major improvements in the past decades, allowing tailor made vectors and engineering of bacteria and eukaryotic cells. Recombinant technology enables cost-efficient production of high value proteins useful in research, therapy and diagnostics.

Numerous expression platforms have been developed, ranging from bacteria, yeasts and fungi to cells of higher eukaryotes. For bacteria, the most common expression system is *E. coli*. It stands for 34% of total biopharmaceutical production (2013) [3], even though many more microbial platforms have been developed. These systems were selected because they are easy to handle and have simple growth requirement as well as known genome sequences and biochemical processes. However, the lack of glycosylation and/or limitations in secretion of proteins restricts the range of usage of these systems. For proteins that need complex glycosylation or presence of several disulfide bonds, higher eukaryotic platforms are generally needed [42]. Eukaryotic systems are also suitable for production of proteins that require multiple post-translational modifications. The yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) is also a commonly used expression host for biopharmaceutial products and stands for 13% of total production (2013) [3]. Fungi, recombinant baculoviruses and insect cells have also been developed for protein production. Mammalian cell lines are other platforms especially used to produce therapeutic proteins and antibodies, and stand for production of 56% of approved biopharmaceuticals (2013) [3]. Disadvantages such as more complex and expensive growth media and other costly and advanced production requirements explain why production of recombinant proteins takes place in higher eukarytoic organisms only when other platforms fail to provide a product of sufficient quality.
This study focuses on bacterial protein expression in *E. coli* and *C. glutamicum*. Description of both microorganisms follows after a brief introduction of bacterial gene expression and cloning vectors.

1.2 Bacterial Gene Expression

Two essential features of living creatures are the ability to replicate their own genome and produce their own energy. To accomplish these features, organism must be able to build proteins using information encoded in their DNA [15]. The central dogma of molecular biology states that information flows from DNA to RNA to protein. Gene expression involves two steps: transcription, which is the transfer of information from DNA to RNA, and translation, the process where information is transferred from RNA to protein [68]. This complex process involves dynamic steps that can be regulated at multiple levels, which include transcriptional, post-transcriptional, translational and post-translational [47]. Tight control of the components of transcription and translation gives rise to an expression system with preferred qualities and characteristics. This section gives a brief explanation of how genes are expressed in prokaryotes, and more particularly in *E. coli*. Figure 1.1 gives an overview of gene expression in prokaryotes. An open reading frame (ORF) is a strech of DNA or corresponding RNA that encodes a protein and does not contain any translation stop codons [15].

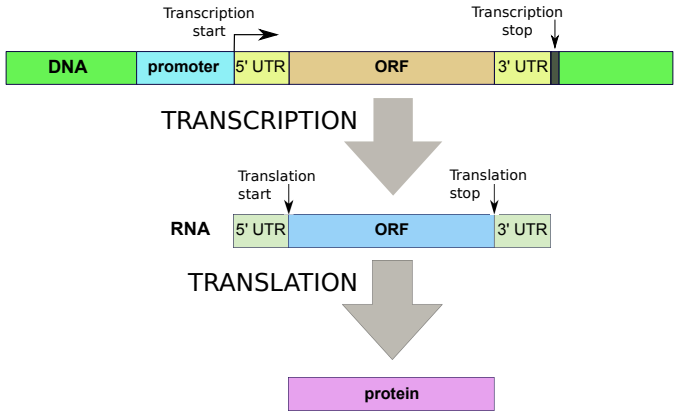


Figure 1.1: Overview of gene expression in prokaryotes. DNA is transcribed to give RNA, and RNA is translated into protein. The figure is adapted from Clark (2012) [15]. UTR: untranslated region, ORF: open reading frame.

1.2.1 Transcription

Transcription can be explained as the production of a single strand RNA copy made from a double helix DNA molecule. This process can be divided into three major steps: initiation, elongation and termination. During initiation, the RNA polymerase recognizes the promoter region in the DNA sequence. If the gene is only expressed under very specific conditions, transcription factors may have to bind to the promoter region before it can be recognized by the RNA polymerase. A promoter is a DNA sequence at the $5'$ end of the coding sequence of a gene, and most bacterial promoters consist of two short, highly

conserved sequences located at about 10 and 35 nucleotide pairs before the transcription-initiation site. These conserved regions are recognized by sigma (σ) factors, subunits of the RNA polymerase, when the RNA polymerase slides along the DNA. RNA polymerase locally unwinds the DNA and one strand of DNA, the template strand, to synthesize a complementary RNA strand called messenger RNA (mRNA) in the $5'$ to $3'$ direction. The RNA strand will hence be identical to the DNA nontemplate strand, except that uridine residues (U) replace thymidines (T).

Elongation of RNA chains is catalyzed by the RNA polymerase, after release of the sigma, σ, subunit. The enzyme slides along the DNA while continuously unwinding the double stranded DNA and synthesizing mRNA by binding nucleotides complementary to the bases in the template strand. After the passage of the transcription complex, DNA rewinds and forms a double stranded molecule again. Elongation continues until the RNA polymerase reaches a termination signal which triggers the termination step. The polymerase then dissociates from the DNA, and the RNA transcript is released.

There are two types of transcription terminators for prokaryotes: rho-dependent and rho-independent. Rho-independent terminators are believed to form hairpin loops due to single-stranded RNA sequences that are complementary. These RNA sequences are produced when the GC-rich regions of the rho-independent terminator are transcribed. The rho-independent terminators contain inverted repeats, sequences of nucleotides in each DNA strand that are inverted and complementary. The hairpin loops brings RNA polymerase to a stop and a sequence of uracils (U) residues after the hairpin region facilitates RNA release. Rho-dependent terminators contain two additional sequences: a rho-binding site called rut and a sequence harboring the termination zone. The rho-protein binds to the rut sequence and follows the RNA polymerase. When the latter encounters the hairpin conforamtion, it pauses and rho catches up, terminates the elongation process and releases the RNA transcript [68].

Transcription can be regulated by a variety of activator and repressor proteins that bind to the DNA in the promoter region. When bound, they either stimulate (activator) or block (repressor) the action of the RNA polymerase [15]. Promoters are one of the most commonly used method for tuning the expression of a desired gene. This is an important feature of recombinant protein production and promoters are either inducible (needs to be activated) or constitutive (continuously active but can be blocked). Promoters cover a wide range of expression levels from weak to strong, based on their affinity for the RNA polymerase and/or σ factors. In addition to the choice of promoters, expression levels can also be adjusted in some cases by varying inducer concentrations [76].

1.2.2 Translation

Translation is also a process that can be divided into three major steps: initiation, elongation and termination. Translation occurs on ribosomes, which are approximately half protein and half RNA. The bacterial ribosome (70S) is made up of two subunits, one small (30S) and one large (50S). When bound to mRNA, each ribosome/mRNA complex contains three binding sites, A, P and E. Figure 1.2 shows the ribosome structure in *E. coli*.

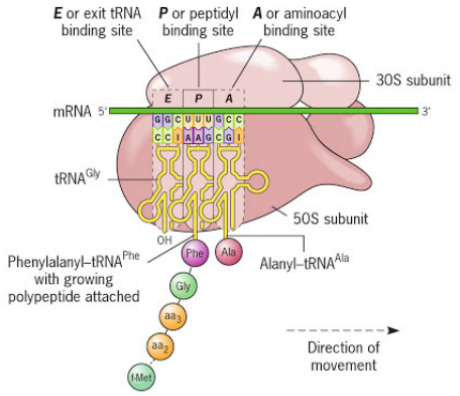


Figure 1.2: Illustration of the ribosome structure (70S) in *E. coli*. Each ribosome/mRNA complex contains three aminoacyl-tRNA binding sites. A: aminoacyl binding site, P: peptidyl binding site and E: exit site. Illustration obtained from *Genetics*, Snustad (2012) [68].

The 5′ untranslated region (5′ UTR) encodes the signal for ribosome binding and is located a few base pairs (bp) before the initiation codon, which is usually AUG encoding the amino acid (aa) methionine. During initiation, the ribosomal subunits assemble on the mRNA at the ribosomal binding site. For bacteria, this binding site is the hexamer AGGAGG, which is called Shine-Dalgarno (SD) sequence, located 4-8 nucleotides upstream from the initiation codon. The nucleotide sequence of mRNA is read as a series of triplets, known as codons. Each codon specifies the insertion of a single aa following a highly conserved code throughout living organsims and referred to as the genetic code. Translation starts at the initiation codon, and ends at a termination codon [68].

Initiation of translation includes all events that precede the formation of a peptide bond between the first two aa of the new polypeptide chain and requires three initiation factors: IF-1, IF-2 and IF-3, as well as one GTP molecule [27]. In the first stage, a free 30S subunit interacts with the mRNA molecule and the initiation factors. The 50S subunit joins to form the 70S ribosome in the final step of the initiation. Synthesis of polypeptides is initiated by a special transfer RNA (tRNA), $tRNA_f^{Met}$, since a vast majority of polypeptides begin with methionine [68].

Elongation, the addition of aa to the growing polypeptide, occurs in three steps. The first is binding of an aminoacyl-tRNA to the A site of the ribosome. Then follows transferring of the growing polypeptide from the tRNA in the P site to the tRNA in the A site by the formation of a new peptide bond. The last step is translocation of the ribosome along the mRNA to position the next codon in the A site. In this way, the ribosome moves along the mRNA and the charged aminoacylated tRNAs recognize complementary codons.

Termination occurs when any of the three chain-termination codons (UAA, UAG or UGA) enters the A-site of the ribosome. These stop codons are recognized by soluble proteins

called release factors (RFs), RF1 and RF2. RF1 responds to UAA and UAG, while RF2 responds to UAA and UGA. Presence of RF in the A site in the ribosome makes the peptidyl transferase add a water molecule to the carboxyl terminus of the nascent polypeptide, which releases the polypeptide from the tRNA molecule in the P site and triggers translocation of that newly freed tRNA to the E (exit) site of the ribosome. Termination is completed when the mRNA molecule is released from the ribosome and the ribosome dissociates to distinct subunits. When dissociated, the ribosome subunits are ready to initiate a new round of protein synthesis [68].

1.3 Cloning vectors for introduction of foreign DNA into host cell

To ensure propagation, replication and expression within the host cell, foreign DNA fragments need to be carried in a vector. Vectors are best described as small carrier molecules that are capable of self-replication [53, 62]. These vectors introduce foreign DNA into host cells, which can then produce molecular copies of the DNA in large quantities [61]. A huge array of different types of vectors is available today, many of them highly specialized and designed to perform specific functions. The four major types of vectors are plasmids, viral vectors, cosmids and artificial chromosomes [62]. In this study, plasmids were used as cloning vectors.

Cloning vectors are used predominantly for amplification of DNA fragments, and contain several essential features. An origin of replication (*ori*), which is required for a plasmid to be maintained without integration in the bacterial chromosome, provides a replication initiation site for cellular enzymes. Bacterial *ori* regions also account for plasmid copy number and compability/incompability with other vectors and replication efficiency in different hosts [59]. Selectable markers, such as genes that confer resistance to specific antibiotics, allow for survival of the cells that contain the recombinant version of the plasmid while the other cells die. Another feature is the presence of specific recognition sequences that are targets for restriction endonucleases, therefore providing sites where the plasmid can be digested to insert foreign DNA [47, 55]. A small vector size facilitates entry into the cells and the biochemical manipulation of DNA in general. The small size of plasmid is obtained by trimming away DNA segments that are not needed from a larger plasmid [53].

Since early 1970s, plasmid vectors have become essential tools of modern biology. Plasmid vectors were initially designed for gene cloning and DNA analysis in *E. coli*, but shuttle vectors for gene transfer between *E. coli* and other organisms for protein production and gene function analysis were quickly developed. A general strategy for construction of shuttle vectors is to combine a replication *ori* from a naturally occuring plasmid in wanted bacterial species with an *E. coli* cloning vector. This will allow the recombinant plasmid to be functional in wanted bacterial species as well as in *E. coli* [47, 55]. Cloning vectors with the transcription and translation sequences needed for regulated expression of a cloned gene are called expression vectors [53].

The RK2 Plasmid

As mentioned, the host range of the plasmid is determined by its *ori* region. This makes the construction of vector systems that function in all bacterial hosts of interest presumably very difficult to achieve. However, some plasmids are able to replicate in several bacterial hosts. These plasmids are called broad-host-range vectors. One of these broad-host-range plasmids that is widely used for construction of expression vectors, especially for use in gram-negative bacteria, is the RK2 plasmid [10]. The RK2 plasmid was first isolated in 1969 in Birmingham from the bacterium *Klebsiella aerogenes* [32]. It belongs in the incompability group IncP due to its ability to replicate in many Gram-negative bacteria, as well as in some Gram-postive bacteria [11, 56]. Two regions are essential for replication in RK2, *oriV*, the origin of vegetative replication, and the *trfA* gene which encodes the replication initation protein. *trfA* encodes two versions of the replication initiation protein, originating from alternative translation starts within the same open reading frame [11, 54]. *trfA* also affects the plasmid copy number, the number of the same plasmid present in the bacterial cell. The copy number is regulated in a process called handcuffing, by interactions between the origin of replication and the TrfA protein [71].

1.4 *Escherichia coli* as a host for recombinant protein production

Escherichia coli (*E. coli*) is a rod-shaped gram-negative bacterium discovered in 1885 by Theodor Escherich. *E. coli* is considered to be the most widely used prokaryotic organism for recombinant protein production due to its requirement for inexpensive media and inducers for relative rapid growth [1]. It is easy to handle and manipulate genetically, and many expression systems have been developed for *E. coli*, enabling efficient production of recombinant proteins [46, 63].

However, not all recombinant genes are expressed efficiently in *E. coli*. Less efficient protein production may be caused by one or several of the following factors: vector and mRNA instability, inefficient translation initiation and differences in codon usage, toxicity of gene products, inappropriate protein folding resulting in inactive protein products and formation of inclusion bodies, degradation of the product by the host cell proteases et cetera. Major drawbacks to using *E. coli* as a host include the inability to perform many post-translational modifications found in proteins from eukaryotic cells and the lack of effective secretion mechanisms for release of recombinant proteins to the culture medium, enabling simplified downstream processing for industrial applications [28, 46, 49, 72].

1.5 *Corynebacterium glutamicum* as a host for recombinant protein production

Corynebacterium glutamicum (*C. glutamicum*) is a rod-shaped, Gram-positive soil bacterium capable of growing on a variety of sugars and organic acids [39]. It is non-pathogenic, do not produce endotoxins and is generally recognized as safe (GRAS). Originally, exploited because of its natural ability to excrete L-glutamate [5], it is now used for large-scale industrial production of various L-amino acids, nucleic acids and vitamins [80, 73].

C. glutamicum has recently attracted attention as a potential host for recombinant protein production since it exhibits numerous ideal features of protein secretion. *C. glutamicum* has the ability to secrete properly folded and functional proteins into the medium, which can ease the purification process and improve the subsequent purification efficiency. It also has minimal secreted protease activity, which makes it suitable to produce protease-sensitive proteins. *C. glutamicum* can consequently be a favorable host for expressing many recombinant proteins, in particular proteins that are difficult to secrete or proteins which activity and purity depend on effective downstream purification [46]. Studies performed by Date and colleagues demonstrated that the human protein hEGF (human epidermal growth factor) could be efficiently secreted in an active form by *C. glutamicum*, and showed the potential for industrial-scale human protein production [19].

However, compared with *E. coli*, *C. glutamicum* has some disadvantages, e.g. a much lower tranformation efficiency and only a few suitable expression vectors available [79]. Considerable effort has been put into genetic modification of several strains of *C. glutamicum* and to date, genetically modified *C. glutamicum* strains showing faster growth, enhanced protein synthesis and efficient protein secretion of heterologous proteins have been reported and validated. There is still a strong demand for genetic and physiological investigation into this species, and areas requiring further research include: optimization of promoters to enhance expression efficiency, construction of plasmid vectors with different features, genetic engineering of host strains to improve growth characteristics and development of more efficient protein secretion pathways [46]. Research on how to increase transformation efficiency would also be of significant interest.

Figure 1.3 shows a circular representation of the *C. glutamicum* ATCC 13032 chromosome. The locations of the prophages (CGP1, CGP2 and CGP3) in the genome are also included [4].

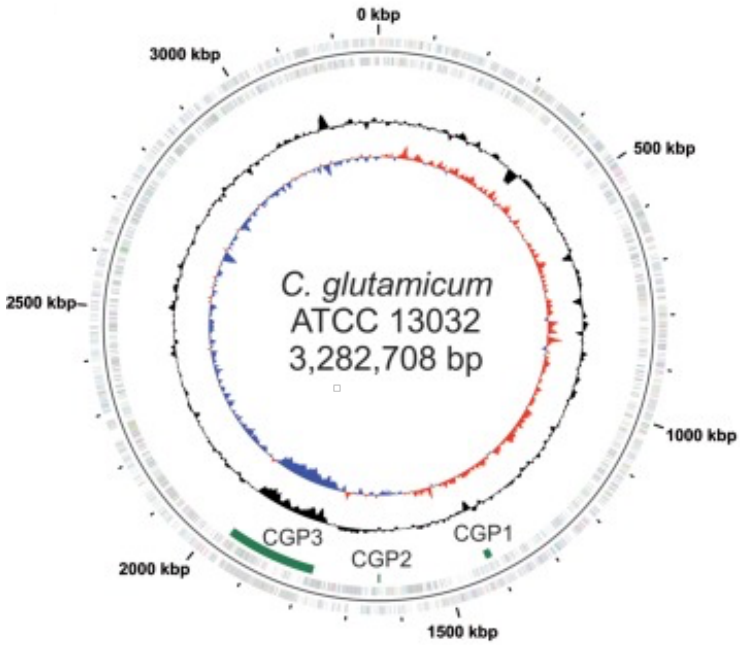


Figure 1.3: Circular representation of the *C. glutamicum* ATCC 13032 chromosome from Baumgart et. al., 2013 [4]

C. glutamicum MB001 is a prophage-free *C. glutamicum* strain with a genome reduced by 6%. The *C. glutamicum* MB001 strain shows the same growth phenotype as ATCC 13032 wild-type strain under standard conditions and improved fitness under conditions triggering prophage induction [4].

C. glutamicum MB001(DE3) is based on the *C. glutamicum* MB001 strain, with part of the DE3 region of *E. coli* BL21(DE3) including the T7 RNA polymerase gene 1 under control of the *lac*UV5 promoter integrated into the chromosome [40]. This results in an isopropyl-β-D-1-thiogalactopyranosid (IPTG)-inducible T7 expression system. The *C. glutamicum* MB001(DE3) strain was the strain mostly used in this study.

As previously mentioned, shuttle vectors allow for functionality in both *E. coli* and wanted bacterial species. pXMJ19 is one of the shuttle vectors constructed for *C. glutamicum/E. coli*. It was constructed by Jakoby and colleagues in 1999 [35] on the basis of high copy number *E. coli* plamsmid pK18 [58], and the cryptic low copy number *C. glutamicum* plasmid pBL1 [65]. The plasmid contains a chloramphenicol resistance cassette which confers resistance up to 50 $\mu g/mL$ [35]. The *lacI*^{q} gene, the IPTG-inducible *tac* promoter, P_{tac}, and the *rrnB* terminators T1 and T2 allow for inducible expression if genes clones under control of P_{tac}. Figure 1.4 shows a physical map of the pXMJ19 plasmid.

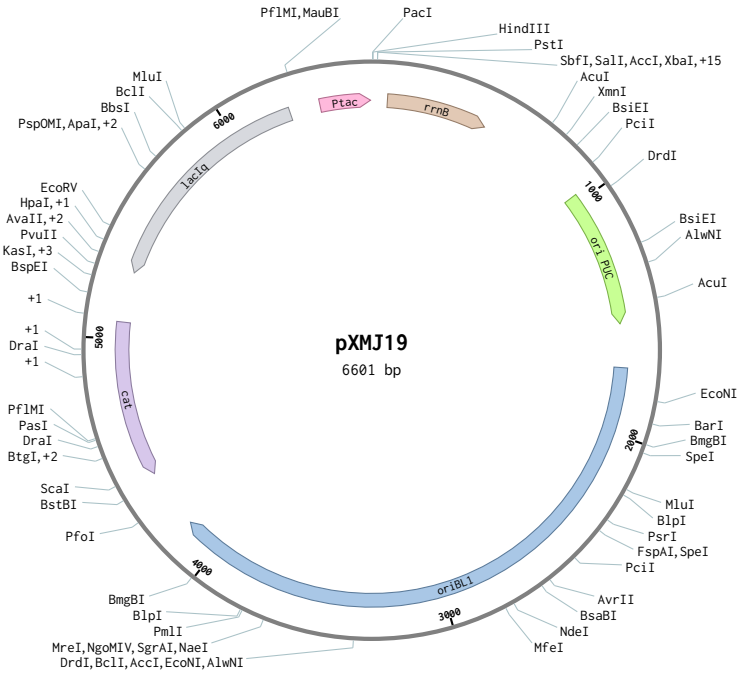


Figure 1.4: Physical map of the pXMJ19 plasmid generated using Benchling. The *lacI*^{q} gene, the IPTG-inducible *tac* promoter and the *rrnB* terminators T1 and T2 allow for inducible expression while the *cat* gene confers chloramphenicol resistance. It also contains *ori* pUC and *ori*BL1 which are the origins of replication in *E. coli* and *C. glutamicum* respectively.

1.6 The XylS/*Pm* Expression System

A tightly controlled expression system is useful for high-level production of recombinant proteins. For the most commononly used hosts, such as *E. coli*, a wide range of different expression systems are available. Ideal expression systems allow for tight control of expression, depencence on cheap inducers and the non-nessicitiy of require particular inducer uptake transport systems. It is also preferable that the expression system works across species barriers [12]. Several positively regulated expression systems have been developed, one of them being the XylS/*Pm* expression system.

The XylS/*Pm* regulator/promoter system originates from the toluene-degradative (TOL) plasmid pWWO from *Pseudomonas putida* [25]. The expression system is found to function in a wide range of bacterial species [11, 12, 21]. The TOL plasmid encodes a pathway for catabolism of toluene and xylenes [78]. The genes involved in this are grouped into the upper- and lower(meta)-pathway operons, positively regulated by the transcription factors XylR and XylS respecively [12]. XylS is a member of the AraC-XylS family which is

composed of positive regulators for recombinant gene expression control in bacteria.

In the lower pathway, the *Pm* promoter requires the positive regulator XylS for activation. Expression of XylS is controlled by two individually regulated promoters, *Ps1* and *Ps2*. XylR can activate transcription from *Ps1*, while transcription from *Ps2* is constitutive and low. This ensures a tightly controlled and balanced expression level of XylS [12]. In the presence of inducer, like benzoate derivatives such as *m*-toluate, XylS activates transcription from *Pm* [25]. If XylS is overexpressed, it can bind to the operator sequence *Om* in the absence of inducer and activate transcription from the meta-pathway *Pm* promoter [51]. Figure 1.5 shows a simplified version of how the expression system works in the cell. Inducer molecules passively enter the cell and bind to XylS, which then becomes activated and dimerizes with another XylS/inducer complex. This complex stimulates transcription from the promoter *Pm* [12]. The expression system has been shown to function well in a wide range of Gram-negative organisms, and recently also in some Gram-positive species [21]. Several elements of the system have been modified and improved, such as the 5′-untranslated RNA region (5′-UTR), the *xylS* coding region, as well as various types of 5′-terminal fusion partners that enhance the expression of recombinant genes [26].

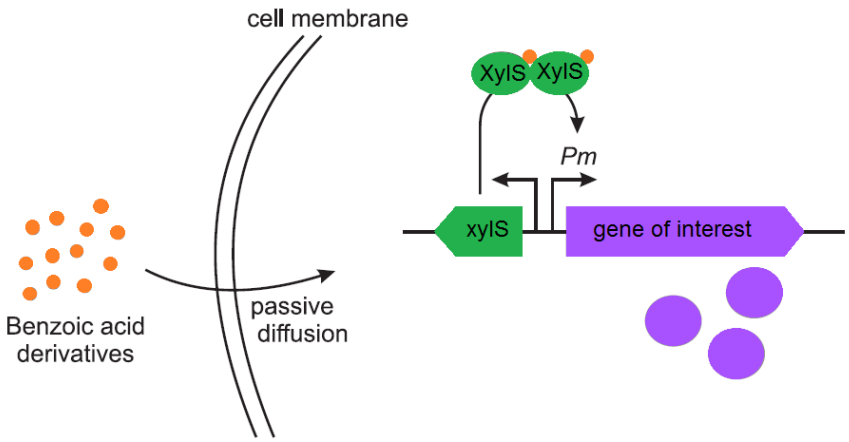


Figure 1.5: The XylS/*Pm* expression system. Inducer molecules (benzoic acid derivatives) passively enter the cell and activate XylS by binding to it. The activated transcription factor XylS dimerizes, stimulates transcription from *Pm*, and the gene of interest downstream of *Pm* is expressed. Illustration obtained from Brautaset et al., 2009 [12].

Some expression vectors used in this study are based on the broad-host-range vector pJB658, also reffered to as pVB-1. pJB658 is an expression vector constructed by Blatny and colleagues in 1997 based on the expression vector pJB653 [11], which itself originates from a minimal replicon of RK2 plasmid fused with the XylS/*Pm* expression cassette [10]. The *xylS* gene is constitutively transcribed from its native *Ps2* promoter, and the gene of interest is placed under transcriptional control of *Pm*, as shown in Figure 1.5. pJB658 is an expression system suitable for expression in Gram-negative bacteria, but since many

Gram-positive species will not be able to replicate RK2, pJB658 is not suitable for those baceteria without alterations [14]. Figure 1.6 shows the physical map of the pJB658 plasmid.

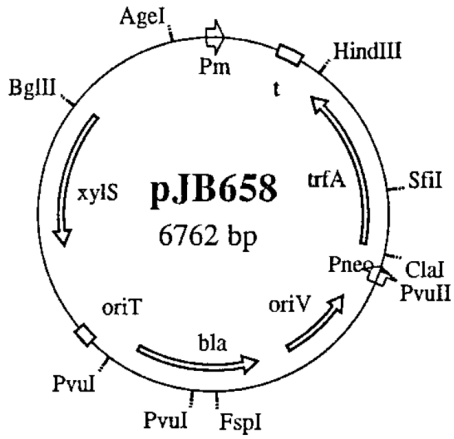


Figure 1.6: Physical map of the expression plasmid pJB658, obtained from Blatny et al., 1997 [10]. The plasmid is based on expression vector pJB653 [11] and consists of the *Pm* promoter upstream from the gene, the *xylS* gene, the *trfA* gene of interest, *oriV* and the *bla* gene which encodes Ampicillin resistance.

1.7 Reporter Genes in Recombinant Protein Production

When transforming bacteria one would want to be able to accurately, quickly and easily select for cells that have taken up the vector. As previously mentioned, this selection can be performed thanks to selection markers or reporter genes. These genes are chosen as reporters because they are easily identified and measured and can therefore report on the presence or absence of a particular genetic element, such as a plasmid [48]. Ideal reporter genes should not be natively expressed in the cell chosen in the study nor result in a phenotype naturally displayed by the host. They should be robust, easy and cheap to use and lack toxicity. Commonly used reporter genes code for characteristics that can be identified visually, for example fluorescent and luminescent proteins such as the green fluorescent protein (GFP) from jellyfish or the enzyme luciferase which catalyzes a reaction that produces light [30]. Other types of selection markers can be antibiotics such as Ampicillin and Chloramphenicol, which are both used in this study. If cells contain the ampicillin resistance (*bla*) gene or chloramphenicol resistance gene, they will be able to grow in the presence of the respective antibiotic while the other celles will not be able to survive.

In this study, *mCherry* is used as a reporter gene. mCherry is a fluorescent protein used to visualize the level of expression of a promoter in an *in vitro* culture [41]. mCherry is monomeric (hence the m in mCherry) and derives from a protein isolated from *Discosoma sp.* It is considered to be the preferred choice among the red monomers as it

combines red-shifted emission with reasonable photostability, brightness and performance in gene fusions [20]. The mCherry protein matures rapidly, making it possible to see results very soon after its translation. The maximum excitation and emission wavelengths from mCherry are 587 nm and 610 nm respectively [67]. Production of mCherry in culture results in a pink coloration, which makes it easy to indicate successful uptake of the plasmid carrying *mCherry* and functional expression from this plasmid. In other words, it is a tool to help demonstrate the functionality of an expression system.

1.8 The Aim of This Study

As mentioned in previous sections, recombinant therapeutic proteins obtained from bacteria are almost exclusively produced in *E. coli*. However, a large fraction of the recombinant proteins produced in *E. coli* are in an insoluble form, which sometimes makes them irrelevant for therapeutic applications. There could be several advantages in producing recombinant proteins in other bacteria, such as *C. glutamicum*, especially when it comes to proteins that are difficult to secrete or dependent on effective downstream processes.

Vectron Biosolutions is a biotechnology company focusing on developing expression vectors for industrial-level production of recombinant proteins. Vectron Biosolutions was founded in 2008 as a result of years of research in Professor Svein Valla's research group at NTNU in Trondheim. At the center of Vectron's technology is the patent-protected XylS/*Pm* expression cassette which is usually used in conjunction with minimal replicons of the RK2 plasmid. The preferred expression host for Vectron is *E. coli*, however, the company is currently working on developing a toolbox of alternative bacterial hosts, including both Gram-negative and Gram-positive species. This thesis was written in collaboration with Vectron Biosolutions, as a part of their work on testing *C. glutamicum* as a potential host for recombinant protein production.

The aim of this study was therefore to test and adapt the expression technology of Vectron Biosolutions, the XylS/*Pm* expression cassette, to the production of recombinant proteins in *C. glutamicum*. A shuttle vector *C. glutamicum*/*E. coli* containing *mCherry* as a reporter gene was constructed and *mCherry* expression from this recombinant plasmid was tested both in *E. coli* and *C. glutamicum*. The expression cassette XylS/*Pm* was then also inserted into the vector, and expression of *mCherry* from the *Pm* promoter was evaluated in both *C. glutamicum* and *E. coli*. Transcription levels of *xylS* and *mCherry* were evaluated to identify bottlenecks for recombinant protein production by *C. glutamicum* when harboring vectors containing the XylS/*Pm* expression cassette. The results from this study will help decide upon suitable alterations to the expression cassette/vectors and might lead to inducible recombinant protein production in *C. glutamicum* at industrial levels.

Chapter 2

Materials and Methods

2.1 Media and Solutions

The media and solutions used in this study are presented in Appendix A.

2.2 Bacterial Strains and Growth Conditions

Bacterial strains and vectors used in this thesis are given in Table 2.1.

Escherichia coli (*E. coli*) DH5α was used as cloning host for construction of expression vectors while *E. coli* BL21 was used as expression strain. Culturing of *E. coli* was conducted in LB medium at 37°C, both when grown in liquid culture (225 rpm) and on agar plates.The choice of antibiotics was based on the antibiotic resistance gene in the plasmids.

C. glutamicum MB001(DE3) and *C.glutamicum* ATCC 13032 were cultivated in BHIS. Both cultivation in liquid culture (225 rpm) and on agar plates were conducted at either 30°C or 37°C. Chloramphenicol was used as selective antibiotic.

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

Bacterial strain	**Description**	**Source or reference**
Escherichia coli		
DH5α	Cloning strain	New England Biolabs (NEB)
BL21(DE3)	Production strain	NEB
BL21	Production strain	NEB
Corynebacterium glutamicum		
MB001(DE3)	Alternative production strain	Kortmann et al. (2015) [40]
ATCC 13032	Wild type strain	
Vector	**Description**	**Source or reference**
pVB-1 (also called pJB658 in other studies)	RK2-based expression vector adapted for *E. coli* harboring XylS/*Pm* inducible promoter system for expression of cloned genes, Amp^r	Blatny et al. (1997) [11]
pVB-1A0B1-mCherry	pVB-1 with a mutant *trfA* gene leading to a slightly increased copy number (15-20), expressing mCherry from the XylS/*Pm* inducible promoter system, Amp^r	Vectron Biosolutions
pXMJ19	shuttle vector *E.coli/C.glutamicum*, Cm^r	Jakoby et al. (1999) [35]
pXMJ19-mCherry	pXMJ19 with insertion of *mCherry* from pVB-1A0B1-mCherry, Cm^r	This study, project thesis [37]
pVB-4A0E1-mCherry	pXMJ19-mCherry with an insertion of expression cassette *XylS/Pm* and *mCherry* from pVB-1A0B1-mCherry and deletion of the original P_{tac} promoter, Cm^r	This study
pMA-T Cgluta-optm-mCherry	pMA-T backbone containing *mCherry* optimized for *C. glutamicum*, Amp^r	GeneArt (Thermo Fisher Scientific)

2.3 Generating Growth Curves

Precultures were prepared for *C. glutamicum* MB001(DE3) and *C. glutamicum* ATCC 13032 in BHIS, incubated at 37°C, 225 rpm overnight. The next day, 500 mL baffled flasks were filled with 100 mL fresh BHIS and inoculated with 4 mL of overnight cultures. Both strains of *C. glutamicum* were incubated at both 30°C and 37°C. The OD_{600} was measured with a spectrophotometer (Helios Unicam) just after inoculation, and then every half hour until the lag phase was over and the exponential phase had started. Then, the OD_{600} was measured every hour until the stationary phase was reached.

2.4 Plasmid Construction

The vector pVB-4A0E1-mCherry was constructed using PCR and the One-Step Sequence- and Ligation-Independent Cloning (SLIC) method.

2.4.1 Plasmid Isolation from *E. coli*

Isolation of plasmid DNA from *E. coli* was performed using Wizard® *Plus* SV Minipreps DNA Purification System (Promega). This is a rapid method, based on alkaline lysis, for isolation of plasmid DNA needed for cloning and verification. Alkaline lysis was first described by Birnboim and Doly [9] and is made up by four basic steps: resuspension, lysis, neutralization and clearing of lysate. Bacteria harboring desired plasmid are grown overnight in appropriate media and antibiotics and the culture is centrifuged to concentrate the cell material into a pellet. The pellet is resuspended in a buffer which contains EDTA (ethylenediaminetetraacetic acid). EDTA forms complexes with divalent cations (Mg_2^+, Ca_2^+), which prevent DNase from damaging the plasmid and help destabilize the cell wall. The second step is lysing, where a strong base (NaOH) will help break down the cell wall and disrupt hydrogen bonding between DNA bases which results in denaturing of chromosomal and plasmid DNA. The lysate is then neutralized by adding potassium acetate which makes the plasmid DNA re-nature to double-stranded (ds) DNA . This part is not possible for the long chromosomal DNA streches. dsDNA dissolves easily in solution, while single-stranded (ss) chromosomal DNA and denatured protein will precipitate, which can be easily separated from the plasmid DNA solution by centrifugation. For isolation of plasmid DNA from the supernatant, the solution is transferred to a column containing a membrane which DNA binds to when centrifuged. The column is then washed and DNA is eluted into sterile eppendorfs by ion free water without nucleases (Wizard® *Plus* SV Minipreps DNA Purification System Protocol). Purified DNA is stored at -20°C.

Concentration determination
Plasmid concentrations were determined with either Nanodrop® ND-1000 Spectrophotometer (Thermo Scientific) or NanoDrop One (Thermo Scientific).

2.4.2 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a technique to amplify genes and other DNA sequences *in vitro*. Two synthetic oligonucleotides, referred to as primers, are complementary to sequences on opposite strands of the double-stranded target DNA and bind at positions defining the extremities of the segment to be amplified. They serve as replication primers that can be extended by a DNA polymerase [53]. Q5 polymerase (NEB), which is developed for high fidelity amplification and ultra-low error rates, was used in this work.

A PCR experiment involves three major steps, which are usually sequentially repeated 30 times:

- **Denaturation of DNA** The double strand of DNA containing the sequence of interest is opened by heating, usually at 92-98°C.
- **Annealing of denatured DNA** Denatured DNA containing the sequence of interest binds to synthetic primers, present in excess in the reaction. The ideal annealing temperature is generally between 50-65°C and depends on the base composition and length of the primer. It must be low enough to enable hybridization between primer and template, but high enough to prevent formation of mismatched hybrids.
- **Replication of the DNA segment** Thermostable polymerase is used to replicate the DNA segment from each primer in the $5' \rightarrow 3'$ direction. The polymerization is usually carried out at 70-72°C.

A typical PCR mix is given in Table 2.2. Primers and template DNA differ from one mix to the other. The primers used in this study are listed in Table B.1 in Appendix B.

Table 2.2: PCR mix for amplification of pXMJ19-mCherry without its original P_{tac} promoter and *mCherry* to construct pVB-4A0E1-mCherry

Component	1x
5X Q5 Reaction buffer (NEB)	10 µL
5X Q5 High GC enhancer (NEB)	10 µL
Primer 17-5 (10 µM)	2.5 µL
Primer 10-16 (10 µM)	2.5 µL
10 mM dNTP mixture (Roche Diagnostics)	1 µL
Template DNA (plasmid - pXMJ19-mCherry)	1 µL
Q5 Hot start high fidelity DNA polymerase (NEB)	0.5 µL
dH_2O	22.5 µL
Total	50 µL

An example of a PCR program used for amplification of segment of interest is given in Table 2.3. This was programmed into the PCR machine C1000 Touch Thermal Cycler (Bio-Rad). In order to get the correct segment as pure as possible, several temperatures for annealing were tested, as well as different elongation times (five or seven minutes).

Table 2.3: PCR program used for amplification of pXMJ19-mCherry without its originial P_{tac} promoter and *mCherry*. This program was used for the PCR mix given in Table 2.2.

Temperature	Time	
98°C	30 s	
98°C	10 s	Repeated
56°C	30 s	35×
72°C	5 min	
72°C	5 min	
4°C	∞	

2.4.3 Gel electrophoresis

Gel electrophoresis is a method used to separate DNA fragments by size. When an electric field is applied, negatively charged DNA moves towards the positive electrode, away from the negative electrode. When DNA migrates it must find its way through a network of tangled chaines of agarose. For this reason, smaller fragments migrate faster than larger fragments [15]. Molecular standards, with known band sizes, are used as a reference to determine the sizes of the different DNA fragments. The loading dye helps to assess how far the samples have run on the gel and is also a reagent to make the DNA samples denser than the running buffer so that the samples will sink in the well.

Procedure
An agarose gel prepared with wells is placed in a 1×TAE buffer-filled tank that has a positive electrode at one end and a negative electrode at the other. DNA samples with added loading dye are transferred into individual wells on the gel. After loading, the power is switched on and the electrophoresis starts. The voltage and duration of the electrophoresis are adjusted according to the size of both the gel and the DNA fragments of interest. The gel is analyzed using a ChemiDoc XRS+ (Bio-Rad).

2.4.4 Extraction of DNA from agarose gel

After desired bands on the gel are identified using ChemiDoc XRS+ (Bio Rad), they are excised using a scapel and the DNA is extracted from the gel fragments. The extraction is done according to the user manual of Zymoclean™ Gel DNA Recovery Kit (Zymo Research). Agarose dissolving buffer (ADB) is added to the gel fragments and incubated at 55°C until the gel is completely dissolved, transferred to a Zymo-Spin™ Column in a collection tube, centrifuged, washed with wash buffer and eluted directly from the column by DNA Elution buffer. The DNA obtained is stored in Eppendorf tubes at -20°C.

2.4.5 One-Step Sequence- and Ligation-Independent Cloning

SLIC is based on the 3'-to 5' exonuclease activity of T4 DNA polymerase and is an alternative to the traditional restriction enzyme/ligase cloning [43]. It does not need specific sequences and can be used to generate recombinant DNA with multiple inserts. The vector-backbone needs to be linearized either with restriction enzymes digestion or PCR. The insert is prepared by PCR with primers with extensions homologous to each end of the linearized backbone. The backbone and insert(s) are then mixed and incubated at room temperature with T4 DNA polymerase to generate 5' overhangs. The reaction mixture is placed on ice for single-strand annealing and then competent *E. coli* is transformed with the annealed DNA mixture[36].

Procedure
Linearized vector and PCR-amplified insert were mixed at a molar ration of 1:2 in a 1.5 ml tube, as shown in Table 2.4. 0.5 µL of T4 DNA polymerase (NEB) was added to the mixture and incubated at room temperature for 2.5 minutes. The reaction mixture was put on ice and icubated for ten minutes to stop the reaction. Competent *E. coli* DH5α cells were thawed on ice and mixed with 1-2 µL of the reaction mixture. The competent *E. coli* DH5α cells were then transformed as described in Section 2.5.2.

An example of a reaction mix for SLIC experiments is given in Table 2.4.

Table 2.4: Reaction mix for SLIC experiments.

Component	SLIC	Control
10X Buffer 2.1 (NEB)	1 µL	1 µL
Linearized vector	X µL	X µL
Insert	Y µL	-
dH_2O	Up to 10 µL	Up to 10 µL

2.4.6 Restriction Site Digestion

A restriction enzyme is an endonuclease that recognizes and cleaves DNA at specific nucleotide sequences called restriction sites [53]. The restriction endonucleases will cleave any DNA, as long as the DNA contains the nucleotide sequence it recognizes. DNA sequences cut with the same restriction enzyme can be covalently fused together regardless of their origin [68]. Genetic engineering often relies on restriction enzymes to isolate DNA sequences of interest. Restriction enzymes either cleave at the center of both strands to yield a blunt end, or they cleave at staggered positions, usually 2 or 4 nucleotides, leaving single-stranded overhangs called sticky ends [13].

Newly constructed plasmids were verified by digestion and sequencing. A typical digestion mix is shown in Table 2.5. The digestion mixes were incubated at 37°C from 1 to 18 hours.

Table 2.5: A typical digestion mix.

Component	Volume
10× CutSmart Buffer (NEB) or appropriate buffer	1 µL
Restriction enzyme 1	0.5 µL
Restriciton enzyme 2	0.5 µL
DNA sample	3-8 µL
dH_2O	up to 10 µL

To each sample 2 µL of 6X purple loading dye (NEB) was added and the DNA fragments were separated by gel electrophoresis.

The restriction enzymes used in this study are listed in Table 2.6.

Table 2.6: The restriction enzymes used in this study.

Restriction enzyme	Recognition sequence[a]	Blunt or sticky end
AhdI (NEB)	GACNNN'NNGTC[b]	Sticky
NcoI-HF (NEB)	C'CATGG	Sticky
SfoI (NEB)	GGC'GCC	Blunt
BamHI-HF (NEB)	G'GATCC	Sticky
HindIII-HF (NEB)	A'GCTT	Sticky
NdeI (NEB)	CA'TATG	Sticky
XbaI (NEB)	T'CTAGA	Blunt
DnpI (NEB)	GA'TC	Blunt

[a] The sequence shown is one strain given in $5' \rightarrow 3'$ direction. Almost all recognition sequences are palindromes: when both strands are considered they read the same in each direction. The position of the cut is indicated with an apostrophe.
[b] N indicates any nucleotide.

2.5 Transformation of *E. coli*

Transformation is the process by which foreign DNA is introduced into the cell. It was first discovered by Griffith in 1928, who showed that transformation of some bacteria will occur with naked DNA [68]. However, this is a rare event occurring with low frequency. Since DNA is highly charged it will not easily pass through the membranes of bacteria [62]. Methods have therefore been developed to obtain competent cells, cells with increased ability to take up naked DNA, and to facilitate transformation [15, 62].

Chemical transformation was discovered in 1970, and cells treated with calcium chloride ($CaCl_2$) have since been used routinely to obtain competent cells [18, 50, 62]. DNA binds to the cell surface, followed by uptake through the membrane when $CaCl_2$ is present in high concentration [75]. However, the function of $CaCl_2$ is not fully understood. It is though to affect the bacterial wall, and may also be responsible for binding DNA to the cell surface. A temperature shock from 0°C to 42°C together with the presence of $CaCl_2$

or other salts is found to be an efficient method to achieve transformation [7]. This heat-shock transformation method is used in this study to transform E. coli. The protocol is adapted from the work performed by Dagert and English [18].

2.5.1 Super Competent *E. coli*

E. coli cells were incubated in 4 mL LB medium (no antibiotic) at 37°C, 225 rpm over night. The following day, 0.5 mL from the overnight culture was used to inoculate 50 mL Psi-medium. The cultures were incubated at 37°C, and OD_{600} was measured until reaching $\simeq 0.4$. The samples were incubated on ice for 15 minutes to stop further growth and centrifuged at 4000 rpm, 4°C for 5 minutes to harvest the cells. Pellets were resuspended in 20 mL cold TFB1, before incubation on ice for 15 minutes. The centrifugation step was repeated and the cells were resuspended in 1.5 mL cold TFB2. The samples were aliquoted in 100 µL volumes into Eppendorf tubes on ice and frozen in liquid nitrogen before storage at -80°C.

2.5.2 Heat Shock Transformation of *E. coli*

Competent *E. coli* (100 µL per transformation) were thawed on ice. Up to 10 µL DNA was added to the thawed cells and gently mixed. The tubes were incubated on ice for 15 to 30 minutes, before the bacteria were heat-shocked in a water bath at 42°C for 45 seconds. Immediately after heat-shock the tubes were transferred to ice and incubated for two minutes. Prewarmed (37°C) SOC medium (900 µL) was added before the cells were incubated at 37°C for at least 1 hour. After incubation, the cells were plated on LA containing a selective antibiotic for the plasmid. The plates were incubated at 37°C until the next day.

The day after transformation, several random colonies were picked from the plates. They were streaked out on a new plate and incubated at 37°C overnight, as well as grown in 5 mL LB with appropriate antibiotics at 37°C at 225 rpm overnight. The next day, plasmids from each of the samples were purified as described in Section 2.4.1. The plasmids were then digested and underwent gel electrophoresis as described in Sections 2.4.6 and 2.4.3 respectively. After analysis of the gel, plasmids that seemed correct were sent for sequencing to an external company, GATC biotech.

2.6 Transformation of *C. glutamicum*

As mentioned before, transformation is the process where cells take up naked DNA. Another method to achieve transformation, other than chemical transformation, is through electroporation [62]. Electroporation is the usage of high-voltage electric shocks to introduce DNA molecules into cells. This was first performed by Wrong and Neumann using fibroblasts in 1982 [77]. The technique has later been further developed and generalized to other cell types. When cell membranes are subjected to a high-voltage electric field, they suffer a temporary break-down and pores large enough to allow macromolecules to pass from one side of the membrane to the other are formed [57]. If a suitable electric field pulse is applied, then the electroporated cells can recover, with the pores resealing spontaneously, and the cells continue to grow. The mechanism behind the electroporation method is not well understood and the development of protocols for particular applications has usually been achieved empirically by adjusting electric pulse parameters [62]. The protocol used in this study was obtained from Tauch et al. [70].

2.6.1 Preparation of Competent *C. glutamicum*

C. glutamicum cells were incubated in 5 mL BHIS medium (no antibiotic) at 30°C, 225 rpm over night. From this pre-culture, 500 µL was used to inoculate 25 mL BHIS, which then was cultivated at 30°C at 225 rpm until OD $\simeq 1.5$. The culture was then centrifuged at 4500 rpm, 4°C for 5 minutes. The pellet was re-suspended in 25 mL TG-buffer and centrifuged at 4500 rpm, 4°C for 5 minutes. The pellet was then washed in 25 mL cold 10% glycerol and then centrifuged again at the same conditions. The supernatant was discarded and the pellet re-suspended in the back-flow and kept on ice.

2.6.2 Electroporation of Competent *C. glutamicum*

0.1 to 10 µg DNA was added to competent *C. glutamicum* cells (100 µL). The cells were transferred to a cold 0.2 cm lectroporation cuvette (BioRad). The cuvette was then electroporated (GenePulser Xcell, BioRad) at 2500 V, 25 µF and 200 Ω. After pulsing the cells were transferred to mL 4 BHIS medium preheated at 46°C, and incubated at 46°C for six minutes. The cells were regenerated first at 37°C at 225 rpm for 1 hour, then at 30°C for 30 minutes. After incubation, the cells were centrifuged at 4500 rpm for 5 minutes and plated out on selective BHIS plates.

2.6.3 Plasmid Isolation from *C. glutamicum*

Lysis of the cell wall will result in nuclear material spilling out from the broken cells. The method for the lysis procedure depends upon the nature of the host cell, some species are harder to lyse than others [62]. It became clear that *C. glutamicum* is a robust soil bacterium, needing altered methods for lysing.

For plasmid isolation, two different kits were tested. For Wizard Plus SV Minipreps DNA Purification System (Promega) the best result was obtained when 15 $\mathrm{mg/mL}$ lysozyme was added to the resuspension buffer along with prolonged incubation time 30-60 minutes, as well as incubation at 37°C instead of room temperature. Exchanging the resuspension buffer from the kit with CelLytic B Plus (Sigma-Aldrich) yielded approximately the same concentrations. However, the highest plasmid concentrations were obtained from ZR Plasmid Miniprep™-Classic (Zymoclean Research) when 15 $\mathrm{mg/mL}$ lysozyme was added to the P1-buffer and samples were incubated for 2 hours at 37°C.

2.7 Colony PCR

Colony PCR is a method for rapidly screening colonies of bacteria that have grown up on selective media following a transformation step, to verify that the desired genetic construct is present [6]. In this study it was used to check for presence of *mCherry*. Presence of *mCherry* would indicate successful transformation.

Cells from a colony were picked from fresh agar plates, re-suspended in 100 µL CelLytic B plus (Sigma-Aldrich) and incubated for 30 minutes at room temperature. The lysate was centrifuged at 8000 rpm for 10 minutes, and the resulting supernatant was subjected to PCR analysis. The PCR mix and the PCR program for the colony PCR are given in Table 2.7 and 2.8 respectively.

Table 2.7: The PCR mix used for colony PCR of *C. glutamicum* bacterial colonies to check for presence of *mCherry*.

Component	**1x**
5X Q5 Reaction buffer (NEB)	10 µL
5X Q5 High GC enhancer (NEB)	10 µL
Primer mCherry fwd (10 µM)	2.5 µL
Primer mCherry rev (10 µM)	2.5 µL
10 mM dNTP mixture (Roche Diagnostics)	1 µL
Template DNA (lysed bacterial colony)	1 µL
Q5 Hot start high fidelity DNA polymerase (NEB)	0.5 µL
dH_2O	22.5 µL
Total	50 µL

Table 2.8: PCR program for colony PCR of *C. glutamicum* bacterial colonies

Temperature	**Time**	
98°C	30s	
98°C	10s	Repeated
50°C	30s	25×
72°C	5min	
72°C	5min	
4°C	hold	

2.8 Expression of mCherry

The production host, either *E. coli* BL21 or *C. glutamicum* MB001(DE3), was transformed with vectors prepared in *E. coli* DH5α.

An overnight culture of said production host was grown in appropriate medium and antibiotics at 37°C and 225 rpm. The OD_{600} value of the culture was determined and the measurement was used to calculate the amount of culture to be inoculated to obtain a starting $OD_{600} \simeq 0.05$ in Hi+Ye medium. Two parallels were prepared of each bacterium and vector and the cultures were incubated for about 4.5 hours at 30°C and 225 rpm until OD_{600} 2.5. Appropriate inducer (1 mM IPTG (30 µL) or 2 mM m-toluate (120 µL)) was added to one of the parallels, resulting in one induced and one uninduced parallel. The cultures were then incubated for almost 18 hours at 30°C. After incubation each culture was centrifuged at 7850 rpm, 4°C for 10 minutes. Supernatant was removed by vacuuming and the wet weight of each pellet was calculated.

The tubes were put on ice and 1 mL 0.9% NaCl was added for each 100 mg pellet. 1 mL from each culture was transferred into 1.5 mL tubes. The samples were centrifuged at 13000 rpm for 5 minutes at 4°C and the supernatant was removed by vacuuming. To lyse the cells, the pellets were resuspended in 500 µL Cell LyticB lysis buffer (Sigma) and incubated for 1 hour on ice at 100 rpm. In order to separate the soluble fraction from the insoluble one, the samples were centrifuged at 13000 rpm for 8 minutes. The supernatant (soluble fraction) was transferred into a clean 1.5 mL tube, while the pellet (insoluble fraction) was resuspended in 500 µL SDS-running buffer. The expression of mCherry in both soluble and insoluble fractions was measured using a fluorometer.

2.8.1 Fluorometry

A fluorescence detector measures the amount of relative fluorescence units (RFU) in the samples and generates data using a computer software. Samples with higher quantities of expressed mCherry have higher RFU values.

For each sample, 2 x 100 µL was loaded from both soluble and insoluble fractions on a 96-well plate, to run each sample in duplicate. For control, two wells were loaded with 100 µL SDS-running buffer. The plate was run in Infinite 200 Quad-4 fluorometer (Tecan). The excitation and emission wavelengths were 584 nm and 620 nm, while the excitation and emission bandwidths were 9 and 20 nm respectively. The shaking duration was 15 seconds, and shaking amplitude was 3 mm. Each well was measured 12 times and the reported values are the means of these measurements.

2.9 Qualitative PCR

The transcript levels of *xylS* and *mCherry* were measured to identify possible hinders when producing mCherry in *C. glutamicum*. The transcript levels in *C. glutamicum* MB001(DE3) harboring pXMJ19-mCherry and pVB-4A0E1-mCherry were compared to the transcript levels in *E. coli* BL21 harboring the same vectors. Droplet digital PCR (ddPCR) was used to evaluate the expression levels.

2.9.1 Isolation of RNA from *E. coli* and *C. glutamicum*

A pre-culture was prepared by growing the bacteria in 5 mL of appropriate medium and antibiotic, at 37°C at 225 rpm over night. 15 mL of Hi+Ye medium was inoculated with the pre-culture to reach $\mathrm{OD}_{600} \simeq 0.1$. Two replicates of each sample were prepared, and at the time of inoculation one of each sample was induced with the appropriate inducer (2 mM m-toluic acid or 1 mM IPTG). Once the OD_{600} had reached $\simeq 1$, a volume of 1 mL from each culture was mixed with 2 mL RNAlater (Ambion). For *E. coli* total RNA from the cultures was isolated as described by the RNAqueous Total RNA Isolation Kit (Ambion) protocol. For *C. glutamicum* an enzyme-pretreatment was added: the cells were resuspended in 100 µL TE (10 mM Tris$-$HCl, 1 mM EDTA) with 1 $\mathrm{mg/mL}$ lysozyme and 100 U mutanolysin, and incubated for 30 minutes at 37°C. Afterwards, the same protocol as for *E. coli* was followed. For both species 50 µL Elution solution it was used to elute the isolated RNA in the final step.

Ambion TURBO DNA-free kit (Thermo Fischer Scientific) was used to remove contaminating DNA by digestion. The supplier's protocol was followed.

2.9.2 Analysis of RNA quality

The Agilent 2100 Bioanalyzer (Agilent Technologies) is a microfluidics-based instrument for sizing, quantification and quality control of DNA, RNA, proteins and cells. RNA samples are separated using electrophoretical separatation, and detected via laser induced fluorescence detection. The Bioanalyzer software generates an electropherogram and displays results such as sample concentration as well as RNA integrity number (RIN). The electropherogram displays fluorescense intensity as a function of time and provides a detailed visual assessment of the quality of the RNA sample. The RIN is a tool developed to help estimate the integrity of the total RNA sample. The scale goes from 0-10, where 10 is completely intact RNA. The software is able to calculate RIN for both eukaryotic and prokaryotic samples, but the prokaryotic RIN has not been sufficiently validated yet [52]. For prokaryotic samples there should be peaks at 16S and 23S. The higher the peaks in this area, the higher RIN value and higher quality RNA.

In this study, the Agilent 2100 Bioanalyzer together with Agilent RNA 6000 Nano Kit (Agilent Technologies) were used for RNA quality assessments. The supplier's protocol was followed with one exception: to save some time, already prepared gel was used.

2.9.3 Preparation of complementary DNA

First-strand complementary DNA (cDNA) is DNA synthesized from single-stranded RNA template, in this case mRNA, in a reaction catalyzed by reverse transcriptase. The DNA produced is single-stranded, and can be converted to double-stranded cDNA molecules with DNA polymerase [68]. In this study, single-stranded cDNA was produced to analyze mRNA expression using ddPCR.

To produce cDNA from mRNA, the First-Strand cDNA Synthesis Kit (GE Healthcare) was used following the provider's protocol.

2.9.4 Detection of *mCherry* and *XylS* mRNA level using Droplet Digital PCR

Digital PCR (dPCR) enables absolute quantification of nucleic acids in a sample. Target DNA molecules are distributed across multiple replicate reactions at a level where there are some reactions that have no template and others have one or more template copies. After amplification with PCR, reactions containing one or more template give positive end-points, while those without template remain negative. The number of DNA molecules present in the initial sample can be extrapolated from the fraction of positive end-point reactions [29]. ddPCR is a method for performing dPCR based on water-oil emulsion. A single initial PCR sample is partitioned into 20,000 monodisperse droplets, thanks to the usage of simple microfluidic circuits and surfactant chemistry. The PCR amplification is then carried out within each droplet, and the droplets are read as positive (harboring target DNA) or negative (no target DNA) to calculate target DNA concentration [8]. Figure 2.1 shows the random distribution of template. The droplets are formed from a mixture of nucleic acid template, oil, primers, polymerase, nucleotides and a fluorescent label. The fluorescent label used in this study is EvaGreen (Bio-Rad), which appears bright when boudn to double-stranded DNA. As the target DNA amplifies, more of the fluorescent dye binds and the droplet appears brighter. The droplets are read by a droplet reader, and their positive or negative status are detected by fluorescence [8].

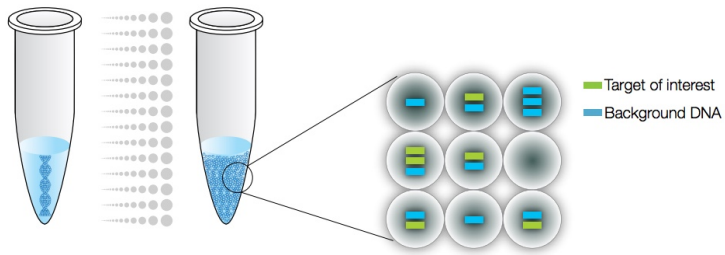


Figure 2.1: In ddPCR, a single PCR is partitioned into 20,000 droplets. Some droplets contain no template, while some have one or more template copies. Illustration obtained from Bio-Rad [8].

Procedure

The sample mix was prepared by mixing the components listed in Table 2.9. The primers used are listed in Table 2.10. A volume of 20 µL of sample mix was loaded to the middle row of DG8 cartridges (Bio-Rad). 70 µL QX200 EvaGreen Droplet Generation Oil (Bio-Rad) was loaded to the lower row. A gasket was attached to the top of the cartridge and placed in the QX200 Droplet Generator (Bio-Rad). After about 2.5 minutes about 20,000 droplets per sample were produced in the top row. The droplets were gently transferred to a 96-well PCR plate. The PCR plate was sealed using PX1™PCR plate sealer (Bio-Rad) and pierceable foil, before being placed in C1000 Touch Thermal Cycler for amplification by PCR. Table 2.11 shows the settings for the PCR. After PCR amplification of the target DNA in the droplets, the plate was read using a QX200 Droplet Reader. The results were analyzed using QuantaSoft Analysis Pro software (Bio-Rad).

Table 2.9: Sample mix for ddPCR.

Component	Volume
Q×200 ddPCR EvaGreen Supermix (Bio Rad)	12 µL
2 µM forward primer	2.4 µL
2 µM reverse primer	2.4 µL
cDNA sample	1.2 µL
dH_2O	6 µL

Table 2.10: Primer sequences used to detect transcript levels of *XylS* and *mCherry* by ddPCR.

Primer	Primer sequence 5′ - 3′
XylS forward	CGCCGAGCCCTATGCA
XylS reverse	CCTTGGGCAGGCGAATAGA
mCherry forward	AAACTGCGTGGCACCAACTT
mCherry reverse	TTCCCAACCCATCGTTTTTTT

Table 2.11: Program used for ddPCR on the C1000 Touch Thermal Cycler (Bio-Rad).

Temperature	Time	
98°C	5 min	
95°C	30 s	Repeated
60°C	1 min	40×
4°C	5 min	
90°C	5 min	
4°C	∞	

2.10 Inducer diffusion study

Precultures were grown in 5 mL medium until they reached exponential phase. Both *C. glutamicum* MB001(DE3) and *C. glutamicum* ATCC 13032 were grown in BHIS, while *E. coli* BL21 was grown in LB. All bacteria were incubated at 37°C. OD_{600} of the precultures were measured, and 50 mL fresh medium was inoculated aiming at reaching $OD_{600} \simeq 0.1$. Cultures (1.2 mL) were aliquoted in a 2 mL deep-well plate. 20 µL inducer, *m*-toluate or RO-water was added o each well as depicted on Table 2.12. The OD_{600} was monitored by measuring every other hour.

For *m*-toluate, the highest stock concentration in EtOH was prepared first, and used to prepare 1 mM stocks of the other concentrations.

Table 2.12: Overview of the deep-well plate. Each number represents the final concentration of *m*-toluate (mM) in the well. Each concentration was tested in triplets, and there also was added a triplet of the control (autoclaved RO-water). For all concentrations, 20 µL was added to the cultures.

	1	**2**	**3**	**4**	**5**
A	Autoclaved RO-water	1	6	15	30
B	Autoclaved RO-water	2	6	15	
C	Autoclaved RO-water	2	8	15	
D	0*	2	8	20	
E	0	4	8	20	
F	0	4	10	20	
G	1	4	10	30	
H	1	6	10	30	

* Added 20 µL EtOH

Chapter 3

Results

3.1 Comparison of *C. glutamicum* Growth at Different Temperatures

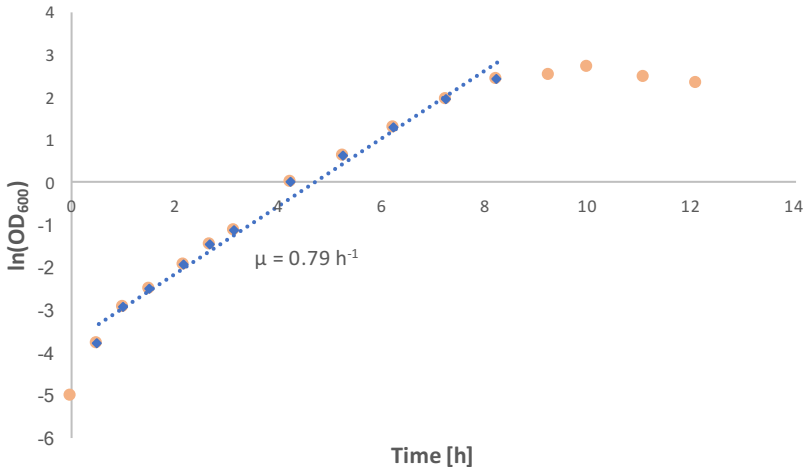
C. glutamicum MB001(DE3) and *C. glutamicum* ATCC 13032 were cultivated in BHIS. Growth curves for both strains of *C. glutamicum* were conducted by measuring the OD_{600} during incubation at either 30°C or 37°C. Three parallels were grown for each strain and temperature tested, and the average of the OD_{600} measurements from those three parallels were plotted using Excel.

Figure 3.1 shows semi-logarithmic plots of OD_{600} measurements as a function of time for *C. glutamicum* MB001(DE3) grown at 37°C (a) and 30°C (b). Figure 3.2 shows semi-logarithmic plots of *C. glutamicum* ATCC 13032 incubated at 37°C (a) and 30°C (b).

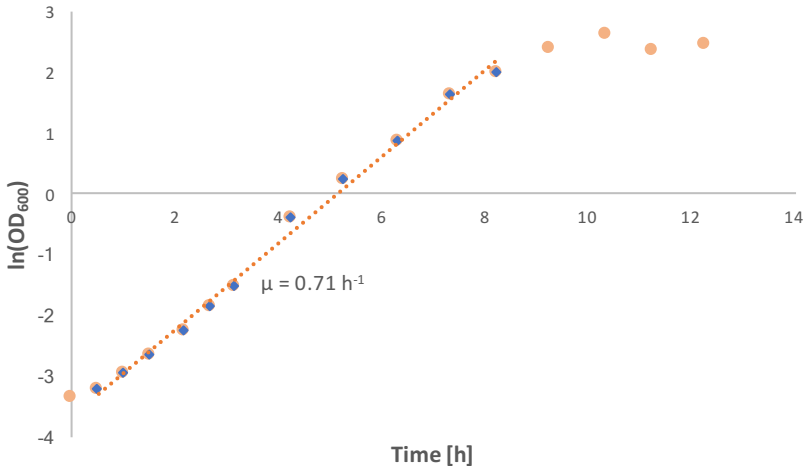
The generation time, the time it takes for the microbial population to double, is calculated based on the exponential phase. Table 3.1 shows the calculated generation times for the strains of *C. glutamicum*. The method used to calculate generation time is shown in Appendix G.

Table 3.1: Generation times calculated for *C. glutamicum* strains MB001(DE3) and ATCC 13032 incubated at 30°C and 37°C.

Strain	Temperature [°C]	Generation time [min]
C. glutamicum MB001(DE3)	37	53
C. glutamicum MB001(DE3)	30	59
C. glutamicum ATCC 13032	37	51
C. glutamicum ATCC 13032	30	57

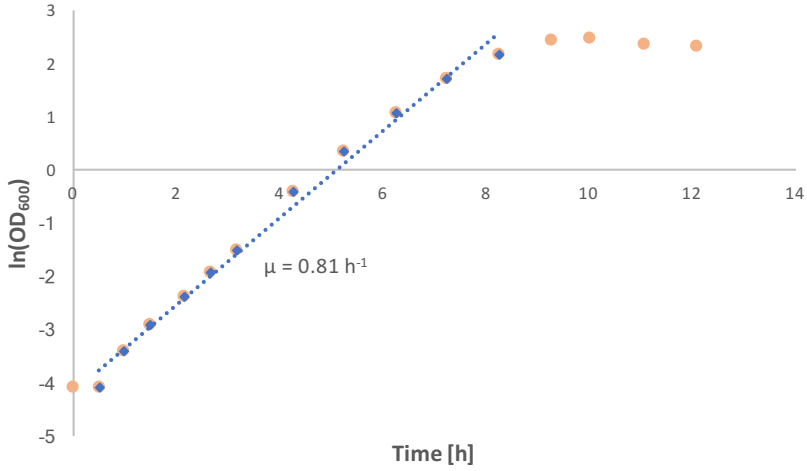


(a) *C. glutamicum* MB001(DE3) at 37°C

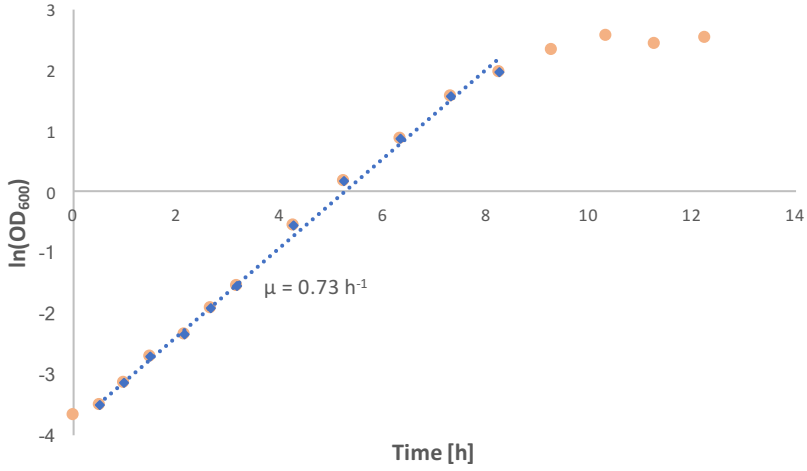


(b) *C. glutamicum* MB001(DE3) at 30°C

Figure 3.1: Semi-logarithmic growth curves of *C. glutamicum* MB001(DE3) incubated at 37°C **(a)** or 30°C **(b)**. The linear regression of the exponential phase and the growth rate μ [h^{-1}] are included.



(a) *C. glutamicum* ATCC 13032 at 37°C



(b) *C. glutamicum* ATCC 13032 at 30°C

Figure 3.2: Semi-logarithmic growth curves of *C. glutamicum* ATCC 13032 incubated at 37°C **(a)** and 30°C **(b)**. The linear regression of the exponential phase and the growth rate μ [h^{-1}] are included.

The generation time for *C. glutamicum* growth at 37°C is six minutes shorter compared to growth at 30°C. This indicates that both strains of *C. glutamicum* grow faster at 37°C. Based on this result, the incubation temperature for the following inducer diffusion study was chosen to be 37°C.

3.2 Inducer Diffusion Study

The expression cassette XylS/*Pm* utilizes *m*-toluate as an inducer. In order find out if *m*-toluate is able to enter the *C. glutamicum* cells, an inducer diffusion study was conducted as described in Section 2.10.

m-toluate, also reffered to as *m*-toluic acid, is a carboxylic acid with $pK_{\mathrm{a}} = 4.27$ [2]. When added in high quantities, this could acidify the medium. To check if the inducer concentration has some kind of effect on the pH, a pH-test was performed at various *m*-toluate concentrations. Results are shown in Table 3.2.

Table 3.2: Measured pH at different inducer concentrations.

Medium	**No inducer**	**1** mM ***m*-toluate**	**10** mM ***m*-toluate**	**30** mM ***m*-toluate**
BHIS	6.96	6.91	6.38	5.27
LB	7.14	6.74	6.37	4.80

For BHIS, pH measured at *m*-toluate concentrations of 0 mM, 1 mM, 10 mM and 30 mM show that the pH is relatively stable from 0 mM to 10 mM, with a slight acidification from 1 mM to 10 mM *m*-toluate. From 10 mM *m*-toluate to 30 mM *m*-toluate, there is a dramatic drop in pH of more than 1 point. For LB, the acidification is already seen at 1 mM of *m*-toluate, however from 1 mM to 10 mM *m*-toluate the pH is relatively stable. Similarly to BHIS, an important drop in pH of over 1.5 points is seen from 10 mM to 30 mM *m*-toluate in LB.

For the inducer diffusion study, *m*-toluate was added in different concentrations (1-30 mM). There were also added two controls, RO-water (No EtOH) and 0 mM *m*-toluate (pure EtOH). Figures 3.3, 3.4 and 3.5 show the mean value for OD_{600} as a function of the incubation time for *E. coli* BL21, *C. glutamicum* MB001(DE3) and *C. glutamicum* ATCC13032 respectively. The mean values for OD_{600} were calculated from three parallels. The standard variation was also calculated and is included in the graphs.

The results show that incerasing concentrations of *m*-toluate seem to have the general effect of slowing down cell growth, until it completely inhibits it and cause cell death. Cell death is seen for inducer concentrations of 8 mM *m*-toluate and higher for both *E. coli* and *C. glutamicum*. pH is realtively stable in both media between 0 mM and 10 mM, which suggests that the cell death observed is indeed caused by a toxic amount of *m*-toluic acid able to enter the cells rather than a pH drop in the medium.

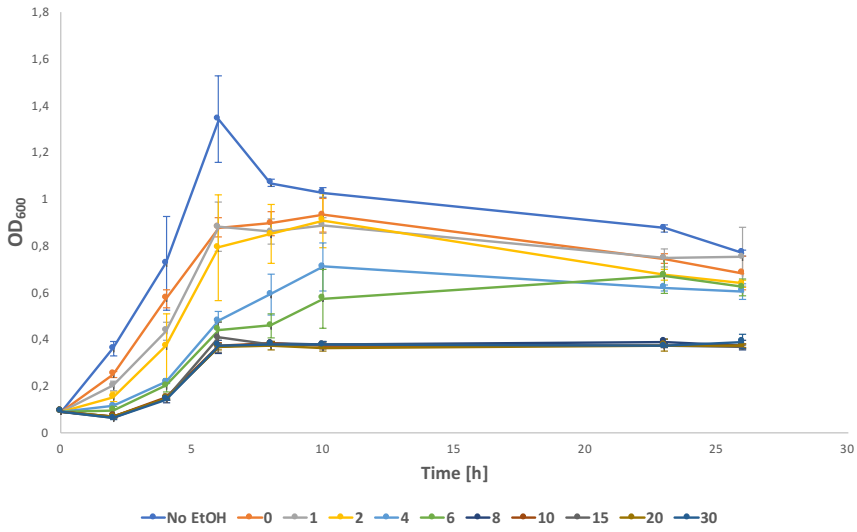


Figure 3.3: Mean values for the OD_{600} measurements of *E. coli* BL21 grown in LB with addition of different concentrations of inducer (0-30 mM), *m*-toluate, plotted as a function of incubation time. Each concentration was tested in three parallels. The standard variation of each value is included.

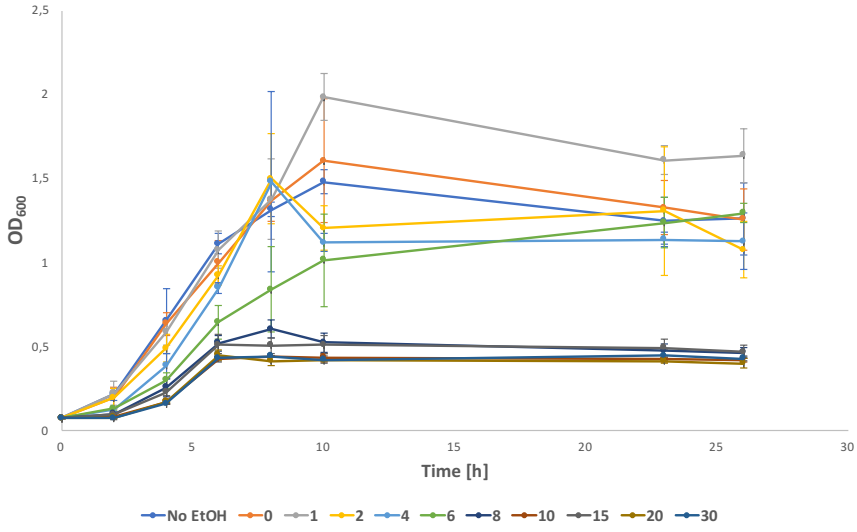


Figure 3.4: Mean values for the OD_{600} measurements of *C. glutamicum* MB001(DE3) grown in BHIS with addition of different concentrations of inducer (0-30 mM), *m*-toluate, plotted as a function of incubation time. Each concentration was tested in three parallels. The standard variation of each value is included.

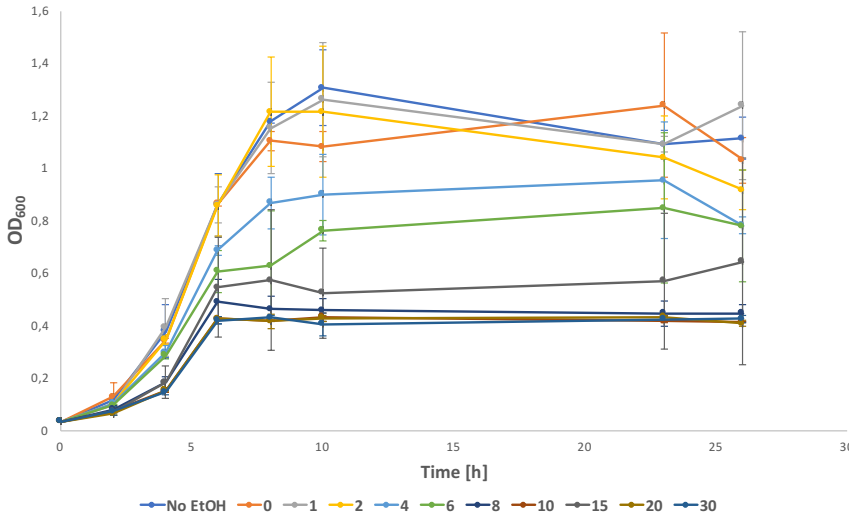


Figure 3.5: Mean values for the OD_{600} measurements of *C. glutamicum* ATCC 13032 grown in BHIS with addition of different concentrations of inducer (0-30 mM), *m*-toluate, plotted as a function of incubation time. Each concentration was tested in three parallels. The standard variation of each value is included.

3.3 Construction of Expression Vectors

For this thesis, the vector pVB-4A0E1-mCherry was constructed. This expression vector is based on the vector pXMJ19-mCherry constructed for the project thesis. Both vectors carry the same backbone from pXMJ19, but pVB-4A0E1-mCherry contains the expression cassette XylS/*Pm* instead of the original promoter from pXMJ19.

3.3.1 Constructing pVB-4A0E1-mCherry

The vector pVB-4A0E1-mCherry was constructed from the plasmids pXMJ19-mCherry and pVB-1A0B1-mCherry using the SLIC-method described in Section 2.4.5. Backbone and insert were both amplified using PCR. Several attempts, different PCR primers and altered methods were needed to construct the vectors.

Unsuccessful attempts
For the first attempt, the PCR primers 4-16 and 10-16 (Table B.1 in Appendix B) were chosen for amplification of the backbone from pXMJ19-mCherry without mCherry, while the primers 8-16 and 9-16 (Table B.1 in Appendix B) were chosen for amplification of a fragment carrying *xylS*, *Pm* and *mCherry* and including overlapping sequences from pVB-1A0B1-mCherry. After the SLIC procedure, all the resulting colonies turned pink both for control and the attempted new vector. The control contained only backbone without addition of insert, and due to the absence of *mCherry* gene, no pink colonies were expected. Because of the pink coloration of the controls, 4-16 and 10-16 were suspected of somehow amplifying the *mCherry* gene together with the rest of the backbone. They were therefore replace with primers 17-5 and 10-16 (Table B.1 in Appendix B). The PCR primers for the insert remained the same. After PCR and SLIC procedure, all the resulting colonies, including the controls, once again turned pink. As before, due to the absence of *mCherry* gene, control colonies were not expected to be pink. It was therefore concluded that the cloning was unsuccessful. The pink coloration of the control colonies indicated an error in the process. One possible explanation could be that template plasmid pXMJ19-mCherry remained in the PCR product after amplification of the backbone. If this template plasmid was cut out of the gel along with the amplified backbone without insert, it would explain the occurrence of pink colonies in the control. Digestion with DpnI, a restriction enzyme which digests methylated DNA, would destroy template DNA only PCR product would be purified from gel. DpnI digestion was first attempted on PCR product purified from gel, and followed by heat inactivation before the SLIC procedure. This time, no colonies were obtained.

Successful strategy
Since none of the previous attempts work, a new strategy was developed. The PCR experiment was repeated using primers 17-5 and 10-6 to amplify the backbone and 8-16 and 9-16 to amplify the insert. This time, DpnI digestion followed directly after PCR before performing gel electrophoresis. The bands corresponding to the backbone (6336 bp) and insert (2889 bp) were cut out and purified. SLIC procedure was then performed on the purified PCR products. The negative control, backbone without insert, resulted in 10-fold

fewer colonies compared to the SLIC plate. Six random colonies from the SLIC plate were picked and grown over night, before the plasmids were isolated. The isolated plasmids from each of the six colonies were digested with SfoI and separated by gel electrophoresis. Figure 3.6 shows the gel after gel electrophoresis with the expected fragment sizes (7021 bp and 2172 bp) present on the gel for five of the six plasmid samples. Sequencing results confirmed that the vector was correct.

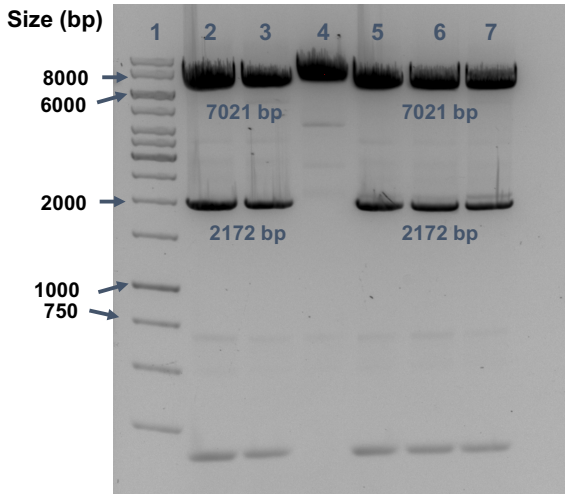


Figure 3.6: Picture of agarose gel after gel electrophoresesis of constructed plasmid expression vector pVB-4A0E1-mCherry digested with SfoI. Lane 1: GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific). Lane 2-7: Isolated plasmid from six random colonies.

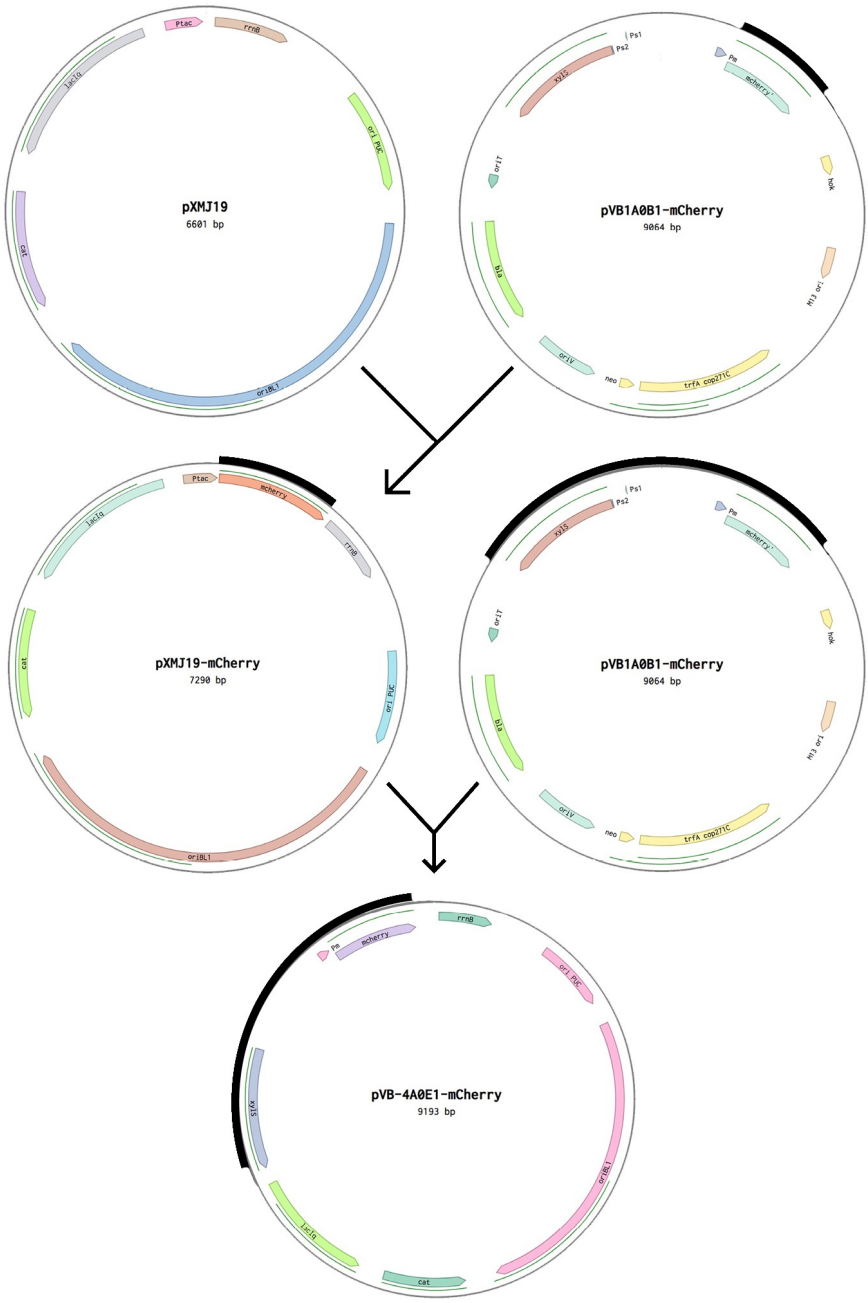


Figure 3.7: Construction map of pVB-4A0E1-mCherry. To construct pXM1J19-mCherry, *mCherry* from pVB-4A0B1-mCherry was inserted into pXMJ19. To construct pVB-4A0E1-mCherry, *mCherry* in pXMJ19-mCherry was replaced with the Xyls/*Pm* expression cassette and *mCherry* from pVB-4A0B1-mCherry. Inserts are highlighted. Plasmid maps were generated using Benchling.

3.3.2 Replacing *mCherry* in Expression Vectors by *mCherry* Codon-Optimized for *C. glutamicum*

More than one codon can code for the same aa. The usage of alternative codons is shown to be non-random, and genes with favorable codons will be translated more efficiently than those genes containing infrequently used codons [62]. This is due to the tRNA levels, meaning that the tRNAs recognizing each codon are not expressed at the same levels across species. Codon optimization is to change codons to match the most prevalent tRNAs without changing the encoded polypeptide, hence increasing the translation efficiency.

In pXMJ19-mCherry
The strategy for replacing *mCherry* with *mCherry* codon-optimized for *C. glutamicum* in pXMJ19 involved using restriction enzymes and ligation. Purified plasmids from overnight cultures of pXMJ19-mCherry and pMA-T Cgluta-optm-mCherry harboring the codon-optimized *mCherry* were digested with HindIII-HF and XbaI. CIP was also added to the digestion mix to prevent self-ligation of the backbone. After purification with electrophoresis and DNA recovery, ligation of backbone and insert was attempted. A control was also included corresponding to backbone fragment without the insert. After heat-shock transformation, both the control and the ligation plates displayed a similar amount of white colonies.

Several attempts, as well as some troubleshooting including testing the enzymes, showed that XbaI might not be working as expected, so XbaI was therefore replaced by BamHI-HF. This attempt followed the same experimental steps as before. The plates, including the control, from this attempt had pink and white colonies. Ten colonies, both white and pink, were purified and digested with restriction enzymes NdeI and AhdI. The digestion mixes were separated by gel electrophoresis. For pXMJ19 containing codon-optimized *mCherry*, it was expected to be one fragment (7925 bp), while the vector pXMJ19-mCherry was expected to have two fragments (4040 bp and 3250 bp). The expected fragments for pXMJ19 containing codon-optimized *mCherry* seemed to be present for two of the samples. These two candidates were sent for sequencing, which showed that the plasmid did not contain the codon-optimized *mCherry* sequence. Due to time restrictions, no more attempts were made.

In pVB-4A0E1-mCherry
The strategy for replacing *mCherry* with codon-optimized *mCherry* for C. glutamicum in pVB-4A0E1 involved using the SLIC-procedure after PCR amplification of backbone and insert.

The purified plasmids pVB-4A0E1-mCherry and pMA-T Cgluta-optm-mCherry were added to PCR mixtures with appropriate primers, Vf-pVB-1A0B1-v1 and Vr-pVB-1A0B1-v1 and 17-9 and 17-10 respectively (Table B.1 in Appendix B). Both DpnI digested PCR products and PCR products straight from the PCR machine were run on electrophoresis gels. The bands with expected sizes were cut out, even though they looked smeary on the gel despite several attempts at adapting the PCR program. SLIC was then attempted. A control was also added, only backbone without the insert. The colony ratio on the SLIC and control plates seemed to indicate that the cloning was successful. Six colonies were

picked and their plasmids were isolated, but neither of the isolated plasmids seemed to be of high quality. They were digested with a restriction enzyme, SfoI, but the patterns observed on the gel did not match what was expected. One of the purified plasmids was sent for sequencing to hopefully help detecting where the issue came from, but its concentration after purification was too low to obtain a reliable result. Due to time restrictions, no more attempts to build this vector were made.

3.4 Constructing Recombinant *C. glutamicum*

To examine if the constructed plasmid vectors pXMJ19-mCherry and pVB-4A0E1-mCherry were functional in *C. glutamicum*, *C. glutamicum* cells were transformed with the vectors. The expression of mCherry protein from these recombinant bacteria was then tested. Several attempts and alterations in the protocols were made for both transformation and plasmid isolation from *C. glutamicum*. Adjustments like adding lysozyme and incubation time and temperature were tested along with different miniprep-kits.

First attempt
In the first attempts at transforming *C. glutamicum*, *C. glutamicum* MB001(DE3) was transformed with pXMJ19-mCherry. There were 10-folds more colonies compared to the control, which seemed promising. The agar plate after transformation contained both pink and white colonies, as well as one yellow. The yellow colony was thought to be contamination from the air. Six white and six pink colonies were picked. The plasmids from each of these colonies were isolated and digested. Six of the twelve samples showed correct bands while the others showed no band after gel electrophoresis. There was no correlation between the color of the colony and the correct plasmid.

When isolating the plasmids, it seemed to be two different bacteria due to visual discrepancies in the lysis step. Half of the samples, the ones harboring the correct plasmids, displayed similar characteristics as *E. coli* during the lysis step. In order to find out if the colonies harboring the correct plasmid were actually *C. glutamicum* and not *E. coli*, two tests were performed: nalidixic acid and Gram-staining. Associated protocols are found in Appendix D. *C. glutamicum* is naturally resistant to the antibiotic nalidixic acid [66], while *E. coli* is not. However, the test gave no conclusive results, as the *E. coli* included as a control did also grow on the plates. The Gram-staining experiment was difficult to conclude from, but it seemed that the ”unknown” bacterium was more similar to *E. coli* than to *C. glutamicum*. It was, therefore, decided to stop further testing, and to try the transformation again.

Successful attempt
An alternative protocol was used for this next attempt at transforming *C. glutamicum* (described in Section 2.6), with some alterations in the preparation of competent *C. glutamicum* cells and the electroporation process. *C. glutamicum* MB001(DE3) cells were transformed with the vectors pXMJ19, pXMJ19-mCherry and pVB-4A0E1-mCherry. A negative control (dH_2O instead of vector) was added. The resulting plates are shown in Figure 3.8.

C. glutamicum transformed with pXMJ19 resulted in a layer of white colonies, while *C. glutamicum* transformed with pXMJ19-mCherry and pVB-4A0E1-mCherry resulted in over a hundred white colonies as seen in Figure 3.8. Since the control only resulted in 19 colonies, it seems that all three vectors were taken up and confered chloramphenicol resistance to *C. glutamicum*, as expected.

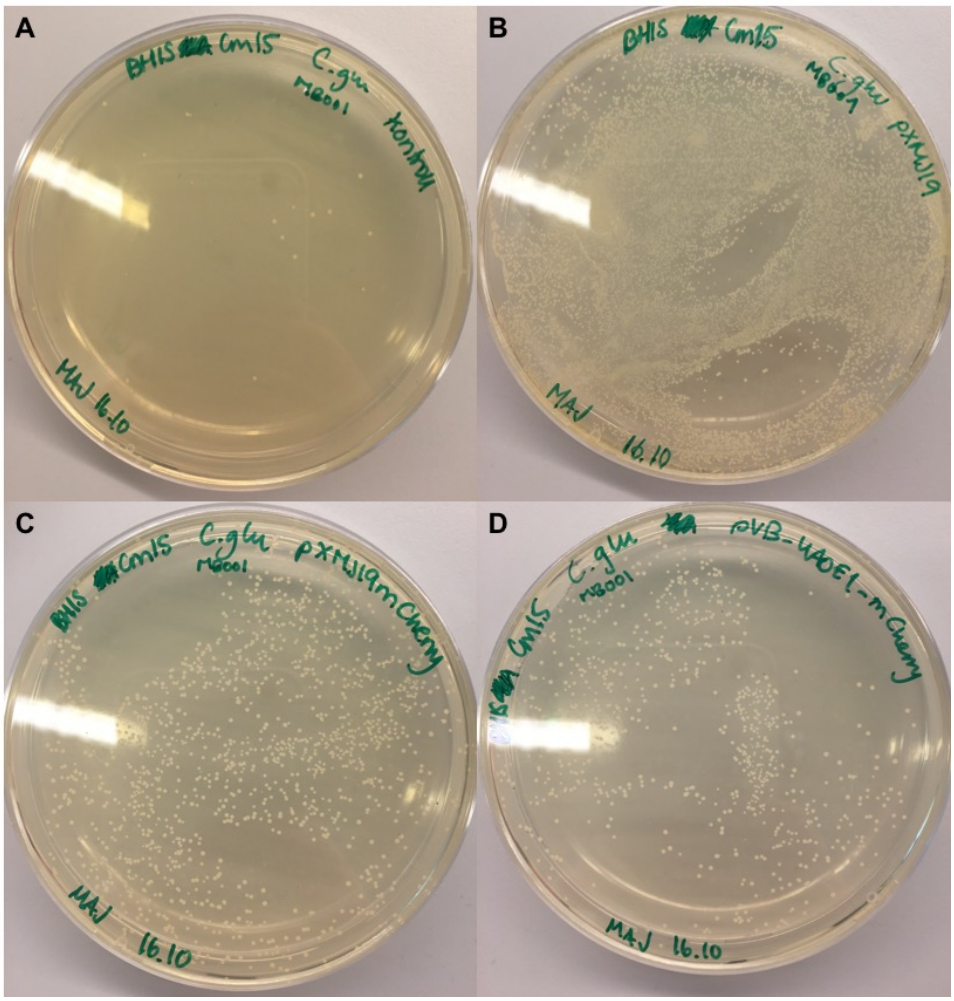


Figure 3.8: *C. glutamicum* MB001(DE3) transformed different with vectors. A: Negative control: *C. glutamicum* without vector B: *C. glutamicum* harboring pXMJ19, C: *C. glutamicum* harboring pXMJ19-mCherry, D: *C. glutamicum* harboring pVB-4A0E1-mCherry. All plates are BHIS agar containing 15 $\mu g/mL$ chloramphenicol.

Two samples of recombinant *C. glutamicum* harboring each vector were randomly chosen and the plasmid isolated. As mentioned above, there were some challenges with isolating plasmid from *C. glutamicum* due to difficulties with lysing the cells because of the thicker layer of peptidoglycans in the cell wall of Gram-positive bacteria. Different kits (Promega and Zymoclean Research) with alterations in incubation time and/or incubation temperature as well as addition of lysozyme to the resuspension solution were tested. The kit from Zymoclean Research with addition of 15 $\mathrm{mg/mL}$ lysozyme to the P1 buffer and an incubation time of 2 hours resulted in the highest plasmid concentrations. Purified plasmid was digested and run on gel electrophoresis. pXMJ19 samples were digested with NdeI and XbaI and expected fragments were 3868 bp and 2733 bp. pXMJ19-mCherry samples were digested with AhdI and NcoI with expected fragment sizes of 5038 bp and 2252 bp. pVB-4A0E1-mCherry samples were digested with SfoI with expected fragment sizes of 7021 bp and 2172 bp. Upon inspection, the two samples from each vector were similar and contained the expected bands, but they also contained 2-3 extra bands. One sample of each vector was sent for sequencing, but no sequence was obtained.

Another method for confirming recombinant *C. glutamicum* harboring the correct plasmids, was to transform *E. coli* DH5α with the purified plasmid. These transformations resulted in few colonies, and when the plasmids were isolated the concentrations were too low to attempt sequencing.

A third strategy to validate recombinant *C. glutamicum* harboring either pXMJ19-mCherry or pVB-4A0E1-mCherry was to use colony PCR with mCherry primers. If the recombinant *C. glutamicum* on the plate contained the *mCherry* gene as expected, there should be a band at 730 bp on the gel after PCR and electrophoresis. In the first attempts, the negative controls (water sample and wt *C. glutamicum* MB001(DE3)) turned positive with a band present at around 0.7 kb. After several rounds of trouble shooting by replacing the components of the PCR mix and chemicals used one by one, a possible contaminated component was identified as the lysis buffer (CelLytic B Plus (Sigma-Aldrich)). When a new bottle of the buffer was used along with filter tips and reduction of the number of cycles in the PCR program from 30 to 25, the negative controls remained negative while the candidates all had a band present at around 0.7 kb. The procedure for colony PCR is described in Section 2.7.

Figure 3.9 shows a picture of the agarose gel after gel electrophoresis. The lower bands seen on the gel are probably primer-dimers. In all but one sample there is a band present at around 0.7 kb, which is the correct size for *mCherry*. As mentioned above, several rounds of the colony PCR experiment was run. In the last run there were two negative controls, both wt *C. glutamicum* MB001(DE3) (lane 1) and purified plasmid pXMJ19 (lane 2). Neither of these should harbor the *mCherry* gene, but a weak band is seen for the sample pXMJ19 at around 0.7 kb. When comparing the intensities of the bands, there are clear differences. The positive controls, purified plasmids from *E. coli* used for transforming *C. glutamicum*: pXMJ19-mCherry (lane 6) and pVB-4A0E1-mCherry (lane 10), are overexposed due to a large quantity of DNA and are clearly the strongest bands as expected. They also show that the experiment works. pXMJ19-mCherry parallel 4 (lane 5) is clearly stronger than the other parallels for pXMJ19-mCherry from *C. glutamicum*. For pVB-4A0E1-mCherry from *C. glutamicum* both parallel 2 (lane 7) and parallel 3 (lane

8) show strong bands. The samples with the strongest bands for each vector, pXMJ19-mCherry parallel 4 and pVB-4A0E1-mCherry parallel 2, were used for expression experiments since those are the ones most likely to be correct.

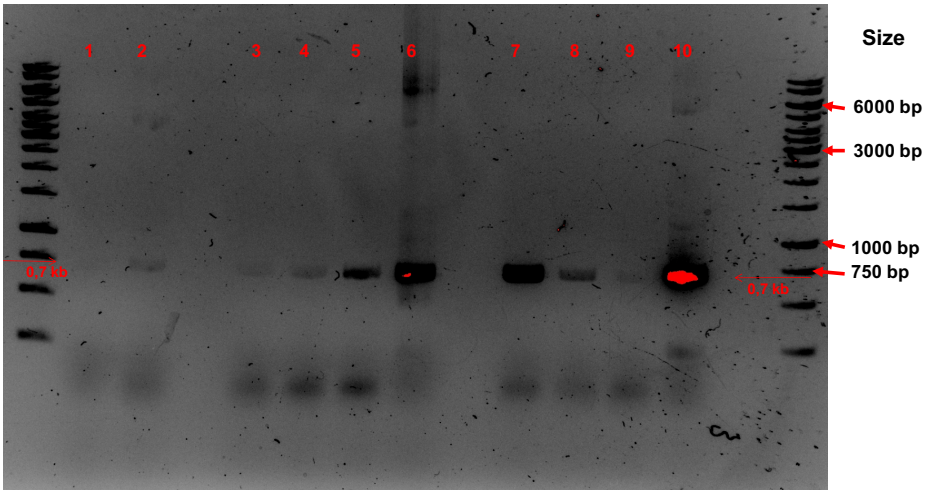


Figure 3.9: Agarose gel after colony PCR and gel electrophoresis pictured by ChemiDoc XRS+ (Bio Rad). The red dots on the bands in lane 6 and 10 are due to overexposure of a large quantity of DNA. Lane 1: wt *C. glutamicum* MB001(DE3) , Lane 2: Purified plasmid pXMJ19, Lane 3: *C. glutamicum* harboring pXMJ19-mCherry (parallel 2), Lane 4: *C. glutamicum* harboring pXMJ19-mCherry (parallel 3), Lane 5: *C. glutamicum* harboring pXMJ19-mCherry (parallel 4) , Lane 6: Positive control: purified plasmid pXMJ19-mCherry from *E. coli* used to transform *C. glutamicum* , Lane 7: *C. glutamicum* harboring pVB-4A0E1-mCherry (parallel 2), Lane 8: *C. glutamicum* harboring pVB-4A0E1-mCherry (parallel 3), Lane 9: *C. glutamicum* harboring pVB-4A0E1-mCherry (parallel 4), Lane 10: Positive control: purified plasmid pVB-4A0E1-mCherry from *E. coli* used to transform *C. gutamicum*. The ladder included on both sides of the gel is the GeneRuler 1kb DNA ladder (Termo Fisher Scientific).

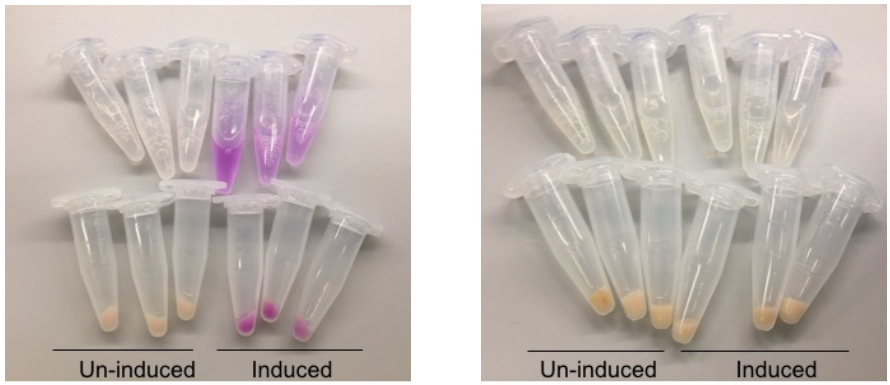
3.5 Expression of *mCherry* in *E. coli* and *C. glutamicum*

To identify if the constructed plasmids were functional in *E. coli* and *C. glutamicum* expression experiments were conducted. For pXMJ19-mCherry, functionality in *E. coli* was established during the project thesis, while functionality in *C. glutamicum* was tested during the work for this thesis. For pVB-4A0E1-mCherry, functionality in both *E. coli* BL21 and *C. glutamicum* MB001(DE3) was assessed. A functional vector would express *mCherry* and, if expressed in large quantities, give the culture a pink color due to the fluorescent properties of the protein.

3.5.1 Production of mCherry in *E. coli* BL21

E. coli BL21 cells were transformed with pVB-4A0E1-mCherry. To test functionality in *E. coli*, an expression experiment was conducted following the protocol described in Section 2.8. Expression of *mCherry* from pVB-4A0E1-mCherry was tested both with and without inducer (2 mM m-toluate). Six parallels were grown, three of them induced. A negative control, that should not display any pink coloration either induced or uninduced, was also included: *E. coli* BL21 harboring pVB-1. The next day, cells were harvested from culture and expressed proteins were separated into soluble and insoluble fractions.

Figure 3.10 (a) shows the presence of pink coloration in both soluble and insoluble fractions in the induced cultures of *E. coli* BL21 harboring pVB-4A0E1-mCherry, compared to the uninduced ones. The presence of pink coloration indicates a functional vector and is visual evidence of production of *mCherry*. As expected, the negative control does not display any color change when induced (Figure 3.10 (b)).



(a) pVB-4A0E1-mCherry **(b)** pVB-1

Figure 3.10: Insoluble (bottom row) and soluble (top row) fractions of produced proteins in *E. coli* BL21 harboring pVB-4A0E1-mCherry and pVB-1.

Even though there is no pink coloration of the uninduced samples of pVB-4A0E1-mCherry there could be mCherry production. A fluorometry analysis is more sensitive than naked eye observations and can confirm and measure production of mCherry. A Tecan fluorometer was used to measure the fluorescence of both fractions of each sample. The results from the analysis for soluble and insoluble protein fractions are given in relative fluorescence unit (RFU) and presented in Figure 3.11 and Figure 3.12 respectively. Production of mCherry from pXMJ19-mCherry in *E. coli* BL21(DE3), both induced with IPTG and uninduced, was measured during the work for the project thesis. In order to be able to compare pXMJ19-mCherry with pVB-4A0E1-mCherry, the results from that experiment are included in Figures 3.11 and 3.12. All measured values are normalized to wet weight of the pellet.

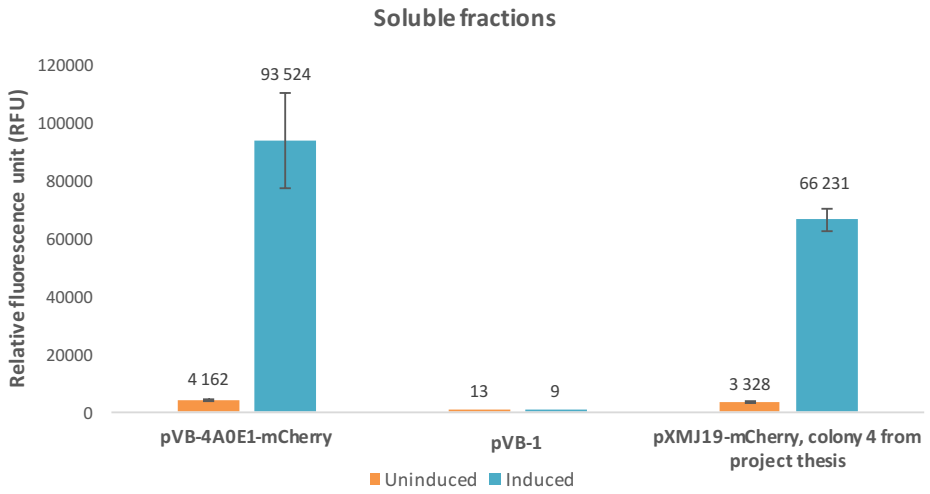


Figure 3.11: mCherry production was measured in *E. coli* BL21 for pVB-4A0E1-mCherry and pVB-1, and *E. coli* BL21(DE3) for pXMJ19-mCherry. Soluble mCherry fractions were measured in RFU by fluorometer (Tecan). Each sample was measured in duplicates. For pVB-4A0E1-mCherry and pVB-1, the value presented here is the average between the three parallels $\pm$ standard deviation. For the pXMJ19-mCherry from the project thesis, the value presented here is the average between the two wells for each sample $\pm$ standard deviation. For pVB-4A0E1-mCherry, each of the three parallels was run in duplicates and each of these duplicate wells was measured 16 times, while for pXMJ19-mCherry, which had only one parallel, each well was measured 12 times.

The fluorometry analysis of the soluble fractions (Figure 3.11) shows that a much higher production of mCherry is measured from the induced samples than uninduced samples for both pVB-4A0E1-mCherry and pXMJ19-mCherry.
For pVB-4A0E1-mCherry, fluorescence measured from uninduced sample is 4162 RFU while from induced sample the measured value is 93524 RFU, which makes fluorescence measured from induced sample about 22.5 times higher than uninduced sample. For pXMJ19-mCherry the measured value for uninduced sample is 3328 RFU while the measured value for induced is 66231 RFU, which makes induced value about 20 times higher than uninduced value. There is close to no fluorescence observed from the negative control, pVB-1, neither from inducer or uninduced sample. When comparing the induced sample of pVB-4A0E1-mCherry to the induced sample of pXMJ19-mCherry, the measured fluorescence from pVB-4A0E1-mCherry is about 1.4 times higher.

The fluorometry analysis of insoluble fractions (Figure 3.12) shows much stronger fluroescence in the induced samples compared to the uninduced ones for both pVB-4A0E1-mCherry and pXMJ19-mCherry. The fluorescence value for induced sample of pVB-4A0E1-mCherry are 50431 RFU while the value for uninduced sample is 1766 RFU, making the fluorescence value measured for induced sample about 28.6 times higher than for uninduced sample. For pXMJ19-mCherry the measured fluorescence for induced sample is 8834 RFU, while the uninduced sample is measured to 301 RFU, making the measured fluroescence in induced sample about 29 times higher than for uninduced sample. When comparing the induced samples of pVB-4A0E1-mCherry and pXMJ19-mCherry the fluorescence measured is about 5.7 times higher for pVB-4A0E1-mCherry. As for the soluble fractions, there is close to no fluorescence observed from the insoluble fractions of the negative control, pVB-1, either when uninduced or induced.

When comparing the soluble and the insoluble fractions, the fluorescence measured is higher in soluble than insoluble for both pVB-4A0E1-mCherry and pXMJ19-mCherry. For pVB-4A0E1-mCherry, the measured fluorescence for induced samples were 1.9 times higher for soluble fractions than insoluble fractions. For pXMJ19-mCherry, the fluorescence values for induced samples were 7.5 times higher for soluble fractions than insoluble fractions. Taken together, the results clearly show that both pVB-4A0E1-mCherry and pXMJ19-mCherry are able to produce mCherry in large amounts when induced, and that most of the protein is found in a soluble state. It also shows that *E. coli* harboring pVB-4A0E1-mCherry produces 5.7 times more insoluble protein than *E. coli* harboring pXMJ19-mCherry, and about 1.4 times more soluble protein, so total production of mCherry (soluble+insoluble) seems higher in pVB-4A0E1-mCherry than pXMJ19-mCherry.

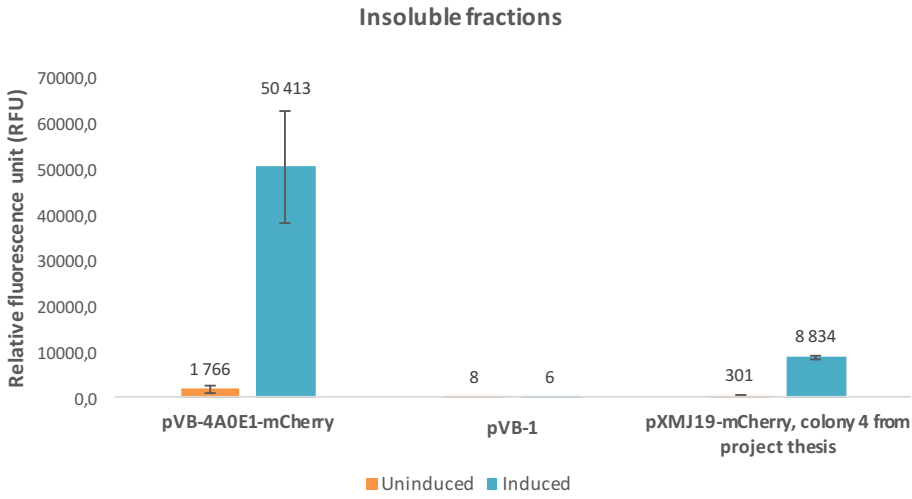


Figure 3.12: mCherry production was measured in *E. coli* BL21 for pVB-4A0E1-mCherry and pVB-1, and *E. coli* BL21(DE3) for pXMJ19-mCherry. Insoluble mCherry fractions were measured RFU by fluorometer (Tecan). Each sample was measured in duplicates. For pVB-4A0E1-mCherry and pVB-1, the value presented here is the average between the three parallels $\pm$ standard deviation. For the pXMJ19-mCherry from the project thesis, the value presented here is the average between the two wells for each sample $\pm$ standard deviation. For pVB-4A0E1-mCherry each of the three parallels was run in duplicates and each of these duplicate wells was measured 16 times, while for pXMJ19-mCherry, which had only one parallel, each well was measured 12 times.

The results show that both vectors pXMJ19-mCherry and pVB-4A0E1-mCherry are functional in *E. coli*. Both vectors are inducible, and *E. coli* BL21 harboring these vectors produces *mCherry* in large amounts after induction.

3.5.2 Production of mCherry in *C. glutamicum* MB001(DE3)

Since both vectors pXMJ19-mCherry and pVB-4A0E1-mCherry were found to be functional in *E. coli*, the next step was to test functionality in *C. glutamicum*. This was performed in the same way as for *E. coli*, with an expression experiment conducted following the protocol described in Section 2.8.

Expression of *mCherry* from the cultures of *C. glutamicum* MB001(DE3) harboring either pXMJ19-mCherry or pVB-4A0E1-mCherry were tested both with and without inducer (1 mM IPTG and 2 mM m-toluate respectively). Two parallels of each recombinant *C. glutamicum* were tested, one of them induced. Two negative controls were also included, *C. glutamicum* MB001(DE3) harboring pXMJ19 and wt *C. glutamicum* MB001(DE3).

The negative control pXMJ19 was added due to the weak band after colony PCR indicating the unexpected presence of *mCherry*. None of the controls were induced. The next day, cells were harvested from the culture and expressed proteins were separated into soluble and insoluble fractions.

Figure 3.13 shows the tubes with harvested pellets. None of these pellets show pink coloration, indicating lack of mCherry production in sufficient amounts to be visible to the naked eye. However, the samples of harvested cells from recombinant *C. glutamicum* are whiter than the harvested cells from untransformed *C. glutamcium*. Wet weight of recombinant *C. glutamicum* pellets are about half the wet weight of wt *C. glutamicum* pellets. This could indicate that harboring the vector is a burden for *C. glutamicum*, causing slower growth. When comparing recombinant *C. glutamicum* to recombinant *E. coli*, the lack of color in *C. glutamicum* cultures indicates that the vectors do not function as well in *C. glutamicum* as they do in *E. coli*.

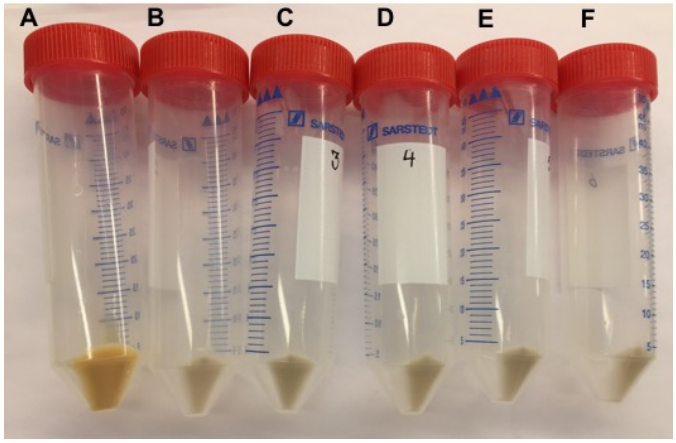


Figure 3.13: Harvested cells after expression of mCherry in *C. glutamicum* MB001(DE3). A: wt *C. glutamicum* MB001(DE3), B: *C. glutamicum* MB001(DE3) harboring pXMJ19, C: *C. glutamicum* MB001(DE3) harboring pVB-4A0E1-mCherry, D: *C. glutamicum* MB001(DE3) harboring pVB-4A0E1-mCherry, induced, E: *C. glutamicum* MB001(DE3) harboring pXMJ19-mCherry, F: *C. glutamicum* MB001(DE3) harboring pXMJ19-mCherry, induced.

To obtain more sensitive and quantified results concerning the mCherry production, fluorometry analysis of both insoluble and soluble fractions were performed. A Tecan flurometer was used to measure RFU of each sample. The results from the analysis for soluble and insoluble protein fractions are presented in Figure 3.14 and Figure 3.15 respectively. All measured values are normalized to wet weight of pellet.

The fluorometry analysis for the soluble protein fractions (Figure 3.14) shows that there is some fluorescence measured in all the samples. The measured fluorescence, 489 RFU, from wt *C. glutamicum* MB001(DE3) represents background fluorescence. For *C. glutamicum* harboring pXMJ19 no expression was expected due to the absence of *mCherry* gene, however, for uninduced sample it was measured to 4069 RFU. *C. glutamicum* harboring pXMJ19 was not induced. For induced sample of pVB-4A0E1-mCherry, the measured value was 4411 RFU, while uninduced sample of pVB-4A0E1-mCherry was 3519 RFU, making the average measured fluorescence from induced sample 1.2 times higher than average fluorescence from uninduced sample. For induced sample of pXMJ19-mCherry the average measured value was 5267 RFU while for uninduced sample of pXMJ19-mCherry the average value was 2857 RFU, making the fluorescence of the induced sample 1.8 times higher in the than uninduced sample. When comparing uninduced samples of pXMJ19, pXMJ19-mCherry and pVB-4A0E1-mCherry the highest average was found for pXMJ19, which was not as expected.

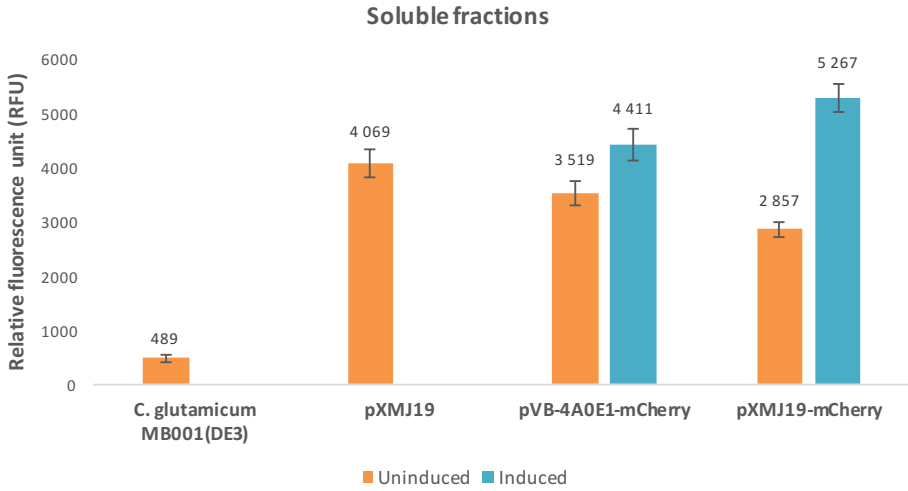


Figure 3.14: Soluble mCherry production in *C. glutamicum* MB001(DE3) measured in RFU by fluorometer (Tecan). Each sample was measured in duplicates. The value for each well was presented as an average between 12 measurements and presented here is the average between the two wells for each sample $\pm$ standard deviation.

For insoluble protein fractions (Figure 3.15), there is also measured some fluorescence from all samples. As for the soluble fractions, the measured fluorescence from wt *C. glutamicum* MB001(DE3) represents background fluorescence. For pXMJ19 uninduced sample, the average value was 7095 RFU, while no fluorescence was expected. For induced sample of pVB-4A0E1-mCherry the average value was 8505 RFU, while the value

for uninduced sample of pVB-4A0E1-mCherry was 11024 RFU, making the average value for induced sample 1.3 times higher than average value for uninduced sample. For induced sample of pXMJ19-mCherry the average fluorescence was found to be 8089 RFU, while uninduced sample of pXMJ19-mCherry was measured to 6217 RFU, making measured value for induced 1.3 times higher than uninduced value. When comparing uninduced samples of pXMJ19, pXMJ19-mCherry and pVB-4A0E1-mCherry, the highest average value was measured for pXMJ19, which was not expected.

For expression of *mCherry* in *C. glutamicum*, the highest measured fluorescence was found in insoluble fractions, rather than soluble fractions which was the case for expression in *E. coli*. For induced sample of pXMJ19-mCherry, fluorescence measured in insoluble fraction was 1.5 times higher than fluorescence measured in soluble fraction. For induced sample of pVB-4A0E1-mCherry, the measured fluorescence in insoluble fractions was 2.5 times higher than fluorescence measured in soluble fraction. When comparing insoluble fraction of pXMJ19 to soluble fraction of pXMJ19, the measured fluorescence value of pXMJ19 was 1.7 times higher in insoluble fraction than soluble fraction.

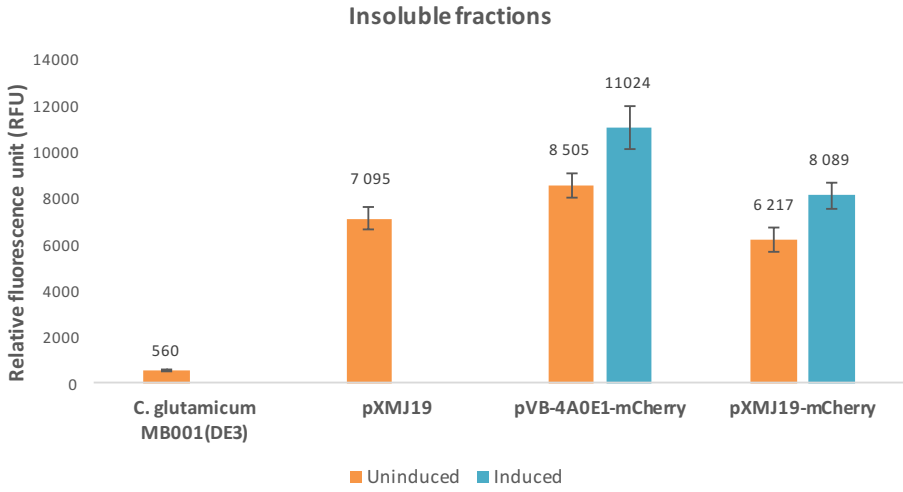


Figure 3.15: Insoluble mCherry production in *C. glutamicum* MB001(DE3) measured in RFU by fluorometer (Tecan). Each sample was measured in duplicates. The value for each well was presented as an average between 12 measurements and presented here is the average between the two wells for each sample ± standard deviation.

Overall, the measured fluorescence values are much lower for expression in *C. glutamicum* compared to *E. coli*. Background fluorescence from wt *E. coli* was unfortunately not measured. *E. coli* harboring pXMJ19 was not measured either. However, *E. coli* harboring pVB-1 resulted in measurements of 13 RFU and 8 RFU for soluble and insoluble

fraction respectively. When comparing those values to the measured background fluorescence from wt *C. glutamicum*, background from *C. glutamicum* is 37 times and 70 times higher for soluble and insoluble fractions respectively. When comparing induced samples of pXMJ19-mCherry in *E. coli* and *C. glutamicum* in soluble fraction, the fluorescence measured for *E. coli* is 12.6 times higher. When comparing insoluble fractions of induced *E. coli* and *C. glutamicum* harboring pXMJ19-mCherry, measured fluorescence is 1.1 times higher in *E. coli*. Insoluble fractions of induced *E. coli* and *C. glutamicum* harboring pVB-4A0E1-mCherry show that measured values are 14.3 times higher in *E. coli*. For pVB-4A0E1-mCherry in induced soluble fractions, the measured values are 22.6 times higher in *E. coli* compared to *C. glutamicum*.

The fluorometry analysis of *C. glutamicum* could indicate that all measured fluorescence from recombinant *C. glutamicum* are variations of background fluorescence. The lysis troubles of *C. glutamicum* could strongly influence the results. Fluorometry analysis together with the lack of visual pink coloration in the culture shows that the vectors do not properly function in *C. glutamicum*. Further studies to investigate the functionality of the vectors in *C. glutamicum* were needed, and the first step was to investigate *mCherry* and *xylS* transcript levels.

3.6 Evaluation of *mCherry* and *xylS* Transcript Levels in *E. coli* and *C. glutamicum*

Evaluation of mCherry production by fluorometry analysis in *C. glutamicum* showed that mCherry could not be identified produced from either of the vectors pXMJ19-mCherry and pVB-4A0E1-mCherry. Another method for investigating functionality is evaluating the transcript levels of *mCherry* in both vectors.

For *C. glutamicum* harboring pVB-4A0E1-mCherry, induction did not seem to have a strong influence on the production of mCherry. A hypothesis for the low production values was that low transcript levels of *xylS* prevented efficient expression of *mCherry* from the XylS/*Pm* expression cassette. To investigate this, the transcript levels of *xylS* were evaluated.

For both vectors, evaluation of transcript levels of *xylS* and *mCherry* were investigated. Both *E. coli* and *C. glutamicum* harboring the vectors pXMJ19, pXMJ19-mCherry and pVB-4A0E1-mCherry as well as wt *E. coli* BL21 and wt *C. glutamicum* MB001(DE3) were grown according to the procedure described in Section 2.9.1. No *xylS* was expected from the vectors pXMJ19-mCherry or pXMJ19, but they were both included as controls in the first round, while the second run of the experiment only used pXMJ19-mCherry as control for *xylS* transcript.

Figure 3.16 clearly illustrates the difference between production of mCherry in *E. coli* and *C. glutamicum*. The pink cultures are induced *E. coli* harboring pXMJ19-mCherry and pVB-4A0E1-mCherry respectively. While the other cultures are induced *C. glutamicum* harboring pXMJ19-mCherry and pVB-4A0E1-mCherry respectively.

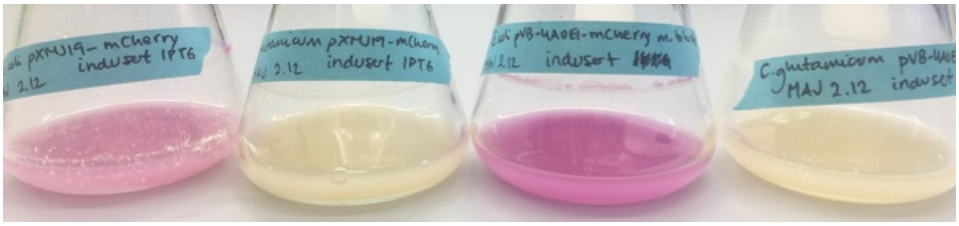


Figure 3.16: The cultures used for isolation of RNA illustrates clear differences of production of mCherry from *E. coli* and *C. glutamicum* harboring the vectors pXMJ19-mCherry and pVB-4A0E1-mCherry. From the left: *E. coli* harboring pXMJ19-mCherry, *C. glutamicum* harboring pXMJ19-mCherry, *E. coli* harboring pVB-4A0E1-mCherry, *C. glutamicum* harboring pVB-4A0E1-mCherry. All cultures are induced.

3.6.1 Evaluating RNA Quality

RNA from *E. coli* and *C. glutamicum* was purified and the quality tested using Bioanalyzer (Agilent Technologies) following description in Section 2.9.2. The results from the Bioanalyzer software showed that all samples, *E. coli* and *C. glutamicum* harboring vectors both induced and uninduced, contained RNA. However, the RIN calculated for *C. glutamicum* were generally lower than for *E. coli*. Calculated concentrations and RIN values are presented in Appendix E, as well as the electropherogram from each sample.

Since all samples contained some RNA, single-stranded cDNA was produced from mRNA samples. The cDNA samples were then used for the following ddPCR experiments. The cDNA samples were quantified using Nanodrop set to ssDNA. Even though RNA concentrations varied a lot, the cDNA concentrations were similar. However, Nanodrop could give unreliable results, as the sample contains a mixture of cDNA, RNA and nucleotides. The difficulties with lysing the *C. glutamicum* cells could also influence the RNA samples, and therefore also the results from the ddPCR.

3.6.2 Quantification of *mCherry* Transcript Level in *E. coli* and *C. glutamicum*

Droplet digital PCR (ddPCR) is a method for performing digital PCR based on water-oil emulsion. It is used for absolute quantification of nucleic acids in a sample [8]. Using the ddPCR method, following description in Section 2.9.4, quantification of *mCherry* transcript levels in *E. coli* BL21 and *C. glutamicum* harboring pXMJ19-mCherry and pVB-4A0E1-mCherry was obtained. For all samples, the experiment was executed twice due to some deviations in handling the samples in the first experiment and to get concentrations the software could work with to yield a value for mRNA [copies/µL]. The results from the second experiment are presented in Figure 3.17, while the results from the first experiment is included in Table F.1 in Appendix F.

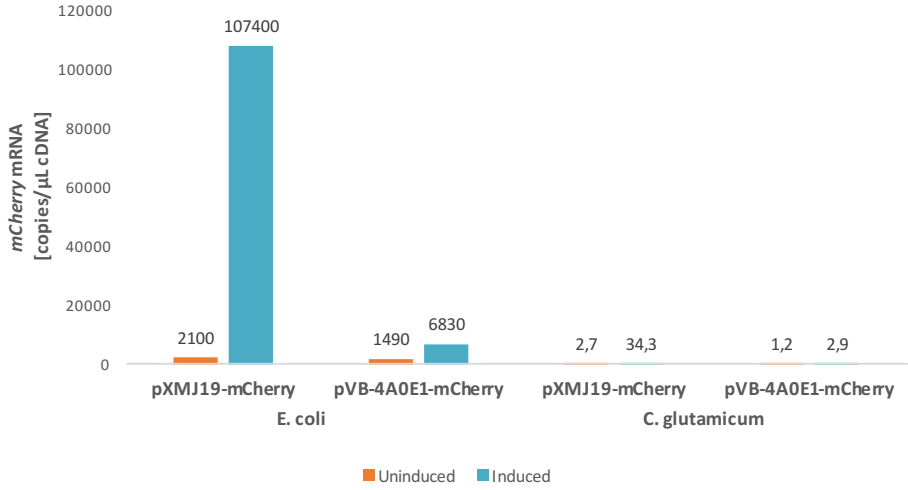


Figure 3.17: Quantification of *mCherry* transcript levels in copies/µL cDNA from *E. coli* BL21 and *C. glutamicum* MB001(DE3) harboring pXMJ19-mCherry and pVB-4A0E1-mCherry.

Evaluation of transcript levels of *mCherry* in *C. glutamicum* were performed to evaluate functionality of the vectors. *E. coli* samples were added for comparison.

The results from ddPCR indicate that a higher level of *mCherry* mRNA is expressed from *E. coli* BL21 harboring pXMJ19-mCherry and pVB-4A0E1-mCherry respectively when the cultures are induced compared to when cultures are uninduced. When comparing uninduced and induced *E. coli* harboring pXMJ19-mCherry the value obtained from induced sample is 51 times higher than uninduced sample. When comparing uninduced and induced *E. coli* harboring pVB-4A0E1-mCherry, the value for induced sample is 4.6 times higher than uninduced sample. For *C. glutamicum* harboring pXMJ19-mCherry, the level of *mCherry* transcript in induced sample is 12 times higher compared to uninduced sample. When comparing induced sample of *C. glutamicum* harboring pVB-4A0E1-mCherry to uninduced sample of *C. glutamicum* harboring pVB-4A0E1-mCherry, the value for induced is 2.4 times higher than uninduced, however, considering the sensitivity of the process, both values are not significant enough to prove the detection of *mCherry* transcripts. Comparison of induced samples of *E. coli* and *C. glutamicum* harboring pXMJ19-mCherry shows the level of *mCherry* transcript is 3131 times higher in *E. coli*.

The results show transcript of *mCherry* in *E. coli* harboring pXMJ19-mCherry as well as in *E. coli* harboring pVB-4A0E1-mCherry. For *C. glutamicum* harboring pXMJ19-mCherry the transcript level of *mCherry* was low, while for *C. glutamicum* haboring pVB-4A0E1-mCherry no transcript of *mCherry* was detected. The transcript levels of *mCherry* in *C. glutamicum* explain why there is no pink coloration seen in the cultures of *C. glutamicum* harboring expression vectors. For pVB-4A0E1-mCherry, expression levels of *mCherry* could be influenced by expression level of *xylS*, which is why they were also evaluated.

3.6.3 Quantification of *xylS* Transcript Level in *E. coli* and *C. glutamicum*

As for the quantification of *mCherry*, the quantification of *xylS* in *E. coli* BL21 and *C. glutamicum* harboring either pXMJ19-mCherry or pVB-4A0E1-mCherry was obtained using the ddPCR method, following description in Section 2.9.4. The full experiment was executed twice, due to some deviations in handling the samples in the first experiment and to get concentrations the software could work with to yield a value for mRNA [copies/µL]. The results from the second experiment are presented in Figure 3.18, while the results from the first experiment are included in Table F.1 in Appendix F.

The ddPCR gives an absolute quantification of the *xylS* transcript levels. For *E. coli* and *C. glutamicum* harboring pXMJ19-mCherry, no transcript of *xylS* was expected due to no *xylS* gene in the sample. It was included as a control of background transcript levels. For both *E. coli* and *C. glutamicum* harboring pXMJ19-mCherry, the background of the induced sample and the uninduced sample are similar, which was expected and indicates a functional experiment.

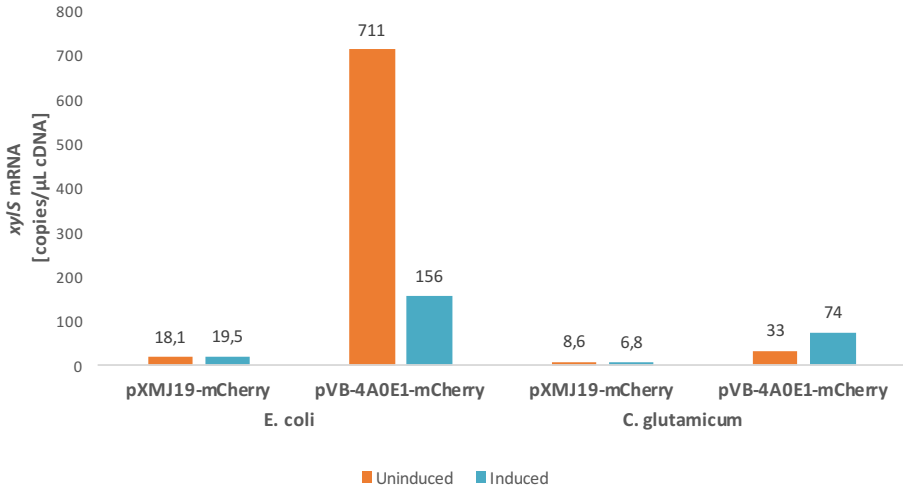


Figure 3.18: Quantification of *xylS* in copies/µL cDNA transcript levels from *E. coli* BL21 and *C. glutamicum* MB001(DE3) harboring pXMJ19-mCherry and pVB-4A0E1-mCherry.

The results show that a higher amount of transcript, 21.5 times higher for uninduced sample and 2.1 times higher for induced sample, is found in *E. coli* compared to *C. glutamicum*. It is also shown that inducer (*m*-toluate) has an influence on *xylS* transcription. When comparing induced *E. coli* habroring pVB-4A0E1-mCherry with uninduced *E. coli* harboring pVB-4A0E1-mCherry in, *xylS* transcript level is 15 times higher in uninduced sample. For *C. glutamicum*, comparison of induced and uninduced pVB-4A0E1-mCherry shows that a double amount of *xylS* transcripts is found in the induced sample compared to the uninduced sample.

3.6.4 Relation between *xylS* and *mCherry* transcription levels?

The vector pVB-4A0E1-mCherry contains the expression cassette XylS/*Pm*. As explained in the introduction, transcription from *Pm* is stimulated by activated transcription factor XylS, and the gene of interest downstream of *Pm* will be expressed. XylS becomes activated when it is bound to inducer (*m*-toluate) and dimerized with another XylS/inducer complex. The expression of XylS should be constitutive and low [12]. However, the results do not show a stable amount of *xylS* transcript in either *E. coli* or *C. glutamicum*.

The experiment was performed twice, and both runs show the same trend, that *xylS* transcript levels varies. As mentioned, for *E. coli* harboring pVB-4A0E1-mCherry, the transcript level of *xylS* decreases when inducer is added while the transcript level of *mCherry* from the *Pm* promoter increases. Comparing induced and uninduced samples of *C. glutamicum* harboring pVB-4A0E1-mCherry, the transcript level of *xylS* increases when induced. It was not found any *mCherry* transcript in *C. glutamicum* harboring pVB-4A0E1-mCherry, which indicates that the amount of *xylS* observed here does not effect the amount of *mCherry* transcript and that there are troubles with *mCherry* transcription in *C. glutamicum*.

From the current reuslts, no obvious correlation can be made between *xylS* and *mCherry* transcript levels.

Chapter 4

Discussion

4.1 Shorter Generation Time at 37°C than at 30°C for *C. glutamicum*

Growth studies of bacteria are an important part of getting to know the species. A fast-growing bacterium is beneficial as it is usually a clue that the bacterium is thriving and it will save time during lab work, so finding its optimal growth conditions is very useful.

For *C. glutamicum*, DSMZ (a biological resource center where the *C. glutamicum* MB001(DE3) was obtained) recommends an incubation temperature of 28°C, while other literature suggests 30°C [22, 34]. Growth studies performed for the project thesis showed that incubation at 37°C, chosen for convenience in the lab, resulted in shorter generation time than for 28°C when *C. glutamicum* was grown in Trypticase Soy Broth (TSB). For this thesis, growth studies were performed for two strains of *C. glutamicum*, *C.glutamicum* ATCC 13032 and *C. glutamicum* MB001(DE3), because it was not known which strain was better suited to electroporate and work with. When grown in BHIS, both species had generations times that were 6 minutes shorter for incubation at 37°C compared to 30°C. The results also showed that the generation time for *C. glutamicum* at 37°C when grown BHIS was shorter than when grown in TSB. Both media are nutrient rich, but different components could cause the differences. Because these conditions allowed for the fastest generation time, incubation at 37°C was performed and BHIS was used as standard medium throughout this study.

4.2 Cell Death Probably caused by *m*-toluate toxicity

The main aim of this thesis was to evaluate if recombinant proteins could be expressed from *C. glutamicum* harboring expression vectors with the inducible XylS/*Pm* expression cassette. In order to induce the expression cassette, the inducer (*m*-toluate) has to be able to enter the cells. It is known that *m*-toluate enters the *E. coli* cell by passive diffusion

[26], and that high concentrations of m-toluic acid are toxic to the cell [21]. To investigate if *m*-toluate is able to enter the *C. glutamicum* cells an inducer diffusion study was performed, where *E. coli* served as a control. Results showed that cell death was observed for concentrations of 8 mM *m*-toluate and above, for *E. coli* as well as both strains of *C. glutamicum*. For both media, LB and BHIS, the pH was measured for *m*-toluate concentrations of 0, 1, 10 and 30 mM. Results showed that between 1 and 10 mM the pH was relatively stable. From this, it is reasonable to say that a change in pH most likely did not cause cell death, and that cell death probably was a result of a toxic amount of *m*-toluic acid entering the cells. Since the pH measurements were conducted after the inducer diffusion experiment, another diffusion experiment with pH-adjusted media for each concentration should be performed to completely eliminate the cell death as a function of pH. It could also be interesting to investigate how a change in pH affects the growth of the bacteria. The similarities in results for both species show that *m*-toluate most likely is able to enter the cells of both *E. coli* and *C. glutamicum* by passive diffiusion. If this had not been the case, testing the XylS/*Pm* expression cassette in *C. glutamicum* would have been useless.

4.3 Difficulties with Constructing and Validating Recombinant *C. glutamicum*

For production of recombinant proteins in *C. glutamicum*, two *C. glutamicum*/*E. coli* shuttle vector were constructed. There were, however, several attempts, revised plans and adjusted protocols behind the successfully constructed vectors pXMJ19-mCherry and pVB-4A0E1-mCherry and the successful transformation of *C. glutamicum*.

Already when constructing the vector pXMJ19-mCherry during the work for the project thesis, there were several issues, such as unexpected patterns observed after gel electrophoresis when digested with restriction enzymes. These issues indicated that the pXMJ19 plasmid map was incorrect. Since both constructed vectors, as well as the unsuccessful attempts at replacing *mCherry* with *mCherry* codon-opitmized for *C. glutamicum* in the vectors, were based on pXMJ19, the incorrect plasmid map probably caused unexpected, extensive amount of cloning troubles.

C. glutamicum is a Gram-positive bacterium, which means that the cell wall of *C. glutamicum* consists of a thicker layer of peptidoglycans than *E. coli*. This makes it harder to lyse. Due to inexperience with lysing *C. glutamicum*, several methods had to be tested. The methods used in this thesis were the ones available in the lab. Since none of the methods used seem optimal, other methods for lysing *C. glutamicum* cells should be tested in further studies. A suggestion is using glass beads: cells and glass beads are placed in a bead mill where the shaken beads disrupt the cells [74]. This was not attempted during the work for this thesis due to lack of equipment. Using ultrasound for lysis of *C. glutamicum* cells was attempted. This was time-consuming and only tested in connection with protein extraction. The extensive duration for the treatment of each sample was the reason this method was not used for plasmid isolation. Miniprep-kits (Promega and Zymoclean Research) with altered protocols, such as addition of lysozyme to resuspension

buffer/P1-buffer and prolonged incubation at higher temperature, were used instead.

The lysis troubles resulted in very low plasmid concentrations when plasmid isolation was performed. Along with unexpected patterns when digested with restriction enzymes, this made it difficult to validate the recombinant *C. glutamicum*. A validation method that did work was colony PCR using *mCherry* primers. Since PCR is a powerful tool and small amounts of template result in a product, there was a challenge with *mCherry* product present for negative samples. Adjustments were made to decrease the amount of contamination, such as using new components and decreasing amount of cycles. However, the contamination could have happened at any step during preparation of the PCR mix, since it was not performed in a template-free room. It could also be that the primers were not specific enough. Finally, it was shown that the vectors pXMJ19-mCherry and pVB-4A0E1-mCherry were successfully transferred to *C. glutamicum*.

4.4 Constructed Vectors Are Expressing High Amounts of *mCherry* in *E. coli* but Not in *C. glutamicum*

Two *C. glutamicum*/*E. coli* shuttle vectors were successfully constructed. To evaluate the functionality of these vectors, expression experiments for both *E. coli* and *C. glutamicum* were performed. Both visual pink coloration of the culture and fluorescence measurements were evaluated.

Both vectors, pXMJ19-mCherry and pVB-4A0E1-mCherry, were found to be functional in *E. coli*. When induced with IPTG and *m*-toluate respectively, the cultures were clearly pink so confirmation of functionality could be made by the naked eye. Neither *C. glutamicum* strains harboring either pXMJ19-mCherry or pVB-4A0E1-mCherry yielded pink cultures when induced with IPTG or *m*-toluate respectively. However, there could be mCherry production even though the pink color was not detected by simply looking at the samples.

A fluorometry analysis is more sensitive than the naked eye, and was performed to confirm and quantify mCherry production. For *E. coli*, fluorometry analysis confirmed the mCherry production. Most mCherry proteins were found in a soluble state, and induction increased RFU values over 20 times compared to uninduced samples. It was also shown that total mCherry production was higher in *E. coli* harboring pVB-4A0E1-mCherry compared to *E. coli* harboring pXMJ19-mCherry. However, since these measurements were conducted at different times and in different strains, it is not ideal to compare the results directly and the experiment should be repeated growing all strains to be compared in the same experiment.

For *C. glutamicum* fluorescence was detected for all vectors, including the negative control *C. glutamicum* harboring pXMJ19. When comparing the results obtained from the induced soluble *C. glutamicum* fractions with the induced soluble *E. coli* fractions, the measured fluoresence for *C. glutamicum* harboring pXMJ19-mCherry is 12.6 times lower than for *E. coli*, while the measured fluroesence from *C. glutamicum* harboring pVB-4A0E1-mCherry is 22.6 times lower than for *E. coli*. The reason for positive fluorescence mea-

surements from *C. glutamicum* harboring pXMJ19 is unknown, but one explanation could be that all fluorescence measurements of *C. glutamicum* are actually only background fluoresence.

The vector pXMJ19-mCherry should be able to function in *C. glutamicum* since the plasmid pXMJ19 is constructed as a *C. glutamicum*/*E. coli* shuttle vector [35], and the *mCherry* gene is only added downstream of the *tac*-promoter using restriction sites, therefore reducing the risk of mutation occuring compared to cloning via PCR. However, as mentioned earlier, there were indications that the plasmid map for pXMJ19 was incorrect. This may have caused a sub-optimal placement of the *mCherry* gene for functioning in *C. glutamicum*, relative to the promoter and the 5′-UTR. It is not the most likely explanation since it works fine for *E. coli*. Another factor that could explain the absence of production, is that the version of *mCherry* inserted (optimized for *E. coli*) might not be easily translated in *C. glutamicum*. There were attempts at constructing both vectors with *mCherry* codon-optimized for *C. glutamicum*, but all attempts unfortunately failed. Therefore there can be drawn no conclusion as to whether codon-optimization of *mCherry* would enhance the mCherry production in *C. glutamicum*. However, as shown later, codon-optimized *mCherry* would not solve all the problems, since low amount of *mCherry* transcripts were observed.

For pVB-4A0E1-mCherry, the expression cassette XylS/*Pm* could be the reason why the vector does not function in *C. glutamicum*. The problems with production of mCherry from *C. glutamicum* harboring pVB-4A0E1-mCherry could be caused by too low expression of XylS due to the *xylS* promoter being inefficient in *C. glutamicum*. Previously, no Gram-positive bacteria have been shown to express proteins from the native XylS/*Pm* expression cassette. However, with some alterations, it has been shown that the expression cassette could be made functional in the non-Gram-negative bacterium *Mycobacterium smegmatis* [21]. It was shown that changing the promoter driving the *xylS* expression sometimes resulted in functional XylS/*Pm* (unpublished data). To investigate if an altered version of the expression cassette for *C. glutamicum* is needed, transcription levels of *xylS* were evaluated. The transcript levels of *mCherry* were also evaluated, to figure out if the bottleneck was at *xylS* or *mCherry* level and also if transcription or translation of these proteins was the main issue.

4.5 High Levels of *mCherry* Transcript in *E. coli* and Low Levels in *C. glutamicum*

To further investigate why no mCherry production could be detected in *C. glutamicum*, the transcript levels of *mCherry* and *xylS* were evaluated and compared in samples of *E. coli* and *C. glutamicum* harboring pXMJ19-mCherry and pVB-4A0E1-mCherry respectively.

RNA samples were purified from both *E. coli* and *C. glutamicum*, and the concentrations obtained from *C. glutamicum* were much lower than from *E. coli*. The lysis issue could potentially have affected concentration and quality of the RNA purified from *C. glutamicum*. RNA quality was tested using Bioanalyzer, and RIN values and concentrations were very

variable (or too low to calculated). However, upon visual inspection of the electropherograms in Appendix E, there were peaks at 16S and 23S for all samples. Old, previously purified gel was used when loading the Bioanlyzer chip, which could have caused the extensive amount of background noise seen in the electropherograms and made it difficult for the software to identify the peaks needed to calculated RIN values. Better quality RNA may be obtained from *C. gltuamicum* through better lysis protocols, and a freshly prepared gel would reduced background noise and help the software to find the peaks.

RNA samples were used to generate cDNA, which were used for evaluation of *mCherry* and *xylS* transcript levels using ddPCR. ddPCR is a method for absolute quantification of nucleic acids in a sample. The ddPCR was performed twice due to irregilarites with handling the samples in the first run. Both experiments show the same trends. There were shown to be high amounts of *mCherry* transcripts in both *E. coli* harboring pXMJ19-mCherry and *E. coli* harboring pVB-4A0E1-mCherry. Both vectors were shown to be functional in *E. coli*, yielding pink coloration of the cultures. It is also shown in the results from the ddPCR, from induced *E. coli* harboring pXMJ19-mCherry and *E. coli* harboring pVB-4A0E1-mCherry, that the amount of *mCherry* transcripts increases over 51 times and 4.5 times respectively compared to uninduced samples.

For *C. glutamicum* harboring pXMJ19-mCherry, induction increased *mCherry* transcript level 12 times compared to uninduced sample. This shows that the expression from this vector seems inducible also in *C. glutamicum*. The presence of *mCherry* mRNA adds to the conclusion that the recombinant *C. glutamicum* harbors the correct vector. However, comparing the transcript level of *mCherry* from induced *C. glutamicum* harboring pXMJ19-mCherry with induced *E. coli* harboring pXMJ19-mCherry, shows that the amount of transcripts obtained from *E. coli* is 3131 times higher than in *C. glutamicum*. From *C. glutamicum* harboring pVB-4A0E1-mCherry no significant values were obtained to prove detection of *mCherry* transcripts. *E. coli* and *C. glutamicum* harboring pXMJ19 were only included in the first round of ddPCR as negative controls. Nevertheless, there were some *mCherry* transcripts detected in *E. coli*. However, compared to the *mCherry* transcript level detected from *E. coli* harboring pXMJ19-mCherry, the *mCherry* transcript level from pXMJ19 was 168 times lower, suggesting that transcripts detected in the pXMJ19 sample were, in fact, unspecific and negligible amplification. This gives an idea of the sensitivity limits of ddPCR in this experiment. Interestingly, no *mCherry* transcript was detected from *C. glutamicum* harboring pXMJ19. This all adds to the conclusion that all fluorescence measured in *C. glutamicum* were variations of background, and that neither of the vectors harboring *mCherry* are functional in *C. glutamicum* at this point.

Several factors could explain why no *mCherry* transcript was detected in *C. glutamicum*: expression of *xylS* might be too low, XylS translation could be inefficient, the sigma (σ) factor and/or RNA polymerase of *C. glutamicum* might not be able to recognize the conserved and *E. coli*-optimized regions of the *Pm* promoter or the activated XylS might not be able to properly interact with the *Pm* promoter.

4.6 *xylS* Transcript Levels Are Affected by Induction

The expression levels of genes controlled by *Pm* depends on the amount of activated XylS [81]. Too low production of XylS will lead to no or little expression of *mCherry* gene from the *Pm* promoter. Therefore, the amount of *xylS* mRNA was investigated for both *E. coli* and *C. glutamicum* harboring pVB-4A0E1-mCherry to find out if low *xylS* expression was the cause of negligible *mCherry* transcription in *C. glutamicum*. Different bacteria use different σ factors, and the difference in *xylS* transcript levels could be due to the adaption of *xylS* promoter to a σ factor present in *E. coli* but absent or down-regulated in *C. glutamicum*. The expression of *xylS* is controlled by the *Ps2* promoter, and the ability of the transcription factors to recognize the conserved sequence of *Ps2* will affect the expression of *xylS* mRNA. An interesting approach would be to modify the promoter for *xylS*. Changing *Ps2* to a mycobacterial promoter resulted in expression from the XylS/*Pm* expression cassette in Mycobacteria [21]. This shows that use of the XylS/*Pm* system can be expanded to non-Gram-negative species. Changing the *Ps2* promoter to a strong promoter originating from *C. glutamicum* could result in sufficient production of XylS to induce expression of *mCherry* in *C. glutamicum*.

For *E. coli* harboring pVB-4A0E1-mCherry, the presence of inducer resulted in less *xylS* transcript. This effect was previously seen in other studies [31](Vectron Biosolutions unpublished). According to literature, *Ps2* is a weak constitutive promoter [12], therefore we do not expect *xylS* transcript level to change upon presence of *m*-toluate. Surprisingly, the results presented here seem to suggest some kind of feedback loop. Conversely, the addition of *m*-toluate seems to increase the amount of *xylS* transcripts in *C. glutamicum*. However, when comparing the level of *xylS* transcripts in *E. coli* and *C. glutamicum*, the induced samples from *E. coli* have an amount of transcripts that is 21.5 times the amount of transcripts from induced *C. glutamicum*, and for uninduced the amount in *E. coli* is 2.1 times the amount in *C. glutamicum*. Studies show that activity of constitutive promoters can be influenced by growth rate [44, 45]. A possibility would then be that induction changes the growth rate, which in turn influences the copy number and transcription rate. If expression from the constitutive promoter *Ps2* is also affected by growth rate, a change in the growth rate caused by induction could result in the observed effect on *xylS* transcript levels. However, for this experiment all samples were harvested when OD_{600} ranged from 1.2 to 1.6, so growth should not have affected the results.

Chapter 5

Conclusion

There could be several advantages to using *C. glutamicum* as an expression host for recombinant protein production. One advantage is *C. glutamicum*'s ability to secrete proteins into the growth medium, which makes downstream processes easier and more effective. *C. glutamicum* also has a different intercellular environment which could result in soluble protein expression when *E. coli* fails. The aim of this study was, therefore, to test and adapt the XylS/*Pm* expression cassette, to the production of recombinant proteins in *C. glutamicum*.

Two *C. glutamicum*/*E. coli* shuttle vectors, pXMJ19-mCherry and pVB-4A0E1-mCherry, have been constructed. Both of these vectors express *mCherry* in *E. coli*, yielding pink cultures when induced with IPTG or m-toluate respectively. For both of them, most of the mCherry protein was found in a soluble state.

Both vectors have been successfully transferred to *C. glutamicum*. However, no production of the mCherry protein was detected.

The transcript levels of *mCherry* and *xylS* were evaluated for *E. coli* and *C. glutamicum* harboring the vectors. For *C. glutamicum* harboring pXMJ19-mCherry, *mCherry* transcripts could be identified and transcript levels increased 12 times when the culture was induced with IPTG. For *C. glutamicum* harboring pVB-4A0E1-mCherry, no *mCherry* transcript could be identified. From this it could not be confirmed that *mCherry* is a suitable reporter gene for *C. glutamicum.* Results also showed that when compared to what was measured in *E. coli*, the amount of *xylS* transcripts identified in *C. glutamicum* was very low. This could explain why no mCherry production was observed in *C. glutamicum* harboring pVB-4A0E1-mCherry.

In conclusion, *xylS* transcription has been identified as a bottleneck for protein expression from the XylS/*Pm* expression cassette in *C. glutamicum*. The work presented here provides a solid basis for further research on adapting Vectron Biosolutions' system to *C. glutamicum*.

Chapter 6

Further work

In the continuation of establishing *C. glutamicum* as an expression host for recombinant proteins, several issues would need to be addressed. First and foremost, one needs to establish expression of a suitable reporter gene from *C. glutamicum*.

For starters, one should try to confirm expression of *mCherry* from pXMJ19. Due to troubles with the pXMJ19 plasmid map, the pXMJ19 plasmid needs to be sequenced for further work involving this vector. Knowing where the theoretical restriction sites are is important in planning cloning work, and to avoid some of the cloning problems seen in this thesis. When a new plasmid map is obtained, the cloning of *mCherry* into the vector should be repeated to make sure the gene is placed in the optimal position relative to the promoter and the 5'-UTR. The 5'-UTR is influencing translation, and has also, in some cases, been shown to influence transcription, so an optimal placement is necessary. If the *mCherry* codon-optimized for *C. glutamicum* is cloned into pXMJ19 as well, comparison in production of mCherry between codon-optimized *mCherry* for *C. glutamicum* and regular (*E. coli* optimized) *mCherry* could be made. Codon-optimization is expected to increase translation efficiency if *mCherry* is inserted in an optimal position and is functional in *C. glutamicum*. If there is still no production of mCherry in *C. glutamicum*, an alternative reporter gene should be tested. One could also change the shuttle vector.

If, after the adjustments, expression experiments show expression of the reporter gene, it could be cloned into a vector harboring the XylS/*Pm* expression cassette where the *Ps2* promoter for *xylS* has been changed to a native *C. glutamicum* promoter. The new promoter could result in higher production of XylS, as was seen in other studies, which in turn could result in increased expression of the reporter gene. Transcript levels of *xylS* could be tested, as it was done in this study, for confirmation of functionality of the new promoter. New expression experiments should follow, to see if the altered expression cassette works better.

Another major cause of troubles in this thesis was the problems with lysing *C. glutamicum*. For further work with *C. glutamicum*, new methods for lysing should be explored. Better lysis methods would yield higher and more reliable plasmid concentrations making it easier to validate recombinant *C. glutamicum*.

References

1. Altenbuchner, J. & Mattes, R. in *Production of recombinant proteins : novel microbial and eukaryotic expression systems* (ed Gellissen, G.) 7–49 (Wiley-VCH, 2005).
2. Aylward, G. H. & Findlay, T. J. V. *SI Chemical Data* 224 (John Wiley & Sons Australia, 2008).
3. Baeshen, N. A. *et al.* Cell factories for insulin production. *Microbial Cell Factories* **13,** 141 (Dec. 2014).
4. 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. *Applied and Environmental Microbiology* **79,** 6006–6015 (Oct. 2013).
5. Becker, J. & Wittmann, C. *Systems and synthetic metabolic engineering for amino acid production - the heartbeat of industrial strain development* Oct. 2012.
6. Bergkessel, M. & Guthrie, C. Colony PCR. *Methods in Enzymology* **529,** 299–309 (2013).
7. Bergmans, H. E. N., Van Die, I. M. & Hoekstra, W. P. M. Transformation in Escherichia coli: stages in the process. *Journal of Bacteriology* **146,** 564–570 (1981).
8. BIO-Rad. *Droplet Digital PCR Applications Guide* 2014.
9. Birnboim, H. C. & Doly, J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic acids research* **7,** 1513–23 (Nov. 1979).
10. Blatny, J. M., Brautaset, T., Winther-Larsen, H. C., Karunakaran, P. & Valla, S. Improved broad-host-range RK2 vectors useful for high and low regulated gene expression levels in gram-negative bacteria. *Plasmid* **38,** 35–51 (July 1997).
11. Blatny, J. M. *et al.* Construction and Use of a Versatile Set of Broad-Host-Range Cloning and Expression Vectors Based on the RK2 Replicon. *Applied and Environmental Microbiology* **63,** 370–379 (1997).
12. Brautaset, T., Lale, R. & Valla, S. Positively regulated bacterial expression systems. *Microbial Biotechnology* **2,** 15–30 (2009).
13. Brown, T. *Gene cloning and DNA analysis: an introduction* 386 (Blackwell Pub, 2010).
14. Caspi, R., Helinski, D. R., Pacek, M. & Konieczny, I. Interactions of DnaA proteins from distantly related bacteria with the replication origin of the broad host range plasmid RK2. *Journal of Biological Chemistry* **275,** 18454–18461 (June 2000).

15. Clark, D. P. & Pazdernik, N. J. *Biotechnology : academic cell update* 750 (Academic Cell Press, 2012).
16. Cohen, S. N., Chang, A. C. Y., Boyer, H. W. & Helling, R. B. Construction of Biologically Functional Bacterial Plasmids In Vitro. *Proceedings of the National Academy of Sciences* **70,** 3240–3244 (1973).
17. Cohen, S. N., Chang, A. C. Y. & Hsu, L. Nonchromosomal Antibiotic Resistance in Bacteria: Genetic Transformation of Escherichia coli by R-Factor DNA*(CaCI2 / extrachromosomal DNA / plasmid). **69,** 2110–2114 (1972).
18. Dagert, M. & Ehrlich, S. D. Prolonged incubation in calcium chloride improves the competence of Escherichia coli cells. *Gene* **6,** 23–38 (1979).
19. Date, M., Itaya, H., Matsui, H. & Kikuchi, Y. Secretion of human epidermal growth factor by Corynebacterium glutamicum. *Letters in Applied Microbiology* **42,** 66–70 (Jan. 2006).
20. Davidson, M. W. & Campbell, R. E. Engineered fluorescent proteins: innovations and applications. *Nature Methods* **6,** 713–717 (Oct. 2009).
21. Dragset, M. S. *et al.* Benzoic acid-Inducible gene expression in mycobacteria. *PLoS ONE* **10** (ed Kremer, L.) e0134544 (Sept. 2015).
22. Eggeling, L. & Bott, M. *Handbook of Corynebacterium glutamicum* 616. arXiv: arXiv:1011.1669v3 (Taylor & Francis, 2005).
23. Emery, A. Recombinant DNA Technology. *The Lancet* **318,** 1406–1409 (1981).
24. FDA. Human insulin receives FDA approval. *FDA drug bulletin* **12,** 18–9 (Dec. 1982).
25. Gallegos, M. T., Marqués, S. & Ramos, J. L. Expression of the TOL plasmid xylS gene in Pseudomonas putida occurs from a σ70-dependent promoter or from σ70- and σ54-dependent tandem promoters according to the compound used for growth. *Journal of Bacteriology* **178,** 2356–2361 (Apr. 1996).
26. Gawin, A., Valla, S. & Brautaset, T. The XylS/ Pm regulator/promoter system and its use in fundamental studies of bacterial gene expression, recombinant protein production and metabolic engineering. *Microbial Biotechnology* **10,** 702–718 (July 2017).
27. Gualerzi, C. O. & Pon, C. L. Initiation of mRNA translation in prokaryotes. *Biochemistry* **29,** 5881–5889 (June 1990).
28. Hannig, G. & Makrides, S. C. Strategies for optimizing heterologous protein expression in Escherichia coli. *Trends in Biotechnology* **16,** 54–60 (Feb. 1998).
29. Hindson, B. J. *et al.* High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Analytical Chemistry* **83,** 8604–8610 (Nov. 2011).
30. Huttly, A. in *Methods in molecular biology (Clifton, N.J.)* June, 39–69 (2009).
31. Hverven, S. K. K. *Evaluation of the Pm / XylS expression system in Bacillus subtilis* Master's Thesis (NTNU, 2017).
32. Ingram, L. C., Richmond, M. H. & Sykes, R. B. Molecular characterization of the R factors implicated in the carbenicillin resistance of a sequence of Pseudomonas aeruginosa strains isolated from burns. *Antimicrobial Agents and Chemotherapy* **3,** 279–288 (1973).
33. Jahn, C. E., Charkowski, A. O. & Willis, D. K. Evaluation of isolation methods and RNA integrity for bacterial RNA quantitation. *Journal of Microbiological Methods* **75,** 318–324 (Oct. 2008).

34. Jakob, K. *et al.* Gene expression analysis of Corynebacterium glutamicum subjected to long-term lactic acid adaptation. *Journal of bacteriology* **189,** 5582–90 (Aug. 2007).
35. Jakoby, M., Ngouoto-Nkili, C.-E. & Burkovski, A. Construction and application of new Corynebacterium glutamicum vectors. *Biotechnology Techniques* **13,** 437–441 (1999).
36. 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* **78,** 5440–5443 (2012).
37. Johnsgaard, M. *First steps towards implementation of C. glutamicum as an alternative production host for Vectron Biosolutions* Report in subject TBT4500 (NTNU, 2017).
38. Johnson, I. S. Human insulin from recombinant DNA technology. *Science (New York, N.Y.)* **219,** 632–7 (Feb. 1983).
39. Kalinowski, J. *et al.* The complete Corynebacterium glutamicum ATCC 13032 genome sequence and its impact on the production of l-aspartate-derived amino acids and vitamins. *Journal of Biotechnology* **104,** 5–25 (2003).
40. Kortmann, M., Kuhl, V., Klaffl, S. & Bott, M. A chromosomally encoded T7 RNA polymerase-dependent gene expression system for Corynebacterium glutamicum: Construction and comparative evaluation at the single-cell level. *Microbial Biotechnology* **8,** 253–265 (Mar. 2015).
41. Lagendijk, E. L., Validov, S., Lamers, G. E. M., De Weert, S. & Bloemberg, G. V. Genetic tools for tagging Gram-negative bacteria with mCherry for visualization in vitro and in natural habitats, biofilm and pathogenicity studies. *FEMS Microbiology Letters* **305,** 81–90 (2010).
42. Lale, R. *The xylS/Pm expression cassette : new functional insights and application potentials* PhD Thesis (NTNU, Trondheim, 2009).
43. Li, M. Z. & Elledge, S. J. Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. *Nature Methods* **4,** 251–256 (2007).
44. Liang, S. T. *et al.* Activities of constitutive promoters in Escherichia coli. *Journal of Molecular Biology* **292,** 19–37 (Sept. 1999).
45. Lin-Chao, S. & Bremer, H. Effect of the bacterial growth rate on replication control of plasmid pBR322 in Escherichia coli. *MGG Molecular & General Genetics* **203,** 143–149 (Apr. 1986).
46. Liu, X. *et al.* Expression of recombinant protein using Corynebacterium Glutamicum: progress, challenges and applications. *Critical reviews in biotechnology* **8551,** 1–13 (2015).
47. Lu, Q. in *Plasmid Biology* 545–566 (American Society of Microbiology, Jan. 2004).
48. Madigan, M. T., Martinko, J. M., Bender, K. S., Buckley, D. H. (H. & Stahl, D. A. *Brock biology of microorganisms* 1030 ().
49. Makrides, S. C. Strategies for Achieving High-Level Expression of Genes in Escherichia coli †. **60,** 512–538 (1996).
50. Mandel, M. & Higa, A. Calcium-dependent bacteriophage DNA infection. *Journal of Molecular Biology* **53,** 159–162 (1970).

51. Marques, S., Manzanera, M., Gonzalez, P. M. M., Gallegos, M. T. & Ramos, J. L. The XylS-dependent Pm promoter is transcribed in vivo by RNA polymerase with sigma32 or sigma38 depending on the growth phase. *Molecular Microbiology* **31,** 1105–1113 (Feb. 1999).
52. Mueller, O. & Schroeder, A. RNA Integrity Number (RIN) – Standardization of RNA Quality Control Application. *Nano,* 1–8 (2004).
53. Nelson, D. L. (L., Cox, M. M. & Lehninger, A. L. *Lehninger principles of biochemistry* (W.H. Freeman and Company, 2013).
54. Perris, S., Helinskij, D. R. & Toukdarian, A. Interactions of Plasmid-encoded Replication Initiation Proteins with the Origin of DNA Replication in the Broad Host Range Plasmid RK2*, 12536–12543.
55. Phillips, G. J. in *Plasmid Biology* 567–588 (American Society of Microbiology, Jan. 2004).
56. Popowska, M. & Krawczyk-Balska, A. Broad-host-range IncP-1 plasmids and their resistance potential. *Frontiers in Microbiology* **4,** 44 (2013).
57. Potter, H. & Heller, R. Transfection by Electroporation. *Current Protocols in Neuroscience.* eprint: NIHMS150003 (2011).
58. Pridmore, R. D. New and versatile cloning vectors with kanamycin-resistance marker. *Gene* **56,** 309–312 (1987).
59. Primrose, S. B. & Twyman, R. M. *Principles of Gene Manipulation and Genomics* 667 (Blackwell Pub, 2006).
60. Rader, R. A. (Re)defining biopharmaceutical. *Nature Biotechnology* **26,** 743–751 (July 2008).
61. Rédei, G. P. Cloning vector. *Encyclopedia of Genetics, Genomics, Proteomics and Informatics,* 247–465 (2001).
62. Reece, R. J. *Analysis of Genes and Genomes* 491 (John Wiley & Sons, 2004).
63. Rosano, G. L. & Ceccarelli, E. A. Recombinant protein expression in Escherichia coli: advances and challenges. *Frontiers in microbiology* **5,** 172 (2014).
64. Sanchez-Garcia, L. *et al.* Recombinant pharmaceuticals from microbial cells: A 2015 update. *Microbial Cell Factories* **15,** 33 (Dec. 2016).
65. Santamaria, R., Gil, J. A., Mesas, J. M. & Martin, J. F. Characterization of an Endogenous Plasmid and Development of Cloning Vectors and a Transformation System in Brevibacterium lactofermentum. *Microbiology* **130,** 2237–2246 (1984).
66. Schafer, A., Kalinowksi, J., Simon, R., Seep-Feldhaus, A. H. & Puhler, A. High-frequency conjugal plasmid transfer from gram-negative Escherichia coli to various gram-positive coryneform bacteria. *Journal of Bacteriology* **172,** 1663–1666 (1990).
67. Shaner, N. C. *et al.* Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nature biotechnology* **22,** 1567–72 (2004).
68. Snustad, D. P. & Simmons, M. J. *Genetics* 766 (Wiley, 2012).
69. Sodowich, B. I., Fadl, I. & Burns, C. Method validation of in vitro RNA transcript analysis on the Agilent 2100 bioanalyzer. *Electrophoresis* **28,** 2368–2378 (2007).
70. Tauch, A. *et al.* Efficient electrotransformation of Corynebacterium diphtheriae with a mini-replicon derived from the Corynebacterium glutamicum plasmid pGA1. *Current Microbiology* **45,** 362–367 (2002).

71. Toukdarian, A. E. & Helinski, D. R. *TrfA dimers play a role in copy-number control of RK2 replication* in *Gene* **223** (Elsevier, Nov. 1998), 205–211.
72. Unzueta, U. *et al.* Strategies for the production of difficult-to-express full-length eukaryotic proteins using microbial cell factories: production of human alpha-galactosidase A. *Applied Microbiology and Biotechnology* **99,** 5863–5874 (July 2015).
73. Vertès, A. A. in *Corynebacterium glutamicum: biology and biotechnology* 351–389 (Springer, Berlin, Heidelberg, 2013).
74. Voges, R., Corsten, S., Wiechert, W. & Noack, S. Absolute quantification of Corynebacterium glutamicum glycolytic and anaplerotic enzymes by QconCAT. *Journal of Proteomics* **113,** 366–377 (Jan. 2015).
75. Weston, A., Brown, M. G., Perkins, H. R., Saunders, J. R. & Humphreys, G. O. Transformation of Escherichia coli with plasmid deoxyribonucleic acid: calcium-induced binding of deoxyribonucleic acid to whole cells and to isolated membrane fractions. *Journal of bacteriology* **145,** 780–7 (Feb. 1981).
76. Winther-Larsen, H. C., Josefsen, K. D., Brautaset, T. & Valla, S. Parameters Affecting Gene Expression from the Pm Promoter in Gram-Negative Bacteria. *Metabolic engineering* **2,** 79–91 (2000).
77. Wong, T.-K. & Neumann, E. Electric field mediated gene transfer. *Biochemical and Biophysical Research Communications* **107,** 584–587 (July 1982).
78. Worsey, M. J. & Williams, P. A. Metabolism of toluene and xylenes by Pseudomonas (putida (arvilla) mt-2: evidence for a new function of the TOL plasmid. *Journal of bacteriology* **124,** 7–13 (1975).
79. Yim, S. S., An, S. J., Choi, J. W., Ryu, A. J. & Jeong, K. J. High-level secretory production of recombinant single-chain variable fragment (scFv) in Corynebacterium glutamicum. *Applied Microbiology and Biotechnology* **98,** 273–284 (Jan. 2014).
80. Yim, S. S. *et al.* Development of a new platform for secretory production of recombinant proteins in Corynebacterium glutamicum. *Biotechnology and Bioengineering* **113,** 163–172 (2016).
81. Zwick, F., Lale, R. & Valla, S. Regulation of the expression level of transcription factor XylS reveals new functional insight into its induction mechanism at the Pm promoter. *BMC microbiology* **13,** 262 (2013).

Appendix A

Media and Solutions

A.1 Antibiotics

Final concentrations for plates:
- 100 µg/mL Ampicillin (PanReac AppliChem)
- 15 µg/mL Chloramphenicol (Sigma)
- 50 µg/mL Kanamycin (PanReac AppliChem)
- 50 µg/mL Nalidaxic Acid (Sigma)

Final concentrations in solutions:
- 100 µg/mL Ampicillin (PanReac AppliChem)
- 10 µg/mL Chloramphenicol (Sigma)

A.2 Growth media

Luria-Bertani Broth (LB) medium
- 5.0 g Tryptone (OXOID)
- 2.5 g Yeast Extract (OXOID)
- 5.0 g NaCl (VWR)
- Up to 500 mL dH_2O

Autoclaved

LB agar (LA) medium
- 5.0 g Tryptone (OXOID)
- 2.5 g Yeast Extract (OXOID)
- 5.0 g NaCl (VWR)
- 7.5 g agar (OXOID)
- Up to 500 mL dH_2O

Autoclaved

Brain Heart Infusion supplemented (BHIS) medium
37 g Brain Heart Infusion (OXOID)
91 g Sorbitol (Sigma)
Up to 1000 mL dH_2O
Autoclaved

BHI and BHIS agar
37 g Brain Heart Infusion (OXOID)
91 g Sorbitol (only in BHIS) (Sigma)
15 g agar (OXOID)
Up to 1000 mL dH_2O
Autoclaved

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

Psi medium
10 g Tryptone (OXOID)
2.5 g Yeast Extract (OXOID)
5.12 g $MgSO_4 \cdot 7\,H_2O$ (VWR)
Up to 500 mL dH_2O
pH adjusted to 7.6 using KOH
Autoclaved

Transformation Buffer (TFB) 1
0.588 g KAc (Merck)
2.42 g RbCl (ACROS Organics)
0.389 g $CaCl_2 \cdot 2\,H_2O$ (Sigma-Aldrich)
3.146 g $MnCl_2 \cdot 4\,H_2O$ (J.T Baker)
30 mL Glycerol (VWR)
Up to 200 mL dH_2O
pH adjusted to 5.8 using acetic acid
Filtered

TFB 2
0.21 g MOPS (Fischer Scientific)
0.121 g RbCl (ACROS Organics)
1.1 g $CaCl_2 \cdot 2\,H_2O$ (Sigma-Aldrich)
15 mL Glycerol (VWR)
Up to 100 mL dH_2O
pH adjusted to 6.5 using NaOH
Filtered

A.4 Media for transformation of competent *E.coli*

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

Super Optimal Broth with Catabolite Repression (SOC) medium
100 mL SOB media
2 mL 20% filtered glucose solution

A.5 Media for preparation of competent *C.glutamicum* and electroporation

Tris-Glycerol (TG) Buffer
60,57 mg Trizma base (Sigma)
100 mL Glycerol (VWR)
Up to 1000 mL dH_2O
pH adjusted to 7.5 using HCl
Autoclaved

A.6 Media for expression of recombinant proteins

Stock solutions Hi+Ye Basis medium
Stock solutions used to make Hi+Ye Basis medium are pre-made by Vectron Biosolutions.

Fe(III) citrate hydrate solution
3 g Fe(III) citrate hydrate (Sigma)
500 µL dH_2O

$CuCl_2 \cdot 2\,H_2O$ solution
0.357 g $CuCl_2 \cdot 2\,H_2O$ (ACROS)
25 µL dH_2O

H_3BO_3 solution
0.75 g H_3BO_3 (Merck)
25 µL dH_2O

$Na_2Mo_4O_4 \cdot 2\,H_2O$ solution
0.625 g $Na_2Mo_4O_4 \cdot 2\,H_2O$ (VWR)
25 µL dH_2O

$MnCl_2 \cdot 4\,H_{20}$ solution
2.5 g $MnCl_2 \cdot 4\,H_2O$ (JT Baker)
250 µL dH_2O

$CoCl_2 \cdot 6\,H_2O$ solution
0.625 g $CoCl_2 \cdot 6\,H_2O$ (Merck)
25 µL dH_2O

EDTA $\cdot 2\,H_2O$ solution
2.1 g EDTA $\cdot 2\,H_2O$ (VWR)
25 µL dH_2O

$Zn(CH_3COO)_2 \cdot 2\,H_2O$ solution
1 g $Zn(CH_3COO)_2 \cdot 2\,H_2O$ (Merck)
250 µL dH_2O

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

Basis medium 1:
- 8.6 g $Na_2HPO_4 \cdot 2\,H_2O$ (Merck)
- 3 g KH_2PO_4 (ACROS)
- g NH_4Cl (Sigma-Aldrich)
- 0.5 g NaCl (VWR)
- 10 mL Fe(III)citratehydrate
- 0.1 mL H_3BO_3
- 1.5 mL $MnCl_2 \cdot 4\,H_2O$
- 0.1 mL EDTA $\cdot 2\,H_2O$
- 0.1 mL $CuCl_2 \cdot 2\,H_2O$
- 0.1 mL $Na_2Mo_4O_2 \cdot 2\,H_2O$
- 0.1 mL $CoCl_2 \cdot 6\,H_2O$
- 2 mL $Zn(CH_3COO)_2 \cdot 2\,H_2O$
- Up to 900 mL dH_2O

Autoclaved

Yeast Extract:
- 10 g Yeast Extract (OXOID)
- Up to 100 mL dH_2O

Autoclaved

Basis medium 1 and Yeast Extract were mixed after autoclavation to make 1 L Hi+YE Basis medium.

1 M $MgSO_4 \cdot 7\,H_2O$
- 12.3 g $MgSO_4 \cdot 7\,H_2O$ (VWR)
- Up to 50 mL dH_2O

Autoclaved

Glucose solution
- 11.35 g Glucose (VWR)
- Up to 50 mL dH_2O

Autoclaved

Glycerol solution
- 50.51 g Glycerol (VWR)
- Up to 100 mL dH_2O

Autoclaved

2.5 mL of 1 M $MgSO_4 \cdot 7\,H_2O$ was added to Hi+Ye Basis medium after autoclavation. 1.82 g/L glucose (240 µL glucose solution/30 mL medium) and 10 g/L glycerol (600 µL glycerol solution/30 mL medium) were added to obtain finished Hi+Ye medium.

A.7 Inducers

1M Isopropyl-β-D-1-thiogalactopyranosid (IPTG)
2.38 g IPTG (VWR)
Up to 10 mL dH_2O
Filtered

1M *m*-Toluic acid
0.68 g *m*-Toluic acid (Aldrich)
10 µL 100% Ethanol
Filtered

A.8 Media for gel electrophoresis

50xTris-acetate-EDTA (50xTAE) buffer
242 g Tris-base (Sigma)
57.1 mL acetic acid (VWR)
100 mL 0.5M EDTA, pH 8 (VWR)
Up to 1000 mL dH_2O
Autoclaved

0.8% Agarose solution
3.2 g Agarose (Lonza)
400 mL 1xTAE

A.9 Media for lysis of *C. glutamicum*

10xTris-EDTA (10xTE) buffer
0.63 g Tris hydrochloride (Sigma)
0.15 mL EDTA (VWR)
40 mL RO-water
pH adjusted to $\sim$ 7.5
Prepared in 50 mL plastic tubes to avoid RNase

Appendix B

Primers

The design of primers is a critical part of a successful PCR experiment. Typically, primers are 18-20 nucleotides long. The length of the primers influences the rate at which they hybridize to the template DNA, with long primers hybriding at a slower rate. If primers are too short they might hybridize to non-target sites which can give undesired amplification products. If the primers are too long, the efficiency of the PCR, estimated by the number of amplified molecules produced during the experiment, is reduced. Ideally the melting temperature (T_m) of the primers should be within 5°C of each other and the GC content should be between 40-60 %. To avoid primer secondary structures such as hairpins, homo-dimers or cross-dimers, the individual primer should not be homologous to itself and the primer pair should not be homologous. Primers should also not contain long runs of a single nucleotide or dinucleotide repeats (CCCC or ATATATAT). For efficient digestion by restriciton enzymes, 3 or 4 nucleotides are typically added in $5'$ of the restriction site if the latter is located at the $5'$ end of the primers. [13, 62].

Table B.1: Primers used in this study.

Primer	Primer sequence 5′ - 3′	Tm [°C]	Template
4-16	TTTTGCGGCCGCACCGAGCTCGAATTCAG	58/76	pXMJ19 fwd
10-16	CGCCAGAAGCATTGGTG		pXMJ19 rev
8-16	GTGCACCAATGCTTCTGGCGTCAAGCCACTTCCTTTTTGCAT		XylS/*Pm* and mCherry fwd
9-16	TTCGAGCTCGGTGCGGCCGCTCCGTGACGCAGTAGCGGTA		XylS/*Pm* and mCherry rev
17-5	ACCGAGCTCGAATTCAG		pXMJ19 fwd
17-9	TGGAGTCATGAACATAAGCTTATGGTTTCTAAGGG	67	Cgluta_optm mCherry fwd
17-10	CTTCACAGGTCAAGCCGGGGATCCTCTAGATC	72	Cgluta_optm mCherry rev
Vf-pVB-1A0B1-v1	GCTTGACCTGTGAAGTGAAA	60	mCherry fwd
Vr-pVB-1A0B1-v1	ATGTTCATGACTCCATTATTATTGTACAT	61	mCherry rev

Appendix C

Molecular weight standard for gel electrophoresis

A molecular weight standard with known band sizes was used to determine the size of the DNA fragments separated by gel electrophoresis. Figure C.1 present the molecular weight standard used in this study: GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific).

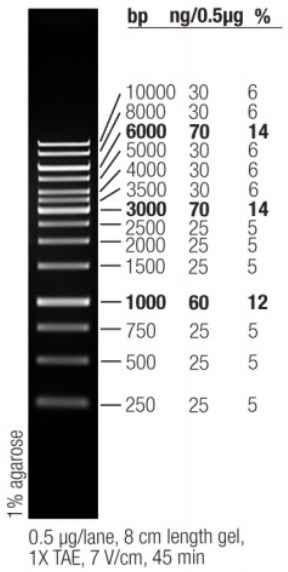


Figure C.1: GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific)

Appendix D

Identifying bacteria

After the first attempts at transforming *C. glutamicum*, some plates seemed to have promising results. To confirm the species of these these "unknown" bacteria, some tests to differentiate between *E. coli* and *C. glutamicum* were performed. From these tests it was concluded that the unknown bacteria was most likely *E. coli*, and the process of transforming *C. glutamicum* was started all over again.

D.1 Nalidixic acid

Nalidixic acid is the first of the synthetic quinolone antibiotics to be discovered. The quinolones are antibacterial compounds that discrupt bacterial metabolism by interfering with bacterial DNA gyrase. DNA gyrase is responsible for supercoiling DNA, a required step for packaging of DNA in the bacterial cell [48]. Nalidixic acid is effective primarily against gram-negative cells, with minor anti-gram-positive activity. In low concentration it inhibits growth and reproduction, while in high concentration it kills the bacteria. It has been used to treat urinary tract infections caused by *E. coli* [48]. *C. glutamicum* ATCC 13032 is found to be naturally resistant to at least 30 μg of nalidixic acid per mL [66]. For this experiment it was therefore expected no growth for *E. coli* in the presence of nalidixic acid, while there was expected some growth for *C. glutamicum* in the presence of nalidixic acid.

Bacteria, both *E. coli* and *C. glutamicum* as well as unknown bacteria, were plated out on BHIS agar plates complemented with nalidixic acid (50 $\mu g/mL$) . After two days of incubation at 30°C, there was growth on all plates. This was not as expected, and no conclusion concerning the unknown bacteria could be drawn from the experiment. This result might be due to a too low concentration of antibiotics than required to induce cell death.

D.2 Gram-staining

Gram-staining is a common technique used to differentiate two large groups of bacteria based on differences in cell wall structure. After Gram-staining, gram-positive bacteria appear purple-violet while gram-negative bacteria appear pink. The Gram-staining involves three processes: coloring with crystalviolet, decolorization with ethanol and counterstaining with safranin. Gram-positive bacteria retain the crystalviolet due to the thicker layer of peptidoglycan in their cell wall. Gram-negative bacteria lose the crystalviolet during the decolorization and are instead colored by safranin in the secondary stain step [48].

Procedure
A small amount of culture was spread in thin film over a slide. The sample was heat fixed to the slide by passing it through a Bunsen burner. The primary stain, crystal violet, was poured over the slide and incubated for 1-2 minutes. The slide was then rinsed with a gentle stream of water for a few seconds. A iodine solution was then added for 1 minute to fix the stain, before decolorizing with 96% ethanol. The sample was then rinsed with a gentle stream of water to avoid leaving ethanol on the sample for too long, since that may also decolorize Gram-postive cells. Safranin, the counterstain, was then added to the slide and incubated for 1 minute. Afterwards, the slide was once again washed with a gentle stream of water and air dried before the slide was inspected under a microscope.

The slides from the Gram-staining process are presented in Figure D.1.

Result
The Gram-staining was performed to examine the unknown bacteria after transformation of *C. glutamicum*. Two controls, *E. coli* and *C. glutamicum*, were also prepared. After inspection with at microscope, the *E. coli* sample looked red/pinkish as expected, while *C. glutamicum* looked purple. The unknown sample looked pinkish, so it was concluded that it most likely was *E. coli*. Unfortunately, no pictures could be taken through the microscope. Since the bacteria was most likely *E. coli*, further work to identify the bacteria was dropped, and the work for another attempt at transforming *C. glutamicum* was started instead.

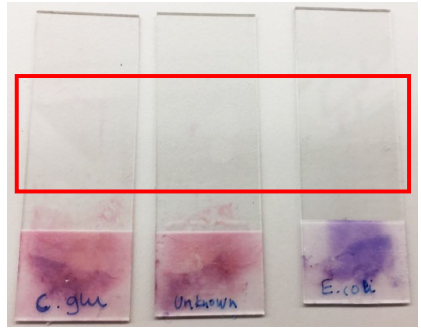


Figure D.1: The slides from the Gram-staining process, from left to right: *C. glutamicum*, uknown bacteria, *E. coli*. After inspection in microscope if was concluded that the unknown sample most likely was *E.coli*. The red square indicates where the bacteria are on the slides.

Appendix E

Total RNA Integrity Analysis

RNA was isolated from both *E. coli* and *C. glutamicum* harboring no vectors as well as harboring pXMJ19-mCherry and pVB-4A0E1-mCherry both induced and not induced. The quality of isolated RNA was analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies). This can be explained as gel electrophoresis on a chip. Samples migrate through individual microchannels with the chip through a gel-dye matrix. The charged RNAs are then separated by size, similar to gel electrophoresis, and small fragments migrates faster than large ones. Dye molecules intercalate into RNA strands, and the complexes are fluoresces as they pass a detector [69].

The RNA integrity number (RIN) describes the quality of the RNA. The scale goes from 0-10, where 10 is completely intact RNA, 1 represents highly degraded RNA, and 0 is no intact RNA. To obtain reliable RIN, the RNA concentration should be above 25 $\mathrm{ng}/\mu\mathrm{L}$ (above 50 $\mathrm{ng}/\mu\mathrm{L}$ is recommended by Agilent Technologies). For qPCR measurements, higher RNA quality is required, and RIN values above 7.0 are required to get reliable data [33].

Table E.1 shows the RIN of each sample as well as the calculated concentration obtained by the software. For some of the samples RIN could not be obtained.

An electropherogram presents fluorescense intensity as a function of migration time and provides a detailed visual assessment of the quality of the RNA sample. Degraded prokaryotic RNA samples show small or no peaks at 23S and 16S rRNA, while for samples with less degraded RNA the peaks are higher. For this experiment, already purified, old gel was used to save some time. This probably caused the noise seen on the electropherograms.

Figure E.1 shows the electropherograms for the *E. coli* samples, while Figure E.2 shows the electrophergorams for the *C. glutamicum* samples.

Table E.1: RIN and calculated concentrations for samples analyzed with Bioanalyzer (Agilent Technologies).

Sample	**RIN**	**Concentration** [ng/µL]
E. coli		
E.coli no vector	N/A	31
pXMJ19	7.2	51
pXMJ19 induced	8.4	24
pXMJ19-mCherry	9.2	54
pXMJ19-mCherry induced	8.4	34
pVB-4A0E1-mCherry	9.1	15
pVB-4A0E1-mCherry induced	5.1	18
C. glutamicum		
wt *C. glutamicum* MB001(DE3)	2.6	27
pXMJ19	N/A	14
pXMJ19 induced	N/A	12
pXMJ19-mCherry	N/A	9
pXMJ19-mCherry induced	N/A	5
pVB-4A0E1-mCherry	N/A	6
pVB-4A0E1-mCherry induced	2.6	14

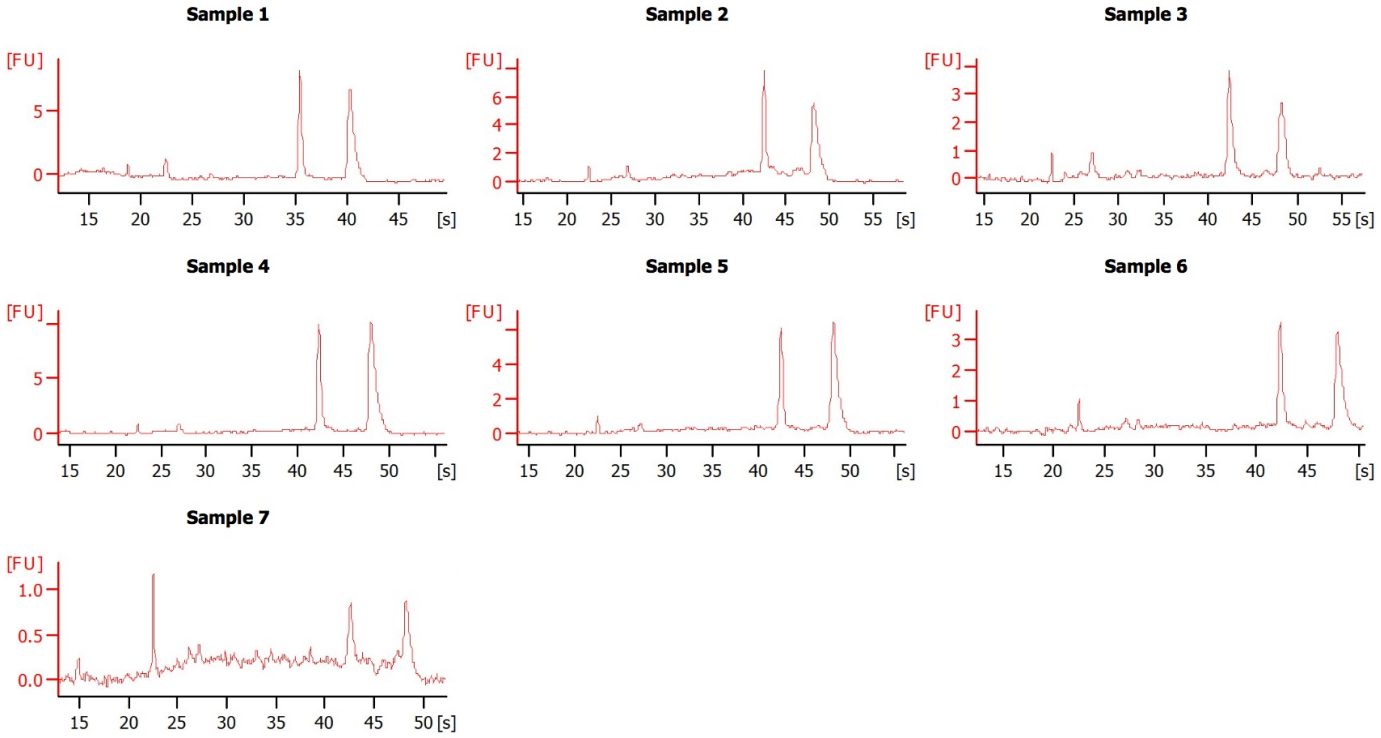


Figure E.1: Electropherograms visualizing RNA separation *E. coli* samples. Sample 1: wt *E. coli* BL21, Sample 2: *E. coli* BL21 harboring pXMJ19, Sample 3: induced *E. coli* BL21 harboring pXMJ19, Sample 4: *E. coli* BL21 harboring pXMJ19-mCherry, Sample 5: induced *E. coli* BL21 harboring pXMJ19-mCherry, Sample 6: *E. coli* BL21 harboring pVB-4A0E1-mCherry, Sample 7: induced *E. coli* BL21 harboring pVB-4A0E1-mCherry.

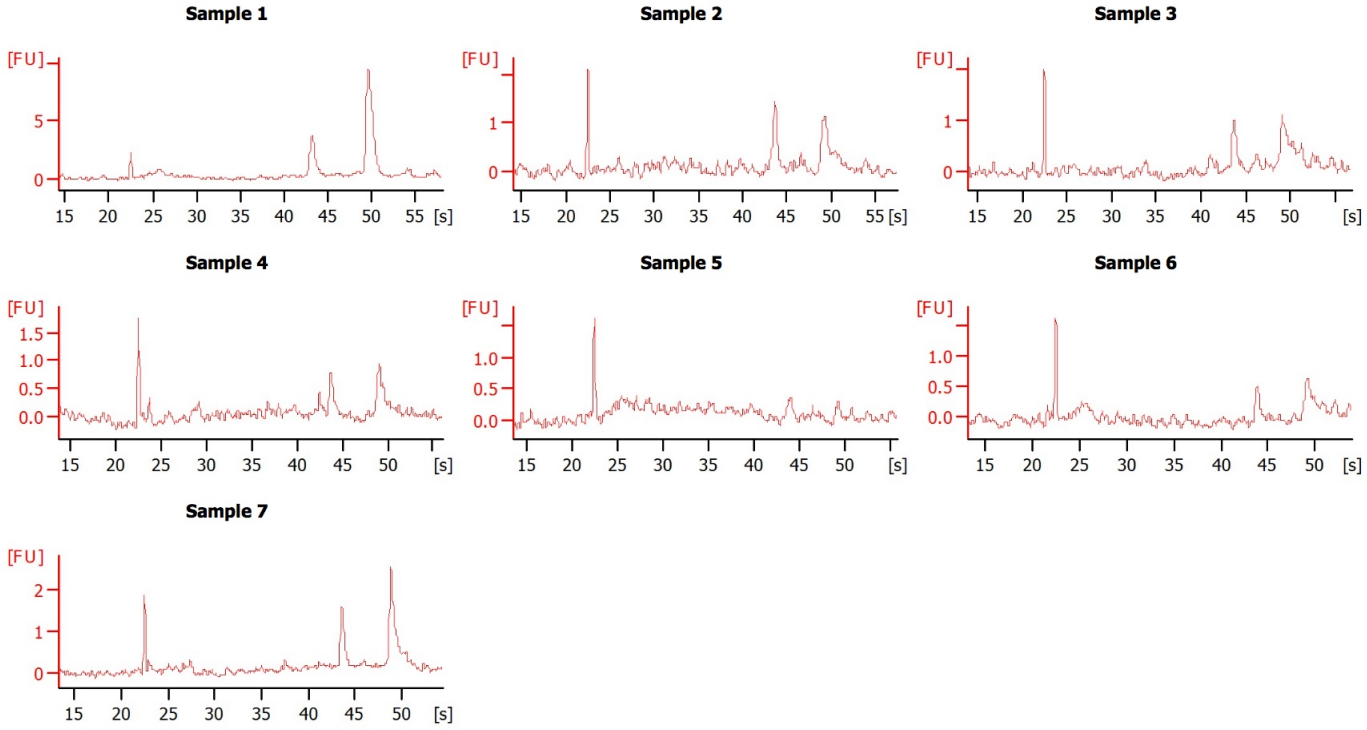


Figure E.2: Electropherograms visualizing RNA separation *C. glutamicum* samples. Sample 1: wt *C. glutamicum* MB001(DE3), Sample 2: *C. glutamcium* MB001(DE3) harboring pXMJ19, Sample 3: induced *C. glutamcium* MB001(DE3) harboring pXMJ19, Sample 4: *C. glutamcium* MB001(DE3) harboring pXMJ19-mCherry, Sample 5: induced *C. glutamcium* MB001(DE3) harboring pXMJ19-mCherry, Sample 6: *C. glutamcium* MB001(DE3) harboring pVB-4A0E1-mCherry, Sample 7: induced *C. glutamcium* MB001(DE3) harboring pVB-4A0E1-mCherry.

Appendix F

ddPCR Raw Data

mCherry and *xylS* mRNA expression levels from pXMJ19-mCherry and pVB-4A0E1-mCherry were measured using ddPCR (QX200™Droplet Digital™PCR System (Bio-Rad)). Samples of *E. coli* and *C. glutamicum* harboring pXMJ19-mCherry, pVB-4A0E1-mCherry as well as wt *E. coli* BL21 and wt *C. glutamicum* MB001(DE3) were analyzed. The ddPCR was performed twice due to differences in sample handling in the first round and to optimize concentrations to obtain readable data. The results from the last round of ddPCR is presented in the Results, Chapter 3.6, while data from the first round is included in Table F.1.

Example results

Figure F.1 presents the ddPCR results when analyzing *mCherry* mRNA level in *E. coli* BL21 harboring pXMJ19-mCherry and pVB-4A0E1-mCherry, induced or not. Positive droplets have presence of *mCherry* RNA, while negative droplets have no *mCherry* RNA. From the results presented in Figure F.1, there is clear that there are higher presence of positive droplets when the *E. coli* harboring either pXMJ19-mCherry or pVB-4A0E1-mCherry are induced.

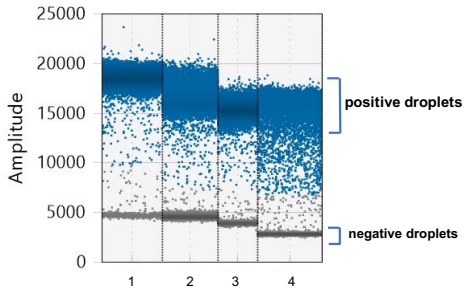


Figure F.1: Example results from analyzing *mCherry* RNA quantity from *E. coli* BL21 harboring pXMJ19-mCherry and pVB-4A0E1-mCherry. 1: pXMJ19-mCherry, 2: pXMJ19-mCherry induced, 3: pVB-4A0E1-mCherry, 4: pVB-4A0E1-mCherry induced.

Table F.1: Data from ddPCR analysis from first round of ddPCR. Values presented here are the quantification of mRNA given as copies/µL cDNA.

	xylS	***mCherry***
E. coli		
E. coli no vector	17	26.4
pXMJ19	5.31	102
pXMJ19 induced	15.9	638
pXMJ19-mCherry	14.7	4750
pXMJ19-mCherry induced	10.4	N/A*
pVB-4A0E1-mCherry	339	2468
pVB-4A0E1-mCherry induced	88.7	3259
E. coli no vector	17.1	24.1
C. glutamicum		
C. glutamicum no vector	9.75	15.5
pXMJ19	11.6	0.858
pXMJ19 induced	0.007	2.47
pXMJ19-mCherry	0.0893	3.62
pXMJ19-mCherry induced	4.4	216
pVB-4A0E1-mCherry	13.7	18.24
pVB-4A0E1-mCherry induced	17.7	7.24
C. glutamicum no vector	21	8.28

* Concentration too high to obtain a value

Presence of *xylS* mRNA was only expected for *E. coli* and *C. glutamicum* harboring pVB-4A0E1-mCherry, since those are the only ones with the *xylS* gene. The values obtained from the other samples gives an idea of the sensitivity limits of ddPCR.

For *C. glutamicum* harboring pVB-4A0E1-mCherry, the values for *mCherry* RNA are not as expected and shows the the vector is not functional in *C. glutamicum*.

Appendix G

Calculating Generation Time

The generation times for *C.glutamicum* MB001(DE3) and *C. glutamicum* ATCC 13032 were calculated from their respective growth curves. The growth curves were plotted as a semi-log plot of OD_{600} measurements as a function of time. An example of these growth curves is given in Figure 3.1a. The exponential phase was identified as the linear part of the growth curve, and a linear regression was performed to fit a line on the form $y = bx + a$. Excel's linear regression built-in function was used to find the straight line through the measured points.

The slope b from the linear regression equals growth rate μ from this equation

$$\mu = \frac{\ln OD_2 - \ln OD_1}{t_2 - t_1}$$

When growth rate is know the generation time, g, can be calculated from the equation below:

$$g = \frac{\ln 2}{\mu}$$

Example calculation

C. glutamicum MB001(DE3) at 37°C (Figure 3.1a) is used for example calculations. Excel calculated the slope to be $\mu = 0.79$ h^{-1}. The generation time was calculated as follows

$$g = \frac{\ln 2}{0.79} = 0.88\text{h} = 53\text{min}$$