Synthetic biology for drug discovery

construction of biosensors for detection of endogenously produced bacterial secondary metabolites

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PREFACE

This study is a continuation of the specialization project performed at the Institute of Biotechnology at NTNU, autumn 2012. The laboratory work and writing of the Master thesis was carried out during the spring semester in 2013. This project is a part of the genome-based bioprospecting research managed by Professor Sergey Zotchev.

During the work with this Master thesis, researcher Olga Sekurova has been my practical supervisor. I will thank her very much for excellent supervision and help in the laboratory. She has taught me many laboratory techniques and given me good advice in the lab as well as with the report. Her kind and positive spirit has helped me to regain my motivation when the results in the lab failed to fulfill my expectations.

I will thank Sergey Zotchev for this interesting project. He has given me useful information about the project idea, and introduced me to useful computer programs as Clone Manager and j5. He has also given me laboratory advice and helpful feedback on the report.

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SUMMARY

Streptomycete bacteria are a great source of natural bioactive secondary metabolites, some of which are being used as antibiotics and anticancer drugs. Many streptomycetes have the genomic potential for producing 20-30 chemically diverse bioactive secondary metabolites, while only 2-4 secondary metabolites are produced under standard laboratory conditions. Activation of otherwise "silent" gene clusters is possible by changing the growth conditions, but detection of new compounds is a challenging and time-consuming process. A biosensor that could detect production of new compounds would greatly assist in discovering new drugs.

Many gene clusters for antibiotic biosynthesis contain genes encoding transporters for antibiotic efflux, thereby conferring resistance to the antibiotic. Expression of these genes is often induced in parallel with antibiotic biosynthesis to avoid intracellular accumulation of the toxic compound. A transcriptional biosensor can be constructed by fusing the promoter, which controls transporter gene expression, to a reporter gene. Expression of the antibiotic may then be detected, since the reporter gene is placed under the same regulatory control as the gene encoding an antibiotic efflux pump.

Streptomyces venezuelae ATCC 10712 (wild type) produces two antibiotics under laboratory conditions: chloramphenicol (Cml) and jadomycin B (JadB). This bacterium also has 27 other gene clusters encoding secondary metabolites. The genes encoding transporters in the Cml gene cluster are most likely expressed in parallel with the genes encoding enzymes for Cml biosynthesis. In order to check this hypothesis, this Master thesis aims at constructing biosensors by placing a reporter gene (*gusA* encoding β-glucuronidase) under the transcriptional control of two promoters upstream of transporter genes (*cmlF* and *kefB*) in the Cml gene cluster. A positive control vector should be constructed by fusing the strong constitutive *ermE** promoter to the reporter gene in a similar vector, while the negative control vector has no promoter.

In order to construct the biosensor plasmids, a vector template (pSOK805), a reporter gene (*gusA*) and different promoters were PCR-amplified to ensure overlapping terminal sequences. Ligation of the overlapping DNA fragments was performed by an *in vitro* isothermal reaction, called 'Gibson' reaction. The reaction mixes were transformed into *Escherichia coli* strains and plasmid DNA was isolated from the transformants.

The biosensors and control vectors were successfully assembled and verified. Three out of four vectors were site-specifically integrated into the genome of *S. venezuelae* by conjugative DNA transfer from *E. coli* ET12567. In order to test the sensitivity of the biosensors, the *S. venezuelae* recombinant strains were cultured in low-production medium with and without addition of ethanol. Samples were collected from the

cultures to measure the chloramphenicol production and reporter enzyme activity. One of the biosensor strains showed a correlation between chloramphenicol production and the enzyme activity, but this trend needs to be verified by further experiments. Only small amounts of chloramphenicol were produced by the cells, thus different cultivation media should be tested in future experiments. The measurements of enzyme activity in the lysates are preliminary, since the protein content has to be adjusted to get comparable results. No final conclusions can be drawn on the functionality of the biosensors, before several independent experiments are performed.

SAMMENDRAG

Streptomycete bakterier er en god kilde til naturlige bio-aktive sekundære metabolitter, hvorav noen blir brukt som antibiotika og kreftmedisiner. Mange streptomyceter har genomisk potensial for å produsere 20-30 ulike bio-aktive sekundære metabolitter, mens bare 2-4 av disse produseres under standard laboratoriebetingelser. Aktivering av "stille" gen-klustre er mulig ved å forandre vekstbetingelsene, men påvisning av nye forbindelser er en utfordrende og tidkrevende prosess. En biosensor som kan oppdage produksjon av nye forbindelser vil kunne bidra til å oppdage nye legemidler.

Mange gen-klustre for biosyntese av antibiotika inneholder gener som koder for transportører av antibiotika, og som dermed gjør bakterien resistent. Disse genene uttrykkes ofte parallelt med biosyntesen av antibiotikumet for å unngå intracellulær akkumuleringen av den giftige forbindelsen. En transkripsjonell biosensor kan konstrueres ved å fusjonere promotoren som kontrollerer transport genet til et reporter gen. Produksjon av antibiotikumet kan dermed oppdages, siden reportergenet er plassert under samme regulatoriske kontroll som genet for antibiotika-transportpumpen.

Streptomyces venezuelae ATCC 10712 (villtype) produserer to antibiotika-typer under laboratoriebetingelser: kloramfenikol (Cml) og jadomycin B (JadB). Denne bakterien har også 27 andre gen-klustre som koder for sekundære metabolitter. Genene som koder for transportører i Cml klusteret blir sannsynligvis uttrykt parallelt med genene som koder for enzymer for Cml biosyntese. For å teste hypotesen, har denne masteroppgaven som mål å konstruere biosensorer ved å plassere et reporter gen (*gusA*) under transkripsjonell kontroll av to promotorer oppstrøms for transporter gener (*cmlF* og *kefB*) i Cml klusteret. En positiv kontroll vektor skal bli konstruert ved å fusjonere den konstitutive *ermE** promotoren til reportergenet i en lignende vektor, mens vektoren for negativ kontroll mangler promotor.

For å konstruere disse vektorene ble en vektor mal (pSOK805), et reporter gen (*gusA*) og ulike promotorer amplifisert med PCR for å lage overlappende ender. De overlappende DNA-fragmentene ble ligert i en *in vitro* isoterm reaksjon, kalt 'Gibson' reaksjon. Reaksjonsblandingene ble transformert inn i *E. coli*-stammer, og plasmid DNA ble isolert fra transformantene.

Biosensorene og kontroll vektorene ble vellykket satt sammen og verifisert. Tre av fire vektorer ble integrert i genomet til *S. venezuelae* ved konjugativ DNA overføring fra *E. coli* ET12567. For å teste sensitiviteten til biosensorene, ble de rekombinante *S. venezuelae* stammene dyrket i et lav-produksjons-medium med og uten tilsats av etanol. Det ble tatt prøver fra kulturene for å måle kloramfenikol produksjonen og aktiviteten til reporter enzymet. En av biosensor stammene viste en sammenheng

mellom kloramfenikol produksjon og enzymaktiviteten, men denne tendensen må verifiseres med flere eksperimenter. Kun små mengder av kloramfenikol ble produsert av cellene. Derfor bør ulike dyrkingsmedier testes i kommende eksperimenter. Målingene av enzymaktivitet i cellelysater er foreløpige, siden proteininnholdet må justeres for å få sammenlignbare resultater. Ingen endelige konklusjoner kan trekkes angående funksjonaliteten til biosensorene før flere uavhengige eksperimenter er utført.

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ABBREVIATIONS

Amp	Ampicillin
bp	base pair(s)
c.	construct
Cml	Chloramphenicol
col.	colony
Crp	Cyclic AMP receptor protein
dsH_2O	distilled sterile water
EHF	Expand high fidelity
EtOH	Ethanol
GBL	γ-butyrolactone
GUS	β-Glucuronidase
JadB	Jadomycin B
Kan	Kanamycin
kb	kilo base pair(s)
Nal	Nalidixic acid
OD	Optical Density
oriT	origin of transfer
PCR	Polymerase chain reaction
pDNA	plasmid Deoxyribonucleic Acid (DNA)
PNPG	p-nitrophenyl-β-D-glucuronide
SDOM	Standard deviation of the mean
Tc	Tetracycline
Thio	Thiostrepton
UPLC	Ultra high Performance Liquid Chromatography
wt	wild type

1 INTRODUCTION

1.1 ACTINOMYCETES

The actinomycetes are a large group of gram positive bacteria that form branching filaments. The network of filaments formed during growth is called mycelium and is similar to the mycelium formed by filamentous fungi (Madigan et al., 2009). These bacteria live mostly in the soil, but are also found in marine environments such as the Trondheim fjord (Bredholdt et al., 2007, Bredholt et al., 2008). Most actinomycetes form spores on solid media after nutrient depletion has occured. When the spores are transferred to more nutrient-rich environments, they germinate to give rise to new colonies. Actinomycetes are considered GC-rich bacteria, since their genomic DNA consists of 63-78 % guanine and cytosine.

Many actinomycetes are able to produce secondary metabolites with diverse biological activities. After extensive screening of actinomycetes, many important antibiotics, anti-cancer agents and cholesterol-lowering drugs have been discovered in this genera (Zotchev et al., 2012). The fact that each actinomycete genome contains approximately 20-30 gene clusters for biosynthesis of secondary metabolites (Siegl and Luzhetskyy, 2012) increases the interest for discovering new potential drugs in these bacteria. However, only 2-4 of the gene clusters are expressed under laboratory conditions, or the compounds are present in too low amounts to be detected. Awakening of these silent gene clusters can presumably be achieved by modifying environmental factors such as temperature, pH, salinity and signaling molecules (Zotchev, 2012). Several other strategies for activation of gene expression will be presented in Chapter 1.4.1.

1.2 STREPTOMYCETES

The genus *Streptomyces* includes gram-positive bacteria belonging to the order *Actinomycetales*. The streptomycetes have GC-rich genomes represented by linear chromosomes of 5-11 Mb in size. Most *Streptomyces* grow in alkaline to neutral soil and they are quite versatile when it comes to nutrition. These bacteria can use a wide variety of carbon sources, such as sugars, amino acids, alcohols, organic acids and aromatic compounds. Many *Streptomyces* produce and secrete enzymes for hydrolytic breakdown of polysaccharides, proteins and lipids (Madigan et al., 2009).

The *Streptomycetes* have a complex life cycle which is presented in Figure 1.1. The bacteria form a large network of multicellular filaments called mycelium, which is similar to fungal growth (Weber et al., 2003). The *Streptomyces* life cycle begins when a spore germinates and continues to grow in a filamentous manner by replication without cell division. As the colony ages, aerial filaments are formed and give rise to

spores by building up cross-walls in the multinucleate filaments (Madigan et al., 2009). When the spores mature, they can be spread to more nutrient- rich environments. *Streptomyces* possibly produce antibiotics as a mechanism to inhibit growth of organisms competing for the same limiting nutrients. This increases the survival chance for *Streptomyces*, because it can complete the sporulation process and spread to new environments before the nutrients are fully consumed (Madigan et al., 2009).

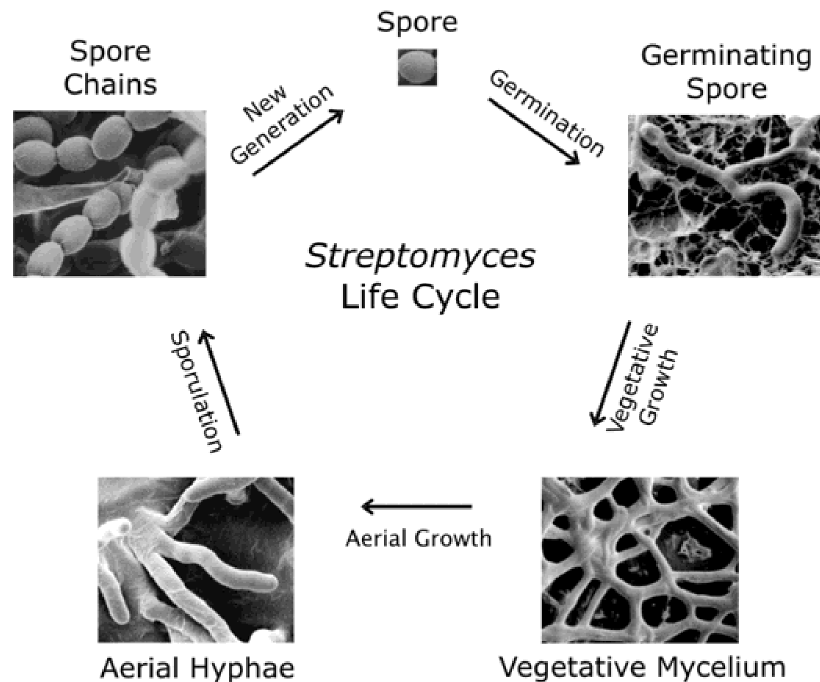


Figure 1.1: The life cycle of *Streptomyces* (Brooks et al., 2012).

Streptomyces are known to produce a wide variety of secondary metabolites, most of which are a great source for new drugs. Over 55 % of the known antibiotics were produced by streptomycetes and 11 % by other actinomycetes (Weber et al., 2003). This increases the interest for finding new natural drugs by expressing silent secondary metabolite gene clusters in these bacteria.

Antibiotic biosynthesis in *Streptomyces* depends on the growth phase. In liquid culture, antibiotic production begins at the end of the exponential growth phase and as the culture enters stationary phase (Kieser et al., 2000). On solid media, the antibiotic biosynthesis coincides with the development of aerial hyphae and continues while sporulation occurs (Bibb, 2005). Several physiological and environmental factors influence the onset of antibiotic biosynthesis including growth rate, imbalances in metabolism, physiological stress and the presence of signaling molecules such as γ-butyrolactones (GBL) (Kieser et al., 2000). The latter are quorum sensing signaling molecules in actinomycetes.

Streptomyces venezuelae is a gram positive soil bacterium, which grows particularly fast under laboratory conditions compared to other streptomycetes. It is known to

produce the antibiotics jadomycin B (JadB) and chloramphenicol (Cml), and has 27 other interesting gene clusters for secondary metabolism. Chloramphenicol biosynthesis depends on the presence of nitrogen and glucose in the medium, while jadomycin biosynthesis is induced under stress conditions, such as heat shock, phage infection or toxic ethanol concentration in the cultivation medium (Doull et al., 1994, Yang et al., 1995). The biosynthesis of jadomycin and chloramphenicol were found to be coordinated by pseudo-γ-butyrolactone (GBL) receptors which assures that only one of the antibiotics is synthesized at any time (Xu et al., 2010b). Most of the 27 other gene clusters in *S. venezuelae* are silent under laboratory conditions, but it might be possible to trigger expression of otherwise silent gene clusters by using different growth media, incubation conditions or signaling molecules. Detection of new compounds are, however, very time-consuming and requires advanced analytical equipment. A biosensor could be used to detect production of new compounds and would be helpful in drug discovery and process development for new antibiotics.

1.3 ANTIBIOTICS

The discovery of penicillin by A. Fleming in 1928 has revolutionized the treatment of bacterial infections. This antibacterial compound was able to kill a wide range of bacteria, and was introduced into medical practice in the early 1940s (Zotchev, 2008). Since then, researchers have discovered a huge number of new antibiotics with different chemical structures and activities. The "golden era of antibiotics" ended with dramatically decreasing discovery of new compounds. At the same time, pathogens are developing resistance shortly after introducing a new antibiotic drug into medical practice. Now, there is an urgent need to find new antibiotics effective against multiresistant life-threatening organisms (e.g. *Staphylococcus aureus, Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*) (Fischbach and Walsh, 2009). However, the big Pharmaceutical companies are no longer developing new antibiotics, because of the low profit compared to drugs for treating chronic diseases (Livermore et al., 2011). New technologies such as screening methods, metabolic engineering and synthetic biology can be useful tools to discover new antibiotics in natural environments.

Antibiotics are chemical substances produced by a microorganism that kills or inhibits growth of other living organisms (Madigan et al., 2009). These antimicrobial, antifungal and/or antiparasitic compounds are used in medical treatment, food industry and in research. Several organisms, including bacteria, fungi, plants and animal species, are able to synthesize antibiotics. Bacteria and fungi can synthesize chemically diverse antibiotics, while plants and animals mostly produce peptides and terpenes with antibiotic activity. Over 60 % of all known antibiotic compounds are

produced by the order *Actinomycetales* which are GC-rich, gram positive bacteria (Zotchev, 2008).

Antibiotics are secondary metabolites, which are not required for growth of the producing organism. Primary metabolites such as amino acids, sugars and lipids are on the other hand necessary for growth and maintenance of the organism. Biosynthesis of antibiotics can be divided into two stages. First, the antibiotic scaffold is synthesized from primary metabolites, catalyzed by enzymes encoded for by the scaffold assembly in Figure 1.2. These antibiotic scaffolds usually possess little or no antimicrobial activity. The second stage includes modification of the scaffolds by specific enzymes that add functional groups, rendering complete and fully active antibiotic molecules (Zotchev, 2008).

Genes encoding enzymes for antibiotic biosynthesis are arranged in gene clusters and their expression is tightly regulated. The organization of a typical gene cluster is presented in Figure 1.2.

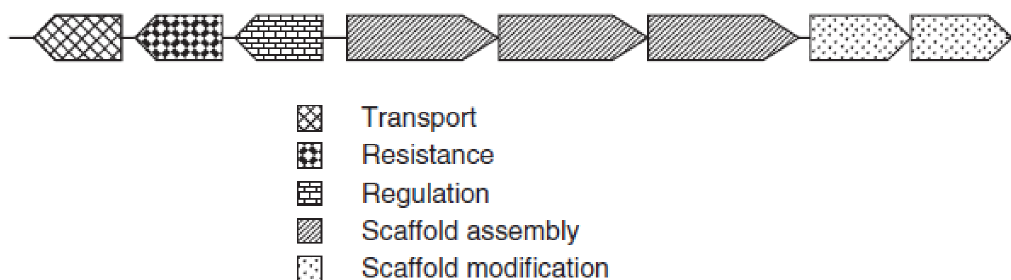


Figure 1.2: Organization of a typical antibiotic biosynthesis gene cluster (Zotchev, 2008).

The gene cluster often contains one or more transporter genes encoding efflux pumps that export antibiotic molecules out of the cell. These transmembrane transporter molecules are important resistance mechanisms for antibiotic producing cells, as well as other bacteria that develop resistance (reviewed in (Piddock, 2006, Higgins, 2007)). Expression of the transporter has to be activated in parallel with antibiotic production, in order to avoid a toxic intracellular antibiotic concentration in the bacteria. The gene cluster may also contain resistance genes encoding enzymes that inactivate accumulated antibiotics by degradation, modification or changing functional groups on the molecule (Zotchev, 2008). Regulatory genes are also important to ensure that certain genes are only expressed when needed. These regulatory genes encode repressors and/or activators that interact with inducers and/or co-repressors as well as operator regions for the genes they regulate (Klug et al., 2009).

Intracellular antibiotic production represents a metabolic burden to the producing organism and is therefore tightly regulated. The biosynthesis can be activated in response to certain environmental stimuli such as nutrient depletion, organic solvents, changes in pH or temperature, phage infection, presence of signaling molecules or

other organisms competing for the same nutrients (reviewed in (Zotchev, 2008)). During exponential growth in liquid culture, the primary metabolites are used to build up biomass. Biosynthesis of antibiotics and other secondary metabolites are initiated when the bacterial growth rate ceases and the cell culture enters the stationary growth phase (Wohlleben et al., 2012).

1.3.1 Chloramphenicol (Cml)

The antibiotic chloramphenicol (Cml) is a broad-spectrum antibiotic produced by the Gram-positive soil bacterium *Streptomyces venezuelae* and some related species. Cml inhibits bacterial growth by binding reversibly to the peptidyl transferase unit of the ribosome, thus preventing protein biosynthesis (Pongs, 1979). Almost all gram-positive and gram-negative bacteria are inhibited by Cml, which became available for clinical use in 1948. However, the use of Cml is restricted due to several unusual and life threatening toxicity syndromes (Shaw and Leslie, 1989).

The molecule structure of Cml is shown in Figure 1.3 and seems quite simple compared to other antibiotics. Chloramphenicol is derived from the shikimate pathway which assembles aromatic metabolites in bacteria (He et al., 2001). The genes encoding enzymes for Cml biosynthesis from chorismic acid are situated in a gene cluster which is presented in Figure 1.6 later in the introduction.

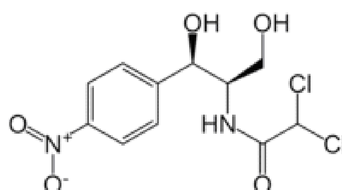


Figure 1.3: The molecule structure of chloramphenicol (Brooks et al., 2012).

Chloramphenicol production in *S. venezuelae* depends on the presence of nitrogen and glucose. The activity of Cml biosynthetic enzymes depends on the concentration of excess glucose in nitrogen-limited media, and conversely, the glucose-suppression of Cml production depends on the residual nitrogen source (Doull and Vining, 1990). High yield of Cml was achieved when using a nitrogen source (e.g. DL-Serine) that resulted in slow and controlled growth of the bacteria (Westlake et al., 1968). Under non-producing conditions, *S. venezuelae* is relatively sensitive to Cml, but resistance is induced by exposure to Cml. Under Cml-producing conditions, the resistance increases with Cml biosynthesis (Mosher et al., 1995), indicating that resistance mechanisms are induced in parallel with Cml production.

Some bacteria evolved resistance mechanisms against Cml within few years after its introduction into clinical use (Shaw and Leslie, 1989). Resistance to Cml has been reported to arise from several different enzymatic modifications, such as dehalogenation, nitro group reduction, hydrolysis of the amide bond and acetylation of one or both of the hydroxyls of Cml (Murray and Shaw, 1997). In most eubacteria,

resistance is mediated by Cml acetyltransferase which acetylates the (C-3) hydroxyl group, yielding inactive 3'*O*-acetyl-Cml (Mosher et al., 1995).

Bacteria have also acquired resistance against Cml by specific and/or multidrug transporters (reviewed in (Schwarz et al., 2004)). One multidrug transporter called Mdfa has been identified in *E. coli*, and overexpression of Mdfa made *E. coli* resistant to many antobiotics, including Cml. However, deletion of the *mdfa* gene barely influenced the cellular resistance, and the real physiological role of Mdfa was found to be a $Na^{+}(K^{+})/H^{+}$ antiporter, which ensures constant intracellular pH under alkaline conditions (reviewed in (Higgins, 2007)).

Interestingly, the Cml producer *S. venezuelae* has developed its own resistance mechanism which includes phosphorylation of the (C-3) hydroxyl group by Cml phosphotransferase (CPT). It is suggested that CPT acts as a carrier that facilitates export of the phosphorylated Cml to an efflux pump. The transmembrane transporter protein then exports Cml-3'-phosphate out of the cell, and the protective phosphate group is removed by an extracellular phosphatase, releasing active Cml to the environment (Izard, 2001). Expression of the transporter protein is presumed to be tightly regulated and will be explained further in Chapter 1.7-1.8.

1.4 SYNTHETIC BIOLOGY: A TOOL FOR DRUG DISCOVERY

A group of European experts proposed the following definition of synthetic biology: "Synthetic biology is the engineering of biology: the synthesis of complex, biologically based (or inspired) systems, which display functions that do not exist in nature" (Serrano, 2007). Synthetic biology brings together engineering and biology to design novel biological devices from natural parts such as genes, promoters, operators, terminators, vectors etc. The well-characterized parts can be combined in a new way to create systems that function in a predictable manner (reviewed in (Neumann and Neumann-Staubitz, 2010)). Synthetic biology is used to reorganize genomes and for improved design of biochemical pathways for the commercial production of desired products. It also has many applications in biosensing, therapeutics, production of biofuels, pharmaceuticals and novel biomaterials (Khalil and Collins, 2010). Synthetic biology is a means to make orthogonal biological systems, explore transcriptional regulation and detect certain environmental compounds. The aim of synthetic biology is to control cellular behavior by applying engineering tools and use characterized parts to achieve desired functions (Mukherji and van Oudenaarden, 2009).

It is complicated and challenging to engineer cells because of intracellular "noise" in the stochastic regime (reviewed in (Andrianantoandro et al., 2006)). Cellular activities have to be seen as random and based on probability, thus most genetic interactions are stochastic and not deterministic when few molecules are involved. Quorum sensing molecules can circumvent the challenges with cell control to make cells react in

synchrony to an induction signal. Good examples of this are construction of pulse generating cells (Basu et al., 2004) and synchronized oscillating cells (Danino et al., 2010). They report on two different systems in synthetic circuits that express the GFP (green fluorescent protein) reporter in a pulse and oscillatory manner, respectively.

The biological devices and synthetic circuits are introduced into 'chassis', which is an engineered organism that ensures optimal performance of the device. These special strains can, for instance, have a reduced genome and reduced number of networks which makes it less complex and less "noisy" in expressing the device (reviewed in (Heinemann and Panke, 2006)). The super-host cell should be able to recognize the promoters and operators used in the devices, as well as detect and respond to induction signals.

1.5 SYNTHETIC BIOLOGY IN *STREPTOMYCES* BACTERIA

Whole genome sequencing of *Streptomyces* species followed by antiSMASH (antibiotics & Secondary Metabolite Analysis Shell) has revealed multiple gene clusters governing biosynthesis of secondary metabolites. The software antiSMASH can rapidly identify and analyze interesting gene clusters in bacterial and fungal genome sequences by detecting known classes of secondary metabolite gene clusters and compare the evolutionary similarities (Medema et al., 2011a). However, most of the detected gene clusters for secondary metabolite biosynthesis are silent or 'cryptic' pathways which are not expressed under laboratory growth conditions.

1.5.1 Activation of silent gene clusters

Silent gene clusters can potentially be expressed by using synthetic biology tools, such as reengineering of the regulatory mechanisms. The native regulation system can be replaced by a synthetic regulation that is predictable, easy to manipulate and possible to fine-tune for the desired function (Medema et al., 2011b). A completely redesign of the gene cluster by changing promoters, ribosome binding sites and possibly also the codon usage, may be necessary in order to achieve this.

One strategy for activating silent gene clusters in *Streptomyces* species is to manipulate the regulatory genes, either by modifying the global regulators or alter the pathway-specific regulators of secondary metabolism (Zerikly and Challis, 2009). It was recently discovered that cyclic AMP receptor protein (Crp), which is known to regulate catabolite repression in *E. coli*, is a global regulator for antibiotic production in *Streptomyces* (Gao et al., 2012). Overexpression of Crp in several *Streptomyces* species increased antibiotic biosynthesis and led to production of new metabolites, while deletion of *crp* in *S. coelicolor* resulted in dramatic reduction of antibiotic production levels.

Expression of a silent gene cluster has also been achieved by placing a pathway-specific positive regulator under the control of a strong constitutive promoter, such as *ermE**p (reviewed in (Baltz, 2010)). The *ermE** promoter could also be inserted in front of transporter genes, to avoid toxic accumulation of the antibiotic due to lack of transporter capacity at high production levels. This constitutive *ermE* promoter was originally found in front of the erythromycin resistance gene in *Saccharopolyspora erythraea*. One base pair mutation was introduced, resulting in the *ermE** promoter with enhanced promoter activity (reviewed in (Medema et al., 2011b)). Another approach for expressing cryptic gene clusters is to delete a pathway-specific repressor. This was performed with a γ-butyrolactone receptor in *S. coelicolor* and resulted in the discovery of a novel bioactive compound (Gottelt et al., 2010).

When expression of a silent gene cluster has been achieved, the remaining task is to isolate the novel compound and elucidate its chemical structure. This depends on sufficient yield of the compound and is often a time-consuming process. It would be very helpful to have a biosensor that expresses a reporter gene when the silent gene cluster is activated and a compound produced. Presence of the reporter can be quantitatively detected and shall ideally indicate the amount of produced secondary metabolite.

1.5.2 Expression of secondary metabolites in heterologous hosts

Expression of secondary metabolite gene clusters can be performed by cloning the gene clusters into heterologous hosts suitable for expressing otherwise silent pathways. Useful *Streptomyces* and *Saccharopolyspora* host strains have been reviewed by Baltz (2010). Manipulation of host strains by elimination or silencing of major secondary metabolite gene clusters prevents the channeling of precursors into competing pathways, thereby improving the yield of the desired product. Such genome-minimized production hosts only waste a minimal amount of resources for cell maintenance, so more of the nutrients are used for production of the desired compound (Medema et al., 2011c). Another advantage is that it simplifies the identification of novel products from cloned heterologous gene clusters (Baltz, 2010). Different species can have different sigma-factors and 16S rRNA sequences, which affect transcription and translation of foreign genes. Therefore, it may be necessary to replace the original promoter and Shine-Dalgarno sequences in the gene cluster in order to achieve successful heterologous expression in a host cell. This replacement can be performed by synthetic biology tools and methods for DNA assembly (reviewed in (Zotchev et al., 2012, Hillson, 2011)). The ideal host cell for heterologous gene expression in *Streptomyces* should have the following features: be genetically tractable (with several vectors and selection markers available), have all (active) secondary metabolite biosynthesis gene clusters deleted, have controllable pathways for biosynthesis of secondary metabolite precursors, be able to utilize a variety of cheap nutrients, have

secondary metabolism independent of growth phase and grow fast without a loss of productivity (Zotchev et al., 2012).

Several factors influence the expression of secondary metabolites both in heterologous hosts and in the native strain. It is important that the precursor pools are sufficient when expression of the secondary metabolites is induced. During constitutive expression, the metabolic requirements for production are competing with the pathways necessary for cellular growth and survival. A synthetic regulatory system can optimize the timing of enzyme production to ensure that the limiting resources are distributed to the right pathways for optimal production of secondary metabolites at the right time (reviewed in (Medema et al., 2011c)). It may be advantageous to induce expression of secondary metabolite biosynthesis pathways when the biomass is high and the growth rate is slowing down, just like the natural induction occurs in *Streptomyces* bacteria. Ideally, the cells should switch to secondary metabolite production before nutrient depletion at the end of the exponential growth phase, so that high-level production can be coupled to slow growth for maintaining the biomass levels.

Other bottlenecks include enzyme concentrations, enzyme affinity for substrates and diffusion of metabolites between the enzymes. These problems can be overcome by synthetic protein scaffolds, which assemble proteins into complexes, thus increasing the local enzyme concentrations. This strategy prevents the build-up of intermediates, increases the metabolic flux through the biosynthetic pathways and results in higher product yield (reviewed in (Medema et al., 2011c)). Fine-tuning of enzyme expression in a pathway is important to optimize flux and avoid accumulation of toxic intermediates. This can be achieved by using synthetic promoters of varying strengths and fuse them to the genes encoding enzymes in the pathway (reviewed in (Neumann and Neumann-Staubitz, 2010).

1.5.3 DNA transfer into *Streptomyces* species

Plasmid DNA (pDNA) can be introduced into *Streptomyces* by transformation, transfection, electroporation or conjugation. DNA transfer by conjugation is simple and usually more efficient than transformation. Depending on the vector used, conjugation can result in autonomous replication of the recombinant plasmid, site-specific integration into the *Streptomyces* genome or integration via homologous recombination between cloned DNA and the *Streptomyces* chromosome (Kieser et al., 2000, Bierman et al., 1992). Conjugative transfer occurs in the early growth phase when *Streptomyces* grows as mycelium, and it only takes place on solid media. When first introduced into one mycelial compartment, the plasmid is soon transferred to all of the mycelial compartments through a protein pore in the crosswalls. This results in stable maintenance of the plasmid during vegetative growth and morphological differentiation. The mechanism for conjugative transfer in *Streptomyces* differs from

the known mechanism for gram-negative bacteria, which involves pili to establish cell-to-cell contact and rolling-circle replication to transfer single-stranded pDNA (Madigan et al., 2009). In *Streptomyces*, the mycelial tips grow together and the conjugal DNA transfer is mediated by a plasmid-encoded DNA translocator, which is a membrane-associated protein localized at the hyphal tip of *Streptomyces* mycelium. This protein is an ATPase that transfers an unprocessed double-stranded DNA molecule into the recipient by ATP hydrolysis (Reuther et al., 2006).

The conjugation method used during this project is based on shuttle vectors that are able to replicate in *E. coli* and integrate site-specifically into the *Streptomyces* genome. The finished constructs are first transferred to the methylation deficient *Escherichia coli* ET12567 strain, which carries a helper plasmid (pUZ8002) that provides transfer function from RP4 origin of transfer (*oriT*). The transformed ET cells are then plated out together with heat-shocked *Streptomyces* spores, since conjugation only takes place on solid media. *E. coli* ET12567 transfers the pDNA containing *oriT* into *S. venezuelae*, while the helper plasmid is not transferred (Flett et al., 1997). Once the constructs are introduced into the recipient, the bacteriophage VWB integrase gene (*int*) and VWB attachment site (*attP*) are responsible for the site-specific integration of pDNA into the *Streptomyces* genome. Plasmids containing *int* and *attP* from the VWB phage have been shown to integrate into the host chromosome by recombination with the chromosomal *attB* locus, which is situated within an arginine tRNA gene (Van Mellaert et al., 1998). This recombination event between *attP* (phage attachment site) and *attB* (bacterial attachment site) results in two new sites called *attL* and *attR*, as shown in Figure 1.4.

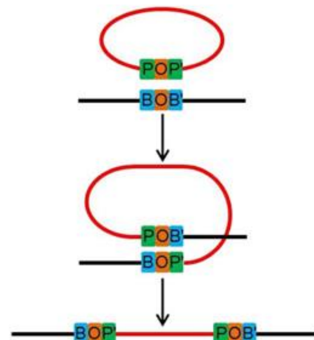


Figure 1.4: Integration of pDNA into chromosomal DNA by recombination between *attP* (POP) and *attB* (BOB) sites, resulting in formation of *attL* (BOP) and *attR* (POB) sites (Myronovskyi and Luzhetskyy, 2013).

Crossover between the *attP* site in the VWB-based vector and the *attB* site inside the *Streptomyces* genome is only possible in the presence of integrase. The VWB integrase belongs to the family of tyrosine recombinases, which recognize the different attachment sites even though they show limited similarity (Myronovskyi and Luzhetskyy, 2013). Tyrosine recombinase recognizes and binds to the specific attachments sites where it catalyzes cleavage, strand exchange and rejoining of the DNA fragments (Grindley et al., 2006).

1.6 BIOSENSORS

One practical application of synthetic biology is to construct biosensors for detection of specific compounds, screening for new drugs and analyze under which conditions a certain gene is expressed and a compound produced. Biosensors are devices that use biological receptors to detect certain signals, and respond by giving a measurable output. Most biosensors consist of two basic parts: a sensory element that detects and recognizes a certain (signal) molecule, and a transducer module that transmits and reports the signals. Cells have several regulatory circuits, including transcription, translation and post-translational mechanisms, for sensing and responding to environmental signals (reviewed in (Khalil and Collins, 2010)).

Transcriptional biosensors are constructed by fusing environment-responsive promoters to a reporter gene. Expression of the reporter gene is in this way controlled by a specific promoter, and reporter product can be quantitatively detected if the expression from this particular promoter is induced. The most commonly used reporters for actinomycetes are green fluorescent protein (GFP) and luciferase. GFP can be detected in real time in living cells and organisms simply by UV-light excitation. The advantage with GFP reporter is that no substrate is required, and the reporter protein is stable. However, GFP has low sensitivity due to background fluorescence of materials used in assays and because autofluorescence often can be observed in actinomycetes. This makes the analysis complicated due to a low signal-to-noise ratio (Myronovskyi et al., 2011).

Whole-cell biosensors are used to detect bioactive compounds in environmental samples (Hansen et al., 2001) and to screen for bioactive compounds interfering with major biosynthetic pathways in bacteria (Urban et al., 2007). A biosensor strain has been developed by fusing an inducible promoter to the luciferase (*luxCDABE*) operon of *Vibrio fischeri*. This biosensor responded to the presence of antibiotics with a certain core structure (macrolides) by expressing the luciferase operon, resulting in light emission from the cells. A suitable application of this biosensor strain is to find new producers of known macrolides or producers of new macrolide core structures, which can result in the discovery of new antibiotics (Möhrle et al., 2007).

The advantage with biosensors is that they can detect compounds even at small concentrations or verify their presence when no standard laboratory procedure for isolation, purification and verification of the compound exists. They are also particularly useful when the compound structure and function is unknown. The biosensor can detect when a certain gene is expressed if the reporter and the desired gene are placed under the same regulatory control. Thus, the genes will be expressed in parallel by the same induction mechanisms.

1.7 REGULATION OF ANTIBIOTIC EFFLUX PUMPS

Bacteria have developed many mechanisms to sense and respond to environmental signals and varying growth conditions. These adaptive responses are mostly mediated by transcriptional regulators which provide control over gene expression. Members of the TetR- family regulatory proteins control genes encoding products that give multidrug resistance and genes for biosynthesis of antibiotics (reviewed in (Ramos et al., 2005)). These repressors are important in antibiotic producing bacteria because they regulate the expression of antibiotic efflux pumps. Antibiotic efflux is only needed when antibiotic compounds accumulate intracellularly, either by diffusing into the cell or by antibiotic biosynthesis.

The TetR repressor in *E. coli* controls expression of the *tetA* gene, which encodes an antiporter efflux pump that transports tetracycline out of the cell. This regulatory network is presented in Figure 1.5. When no tetracycline is present in the cell, the TetR repressor is bound to the operator regions for *tetA* and *tetR*, thereby inhibiting expression of *tetA* by blocking for RNA polymerase. Expression of the repressor gene, *tetR*, is only slightly reduced when TetR is bound to its operator region. When tetracycline (Tc) is present in the cytosol, it interacts with the C-terminal domain of TetR, preventing DNA binding and thereby activating expression of the *tetA* resistance gene (Tahlan et al., 2008). The *tetA* gene will then be expressed and the transporter protein becomes integrated into the cell membrane where it starts to pump tetracycline out of the cell. After a while, most of the intracellular tetracycline is removed from the cell and the remaining Tc will diffuse from the TetR repressor, which then can block the *tetA* operator. This results in fewer transmembrane transporters, because they will be degraded after a while and the synthesis of new TetA molecules has stopped.

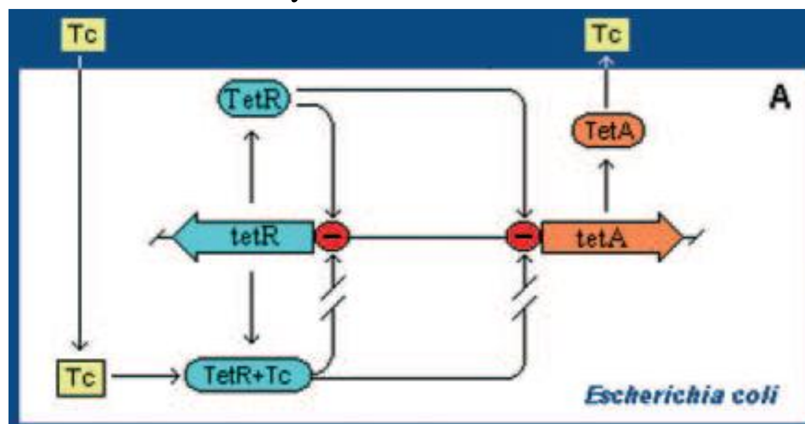


Figure 1.5: Regulation of *tetA* expression by the TetR repressor in *E. coli.* TetA is a transmembrane protein that transports tetracycline (Tc) out of the cell. Tetracycline acts as an inducer by binding to TetR and inhibiting its repression of the *tetA* and *tetR* genes (Ramos et al., 2005).

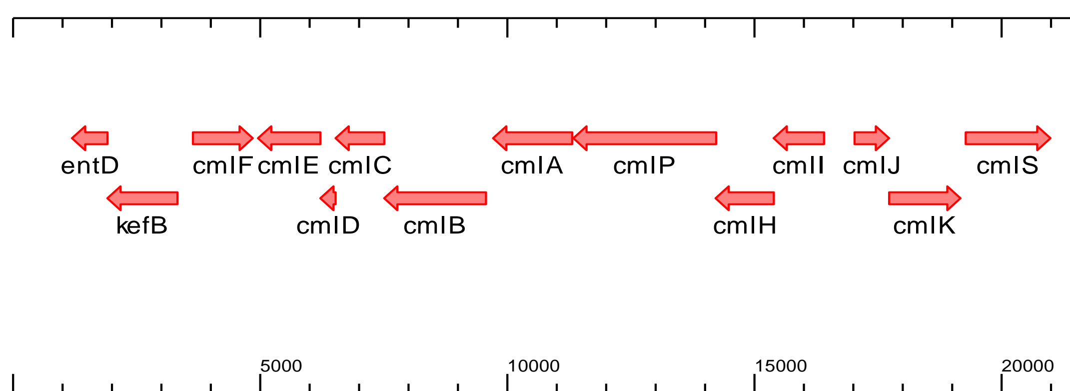
Several TetR-like regulators are present in different bacteria. ActR is a TetR-like protein in *S. coelicolor* which controls the expression of two actinorhodin exporters.

Many ligands, including the antibiotic actinorhodin, can bind to ActR and prevent its interaction with DNA, thereby inducing expression of antibiotic efflux pumps. This indicates that the *actR* locus can be activated by, and maybe evolved to confer resistance to other antibiotics (Tahlan et al., 2008).

1.8 PROJECT BACKGROUND AND IDEA

1.8.1 The Chloramphenicol cluster in *S. venezuelae*

The *S. venezuelae* genome contains 29 gene clusters for secondary metabolite biosynthesis, but most of them are silent under laboratory conditions. Only the gene clusters for biosynthesis of chloramphenicol (Cml) and jadomycin B (JadB) are expressed under laboratory conditions. The cluster for Cml biosynthesis is shown in Figure 1.6, and contains among others, genes encoding enzymes for Cml biosynthesis (*cmlD-cmlS*) (Piraee et al., 2004). Chloramphenicol biosynthesis starts from Chorismic acid which is made in the shikimate pathway. The *cmlE* gene encodes an enzyme that initiates the shikimate pathway and may therefore have a role in regulating this pathway to make precursors for Cml (He et al., 2001).



Cml cluster (21607 bps)

Figure 1.6: The chloramphenicol gene cluster contains genes encoding transporter proteins (*cmlF*, *kefB*) and enzymes for biosynthesis of chloramphenicol (*cmlD-cmlS*).

The amino acid sequence of the *cmlF* gene was analyzed in BLASTP by He *et al.* (2001). They showed that the sequence was strikingly similar to proteins encoded by Cml efflux genes in three other bacteria and the product of *cmlV*, which is located in another region of the *S. venezuelae* genome than the Cml gene cluster. By topological analysis of the CmlF product, they also showed that the protein contained 12-13 transmembrane domains similar to other Cml efflux proteins. These similarities suggest that the *cmlF* gene encodes a Cml efflux pump that releases the antibiotic into the environment and protects the cell from intracellular accumulation of this toxic compound (He et al., 2001). The CmlF transporter belongs to the major facilitator superfamily (MFS) of multidrug-resistance efflux pumps. Previous research has

demonstrated that Cml production in *S. venezuelae* was only marginally affected by disrupting the *cmlF* gene (He et al., 2001). This indicates the presence of other genes mediating Cml efflux or conferring resistance to Cml by inactivating the antibiotic. One reported resistance mechanism in *S. venezuelae* includes phosphorylation of chloramphenicol by Cml phosphotransferase (CPT), which also binds its product and transports it to the efflux pump (Izard, 2001).

The promoter sequence of the *cmlF* gene is divergent with the promoter for the *kefB* gene on the complementary strand. It is possible that these genes are controlled by the same regulatory mechanisms. The *kefB* gene is homologous to transmembrane Na^+ or K^+ antiporters in other organisms, but the same function is not verified for this gene in *S. venezuelae*. As mentioned previously in Chapter 1.3.1, the Mdfa multidrug transporter found in *E. coli* turned out to be a $Na^+(K^+)/H^+$ antiporter (reviewed in (Higgins, 2007)), so it remains to investigate whether KefB also can have several physiological roles in *S. venezuelae*. It is unknown under which conditions the *kefB* gene is expressed and whether it is coupled to expression of Cml. It would be interesting to explore how these two promoters are regulated, thus each promoter will be used in biosensor constructs.

Since genes encoding biosynthetic enzymes and transporter proteins have to be expressed in parallel to avoid toxic intracellular accumulation of antibiotics, these genes may be regulated by the same protein. It is therefore possible that expression of the CmlF and KefB transporters is regulated by a TetR-like protein, possibly (JadR$_2$ through JadR$_1$) from the JadB gene cluster. This regulation is explained in Chapter 1.8.4.

1.8.2 Regulation of JadB biosynthesis in *S. venezuelae*

Two distinct antibiotics are produced in *S. venezuelae* under different conditions. Cml production depends on the presence of nitrogen and glucose, while JadB is produced under stress conditions, such as addition of ethanol in the growth medium, heat shock or the presence of bacteriophage. The production of these two antibiotics is regulated by a pair of regulators situated in the jadomycin gene cluster.

JadB production is regulated by the TetR-like repressor $JadR_2$ (Yang et al., 1995) and by the $JadR_1$ activator (Yang et al., 2001). The $JadR_1$ activator seems to be required for jadomycin B production, but it is not expressed in the wild type strain under unstressed conditions, possibly due to repression by $JadR_2$. These regulatory interactions between $JadR_1$ and $JadR_2$ are presented in Figure 1.7. The $JadR_2$ protein is a TetR-like repressor (and a "pseudo" GBL-receptor) that recognizes and binds to the operator upstream of the *jad*R_1 gene, which encodes an activator for JadB biosynthesis genes. In that way, $JadR_2$ indirectly represses JadB production by inhibiting expression of the $JadR_1$ activator which is required for JadB biosynthesis.

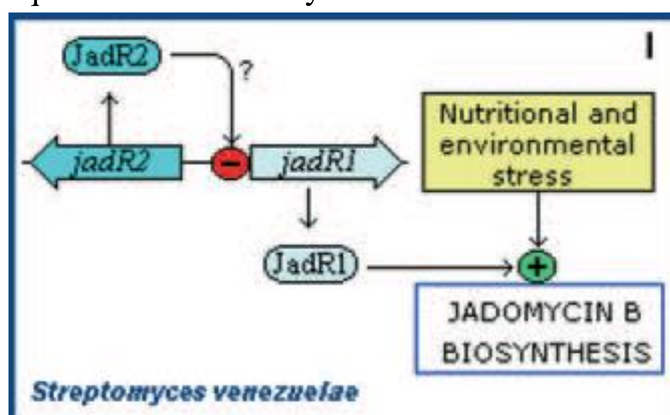


Figure 1.7: The TetR-like repressor $JadR_2$ in *Streptomyces venezuelae* regulates the expression of Jadomycin B biosynthesis genes by controlling expression of the *$jadR_1$* gene. $JadR_1$ acts as an activator for jadomycin B biosynthesis (Ramos et al., 2005).

1.8.3 γ-butyrolactone signaling molecules

Secondary metabolite biosynthesis in *Streptomyces* is often regulated by small signaling molecules called γ-butyrolactones (GBL), which can bind to cytoplasmic receptor proteins (reviewed in (Takano, 2006)). These GBL receptors often act as repressors by binding to operator regions and thereby inhibiting transcription of certain genes. When the diffusible GBL molecules bind to their respective receptors, gene expression is induced because the repressor can no longer bind to its operator site. The GBL-receptor is a regulatory protein that responds to external signals (GBL-molecules) and regulates genes encoding pathway-specific regulatory genes for antibiotic biosynthesis, collectively known as SARPs (*S*treptomyces *a*ntibiotic *r*egulatory *p*roteins), most of which are activators. This cascade of regulatory networks

may be different even for antibiotics with similar structure, because the regulatory mechanisms are more diverse than biosynthetic genes (Martín and Liras, 2010).

It has been speculated whether the GBLs act as quorum sensing molecules like the homoserine lactone autoinducers in Gram-negative bacteria (reviewed in (Bibb, 2005)). Quorum sensing is the cellular response to bacterial population density, which is detected by production and recognition of autoinducer molecules. When a high concentration of autoinducers is present in a population, they bind to receptors which activate the transcription of specific genes. However, the GBLs in *Streptomyces* are not only a communication method between members of the same species or an indication of the population density. The interaction between GBL signals and their respective receptors influences both antibiotic biosynthesis and sporulation, and perhaps they have a role in coordinating both secondary metabolism and morphological differentiation during the developing mycelial colony (Bibb, 2005). In *S. venezuelae*, one gene (*jadW*$_1$) found in the JadB gene cluster, is associated with production of GBL signaling molecules and was shown to control sporulation and antibiotic production (Wang and Vining, 2003). The jadW$_1$ component in the GBL system probably acts as a positive regulator for cellular differentiation, while the mechanism of influencing GBL synthesis is unknown.

1.8.4 Cross-regulation of JadB and Cml biosynthesis in *S. venezuelae*

Some antibiotics in sub-inhibitory concentrations have a general signaling role to induce changes in gene transcription in a bacterial population. The mechanisms by which they act are not completely revealed yet, but Xu et al. (2010b) demonstrated that antibiotics can act as signaling molecules just like the quorum sensing auto-inducers. Unlike usual GBL receptors, which only bind specific GBL molecules, "pseudo"-GBL receptors, such as JadR$_2$, coordinate antibiotic biosynthesis by binding and responding to different antibiotics. In *S. venezuelae*, JadR$_2$ was found to bind jadomycin A and B, which led to its dissociation from the *jadR*$_1$ promoter. Cml was, however, less effective than jadomycin A and B in inhibiting the DNA-binding properties of JadR$_2$ (Xu et al., 2010b).

The Cml biosynthetic gene cluster in *Streptomyces venezuelae* (Figure 1.6) has no cluster-situated regulators, so Xu et al. (2010b) investigated whether JadR$_2$ also regulates the Cml biosynthesis. They demonstrated that JadR$_2$ is a GBL receptor homologue in *S. venezuelae* that coordinates Cml and JadB biosynthesis by direct repression of *jadR*$_1$ expression. JadR$_2$ was found to indirectly activate chloramphenicol biosynthesis by inhibiting expression of *jadR*$_1$, which represses Cml production. The JadR$_1$ was shown to directly regulate both JadB and Cml biosynthetic pathways as shown in Figure 1.8.

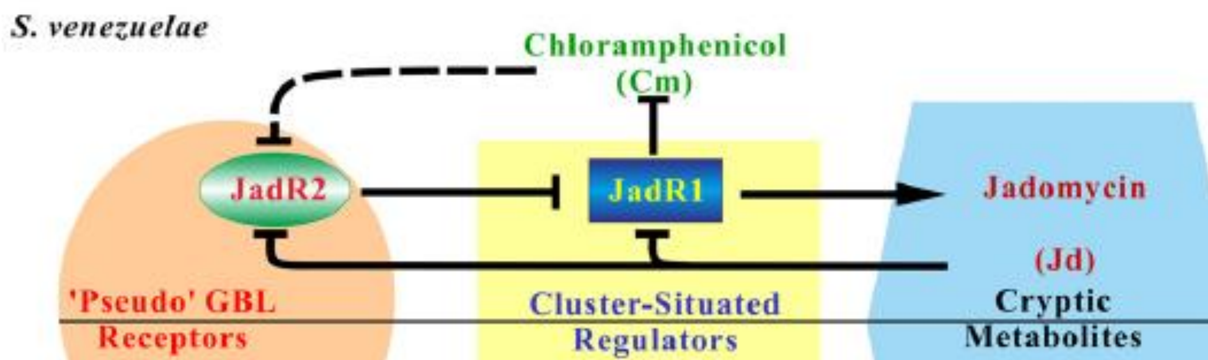


Figure 1.8: Coordination of chloramphenicol (Cml) and jadomycin B (JadB) biosynthesis by the pseudo γ- butyrolactone (GBL) receptor JadR$_2$ which represses the transcription of *jadR$_1$*. The cluster situated regulator JadR$_1$ activates the biosynthesis of JadB and represses the Cml biosynthetic genes (Xu et al., 2010b).

In the regulatory network of JadB and Cml biosynthesis, JadR$_2$ is the signal coordinator that senses metabolites and responds by regulating the transcription of *jadR*$_1$, which directly controls the antibiotic biosynthesis (Xu et al., 2010b). JadB was found to feedback regulate its own biosynthesis by interacting with JadR$_1$ (Wang et al., 2009). The "pseudo" GBL receptor JadR$_2$ could bind to Cml and JadB, and the interactions led to derepression of *jadR*$_1$, thus inducing expression of this cluster-situated regulator (Xu et al., 2010b). This feedback control is a mechanism for tight regulation and coordination of antibiotic biosynthesis, which ensures that only one of the two antibiotics, JadB or Cml, is synthesized at any time.

1.8.5 The aim of this Master thesis

Expression of genes encoding transporters for chloramphenicol (Cml) efflux is most likely induced in parallel with the genes encoding enzymes for Cml biosynthesis. In order to check this hypothesis, this Master thesis aims at constructing biosensors by placing a reporter gene under transcriptional control of the *cmlF* and *kefB* promoters. If the hypothesis is correct, the reporter gene will be expressed under Cml-producing conditions, and will be repressed when Cml-production is inhibited by addition of ethanol.

The project idea is illustrated in Figure 1.9. Promoters from the Cml cluster in *S. venezuelae* will be combined with a vector and a reporter gene to construct biosensor plasmids. A positive and a negative control plasmid will also be made to make sure that the results from the reporter assay are not random. These constructs are shuttle vectors that can replicate in *E. coli* strains and provide site-specific integration into the genome of *S. venezuelae*. The recombinant *S. venezuelae* strains will be grown under different conditions, and expression of the reporter gene will be analyzed by performing reporter assays as described in Chapter 1.9.2. Cml production will be measured by Ultra high Performance Liquid Chromatography (UPLC) analysis in parallel with the reporter assay.

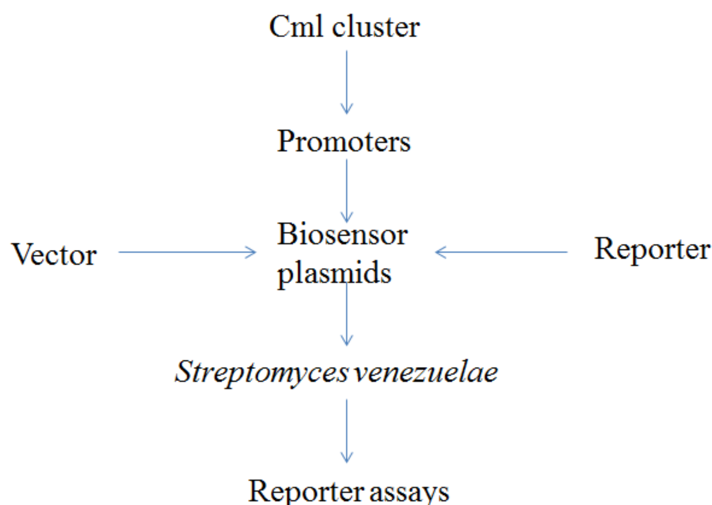


Figure 1.9: The project aims at constructing biosensor plasmids based on a shuttle vector, promoters from the chloramphenicol cluster and a reporter gene. These biosensors will be introduced into *S. venezuelae* and then reporter expression will be analyzed under different conditions by performing reporter assays.

The plasmids are constructed as model biosensors which principle of operation, if proven functional, can be used for analysis of other interesting gene clusters in *Streptomyces*. If the biosensors function as predicted, other promoters for efflux pump expression in silent gene clusters can be introduced into new biosensors. Then it may be easier to check under which conditions a silent secondary metabolite gene cluster is expressed. This can be a useful strategy to discover new drugs in *Streptomyces,* which are important sources for bioactive secondary metabolites.

1.9 METHODS TO BE EMPLOYED

1.9.1 DNA assembly method ('Gibson' reaction)

Methods for DNA assembly are important tools in synthetic biology. These techniques enable the reconstruction of natural pathways as well as combination of individual parts to create new genetic circuits with predictable properties. Modern DNA assembly techniques can be divided into methods that use restriction enzymes such as Golden Gate and Bio Brick, and the sequence independent protocols such as Gibson isothermal assembly and SLIC (Sequence and Ligase Independent Cloning) (reviewed in (Zotchev et al., 2012)). Recently, a novel cloning method called SLiCE (Seamless Ligation Cloning Extract) was reported to assemble several DNA fragments in a single *in vitro* reaction involving bacterial cell extract (Zhang et al., 2012).

In this work, DNA fragments are joined by the so-called 'Gibson' reaction, described by Gibson *et* al. (2009). They combined several linear DNA molecules with overlapping terminal sequences in a one-step isothermal reaction. The DNA fragments are added in equimolar amounts to a mix containing three enzymes, and this mix is incubated at 50 °C for one hour. The enzyme T5 exonuclease removes nucleotides from the 5´ends of double stranded DNA, leaving the complementary sequences open for annealing. Incubation at 50 °C inactivates the heat-labile T5 exonuclease after a while, and the overlapping fragments can anneal. Finally, Phusion polymerase will introduce nucleotides in the gap and *Taq* ligase seals the nicks, resulting in a seamless DNA molecule. This 'Gibson' reaction is described in Figure 1.10.

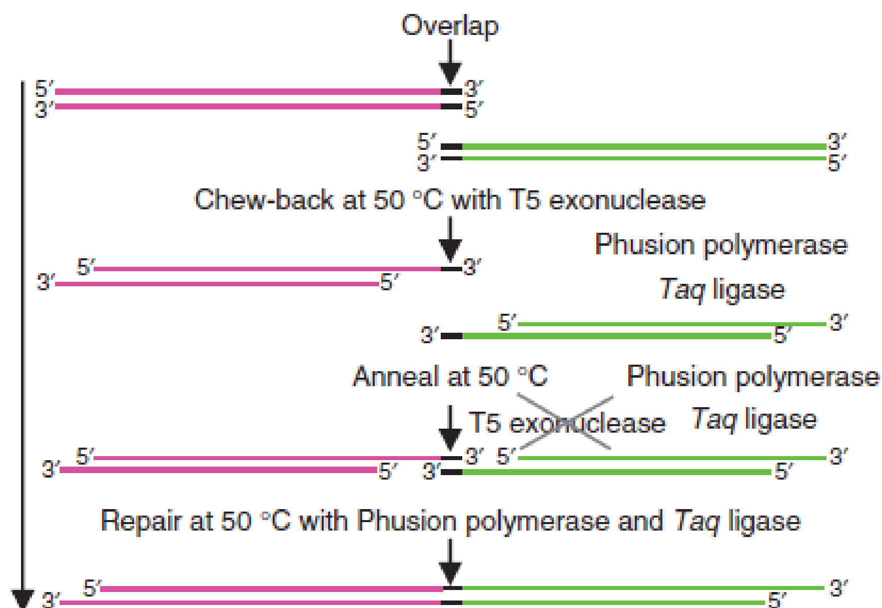


Figure 1.10: One-step isothermal *in vitro* recombination. DNA fragments with terminal sequence overlaps (black) were joined into one molecule in a one-step reaction. Three enzymes contribute to the reaction. T5 exonuclease chews back nucleotides from the 5´ends of double stranded DNA molecules until the enzyme is inactivated. The complementary single-stranded DNA anneal, Phusion DNA polymerase filles the gaps with nucleotides and *Taq* DNA ligase seals the nicks (Gibson et al., 2009).

1.9.2 Reporter assay

Ideal reporter assays are sensitive, quantitative, reproducible, easy, rapid and safe (reviewed by (Schenborn and Groskreutz, 1999)). The most commonly used reporters for actinomycetes are GFP and luciferase. However, the GFP reporter gene is not ideal for actinomycetes, because of low sensitivity. The luciferase assays are not optimal either, due to the complexity of enzymatic reactions, which require multiple reagents. In addition, the transcriptional level cannot be quantitatively detected because there is no enzymatic amplification of the light emitting signal (Myronovskyi et al., 2011).

In this project, the reporter gene *gusA* encoding β-Glucuronidase (GUS) will be used in the biosensors. Using GUS as a reporter has many advantages: it is highly sensitive, stable and offers high specific enzyme activity without any cofactors. In addition, the enzyme is tolerant to commonly used chemicals and assay conditions, and most streptomycetes do not possess any endogenous GUS activity. The GUS reporter assay is simple, sensitive and inexpensive with many available substrates for different types of assays (Myronovskyi et al., 2011). A spectrophotometric assay will be used in this project, but the GUS assay can also be fluorometric and chemiluminescent, depending on the substrate. The substrate for analyzing expression of *gusA* in this project is p-nitrophenyl-β-D-glucuronide (PNPG). The GusA enzyme cleaves PNPG, yielding β-D-glucuronic acid and *p*-nitrophenol as described in Figure 1.11. The latter is a chromogenic compound that has a maximum absorbance at 405 nm (Aich et al., 2001).

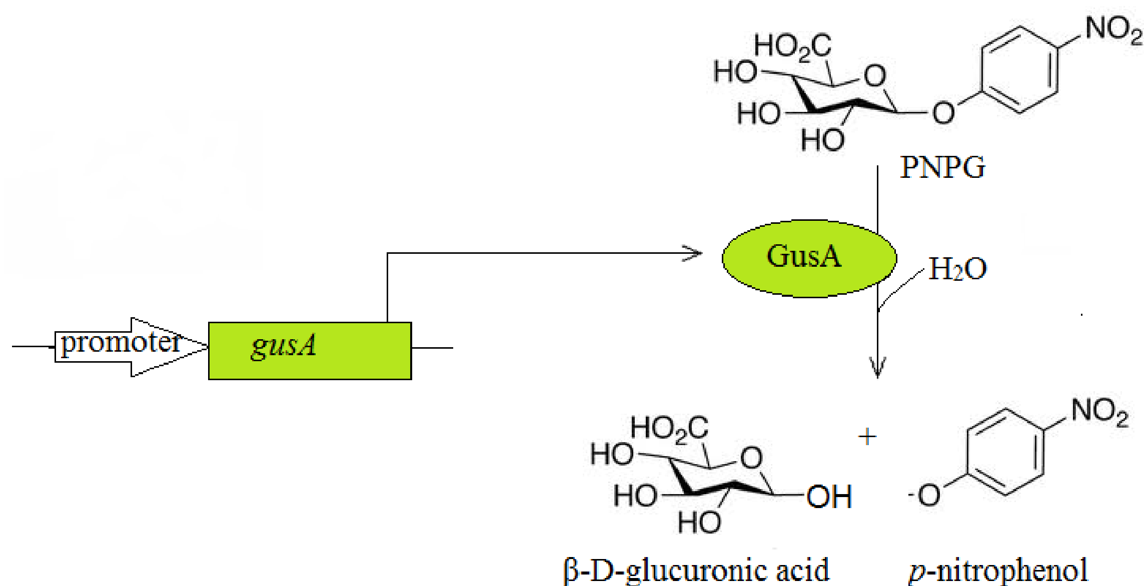


Figure 1.11: The GusA reporter assay. If the *gusA* gene is expressed in one of the biosensor constructs, the GusA enzyme will catalyze the reaction from p-nitrophenyl-β-D-glucuronide (PNPG) to β-D-glucuronic acid and p-nitrophenol, which has a maximum absorbance at 405 nm.

2 MATERIALS AND METHODS

2.1 CHEMICALS AND EQUIPMENT

The chemicals and laboratory equipment that were utilized are given in Table 2.1.1 and 2.1.2, respectively.

Table 2.1.1: Chemicals and enzymes used for the laboratory work.

Chemicals	Producer
*Aat*II	New England Biolabs Inc.
Acetic Acid	SdS
Agar Bacteriological	OXOID LTD
Ampicillin sodium salt	BioChemica, AppliChem
BSA	New England Biolabs Inc.
Chloramphenicol	AppliChem
Difco ISP Medium 4	Becton, Dickinson and Company
DMSO	Sigma-Aldrich
dNTP's	Promega
*Dpn*I	New England Biolabs Inc.
DTT (Dithiothreitol)	VWR
*Eco*O109I	New England Biolabs Inc.
EDTA (0.5 M)	Merck
Ethanol (96 %)	VWR
Ethyl acetate HiPerSolv Chromanorm for HPLC	VWR
Expand High Fidelity (EHF) DNA polymerase	Roche
GC-rich PCR buffer	Roche
GC-rich resolution solution	Roche
GelGreen Nucleic Acid Stain (10 000x) (Cat: 41005)	Biotium
Gene ruler™ DNA ladder mix (Lot. 2702)	Fermentas
Glycerol bidistillied (99.5 %)	AnalaR NORMAPUR, VWR Prolab
High Fidelity 2x Long PCR premixes (1-9)	Epicentre
Isopropanol	Arcus
Kanamycin	AppliChem
Lysozyme (> 30 000 FIP U/mg)	Merck
Malt extract	Sigma-Aldrich
Maltose (Lot 109H1049)	Sigma-Aldrich
MasterAmp Extra-Long DNA Pol. Mix (2.5 U/µl)	Epicentre
Methanol LC-MS Chromasolv (> 99.9 %)	Sigma-Aldrich
$MgCl_2$ (25mM)	Roche
MOPS sodium salt (99 %)	AppliChem
NaCl	VWR
$Na_2HPO_4 * 2H_2O$ (99.5 %)	Merck
NAD (100 mM)	Sigma-Aldrich
$NaH_2PO_4 *H_2O$ (99.0 %)	Merck

Chemicals	Producer
Nalidixic acid sodium salt	Sigma-Aldrich
NaOH (99 %)	Merck
NEB buffer 3 and 4	New England Biolabs Inc.
Phusion HF DNA polymerase (2 000 U/ml)	New England Biolabs Inc.
p-nitrophenyl-β-D-glucuronide (PNPG) (99.4 %)	CalbioChem
Polyethyleneglycol (PEG8000)	FLUKA
Primers (Attachment C)	Sigma-Aldrich
*Pst*I	New England Biolabs Inc.
SeaKem LE Agarose (Catalog no. 50004)	Cambrex Bio Science Rockland, Inc.
T5 exonuclease (10 U/µl)	New England Biolabs Inc.
Taq DNA ligase (40 000 U/ml)	New England Biolabs Inc.
Thiostrepton from *Streptomyces aureus* (min. 90 % HPLC)	Sigma-Aldrich
Tris-base	Roth
Triton X-100 SigmaUltra	Sigma-Aldrich
Tryptone	OXOID LTD
Tryptone soya broth (TSB)	OXOID LTD
*Xma*I	New England Biolabs Inc.
Yeast extract	OXOID LTD

Table 2.1.2: Equipment used in the laboratory.

Equipment	Specification	Producer
Autoclave	SX-500E	Tomy
Cryo vials		Greiner bio-one
Cyvettes	(0.1 cm gap)	Bio-Rad
DNA gel electrophoresis power source	Power PAC	Bio-Rad
DNA gel electrophoresis systems	Owl Easycast B1A Mini	Thermo scientific
Eppendorf tubes		Sarstedt
Freezer (- 20 °C)		Electrolux
Freezer (- 80 °C)	C66085	New Brunswick Scientific
GelDoc 2000		Bio-Rad
Heat incubators (30 °C, 37 °C)		ASSAB
Microcentrifuge	5415 R	Eppendorf AG
PCR machine		VWR
Petri plates		Gosselin
pH-meter	PHM92	Unigen
Pipette tips	10 µl	Molecular BioProducts
Pipette tips	200 µl, 1 ml	Sarstedt
Pipettes		Eppendorf
Pyrex baffled Erlenmeyer flask	250 ml	Sigma-Aldrich
QIAEX II Suspension	Lot no. 133214960	QIAGEN
QIAquick Gel Extraction Kit		QIAGEN
QIAquick PCR purification kit		QIAGEN
QIAquick spin columns, collection tubes		QIAGEN
Shaking incubators (30 °C, 37 °C)	28573	Infors HT multitron
Spectrophotometer	SpectraMax Plus 384	Molecular Devices

Equipment	Specification	Producer
SpeedVac Concentrator	Savant SPD 2010	Thermo Electron Corporation
Vortex		Heidolph
Wizard Genomic DNA Purification Kit		Promega
Wizard *Plus* SV Minipreps DNA Purification Kit		Promega
Wizard SV Minicolumns		Promega

The bacterial strains and plasmids that were used are given in Table 2.1.3 and 2.1.4, respectively. Plasmid maps are given in Attachment D (page 81).

Table 2.1.3: The characteristics of bacterial strains used.

Bacterial strains	Genotype/ phenotype	Source/ reference
Escherichia coli DH5α	High efficiency transformation strain. Genotype: *supE44 ΔlacU169 recA1 endA1 gyrA96 thi-1 relA1*	(Reisner et al., 2003)
Escherichia coli DH10B	High transformation efficiency and maintenance of large plasmids. Genotype: *F^- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ^- rpsL (Str^R) nupG*	(Wu et al., 2010)
Escherichia coli EC100	TransforMax™ EC100™ Electrocompetent E. coli from Epicentre, Catalog No. EC10010 Genotype: *F^- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ^- rpsL (Str^R) nupG*	Epicentre
Escherichia coli ET125671 (pUZ8002)	Methylation deficient (*dam^-*, *dcm^-*, *$hsdM^-$*), contains helper plasmid pUZ8002 (Kan^R, Cml^R) which mediates conjugative DNA transfer from RP4 oriT.	(MacNeil et al., 1992)
Streptomyces venezuelae ATCC 10712 (ISP5230)	Wild type, GC-rich, linear chromosomes, produces Chloramphenicol and Jadomycin B.	

Table 2.1.4: A list of the plasmids used in this master thesis.

Plasmids	Characteristics	Source
pUC59 (4750 bp)	T7.3_GUS Reporter gene (*gusA*), Amp^R	Synthetic gene, UC Berkeley USA
pGEM7ermLi	Strong constitutive promoter for the gene *ermE* (resistence to erythromycin)	C.R. Hutchinson, Wisconsin Madison USA
pSOK805 (6562 bp)	Based on pKT02, with *oriT* from pSOK804 Amp^R, $Thio^R$, *RP4 oriT*, *attP, int, ColElori*	(Van Mellaert et al., 1998, Sekurova et al., 2004) Made during the project work
pSOK807 (6861 bp)	pSOK805-*ermE**p: Amp^R, $Thio^R$, *RP4 oriT*, *attP, int, ColElori*	This work
Construct 1 (8803 bp)	pSOK805-*ermE**p-*gusA*, Amp^R, $Thio^R$, *RP4 oriT*, *attP, int, ColElori*	This work
Construct 2 (8781 bp)	pSOK805-*cmlF*p-*gusA*, Amp^R, $Thio^R$, *RP4 oriT*, *attP, int, ColElori*	This work
Construct 3 (8738 bp)	pSOK805_*kefB*p-*gusA*, Amp^R, $Thio^R$, *RP4 oriT*, *attP, int, ColElori*	This work
Construct 4 (8401 bp)	pSOK805-*gusA*, Amp^R, $Thio^R$, *RP4 oriT*, *attP, int, ColElori*	This work

2.2 PROCEDURES

The aim of this Master thesis is to make biosensor plasmids by placing a reporter gene under the transcriptional control of promoters upstream of transporter genes in the Cml gene cluster. A list of the procedure and specific methods used is given below. All the methods are described in detail in Chapter 2.3 and the recipes for making media, buffers and stock solutions are given in Attachment A and B (at page 74 and 76).

2.2.1 Construction and verification of the biosensor vectors

Four vectors with the following characteristics were constructed, and their plasmid maps are presented in Figure 2.2.1 and in Attachment D (page 81). These vectors will hereby be referred to as construct 1, 2, 3 and 4, respectively.

1. Positive control: pSOK805– *ermE** (constitutive) promoter– *gusA* reporter gene
2. Biosensor: pSOK805 – *cmlF* promoter – *gusA* reporter gene
3. Biosensor: pSOK805 – *kefB* promoter – *gusA* reporter gene
4. Negative control: pSOK805 – *gusA* reporter gene

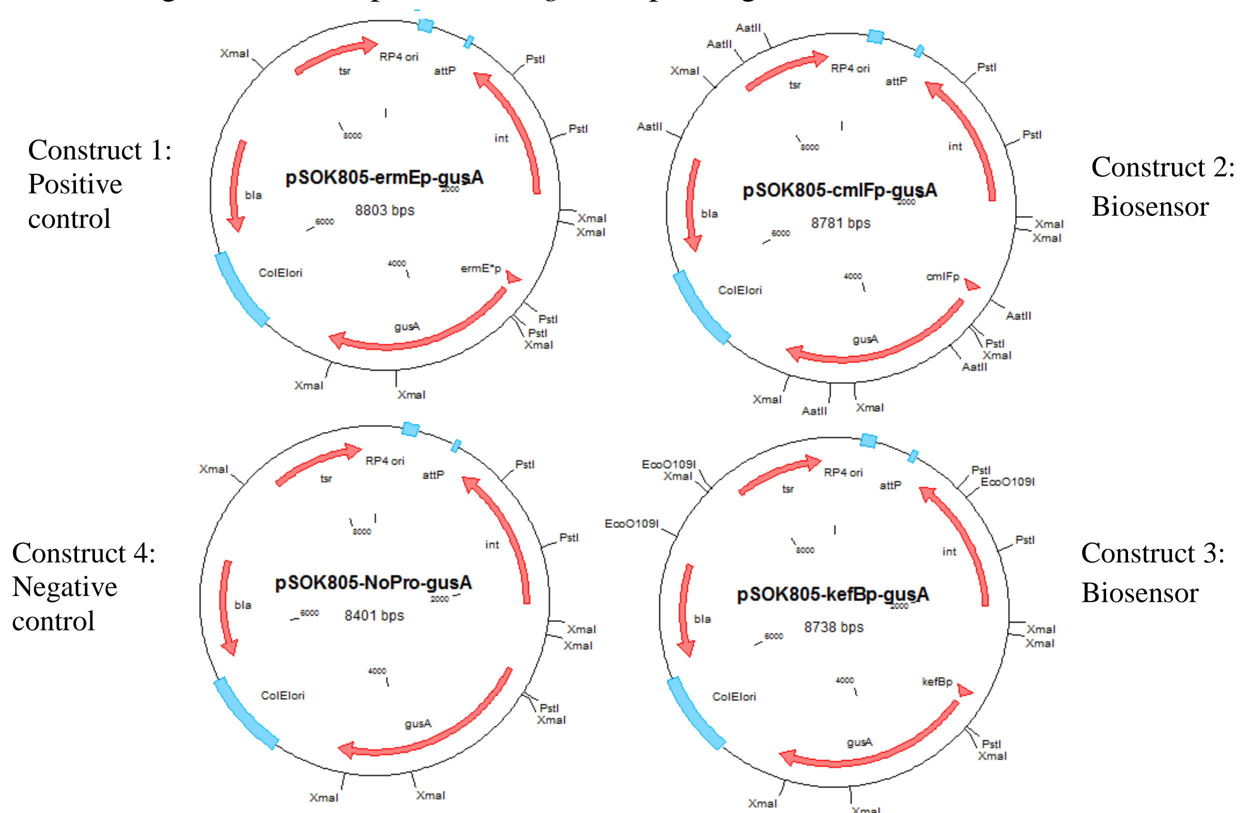


Figure 2.2.1: Biosensor and control constructs for reporter assay analysis. The upper and lower left constructs are for positive and negative control, containing a strong promoter and no promoter, respectively. The plasmids to the right are biosensor constructs which contain promoters controlling transporter genes in the Cml gene cluster. All the constructs contain origin of replication (*ColElori*) and Ampicillin resistance gene (*bla*) for replication and selection in E. *coli* strains. They also contain origin of transfer (*RP4 oriT*) as well as an integrase gene (*int*) and attachment site (*attP*) which provides site-specific integration of the plasmid DNA into the genome of *S. venezuelae*. The *tsr* gene confers resistance to thiostrepton for selection of *S. venezuelae* transconjugants, and the *gusA* reporter gene encodes a β-Glucuronidase enzyme.

Due to some problems with assembling construct 1 in a one-step reaction, a new strategy was proposed to ensure the construction of this plasmid. The three fragments should be assembled together in two steps. First vector and promoter were assembled to result in pSOK807 (pSOK805-*ermE**p). This new plasmid was PCR-amplified to ensure overlapping terminal sequences with the reporter gene, and the fragments were combined by the 'Gibson' reaction. The general procedure for construction and verification of the biosensor constructs is described below.

1. **Amplification of DNA fragments by PCR:**
   Templates for PCR-amplification of the *cmlF* and *kefB* promoters were made by isolation of genomic DNA from *S. venezuelae* (wild type), as described in Chapter 2.3.3. The PCR-mixes were made as described in section 2.3.4 and the PCR-programs are given in Table 2.3.5 and 2.3.6. Primer specifications are given in Attachment C (page 80). A small amount (3 µl) of each PCR-product was analyzed by DNA gel electrophoresis, described in section 2.3.5, to check for byproducts.
2. **Isolation and purification of DNA fragments from PCR:**
   The PCR-product with vector and reporter gene fragments also contained several byproducts, as indicated by several bands on the agarose gel after DNA gel electrophoresis. These fragments were therefore isolated from the agarose gel as described in Chapter 2.3.6. The promoter fragments were pure enough to be isolated directly from the PCR mixes, as described in Chapter 2.3.7.
3. ***Dpn*I-treatment of the purified vector and reporter fragments:**
   The PCR-amplified pSOK805 vector and the *gusA* reporter fragments were digested with *Dpn*I in order to digest the original, methylated DNA templates. This is important to ensure that the PCR-templates were removed, because they contained an ampicillin resistance gene. The procedure for *Dpn*I-treatment is described in section 2.3.12.
4. **'Gibson' assembly and transformation of *E. coli*:**
   'Gibson' ligation of the fragments is described in Chapter 2.3.11 (and 1.9.1). After the isothermal reaction, the ligation mixes were transformed into *E. coli* cells (DH5α, DH10B or EC100), as described in Chapter 2.3.9. The transformation mixes were plated on LA with ampicillin (Amp), and placed for overnight incubation at 37 °C. The chemically competent and electrocompetent *E. coli* DH10B cells were prepared as described in Chapter 2.3.8.
5. **Isolation and verification of constructs:**
   Ampicillin-resistant clones were selected and inoculated overnight as described in Chapter 2.3.1. Plasmid DNA (pDNA) was isolated from the overnight cultures as described in Chapter 2.3.10, and then digested with one or several restriction enzymes as described in Chapter 2.3.12. In order to verify the plasmids, the digestion mixes were analyzed by gel electrophoresis as described

in Chapter 2.3.5. The resulting fragments were compared to the ladder band sizes (given in Attachment E, page 83) and to the expected fragment sizes which are given in Table 2.3.12 - 2.3.14 in Chapter 2.3.12.

2.2.2 Conjugative DNA transfer from *E. coli* ET12567 into *S. venezuelae*

1. **Cloning the constructs into *E. coli* ET12567 cells:**
   Preparation of chemically competent *E. coli* ET12567 (pUZ8002) cells was performed as described in section 2.3.8. The constructs were first introduced into ET12567 cells as described in Chapter 2.3.9. The transformation mixes were plated on LA with Amp, Cml and kanamycin (Kan) to select for the two plasmids (original pUZ8002 and construct 1, 2, 3 or 4) and placed at 37 °C for overnight incubation. Three resistant clones were selected and plated on LA with Amp, Cml and Kan. The next day, one well-grown ET12567 clone was chosen for conjugation. Overnight cultures were also prepared from these three colonies in order to make glycerol stocks as described in Chapter 2.3.2.
2. **Conjugative DNA transfer:**
   An ISP4 plate with *S. venezuelae* wild type (wt) was prepared 1-2 days ahead of the planned conjugation, in order to prepare a fresh spore suspension for this procedure. A glycerol stock of *S. venezuelae* wt could also be used for conjugation, but it needs more time to grow prior to selection with antibiotics. The conjugative DNA transfer from *E. coli* ET12567 into *S. venezuelae* is described in Chapter 2.3.13. The transconjugants were picked and transferred to ISP4 medium supplemented with nalidixic acid (Nal) to select against *E. coli* cells and thiostrepton (Thio) to select for *S. venezuelae* with the construct inserted into its genome. Glycerol stocks of the transconjugants were made as described in Chapter 2.3.2, in order to store the strains at – 80 °C.

2.2.3 Analysis of chloramphenicol production in cultured *S. venezuelae*

Overnight cultures of *S. venezuelae* transconjugants with the inserted constructs were made by inoculating a strain (either from the glycerol stock or from a fresh spore suspension) in TSB medium (10 ml). The conjugative transfer of construct 1 (positive control) into *S. venezuelae* did not result in any real transconjugants that could grow on ISP4 medium supplemented with Thio. Time was running out, so some spores were picked up from an ISP4 plate with Nal and Thio, and inoculated in TSB (10 ml) supplemented with Thio (30 μg/ml). The strain with construct 1 did not grow at 30 °C overnight, so it was not cultured as the other strains. The overnight cultures of *S. venezuelae* with introduced construct 2, 3 and 4 were inoculated in MYM medium (2 × 50 ml) and grown in baffled flasks at 30 °C for 10 hours, before ethanol (6 %, v/v) was added to half of the cultures. Twelve hours after addition of ethanol, 6 × 1 ml samples were collected from each flask. Three of them were prepared for Ultra high Performance Liquid Chromatography (UPLC) analysis as explained in Chapter 2.3.14,

and the other 3 parallels were used for reporter assay analysis. Four days after inoculation into MYM medium, the same amount of samples were collected once more and prepared for the two analysis methods.

2.2.4 Reporter assay

While culturing the different transconjugants to analyze the Cml production, samples were also collected for the reporter assay analysis and stored at -20 °C. This was performed in order to give comparable results. The protocol for analysis of reporter gene expression is described in Chapter 2.3.15.

2.3 PROTOCOLS

The laboratory protocols are described in the following subchapters. Recipes for the media, buffers and stock solutions used are listed in Attachment A and B (page 74 and 76), respectively.

2.3.1 Overnight cultures

Overnight cultures are used to increase the cell concentration in order to isolate genomic DNA, pDNA or make a glycerol stock. The following procedure describes how to make overnight cultures of *S. venezuelae* and different *E. coli* strains.

Materials:

- ✓ Bacterial strain from freezer, colony from an agar plate or previous overnight culture
- ✓ Growth medium: LB for *E. coli*, TSB for *S. venezuelae* ATCC 10712
- ✓ Antibiotics for selection: Ampicillin (Amp), Chloramphenicol (Cml), Kanamycin (Kan)
- ✓ Sterile toothpicks or loops

Overnight cultures can be made by inoculating a strain from the freezer, a colony from an agar plate, or from a previous overnight culture. *E. coli* was inoculated in LB medium (2 ml), with a certain antibiotic for plasmid selection, and incubated overnight in a 37 °C shaker (225 rpm). In order to cultivate *E. coli* strains transformed with 'Gibson' ligation mixes, LB medium (2 ml) was added Amp (100 mg/ml, 2 µl) to select for cells containing pDNA with an Amp^R gene. *E. coli* ET12567 contains a helper plasmid which has to be selected for by adding Cml (30 mg/ml, 2 µl) and Kan (40 mg/ml, 1 µl) to 2 ml of LB medium, in addition to Amp (100 mg/ml, 2 µl).

S. venezuelae was inoculated in TSB medium (2 ml) and incubated overnight in a 30°C shaker (225 rpm).

2.3.2 Glycerol stocks

Bacterial strains can survive for several years if they are stored at -80 °C in a glycerol solution. The following procedure describes how to make glycerol stocks of *E. coli* and *S. venezuelae* strains.

Materials:

- ✓ Overnight culture (*E. coli*) or fresh spores from an ISP4 plate (*S. venezuelae*)
- ✓ Sterile 20 % Glycerol solution
- ✓ Sterile cryo vials
- ✓ Sterile cotton wool filters (*S. venezuelae*)
- ✓ Centrifuge

Protocol for *E. coli* glycerol stocks:

1. The overnight culture (1.5 ml) was transferred to an Eppendorf tube under sterile conditions.
2. The tube was centrifuged (13 000 rpm, 4 min), followed by removing the supernant.
3. The cells were resuspended in 20 % Glycerol (1.5 ml) and the cell suspension was transferred to a cryo vial for storage at -80 °C.

Protocol for *S. venezuelae* glycerol stocks:

1. A glycerol solution (20 %, 4 ml) was applied onto an ISP4 plate with fresh *S. venezuelae* spores and the plate was rubbed with light movements to detach the spores from the plate.
2. The spore suspension was filtered through a sterile cotton wool filter and the filtrate was transferred to a cryo vial for storage at -80 °C.

2.3.3 Isolation of genomic DNA

Isolation of genomic DNA from *Streptomyces venezuelae* ATCC 10712 was performed by using the Wizard® Genomic DNA Purification Kit from Promega. The protocol for Gram positive Bacteria was followed as described in Attachment F1.

Materials:

- ✓ Wizard Genomic DNA Purification Kit
- ✓ Overnight cell culture of *Streptomyces venezuelae* ATCC 10712
- ✓ Sterile Eppendorf tubes (1.5 ml)
- ✓ Isopropanol
- ✓ Ethanol (70 %)
- ✓ Lysozyme (10 % in 50 mM EDTA)
- ✓ Water bath at; 37 °C, 65 °C and 80 °C.
- ✓ Centrifuge

2.3.4 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is used for *in vitro* amplification of certain DNA fragments. The reaction is based on temperature cycles where DNA is denatured, annealed to primers and then elongated by DNA polymerase (Madigan et al., 2009). Different polymerase mixes were used to amplify fragments of varying lengths. Most fragments were amplified with Expand High Fidelity DNA polymerase. This enzyme mix contains thermostable Taq DNA polymerase and thermostable Tgo DNA polymerase which has proofreading activity (Roche, 2011). Long vector fragments (6-8 kb) were sometimes problematic to amplify. In those cases, MasterAmp Extra-Long DNA Polymerase Mix was used to increase the amount of PCR product. This polymerase mix also contains thermostable Taq DNA polymerase and unspecified proofreading polymerase(s).

Materials:

- ✓ Template: plasmid DNA, genomic DNA etc. which contains the desired sequence
- ✓ Primers (10 μM): forward and reverse (given in Attachment C)
- ✓ Thermostable DNA polymerases
- ✓ Deoxyribonucleotides (dNTP's, 10mM): 4 types
- ✓ Buffers: GC-rich for amplification of *Streptomyces* genes, High Fidelity 2× Long PCR premixes for long fragments
- ✓ PCR machine

Reaction mixes:

The PCR-mixes in Table 2.3.1 were used for amplification of (pSOK805) vector fragments for the biosensor constructs. These long vector fragments were amplified with the PCR-program "Ingrid2", which is described in Table 2.3.5.

Table 2.3.1: PCR-mixes for amplification of vector fragments with PCR-program "Ingrid2".

Vector fragments: **Ingredients**	**Construct 1: pSOK805 amount [μl]**	**Construct 2: pSOK805 amount [μl]**	**Construct 3: pSOK805 amount [μl]**	**Construct 4: pSOK805 amount [μl]**
Template (pSOK805)	1	1	1	1
Primers (see Attachment C)	1+1	1+1	1+1	1+1
ds H_2O	8	7.5	8	8
DMSO	0.5	0.5	0.5	0.5
$MgCl_2$		0.5		
High Fidelity 2x Long PCR premix:	nr. 5: 12.5	nr. 9 : 12.5	nr. 9: 12.5	nr. 9: 12.5
EHF Polymerase	1		1	1
MasterAmp Extra-Long Pol. Mix		1		
Total volume	25	25	25	25

The different promoters for construct 1, 2 and 3 were amplified by using the PCR-mixes given in Table 2.3.2. The PCR-programs "Ingrid1" and "SZ3" were used as indicated in the table and these programs are described in Table 2.3.5 and 2.3.6, respectively. The pGEM7ermLi plasmid was used as template for the strong constitutive promoter (*ermE**p). The genomic DNA of *Streptomyces venezuelae* was isolated as described in section 2.3.3, and used as template for the promoters in construct 2 and 3 (*cmlF*p and *kefB*p). Construct 4 has no promoter since it is the negative control plasmid and should not express the reporter gene under any conditions.

Table 2.3.2: PCR-mixes for amplification of the promoters for construct 1, 2 and 3.

Promoter fragments: **Ingredients**	**Construct 1:** ***ermE*p amount [µl]**	**Construct 2:** ***cmlF*p amount [µl]**	**Construct 3:** ***kefB*p amount [µl]**
Template (pGEM7ermLi (c.1), *S. venezuelae* genomic DNA (c.2, 3))	1	1	1
Primers (see Attachment C)	1+1	1+1	1+1
ds H_2O	8	6.5	6.5
DMSO	0.5	0.5	0.5
High Fidelity 2x Long PCR premix nr. 7	12.5		
dNTP's (10 mM)		1	1
GC-rich buffer (10x) with $MgCl_2$		4	4
GC-rich resolution solution		4	4
EHF Polymerase	1	1	1
Total volume	25	20	20
PCR-program	"Ingrid1"	"SZ3"	"SZ3"

Amplification of the reporter genes was performed with the PCR-mixes given in Table 2.3.3. The plasmid template (pUC59) was diluted 5 times prior to the PCR-mix for construct 2, due to a high plasmid concentration. A plasmid map of this template is presented in Attachment D (page 81). The PCR-programs "Ingrid1" and "Ingrid4" were used to amplify the *gusA* gene fragments, and these programs are described in Table 2.3.5 and 2.3.6, respectively.

Table 2.3.3: PCR-mixes for amplification of the *gusA* reporter gene fragments. Different PCR-programs were used as indicated in the last row.

Reporter fragments: **Ingredients**	**Construct 1: GusA_erm amount [µl]**	**Construct 2: GusA_cml amount [µl]**	**Construct 3: GusA_kef amount [µl]**	**Construct 4: GusA_noPro amount [µl]**
Template (pUC59)	1	1 (5 x diluted)	0.5	0.5
Primers (see Attachment C)	1+1	1+1	1+1	1+1
ds H_2O	8	8	8.5	9
DMSO	0.5	0.5	0.5	
HF 2x Long PCR premix	nr. 8: 12.5	nr. 8: 12.5	nr. 5, 7: 12.5	nr. 8: 12.5
EHF Polymerase	1	1	1	1
Total volume	25	25	25	25
PCR-program:	"Ingrid4"	"Ingrid4"	"Ingrid1"	"Ingrid4"

Construct 1 (the positive control plasmid) was assembled in two steps. First, the PCR-amplified pSOK805 vector fragment was assembled with the *ermE** promoter by the 'Gibson' reaction, resulting in a new vector called pSOK807. Then, this new vector was PCR-amplified to enable introduction of the *gusA* gene fragment downstream of the promoter, resulting in the right construct 1. The PCR-mix for amplification of the assembled pSOK807 plasmid is given in Table 2.3.4.

Table 2.3.4: The PCR-mix for amplification of pSOK807 vector to ensure the final assembly of construct 1 in two steps.

Ingredients / Vector fragments:	Construct 1: pSOK807 amount [µl]
Template: pSOK807 colony 11	1
Primers: pSOK807-F and -R	1+1
ds H_2O	8
DMSO	0.5
High Fidelity 2x Long PCR premix nr. 7	12.5
MasterAmp Ex.-Long DNA Pol. Mix	1
Total volume	25
PCR-program:	"Ingrid2"

These PCR-amplified DNA fragments were analyzed by DNA gel electrophoresis and assembled with the 'Gibson' reaction as described in Chapter 2.3.11.

PCR-programs used:

The PCR-programs that were used to amplify different DNA fragments are specified in Table 2.3.5 and 2.3.6.

Table 2.3.5: The PCR-programs used for amplification of several DNA fragments. "Ingrid1" was used for amplification of small DNA fragments, while "Ingrid2" was used for longer vector fragments as pSOK805.

		Ingrid1		Ingrid2	
Step	Process	Temperature [°C]	Time [min]	Temperature [°C]	Time [min]
1	Denaturation	94	2	94	1
2	Continued denaturation	94	0.75	95	0.5
3	Annealing	58	1	56	1
4	Elongation	68	4	68	7
5	Continued elongation	72	6	68	8
6	Cool down	4	∞	4	∞
Repeated cycles (step 2-5)		35		30	

Table 2.3.6: PCR-programs used for amplification of DNA fragments. "SZ3" was used for amplification of the promoters for construct 1, 2 and 3, while "Ingrid4" was used for amplification of some *gusA* reporter genes.

		SZ3		Ingrid4	
Step	Process	Temperature [°C]	Time [min]	Temperature [°C]	Time [min]
1	Denaturation	95	5	95	1
2	Continued denaturation	95	1	95	0.75
3	Annealing	60	1	70	5
4	Elongation	72	4	70	5
5	Continued elongation	72	7	72	10
6	Cool down	4	∞	4	∞
Repeated cycles (step 2-5)		35		25	

2.3.5 DNA gel electrophoresis

Gel electrophoresis is a widely used technique to separate differently sized biological macromolecules, such as nucleic acids and proteins. DNA is negatively charged due to the phosphate groups, and will therefore migrate from the negative to the positive pole in an electric field (Madigan et al., 2009). Small fragments will migrate faster through the agarose gel than larger fragments because differently sized pores in the gel restrict migration of larger molecules the most (Klug et al., 2009). Hence, the DNA mix will be separated according to fragment sizes. During this work, gel electrophoresis was performed to check if the correct PCR product was amplified, and to check the purity and amount of isolated DNA.

Materials:

- ✓ 0.8 % Agarose gel
- ✓ 1 × TAE-buffer
- ✓ Gel electrophoresis equipment
- ✓ Gel Doc 2000
- ✓ Loading dye
- ✓ DNA ladder

Protocol:

Agarose solution was prepared as described in Attachment B3 (page 76). The gel was made by filling liquid 0.8 % agarose solution in a gel-form with an appropriate sized well-maker. After the gel had cooled down and stiffened, the gel was covered with 1×TAE-buffer. A DNA ladder (2 - 3 µl) was filled in the first well. DNA samples with loading dye were added to the following wells. To check a PCR-product, 3 µl of the PCR product was mixed with dsH_2O (7 µl) and loading dye (10x, 1 µl), before loading the suspension into a well in the gel. In order to isolate DNA from the agarose gel or analyze digested pDNA, the whole amount of sample was loaded in one well. The gel was run at 80-110 V for 40-90 minutes until sufficient separation of fragments was achieved. DNA bands were visualized with UV-light in Gel Doc 2000. By comparing the DNA bands with the ladder, the approximate size of the fragment can be estimated. The band sizes of the ladder used is given in Attachment E (page 83).

2.3.6 Isolation of DNA fragments from agarose gel

The DNA band of expected size was cut from the gel and purified with QIAquick Gel Extraction Kit or QIAEX II suspension from QIAGEN. The QIAEX II suspension was used for smaller amounts of PCR products. The two protocols are described in Attachment F2 (page 85).

Materials:

- ✓ QIAquick Gel Extraction Kit
- ✓ QIAGEN spin columns and collection tubes
- ✓ Isopropanol
- ✓ QIAEX II suspension
- ✓ Centrifuge

2.3.7 Purification of PCR product

When the PCR product was pure (indicated by only one DNA band after gel electrophoresis), the DNA could be purified directly from the PCR tube. For this purpose, QIAquick PCR Purification Kit from QIAGEN was used and the protocol is described in Attachment F3 (page 86).

Materials:

- ✓ QIAquick PCR Purification Kit
- ✓ QIAGEN spin columns and collection tubes
- ✓ Centrifuge

2.3.8 Preparation of competent *E. coli* cells

Competent cells are highly capable of accepting plasmids. *E. coli* DH10B was used for intracellular plasmid replication and *E. coli* ET12567 was used for conjugative DNA transfer into *S. venezuelae*.

Preparation of chemically competent *E. coli* DH10B and *E. coli* ET12567:

Materials:

- ✓ Glycerol stock of *E. coli* cells from the -80 °C freezer: DH10B or ET12567
- ✓ LB medium
- ✓ Plasmid DNA
- ✓ TSS-buffer
- ✓ Ice
- ✓ Cold centrifuge

Protocol:

1. *E. coli* DH10B or ET12567 cells from a glycerol stock were inoculated in LB medium (2 ml). The ET12567 cells were incubated with Cml (30 mg/ml, 2 µl) and Kan (40 mg/ml, 1 µl) to select for the helper plasmid. The cultures were incubated in a shaker overnight (37 °C, 225 rpm).
2. Some of the overnight culture (0.4 ml) was inoculated in 40 ml LB medium (added Cml (40 µl) and Kan (20 µl) for preparation of *E. coli* ET12567 competent cells) and incubated for approximately 2 h in a shaking incubator (37 °C, 225 rpm) until OD_{600} was between 0.4– 0.6.
3. The cell suspension was centrifuged (4500 rpm, 5 minutes, 4 °C), and the supernatant was removed. The cells were resuspended in 4 ml cold TSS- buffer.
4. The cell suspension was kept on ice for 1 hour, then distributed into several Eppendorf tubes and used for transformation or stored at -80 °C.

Preparation of electrocompetent *E. coli* DH10B:

Materials:

- ✓ dsH_2O
- ✓ 10 % v/v glycerol solution
- ✓ LB medium
- ✓ Overnight culture of desired cells
- ✓ 50 ml cubic tubes
- ✓ Cold centrifuge

Protocol:

1. An overnight culture (3 ml) was inoculated in LB medium (300 ml). The culture was grown in a 37 °C shaker until the OD_{600} = 0.35 -0.4.
2. The cells were put on ice and chilled for 20-30 minutes and then distributed into 6 cold 50 ml cubic tubes.
3. The bottles were centrifuged at 2400 rpm for 20 min at 4 °C.
4. Supernatant was decanted and each pellet was resuspended in 40 ml of ice cold dsH_2O.
5. Step 3 and 4 was repeated and the pellet was resuspended in 20 ml of ice cold dsH_2O, before centrifuging under the same conditions.
6. The supernatant was decanted and each pellet was resuspended in 8 ml of ice cold 10 % glycerol solution. Suspensions were combined, resulting in 2 bottles with 24 ml in each.
7. The bottles were centrifuged again under the same conditions.
8. The supernatant was carefully aspirated with a pipette and the remaining cells were resuspended in 200 ml of ice cold 10 % glycerol solution by swirling the tube gently.
9. The competent cells were distributed into eppendorf tubes, 100 µl in each, and stored at -80 °C.

2.3.9 Transformation

Transformation is a process where extracellular DNA is introduced into a (competent) host cell. The extracellular DNA is a plasmid/vector containing a gene that confers antibiotic resistance to the transformed cells. This makes it possible to select for transformed cells by growing them on media with antibiotic(s), where only the plasmid-containing cells can grow. The following procedure for transformation was used for chemically competent *E. coli* strains: DH10B and ET12567. Electroporation with electrocompetent *E. coli* DH10B and EC100 cells is also described below. This procedure introduces vectors into the cells by applying a small voltage on the cell suspension, which makes the cells permeable to DNA (Chassy et al., 1988). Electroporation is generally more efficient than heat shock transformation.

Materials:

- ✓ Competent cells: 100 µl in Eppendorf tubes
- ✓ Plasmid(s)
- ✓ Ice
- ✓ Water bath at 42 °C or electroporator and cuvettes
- ✓ LB medium
- ✓ Shaking incubator (37 °C)
- ✓ Agar plates with antibiotic(s) for selection

Heat shock transformation:

1. The frozen competent cells were melted slowly on ice.
2. Plasmid DNA or ‘Gibson’ ligation mix (1-3 µl per 100 µl competent cells) was added and the cells were kept it on ice for approximately 15 minutes.
3. Heat shock was performed at 42 °C for 45 seconds to destabilize the cell wall so that plasmids could enter the cells.
4. The cells were placed on ice for 2-10 minutes.
5. LB media (400 µl) was added to each tube and the cells were incubated for 1-2 hours in a shaking incubator (37 °C, 225 rpm).
6. 100 µl of the transformation mixes were plated on each agar plate with appropriate antibiotic(s) for plasmid selection. The plates were incubated overnight at 37 °C.

Electroporation:

1. Plasmid DNA or ‘Gibson’ ligation mix (2 µl) was added to electrocompetent cells (100 µl) in an eppendorf tube and mixed carefully without pipetting. The cells were kept on ice for 10-30 min.
2. The cells were transferred to a cold cuvette and placed on ice.
3. Ice and water was wiped off the cuvette before it was placed into the electroporator device. Electroporation was performed by running the protocol (voltage: 2500 V, capacitance: 25 C, resistance: 100 Ω, cuvette: 1mm).
4. After the electroporation, 500 µl of LB medium was added to the cuvette. The content was mixed by pipetting and transferred to an eppendorf tube, which was incubated for 1-2 hours (37°C, 225 rpm).
5. 100 µl of the transformation mixes were plated on each agar plate with appropriate antibiotic(s) for plasmid selection. The plates were incubated overnight at 37 °C.

2.3.10 Isolation of plasmid DNA (pDNA)

Plasmid DNA was isolated from overnight cultures of transformed cells by using Wizard® *Plus* SV Minipreps DNA Purification System from Promega. The protocol is described in Attachment F4 (page 87).

Materials:

- ✓ Wizard® *Plus* SV Minipreps DNA Purification Kit
- ✓ Wizard® SV Minicolumns
- ✓ Overnight culture of transformed cells

2.3.11 'Gibson' reaction

As described in Chapter 1.9.1, the 'Gibson' one-step isothermal reaction was used to join DNA fragments for construction of new vectors. By specific designed primers, overlapping terminal sequences of 25 bp were added to the DNA fragments and amplified by PCR. Vector and reporter fragments were treated with *Dpn*I (described in Chapter 2.3.12) to digest methylated PCR template. The plasmids pSOK805 and pUC59, which were templates for the vector and *gusA* reporter gene, both contained an ampicillin resistance gene. It was therefore important to remove all traces of the plasmids before the 'Gibson' reaction was performed.

The DNA fragments were added in equimolar amounts to 15 µl of the 'Gibson' master mix, described in Attachment B7, until a total volume of 20 µl. The reaction mixes (Table 2.3.8 and 2.3.9) were incubated at 50 °C for 1 hour in the PCR-machine by using program "ingib" described in Table 2.3.7.

Table 2.3.7: PCR-program details for the 'Gibson' reaction.

Step	Process	ingib Temperature [°C]	Time [min]
1	'Gibson' ligation	50	60
2	Cool down	4	∞

Composition of the 'Gibson' reaction mixes for assembling the construct 2, 3 and 4 in one step is described in Table 2.3.8. Negative control reactions were made by adding the same amount of fragments, but with distilled sterile water (ds H_2O) instead of the promoter. 'Gibson' assembly should not be possible without the promoter fragments. The negative control mixes were transformed into *E. coli*, in order to detect possible background "noise" created by transformation of un-digested templates.

Table 2.3.8: 'Gibson' reaction mixes for assembling construct 2, 3 and 4 in one step. Reaction mixes without the promoter was performed as a negative control.

Construct nr.	2	Control for construct 2	3	Control for construct 3	4	Control for construct 4
Vector [µl]	1	1	1	1	0,5	0,5
Promoter [µl]	2	2 µl ds H_2O	1,5	1,5 µl ds H_2O	-	-
Reporter [µl]	2	2	2,5	2,5	4,5	4,5 µl ds H_2O
Gibson master mix [µl]	15	15	15	15	15	15
Total volume [µl]	20	20	20	20	20	20

Construct 1 was assembled in two steps. The composition of the two 'Gibson' reaction mixes is described in Table 2.3.9.

Table 2.3.9: The 'Gibson' reaction mixes for assembling construct 1 in two steps.

Construct:	pSOK807	Control for pSOK807	**Construct nr.**	1	Control for construct 1
Vector [µl]	3	3	Vector [µl]	2	2
Promoter [µl]	2	2 µl ds H_2O	**Reporter [µl]**	3	3 µl ds H_2O
Gibson master mix [µl]	15	15	Gibson master mix [µl]	15	15
Total volume [µl]	20	20	Total volume [µl]	20	20

2.3.12 Enzyme digestion

***Dpn*I-treatment:**

The PCR-amplified vector and reporter fragments were treated with *Dpn*I in order to digest original, methylated pDNA, and make sure that only the PCR product was present before the ligation reaction. DNA from common *E. coli* strains is Dam-methylated and therefore susceptible to *Dpn*I digestion (Weiner et al., 1994). The *Dpn*I-treatment was performed by incubating the following mixture in the PCR machine, using the PCR-program "osdpnI" or "DpnLong" as described in Table 2.3.10.

- ✓ 17 µl purified vector
- ✓ 1 µl *Dpn*I
- ✓ 2 µl NEB buffer 4

Table 2.3.10: PCR-program for *Dpn*I-treatment of vector and reporter fragments.

Step	**Process**	**osdpnI Temperature [°C]**	**Time [min]**	**DpnLong Temperature [°C]**	**Time [min]**
1	Enzyme digestion	37	120	37	480
2	Enzyme inactivation	80	20	80	20
3	Cool down	4	∞	4	∞

Enzyme-digestion of the constructs:

In order to verify the constructs, pDNA was first isolated from overnight cultures of transformants, as described in Chapter 2.3.10. The pDNA was then digested with restriction enzymes, and analyzed by gel electrophoresis. The pDNAs were cut by incubating the digestion mixes, described in Table 2.3.11, in a water bath at 37 °C for 1 hour.

Table 2.3.11: Enzyme mixes for digestion of pDNA.

	***Pst*I-digestion Amount [µl]**	***Eco*0109-digestion Amount [µl]**	***Aat*II-digestion Amount [µl]**	***Xma*I-digestion Amount [µl]**
ds H_2O	13.5	13.5	14	13.5
NEB buffer	nr. 3: 2 µl	nr. 4: 2 µl	nr. 4: 2 µl	nr. 4: 2 µl
BSA	0.5	0.5	-	0.5
pDNA	3	3	3	3
Enzyme	1	1	1	1

The expected band sizes after proper enzyme digestion of the constructs are given in Table 2.3.12-2.3.14.

Table 2.3.12: Expected fragment sizes after *Pst*I-digestion of the constructs and pSOK805.

Construct	**1 (*ermE*p)**	**2 (*cmlF*p)**	**3 (*kefB*p)**	**4 (noPro)**	**pSOK807**	**pSOK805**
Restriction enzyme	***Pst*I**	***Pst*I**	***Pst*I**	***Pst*I**	***Pst*I**	***Pst*I**
Band sizes [bp]	6698 1384 595 126	6654 1532 595	6654 1489 595	6654 1152 595	4882 1384 595	5967 595

Table 2.3.13: Expected fragment sizes after *Xma*I-digestion of construct 2, 3, 4 and pSOK805.

Construct	**2 (*cmlF*p)**	**3 (*kefB*p)**	**4 (noPro)**	**pSOK805**
Restriction enzyme	***Xma*I**	***Xma*I**	***Xma*I**	***Xma*I**
Band sizes [bp]	3387 2835 1049 877 525 108	3387 2835 1049 834 525 108	3387 2835 1049 525 497 108	3387 3067 108

Table 2.3.14: Expected fragment sizes after *Aat*II- digestion of construct 2, pUC59 and pSOK805, and *Eco*O109I-digestion of construct 3 and pSOK805.

Construct	**2 (*cmlF*p)**	**pUC59**	**pSOK805**	**3 (*kefB*p)**	**pSOK805**
Restriction enzyme	***Aat*II**	***Aat*II**	***Aat*II**	***Eco*O109I**	***Eco*O109I**
Band sizes [bp]	3548 2688 1032 786 484 243	2782 1032 936	5533 786 243	5991 2278 469	3815 2278 469

2.3.13 Conjugative DNA transfer from *E. coli* ET 12567 to *S. venezuelae*

Conjugation is a simple and efficient method for transferring pDNA from *E. coli* ET12567 into the genome of *S. venezuelae* ATCC 10712. The *E. coli* ET12567 (pUZ8002) strain– later just ET – is a non-methylating host (*dam*$^-$, *dcm*$^-$) carrying pUZ8002 helper plasmid which provides transfer functions from RP4. The strain is resistant to both Cml (30 µg/ml) and Kan (40 µg/ml). This strain allows mobilization of any plasmid carrying *RP4 oriT* into the recipient. For laboratory conjugation, both fresh *S. venezuelae* spores as well as frozen glycerol stock of spores can be used.

Materials:

- ✓ *E. coli* ET12567 (pUZ8002) cells with introduced constructs on a fresh LA plate with Amp, Cml and Kan
- ✓ ISP4 plate with *S. venezuelae* ATCC 10712 spores
- ✓ Glycerol stock of *S. venezuelae* ATCC 10712 spores
- ✓ ISP4 + $MgCl_2$ plates
- ✓ LB medium
- ✓ 2×YT medium
- ✓ Sterile cotton wool filters

Protocol:

1. Prepared spore suspension of *S. venezuelae* in distilled sterile water by washing off spores from a fresh ISP4 plate with 5.0 ml of water and filtering it through the sterile cotton wool. Added 50 µl of this spore suspension or frozen spore suspension to 2×YT medium (500 µl), mixed and incubated for 5 min at 50°C (germination of spores are induces by this heat shock). Allowed the heat-shocked spore suspension to cool down at room temperature (ca. 15-20 min).
2. Prepared ET cell suspension by sampling cells from a plate and resuspending them in 2×YT medium (500 µl).
3. Mixed heat-shocked spore suspension of *S. venezuelae* (550 µl) with 100 µl of the ET cell suspension by pipetting. The mix was spun down at a table centrifuge for 1 min, and 550 µl of the supernatant was removed. The cells were resuspended in the rest of the medium (100 µl) and plated on ISP4 + $MgCl_2$ medium. The cells were grown at room temperature on the laboratory bench for 14-23 h until a thin mycelium layer was observed (less time for fresh spores than for glycerol stock).
4. Made antibiotic solution for selection of transconjugants by mixing nalidixic acid (0.9 mg/ml) and thiostrepton (0.9 mg/ml) with sterile distilled water. Each ISP4 + $MgCl_2$ plate with conjugation mix was added 1 ml of the antibiotic mix (Nal + Thio), which was evenly distributed over the surface of the plate using a sterile glass triangle. The resulting concentration of each antibiotic was 30 µg/ml medium. The closed plate was left on the bench for 1 h to dry out, before

it was placed at 30°C for further growth. Nalidixic acid (Nal) was used to select against contaminating *E. coli*, since *Streptomycetes* are naturally resistant to Nal, while *E. coli* is sensitive to it. Thiostrepton (Thio) was used to select for transconjugants, since the plasmid contained the *tsr* gene, which confers resistance to Thio. The antibiotic stock solution recipes can be found in Attachment B (page 76).

5. Approximately one week after selection, some transconjugants were selected and transferred onto ISP4 plates with Nal (30 µg/ml) and Thio (30 µg/ml), and placed at 30 °C for further growth.
6. After 2-4 days of growth, each colony was distributed on an agar plate with ISP4 medium and Thio. This plate was placed at 30 °C for 2-3 days until sporulation. Then, glycerol stocks were made from the spore suspension as described in Chapter 2.3.2.

2.3.14 Analysis of chloramphenicol production in cultured *S. venezuelae*

S. venezuelae recombinant strains with introduced construct 2, 3 and 4 were cultured for several days in order to analyze the production of chloramphenicol (Cml). Half of the cultures were added ethanol (6 % v/v) to stop the production of Cml. The samples taken from the culture broth were prepared for Ultra high Performance Liquid Chromatography (UPLC) analysis as described below.

Materials:

- ✓ *S. venezuelae* with introduced constructs
- ✓ MYM medium and TSB medium
- ✓ Ethanol (96 %)
- ✓ 250 ml baffled flasks (Pyrex)
- ✓ Shaking incubator (30 °C)
- ✓ Centrifuge
- ✓ Ethyl acetate
- ✓ SpeedVac Concentrator
- ✓ Methanol

Procedure for culturing the *S. venezuelae* strains:

1. Spore suspension or glycerol stock (50 µl) of *S. venezuelae* strains were inoculated in TSB (10 ml) and grown overnight at 30 °C in a shaking incubator.
2. For each strain, two baffled flasks (250 ml) were added 50 ml MYM medium. Overnight culture (2.5 ml) of *S. venezuelae* strain was added in each baffled flask and grown at 30 °C in a shaking incubator.
3. After 10 hours of growth, ethanol (3.5 ml) was added to one flask of each strain. This resulted in a total ethanol concentration of 6 % (v/v). Addition of

ethanol should stop the production of Cml. The cultures were grown further under the same conditions.

4. About 12 hours after addition of ethanol, six samples (1 ml) were taken from each culture and three of them were prepared for UPLC analysis as described below. The three other parallels were frozen down at -20 °C.
5. On the fourth day of growth (72 h after the last samples were taken), the same amount of samples were collected once more. Three parallels were prepared for UPLC analysis, while the remaining three parallels were used for reporter assay as described in Chapter 2.3.15.

Preparation of samples for UPLC analysis:

1. The samples were centrifuged (13 000 rpm, 5 min) and the supernatant (0.8 ml) was carefully transferred to a clean tube.
2. Ethyl acetate (0.3 ml) was added to extract Cml, and the sample was vortexed for 1 minute.
3. Centrifugation (13 000 rpm, 2 minutes) resulted in a phase separation. The top layer consisted of ethyl acetate and Cml, while the bottom layer was the remaining medium. Only 0.1 ml of extract was taken from the top layer. This was performed fast and carefully because ethyl acetate is very volatile.
4. Step 3 and 4 were repeated and 0.2 ml of the ethyl acetate layer was extracted and added to the first 0.1 ml of extract.
5. The ethyl acetate was removed by evaporation in a SpeedVac Concentrator (5.0 Torr, 45 °C, 40 min) and chloramphenicol was diluted in methanol (400 µl).
6. The methanol solution (100 µl) was transferred to small brown flasks prior to the UPLC analysis. Only 1 µl of this solution was injected into the UPLC machine.

The UPLC analysis was performed as described in (Shah et al., 2012).

Results from the UPLC analysis are presented as nM Cml in Attachment G. Calculation of the Cml concentration per ml of sample was performed by using the equation below, which was developed by considering the following facts:

1. Only 0.8 ml out of the 1 ml sample was used for extraction of Cml, since the remainder contained pelleted cells. Thus the concentration should be multiplied by the factor 5/4 to get the Cml concentration in 1 ml media.
2. Only half of the ethyl acetate added to extract Cml was collected for analysis, it should therefore be multiplied by 2.
3. The ethyl acetate was evaporated and the remaining Cml powder was diluted in 400 µl of methanol, which was analyzed by UPLC.
4. Chloramphenicol has a molar mass of 323.14 g/mol.

$$C_{Cml}[\mu\text{g/ml}] = \frac{5}{4\text{ ml}} \cdot 2 \cdot c_{Cml}[10^{-9}\text{mol/L}] \cdot 400 \cdot 10^{-6}\text{L} \cdot 323.14\text{ g/mol} \cdot 10^{6}\,\frac{\mu\text{g}}{\text{g}}$$

2.3.15 Measurement of β-glucuronidase activity in cell lysates

In order to investigate under which conditions the reporter gene was expressed, a reporter assay was performed as described below. Preparation of the buffers and solutions is described in Attachment B (page 76).

Materials:

- ✓ Samples from cultured *S. venezuelae* strains
- ✓ Dilution buffer
- ✓ Lysis buffer
- ✓ p-nitrophenyl-β-D-glucuronide (PNPG) (0.2 M)
- ✓ 96 well plate
- ✓ Spectrophotometer
- ✓ Centrifuge

Protocol:

1. While collecting samples from the cultured strains for HPLC analysis, 3 × 1 ml was collected from each culture to use in the reporter assay. The samples were stored at - 20 °C and melted before use.
2. The samples were centrifuged to pellet the cells (4 °C, 8 000 rpm, 1 minute).
3. Supernatant was discarded, and the cells were washed in dsH_2O.
4. The cells were resuspended in lysis buffer (1 ml) and incubated at 37 °C for 15 minutes.
5. Samples were centrifuged again (4 °C, 4 000 rpm, 10 min).
6. The dilution buffer (5 ml) was mixed with PNPG substrate (50 µl, 0.2 M) and 50 µl of this mix was distributed in a 96 well plate.
7. Cell lysate (50 µl) was distributed into the wells which were already added dilution buffer and substrate. A 1:1 mixture of dilution buffer and lysate was used as blank references. The 96 well plate was incubated at 37 °C for 40 minutes.
8. The optical density (OD) was measured at light wavelengths of 405 nm and 415 nm in a spectrophotometer.
9. The plate was left at room temperature overnight and new OD measurements were performed the next morning, about 17 hours after the first measurements.

3 RESULTS AND DISCUSSION

3.1 CONSTRUCTION AND VERIFICATION OF THE VECTORS

In order to construct the biosensor plasmids, DNA fragments were PCR-amplified to render overlapping terminal sequences. These overlaps could then anneal during the 'Gibson' assembly, resulting in a seamless vector. pSOK805 is a shuttle vector that can be transferred from *E. coli* to *S. venezuelae* by conjugation. DNA fragments for all the constructs were amplified by PCR as described in Chapter 2.3.4 with the specific primers given in Attachment C (page 80). Optimization of conditions for PCR reactions was performed for different DNA fragments. The vector and *gusA* reporter gene fragments were *Dpn*I-treated to ensure that the PCR-template was removed, because the templates conferred resistance to ampicillin which was used for selection of transformants.

3.1.1 Assembling construct 3: pSOK805-*kefB*p-*gusA*

Construct 3 was the first vector to be successfully assembled by the 'Gibson' reaction and then transformed into *E. coli* cells. The purified DNA fragments for making construct 3 (pSOK805-*kefB*p-*gusA*) are shown in Figure 3.1.1. The band sizes of the ladder are given in Attachment E (page 83).

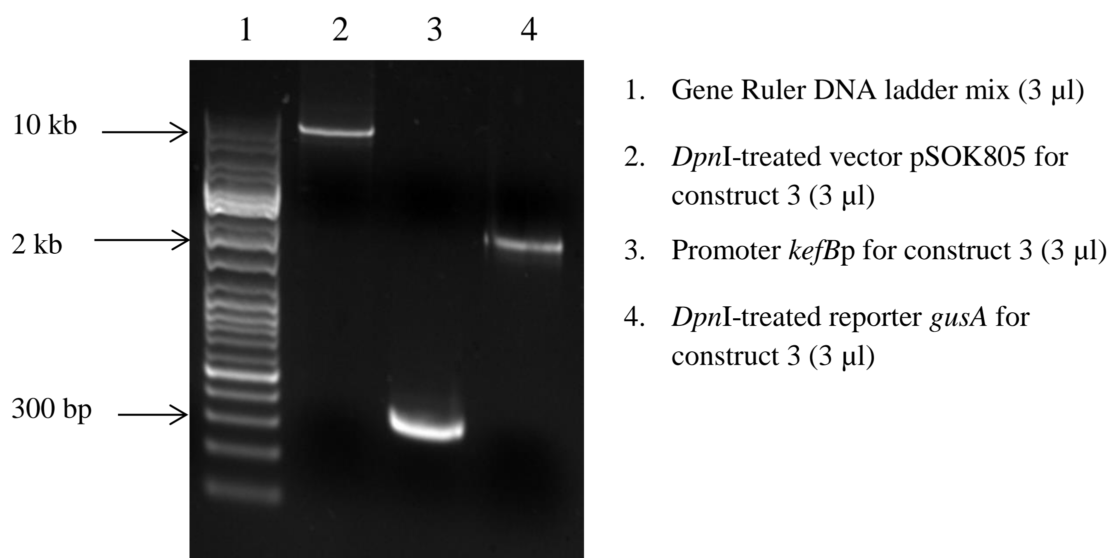


Figure 3.1.1: Gel electrophoresis of the DNA fragments for making construct 3 (pSOK805_*kefB*p_*gusA*). The content of each well is specified to the right and the ladder bands closest to the DNA fragments are indicated to the left.

The DNA bands have the estimated sizes. The vector fragment in well number 2 is expected to be around 6.6 kb long (6562 bp + overlapping regions), while is seems to be 10 kb on the gel. This deviation is probably caused by the slower migration of high-

concentration DNA fragments and GC-rich DNA through the agarose gel. The promoter and reporter gene are 300 and 1900 bp in size, respectively.

The amount of each fragment in the 'Gibson' ligation mix is given in Table 2.3.8 and the rest of the procedure is presented in Chapter 2.3.11. A negative control mix was made by adding the same amount of vector and reporter, but water instead of the promoter fragment.

Electrocompetent *E. coli* DH10B cells were made as described in Chapter 2.3.8, because electroporation is usually a more efficient transformation method. After introducing the 'Gibson' ligation mix for construct 3 and negative control into *E. coli* DH10B cells by electroporation, a total of 12 colonies appeared and no colonies were found on the negative control plate. Plasmid DNA was isolated from the overnight cultures and then cut with a restriction enzyme. The colonies were first digested with *Eco*O109I and the resulting fragments were analyzed by gel electrophoresis as shown in Figure 3.1.2.

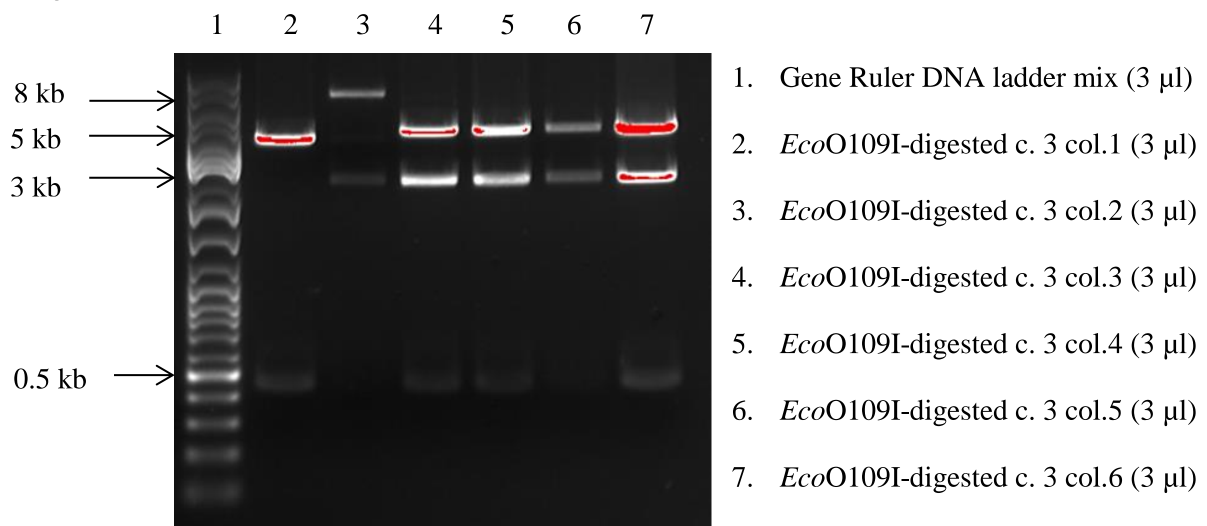


Figure 3.1.2: Gel electrophoresis of *Eco*O109I-digested pDNA from clones transformed with 'Gibson' ligation mix for construct 3.

The expected band sizes after *Eco*O109I-digestion of construct 3 and the vector template (pSOK805) are given in Table 2.3.14 in Chapter 2.3.12. The second colony of construct 3 (c. 3 col. 2) in well 3 has the right restriction pattern. The expected band sizes of *Eco*O109I-digested construct 3 are 6 kb, 2.3 kb and 0.47 kb. The pDNAs in well 4-7 are probably the pSOK805 vector template, since the restriction pattern is as expected for pSOK805.

In order to further verify construct 3, it was digested with *Pst*I as well. Gel electrophoresis of the DNA fragments resulted in the picture shown in Figure 3.1.3.

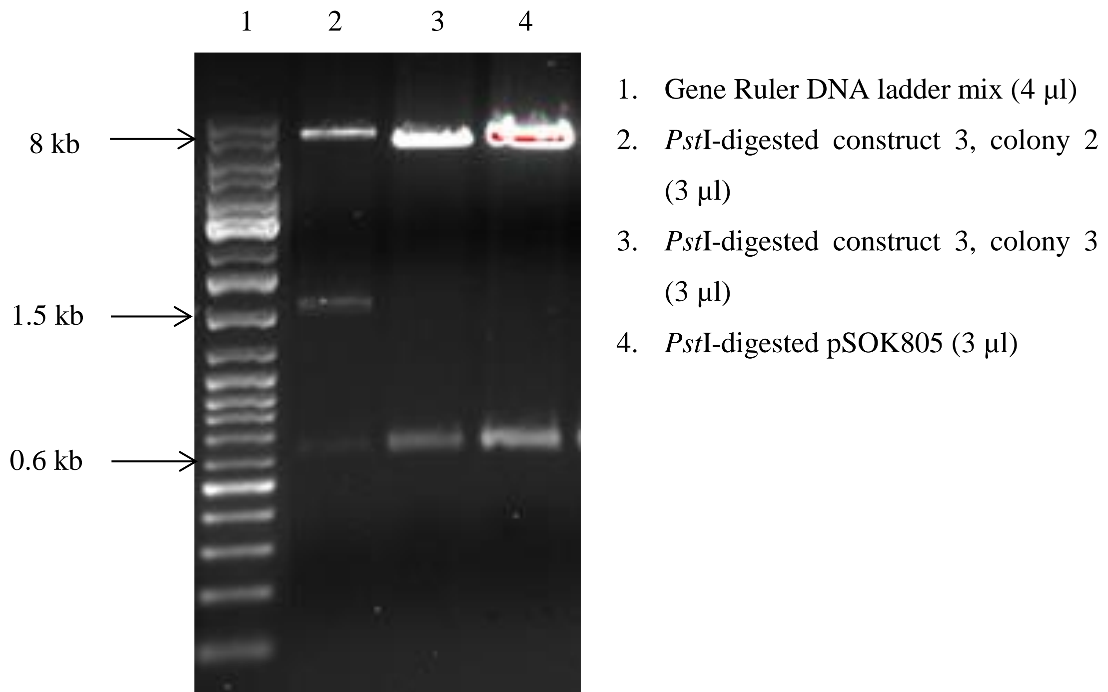


Figure 3.1.3: Gel electrophoresis of *Pst*I-digested pDNA from clones transformed with the 'Gibson' ligation mix for construct 3.

The expected fragment sizes of *Pst*I-digested construct 3 are 6.7 kb, 1.5 kb and 0.6 kb, which correspond well to the bands in well 2. The pDNA in well 3 (construct 3, colony 3) is clearly pSOK805, since it is similar to the restriction pattern of pSOK805 in well 4. This second digestion confirmed that clone 2 contains the correct construct 3.

3.1.2 Assembling construct 4: pSOK805-*gusA* (negative control)

Construct 4 was the second plasmid to be successfully assembled and cloned into *E. coli.* The DNA fragments for assembling this negative control vector, were PCR-amplified and purified as described in Chapter 2.3.4-2.3.7. The fragments were analyzed by gel electrophoresis in order to estimate their concentrations (Figure 3.1.4). Construct 4 is the negative control which contains only vector and reporter gene.

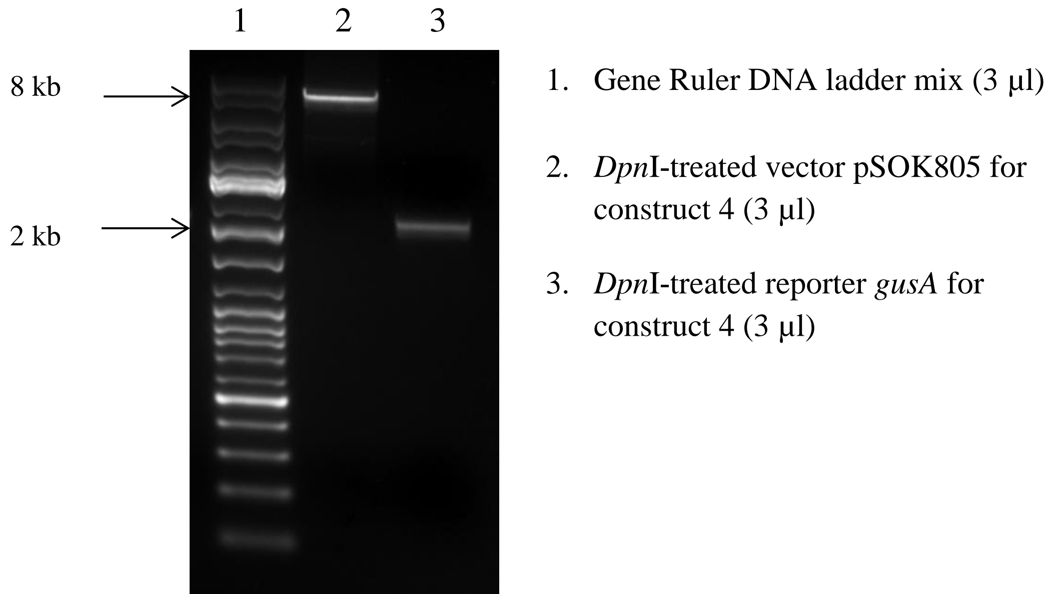


Figure 3.1.4: Gel electrophoresis of the DNA fragments for making construct 4, the negative control.

The DNA fragments is Figure 3.1.4 were assembled by the 'Gibson' reaction, as explained in Chapter 2.3.11. The 'Gibson' reaction mix was transformed into electrocompetent *E. coli* DH10B cells, resulting in a total of 69 clones. Plasmid DNA was isolated from 12 clones, *Pst*I-digested and analyzed by gel electrophoresis (Figure 3.1.5).

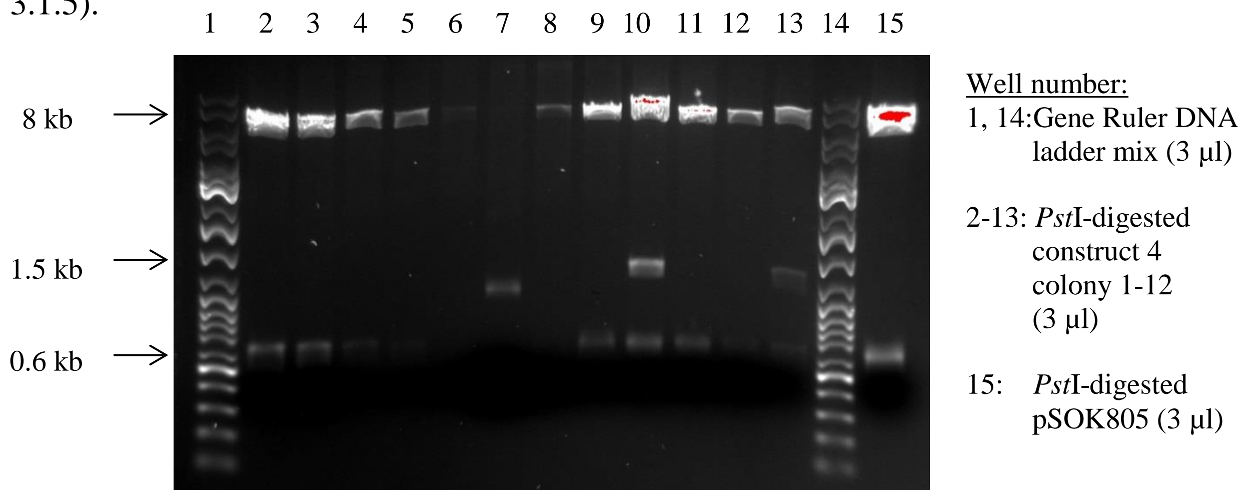


Figure 3.1.5: Gel electrophoresis of *Pst*I-digested pDNA from clones transformed with the 'Gibson' ligation mix for construct 4 (negative control).

The restriction pattern in well 10 and 13 correspond well to the expected band sizes of *Pst*I-digested construct 4, which are 6.7 kb, 1.2 kb and 0.6 kb. This indicates that the isolated pDNA from colony 9 and 12 are the desired construct 4. Most of the other restriction patterns resemble the control pSOK805 in well 15, thus they are vector templates.

3.1.3 Assembling construct 1: pSOK805-*ermE**p-*gusA* (positive control)

Construct 1 consists of the strong constitutive promoter, *ermE**p, upstream of the *gusA* reporter gene. This should give constant production of the reporter protein and can therefore be used as a positive control when performing the reporter assay. The assembly of construct 1 was first tried in a one-step reaction as with the other constructs. Since this would not work after several attempts, a new strategy was developed. This strategy involved a two-step assembly in which the pSOK805 vector and *ermE** promoter should first be assembled, resulting in a new vector called pSOK807. The new vector should be PCR-amplified to give overlapping terminal sequences with the reporter gene. The second assembly should introduce the *gusA* gene downstream of the promoter, to ensure that the promoter regulates expression of the reporter gene. New primers for this two-step assembly were designed in j5 (Hillson) and ordered from Sigma-Aldrich.

In order to assemble the first vector, pSOK807, the vector template and promoter were PCR-amplified and purified. The fragments were analyzed by gel electrophoresis to compare their concentrations (Figure 3.1.6).

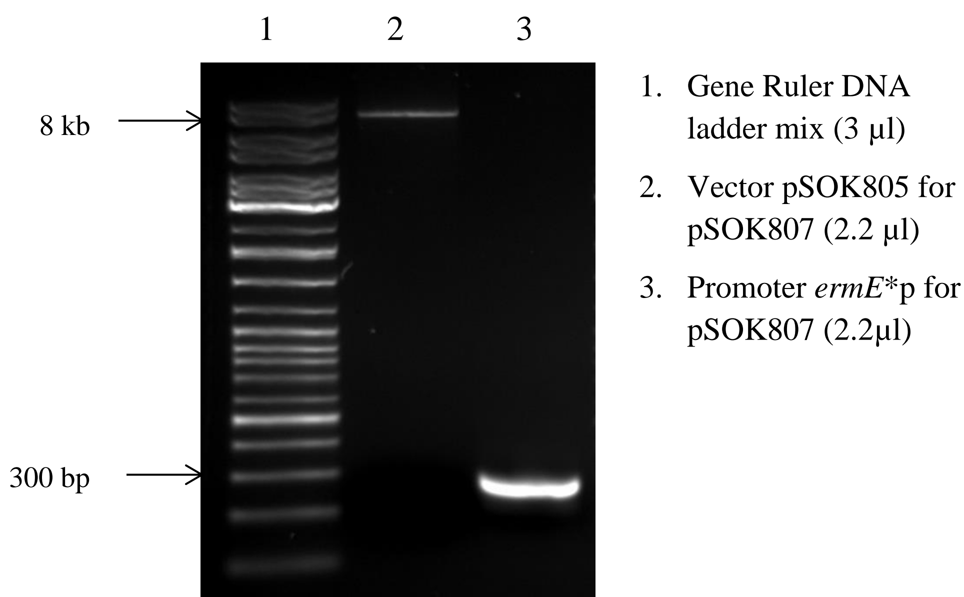


Figure 3.1.6: Gel electrophoresis of the DNA fragments for making pSOK807 (pSOK805-ermE*p), the first step in the two-step-assembly to make construct 1 (the positive control).

The fragments in Figure 3.1.6 seem pure and have the same size as the other vector and promoter for making construct 3. A 'Gibson' ligation mix containing these

fragments was transformed into chemically competent *E. coli* DH10B cells, resulting in 12 transformants which were able to grow on selective media. Plasmid DNA was isolated from the clones, *Pst*I-digested and analyzed be gel electrophoresis (Figure 3.1.7).

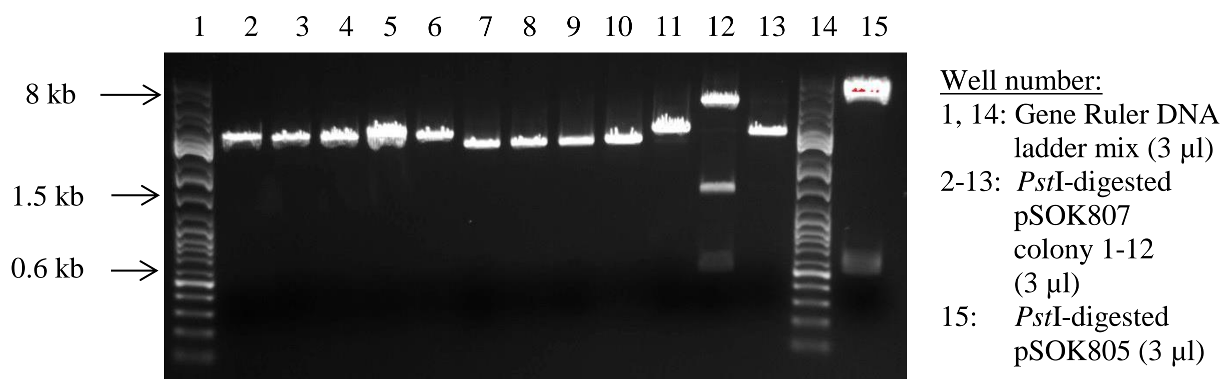


Figure 3.1.7: Gel electrophoresis of *Pst*I-digested pDNA from clones transformed with pSOK807 (construct 1, step 1) 'Gibson' ligation mix.

The expected band sizes after *Pst*I-digestion of pSOK807 are 4.9 kb, 1.4 kb and 0.6 kb, while *Pst*I-digestion of the vector template pSOK805 should give two bands of 6.0 kb and 0.6 kb in size. The pDNA from colony 11 (well 12 on the gel) has the expected restriction pattern for pSOK807, thus it is probably the desired plasmid.

In order to perform the second assembly step and make construct 1, the correct pSOK807 isolated from colony 11 was used as template for PCR-amplification of this new vector fragment. The reporter gene was also PCR-amplified to give overlapping terminal sequences with the vector fragment. The purified fragments were analyzed by gel electrophoresis, and the gel picture is given in Figure 3.1.8.

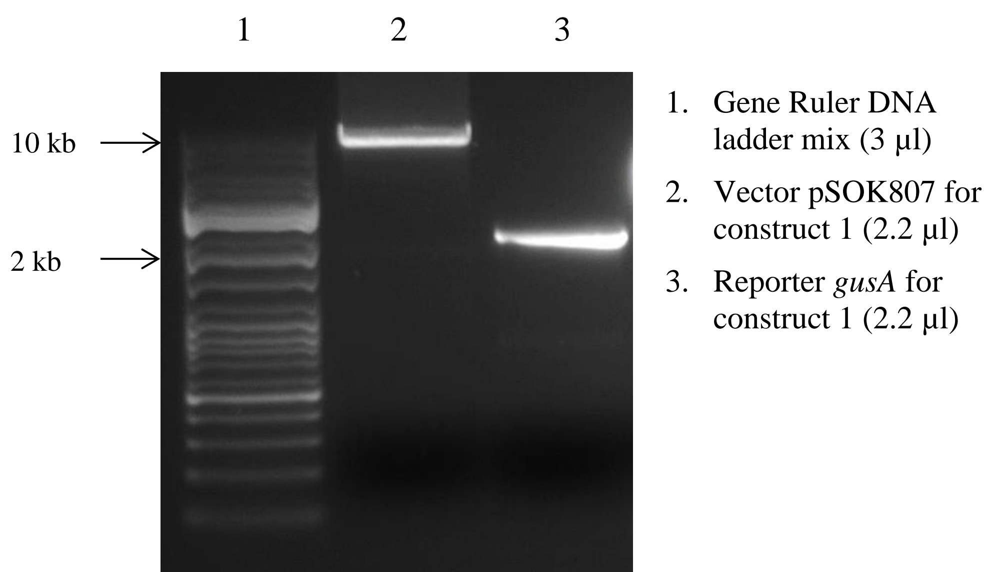


Figure 3.1.8: Gel electrophoresis of the DNA fragments for assembling construct 1 (the positive control) in the second step.

The vector and reporter fragment in Figure 3.1.8 seem pure, concentrated and have the expected size. The 'Gibson' ligation mix was transformed into *E. coli* DH10B and EC100 by electroporation and plated on LA with Amp. A total of 44 DH10B clones appeared on the plates, while over 200 clones were observed on each plate with EC100 cells. Plasmid DNA was isolated from 12 clones of each strain, digested with *Pst*I and analyzed by gel electrophoresis as shown in Figure 3.1.9 and 3.1.10.

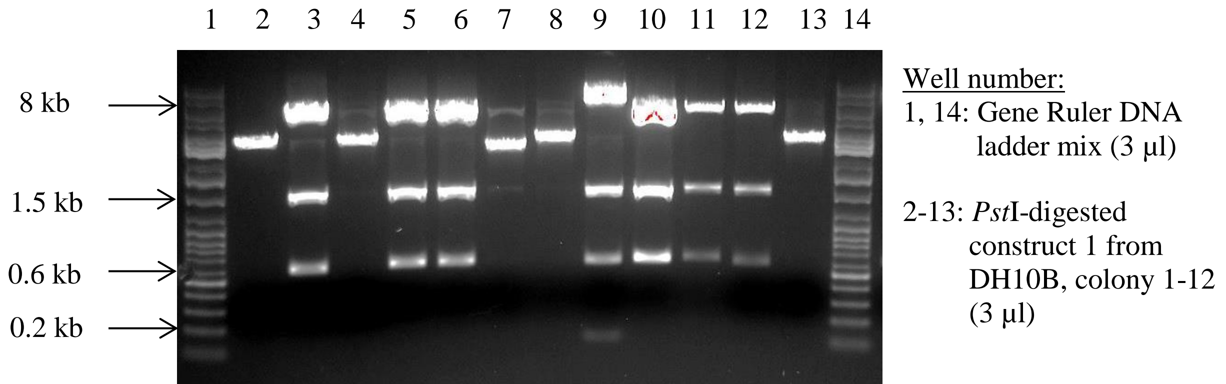


Figure 3.1.9: Gel electrophoresis of *Pst*I-digested pDNA from *E. coli* DH10B clones transformed with the 'Gibson' ligation mix for construct 1 (positive control), step 2.

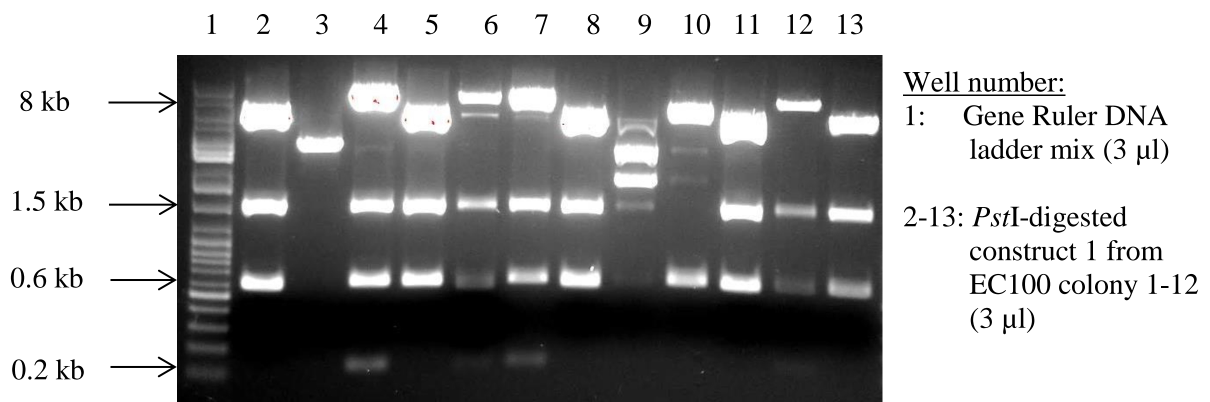


Figure 3.1.10: Gel electrophoresis of *Pst*I-digested pDNA from *E. coli* EC100 clones transformed with the 'Gibson' ligation mix for construct 1 (positive control), step 2. Colony 3, 5, 6 and 11 in well 4, 6, 7 and 12 have the right restriction patterns.

The expected band sizes of *Pst*I-digested construct 1 are 6.7, 1.4, 0.6 and 0.13 kb. This particular restriction pattern can be seen in well 9 in Figure 3.1.9 and in well 4, 6, 7 and 12 in Figure 3.1.10. These results indicate that 1/12 of the DH10B colonies, and as much as 4/12 of the EC100 colonies that were checked, contained the desired construct. Some of the other restriction patterns are similar to pSOK807 in Figure 3.1.7 (well 12), thus the *Dpn*I-treatment was not sufficient in this case. In Figure 3.1.10, the isolated pDNAs in well 9 and 10 have the expected restriction pattern of the reporter gene template (pUC59) and the original vector template (pSOK805), respectively.

3.1.4 Assembling construct 2: pSOK805-*cmlF*p-*gusA*

Construct 2 contains the *cmlF* promoter, which controls the expression of the chloramphenicol transporter protein. Several attempts to assemble this construct failed, and 2 sets of primers were ordered for different strategies. The primer combination resulting in proper construction of the vector is given in Attachment C. The purified PCR products for a final 1-step assembly of construct 2 were analyzed by gel electrophoresis, as shown in Figure 3.1.11.

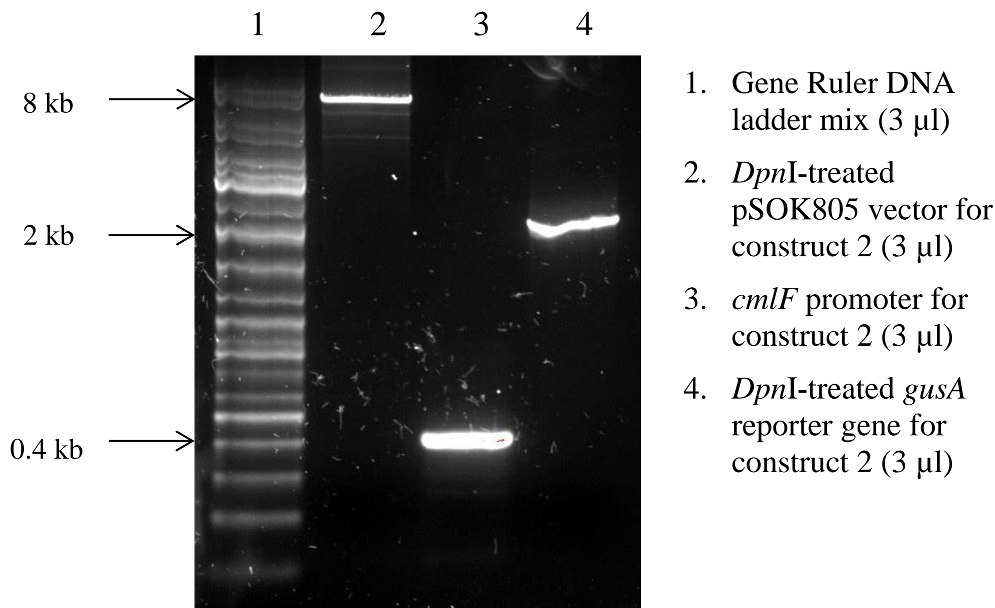


Figure 3.1.11: Gel electrophoresis of the purified DNA fragments for assembling construct 2.

The vector, promoter and reporter gene fragment in Figure 3.1.11 are pure, concentrated and have the expected sizes. These DNA fragments with overlapping ends were assembled by the 'Gibson' reaction. The reaction mix was transformed into *E. coli* DH10B by electroporation. A negative control 'Gibson' ligation mix was made of the same amount of fragments, but with water instead of the promoter. The transformation mixes were plated out on LA with Amp, and resulted in a total of 13 colonies, while only 2 colonies appeared on the negative control plate. Plasmid DNA was isolated from overnight cultures and digested with *Pst*I restriction enzyme. The digested pDNAs were analyzed by gel electrophoresis and the resulting gel picture is shown in Figure 3.1.12.

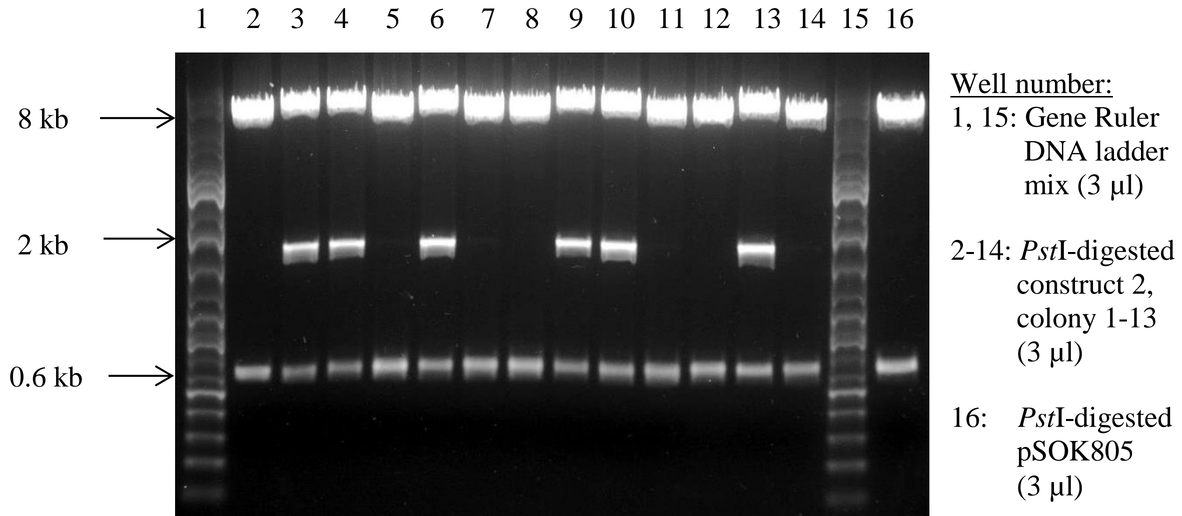


Figure 3.1.12: Gel electrophoresis of *Pst*I-digested pDNA from *E. coli* DH10B clones transformed with the 'Gibson' ligation mix for construct 2. Colony 2, 3, 5, 8, 9 and 12 in well 3, 4, 6, 9, 10 and 13 seem to have the right restriction patterns.

The expected band sizes after *Pst*I-digestion of construct 2 is 6.6, 1.5 and 0.6 kb, which corresponds well with 6 out of 13 colonies, considering that concentrated DNA migrates slower through the gel than the ladder bands. The remaining 7 colonies are pSOK805, since the restriction patterns are similar to the *Pst*I-treated pSOK805 in well 16.

In order to further verify that construct 2 finally was made, the pDNA from colony 2, 3, 5, 8, 9 and 12 were also digested with *Aat*II and *Xma*I restriction enzymes. These enzymes should cut six times in construct 2, while digestion of the pSOK805 vector template only should result in three fragments. All of the expected band sizes after enzyme digestion are given in Table 2.3.12-2.3.14 in Chapter 2.3.12. After enzyme digestion of the pDNA's and pSOK805 as control, the fragments were analyzed by gel electrophoresis and the result is shown in Figure 3.1.13.

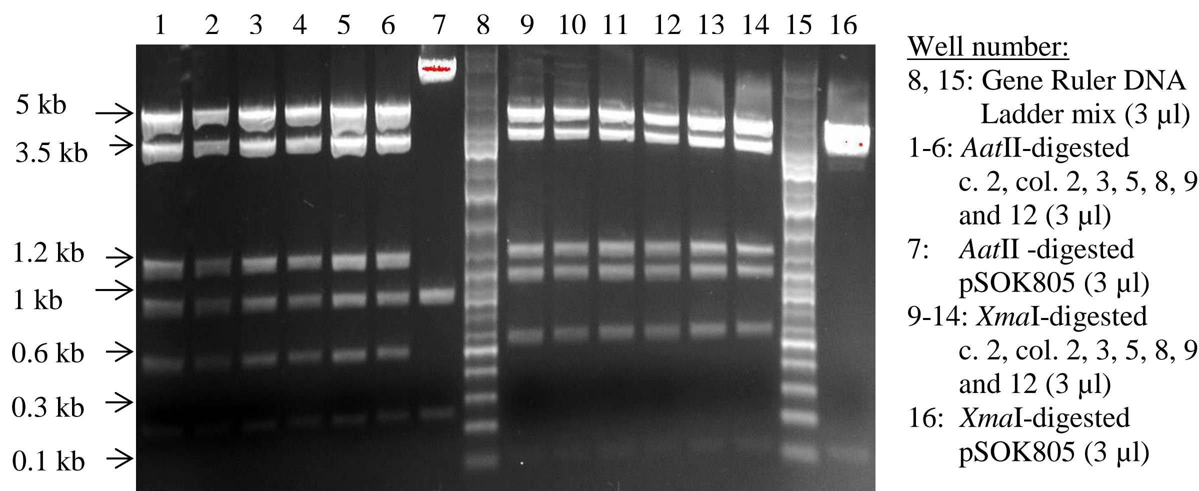


Figure 3.1.13: Gel electrophoresis of *Aat*II- and *Xma*I-digested construct 2, colony 2, 3, 5, 8, 9 and 12 which seemed right on the previous gel picture. The ladder bands closest to most of the DNA fragments are indicated to the left and the content in each well is specified to the right.

The fragment sizes after *Aat*II-digestion of construct 2 are as expected (3.5 kb, 2.7 kb, 1.0 kb, 0.8 kb, 0.5 kb and 0.25 kb). The *Xma*I-digested construct 2 also have the expected fragment sizes as given in Table 2.3.13. The expected fragments after *Xma*I-digestion of pSOK805 (3.4 kb, 3.1 kb and 0.1 kb) correspond well with the bands seen in well 16, considering that the double-band was not sufficiently separated during gel electrophoresis. These results confirm that construct 2 finally was assembled.

A summary of the transformations that led to isolation and verification of the correct constructs is given in Table 3.1.1. The vectors with their respective promoters are listed in this table. The number (#) of colonies that appeared after transformation into the specific strains are also listed, as well as how many correct constructs that were verified from the isolated plasmid DNA.

Table 3.1.1: Summary of the transformation and verification of the four constructs. The total number (#) of colonies that appeared after transformation, number of isolated plasmid DNA and how many correct constructs that were among them are listed in this table.

Vector	Promoter	*E. coli* strain	Transformation method	# colonies	# isolated pDNA's	# correct constructs
pSOK807	*ermE**p	DH10B	heat-shock	12	12	1
construct 1	*ermE**p	DH10B	electroporation	44	12	1
construct 1	*ermE**p	EC100	electroporation	> 500	12	4
construct 2	*cmlF*p	DH10B	electroporation	13	13	6
construct 3	*kefB*p	DH10B	electroporation	12	6	1
construct 4	-	DH10B	electroporation	69	12	2

3.2 TRANSFERRING THE CONSTRUCTS INTO *S. VENEZUELAE*

In order to transfer the constructs into *S. venezuelae*, the vectors were first introduced into chemically competent *E. coli* ET12567 cells by transformation. ET cells with the constructs were mixed with heat-shocked spores of *S. venezuelae* to promote conjugative transfer of pDNA into the genome of *S. venezuelae*. The detailed mechanism for conjugative DNA transfer is described in Chapter 1.5.3, while the laboratory procedure is described in Chapter 2.3.13.

The conjugative transfer of construct 3 (pSOK805-*kefB*p-*gusA*) was performed by using fresh *S. venezuelae* spores, and the cell mix was grown at room temperature for 15 hours prior to selection with antibiotics. Some transconjugants appeared six days after selection and were then transferred onto ISP4 plates supplemented with nalidixic acid (Nal) and thiostrepton (Thio). Colonies which were able to grow on selective media were distributed over an ISP4 plate with Thio in order to make a glycerol stock of the strain.

Construct 4 (negative control) was transferred into *S. venezuelae* from a frozen glycerol stock. Selection for transconjugants was performed 15 hours after plating the cell suspension. The plates were placed at 30 °C for further growth, and after 15 days one small colony was observed. This colony was transferred onto ISP4 medium with Nal and Thio to select further, and 11 days later, colonies with *Streptomyces* morphology was observed. Since only one transconjugant arised from the first conjugation with construct 4, a second conjugation was performed, this time with fresh spores of *S. venezuelae*. The plates had a matt white surface when selection of transconjugants was performed 16 hours after plating the cell suspension. One week after selection, transconjugants were transferred onto ISP4 with Nal and Thio for further selection, followed by final selection on ISP4 with Thio.

The conjugative transfer of construct 1 (positive control) into *S. venezuelae* did not result in any well-growing clone even after 3 independent conjugations with fresh spore suspensions. During the conjugations, the mixtures of donor cells and recipient spores were grown for 16 hours at room temperature before Nal and Thio was distributed over the plates to select for transconjugants. One week after selection, some colonies were transferred onto ISP4 plates supplemented with Nal and Thio for further selection, but they did not grow after six days of incubation. The original colonies on the conjugation plate were yellow and did not have the characteristic morphology of *S. venezuelae*, indicating that they were not real recombinant strains with construct 1. This can be due to the wrong choice of ET cells, which maybe did not contain construct 1.

Construct 2 (pSOK805-*cmlF*p-*gusA*) was introduced into *S. venezuelae* by growing the mixtures of donor cells and recipient spores for 19 hours prior to selection with antibiotics. Several recombinant strains appeared four days after selection, and were selected further by growth on selective media.

In summary, the biosensor constructs number 2 and 3, as well as the negative control construct number 4 were introduced into the genome of *S. venezuelae* by conjugative DNA transfer from *E. coli* ET12567, resulting in recombinant strains. Construct 1 (positive control) was however not successfully introduced into *S. venezuelae* even after 3 independent attempts.

3.3 ANALYSIS OF CML PRODUCTION IN CULTURE BROTH

Recombinant *S. venezuelae* strains with introduced construct 2, 3 and 4 were cultured low-production medium to test the sensitivity of the biosensors. Ethanol was added to half of the cultures 10 hours after inoculation and 12 hours before the first samples were collected on day 1. The procedure for Cml extraction and preparation for UPLC analysis is described in Chapter 2.3.14.

After pelleting the cells by centrifugation, it was observed about 4-5 times more cells in the samples from cultures without ethanol, than from the cultures with ethanol. This indicates that addition of ethanol inhibits cell growth significantly. The cell concentration affects the amount of GusA enzyme in the cell lysates and may also affect the Cml production level. Cml was extracted from the cell supernatant by ethyl acetate, which was later evaporated. The residue was dissolved in methanol and analyzed by UPLC. Raw data from the UPLC analysis is given in Attachment G (page 88), while the calculation of final Cml concentration in µg/ml is described in Chapter 2.3.14. The mean Cml concentration in each culture is presented in Figure 3.3.1 and the error bars indicate the standard deviation of the mean (SDOM).

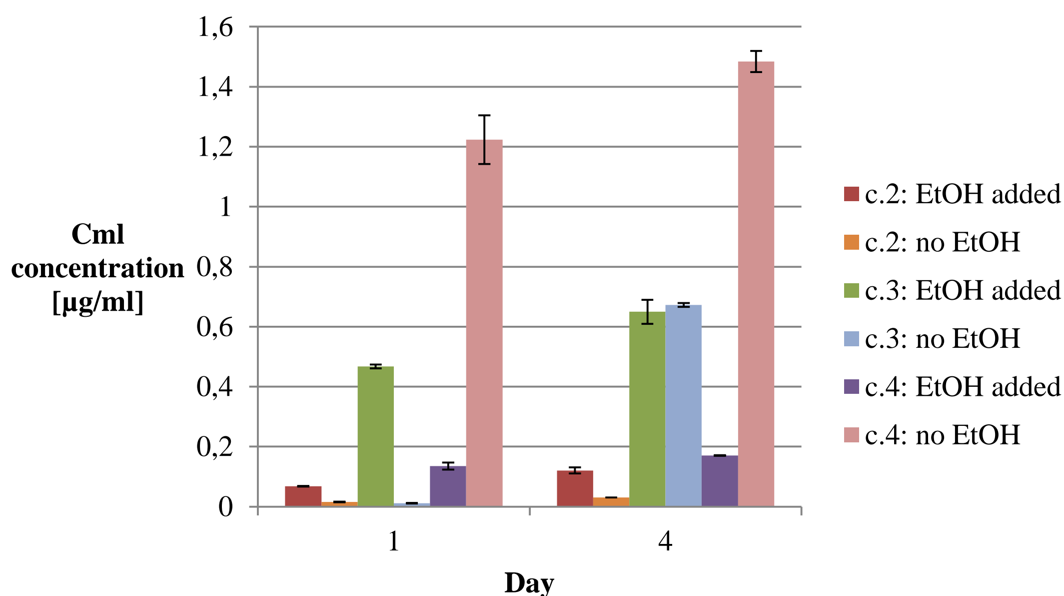


Figure 3.3.1: Chloramphenicol production in *S. venezuelae* transconjugants with introduced construct 2 (*cmlF*p), 3 (*kefB*p) and 4 (noPro). The strains were cultured with and without addition of ethanol (EtOH), and three parallel samples were taken from each culture on day 1 and 4 after inoculation.

The results from this first experiment are mostly not as expected. Addition of ethanol to the cultures should stop the production of Cml. The opposite is observed for the *S. venezuelae* transconjugant with introduced construct 2, which also shows a particularly low Cml productivity.

Chloramphenicol biosynthesis in the recombinant strain with construct 3 cultured with ethanol, seems to begin already before the first samples were collected on day one. Finally, on day 4 there was no difference in Cml production by this strain growing with or without ethanol shock, even though biosynthesis in the non-shocked strain started later. This may indicate that addition of ethanol decreased the rate of Cml production in this strain, since the non-shocked strain produced more Cml from day 1 to day 4 than the ethanol-shocked strain did during these days.

The only recombinant strain showing expected Cml production under different conditions is the strain that contains construct 4, which has no promoter controlling the *gusA* reporter gene. This strain showed low Cml production in the culture with ethanol added and high Cml production in cultures without ethanol. Unfortunately, this was not a biosensor strain, but a negative control strain for the reporter assay, so the Cml production could not be correlated to the GusA enzyme activity.

The Cml titer is expected to increase with longer production time, and this is also the case for all of the recombinant strains shown in Figure 3.3.1. Some of the strains show less difference in produced Cml than others, but there is an overall increase in Cml produced from day 1 to day 4. The recombinant strain with construct 3 shows the highest increase in Cml concentration from day 1 to day 4 in the culture without ethanol. Negligible amounts of Cml was produced until day 1, compared to the higher Cml concentration on day 4, which indicates that the onset of Cml production was induced later than for the other strains.

The total Cml production titer in this experiment is low compared to Cml yields reported previously by others. *S. venezuelae* wild-type strain cultured in medium containing glucose (3 % w/v), isoleucine (0.75 %) and basal salts produced 50 µg/ml Cml after three days of growth (Xu et al., 2010b). In comparison, the highest Cml concentration achieved in this experiment was 1.5 µg/ml after 4 days of growth. This deviation is probably due to the low-production medium used in this experiment in order to investigate the sensitivity of the biosensors. More experiments should be performed with other media, in order to increase the Cml production levels.

3.4 RESULTS FROM THE REPORTER ASSAY

The samples collected from the cultures were frozen down until the reporter assay analysis was performed, as described in Chapter 2.3.15. The cells were lysed to release the intracellular GusA enzyme, which converts PNPG substrate to a chromogenic compound that has maximum absorbance at 405 nm (Aich et al., 2001). The PNPG substrate was added to the cell lysates and incubated at 37 °C for 40 minutes until the optical density (OD) was measured in a spectrophotometer at 415 nm and 405 nm of light wavelength. The plate was left at room temperature for about 17 hours before new measurements were performed the next morning. These last results are presented graphically in Figure 3.4.1 and 3.4.2, and the OD measurements are given in Attachment H (page 89).

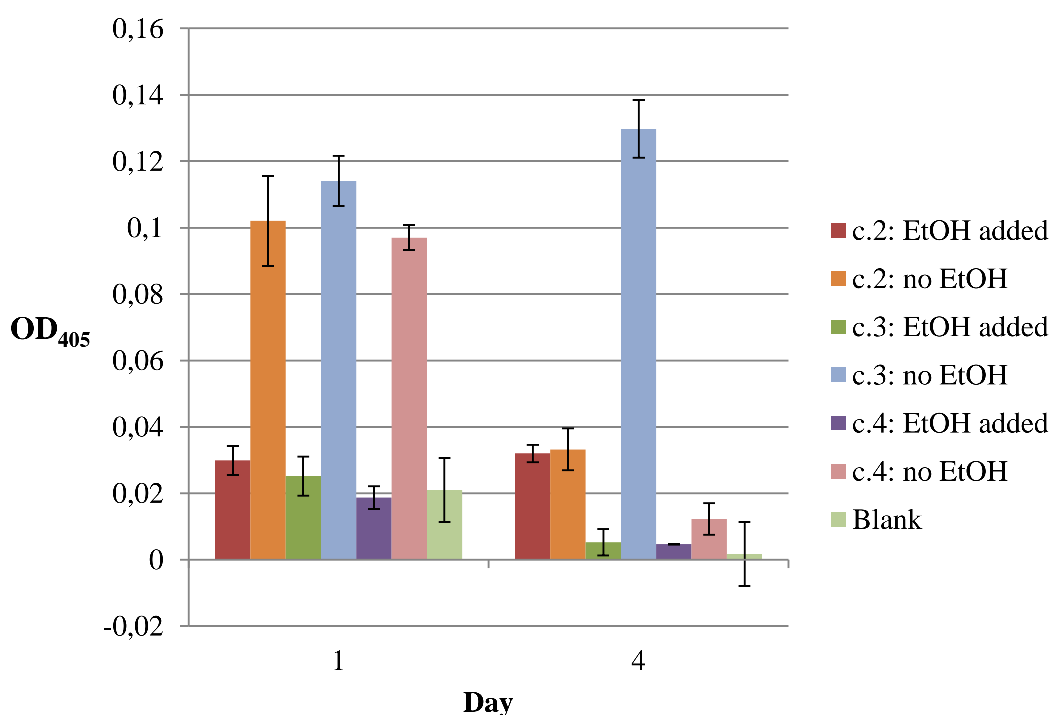


Figure 3.4.1: Glucuronidase activity measured as OD_{405} in cell lysates of *S. venezuelae* transconjugants with introduced construct 2 (*cmlF*p), 3 (*kefB*p) and 4 (noPro), which were cultured with and without addition of ethanol (EtOH). The error bars indicate the means ± 1 SDOM.

The results from OD_{405} and OD_{415} analysis have similar trends, although the OD_{405} measurements are about 0.02 values higher than the OD_{415} measurements. This is in agreement with the fact that the maximum absorbance of p-nitrophenol (one of the reaction products) is at 405 nm (Aich et al., 2001). The absorbance at 415 nm was also measured since this wavelength was used by Myronovskyi et al. (2011) when PNPG was used as a substrate for glucuronidase.

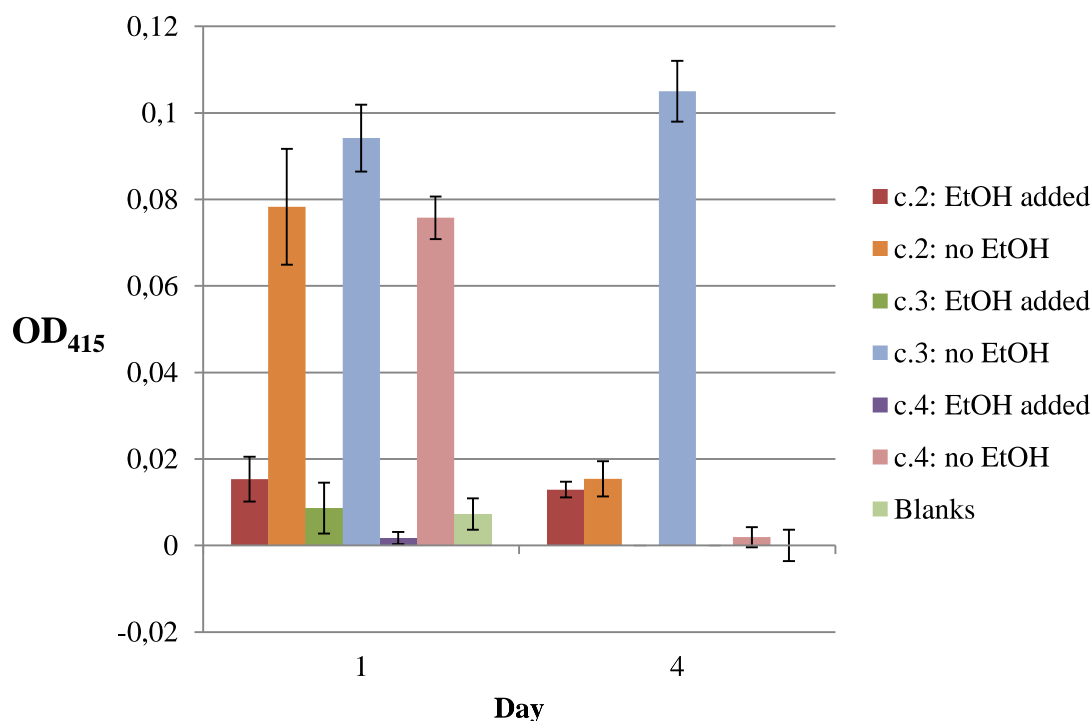


Figure 3.4.2: Glucuronidase activity measured as OD_{415} in cell lysates of *S. venezuelae* transconjugants with introduced construct 2 (*cmlF*p), 3 (*kefB*p) and 4 (noPro). The strains were cultured with and without addition of ethanol (EtOH) as indicated to the right. The error bars indicate the means ± 1 SDOM.

The samples collected on day 1 show lower enzyme activity in the cultures with ethanol, than in those without. This deviation may be due to the much lower cell concentration in the samples added ethanol, since the GusA concentration in the lysate is proportional to the amount of cells. In future experiments, the enzyme activity should be adjusted and correlated to the protein concentration in the lysates in order to get comparable results.

Construct 4 lacks a promoter for the *gusA* gene and is therefore considered as a negative control. Unexpectedly, the glucuronidase activity in lysate from the recombinant strain with construct 4 is as high as the activity of lysate from the strain with construct 2 (on day 1). This may be due to read-through from a promoter in the genome of *S. venezuelae* at the site of construct integration.

The *S. venezuelae* recombinant strain with introduced construct 3 (*kefB*p) showed the highest glucuronidase activity. The activity increases from the first day until the fourth day, which corresponds to the higher Cml concentration achieved on day 4. However, the increase in Cml production from day 1 to day 4 does not fully correlate with the increase in GusA activity. Several experiments are required to verify this trend.

4 GENERAL DISCUSSION

4.1 CONSTRUCTION AND VERIFICATION OF THE VECTORS

While analyzing the overlapping fragments for 'Gibson' ligation by gel electrophoresis, a deviation from the expected band size was observed for the largest fragments. This may be due to the slower migration rate of GC-rich DNA and fragments with higher concentration than the ladder band.

*Dpn*I treatment of PCR products is used to digest methylated PCR templates and retain the un-methylated PCR product. Common *E. coli* strains Dam-methylate their DNA, which is therefore susceptible to the *Dpn*I digestion (Weiner et al., 1994). During verification of the constructs, many of the isolated pDNA's were identified as original vector or reporter gene plasmid templates. This insufficient digestion of methylated DNA templates may be due to high template concentrations compared to the enzyme concentration, or too short digestion times. Dilution of the PCR-template was probably the most efficient approach in order to reduce pDNA templates in the transformants, since longer *Dpn*I treatment was used for several other fragments without eliminating the templates completely.

Verification of the constructs can also be performed by DNA sequencing in order to ensure that no mutations had occurred in important parts of the vector. A mutation in the origin of transfer, attachment site or integrase gene could affect the conjugative transfer of DNA and result in no transconjugants, while mutations in the inserted promoters and/ or *gusA* gene could result in unexpected reporter assay results.

4.2 CONJUGATIVE DNA TRANSFER

Optimization of the conjugation protocol for streptomycetes is in progress, and the protocol was recently changed. The main differences between the protocol used for the project work and this Master thesis are: (i) centrifugation of the bacterial mix to achieve better cell-to-cell contact, (ii) overnight incubation at room temperature instead of 30 °C, (iii) letting the antibiotic suspension diffuse into the plate with the lid closed instead of drying it with an open lid inside the sterile hood.

A critical point in the conjugation procedure is the selection of transconjugants by adding a solution of nalidixic acid and thiostrepton to the plate. Thiostrepton was used to select for recombinant strains with the inserted constructs, while nalidixic acid was used to select against *E. coli* since streptomycetes are naturally resistant. If the antibiotic mix is added too early in the growth phase, it will probably affect the outcome, since the bacteria needs to be robust to survive the antibiotic stress imposed

upon them. After using trial-and-error method it was observed that more transconjugants were obtained when the antibiotic solution was added after a visible layer of mycelia had appeared on the plate.

4.3 CHLORAMPHENICOL PRODUCTION IN CULTURED STRAINS

In order to achieve higher Cml production, different growth conditions for *S. venezuelae* should be tested in future experiments. Cml production has been reported to depend on the combination of carbon- and nitrogen source in the growth medium (Doull and Vining, 1990) and nitrogen sources that resulted in a slow, controlled growth (e.g. DL-Serine) were shown to increase the Cml yield (Westlake et al., 1968). A common medium used to promote Cml biosynthesis contains glucose (3 % w/v), isoleucine (0.75 %) and basal salts (Doull et al., 1985, Brown et al., 1996, Facey et al., 1996). In this experiment, a low-production medium containing maltose, yeast extract and malt extract was used in order to test the sensitivity of the biosensors. Other media and growth conditions may be tested in future experiments.

4.4 REPORTER ASSAY

The GusA activity was investigated by incubating the PNPG substrate with cell lysates from the recombinant strains that were cultured under different conditions. Enzyme concentration in cell lysates is expected to be proportional to the amount of cells. The cell concentration in cultures without ethanol was 4-5 times larger than in the cultures added ethanol, thus the protein content must be adjusted in order to give comparable results. The results from this first reporter assay are therefore preliminary. Future experiments should also include the positive control construct and measurements of protein content in the cell lysates, in order to compare the enzyme activities measured in the reporter assay.

Other sources of error in the reporter assay may be caused by freezing down the samples collected from the cell cultures, since it is unknown whether the enzyme tolerates to be stored at -20 °C. Another source of error may be insufficient lysis of the cells, which results in less GusA enzyme in the cell lysate. It may also be that storing the lysate at room temperature, instead of on ice, can influence the enzyme activity and thus also the OD measurements. The reporter assay protocol can be developed to circumvent such sources of error and perhaps lead to better results. The main source of error during the OD measurements was bubbles in the wells: light reflection from the bubble surface may have affected the measured absorbance. Careful pipetting can reduce the amount of bubbles in future experiments.

4.5 FUTURE WORK

The positive control for the reporter assay, construct 1, remains to be introduced into *S. venezuelae*. Modified versions of the conjugation protocol, as well as using other colonies of *E. coli* ET12567 cells containing the construct, may result in site-specific integration of the construct into the genome of *S. venezuelae*. However, if future observations indicate that the strong *ermE** promoter inhibits cell growth by constitutive expression of the GusA enzyme, an inducible promoter may be used instead. Examples of inducible promoters used in streptomycetes are: *tipA*, which is induced by addition of thiostrepton, and *tetR*, which is induced by tetracycline and anhydrotetracycline (Medema et al., 2011b). The limitation with these inducible systems is that they are leaky. Low-level transcription from the promoter occurs even in the absence of an inducer (Myronovskyi et al., 2011).

The culturing of transconjugant strains should be repeated and different culture conditions can be tested, as discussed previously. The positive control construct should be included and the protein content in lysates should be measured to get comparable results from the reporter assay.

If future experiments result in the expected correlation between Cml production and GusA activity in the strains containing biosensor constructs, this model may be used to detect when silent gene clusters are expressed. New biosensors may be constructed by fusing promoters that control transporter gene expression in silent secondary metabolite gene clusters to the *gusA* reporter gene. Expression of the transporter gene may be detected by the reporter assay, since the genes are placed under the same regulatory control mechanisms. This may help to detect under which conditions expression of a silent secondary metabolite gene cluster is activated, which may result in the discovery of new drugs.

5 CONCLUSION

The four vectors were successfully assembled by the 'Gibson' reaction and verified by enzyme digestion followed by gel electrophoresis. Three out of four constructs were site-specifically integrated into the genome of *S. venezuelae* by conjugative DNA transfer from *E. coli* ET12567. The recombinant strains were verified by further selection with antibiotics.

Culturing of the *S. venezuelae* recombinant strains in low-production medium was followed by the reporter assay and detection of chloramphenicol production by UPLC analysis. One of the biosensor strains showed a correlation between production of chloramphenicol and the reporter assay results, but this trend needs to be verified by further experiments. The results from the reporter assay are preliminary, since the protein content has to be adjusted when measuring enzyme activity in the lysates. The cells produced only small amounts of chloramphenicol, thus different media and culture conditions should be tested in future experiments. Several independent experiments are required before any final conclusions can be drawn on the functionality of the biosensors.

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ATTACHMENT A: MEDIA RECIPES

A1 LB medium

LB medium for bacterial growth in liquid culture was prepared by weighing out the ingredients listed in Table A1. The ingredients were dissolved in distilled water, and then autoclaved at 121 °C for 20 min.

Table A1: Recipe for making LB-medium.

Ingredients	Concentration [g/L]
Tryptone	10
Yeast extract	5
NaCl	5

A2 LA medium

The prescription on how to prepare LA medium for bacterial growth on a solid plate is given in Table A2. The ingredients were dissolved in distilled water before sterilization at 121 °C for 20 min. Different antibiotic(s) were added to the medium when it had cooled down to 50-60 °C: 1µl antibiotic stock solution per 1 ml LA medium. The solution was mixed well before distributing the hot liquid into plates.

Table A2: Recipe for making LA medium.

Ingredients	Concentration [g/L]
Tryptone	10
Yeast extract	5
NaCl	5
Agar	15

A3 ISP4 medium

ISP4 medium for *Streptomyces* growth and conjugation was prepared as suggested on the package (*Difco* ISP Medium 4): 37 g media/L distilled water. The mixture was autoclaved at 121 °C for 20 min. $MgCl_2$ was added to some of the ISP4 medium (1 ml 1 M $MgCl_2$ / 100 ml ISP4 medium) to increase the conjugation efficiency. ISP4 plates with only thiostrepton, and some with both nalidixic acid and thiostrepton were also made. The final concentration of each antibiotic was in both cases 30 µg/ml medium.

A4 TSB medium

Tryptone Soya Broth (TSB) medium for culturing Streptomyces was prepared as suggested on the package: 30 g/L distilled water. The mix was autoclaved at 121 °C for 20 min.

A5 2×YT medium

The 2×YT medium for conjugation between *Streptomyces venezuelae* and *E. coli* ET12567 was prepared by adding the ingredients listed in Table A5 to 9/10 of the total volume distilled water. Then the pH was adjusted to 7.0 by adding 5 M NaOH. The volume was adjusted to 1L with distilled water before sterilization at 121 °C for 20 min.

Table A5: Recipe for 2×YT medium.

Ingredients	Concentration [g/L]
Bacto Tryptone	16
Bacto Yeast extract	10
NaCl	5

A6 MYM medium

The MYM medium for culturing Streptomyces was prepared my mixing the ingredients listed in Table A6 with 1 L distilled water before autoclaving the medium at 121 °C for 20 min.

Table A6: Recipe for MYM medium

Ingredients	Concentration [g/L]
Maltose	4
Yeast extract	4
Malt extract	10
MOPS Na-salt	1,9

ATTACHMENT B: BUFFERS AND STOCK SOLUTIONS

B1 TSS buffer

The TSS buffer is used to prepare chemically competent cells. It was made by adding the three first ingredients in Table B1, adjusting the pH to 6,5 and autoclaving before addition of DMSO.

Table B1: Recipe for TSS buffer.

Ingredients	Total concentration	Amount for 50 ml buffer
LB-media	85 %	42.5 ml
PEG_{6000}	10 %	5 g
$MgCl_2$ (1 M)	20 mM	2.5 ml
DMSO	5 %	2.5 ml

B2 50× TAE buffer

TAE buffer is used for DNA gel electrophoresis. The 50× TAE buffer was made according to the recipe in Table B2, and diluted to 1× TAE buffer.

Table B2: Recipe for 50× TAE buffer.

Ingredients	Amount
Tris-base	242.0 g
Acetic acid (100 %)	57.1 ml
EDTA (0.5 M) pH 8	100 ml
ds H_2O	up to 1 L total volume

B3 Agarose (0,8 %)

Agarose solution for gel electrophoresis was made by mixing the ingredients in Table B3 together and warming it in the microwave for 5 min until the powder was dissolved and the solution was clear. The agarose solution was stored at 60 °C.

Table B3: Recipe for agarose solution (0,8 %).

Ingredients	Amount
SeaKem LE Agarose	2.4 g
1× TAE buffer	300 ml
GelGreen Nucleic Acid Stain (10 000×)	30 µl

B4 Glycerol stock solution (20 %)

The 20 % Glycerol solution was made by diluting glycerol solution (99.5 %) in distilled water with ratio 1: 5 as indicated in Table B4.

Table B4: Recipe for 20 % Glycerol stock solution.

Ingredients	Concentration [ml/L]
Glycerol (99.5 %)	200
distilled H_2O	800

B5 Lysozyme (10 %) in EDTA

A 10 % Lysozyme solution was made by mixing the ingredients listed in Table B5.

Table B5: Recipe for 10 % Lysozyme in EDTA.

Ingredients	Amount for 5 ml
Lysozyme	0.05 g
EDTA (50 mM, diluted from 0.5 M)	5 ml

B6 Antibiotic stock solutions

All the antibiotic stock solutions were made by dissolving proper amount of the antibiotic salt in a solvent. The ingredients are listed in Table B6.1 - B6.5. All the antibiotics were stored at - 20 °C.

Table B6.1: Ampicillin stock solution (100 mg/ml dsH_2O).

Ingredients	Amount
Ampicillin	0.5 g
dsH_2O	5 ml

Table B6.2: Chloramphenicol stock solution (30 mg/ml dsH_2O).

Ingredients	Amount
Chloramphenicol	150 mg
Absolute ethanol	5 ml

Table B6.3: Kanamycin stock solution (40 mg/ml dsH_2O).

Ingredients	Amount
Kanamycin	200 mg
dsH_2O	5 ml

Table B6.4: Nalidixic acid stock solution (30 mg/ml 0.1 M NaOH).

Ingredients	Amount
Nalidixic acid sodium salt	150 mg
NaOH (0.1 M)	5 ml

Table B6.5: Thiostrepton stock solution (30 mg/ml DMSO).

Ingredients	Amount
Thiostrepton	150 mg
DMSO	5 ml

B7 ‘Gibson’ reaction solutions

The recipes for the Gibson 5× isothermal reaction buffer and Gibson ligation master mix are given in Table B7.1 and B7.2, respectively.

Table B7.1: Gibson 5× isothermal reaction buffer.

Ingredients	Amount
Tris-HCl (1 M)	3 ml
$MgCl_2$ (2 M)	150 µl
dGTP (100 mM)	60 µl
dATP (100 mM)	60 µl
dTTP (100 mM)	60 µl
dCTP (100 mM)	60 µl
DTT (1 M)	300 µl
Polyethyleneglycol (PEG-8000)	1.5 g
NAD (100 mM)	300 µl

Table B7.2: Gibson ligation master mix.

Ingredients	Amount
5 × isothermal reaction buffer	80 µl
T5 exonuclease (1 U/µl, diluted 10x from 10 U/µl)	1.6 µl
Phusion DNA polymerase	5 µl
Taq DNA ligase	40 µl
dsH_2O	174.4 µl

B8 Buffers for the reporter assay

Recipes for buffers used in the reporter assay are given in Table B8.1- B8.6. The sodium phosphate buffer was adjusted to pH 7.0 before it was used to prepare the dilution buffer.

Table B8.1: Recipe for making the sodium phosphate buffer (100 mM, pH 7.0).

Ingredients	Amount [ml]
Na_2HPO_4 (1 M)	5.78
NaH_2PO_4 (1 M)	4.24
dsH_2O	90
Total volume	100

This phosphate buffer was used as a basis to make the dilution buffer as described in Table B8.2. The DTT (1 M) and Triton X-100 (10 %) solutions were made prior to making the dilution buffer. The dilution buffer was autoclaved at 120 °C for 20 min to sterilize it.

Table B8.2: Recipe for making the dilution buffer.

Ingredients	Total concentration	Amount
Phosphate buffer (100 mM, pH 7,0)	50 mM	100 ml
Dithiothreitol (DTT, 1M)	5 mM	1 ml
Triton-X-100 (10 %)	0,1 %	2 ml
dsH_2O		97 ml
Total volume		200 ml

The sterilized dilution buffer was used to make the lysis buffer, which was prepared fresh right before use by mixing the ingredients in Table B8.3.

Table B8.3: Recipe for making the lysis buffer (1 mg lysozyme/ml dilution buffer).

Ingredients	Amount
Dilution buffer	58 ml
Lysozyme	0.0577 g

A stock solution of p-nitrophenyl-β-D-glucuronide (PNPG) substrate for the reporter assay was also freshly prepared by mixing the ingredients listed in Table B8.4.

Table B8.4: Recipe for making PNPG substrate stock solution (0.2 M).

Ingredients	Total concentration	Amount
p-nitrophenyl-β-D-glucuronide (PNPG)	0.2 M	0.0323 g
Sodium phosphate buffer (100 mM, pH 7.0)	50 mM	256 µl
dsH_2O		256 µl

ATTACHMENT C: PRIMERS

The primers were designed in j5 (Hillson).

Construct 1: pSOK805::*ermE*p::*gusA* assembled in two steps

1) Assembling pSOK807 (pSOK805 + ermEp)

SOK805e-F: GATCTGCAGCCAAGCGATGAATTCGTAATCATGGTCATAGC
SOK805e-R: GAGCTCGAATTCCGATATCTAGATCTCGAGCTCGCG
ermEp7-F: AGATCTAGATATCGGAATTCGAGCTCGGTACC
ermEp7-R: GATTACGAATTCATCGCTTGGCTGCAGATCCTACC

2) Assembling construct 1 (pSOK807 + GusA)

SOK807-F: CCGGTATCCGACCGATGAATTCGTAATCATGGTCATAGC
SOK807-R: CCGGCCTCAGCATGCTTGGCTGCAGATCCTACC
GusA7-F: TCTGCAGCCAAGCATGCTGAGGCCGGTCGAG
GusA7-R: TTACGAATTCATCGGTCGGATACCGGTGGAAAC

Construct 2: pSOK805::*cmlF*p::*gusA*

SOK805-F1: use SOK805_kef-F
SOK805-R1: TGTTCATGACGACCCGAAGTTCACCGAAGAGC
cmlFpN-F: CGGTGAACTTCGGGTCGTCATGAACACTCCTTCTCC
cmlFpN-R2: CCGGCCTCAGCATGAATTCCGACGTTCCCTGG
GusAcp-F1: AACGTCGGAATTCATGCTGAGGCCGGTCGAG
GusAcp-R1: GGAATTGTGAGCGGATAACGGTCGGATACCGGTGGAAAC

Construct 3: pSOK805::*kefB*p::*gusA*

SOK805_kef-F: CCGGTATCCGACCGTTATCCGCTCACAATTCCACAC
SOK805_kef-R: ATCTACGTTCTCGCCGAAGTTCACCGAAGAGC
KefBp-F: CGGTGAACTTCGGCGAGAACGTAGATGGCGAATGG
KefBp-R: CCACAGGTCTCAGCATGAACACTCCTTCTCCGCG
GusA_kef-F: AGAAGGAGTGTTCATGCTGAGACCTGTGGAAAC
GusA_kef-R: GGAATTGTGAGCGGATAACGGTCGGATACCGGTGGAAAC

Construct 4: pSOK805::*gusA* (NoPro)

SOK805n-F: use SOK805kef-F
SOK805n-R: GGTTGGTGACTGCCCGAAGTTCACCGAAGAGC
SD_gusA-F: CGGTGAACTTCGGGCAGTCACCAACCGCATC
GUSAn-R: GGAATTGTGAGCGGATAACGGTCGGATACCGGTGGAAAC
SD_gusA-F + GUSAn-R

ATTACHMENT D: PLASMID MAPS

The plasmid maps with relevant restriction sites are presented in Figure D1-D5. All the plasmids contain a *bla* gene which confers resistance to ampicillin, and most of the plasmids contain a *gusA* gene which encodes the reporter enzyme (β-glucuronidase). The constructs in Figure D3-D5 and pSOK805 in Figure D1 contain: *RP4 oriT*, origin of conjugal transfer; *attP*, attachment site; *int*, integrase gene; *ColElori*, origin of replication in *E. coli*; *tsr*, thiostrepton resistance gene for selection of *S. venezuelae* transconjugants.

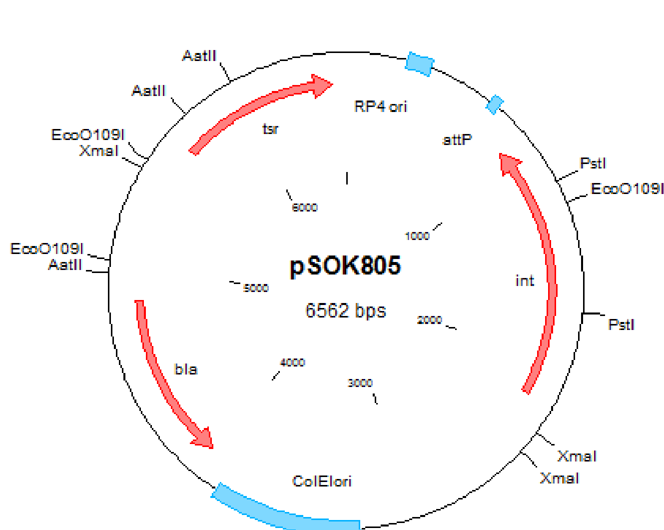


Figure D1: Template vector for construction of the biosensors.

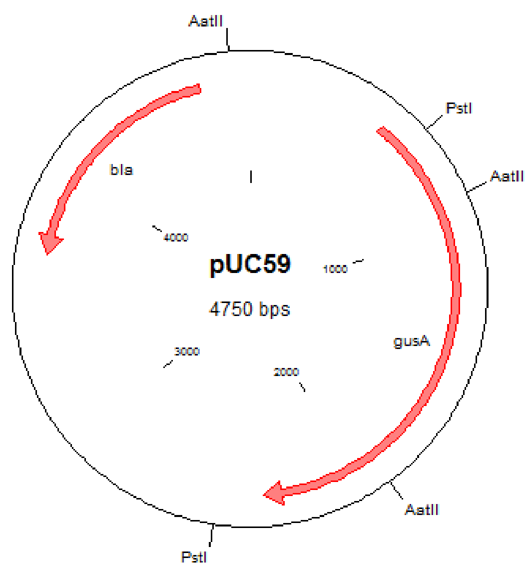
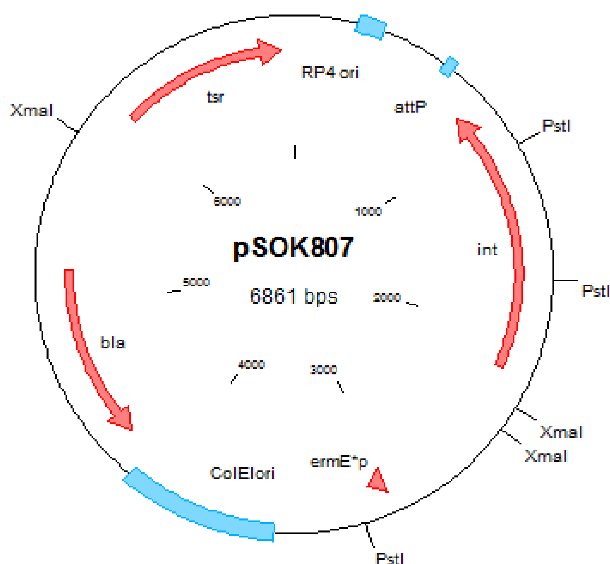


Figure D2: Template plasmid for the *gusA* reporter gene. It also contains a *bla* gene which confers resistance to ampicillin.



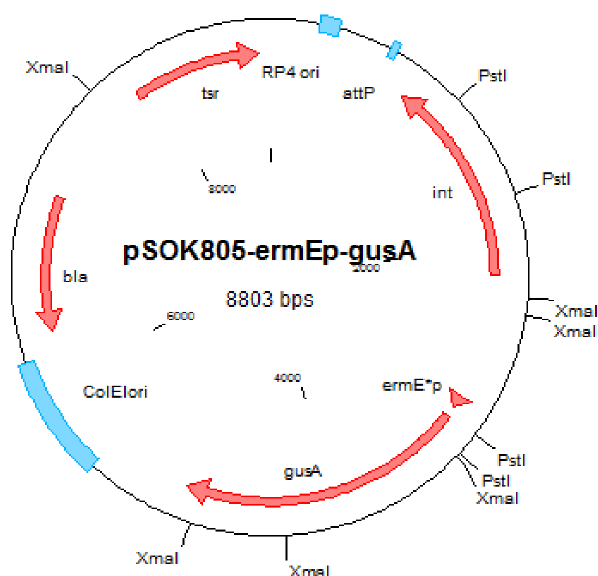


Figure D3: Left: the intermediate (pSOK807) plasmid when constructing the positive control vector in two steps. Right: construct 1 (positive control).

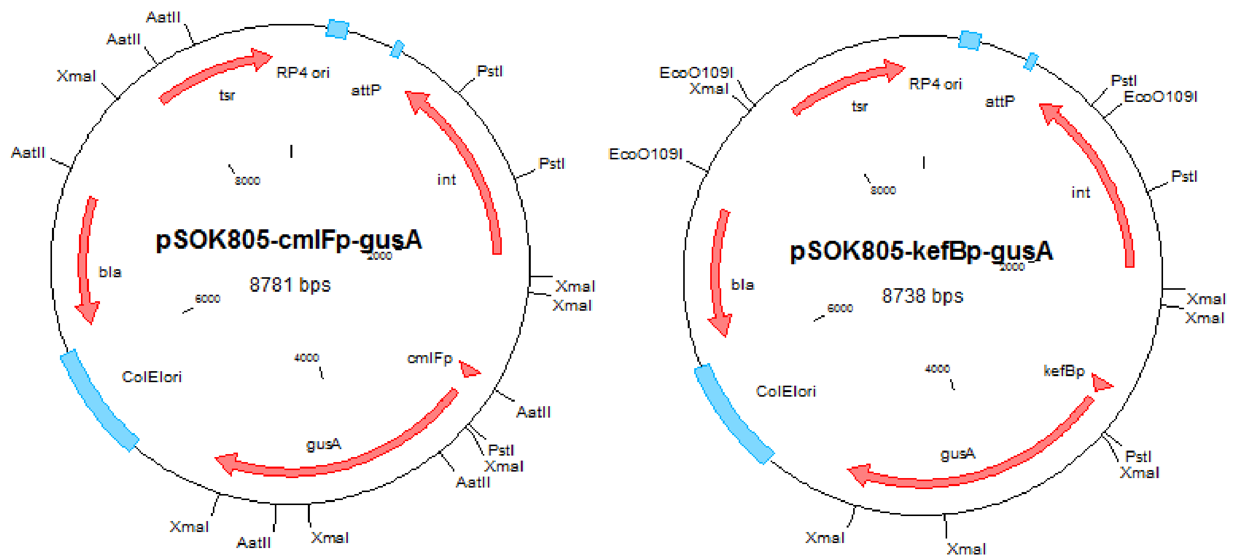


Figure D4: The biosensor plasmids. Construct 2 to the left, construct 3 to the right.

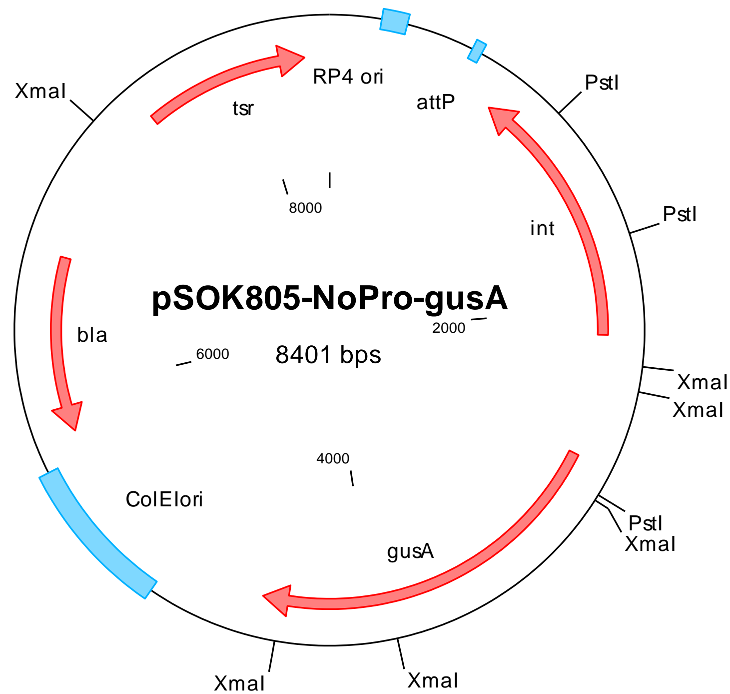


Figure D5: The negative control construct 4 without any promoter upstream of the *gusA* reporter gene.

ATTACHMENT E: GEL ELECTROPHORESIS LADDER

The ladder used in gel electrophoresis is shown in Figure E1.

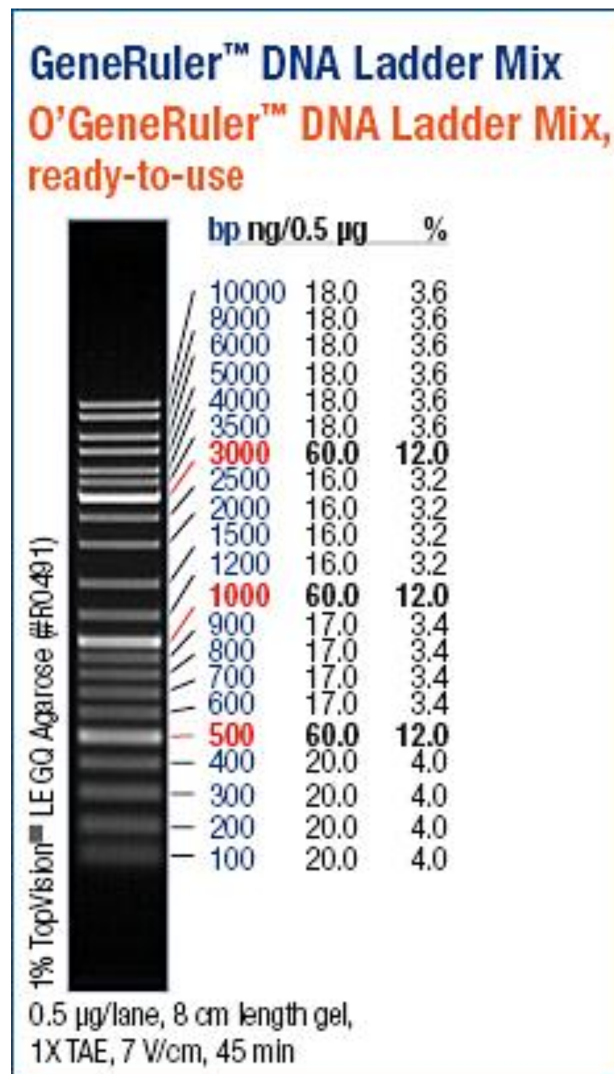


Figure E1: The band sizes of Gene Ruler 1 kb DNA ladder mix from Fermentas.

ATTACHMENT F: STANDARD LAB PROTOCOLS

F1 Isolation of genomic DNA

The protocol for isolation of genomic DNA with Wizard® Genomic DNA Purification Kit from Promega is described below.

1. 1,5 ml of an overnight culture was transferred to an Eppendorf tube and centrifuged (13 000 rpm, 4 min).
2. The supernatant was removed and the cells were resuspended in 600 µl of lysozyme (10 % in 50mM EDTA) and then incubated at 37 °C for 30 – 60 min. Lysozyme inhibits the cross-bonding in peptidoglycan, thus weakening the cell wall of Gram positive bacteria.
3. After incubation, the suspension was centrifuged under the same conditions and the supernatant was removed.
4. The cells were gently resuspended in 600 µl of Nuclei Lysis Solution. This mix was incubated at 80 °C for 5 min to lyse the cells, and then cooled down to room temperature.
5. RNase Solution (3 µl) was added, and mixed by inverting the tubes 4 times. It was incubated at 37°C for up to 60 min, before cooling it down to room temperature.
6. Proteins were precipitated from the solution by adding Protein Precipitation Solution (200 µl) and vortexing for 20 seconds.
7. The sample was incubated on ice for 5 min, and centrifuged (13 000 rpm, 3 min).
8. The supernatant was transferred to a clean Eppendorf tube containing 600 µl of isopropanol. The solution was gently mixed by inversion until DNA precipitated.
9. When DNA had formed a visible mass, the tube was centrifuged again (13 000 rpm, 2 min). The supernatant was discarded and the tube was drained on absorbent paper.
10. Ethanol (600µl, 70 %) was added and the tube was mixed by inversion to wash the DNA pellet.
11. The sample was centrifuged (13 000 rpm, 3 min) and the ethanol was carefully aspirated.
12. The tube was drained on absorbent paper and the pellet was air-dried for 10-15 min.
13. The DNA-pellet was rehydrated by adding 100 µl DNA Rehydration Solution and incubating it at 65°C for 1 hour. It was mixed several times during incubation by tapping the tube.
14. Purified genomic DNA was stored at -20 °C.

F2 Isolation of DNA fragments from agarose gel

QIAquick Gel Extraction Kit:

The protocol for isolation of DNA from agarose gel by using QIAquick Gel Extraction Kit from QIAGEN is described below.

1. The DNA band was cut from the gel with a clean, sharp scalpel while visualized by trans UV-light in Gel Doc 2000.
2. The gel slice was weighed and added 3 volumes of Buffer QG to 1 volume of gel in an Eppendorf tube (ml Buffer QG = 3* mg of gel).
3. The solution was incubated at 50 °C until the gel was dissolved.
4. The solution was yellow which indicated right pH for optimal DNA binding. If the solution had been orange or violet, 10 μl of 3 M sodium acetate should be added to achieve pH ≤ 7.5.
5. One gel volume of isopropanol was added to increase the yield of DNA fragments, and mixed by vortexing.
6. The mix was transferred to a QIAquick spin column in a collection tube and centrifuged for 1 min at full speed.
7. The flow-through was discarded, and Buffer QG (500 μl) was added to remove all traces of agarose. The tube was centrifuged again and the flow-through was discarded.
8. Buffer PE (750 μl) was added to wash the DNA. The column was centrifuged, the flow-through discarded and a second centrifugation was performed (1 min, full speed).
9. The spin column was transferred to a clean Eppendorf tube and warm (50°C) Buffer EB (30 μl) was added to elute DNA. The tube was centrifuged again.
10. The purified DNA was stored at -20°C, and a small amount of it was checked by gel electrophoresis.

QIAEX II Suspension:

The QIAEX II suspension contains silica particles which absorb DNA in the presence of high salt solution. Impurities like agarose, proteins and salts are removed during the washing steps, while nucleic acids are bound to the silica particles. The protocol for isolation of DNA from agarose gel by using QIAEX II suspension from QIAGEN is described below.

1. The DNA band was cut from the gel with a clean, sharp scalpel while visualized by trans UV-light in Gel Doc 2000.
2. The gel slice was weighed and added 3 volumes of Buffer QG to 1 volume of gel in an eppendorf tube (ml Buffer QG = 3*mg of gel).
3. QIAEX II suspension (10 μl) was added to the tube and mixed well by vortexing (for fragments < 10 kb) or flicking the tube (for isolation of larger

fragments > 10 kb). The solution was incubated at 50 °C for 10 min, while flicking the tube once in a while to mix and enhance DNA-absorption by the silica particles in the QIAEX II suspension.

4. The tube was centrifuged at top speed for 30 seconds, before aspirating the supernatant with a pipette. Buffer QG (500 µl) was added to the pellet and resuspended by flicking the tube gently.
5. Step 4 was repeated two times with 500 µl of Buffer PE.
6. The tube was centrifuged at top speed for 30 seconds, and the supernatant was removed with a thin pipette to take out as much liquid as possible. The pellet was dried out for 15 min until it turned white.
7. Buffer EB (20 µl) was added to resuspend the pellet and elute DNA from the silica particles. The tube was incubated at 50 °C for 10 min, followed by centrifugation at top speed for 30 seconds. The DNA-containing liquid was transferred to a clean Eppendorf tube.
8. The purified DNA was stored at -20°C, and a small amount of it was checked by gel electrophoresis.

F3 Purification of PCR product

The protocol for purification of PCR product by using QIAquick PCR Purification Kit from QIAGEN is described below.

1. Five volumes of Buffer PB were added to 1 volume of the PCR sample. The solution was mixed and then transferred to a QIAquick spin column placed in a collection tube.
2. The sample was centrifuged (13 000 rpm, 1 min) to bind DNA to the column.
3. The flow-through was removed and Buffer PE (750 µl) was added to wash the column. The tube was centrifuged again and the flow-through was discarded. One additional centrifugation was performed to remove all traces of washing solution.
4. The column was placed in a clean Eppendorf tube.
5. The DNA was eluted from the column by adding Buffer EB (50 µl) and centrifuging.
6. The concentration and size of purified DNA was checked by gel electrophoresis. Purified DNA was stored at -20 °C.

F4 Isolation of plasmid DNA (pDNA)

The protocol for isolation of pDNA by using Wizard® *Plus* SV Minipreps DNA Purification System from Promega is described below.

1. The overnight culture was transferred to an Eppendorf tube and centrifuged (10 000 rpm, 5 min). The supernatant was discarded and excess media was removed by drying the tube on a paper towel.
2. The cell pellet was resuspended in Cell Resuspension Solution (250 µl). The suspension should not be vortexed after step 2 to prevent splitting of chromosomal DNA.
3. Cell Lysis Solution (250 µl) was added and mixed by inverting the tube 4 times. The cells were incubated for up to 5 min until the cell suspension cleared, which indicated that the cells were lysed.
4. Alkaline Protease Solution (10 µl) was added to inactivate endonucleases and other proteins. Mixed by inverting the tube 4 times, before incubating at room temperature for 5 min. (This step is not necessary for a good product and Alkaline Protease can in some cases destroy large vectors.)
5. The suspension was neutralized by adding Neutralization Solution (350 µl), and mixed by inverting the tube 4 times, followed by centrifugation (13 000 rpm, 10 min).
6. Spin Columns were placed in the Collection Tubes and the DNA-containing supernatant was transferred to the Spin Columns.
7. DNA was bound to the column by centrifugation (13 000 rpm, 1 min), and the flow-through was discarded from the Collection Tube. The DNA was washed by adding Column Wash Solution (750 µl) to the Spin Column, followed by centrifugation (13 000 rpm, 1 min).
8. The flow-through was discarded and Column Wash Solution (250 µl) was added to the Column. The tube was centrifuged again (13 000 rpm, 2 min).
9. The Spin Column was transferred to a clean Eppendorf tube and Nuclease-Free Water (100 µl) was added to elute the DNA bound to the Column.
10. The Eppendorf tube was centrifuged (13 000 rpm, 1 min) and the purified plasmid DNA was stored at -20 °C.
11. The plasmid size and purity was checked by enzyme digesting, followed by DNA gel electrophoresis.

ATTACHMENT G: RESULTS FROM UPLC ANALYSIS

The raw data from measurements of chloramphenicol concentration in three parallel samples from each transconjugant strain is presented in Table G1. The number marked in red was considered as a statistical outlier and was therefore not included in further calculations.

Table G1: Chloramphenicol concentration in 400 µl of methanol suspension analyzed by UPLC.

EtOH	day	Strain	Parallel	Cml concentration [nM]
+	1	c.2	1	218,9
+	1	c.2	2	210,7
+	1	c.2	3	203,6
+	4	c.2	1	311,2
+	4	c.2	2	415
+	4	c.2	3	396,3
-	1	c.2	1	47,5
-	1	c.2	2	46
-	1	c.2	3	52,4
-	4	c.2	1	16,5
-	4	c.2	2	95,6
-	4	c.2	3	94,3
+	1	c.3	1	1484,6
+	1	c.3	2	1425,6
+	1	c.3	3	1425,7
+	4	c.3	1	1763,5
+	4	c.3	2	2093,2
+	4	c.3	3	2173,5
-	1	c.3	1	36,9
-	1	c.3	2	31,3
-	1	c.3	3	38,9
-	4	c.3	1	2050
-	4	c.3	2	2078,8
-	4	c.3	3	2115,3
+	1	c.4	1	453,5
+	1	c.4	2	458,5
+	1	c.4	3	344,4
+	4	c.4	1	521,6
+	4	c.4	2	531,7
+	4	c.4	3	531,5
-	1	c.4	1	3863,2
-	1	c.4	2	4175,8
-	1	c.4	3	3320,5
-	4	c.4	1	4717,8
-	4	c.4	2	4376,9
-	4	c.4	3	4686,2

ATTACHMENT H: RESULTS FROM THE REPORTER ASSAY

The OD measurements which were performed after 17 hours incubation at room temperature (in addition to the first 40 minutes of incubation at 37 °C) are given in Table H1-H2. The numbers marked in red were considered as statistical outliers and were therefore kept out when calculating the mean and standard deviation of the parallels. The negative measurements were set equal to zero, before drawing the graphs. Calculation of the mean and standard deviation (SD) was performed by standard excel formulas (MEAN and STDAV.S). Standard deviation of the mean was calculated by this formula: $SD/\sqrt{3}$.

Table H1: Results from OD_{405} measurements of the samples collected from recombinant *S. venezuelae* strains with introduced construct 2, 3 and 4.

EtOH	day	construct 2 (*cmlF*p)			construct 3(*kefB*p)			construct 4 (noPro)		
+	1	0,0225	0,0296	0,0375	0,0369	0,0183	0,0203	0,0145	0,0229	-
+	4	0,0346	0,0267	0,0346	0,0026	0,0001	0,0129	0,0002	-0,0006	0,0137
-	1	0,1191	0,0753	0,1118	0,1021	0,1280	0,1122	0,0855	0,0946	0,1109
-	4	0,1461	0,0395	0,0269	0,1215	0,1206	0,1472	0,0118	0,0234	0,0016
Blanks:		0,0210	0,0017							

Table H2: Results from OD_{415} measurements of the samples collected from recombinant *S. venezuelae* strains with introduced construct 2, 3 and 4.

EtOH	day	construct 2 (cmlFp)			construct 3(kefBp)			construct 4 (neg. control)		
+	1	0,0057	0,0170	0,0234	0,0204	0,0017	0,0040	-0,0023	0,0035	-
+	4	0,0148	0,0093	0,0148	-0,0137	-0,0163	-0,0063	-0,0145	-0,0170	-0,0026
-	1	0,0966	0,0523	0,0862	0,0800	0,1065	0,0962	0,0629	0,0750	0,0895
-	4	0,1216	0,0195	0,0114	0,0980	0,0980	0,1191	-0,0054	0,0058	-0,0144
Blanks:		0,0073	-0,0073							