Diplomarbeit

Reconstruction of the *alk*-operon from *Pseudomonas putida* using Golden Gate Shuffling for the production of adipic acid in *Escherichia coli*

ausgeführt am
Institut für angewandte Mikrobiologie
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eingereicht von
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Wien, im April 2014

Danksagung

Ich möchte mich herzlich bei allen Personen bedanken, die mich bei dieser Diplomarbeit und während des Studiums unterstützt haben. Spezieller Dank gilt hierbei meiner Familie und besonders meinen Eltern Anna und Jürgen.

Für die wissenschaftliche Betreuung und die Unterstützung bei der Durchführung dieser Arbeit möchte ich mich bei **Priv.-Doz. FH-Prof. Dr. Michael Sauer**, sowie **Dipl-Ing. Dr. Hans Marx** bedanken. Des Weiteren gilt mein Dank allen Mitarbeitern des Bioengineering-Labors des Instituts für angewandte Mikrobiologie in der Muthgasse 107, 1190 Wien.

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1. Abstract

Increasing environmental concerns and limited resources for fossil petroleum make it inevitable to search for sustainable alternatives based on renewable resources to produce energy and industrial commodities.

With an annual production volume of 2,5 million tons per year adipic acid (1,6-hexanedioic acid) is one of the most important dicarboxylic acids for chemical industries and down to the present day conventional production methods rely on petroleum based raw materials. Metabolic engineering of microbes could provide a sustainable production way for adipic acid based on renewable resources like sugar. The *alk*-operon found on the OCT plasmid in *Pseudomonas putida* has been described to provide all the enzymes required to convert medium n-alkanes into carboxylic and dicarboxylic acids.

However, the engineering and optimization of microbial metabolic pathways is associated with the construction of large plasmid libraries, where single genes are tested for different expression levels and these processes require a lot of cloning steps in terms of classical cloning procedures. Golden Gate Shuffling represents a novel high-throughput cloning method and serves as a powerful tool for metabolic engineering. Within this work, we successfully established a modular cloning system based on Golden Gate cloning with two type IIS restriction enzymes *Bbs*I and *Bsp*MI.

As a second part, genes forming the *alk*-operon were re-assembled using the Golden Gate cloning method, put under the control of different promoters and proven to be functional on an extra-chromosomal plasmid in *Escherichia coli*. The biocatalytical conversion of n-hexane to hexanoic acid, as well as conversion of hexanoic acid to adipic acid was accomplished in this project.

2. Kurzzusammenfassung

Das wachsendes Bewusstsein unserer Gesellschaft für industrielle Umweltschäden und die limitierten Reservoirs fossilen Erdöls lassen die Suche nach alternativen Produktionswegen für Energie und Industriegüter in den Fokus der Wissenschaft rücken.

Adipinsäure (1,6-Hexandisäure) ist mit einem Produktionsvolumen von 2,5 Millionen Tonnen pro Jahr eine der wichtigsten industriellen Dicarbonsäuren für die chemische Industrie. Bis heute beruhen klassische Produktionsmechanismen jedoch auf Erdöl als Ausgangsrohstoff. Metabolic Engineering in Mikroorganismen könnte eine alternative Produktionsroute basierend auf erneuerbaren Rohstoffen liefern. Unter Metabolic Engineering versteht man die Veränderung, bzw. den Transfer von Biosynthesewegen zur Produktion gewünschter Moleküle in Mikroorganismen. Das *alk*-Operon des OCT-Plasmids aus *Pseudomonas putida* könnte als möglicher Lieferant für die notwendigen Enzyme dienen, um mittelkettige n-Alkane in Dicarbonsäuren zu verwandeln.

Die Veränderung und Optimierung ganzer Synthesewege ist jedoch mit der Klonierung einer großen Anzahl von Plasmiden verbunden und erfordert viel Zeit. Golden Gate Cloning, eine kürzlich vorgestellte Klonierungsmethode, könnte dazu dienen den Aufwand für erfolgreiche Stammveränderungen zu senken. In diesem Projekt ist es uns gelungen unter der Verwendung der beiden Typ IIS Restriktionsenzyme *Bbs*I und *Bsp*MI ein modulares Klonierungsverfahren auf Grundlage des Golden Gate Cloning zu etablieren.

Der zweite Teil der Arbeit bestand darin das *alk*-Operon unter der Verwendung von Golden Gate Cloning in ein neues extra-chromosomales Plasmid einzubauen, mit verschiedenen Transkriptionspromotoren zu testen und in *Escherichia coli* zu exprimieren. Dadurch konnte die biokatalytische Konversion von n-Hexan zu Hexansäure, sowie von Hexansäure zu Adipinsäure erreicht werden.

3. Introduction

3.1. Industrial Biotechnology as a main contributor to "green chemistry"

During the last 200 years, since the industrial revolution, the demand of mankind for energy and resources increased exponentially. Society of the 21st century and our way of living is based on the findings of crude oil for the production of energy and industrial commodities. Exploitation and utilisation of oil reserves had a great impact on ecological systems and in 2013 the Mauna Loa Observatory in Hawaii for the first time noted a carbon dioxide concentration of 400 ppm (parts per million) in earth's atmosphere, which represents an increase of 25% since the commissioning of the observatory in 1958 (Keeling et al., 2014). The arising problems of global warming, declining reservoirs of fossil fuels, a growing world population as well as growing demands for raw materials and energy make it more and more important to establish alternative and sustainable ways to produce industrial commodities. A change of thinking is currently taking place amongst policymakers towards clean (or "green") chemical technologies starting from renewable raw materials and energy with minimal impacts on nature (Soetaert and Vandamme, 2006). Industrial microbiology (white biotechnology) provides promising opportunities for a modern society with ecological sensibility. It is a multi-disciplinary field of biochemistry, microbiology, process technology and molecular genetics, which is steadily extending, or replacing classical production processes in the chemical industry (Hatti-Kaul et al., 2007).

The most important feed-stocks for renewable raw materials are sugar cane, sugar beet, wood, oil plants and waste from agriculture and other industries, but utilisation of these raw materials is problematic in conventional chemical processes (Willke and Vorlop, 2004). Hence, a lot of research is going into preparation processes like enzymatic digestion of complex raw materials to simple sugar molecules, which are ideal starting materials for microbial fermentations. A wide variety of products can be produced by microorganisms like bacteria, yeast and fungi and due to advances in recombinant DNA-technologies the product spectrum is expanding. Mild reaction conditions, less input of energy and the ability to synthesise complex materials in a single step process are further advantages of biotechnological processes (Hatti-Kaul et al., 2007).

For the successful commercialization of bio-based chemicals several requirements have to be fulfilled. The titer (g/L product), yield (g product /g substrate) and the productivity (g/L h)

are essential characteristics for the efficiency of a biotechnological process and have to compete with petroleum based production methods. Additionally, the purification of microbial fermentation products creates significant downstream costs, requires high product titers and has to be considered during process development (Sauer et al., 2008). Therefore, metabolic engineering of microbial production strains aims to reduce the formation of undesired by-products, to maintain the redox and energy balance of the organism and to direct the carbon flux away from cell growth towards product synthesis. Many of these approaches are based on empirical experiments, since the accuracy and functionality of computational prediction methods is still limited. However, scientists nowadays benefit of remarkable developments in new generation sequencing methods and novel cloning methods (e.g. Golden Gate cloning see 3.4), which enable the screening of large libraries of different constructs in a short period of time. With DNA synthesis methods becoming cheaper and faster, even the artificial preparation of a whole genome might become a reality in a couple of years (reviewed by Sauer and Mattanovich, 2012; Van Dien, 2013).

The commercialization of biotechnological production processes for succinic acid, lactic acid and 1,3-Propanediol are three examples for successful strain engineering and the spectrum of microbial produced chemicals is constantly increasing (Lee et al., 2011). Especially the bio-based production of adipic acid is considered to be a future market for industrial biotechnology.

3.2. Adipic acid

Adipic acid (1,6-hexanedioic acid) is one of the most important dicarboxylic acids for chemical industries. With an annual production volume of 2,5 million tons (t) the market for adipic acid is greater than the market for microbial citric acid (1,6 million t/a) (Sauer et al., 2008). A large part is used in a polycondensation reaction with hexamethylene diamin resulting in 6,6-nylon, used for the production of synthetic fibers (Fig. 3-1). Adipic acid is also used as acidifier, buffer and leavening agent for products in food and pharmaceutical companies, as well as a lubricant component and a compound for the formation of polyurethane foam and plasticizers (Polen et al., 2013).

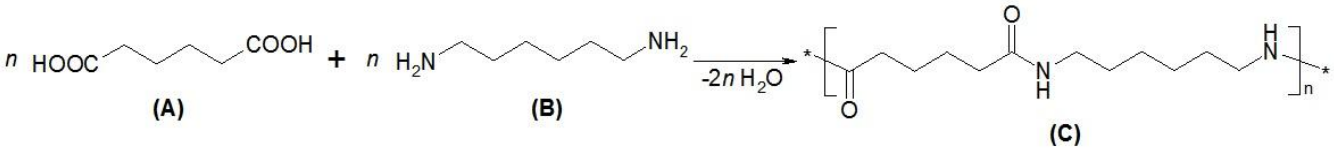

Figure 3-1: Polycondensation reaction of adipic acid (A) and hexamethylene diamine (B) to nylon-6,6 (C) (taken from Daraban, 2012)

Conventional production of adipic acid is based on the oxidation of ketone-alcohol oil ("KA oil"), a mixture of cyclohexanone and cyclohexanol obtained from benzene, a fraction of non-renewable petroleum (Fig. 3-2). Oxidation is conducted with nitric acid (HNO_3) under the release of nitrogen oxide (N_2O), which is a green house gas involved in ozone depletion and global warming processes (Thiemens and Trogler, 1991). A few years ago about 10% of the annual nitrogen oxide emissions were related to this process (Alini et al., 2007).

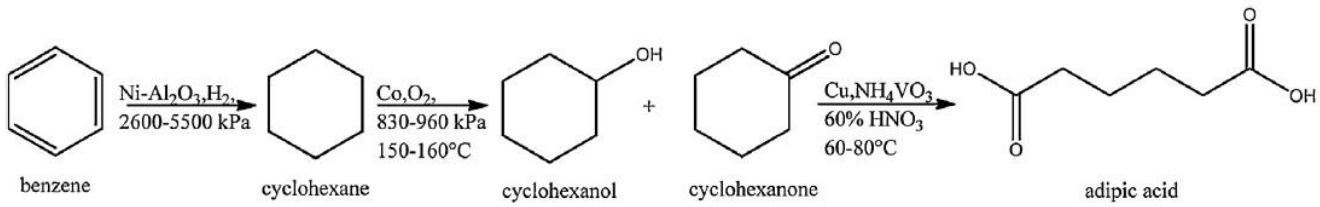

Figure 3-2: Chemical route of adipic acid synthesis from benzene (taken from Niu et al., 2002)

There are several alternative ways for the production of adipic acid. The oxidation of cyclohexene, or cyclohexane under mild reaction conditions with hydrogen peroxide (Sato,

1998) and acetylperoxyborate (Raja et al., 2006), respectively, avoid the emission of nitrogen oxide.

Biotechnological approaches in scientific literature include bacterial fermentation of petroleum based cyclohexanol or cyclohexane (Brzostowicz et al., 2005) and chemical conversion of petroselinic acid from plants with ozone, which is a very expensive process (Metzger and Bornscheuer, 2006). Fermentation of long chain carbon substrates like alcohols and fatty acids in a fed-batch cultivation with engineered yeast (e.g. *Candida tropicalis*) resulted in α,ω-dicarboxylic acids and also in the production of adipic acid (Picataggio et al., 1992). Compared to glucose and other sugars long chain carbon substrates are still expensive and also *Candida tropicalis* is a possible pathogen. More promising is the hydrogenation of bio-based *cis, cis*-muconic acid, which can be obtained by microbial conversion of D-Glucose via 3-dehydroshikimic acid (DHS) an intermediate in aromatic amino-acid biosynthesis pathways. Three enzymatic steps are required to convert DHS to *cis, cis*-muconic acid and can be plasmid-mediated in *Escherichia coli.* Cost-intensive steps are the removal of cells and purification of *cis, cis*-muconic acid before hydrogenation as well as the disposal of platinum catalysts. Chemical conversion is achieved by 10% Pt on carbon and a process pressure of 3400 kPa at ambient temperature for 2.5 hours. Microbial conversion would bypass these issues, but up to date there is no known bio-catalytic conversion pathway of *cis, cis*-muconic acid to adipic acid (Niu et al., 2002; Polen et al., 2013).

Another pathway to synthesise adipic acid from glucose is via 2-oxoadipate, an intermediate in α-aminoadipate pathways for L-Lysine biogenesis found in fungi, higher plants and some bacteria. It is synthesised in three enzymatic steps from 2-oxoglutarate and acetyl-CoA. 2-oxoadipate is then converted chemo-catalytically, or enzymatically by engineered cells. Alternatively, 2-oxoadipate is also obtained by degradation of L-Lysine in three enzymatic steps. L-Lysine is widely used as animal feed and there are well established production processes with high-performance strains of *Corynebacterium glutamicum* and *E. coli*. Unfortunately there is no established metabolic pathway for the bioconversion of 2-oxoadipate to adipic acid. Two possible ways were proposed recently, derived from biosynthetic route for glutaconic acid in *Clostridium symbiosum* and from mamalian L-Lysine degradation pathways, but both are still under investigation and need to be specified (reviewed by Polen et al., 2013)

3.3. A sustainable way of adipic acid production

The microbial production pathway approached in this work is based on the production of hexanoic acid from glucose, e.g. by *Megasphera elsdenii* (Sauer et al., 2012), with a further oxidization step to adipic acid. The second step in this pathway, the oxidation of hexanoic acid, is possibly performed by genes found on the *alk*-operon of the extrachromosomal OCT plasmid from *Pseudomonas putida* (Kunz and Weimer, 1983) which enables them to grow on C6 to C12 n-alkanes. The *alk*-operon consists of nine genes grouped into two clusters and is mainly induced by n-Octane, but also by other alkanes with a chain length between six and ten carbon atoms. Proteins encoded on the first cluster of the OCT plasmid are a membrane-bound monooxygenase (*alkB*) followed by two rubredoxins (electron carriers, *alkF* and *alkG*), an aldehyde dehydrogenase (*alkH*) and a membrane-bound alcohol dehydrogenase (*alkJ*). The two remaining genes represent an acyl-CoA synthetase (*alkK*) and an outer membrane protein with unknown function (*alkL*) (van Beilen et al., 2001). The *alkK* protein specifically directs medium chain length alkanoic acids towards β-oxidation and therefore is undesirable for the synthesis of carboxylic acids. Most important protein of the second gene cluster is encoded by *alkT*, a rubredoxin reductase, which regenerates the rubredoxins (*alkG* and *alkF*) and ensures the sustenance of *alkB* monooxygenase with electrons (see Fig. 3-3). Final protein of the OCT plasmid is *alkS*, a transcriptional regulator protein for *alk*-operon promoters (van Beilen et al., 2001).

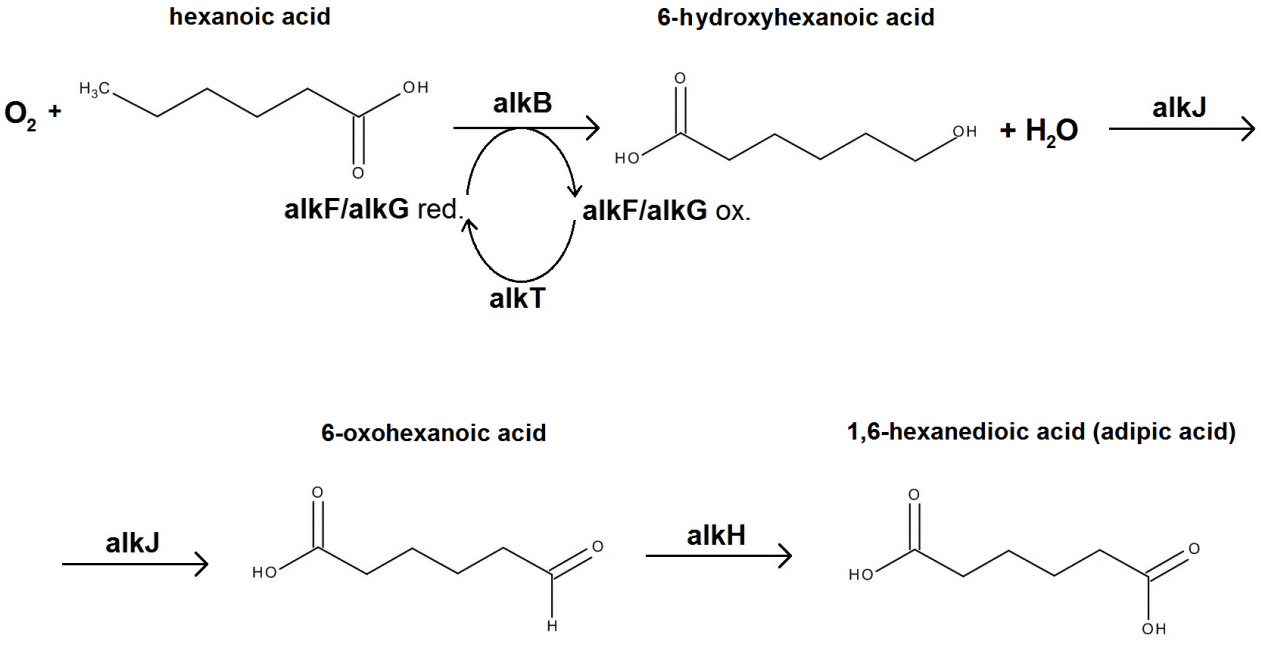

Figure 3-3: Hexanoic acid oxidation pathway of *alk*-operon genes

In 2012, Daniel Daraban managed to produce up to 47 mg/L of adipic acid from hexanoic acid as a substrate (ω-oxidation) using process optimization for the induction of the *alk*-operon with n-octane in *Escherichia coli*. However, the reproducibility of this process was very poor and the *alk*-operon was not inducible with n-hexane in *E. coli* for the production of hexanoic acid. Also the replacement of the endogenous promoter by an IPTG-inducible *trc* promoter resulted in no conversion of n-hexane, hexanol, or hexanoic acid in *E. coli.* He concluded with a suggestion to rearrange the entire *alk*-operon and to optimize the expression ratios for every single *alk* gene (Daraban, 2012).

Following this conclusion, the reconstruction of the *alk*-operon in *E. coli* was set as the main goal within this work and for that reason the modular cloning system of Golden Gate cloning was chosen as an effective tool for the rearrangement. Otherwise, the generation of a plasmid library with six distinct genes, where every single gene is tested for their expression under different promoters, would take up a lot of time in terms of classical cloning methods (Engler et al., 2009).

3.4. Golden Gate Shuffling

This cloning method was presented in 2008 by Carola Engler and her colleagues as a “one pot, one step” process “with high throughput capability” and later on described as a modular cloning system by Weber et al. (2011). It is based on the special feature of type IIS restriction enzymes to cut outside the enzyme recognition sequence, independent from the cleaved DNA sequence creating single-stranded nucleotide overhangs (sticky ends). The introduction of enzyme recognition sites via primer-directed PCR allows the generation of freely selectable 3’ or 5’ overhangs of three or four nucleotides (depending on the restriction enzyme). In case of four nucleotide overhangs it is possible to generate 256 hypothetical recombination sites (fusion sites). Anyway, the 16 palindromic sequences would lower the cloning efficiency and should be avoided, which results in 240 possible fusion sites. With compatible (reverse) fusion sites on two DNA fragments Golden Gate cloning facilitates site-directed re-ligation of these fragments in a linear and hierarchical manner (Engler et al., 2008). Up to nine separate fragments were cloned into one expression vector within one restriction-ligation reaction, with a cloning efficiency of more than 90% for the desired product (Weber et al., 2011).

The key feature of Golden Gate cloning lies in the self-elimination of the recognition sequence by the restriction enzyme. Ligated DNA fragments are no longer recognized by the restriction enzyme, which leads to an accumulation of the desired products during restriction-ligation circles. Re-ligated template DNA reconstitutes restriction sites and gets digested again (Engler et al., 2008). The principles of Golden Gate cloning are shown in figure 3-4.

Another key feature is the change in resistance for antibiotics between the levels of the modular cloning system. For example, level-0 modules contain a Kanamycin resistance cassette, whereas the level-1 recipient vector provides a resistance to Ampicillin. After transformation only clones with a level-1 vector are able to grow on selective agar-plates containing Ampicillin. One stage later Kanamycin can be used again as selective agent for level-2 modules holding the Kanamycin resistance gene (Engler et al., 2009).

The recipient vectors also include a l*acZ* gene for blue/white screening, to distinguish undigested vectors from correctly assembled cloning products with insert. After transformation into competent cells and incubation with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), negative clones appear blue, because of the β-galactosidase activity of the l*acZ* gene product. Positive clones, or clones with at least some kind of insert are white, since the coding sequence of *lacZ* is cut out and replaced with the insert in the restriction-ligation reaction (Engler and Marillonnet, 2011).

The creation of a Golden Gate library requires several preparation steps. There have to be no other restriction sites for the chosen type IIS restriction enzyme than the two sites flanking the fusion sites. All other recognition sequences on DNA fragments, but also on the plasmid backbones have to be eliminated by silent mutations. This can be achieved by PCR assemblies of genes with miss-matching primers. Golden Gate assemblies can be done with a consistent incubation temperature, or in a thermo-cycler with changing temperature optimums regarding the restriction enzyme and the DNA ligase. Therefore, it is important to choose a compatible combination of restriction enzyme and DNA ligase. For example, *Bbs*I has a temperature optimum of 37°C and can be combined with T4 ligase for a consistent incubation temperature. Anyway, the temperature optimum for T4 ligase is 16°C, so it is crucial that *Bbs*I is stable at lower temperatures, in case Golden Gate assemblies are performed in a thermocycler. Nonetheless, both enzymes have to be functional in the same buffer solution (Engler and Marillonnet, 2011).

Major advantages of a modular cloning system are the flexibility and standardization of the basic modules. Previously validated elements can be reused and exchanged between different users to reduce the number of cloning steps and to simplify cloning strategies. Directional cloning and the full control over recombination sites make it applicable for large genes, including the possibility for exon shuffling and the creation of large construct libraries. The adjustment of different expression ratios serves as a useful tool for metabolic engineering and the optimization of entire biochemical pathways (Weber et al., 2011). Also the number of procedures performed on DNA is brought to a minimum, avoiding gel purification steps and PCR for the assembly of final expression constructs. Since the determination of DNA concentration is more accurate for undigested plasmids, it is another advantage that Golden Gate assemblies can be set up directly from plasmid stocks, because equimolar amounts of DNA fragments are important for successful cloning procedures. Finally, no buffer incompatibilities occur between restriction enzymes, since there is need for only one restriction enzyme (Engler and Marillonnet, 2011).

Since this method hasn't been tested in our lab before, the first step was to establish a variant of Golden Gate cloning sufficient for our lab, with an appropriate combination of restriction enzymes and buffer solutions and also to create a standard operating procedure, which can be also used by other persons in the lab. *Bbs*I and *Bsp*MI were chosen as type IIS restriction enzymes. Two reporter proteins (GFP and DsRed) were used to test various promoters and to determine the ideal incubation conditions for a successful restriction-ligation reaction.

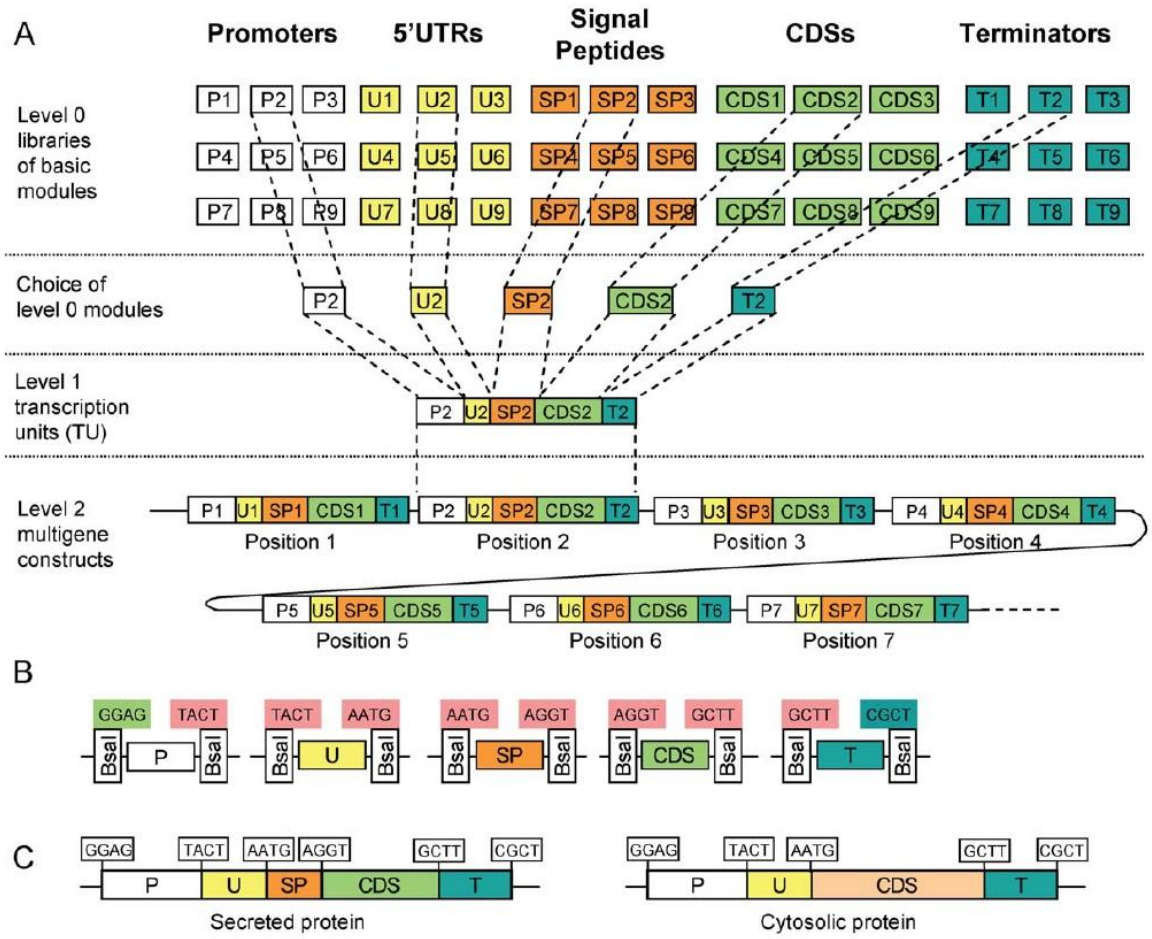

Figure 3-4: General overview of the hierarchical and modular cloning system

(A) Libraries of basic (level-0) modules contain cloned and sequenced genetic elements such as promoters (P), 5` untranslated regions (U), signal peptides (SP), coding sequences (CDS) and terminators (T). Transcription units are assembled from selected level-0 modules using a one-pot one-step cloning reaction. Multigene constructs are then assembled in a second cloning step (and optionally further steps) from the transcription units.

(B) Level-0 modules of different classes are flanked by compatible fusion sites. Each fusion site consists of 4 nucleotides of choice (boxed) flanked by a type IIS enzyme recognition site on the left or right side (vertical box drawn under the fusion site).

(C) Examples of assembled transcription units for secreted or cytosolic proteins. The transcription unit for the cytolic protein was assembled from 4 modules rather than 5, using a CDS module cloned between fusion sites AATG and GCTT.

(figure and description reprinted from Weber et al., 2011)

doi:10.1371/journal.pone.0016765.g001

4. Materials and Methods

4.1. Strains, Media, Plasmids

4.1.1. E. coli strains

Strain	Genotype	Source
TOP10	F- *mcr*A Δ(*mrr*-*hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara,leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G λ-	Invitrogen
DH10B	F- *mcr*A Δ(*mrr*-*hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara,leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G /pMON14272 / pMON7124	Invitrogen
HMS174(DE3)	F^- *rec*A1 *hsd*R(r_{K12}^- m_{K12}^+) (DE3) (Rif R)	(G. Striedner)

4.1.2. Media

Name	Ingredients
LB	1% Tryptone 0.5% Yeast Extract 0.5% NaCl
LB-Amp	1% Tryptone 0.5% Yeast Extract 0.5% NaCl 100 mg/L Ampicillin
LB-Kan	1% Tryptone 0.5% Yeast Extract 0.5% NaCl 100 mg/L Kanamycin
M9	12,8 g/L Na_2HPO_4 3,0 g/L KH_2PO_4 0,5 g/L NaCl 1,0 g/L NH_4Cl 1/10 of final volume 10x M9 trace stock

M9 trace stock	40,0 g/L glucose 9,88 g/L $MgSO_4$ * 7 H_2O 0,30 g/L $CaCl_2$ * 2 H_2O 0,22 g/L Thyamine * HCl 0,20 g/L$FeSO_4$ * 7 H_2O

For the preparation of plates 2% Agar was added to the media.

4.1.3. Plasmids

pSTBlue-1 Vector

The initial plasmid for all cloning steps and Golden Gate assemblies was the pSTBlue-1 vector from Novagen.

pSTBlue-1 sequence landmarks	
lacZ start codon	1
lacZ α-peptide ORF	1-378
T7 promoter	24-40
T7 transcription start	41
multiple cloning region (*Kpn* I - *Not* I)	51-178
SP6 promoter	185-202
SP6 transcription start	184
f1 origin	373-828
bla coding sequence	960-1817
Kan coding sequence	1966-2778
pUC origin	3453

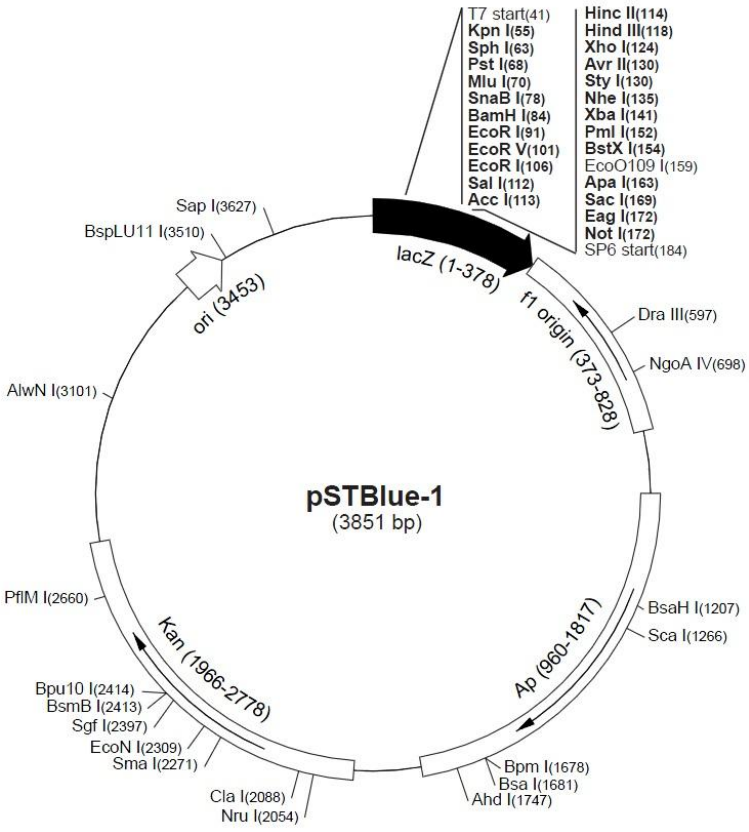

Figure 4-1: pSTBlue-1 vector map (www.lifetechnologies.com)

Level-0 modules (backbone 1)

This plasmid was derived from the pSTBlue-1 vector. The Ampicillin resistance cassette has been removed by PCR amplification of the rest of the plasmid, simultaneously introducing a *Bgl*II recognition sequence. Followed by enzymatic digestion of the PCR products with *Bgl*II, purification and re-ligation of the DNA fragment.

For the creation of level-0 recipient vectors the plasmid is cut with *Eco*RV, dephosphorylated with Calf Intestinal Alkaline Phosphatase (CIP) and blunt ligated with an insert flanked by *Bbs*I and *Bsp*MI restriction sites and the desired fusion sites using the NOVAGEN Perfectly Blunt® Cloning Kit. The kit enables blunt ligation of PCR fragments with any kind of end, since they are converted to blunt, phosphorylated ends during End Conversion step. A list of fusion sites used for linker elements can be found in table 4-1.

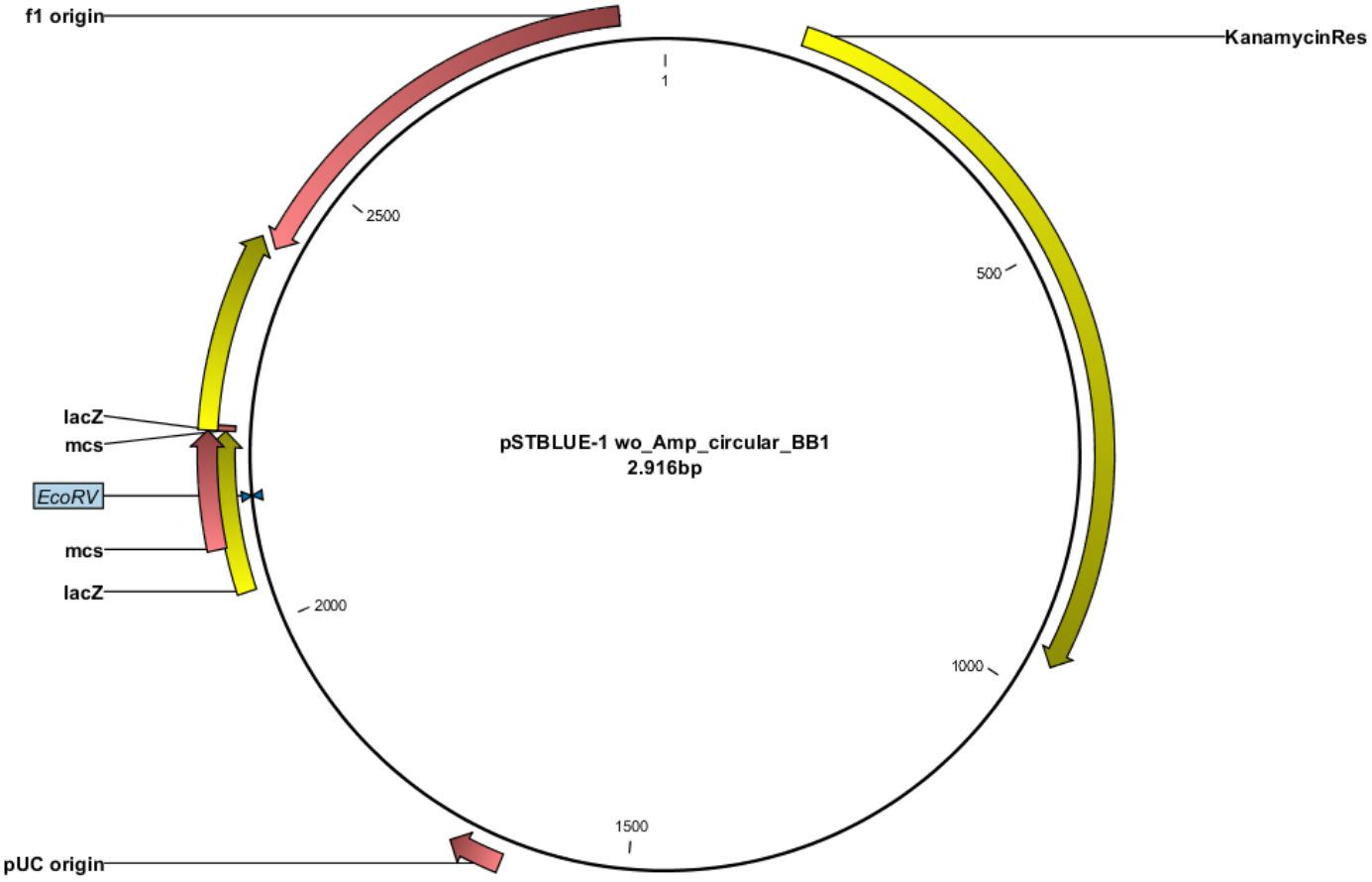

Figure 4-2: Level-0 modules derived from pSTBlue-1

Level-1 recipient vector (backbone 2)

This plasmid was derived from the pSTBlue-1 vector. The Kanamycin resistance cassette has been removed by PCR amplification of the rest of the plasmid, simultaneously introducing a *Bgl*II recognition sequence. Followed by enzymatic digestion of the PCR products with *Bgl*II, purification and re-ligation of the DNA fragment.

For the creation of level-1 recipient vectors the plasmid is cut with *Eco*RV, dephosphorylated with Calf Intestinal Alkaline Phosphatase (CIP) and blunt ligated with a linker insert (fig. 4-3) flanked by *Bbs*I and *Bsp*MI restriction sites (orange/red arrows) and the desired fusion sites (FS) using the NOVAGEN Perfectly Blunt® Cloning Kit. A list of fusion sites used for linker elements can be found in table 4-1.

level-0 fusion sites		**Restriction enzyme**	**Recognition site / fusion site**
1	GGAG	*Bbs*I	GAA GAC GC / GGAG
2	CATG	*Bbs*I	GAA GAC GC / CATG
3	GCTT	*Bbs*I	GAA GAC GC / GCTT
4	CGCT	*Bbs*I	GAA GAC GC / CGCT
level-1 fusion sites		**Restriction enzyme**	**Recognition site / fusion site**
A	GATC	*Bsp*MI	ACC TGC GCGC / GATC
B	CCGG	*Bsp*MI	ACC TGC GCGC / CCGG
C	AATT	*Bsp*MI	ACC TGC GCGC / AATT
D	AGCT	*Bsp*MI	ACC TGC GCGC / AGCT
E	GGAG	*Bsp*MI	ACC TGC GCGC / GGAG
F	GCTT	*Bsp*MI	ACC TGC GCGC / GCTT
G	CGCT	*Bsp*MI	ACC TGC GCGC / CGCT
level-2 fusion sites		**Restriction enzyme**	**Recognition site / fusion site**
α	GGAG	*Bbs*I	GAA GAC GC / GGAG
β	CATG	*Bbs*I	GAA GAC GC / CATG
γ	GCTT	*Bbs*I	GAA GAC GC / GCTT
δ	CGCT	*Bbs*I	GAA GAC GC / CGCT

Table 4-1: Fusion sites for Golden Gate cloning method

Figure 4-3: Linker element for level-1 recipient vector carrying one expression cassette (FS1-FS4), flanked by fusion sites E and F

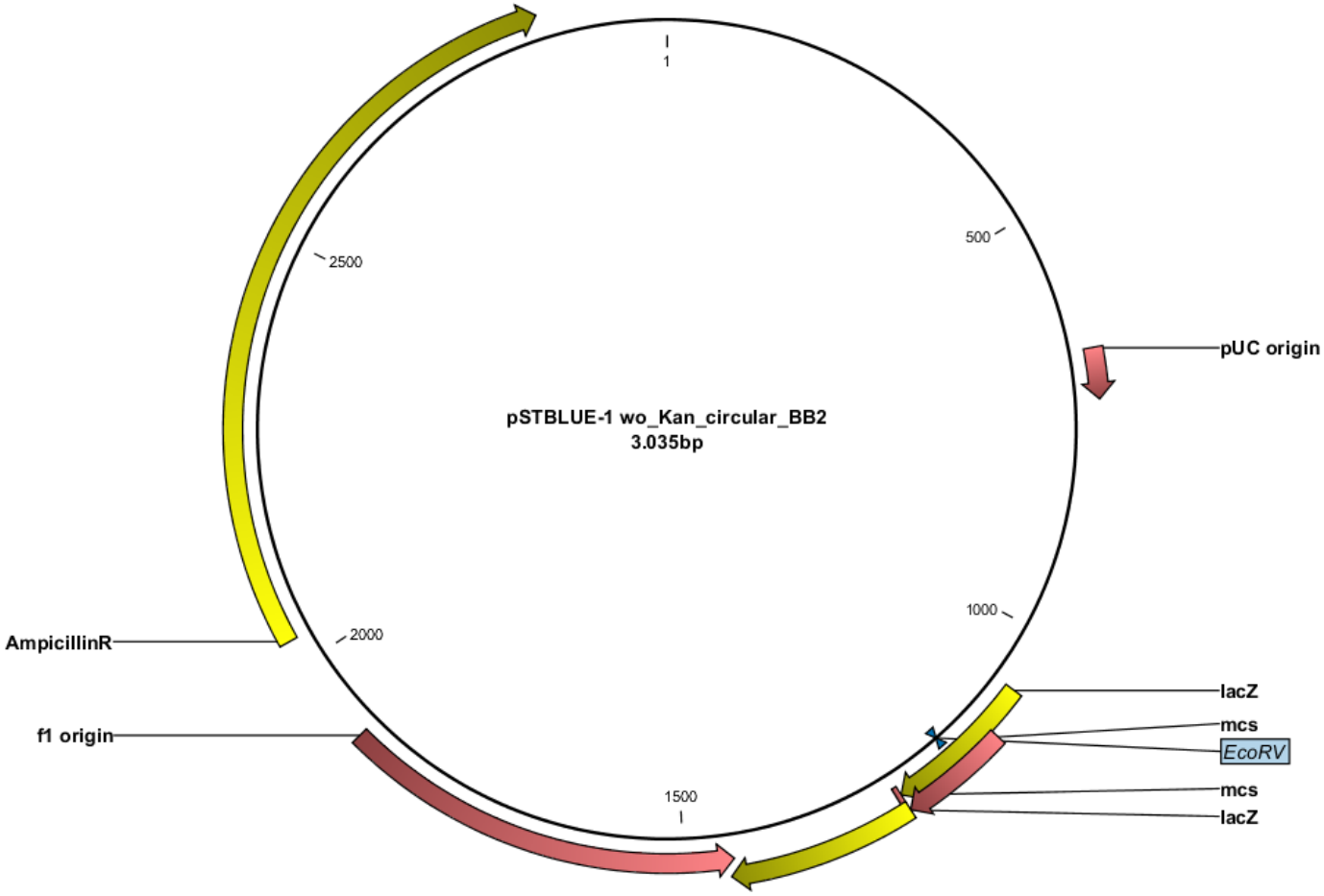

Figure 4-4: Level-1 modules derived from pSTBlue-1

Level-2 final recipient vector (backbone 3)

This plasmid was build in a Golden Gate assembly of an origin of replication (ORI), an antibiotic resistance cassette, a non-transcribed intergenic spacer from *Pichia pastoris* (NTS) and a linker fragment with the desired fusion sites for further cloning steps. All these fragments were originally PCR amplified with *Bbs*I restriction sites, blunt ligated into level-1 modules using the NOVAGEN Perfectly Blunt® Cloning Kit and are listed in table 5-3.

4.1.4. Primers

The following primers were used for sequencing of sub-cloned PCR products into pSTBlue-1 derived vectors backbone 1 and backbone 2.

Name	T_m	Sequence
pSTBlue_Seq_Fw	53,9°C	5‘-GAGTCAGTGAGCGAGGAA-3‘
pSTBlue_Seq_Bw	52,2°C	5‘-GAAACAACACTCAACCCT-3‘

The following primers were used for sequencing of recipient plasmids and final constructs.

Name	T_m	Sequence
Seq_pUC_ORI_to_LacZ_FW	55.4°C	5‘-GGTAGCTCTTGATCCGGC-3‘
Seq_KanHyg_to_LacZ_BW	55.7°C	5‘-CATGGGGATGTATGGGCTAAATG-3‘

A list of all used primers for PCR amplification of desired genes can be found in the appendix. They were obtained from IDT® Integrated DNA Technologies, Inc. (www.idtdna.com).

4.1.5. Strain conservation

Bacterial cells were stored on agar plates at 4°C for maximally 2 weeks. For long time preservation of strains cryo culture conservation was used.

Procedure:

1350 µL of a liquid overnight culture were mixed with 150 µL water-free glycerol to an end-concentration of 10% glycerol and immediately frozen at -80°C.

4.2. DNA Manipulation

4.2.1. DNA restriction

Restriction enzymes (restriction endonucleases) cut DNA at specific sequences, also known as restriction sites. These enzymes were originally found in bacteria and archea as defence mechanisms against invading viral DNA. Restriction sites usually vary between 4 and 8 nucleotides, are often palindromic and differ for each restriction endonuclease. DNA restriction enzymes can produce „blunt“ ends (double-stranded), or they produce „sticky“ ends (single-stranded overhang) (Berg et al., 2002).

These enzymes are widely used as laboratory tools with specific buffers and reaction conditions (mostly 37°C). In case of a double digestion a buffer with the highest activity of both enzymes was used.

All used enzymes and buffers were purchased either from New England Biolabs (NEB) or from Thermo Scientific.

Procedure:

Restriction enzyme digestion (20µL preparation)	
2 µL	DNA-Template (~ 100 ng/µL)
2 µL	10x Reactionbuffer
1 unit/µg DNA	Restriction Enzyme
2 µL	10x BSA (1 mg/mL) optional
to 20 µL	RO-Water
Gently mixed and incubated at specific temperature for 1 hour.	

Table 4-2: Restriction enzyme digestion conditions

4.2.2. Dephosphorylation of DNA

Calf Intestinal Alkaline Phosphatase (CIP) is used to dephosphorylate linear DNA ends and therefore cleaves phosphate groups from 5’ends enzymatically. This is done to prevent recirculation of plasmids, which can only build a phosphodiester bond between a 5’-phosphate group and a 3’-hydroxy group.

The enzyme from New England Biolabs was used according to their protocol.

Procedure

The suspended DNA is diluted in NEBuffer 3 to a maximum-concentration of 0.5 µg/10µL and 0.5 units of CIP/µg vector DNA are added. The mixture is incubated for 1 hour at 37°C.

4.2.3. DNA ligation

DNA fragments can be recombined with the enzyme DNA-ligase, which forms phosphodiester-bonds between a 5'-phosphate-group and a 3'-hydroxy-group of open ends of double-stranded DNA fragments. In this work the NOVAGEN Perfectly Blunt® Cloning Kit designed for simplified cloning of DNA generated by PCR has been used, according to the user protocol [TB 183]. The kit enables blunt ligation of DNA fragments with any kind of end, since they are converted to blunt, phosphorylated ends during End Conversion step.

End conversion and ligation:

For a standard reaction 0,05 pmol PCR product or restriction fragment are used in the end-conversion reaction when cloning into 0,02 pmol pSTBlue-1 derived vectors.

DNA Ligation	
0,05 pmol	DNA product
5,0 µL	End Conversion Mix
up to 10 µL	nuclease-free water
incubate the reaction at 22°C for 15 min, inactivate by heating at 75°C for 5 min	
cool the reaction briefly on ice for 2 min, centrifuge	
collect condensate and add the following components:	
0,02 pmol	blunt recipient vector (level-0 or level-1)
1 µL	T4 DNA Ligase (4 U)
incubate at 22°C for 15 min to 2 hours	

Table 4-3: DNA ligase conditions

4.3. DNA Amplification

The technique of DNA amplification by the polymerase chain reaction (PCR) is a sensitive and rapid method to amplify pieces of double-stranded DNA out of small amounts of template DNA (Mullis and Faloona, 1987).

The enzyme DNA polymerase provides elongation of the newly synthesised DNA strand and has a proof-reading function for the correction of errors. In vivo it serves in the process of semi-conservative DNA replication during cell cycle phases. For this work the Phusion™ High-Fidelity DNA Polymerase from New England Biolabs was used, according to the manufacturer protocol.

Deoxy-nucleoside triphosphates (dNTPs) are the building blocks of the DNA amplification process, which are attached to the newly synthesised DNA strand by the DNA polymerase.

Short and specific DNA pieces of known sequence, called primers, determine the starting point of DNA amplification processes. For double-stranded PCR products two primers are required, a forward and a backward version. They are synthetically produced oligo-nucleotides and often contain additional modifications like restriction sites and desired point mutations. All primers were obtained by IDT® Integrated DNA Technologies, Inc. (www.idtdna.com) and are listed in the appendix.

A polymerase chain reaction runs in circles. The first step is to denaturate the double-stranded DNA at a temperature of 98°C to make it accessible for the primers. Every primer has a specific melting temperature (T_m). For the annealing-step the mixture is cooled down to a temperature about 5°C lower than T_m. After this hybridisation the DNA polymerase can attach to the primer and starts to synthesise the complementary strand at a specific temperature, which is 72°C for Phusion polymerase. These steps are repeated about 25 times, which leads to an exponential increase in PCR-product.

Procedure:

Component	Volume / 50 µL reaction	Final concentration
H2O	add to 50 µL	
5x Phusion HF Buffer	10 µL	1x
10 mM dNTPs	1 µL	200 µM each
forward primer	x µL	0.5 µM
backward primer	x µL	0.5 µM
template DNA	x µL	1pg – 10ng / 50 µL
(DMSO, optional)	(1.5 µL)	(3%)
Phusion DNA Polymerase	0.5 µL	0.02 U/µL

Table 4-4: PCR pipetting instructions

Cycle step	Temperature	Time	Cycles
Denaturation	98°C	30 sec	1
Denaturation Annealing Extension	98°C T_m -5°C 72°C	5 – 10 sec 10 – 30 sec 15 – 30 sec / 1 kb	25 – 30
Final extansion	72°C 4°C	5 – 10 min hold	1

Table 4-5: PCR cycling instructions

4.4. Golden Gate Assembly

4.4.1. Preparation of plasmid stocks

Equimolar amounts of DNA for each module as well as the recipient vector are very important for efficient Golden Gate cloning. In their protocol Ernst Weber and Carola Engler recommended an approximate concentration of 40 fmol for each DNA component (PCR product or plasmid). This corresponds roughly to 100 ng of DNA for a 4 kb plasmid (Weber et al., 2011).

Therefore, all obtained plasmid stocks in this work were quantified by NanoDrop ND1000 and diluted to a final concentration of 40 nmol/L. One µL of these diluted stocks was used in Golden Gate assemblies, which equals 40 fmol in total.

4.4.2. Golden Gate assembly procedure

A Golden Gate assembly contains the following components:

Volume	Component	Comment
1 µL	Insert #1 (40 nM)	Plasmid DNA or PCR product
1 µL	Insert #2 (40 nM)	Plasmid DNA or PCR product
up to 9 inserts can be processed in one assembly step		
1 µL	Recipient vector (40 nM)	Plasmid DNA or pre-cut vector
2 µL	10x T4 Ligase buffer	
2.5 µL	T4 ligase (40 U/µL)	1:10 diluted enzyme stock with 400 U/µL
2 µL	Restriction enzyme type IIS	e.g. *Bbs*I, *Bsp*MI
fill up to 20 µL	RO-H_2O or 10 mM TRIS-HCl pH 8.0	

Table 4-6: Golden Gate assembly components

The mixture is incubated in a thermo-cycler either under a continuous temperature of 37°C, or under switching from optimum temperature for the restriction enzyme (e.g. 37°C for *Bbs*I and *Bsp*MI) to the optimum temperature for T4 ligase (16°C) in 45 repeats.

After that, incubation at 50°C for 5 minutes serves as a final digestion step for plasmids still containing restriction sites, as well as 10 minutes at 80°C for inactivation of the restriction enzymes and DNA ligase.

Continuous temperature		Changing temperature	
6 hours	37°C	2 min	37°C
		5 min	16°C
		45 repeats	
5 min	50°C	5 min	50°C
10 min	80°C	10 min	80°C

Table 4-7: Golden Gate assembly conditions

As seen in the table above, Weber and Engler recommend an incubation time of 2 minutes for the restriction enzyme and 5 minutes for T4 ligase (Weber et al., 2011). For *Bbs*I half of the time is also sufficient.

The obtained products (plasmids) can be directly transformed into chemically competent *E. coli*, used for further DNA manipulation steps, or stored at -20°C. The fusion sites used in our variant of Golden Gate cloning can be found in table 4-1.

For the creation of level-1 modules in a Golden Gate assembly reaction, level-0 modules with corresponding fusion sites are mixed with *Bbs*I, T4 ligase and a level-1 recipient vector also containing the corresponding fusion sites of the first and the last insert. Figure 4-5 shows the Golden Gate assembly of an expression cassette built of three inserts (promoter, coding sequence and a terminator), which were blunt ligated into level-0 vector plasmids containing a Kanamycin resistance cassette. The promoter is flanked by fusion sites 1 and 2 (FS1 and FS2), whereas the coding sequence is flanked by FS2 and FS3, and the terminator by FS3 and FS4. All three inserts are ligated in hierarchical manner into the recipient vector containing fusion sites 1 and 4. After transformation of the ligation products into competent *E. coli*, cells with level-1 recipient vectors containing an Ampicillin resistance marker can be selected on agar-plates containing the antibiotic.

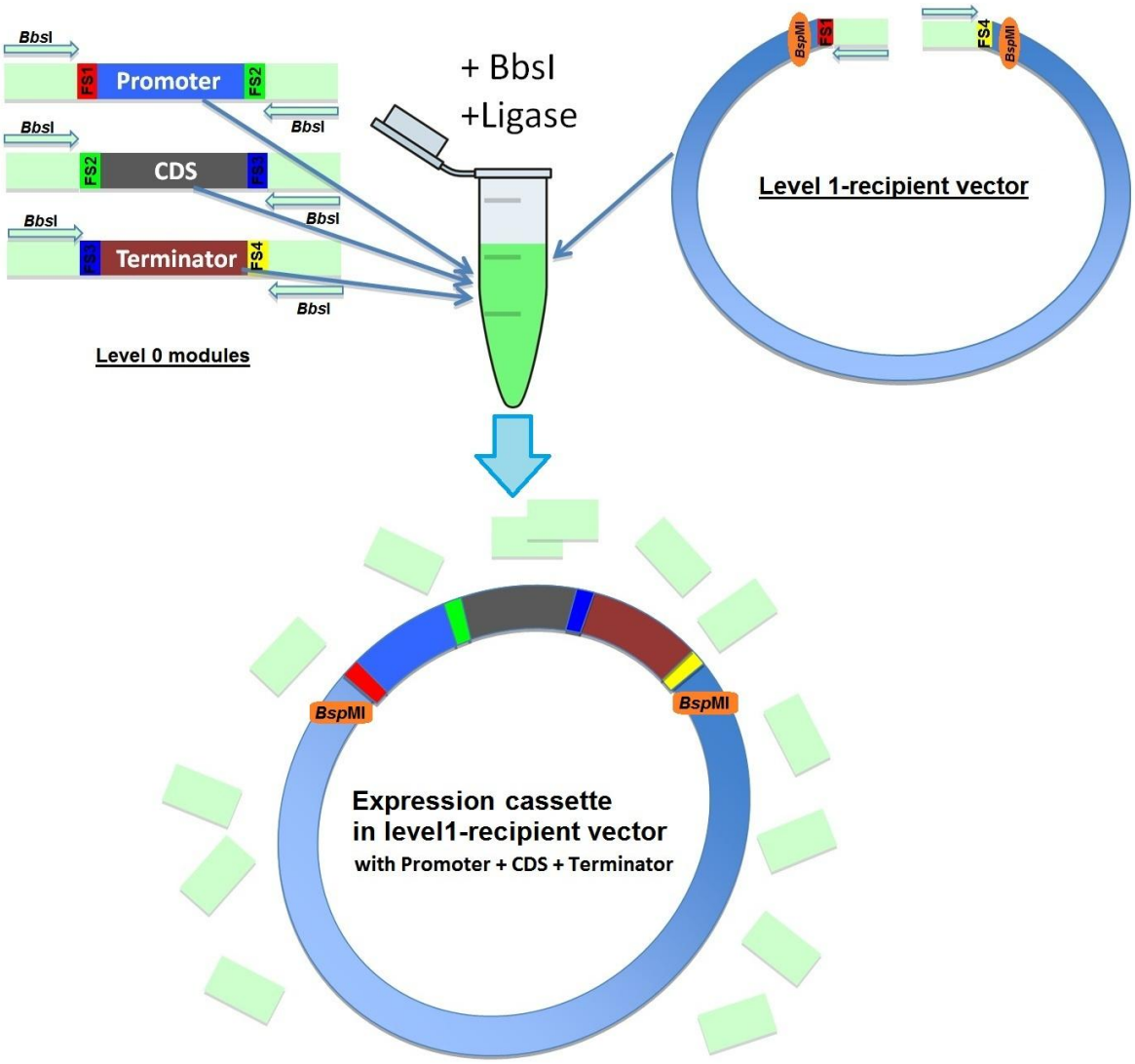

Figure 4-5: Golden Gate assembly of level-1 vector plasmids

Also level-1 modules are flanked by fusion sites A – G and *Bsp*MI restriction sites. In a second Golden Gate assembly, up to six expression cassettes can be ligated in tandem into level-2 recipient vectors, enabling the engineering and artificial creation of entire metabolic pathways. Therefore, level-2 recipient plasmids contain a small linker element with the corresponding fusion sites for the creation of level-2 vectors with 1 insert (linker A-B), 2 inserts (linker A-C), up to 6 inserts (A-G), as can be seen in figure 4-6.

Level-2 recipient vectors are built by Golden Gate assembly of linker elements (fusion sites α and β), a desired resistance marker (FSβ and FSγ), NTS sequence (FSγ and FSδ) and an origin of replication (FSδ and FSα), using *Bbs*I as restriction enzyme.

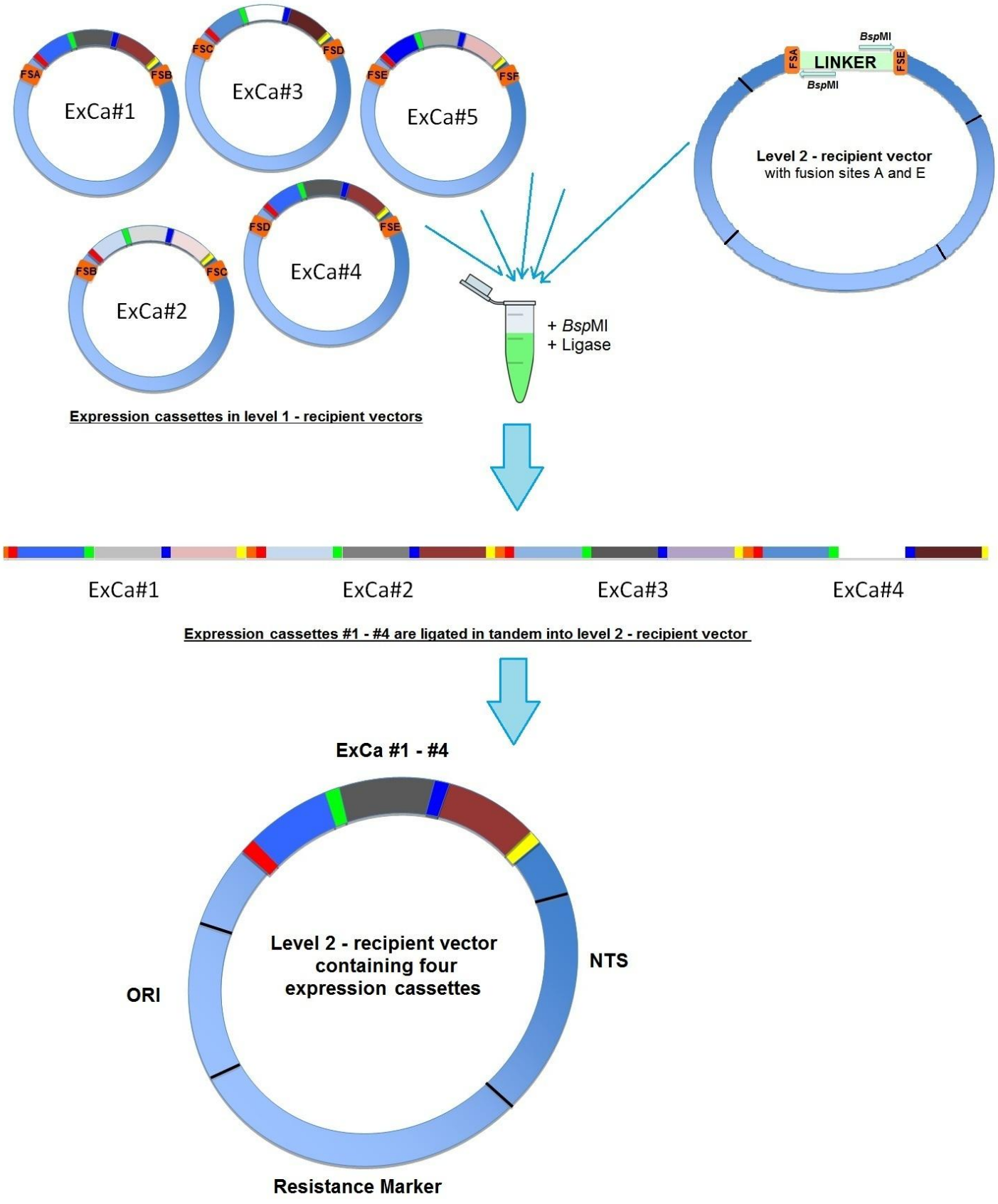

Figure 4-6: Golden Gate assembly of level-2 vector plasmids

4.5. Transformation

4.5.1. Preparation of chemically competent *Escherichia coli*

Materials:

- *E. coli* TOP10, DH10B, or HMS174(DE3)
- LB Medium
- FB buffer
  - 7.4 g/L KCl
  - 7.5 g/L $CaCl_2 * 2 H_2O$
  - 100 g/L Glycerol
  - 10 mL/L K-Acetate 1M pH 7.5
- pre-chilled 1.5 mL eppis (-20°C)
- cooling centrifuge

Procedure:

Pre-culture: Inoculate 5 mL of LB medium with *E. coli* TOP10, DH10B, or HMS174(DE3) in a 50 ml shake flask and incubate overnight at 37°C under shaking (180 rpm).

Main-culture: Use the overnight culture to inoculate 200 mL of LB medium and incubate this main culture at 37°C under shaking (180rpm) for 2 to 4 hours up to an OD_{600} of approximately 0.5. Transfer the culture to 4 sterile 50 mL centrifuge tubes and put them on ice for 10 min. In the meanwhile, cool down the centrifuge to 4°C and put the FB buffer on ice.

Centrifuge at 3000 rpm for 15 min at 4°C. Remove the supernatant and re-suspend all 4 pellets in a total volume of 100 mL pre-chilled FB buffer as they are pooled together in 50 ml. Put the cells on ice for 45 min. Centrifuge the cells at 2200 rpm for 15 min at 4°C and remove the supernatant. Re-suspend the cells in 15 mL pre-chilled FB buffer and transfer 100 µL aliquots into pre-chilled 1.5 mL eppis and immediately store them at -80°C.

4.5.2. Transformation of chemically competent bacteria

Materials:

- chemically competent *E. coli* (100 µL per transformation)
- LB Medium
- incubator (42°C and 32°C)
- selective agar plates

Procedure:

Thaw the competent cells on ice. Add 20 µL of ligation mix, or Golden Gate assembly mix to 100 µL of competent cells. Tap the tube gently and put it on ice for 5 min. Incubate the mix for exactly 90 sec at 42°C (heat-shock) and put the mix back on ice for 5 min. Add 1 mL LB medium and incubate at 37°C for 30 min (Ampicillin resistance), or 2 hours (Kanamycin resistance). Plate the cells on selective agar and incubate overnight at 37°C.

4.6. DNA Preparation

4.6.1. Preparation of plasmid DNA

For the preparation of small scale plasmid DNA from a 2 mL *E. coli* overnight culture (incubated at 37°C and 180rpm shaking) the commercially available “HiYield® Plasmid Mini Kit” from SLG® (Cat.No. 30 HYPD100) was used, according to the manufacturer protocol.

For the preparation of medium scale plasmid DNA from a 100 mL *E. coli* overnight culture (incubated at 37°C and 180rpm shaking) the commercially available “HiSpeed Plasmid Midi Kit” from QIAGEN (Cat.No. 12643) was used, according to the manufacturer protocol.

For the elution of purified plasmid DNA 10 mM TRIS-HCl pH 8.0 was preferred for both kits, since the elution buffer of the kit contains EDTA, which might interfere in further steps like PCR or Golden Gate assemblies.

4.6.2. Separation of DNA fragments (agarose gel electrophoresis)

Agarose gel electrophoresis is an effective tool for seperation of DNA fragments in a range of 100 bp to 25 kbp. Agarose consists of repeated L- and D-galactose subunits, is isolated from seaweed and used in a range between 1% and 3% in a gel. The subunits associate non-covalently, generating a three-dimensional matrix.

DNA molecules are negatively charged, due to phosphate residues on their backbone. When placed in an electric field DNA fragments migrate towards the anode and are separated by size. A uniform mass/charge ratio determines the distance travelled by a DNA fragment, which is inversely proportional to the log of its molecular weight (Lee et al., 2012). Seperated DNA can be visualized through SYBR® Safe DNA Gel Stain under UV light (260nm – 320nm). Quantification of size is achieved by Thermo Scientific MassRuler DNA Ladder Mix (80 – 10000bp).

DNA samples are mixed with Thermo Scientific 6X MassRuler DNA Loading Dye before transfer into gel pockets. It contains bromophenol blue for visual tracking of DNA migration during electrophoresis, EDTA and glycerol to ensures that the DNA forms a layer at the bottom of the well.

Materials:

- 50x TAE buffer
  242 g Tris base
  57.1 mL acetic acid
  100 mL 0.5 M EDTA pH 8.0
  up to 1000 mL with RO-H_2O
- 1% agarose gel (150mL):
  1.5 g agarose
  3 mL 50x TAE buffer
  filled up to 150 mL with RO-H_2O

  To melt the agarose the mixture is heated to boiling in a microwave and afterwards 15 µL SYBR® Safe DNA Gel Stain is added.

- Thermo Scientific 6X MassRuler DNA Loading Dye
- Thermo Scientific MassRuler DNA Ladder Mix (80 – 10000bp)

4.6.3. Agarose gel extraction

For the purification of DNA from agarose gels the commercially available Promega Wizard® SV Gel and PCR Clean-UP System was used, according to the protocol for gel extraction.

Samples were eluted in 50 µL nuclease free water and stored on ice for further processing, or at -20°C for long-time storage.

4.6.4. Quantification of plasmid DNA (NanoDrop)

Concentration of plasmid DNA was measured by Thermo Scientifc NanoDrop 1000 Spectrophotometer, according to the protocol for nucleic acid quantification. The detection range lies between 2 ng/µL and 3700 ng/µL.

4.6.5. Sequencing

For checking purpose many plasmids obtained by Golden Gate cloning were sent for sequencing to the company microsnyth (www.microsynth.ch). The samples were pre-mixed with the desired sequencing-primer according to their protocol of preparation.

DNA	Template Concentration	Effective Amount
Plasmid	60-100 ng/µl	720-1200 ng
Primer	2 pmol/µl	30 pmol
RO-H_2O or 10 mM TRIS-HCl pH 8.0	fill up to 15 µL	

Table 4-8: Preparation of samples for sequencing

4.7. Cultivation

4.7.1. Determination of optical density

The determination of optical density (OD) is an essential tool for cultivation experiments. Therefore, 1 mL of cell suspension is measured through a photometer at a wavelength of 600 nm. Samples have to be diluted to an OD_{600} value between 0.1 and 0.6 (linear range of the photometer). The obtained OD_{600} value is directly proportional to the biomass concentration of the sample. For *E. coli* an OD_{600} value of 3 accords to 1 g/L of biomass dry weight (BDW) (experimentally determined factor in house by G. Striedner).

4.7.2. Expression of reporter protein GFP and DsRed

A single colony from a streaked plate is incubated overnight in 5 mL LB medium with appropriate antibiotic at 37°C and under shaking (180 rpm). This culture is diluted 1:100 into fresh, pre-warmed LB medium with same antibiotic and incubated under the same conditions described above. After 3 hours OD_{600} and protein expression can be measured.

4.7.3. Production of carboxylic acids

Pre-culture

A single colony from a streaked plate is incubated overnight in a shake-flask of 100 mL LB medium with appropriate antibiotic at 37°C and under shaking (180 rpm). For the cultivation of clones containing two plasmids with different antibiotic resistance markers (eg. *alkBTFG* and *alkJ* – see result section) both antibiotics are added in appropriate amounts.

Main-culture

A shake flask of 500 mL fresh, pre-warmed LB medium with the same antibiotic(s) are inoculated with an OD_{600} of 0,1 from the pre-culture. The desired amount of sterile-filtered Isopropyl-β-D-thiogalactopyranosid (IPTG) for induction of the *lac*-operon is added (2,3 µmol/g BDW or 23 µmol/g BDW) and the culture is incubated overnight to a final OD_{600} of 4. Strong induction can cause growth inhibition; in this case an OD_{600} of 2 is also sufficient.

Production

The cultures are harvested and centrifuged (6000g, 10 min), washed in 200 mM sodium phosphate buffer (pH 7, sterile-filtered) and centrifuged again (6000g, 10 min). For the preparation of a 200 mM sodium phosphate buffer two solutions of 200 mM NaH_2PO_4 (27,6 g/L) and 200 mM Na_2HPO_4 (35,6 g/L) are mixed to a final pH of 7,0.

The cells are re-suspended in 25 mL (for OD_{600} =4), or 12,5 mL (OD_{600} =2) of the tested media (LB, M9, or phosphate buffer). Sterile-filtered $FeSO_4$ to a final concentration of

100mg/L is added, as well as the same amount of sterile-filtered Isopropyl-β-D-thiogalactopyranosid (IPTG) as for the main-culture (2,3 µmol/g BDW or 23 µmol/g BDW). Cultivation occurs in baffled shake-flasks at 37°C under shaking (180 rpm). For the production of hexanoic acid 0,5 g/L n-hexane is added and for overnight cultivation another 5 g/L n-hexane is added.

For the production of adipic acid 0,5 g/L hexanoic acid is added and for overnight cultivation another 0,5 g/L is added.

Sampling:

After 2, 4 and 6 hours, as well as overnight cultivation (~20 hours) 2 mL samples are taken. After centrifugation (13.000 rpm, 5 min) the supernatant can be either prepared for HPLC measurements, or stored at -20°C.

4.8. Fluorescence Spectroscopy

Expression of reporter proteins GFP and DsRed were measured with a fluorometer (Tecan Infinite® 200) in a 96-well plate (Nunclon 96 Flat Bottom Black Polystyrol LumiNunc Fluoronunc) with an UV Xenon flash lamp.

The excitation wavelengths for GFP and DsRed were 488 nm and 554 nm, respectively. The emission wavelengths were measured at 520 nm and 585 nm for GFP and DsRed, respectively. The values obtained are “relative fluorescence units” (RFU).

All samples were measured in duplicate under reproducible conditions with a sample volume of 100 µL per well.

4.9. HPLC measurement

Metabolites produced during cultivations were quantified by HPLC analysis (Shimadzu, Korneuburg, Austria) with a Rezex ROA-Organic Acid H^+ column (300mm x 7.8 mm, Phenomenex, USA) and a refraction index detector (RID-10A, Shimadzu, Korneuburg, Austria). The column was operated at 60 °C, 1.0 ml min^{-1} flow rate and 4 mM H_2SO_4 as mobile phase.

All samples were adjusted to 4 mM H_2SO_4 and filtered through a 0,22 µm filter prior to measurement.

5. Results

5.1. A variant of Golden Gate Cloning

The characteristics of Golden Gate cloning enable the constitution of a modular cloning system for the reconstruction and optimisation of entire metabolic pathways (see 3.4) and the first part of this work was to establish a variant of Golden Gate cloning, which is sufficient for the engineering of metabolic pathways in our lab. With our method up to six different DNA sequences can be ligated in a hierarchical manner into a recipient vector. DNA sequences are flanked by type IIS restriction enzyme recognition sequences (obtained by primer-directed PCR). The restriction enzymes used in this variant of the modular cloning system are *Bbs*I and *Bsp*MI which both create freely selectable four nucleotide overhangs outside their recognition sequence. This feature enables site-directed digestion and ligation within one reaction tube. Also, the recognition sequence is missing in the final ligation product, so no further restriction events occur and the ligation product is stable and accumulates during the reaction.

Basic DNA fragments like promoters, transcription terminators and coding sequences flanked by *Bbs*I restriction sites are blunt-ligated into modified pSTBlue-1 vectors, called level-0 modules. Up to nine of these modules can be combined in one reaction and ligated into a level-1 recipient vector also derived from pSTBlue-1 vectors. These vectors contain *Bsp*MI restriction sites at the flanks of the level-0 insert which are used in a second restriction-ligation step to put level-1 inserts into another pSTBlue-1 derived level-2 recipient vector.
The designed recipient vectors contain alternating antibiotic resistance markers and *E. colis* carrying the plasmid can be selected on agar-plates containing the respective antibiotic. Level-0 vectors provide a resistance to Kanamycin, whereas level-1 vectors contain an Ampicillin resistance cassette. Level-2 vectors have a Kanamycin resistance cassette again. Additionally, blue/white screening is used to distinguish vectors without insert from the desired ligation products.

Several optimisation experiments were carried out to find the ideal conditions for Golden Gate reactions using *Bbs*I and *Bsp*MI as restriction enzymes.

5.1.1. Optimization experiments for *Bbs*I

To establish the Golden Gate cloning method and to determine the optimal conditions for the restriction enzyme *Bbs*I several assemblies with different parameters were tested:

- incubation method (constant and switching temperatures – see 4.4.2.)
- circular vector plasmid / linearised vector plasmid (pre-cut with *Bbs*I)
- 10 units / 100 units of T4 ligase

To test these parameters a vector with GFP as coding sequence, a medium strength constitutive promoter and a transcription terminator was assembled, transformed into chemically competent *E. coli* TOP10 and plated on selective LB agar plates containing 70 µg/mL X-gal and 80 µM IPTG. Correctly assembled plasmid constructs could be observed as green colonies under a UV lamp. White colonies which showed no green fluorescence contain incorrectly assembled plasmids. No blue colonies were observed in this experiment.

All assemblies were performed with 10 units *Bbs*I.

	volume plated [µL]	**switching temperature**				**constant temperature**			
		total	**white**	**green**		**total**	**white**	**green**	
circular vector, 10 U Ligase	10	18	1	17	94%	8	4	4	50%
	100	176	25	151	86%	190	83	107	56%
circular vector, 100 U Ligase	10	50	11	39	78%	130	116	14	11%
	100	>>300	>300	>300	n/a	>>300	>300	134	n/a
linear vector, 10 U Ligase	10	12	0	12	100%	2	0	2	100%
	100	161	5	156	97%	23	3	20	87%
linear vector, 100 U Ligase	10	3	0	3	100%	14	1	13	93%
	100	116	6	110	95%	169	19	150	89%

Table 5-1: Results Golden Gate optimization experiment with *Bbs*I

Two different plasmid templates were tested as recipient vectors (linear and circular form), as well as two different concentrations of T4 DNA Ligase were used (10 units and 100 units). After transformation of Golden Gate products, colonies were examined under UV light. Green colonies represent successful plasmid assemblies containing a GFP expression cassette. White colonies carry incorrectly assembled plasmids.

As seen in table 5-1, the results were very satisfying. The cloning efficiency of *Bbs*I with switching temperature conditions is between 80 and 100%. Restriction digests proved, that almost 100% of green colonies contained a perfectly assembled GFP expression cassette.

5.1.2. Optimization experiments for *Bsp*MI

The second restriction enzyme could not fulfil the high expectations after successful *Bbs*I experiments.

Optimization experiments for *Bsp*MI brought no reproducible results after Golden Gate assemblies whether with switching temperature conditions, nor with a constant incubation temperature. Randomly selected samples were sent for sequencing and showed that Golden Gate assemblies with *Bsp*MI resulted mostly in uncut recipient plasmids, or in plasmids containing multiple randomly assembled inserts.

Several commercially available enzymes from different companies cutting a *Bsp*MI recognition sequence were tested in different buffers, as can be seen in the figure below. A DsRed expression cassette with a medium constitutive promoter in a level-1 module served as test-plasmid. A restriction digest with *Bsp*MI should result in two bands with 3063 base pairs and 787 base pairs respectively.

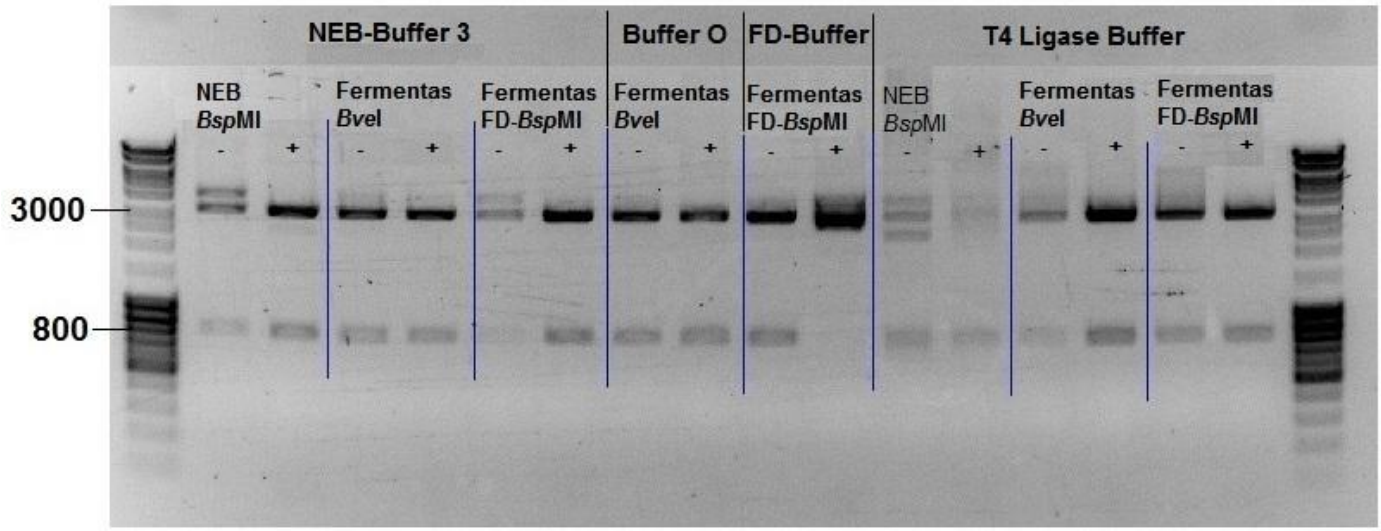

Figure 5-1: Restriction digest of test-plasmid with *Bsp*MI (3063 bp and 787 bp)

Several commercially available enzymes from different companies cutting a *Bsp*MI recognition sequence were tested in different buffers (listed in table 5-2). A DsRed expression cassette served as test-plasmid and a restriction digest with *Bsp*MI should result in two bands with 3063 base pairs and 787 base pairs respectively. Right lanes (+) show a digest where oligonucleotides were added, as recommended in the manufacturer protocol. Enzymes in lane 2 and 4 (both *Bve*I) and lane 9 (Fast Digest *Bsp*MI) showed restriction efficiency, but the cloning efficiencies after Golden Gate assemblies were very poor.

All the enzymes listed in table 5-2 were used for Golden Gate assemblies of alk operon plasmids and for multiple GFP constructs. In the manufacturer manual oligonucleotides are added to increase efficiency of enzymatic digest, but simultaneously they may interfere with

Golden Gate assemblies and lead to undesired ligation products. Therefore, oligonucleotides are not used in Golden Gate assemblies later on.

Although two enzyme-buffer combinations showed a promising result in this experiment (BveI and Fast Digest *Bsp*MI – both of Fermentas), the cloning efficiency results were very poor; therefore the last step in the Golden Gate cloning method brought no reproducible results. Anyhow, two final constructs containing genes of the *alk*-operon were generated after several cloning attempts and used for cultivation experiments (see 5.2 and 5.4).

Nr.	Enzyme	Company	Buffer
1	*Bsp*MI	New England Biolabs	NEB 3
2	*Bve*I (*Bsp*MI)	Fermentas	NEB 3
3	Fast Digest *Bsp*MI	Fermentas	NEB 3
4	*Bve*I (*Bsp*MI)	Fermentas	Buffer O
5	Fast Digest *Bsp*MI	Fermentas	FD – Buffer
6	*Bsp*MI	New England Biolabs	T4 Ligase Buffer
7	*Bve*I (*Bsp*MI)	Fermentas	T4 Ligase Buffer
8	Fast Digest *Bsp*MI	Fermentas	T4 Ligase Buffer
in the right lane 0,5 µM of oligonucleotides were added to the reaction mix			

Table 5-2: Restriction digest of test-plasmid with *Bsp*MI

The results of optimization experiments showed, that the variant of Golden Gate cloning set up in our lab is working properly, especially for *Bbs*I. During these experiments three inserts were successfully assembled to a functional expression cassette into a recipient vector. Only the second restriction enzyme of our method *Bsp*MI did not meet the requirements indicated by the manufacturer catalogue. To bypass the poor efficiency of *Bsp*MI the second restriction enzyme of the modular cloning system has to be changed to another more efficient enzyme. *Bsa*I from New England Biolab has been chosen to replace *Bsp*MI, which will be executed within another project.

5.2. Golden Gate Library

The plasmid constructs obtained during this work are listed in the tables below.

BASIC MODULES			
Level-0	**Fusion sites**	**Description**	
Promoter	1_pW_2	BBa_J23112 weak constitutive (pW)	source: http:// parts.igem.org
	1_pM_2	BBa_J23105 medium constitutive (pM)	
	1_pS_2	BBa_J23119 strong constitutive (pS)	
	1_pT7_2	IPTG inducible (pT7)	
	1_pT7lacI_2	IPTG inducible with repressor (pT7lacI)	
Coding sequence	2_GFP_3	green fluorescent protein (GFP)	
	2_DsRed_3	red fluorescent protein (DsRed)	
Terminator	3_TT_4	transcription terminator (TT)	
Linker cassettes	X_1_LacZ_4_Y	X-Y … Fusion sites: A-B, B-C, C-D, D-E, E-F, F-G	
Level-1	**Fusion sites**	**Description**	
Recipient cassettes	α_A_LacZ_Y_β	A-Y … Fusion sites: A-B, A-C, A-D, A-E, A-F, A-G	
Resistance	β_KanR_γ	Kanamycin (KanR)	
	β_ZeoR_γ	Zeocin (ZeoR)	
	β_HygroR_γ	Hygromycin (HygroR)	
NTS	γ_NTS_δ	non-transcribed intergenic spacer from *P.pastoris*	
Origin of replication	δ_pUC19_α	pUC19_ORI (high copy number plasmid)	
	δ_p15_α	p15_ORI (low copy number plasmid)	
Insert position No.1	A_pW_GFP_TT_B	weak GFP expression at position 1	
	A_pM_GFP_TT_B	medium GFP expression at position 1	
	A_pT7_GFP_TT_B	inducible GFP expression at position 1	
	A_pT7lacI_GFP_TT_B	inducible GFP expression with repressor at position 1	
	A_pW_DsRed_TT_B	weak DsRed expression at position 1	
	A_pM_DsRed_TT_B	medium DsRed expression at position 1	
	A_pT7_DsRed_TT_B	inducible DsRed expression at position 1	
Insert position No.2	B_pW_GFP_TT_C	weak GFP expression at position 2	
	B_pM_GFP_TT_C	medium GFP expression at position 2	
	B_pW_DsRed_TT_C	weak DsRed expression at position 2	
	B_pM_DsRed_TT_C	medium DsRed expression at position 2	
Insert position No.3	C_pW_GFP_TT_D	weak GFP expression at position 3	
Insert position No.4	D_pW_GFP_TT_E	weak GFP expression at position 4	
Insert position No.5	E_pW_GFP_TT_F	weak GFP expression at position 5	
Insert position No.6	F_pW_GFP_TT_G	weak GFP expression at position 6	
Level-2 Recipient vectors	**Fusion sites**	**Description**	
for 1 insert	KanR_NTS_pUC19_A-LacZ-B	high copy plasmid with kanamycin resistance	
for 2 inserts	KanR_NTS_pUC19_A-LacZ-C	high copy plasmid with kanamycin resistance	
	ZeoR_NTS_pUC19_A-LacZ-C	high copy plasmid with zeocin resistance	
for 3 inserts	KanR_NTS_pUC19_A-LacZ-D	high copy plasmid with kanamycin resistance	
	ZeoR_NTS_pUC19_A-LacZ-D	high copy plasmid with zeocin resistance	

for 4 inserts	KanR_NTS_pUC19_A-LacZ-E	high copy plasmid with kanamycin resistance
for 5 inserts	KanR_NTS_pUC19_A-LacZ-F	high copy plasmid with kanamycin resistance
for 6 inserts	KanR_NTS_pUC19_A-LacZ-G	high copy plasmid with kanamycin resistance
Final construct		**Description**
with 2 inserts	KanR_NTS_pUC19_ pM_GFP_TT_pM_DsRed_TT	medium expression of GFP and DsRed in a high copy plasmid with kanamycin resistance

Table 5-3: Golden Gate library - basic modules

ALK OPERON		
Level-0	**Fusion sites**	**Description**
alk operon gene	2_alkB_3	alkB – monooxygenase
	2_alkT_3	alkT – rubredoxin reductase
	2_alkF_3	alkF – rubredoxin 1
	2_alkG_3	alkG – rubredoxin 2
	2_alkH_3	alkH – aldehyde dehydrogenase
	2_alkJ_3	alkJ – alcohol dehydrogenase
Level-1	**Fusion sites**	**Description**
Insert position No.1	A_pW_alkB_TT_B	weak alkB expression at position 1
	A_pM_alkB_TT_B	medium GFP expression at position 1
	A_pT7lacI_alkB_TT_B	inducible alkB expression with repressor at position 1
Insert position No.2	B_pM_alkT_TT_C	medium alkT expression at position 2
	B_pT7_alkT_TT_C	inducible alkT expression at position 2
Insert position No.3	C_pM_alkF_TT_D	medium alkG expression at position 3
	C_pT7_alkF_TT_D	inducible alkG expression at position 3
Insert position No.4	D_pM_alkG_TT_E	medium alkG expression at position 4
	D_pT7_alkG_TT_E	inducible alkG expression at position 4
	D_pT7_alkH_TT_E	inducible alkH expression at position 4
Insert position No.5	E_pW_alkH_TT_F	weak alkH expression at position 5
	E_pT7_alkH_TT_F	inducible alkH expression at position 5
	E_pT7_alkG_TT_F	inducible alkG expression at position 5
Insert position No.6	F_pW_alkJ_TT_G	weak alkJ expression at position 6
	F_pM_alkJ_TT_G	medium alkJ expression at position 6
	F_pT7_alkJ_TT_G	inducible alkJ expression at position 6
Level-2 (Final construct)		**Description**
with 4 inserts	KanR_NTS_pUC19_T7(lacI)_alkBTFG	IPTG inducible expression of alkBTFG in a high copy plasmid with kanamycin resistance

Table 5-4: Golden Gate library - alk operon

Despite the IPTG inducible plasmid with *alkBTFG* also two IPTG inducible *alkBTFGHJ* constructs were obtained. These constructs could not be used for cultivation experiments, because of an IS1 transposase in the *alkH* gene (see 5.5).

5.3. Cultivation experiments with reporter proteins

After establishing the Golden Gate cloning method for *Bbs*I expression cassettes with reporter proteins GFP and DsRed in level-1 recipient vectors were built.

5.3.1. Expression of reporter protein GFP

During the construction of GFP expression cassettes we noted, that some of the colonies were greener than others, although GFP expression was under the control of the same weak constitutive promoter. This led us to the assumption that insertion direction of genes into recipient vectors derived from pSTBlue-1 vectors might play a role in gene expression. Since level-1 recipient vectors were obtained by blunt-ligation of linker elements containing fusion sites and recognition sequences for restriction enzyme *Bbs*I, some of the inserts ended up in backward direction, which we thought might not be important for further cloning steps. To determine the influence of the insertion direction of a gene into vectors derived from pSTBlue-1 two different clones with weak constitutive GFP expression were tested. (see 4.7.2). The expression cassette is inserted backward in B_pW_GFP_TT_C (BC) and forward in E_pW_GFP_TT_F (EF).

	insertion direction	**OD_{600}**	**biomass concentration [g/L]**	**relative fluorescence unit**
B_pW_GFP_TT_C #1	backward	0,580	0,193	5796,5
B_pW_GFP_TT_C #2	backward	0,450	0,150	4773,0
B_pW_GFP_TT_C #3	backward	0,548	0,183	5419,5
E_pW_GFP_TT_F #1	forward	0,205	0,068	31596,0
E_pW_GFP_TT_F #2	forward	0,197	0,066	38054,5
E_pW_GFP_TT_F #3	forward	0,152	0,051	28516,5

Table 5-5: Expression of GFP in level-1 modules with fusion sites B-C and E-F

Two different clones with weak constitutive GFP expression were tested for GFP expression. Relative fluorescence units for expression cassettes inserted backward in B_pW_GFP_TT_C (BC) showed lower expression rates than forward inserted cassettes in E_pW_GFP_TT_F (EF). Contrary effects on growth rates were observed, as can be seen in biomass concentration of the samples.

Vectors with forward inserted expression cassettes show higher expression rates than backward vectors (see figure 5-2). This has an influence on growth rates, as clones containing a forward vector attain lower biomass concentrations (see figure 5-3).

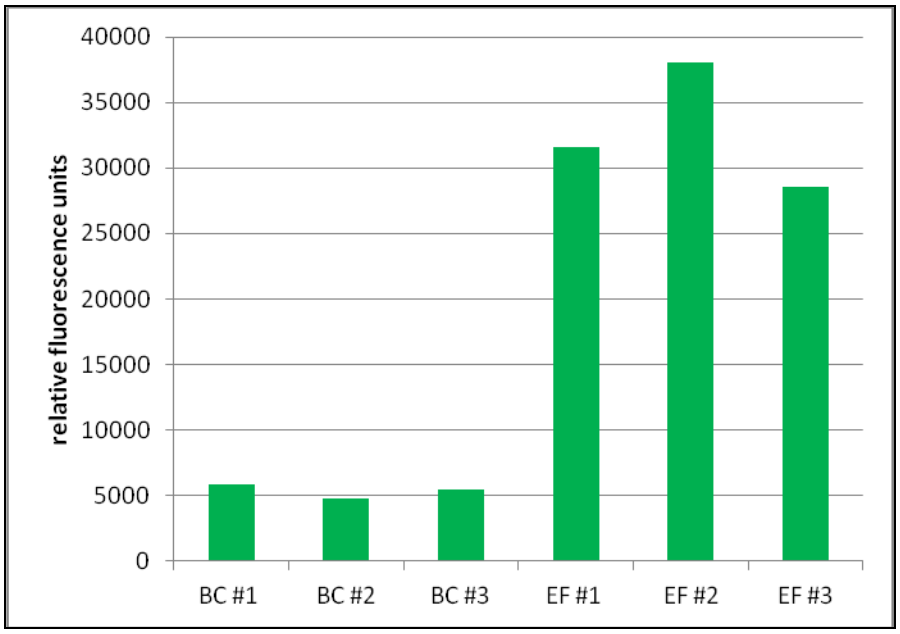

Figure 5-2: Expression of GFP in forward and backward vector

Three clones containing expression cassettes inserted backward (BC#1, BC#2, BC#3) and three clones containing expression cassettes inserted forward (EF#1, EF#2, EF#3) were incubated in LB media (see 4.7.2). Expression rates were measured by fluorescence spectroscopy (see 4.8) Biomass concentration of the cultures was derived from optical density (see 4.7.1). Expression cassettes inserted backward showed lower expression rates than forward inserted cassettes. Contrary effects on growth rates were observed, as can be seen in the figure below.

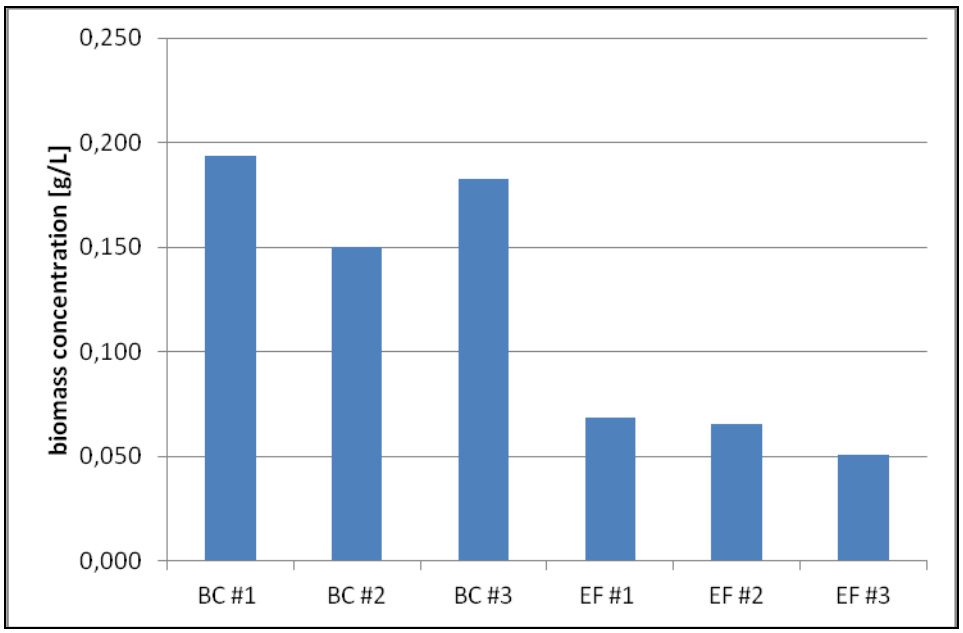

Figure 5-3: Influence on growth of forward and backward vector

Consistent and reproducible expression of the desired gene product is a key feature for successful metabolic engineering, especially when it comes to the testing of different promoters and their specific expression rates. To avoid undesired gene expression the promoter elements for Golden Gate cloning have to be modified and a transcription termination sequence will be added upstream of the promoter sequence as part of another project.

5.3.2. Expression of reporter protein DsRed

Several plasmid constructs containing different promoters (BBa_J23112 weak strength and BBa_J23105 medium strength) were built with the established variant of Golden Gate cloning, transformed into *E. coli* TOP10 and incubated in LB medium (see 4.7.2). Fluorescence spectroscopy of these clones brought no reproducible results, as the measured fluorescence intensities were not significantly different from negative controls. Expression rate of promoters BBa_J23112 and BBa_J23105 are possibly not high enough for adequate production of DsRed.

Also, DsRed has a long maturation time during which fluorescence intensity is slowly switching from green to red (Baird et al., 2000). Therefore, it is not practicable for the comparison of expression rates of different promoters.

5.4. Cultivation experiments with *alk* operon genes

After establishing the Golden Gate Cloning method the *alk* operon was re-assembled in pSTBlue-1 derived vectors. First step was to build a plasmid with *alkB* monooxygenase as key enzyme, supported by *alkT* rubredoxin reductase and two coding sequences for rubredoxins (*alkG* and *alkH).* This plasmid construct was called *alkBTFG*.

One construct of T7*_alkBTFG* was successfully generated and transformed into *E. coli* TOP10. This plasmid consists of the first four genes of the *alk* operon and every single gene is under the control of an IPTG-inducible T7 promoter. Additionally a *lacI* repressor is put in front of *alkB* monooxygenase expression cassette to suppress basal expression of proteins, since expression of *alkB* is thought to cause stress to the cell because of its insertion into the cell membrane of the host (Eggink et al., 1987).

With *alkJ* and *alkH* missing the production of carboxylic acids would be expected to stop at the step of alcohol oxidation (hexanol from hexane, or 6-hydroxyhexanoic acid from hexanoic acid).

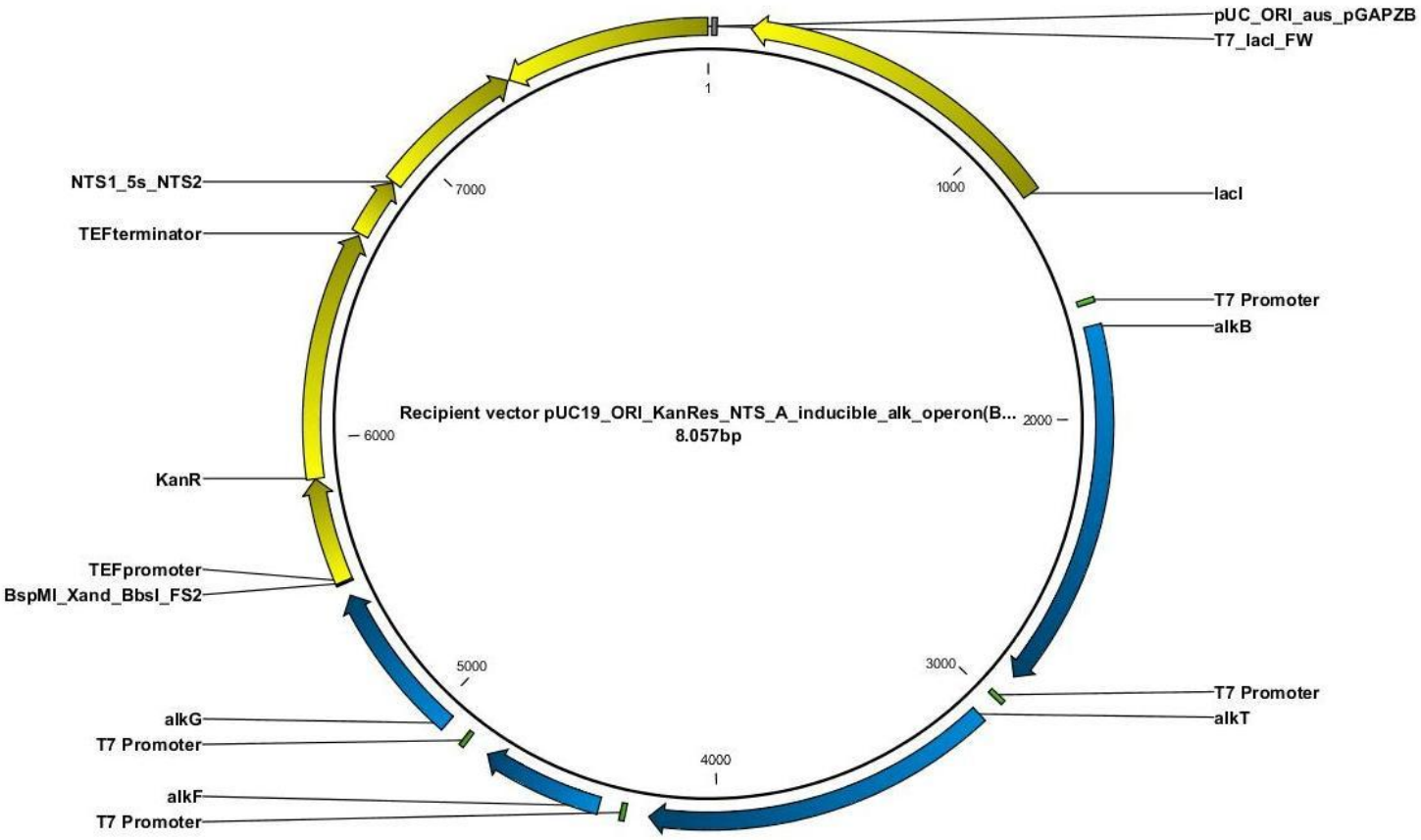

Figure 5-4: Level-2 recipient vector expressing four genes of the *alk*-operon (*alkBTFG*)

5.4.1. Production of hexanoic acids with *alkBTFG*

The cultivation experiments were accomplished in 25 mL of either LB medium (C2.1), M9 medium (C2.2), or 200 mM Na-phosphate buffer (C2.3) with 100mg/L Kanamycin and with 64 µmol/L of IPTG, which refers to 2,3 µmol IPTG/g biomass dry weight with a biomass concentration for the main culture of approximately 1,3 g/L. An *E. coli* Top10 clone containing the final construct plasmid KanR_NTS_pUC19_T7(lacI)_*alkBTFG* was incubated with 0,5 g/L n-hexane.

	hexanoic acid [mg/L]		
incubation time [h]	**C2.1 LB medium**	**C2.2 M9 medium**	**C2.3 PO_4 buffer**
0	14,39	0,00	0,00
2	43,84	21,59	30,98
4	44,24	20,35	30,90
6	43,10	20,66	32,29

Table 5-6: Production of hexanoic acid with *alkBTFG*

Cultivation experiments of *E. coli* TOP10 carrying the plasmid T7_*alkBTFG* for IPTG inducible expression of *alkB* monooxygenase, *alkT* rubredoxin reductase and two rubredoxins (*alkG* and *alkH)* resulted in production of hexanoic acid from n-hexane as substrate. Most of the hexanoic acid has been produced after two hours of incubation. An IPTG concentration of 64 µmol/L was applied.

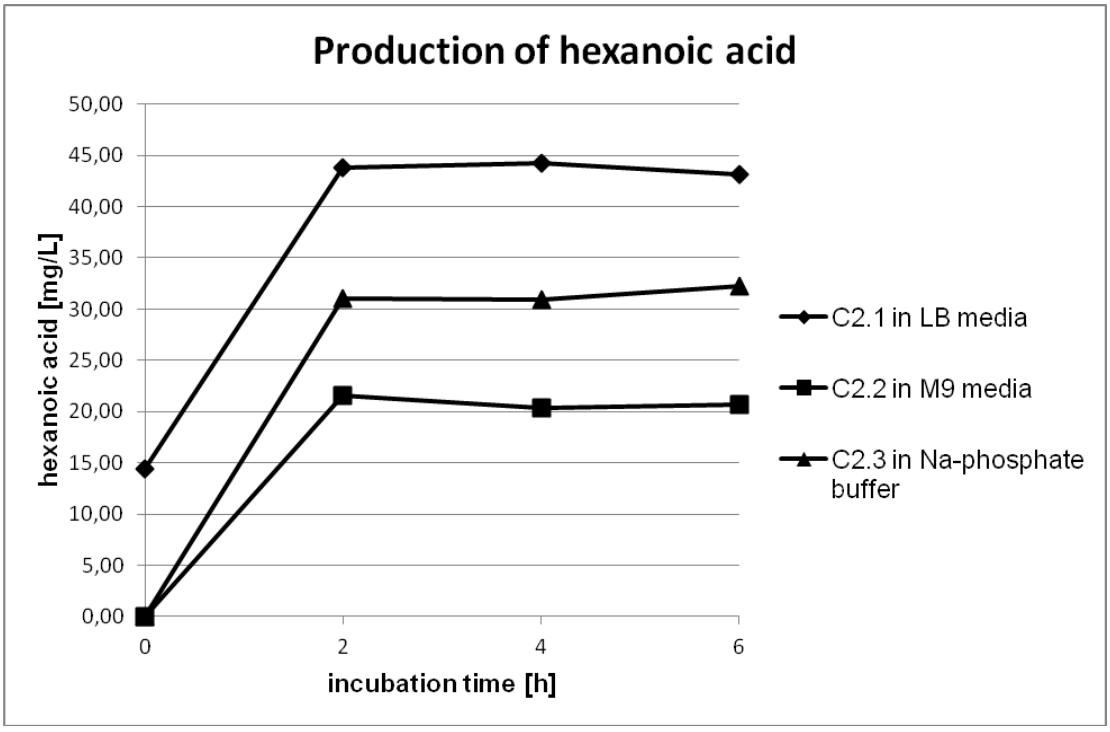

Figure 5-5: Production of hexanoic acid with *alkBTFG* (incubation at 37°C shaking)

In all three media hexanoic acid could be produced from n-hexane. Hexanoic acid was also detected in LB medium before the addition of n-hexane. It seems to contain other substances with a similar retention time to hexanoic acid for the chosen HPLC measurement conditions. However, production of hexanoic acid stopped after two hours.

Apparently *E. coli* is able to complete the oxidation of n-hexane to hexanoic acid with endogenous enzymes, since the conversion of n-hexane did not stop at the step of hexanol oxidation, although genes encoding *alkJ* alcoholdehydrogenase and *alkH* aldehyde dehydrogenase for the final conversion step were missing

The production of adipic acid from n-hexane or hexanoic acid could not be achieved with this clone.

5.4.2. Production of carboxylic acids with *alkBTFG* and *alkJ*

In the previous experiment the introduction of *alkB*-monooxygenase resulted in conversion of n-hexane to hexanoic acid. Anyway, the dicarboxylic acid (adipic acid) could not be produced with this plasmid construct. With *alkJ* and *alkH* missing, the conversion of hexanoic acid may have stopped at the step of alcohol oxidation (6-hydroxyhexanoic acid).

AlkJ-alcoholdehydrogenase was added for the next experiment, to increase conversion of 6-hydroxyhexanoic acid to adipic acid. Therefore, *E. coli* containing two plasmids with *alkBTFG* and *alkJ* were cultivated and induced with two different concentrations of IPTG.

<u>Induced with 64 µmol/L of IPTG</u>

The cultivation experiments were accomplished in 25 mL of 200 mM Na-phosphate buffer with 64 µmol/L of IPTG, which refers to 2,3 µmol IPTG/g biomass dry weight with a biomass concentration for the main culture of approximately 1,3 g/L. An *E. coli* Top10 clone containing two plasmids (final construct KanR_NTS_pUC19_T7(lacI)_*alkBTFG* and F_pT7_*alkJ*_TT_G in a level-1 recipient plasmid) was incubated with 0,5 g/L hexanoic acid during the day and with 1,0 g/L overnight (see 4.7.2).

C3.1 in Na-phosphate buffer		
incubation time [h]	hexanoic acid [mg/L]	adipic acid [mg/L]
0	507,96	0,00
2	289,42	0,00
4	162,06	0,00
6	98,27	0,00
21	494,14	2,08

Table 5-7: Production of adipic acid with *alkBTFG* and *alkJ*

Cultivation experiments of *E. coli* TOP10 carrying the plasmid T7_*alkBTFG* for IPTG inducible expression of *alkB* monooxygenase, *alkT* rubredoxin reductase and two rubredoxins (*alkG* and *alkH),* as well as a second plasmid T7_*alkJ* expressing *alkJ* alcoholdehydrogenase resulted in continuous consumption of hexanoic acid. Small amounts of adipic acid were detected after 21 hours of cultivation. An IPTG concentration of 64 µmol/L was applied.

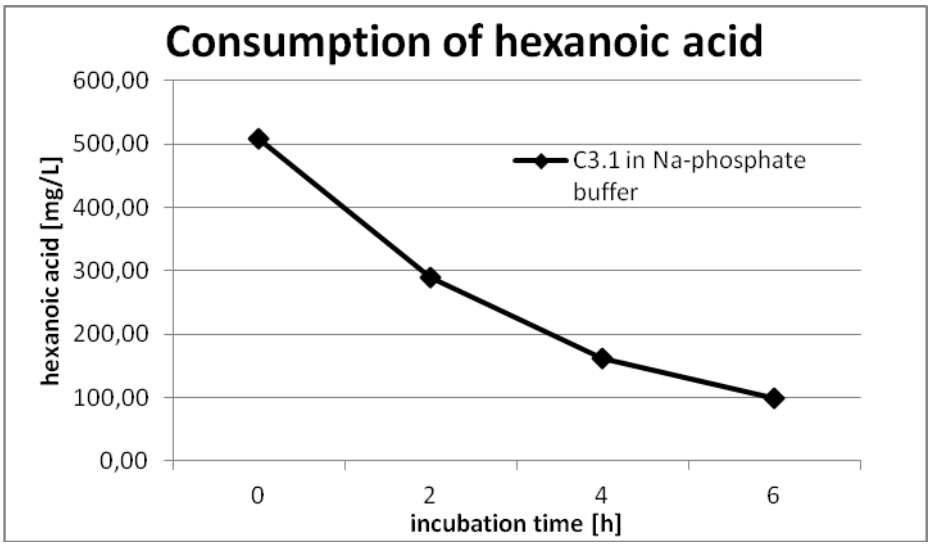

Figure 5-6: Consumption of hexanoic acid with *alkBTFG* and *alkJ* (incubation at 37°C shaking)

The data show a continuous consumption of hexanoic acid during the first six hours of incubation but no production of adipic acid. After 21 hours of incubation 500 mg/L of hexanoic acid were consumed, but only 2 mg/L of adipic acid produced.

With *alkJ* alcohol dehydrogenase added to the set of *alk*-enzymes conversion of hexanoic acid to adipic acid becomes possible.

Induced tenfold with 640 µmol/L of IPTG

To increase the outcome of adipic acid the *alk*-operon was induced with a 10-times amount of IPTG. This resulted in a slower growth rate of bacterial cultures, but the overall conversion of hexanoic acid was better.

The cultivation experiments were accomplished in 12,5 mL of 200 mM Na-phosphate buffer with 640 µmol/L of IPTG, which refers to 23 µmol IPTG/g biomass dry weight with a biomass concentration for the main culture of approximately 0,65 g/L. An *E. coli* Top10 clone containing two plasmids (final construct KanR_NTS_pUC19_T7(lacI)_*alkBTFG* and F_pT7_*alkJ*_TT_G a level-1 recipient plasmid) was incubated with 0,5 g/L hexanoic acid during the day and with 1,0 g/L overnight (see 4.7.2).

C5.1 in Na-phosphate buffer		
incubation time [h]	hexanoic acid [mg/L]	adipic acid [mg/L]
0	488,17	0,00
3	394,95	0,00
19	900,30	10,49
C5.2 in Na-phosphate buffer		
incubation time [h]	hexanoic acid [mg/L]	adipic acid [mg/L]
0	469,81	0,00
3	363,66	0,00
19	835,59	7,99

Table 5-8: Production of adipic acid with *alkBTFG* and *alkJ*

Cultivation experiments of *E. coli* TOP10 carrying the plasmid T7_*alkBTFG* for IPTG inducible expression of *alkB* monooxygenase, *alkT* rubredoxin reductase and two rubredoxins (*alkG* and *alkH),* as well as a second plasmid T7_*alkJ* expressing *alkJ* alcoholdehydrogenase resulted in continuous consumption of hexanoic acid. Up to 10 mg/L of adipic acid were detected after 19 hours of cultivation. An IPTG concentration of 640 µmol/L was used.

The table above shows approximately 100 mg/L hexanoic acid is consumed during the first three hours of incubation, which is significantly less than before. Overnight no further hexanoic acid has been consumed in C5.1 and C5.2. After 19 hours 8-10 mg/L of adipic acid has been produced, this is significantly higher compared to the previous experiment.

LB and M9 media brought no reproducible results for this method of cultivation.

The production of adipic acid from n-hexane as a substrate could not be achieved with this clone.

5.5. *AlkH* aldehyde-dehydrogenase – A metabolic burden for *E. coli*

Despite the satisfying outcome for Golden Gate assemblies with *Bbs*I (see 5.1.1) the construction of *alkH* transcripition units brought deviant results. Several attempts were necessary to build a level-1 module with a weak constitutive promoter, as well as an IPTG-inducible expression cassette for *alkH* with a T7 promoter. The number of colonies after transformation was significantly lower compared to other *alk* genes and the colonies were much smaller. The construction of a medium strength constitutive expression cassette was not successful.

After five attempts to build a level-2 plasmid for constitutive expression of the entire alk operon (*alkBTFGHJ*) the experiment brought no reproducible results. For IPTG-inducible expression two clones seemed to have all six genes, but cultivation experiments showed no conversion of n-hexane or hexanoic acid.

As a consequence, the T7_ *alkH*-expression cassette was re-sequenced and the results showed the insertion of an IS1-transposase of *E. coli* after 27 base-pairs in the *alkH* coding sequence. After revisiting the plasmid map for *alkH* one characteristic IS1 repeat was found in the coding sequence of this gene (Fig. 5-6: 9 base-pairs of GTTAAACTC). Alignment of the sequenced *alkH* expression cassette with IS1 transposase derived from the National Centre of Biotechology Information (NCBI - http://www.ncbi.nlm.nih.gov/) supported our assumption for the insertion of an IS1 transposase (Fig. 5-8).

The level-0 module containing the coding sequence for *alkH* aldehyde-dehydrogenase has also been re-sequenced and showed no IS1 insertion. As a consequence, the transpositional event must have occurred after the transformation of the level-1 Golden Gate cloning product.

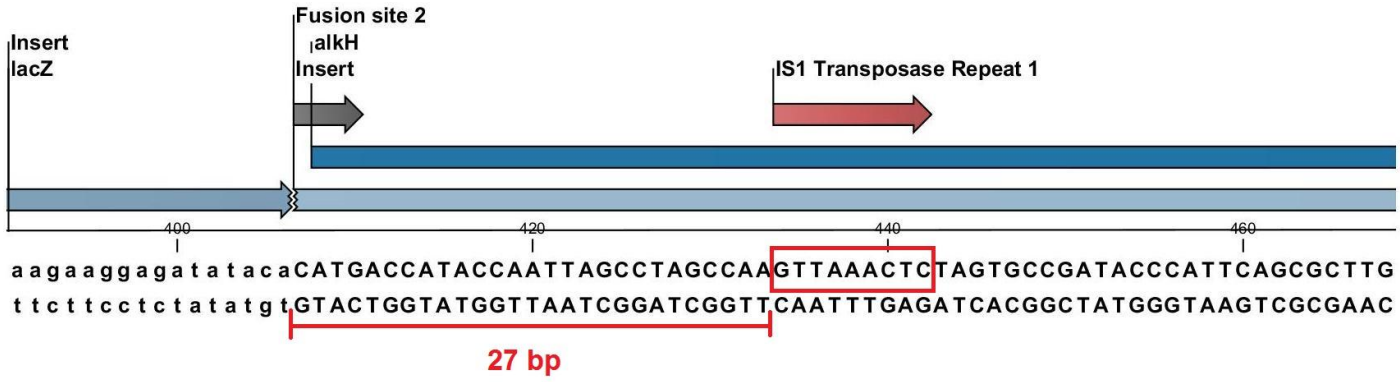

Figure 5-7: IS1 Transposase repeat sequence in *alkH* gene

In conclusion, weak constitutive expression of *alkH* aldehyde dehydrogenase seems to be a metabolic burden for *E. coli*, and even non-induced basal expression of the *lac-operon* has an impact on cell viability. There are reports in scientific literature that transposons are used by *E. coli* to knock out genes causing metabolic stress (Escoubas et al., 1994) and in the past a*lkB* monooxygenase has been thought to cause stress to the cell since its insertion into the cell membrane of the host (Eggink et al., 1987). After these results showing the impact of *alkH* expression focus should also be laid on *alkH* regarding a tighter control of expression for this protein.

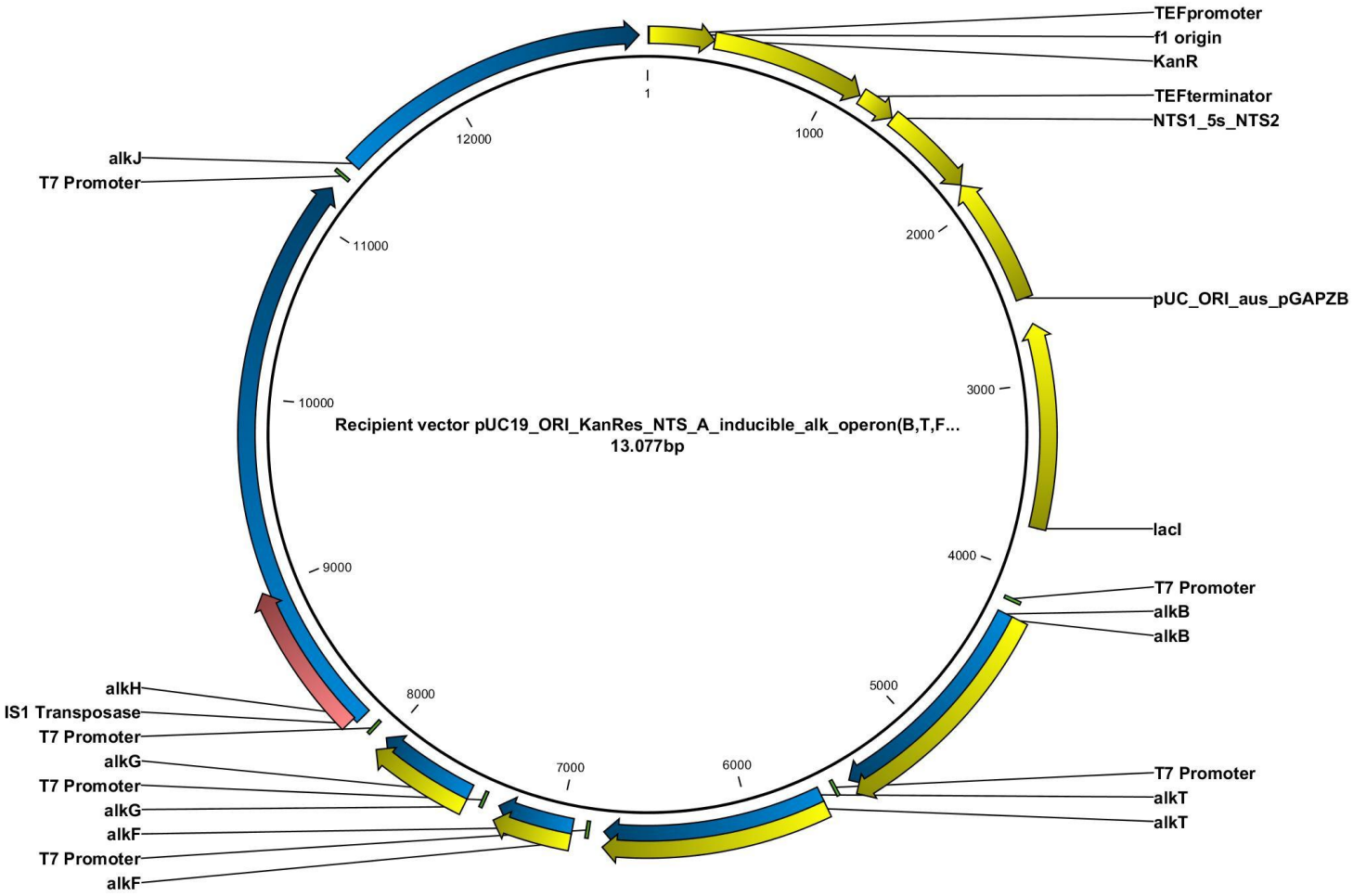

Figure 5-8: T7_*alkBTFGHJ* construct with IS1 transposase in *alkH*

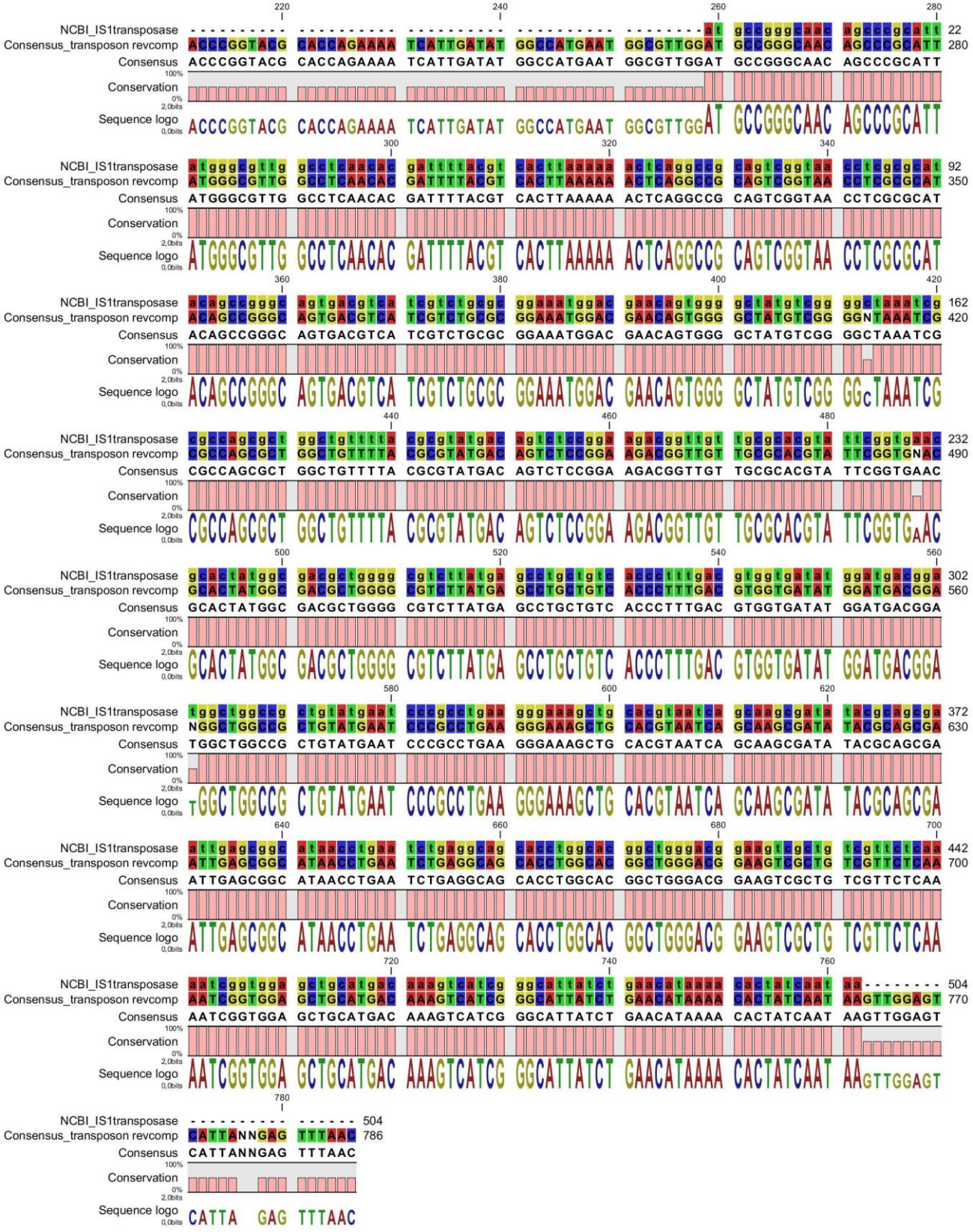

Figure 5-9: Positive alignment of IS1 transposase sequences from NCBI and sequenced Golden Gate assembly product T7_*alkH*

6. Discussion

Within this work, a modular cloning system was successfully established in our lab based on the principles of Golden Gate cloning. It is dependent on the properties of type IIS restriction enzymes *Bbs*I and *Bsp*MI to cut outside their enzyme recognition sequence and to create freely selectable nucleotide overhangs (Engler et al., 2008). Up to four inserts were successfully cloned in a “one pot-one step” reaction with *Bbs*I as restriction enzyme and T4 DNA ligase into a recipient vector derived from pSTBlue-1. The procedure showed high reproducibility and cloning efficiencies between 80% and 100%. A protocol with operating instructions was created (see appendix) and the method is already used by other operators in the lab. However, the cleavage efficiency of the second type IIS restriction enzyme (*Bsp*MI) did not meet the high expectations after establishing *Bbs*I. Several commercially available *Bsp*MI enzymes from different companies were tested and also isoschizomers like *Bfu*AI. All these enzymes were not effective. As a consequence, *Bsp*MI will be replaced by another type IIS restriction enzyme *Bsa*I to further optimize the described variant of Golden Gate cloning.

Expression cassettes with green fluorescent protein (GFP) and different types of promoters were built to observe their expression rates. Results showed that the insertion direction of the insert into pSTBlue-1 derived vectors has an influence on expression patterns. Higher expression rates for inserts integrated in forward direction were observed, which concurrently resulted in lower growth rates. One explanation would be a read-through from another promoter upstream of the weak constitutive promoter BBa_J23112. As a consequence of these observations a precautionary transcription termination sequence will be added outside both ends of the Golden Gate fusion sites.

Moreover, no reproducible results were obtained for the expression of the second reporter protein DsRed. Baird et al. (2000) reported that DsRed requires a maturation time of several days at room temperature to acquire its full potential. The protein shows a green fluorescence during the initial 7 hours only then to gradually shift towards its characteristic red colour. Therefore, it is not suitable for the rapid screening of short-term protein expression and another marker protein might be more useful.

After determination of the optimal working conditions, Golden Gate cloning was used for the reconstruction of the *alk*-operon to build expression cassettes for each gene of the operon. A weak and a medium strength constitutive promoter were applied, as well as an IPTG inducible T7 promoter. After several attempts one functional final plasmid, containing the first

four genes of the operon (*alkBTFG)* was obtained (see figure 5-4), expressing *alkB* monooxygenase as key enzyme, supported by *alkT* rubredoxin reductase and two coding sequences for rubredoxins (*alkG* and *alkH).*

Cultivations of *Escherichia coli* containing the IPTG inducible plasmid with *alkBTFG* genes in different media with n-hexane provided evidence for the production of hexanoic acid and the functionality of the remodelled *alk*-operon. Although the genes for aldehyde dehydrogenase (*alkH*) and alcohol dehydrogenase (*alkJ*) were missing, a full conversion of n-hexane to hexanoic acid could be achieved. We assume that the final conversion steps were performed by endogenous enzymes of the host. The production of hexanoic acid stopped after two hours at approximately 30 mg/L, although higher product titres were expected. Since β-oxidation is not induced in *E. coli* under aerobic conditions by medium chain fatty acids, metabolization of hexanoic acid is deactivated (Favre-Bulle and Witholt, 1992; Rothen et al., 1998). Evaporation of most of the n-hexan during cultivation as well as a limited uptake-rate of n-hexane by *E. coli* could be explanations for the observed outcome. The ω-oxidation of adipic acid was not practicable with this plasmid construct.

As a consequence, cultivation experiments with clones containing two different plasmids (*alkBTFG* + *alkJ*, both with IPTG inducible T7 promoters) were performed, adding *alkJ* alcohol-dehydrogenase to the set of expressed *alk*-genes. The results showed a continuous consumption of hexanoic acid of up to 50% of the substrate, but only very small amounts of adipic acid (2 mg/L) were detected in the culture media. Again, metabolization of hexanoic acid through β-oxidation is deactivated under the performed cultivation conditions. A potential integration of hexanoic acid into the cell membrane could be an explanation.

With stronger expression of target genes the amounts of adipic acid increased to 10 mg/L, but the bacterial cultures had a significantly lower growth rate and consumed less hexanoic acid than before. This might be a result from enhanced metabolic stress due to the higher expression rate of *alk*-operon genes. Despite the fact, that the gene for *alkH* aldehyde dehydrogenase was completely missing in these experiments we successfully achieved the production of adipic acid from hexanoic acid as a substrate.

Difficulties assembling a functional *alkH* expression cassette and the insertion of an IS1 transposase into a correctly assembled *alkH* transcription unit led to the assumption that *E. coli* might have used this transpositional event to knock out the *alkH* gene. In 1984, McClintock hypothesised that stress conditions might induce the transposition of mobile elements and DNA rearrangement and Escoubas et al. (1994) assumed a similar effect for IS1 transposases in *E. coli*. The aldehyde dehydrogenase encoded by *alkH* seems to have an effect on the growth rate of the host organisms. Colonies expressing *alkH* were much

smaller on agar plates compared to clones containing other plasmid constructs and the disruption of the coding sequence would probably generate a growth advantage. It has been demonstrated that high expression rates of *alkB* have negative effects on the cell growth (Nieboer et al., 1993), so the first intention was to build an *alkB* expression cassette with T7 promoter and the *lacI* repressor to eliminate basal expression of *alk* genes. The problems arising with *alkH* expression suggest a more stringent control of expression for the aldehyde dehydrogenase.

Golden Gate cloning proved to be a very useful method for the engineering of a metabolic pathway and also the partial functionality of the *alk*-operon after modular rearrangements was evidenced. To increase the efficiency of Golden Gate cloning *Bsp*MI will be changed to another type IIS restriction enzyme *Bsa*I. Showing the conversion of hexanoic acid to adipic acid only with parts of the *alk*-operon, construction of a vector containing the entire *alk*-operon and the optimization of expression ratios of each and every one of the *alk* genes will be the next steps towards the introduction of a sustainable way of adipic acid production.

7. Bibliography

Alini, S., Basile, F., Blasioli, S., Rinaldi, C., Vaccari, A., 2007. Development of new catalysts for N2O-decomposition from adipic acid plant. Appl. Catal. B Environ. 70, 323–329. doi:10.1016/j.apcatb.2005.12.031

Baird, G.S., Zacharias, D.A., Tsien, R.Y., 2000. Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. Proc. Natl. Acad. Sci. U. S. A. 97, 11984–11989. doi:10.1073/pnas.97.22.11984

Berg, J.M., Tymoczko, J.L., Stryer, L., 2002. Biochemistry. 5th edition. New York: W H Freeman; 2002. Section 9.3, Restriction Enzymes: Performing Highly Specific DNA-Cleavage Reactions.

Brzostowicz, P.C., Walters, D.M., Jackson, R.E., Halsey, K.H., Ni, H., Rouviere, P.E., 2005. Proposed involvement of a soluble methane monooxygenase homologue in the cyclohexane-dependent growth of a new Brachymonas species. Environ. Microbiol. 7, 179–190. doi:10.1111/j.1462-2920.2004.00681.x

Daraban, I.D., 2012. Microbial production of adipic acid (PhD Thesis). Universität für Bodenkultur, Wien.

Eggink, G., van Lelyveld, P.H., Arnberg, A., Arfman, A., Witteveen, C., Witholt, B., 1987. Structure of the Pseudomonas putida alkBAC operon. Identification of transcription and translation products. J. Biol. Chem. 262, 6400–6406.

Engler, C., Gruetzner, R., Kandzia, R., Marillonnet, S., 2009. Golden Gate Shuffling: A One-Pot DNA Shuffling Method Based on Type IIs Restriction Enzymes. PLoS ONE 4, e5553. doi:10.1371/journal.pone.0005553

Engler, C., Kandzia, R., Marillonnet, S., 2008. A One Pot, One Step, Precision Cloning Method with High Throughput Capability. PLoS ONE 3, e3647. doi:10.1371/journal.pone.0003647

Engler, C., Marillonnet, S., 2011. Generation of families of construct variants using Golden Gate Shuffling. Methods Mol. Biol. Clifton NJ 729, 167–181. doi:10.1007/978-1-61779-065-2_11

Escoubas, J.M., Lane, D., Chandler, M., 1994. Is the IS1 transposase, InsAB', the only IS1-encoded protein required for efficient transposition? J Bacteriol 176, 5864–5867.

Favre-Bulle, O., Witholt, B., 1992. Biooxidation of n-octane by a recombinant Escherichia coli in a two-liquid-phase system: Effect of medium components on cell growth and alkane oxidation activity. Enzyme Microb. Technol. 14, 931–937. doi:10.1016/0141-0229(92)90058-V

Hatti-Kaul, R., Törnvall, U., Gustafsson, L., Börjesson, P., 2007. Industrial biotechnology for the production of bio-based chemicals – a cradle-to-grave perspective. Trends Biotechnol. 25, 119–124. doi:10.1016/j.tibtech.2007.01.001

Keeling, R.F., Walker, S.J., Piper, S.C., Bollenbacher, A.F., 2014. Scripps CO2 Program.

Kunz, D.A., Weimer, P.J., 1983. Bacterial Formation and Metabolism of 6-Hydroxyhexanoate: Evidence of a Potential Role for w-Oxidation. J Bacteriol 156, :567.

Lee, J.W., Kim, H.U., Choi, S., Yi, J., Lee, S.Y., 2011. Microbial production of building block chemicals and polymers. Curr. Opin. Biotechnol. 22, 758–767. doi:10.1016/j.copbio.2011.02.011

Lee, P.Y., Costumbrado, J., Hsu, C.-Y., Kim, Y.H., 2012. Agarose Gel Electrophoresis for the Separation of DNA Fragments. J. Vis. Exp. doi:10.3791/3923

McClintock, B., 1984. The significance of responses of the genome to challenge. Science 226, 792–801. doi:10.1126/science.15739260

Metzger, J.O., Bornscheuer, U., 2006. Lipids as renewable resources: current state of chemical and biotechnological conversion and diversification. Appl. Microbiol. Biotechnol. 71, 13–22. doi:10.1007/s00253-006-0335-4

Mullis, K.B., Faloona, F.A., 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol. 155, 335–350.

Nieboer, M., Kingma, J., Witholt, B., 1993. The alkane oxidation system of Pseudomonas oleovorans: induction of the alk genes in Escherichia coli W3110(pGEc47) affects membrane biogenesis and results in overexpression of alkane hydroxylase in a distinct cytoplasmic membrane subtraction. Mol. Microbiol. 8, 1039–1051. doi:10.1111/j.1365-2958.1993.tb01649.x

Niu, W., Draths, K.M., Frost, J.W., 2002. Benzene-Free Synthesis of Adipic Acid. Biotechnol. Prog. 18, 201–211. doi:10.1021/bp010179x

Picataggio, S., Rohrer, T., Deanda, K., Lanning, D., Reynolds, R., Mielenz, J., Eirich, L.D., 1992. Metabolic Engineering of Candida Tropicalis for the Production of Long–Chain Dicarboxylic Acids. Bio/Technology 10, 894–898. doi:10.1038/nbt0892-894

Polen, T., Spelberg, M., Bott, M., 2013. Toward biotechnological production of adipic acid and precursors from biorenewables. J. Biotechnol. 167, 75–84. doi:10.1016/j.jbiotec.2012.07.008

Raja, R., Thomas, J.M., Xu, M., Harris, K.D.M., Greenhill-Hooper, M., Quill, K., 2006. Highly efficient one-step conversion of cyclohexane to adipic acid using single-site heterogeneous catalysts. Chem. Commun. 448. doi:10.1039/b513583d

Rothen, S.A., Sauer, M., Sonnleitner, B., Witholt, B., 1998. Biotransformation of octane byE. coli HB101[pGEc47] on defined medium: Octanoate production and product inhibition. Biotechnol. Bioeng. 58, 356–365. doi:10.1002/(SICI)1097-0290(19980520)58:4<356::AID-BIT2>3.0.CO;2-I

Sato, K., 1998. A “Green” Route to Adipic Acid: Direct Oxidation of Cyclohexenes with 30 Percent Hydrogen Peroxide. Science 281, 1646–1647. doi:10.1126/science.281.5383.1646

Sauer, M., Marx, H., Mattanovich, D., 2012. From rumen to industry. Microb. Cell Factories 11, 121. doi:10.1186/1475-2859-11-121

Sauer, M., Mattanovich, D., 2012. Construction of microbial cell factories for industrial bioprocesses. J. Chem. Technol. Biotechnol. 87, 445–450. doi:10.1002/jctb.3711

Sauer, M., Porro, D., Mattanovich, D., Branduardi, P., 2008. Microbial production of organic acids: expanding the markets. Trends Biotechnol. 26, 100–108. doi:10.1016/j.tibtech.2007.11.006

Soetaert, W., Vandamme, E., 2006. The impact of industrial biotechnology. Biotechnol. J. 1, 756–769. doi:10.1002/biot.200600066

Thiemens, M.H., Trogler, W.C., 1991. Nylon Production: An Unknown Source of Atmospheric Nitrous Oxide. Science 251, 932–934. doi:10.1126/science.251.4996.932

Van Beilen, J.B., Panke, S., Lucchini, S., Franchini, A.G., Röthlisberger, M., Witholt, B., 2001. Analysis of Pseudomonas putida alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the alk genes. Microbiology 147, 1621–1630.

Van Dien, S., 2013. From the first drop to the first truckload: commercialization of microbial processes for renewable chemicals. Curr. Opin. Biotechnol. 24, 1061–1068. doi:10.1016/j.copbio.2013.03.002

Weber, E., Engler, C., Gruetzner, R., Werner, S., Marillonnet, S., 2011. A Modular Cloning System for Standardized Assembly of Multigene Constructs. PLoS ONE 6, e16765. doi:10.1371/journal.pone.0016765

Willke, T., Vorlop, K.-D., 2004. Industrial bioconversion of renewable resources as an alternative to conventional chemistry. Appl. Microbiol. Biotechnol. 66, 131–142. doi:10.1007/s00253-004-1733-0

8. List of figures

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10. Appendix

10.1. Primer list

Description	Name	Sequence
weak const. promoter	FS1_BBa_J23112_FS2_FW	5'-GAAGACGCGGAGCTGATAGCTAGCTCAGTCCTAGGGATTATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGACCATGGCGTCTTC-3'
	FS1_BBa_J23112_FS2_BW	5'-GAAGACGCGGAGTTGACAGCTAGCTCAGTCCTAGGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGACCATGGCGTCTTC-3'
medium const. promoter	FS1_BBa_J23105_FS2_FW	5'GAAGACGCGGAGTTTACGGCTAGCTCAGTCCTAGGTACTATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGACCATGGCGTCTTC-3'
	FS1_BBa_J23105_FS2_BW	5-'GAAGACGCCATGGTCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACATAGTACCTAGGACTGAGCTAGCCGTAAACTCCGCGTCTTC-3'
strong const. promoter	FS1_BBa_J23119_FS2_FW	5'- AAGACGCGGAGTTGACAGCTAGCTCAGTCCTAGGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGACCATGGCGTCTTC-3'
	FS1_BBa_J23119_FS2_BW	5'-GAAGACGCCATGGTCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACATTATACCTAGGACTGAGCTAGCTGTCAACTCCGCGTCTTC-3'
T7 promoter	FS1_T7prom_FW	5'-GAAGACGCGGAGGGATCGAGATCTCGATCCCGCC-3'
	FS2_T7prom_BW	GAAGACGCCATGTGTATATCTCCTTCTTAAAG
T7 promoter + repressor	FS1_T7prom_lacI_FW	5'-GAAGACGCGGAGGTGCGGCGACGATAGTC-3'
	lacI_plus_T7prom_inner_1_FW	5'-GATATAACATGAGCTATCTTCGGTATC-3'
	lacI_plus_T7prom_inner_1_BW	5'-GATACCGAAGATAGCTCATGTTATATC-3'
	lacI_plus_T7prom_inner_2_FW	5'-CAGTCGCGTACCATCTTCATGGGAG-3'
	lacI_plus_T7prom_inner_2_BW	5'-CTCCCATGAAGATGGTACGCGACTG-3'
Terminator	FS3_BBa_B1001_FS4_FW	5'-GAAGACGCGCTTAAAAAAAAACCCCGCTTCGGCGGGGTTTTTTTTTCGCTGCGTCTTC-3'
	FS3_BBa_B1001_FS4_BW	5'-GAAGACGCAGCGAAAAAAAAACCCCGCCGAAGCGGGGTTTTTTTTTAAGCGCGTCTTC-3'
GFP	FS2_GFP_FW	5'-GAAGACGCCATGAGCAAAGGCGAAGAACTGTTTA-3'
	FS3_GFP_BW	5'-GAAGACGCAAGCTTATTTATACAGTTCATCCATGCCA-3'
DsRed	FS2_DsRed_FW	5'-GAAGACGCCATGGCCTCCTCCGAGGACGTC-3'
	FS3_DsRed_BW	5'-GAAGACGCAAGCCTACAGGAACAGGTGGTGGCG-3'
	DsRed_innerPrimer_FW	5'-GTAATGCAGAAGAAAACTATGGGCTG-3'
	DsRed_innerPrimer_BW	5'-CAGCCCATAGTTTTCTTCTGCATTAC-3'
pUC ORI	FS4_pUC_ORI_FW	5'-GATCGAATTCGAAGACGCCGCTGTGAGCAAAAGGCCAGCAAAAG-3'
	FS1_pUC_ORI_BW	5'-GATCGAATTCGAAGACGCCTCCACCCCGTAGAAAAGATCAAAGGATC-3'
p15 ORI	p15_ORI_FW	5'-TTGAGATCGTTTTGGTCTGCGCGTA-3'
	p15_ORI_BW	5'-TTTCCATAGGCTCCGCCCCCCT-3'
Zeocin resistance	FS2_ZeoR_FW	5'-GAAGACGCCATGCACACACCATAGCTTCAAAATGTTTC-3'
	FS3_ZeoR_BW	5'-GAAGACGCAAGCACATGTTGGTCTCCAGCTTGCAAATTAAAG-3'
	ZeoR_inner_FW	5'-GTTAATAAACGGTCATCAATTTCTCAAG-3'
	ZeoR_inner_BW	5'-CTTGAGAAATTGATGACCGTTTATTAAC-3'
Kanamycin / Hygromycin resistance	FS2_KanHyg_FW	5'-GAAGACGCCATGGACATGGAGGCCCAGAATACCC-3'
	FS3_KanHyg_BW	5'-GAAGACGCAAGCCAGTATAGCGACCAGCATTCACATAC-3'
	Kan_Hyg_inner_FW	5'-CGCTGCAGACGTGCGAGCAGGGAAA-3'
	Kan_Hyg_inner_BW	5'-TTTCCCTGCTCGCACGTCTGCAGCG-3'
	hph_inner_FW	5'-GTTGCAAGACCTCCCTGAAACCGAAC-3'
	hph_inner_BW	5'-GTTCGGTTTCAGGGAGGTCTTGCAAC-3'
NTS	FS3_NTS_FW	5'-GAAGACGCGCTTGTAAGCAATCTGGACAATTATGTAAG-3'

NTS	FS4_NTS_BW	5’-GAAGACGCAGCGGTCCACTAGGCCACAAGAGTGGATTC-3’
LacZ Cassettes - with fusion sites	FS1_FSA_LacZ_FW	5’-GAAGACGCGGAGGATCGCGCGCAGGTCGCAATTAATGTG AGTTAGCTCACTC-3’
	FS2_FSB_LacZ_BW	5’- GAAGACGCCATGCCGGGCGCGCAGGTCGGCCTATTGGT TAAAAAATGAGCTG -3’
	FS2_FSC_LacZ_BW	5’- GAAGACGCCATGAATTGCGCGCAGGTCGGCCTATTGGTT AAAAAATGAGCTG -3’
	FS2_FSD_LacZ_BW	5’- GAAGACGCCATGAGCTGCGCGCAGGTCGGCCTATTGGTT AAAAAATGAGCTG -3’
	FS2_FSE_LacZ_BW	5’- GAAGACGCCATGCTCCGCGCGCAGGTCGGCCTATTGGTT AAAAAATGAGCTG -3’
	FS2_FSF_LacZ_BW	5’- GAAGACGCCATGAAGCGCGCGCAGGTCGGCCTATTGGTT AAAAAATGAGCTG -3’
	FS2_FSG_LacZ_BW	5’- GAAGACGCCATGAGCGGCGCGCAGGTCGGCCTATTGGT TAAAAAATGAGCTG -3’
Linker - with fusion sites	A_1_4_B_BW	5’- ACCTGCGCGCCCGGAGCGGCGTCTTCGAAGACGCCTCC GATCGCGCGCAGGT -3’
	A_1_4_B_FW	5’- ACCTGCGCGCGATCGGAGGCGTCTTCGAAGACGCCGCT CCGGGCGCGCAGGT -3’
	B_1_4_C_BW	5’- ACCTGCGCGCAATTAGCGGCGTCTTCGAAGACGCCTCC CCGGGCGCGCAGGT -3’
	B_1_4_C_FW	5’- ACCTGCGCGCCCGGGGAGGCGTCTTCGAAGACGCCGCT AATTGCGCGCAGGT -3’
	C_1_4_D_BW	5’- ACCTGCGCGCAGCTAGCGGCGTCTTCGAAGACGCCTCCA ATTGCGCGCAGGT -3’
	C_1_4_D_FW	5’- ACCTGCGCGCAATTGGAGGCGTCTTCGAAGACGCCGCTA GCTGCGCGCAGGT -3’
	D_1_4_E_BW	5’- ACCTGCGCGCCTCCAGCGGCGTCTTCGAAGACGCCTCCA GCTGCGCGCAGGT -3’
	D_1_4_E_FW	5’- ACCTGCGCGCAGCTGGAGGCGTCTTCGAAGACGCCGCT GGAGGCGCGCAGGT -3’
	E_1_4_F_BW	5’- ACCTGCGCGCAAGCAGCGGCGTCTTCGAAGACGCCTCC CTCCGCGCGCAGGT -3’
	E_1_4_F_FW	5’- ACCTGCGCGCGGAGGGAGGCGTCTTCGAAGACGCCGCT GCTTGCGCGCAGGT -3’
	F_1_4_G_BW	5’- ACCTGCGCGCAGCGAGCGGCGTCTTCGAAGACGCCTCC AAGCGCGCGCAGGT -3’
	F_1_4_G_FW	5’- ACCTGCGCGCGCTTGGAGGCGTCTTCGAAGACGCCGCT CGCTGCGCGCAGGT -3’
alkB gene	FS2_alkB_FW	5’-GAAGACGCCATGCTTGAGAAACACAGAGTTCTGG-3’
	FS3_alkB_BW	5’-GAAGACGCAAGCCTACGATGCTACCGCAGAGG-3’
alkT gene	FS2_alkT_FW	5’-GAAGACGCCATGGCAATCGTTGTTGTTGGC-3’
	FS3_alkT_BW	5’-GAAGACGCAAGCCTAATCAGGTAATTTTATACTCCCTGCAAG-3’
	alkT_inner_FW	5’-GGGGTCTGAACTGTCTGGGGTCTGCTATTTACGCAGTATGGAGG ACGCCAAAAATTTACG-3’
	alkT_inner_BW	5’-GCAGACCCCAGACAGTTCAGACCCCTCGCACGTTAACCTACGTG CGCTAGCTGGTGTTGC-3’
alkF gene	FS2_alkF_FW	5’-GAAGACGCCATGTCAAGGTACCAGTGTCCAG-3’
	FS3_alkF_BW	5’-GAAGACGCAAGCTTATTTATTTCTCATCTTCTTCCTTGTGGAAG-3’
alkG gene	FS2_alkG_FW	5’-GAAGACGCCATGGCTAGCTATAAATGCCCGG-3’
	FS3_alkG_BW	5’-GAAGACGCAAGCTCACTTTTCCTCGTAGAGCACATAAT CTTCTTTCG-3’
alkH gene	FS2_alkH_FW	5’-GAAGACGCCATGACCATACCAATTAGCCTAGCC-3’
	FS3_alkH_BW	5’-GAAGACGCAAGCTCAGCTCAAATACTTAACTGTGATACC-3’

alkJ gene	FS2_alkJ_FW	5’-GAAGACGCCATGTACGACTATATAATCGTTGGTGCTG-3’
	FS3_alkJ_BW	5’-GAAGACGCAAGCTTACATGCAGACAGCTATCATGG-3’
	alkJ_inner_1_FW	5’-CCGAGGGCATGAGGACGATTACCAC-3’
	alkJ_inner_1_BW	5’-GTGGTAATCGTCCTCATGCCCTCGG-3’
	alkJ_inner_2_FW	5’-GCATCAAAAATTCTTTTTGAGGACCGTAAGGCGG-3’
	alkJ_inner_2_BW	5’-CCGCCTTACGGTCCTCAAAAAGAATTTTTGATGC-3’

10.2. Fusion sites for Golden Gate cloning

level-0 fusion sites		**Restriction enzyme**	**Recognition site / fusion site**
1	GGAG	*Bbs*I	GAA GAC GC / GGAG
2	CATG	*Bbs*I	GAA GAC GC / CATG
3	GCTT	*Bbs*I	GAA GAC GC / GCTT
4	CGCT	*Bbs*I	GAA GAC GC / CGCT
level-1 fusion sites		**Restriction enzyme**	**Recognition site / fusion site**
A	GATC	*Bsp*MI	ACC TGC GCGC / GATC
B	CCGG	*Bsp*MI	ACC TGC GCGC / CCGG
C	AATT	*Bsp*MI	ACC TGC GCGC / AATT
D	AGCT	*Bsp*MI	ACC TGC GCGC / AGCT
E	GGAG	*Bsp*MI	ACC TGC GCGC / GGAG
F	GCTT	*Bsp*MI	ACC TGC GCGC / GCTT
G	CGCT	*Bsp*MI	ACC TGC GCGC / CGCT
level-2 fusion sites		**Restriction enzyme**	**Recognition site / fusion site**
α	GGAG	*Bbs*I	GAA GAC GC / GGAG
β	CATG	*Bbs*I	GAA GAC GC / CATG
γ	GCTT	*Bbs*I	GAA GAC GC / GCTT
δ	CGCT	*Bbs*I	GAA GAC GC / CGCT