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„CO_2 fixation with genetically modified *E. coli* W“

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Alexander Gill

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Chapter 1

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Chapter 2

Abstract

Our modern world struggles with increasing CO_2 emissions. The best way to lower them is the usage of renewable energy-sources. Since autotrophic organisms are growing very slow and need more complex bio reactors, it was successfully tested if genetically engineered *E. coli* expressing Rubisco, are able to fix CO_2 and build up biomass with it. In this work it was tried to transfer this method into *E. coli* W a more stress resistant strain.

First growth experiments with *E. coli* W Δ *pfkA*, Δ *pfkB* and Δ *zwf* (WKO^3) were performed. These knockouts cut off the oxidative pentose phosphate pathway from the glycolysis and should create a metabolic shunt if Rubisco is expressed. The results of growth experiments showed that these knockouts are not sufficient to force *E. coli* W into Rubisco dependent growth. So it was decided to add an additional knockout in *gapA*.

The initial plan of transforming a plasmid encoding Rubisco, Phosphoribulokinase and Carbonic Anhydrase into *E. coli* WKO^3 did not work because the plasmid was not stable in this strain. One nearly successful attempt showed a point mutation in the promotor sequence of *prk*. This leads to the assumption that the toxicity of ribulose 1,5 bisphosphate, the product of Prk causes these problems. In order to overcome this problem it was tried to integrate an anhydrotetrycycline-inducible promoter (tetP) for regulating the expression of *prk*.

In the end the knockout of *gapA* could not be realized within the time of this work, as well as the integration of tetP as promoter for *prk*.

Contents

Chapter 3

Introduction

During the last few decades it became more and more clear that the climate is changing and human-caused green-house gas emissions play a big role for that. Especially CO_2 emissions cause beside global warming also ocean acidification. Now we have the highest atmospheric CO_2 concentration of the last 800.000 years [1].

Since CO_2-emissions strongly correlate with economic power and prosperity [2] it is not very advisable only to reduce CO_2 emitting processes within one country. To overcome this problem renewable energy sources need to be used like wind energy or solar energy. One attempt for lowering CO_2 concentrations is to fix CO_2 with biotechnological processes by using different energy sources and re-use it for example as biofuel, so a cycle is created and no additional CO_2 is released into the atmosphere [3]. Autotrophic organisms like plants, algae and microalgae are capable of fixing CO_2 and build up biomass from it. This is the central gateway of supplying the organic world with carbon. Microalgae are capable of fixing CO_2 at the highest rate of these named organisms, so they are of big interest in green biotechnology. But to use these photoautotrophic organisms in big-scale very complex bio reactors are needed and no optimal solution has been developed up to now [3]. Therefore Antonovsky et al addressed the question if heterotrophic organisms like *Escherichia coli* are able to switch to autotrophic growth, so the CO_2 fixing process could be performed in a standard bio reactor. This attempt was successful by expressing some genes of the Calvin-Benson-Bassham-Cycle in an *E. coli* knockout strain [4]. The aim of this work is to reproduce his work in a different, more stress-resistant strain: *E. coli* W. The key for that is the expression of Phosphoribulokinase (Prk) and Ribulose-1,5-bisphosphate-carboxylase/-oxygenase (Rubisco) from the Calvin-Benson-Bassham-Cycle (CBB-Cycle) and Carbonic Anhydrase (CA) in *E. coli* cells in which the pentose-phosphate-pathway is cut off from glycolysis.

3.1 Calvin-Benson-Bassham-Cycle

The Calvin-Benson-Bassham-Cycle (CBB-cycle) occurs in photosynthetic organisms like green plants and cyanobacteria. It represents the second step of photosynthesis, where the energy collected from light is used to reduce carbon and build up biomass from it. It is the most important metabolic pathway to integrate

inorganic carbon from CO_2 into organic sugars. Since the Pentosephosphate-Pathway, which occurs in nearly all living organisms, is strongly correlated to the CBB-Cycle [5] it is used as attaching point for the new metabolic pathway in this project.

Ribulose-1,5-bisphosphate-carboxylase/-oxygenase (Rubisco) is the central enzyme of the CBB-cycle, it catalyzes the carboxylation of Ribulose 1,5 Bisphosphate (Ribulose-BP) with CO_2 yielding 3-Phosphoglycerate (figure 3.1). Rubisco is highly conserved and the vast majority of organic carbon on our planet was once fixed by Rubisco [5]. An estimation by Ellis et al. shows that it is probably the most abundant protein on our planet [6]. It occurs in photoautotrophic organisms like higher plants, algae, cyanobacteria and many autotrophic bacteria and represents up to 50 % of the biomass from these organisms [7]. Despite of its important role for the living world Rubisco is very inefficient, it not only carboxylates Ribulose-BP, but is also capable of oxigenizing it by producing phosphoglucolate (figure 3.1), which has to be recycled by the energy consuming process of photorespiration [8].

There are two major types of functional Rubisco-complexes, type II is a simple, dimeric complex which occurs in autotrophic bacteria, type I is a hexadecameric complex which occurs in Cyanobacteria and in higher plants. The hexadecameric complex consists out of eight large sub-units (*rbcL*) and eight small sub-units (*rbcS*). The large sub-units are homologues to the two sub-units of typ II Rubisco [9]. For this project Rubisco from *Rhodospirillum rubrum* as dimeric example and *Synechococcus elongatus* as hexadecameric example were used in order to test which of them fits best for CO_2 fixation in *E. coli*. Since even the sub-units of type II Rubisco are very complex in structure chaperones are needed to fold them in a correct way to yield functional enzymes [10].

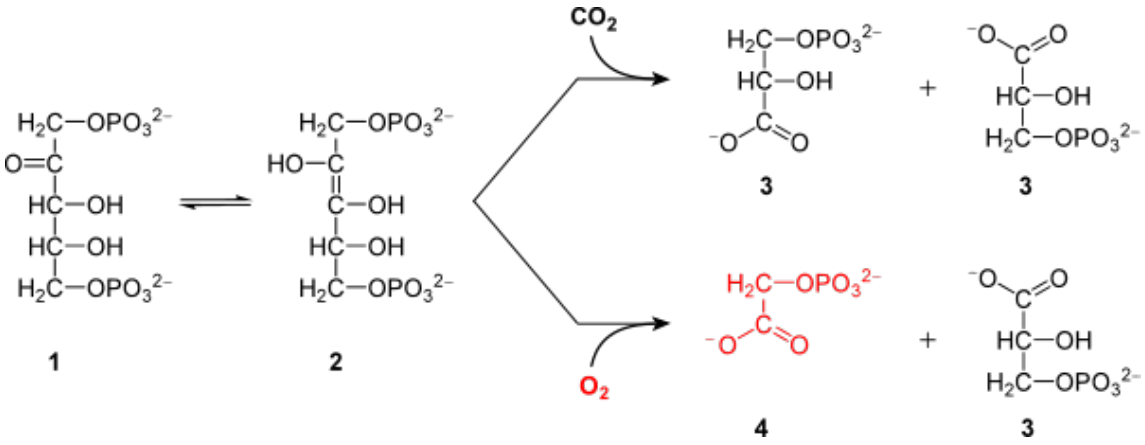

Figure 3.1: The carboxylation of Ribulose 1,5 bisphosphate (1,2) to two 3-Phosphoglycerate (3) and the oxygenisation of Ribulose 1,5 bisphosphate (1,2) to phosphoglucolate and 3-Phosphoglyzerate (3). Both reactions are catalysed by Ribulose-1,5-bisphosphate-carboxylase/-oxygenase. [11]

Also for this project Rubisco represents the central enzyme for CO_2 fixation. The product, 3-Phosphoglycerate can be used further for building up biomass. To supply Rubisco with Ribulose 1,5 bisphosphate an additional enzyme called Phosphoribulokinase (Prk) is needed. Prk converts Ribulose 5 phosphate which occurs in the native metabolism of *E. coli* W into Ribulose 1,5 bisphosphate which

is used by Rubisco as substrate [5]. Since Rubisco is a very slow working enzyme a high availability of CO_2 is essential for this pathway to work efficient. So a carbonic anhydrase is added to this construct, it solubilises CO_2 and increases the availability for Rubisco. In figure 6.1 the constructs used for the insertion of CBB-genes are shown.

3.2 *Synechococcus elongatus*

Synechococcus belongs to the Phylum of Cyanobacteria and is a small, unicellular, photoautotroph living organism. It can be found in freshwater as well as marine environments. Marine *Synechococcus* strains are responsible for about 25% of the primary production in the world [12]. *Synechococcus elongatus* was first found in 1925 by Geitler [13] it occurs in freshwater environments like rain-filled pools at mesophile or moderate temperature. Later Frenkl et. al showed its ability for photosynthesis in 1950 [14]. Caused by its simplicity and the fact that the genome is already sequenced [15] it is used as model organism for understanding the circadian clock [16] and the detailed function of photosynthesis [17]. *Synechococcus elongatus* possesses a type I Rubisco, that means it is built out of eight large and eight small subunits. But compared to Rubisco of higher plants it possesses also similarities to type II Rubisco like in *Rhodospirillum rubrum* referring to specificity and turnover. It has about half the specificity for carboxylation of Ribulose 1,5 bisphosphate compared to Rubisco from tobacco or spinach but it has a higher turnover. The structure was resolved in 1993 by Newman et.al. [18]. Rubisco from *Synechococcus elongatus* was the first type I Rubisco which could be functionally expressed in *E. coli* using the native Chaperon system Hsp70/DnaK [9][10]. In some organisms a Rubisco Chaberonin (RbcX) is needed to gain functional Rubisco. In *Synechococcus elongatus* it is not within the locus of the other Rubisco genes (*rbcL* and *rbcS*) and not essential to form a functional Rubisco [19]. It is involved in the formation of the RbcL octamer and part of the resulting Carboxysome [20]. RbcX deficient cells does not contain less active Rubisco, but more inactive subunits [21].

3.3 *Rhodospirillum rubrum*

The Proteobacteria *Rhodospirillum rubrum*, previously known as *Spirillum rubrum* was first described by Esmarch in 1887 [22]. It is facultative anaerobe, during aerobic growth the photosynthesis is genetically suppressed [23]. Its growth optimum is at mesophilic temperatures between $22°$C and $35°$C [24] and occurs in lakes, streams and standing water [25]. As electron donor it uses sulfide, so the byproduct of photosynthesis is sulfur instead of oxygen, and it is also capable of fixing elemental nitrogen [23] and is able to use CO as energy source in dark anaerobic conditions [26].

Rhodospirillum rubrum is used as model organism for photosynthesis because of its simple photosynthetic apparatus [23] and for nitrogen fixation [27]. It is also able to produce Poly-β-hydroxyalkanoates which can be used to produce biodegradable plastic [28]. For autotrophic growth it uses a type II Rubisco,

that means it consists of two subunits which are homologue to the large subunits of type I Rubisco [7]. The autotrophic system of *Rhodospirillum rubrum* is only active under anaerobic conditions because it is about 7-14 times less specific for carboxylation instead of oxygenisation than type I Rubisco, but it is also twice as fast [29][30].

3.4 *Escherichia coli* W

E. coli W was first isolated in 1943 by Selman A. Waksman from graveyard soil [31]. It is one of only five as safe declared *E. coli* strains [32][33] and belongs to the mostly pathogenic phylogroup B1. Archer et al claimed it should be used as type strain of this group. Further the genome of *E. coli* W was sequenced in 2011. It possesses two plasmids and the largest chromosome of all non-pathogenic *E. coli* strains with 4,901 kbp [31]. *E. coli* W possesses many good properties for industrial biotechnology. It is able to grow on high NaCl and ethanol concentrations, on low pH-value and is highly stress tolerant compared to other non-pathogenic *E. coli*-strains [34][35]. Further its the only safe *E. coli* strain that is able to utilize sucrose as sole carbon-source [36], which is a good an cheap energy source for industrial biotechnology [37].

3.5 Hypothesis

A computersimulation, performed by Antonovsky et al showed that *E.coli* with knockouts in *pfkA*, *pfkB* and *zwf* should not be able to grow on minimal-medium with a pentose as sole carbon source, but by expressing Prk and Rubisco this ability should be restored [4]. In figure 3.2 can be seen how the native metabolism of *E. coli* W works together with the newly added genes in theory.

So the plan was to proof that *E.coli* Δ*pfkA*, Δ*pfkB* and Δ*zwf* (further called *E.coli* WKO^3) is not able to grow on minimal-medium supplemented with a pentose as sole carbon-source first. Followed by the transformation of the constructs CC-Selo and CC-Rhodo (figure 6.1) into the knockout-strain and proof that they restore their ability of growing under these restricted conditions. During the experiments we realized that *E. coli* WKO^3 is able to grow in these restricted conditions without Rubisco, so we decided to knock out *gapA* to ensure the growth of the final strain is dependent on Rubisco flux.

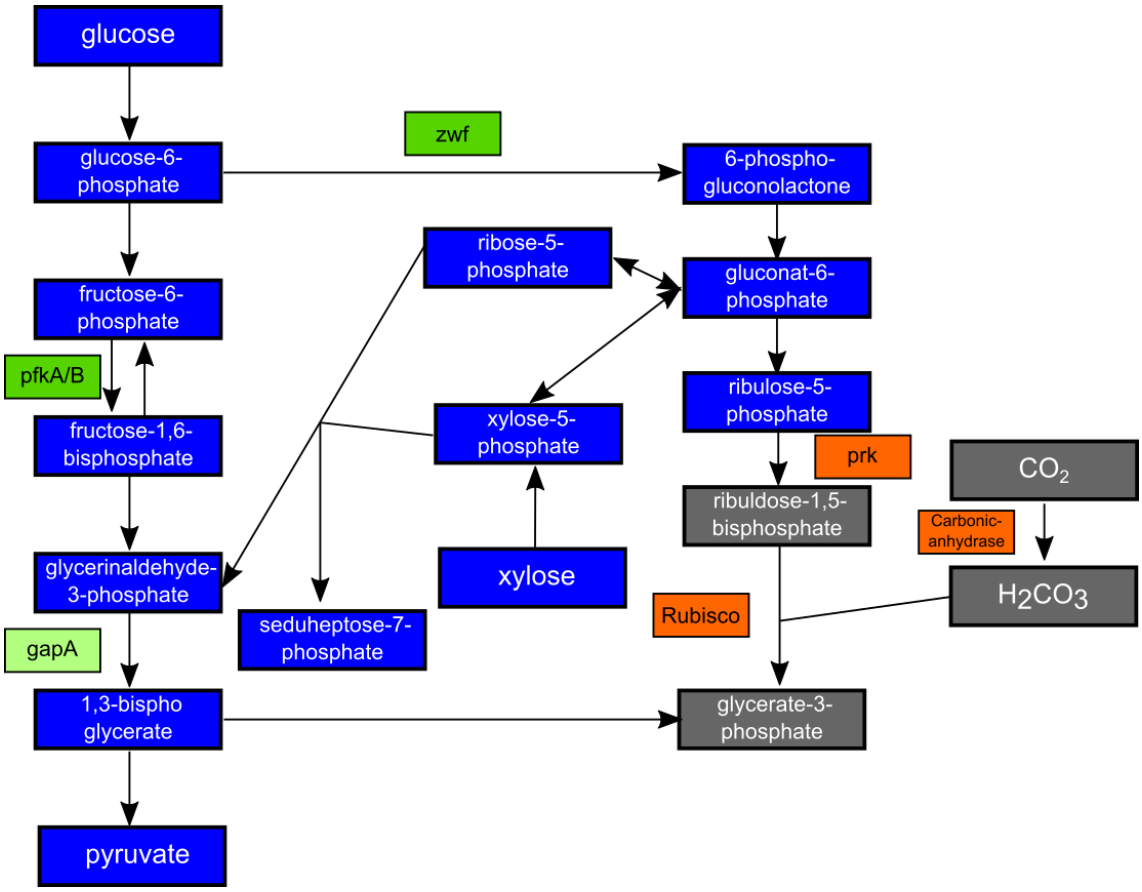

Figure 3.2: Overview of the engineered metabolic pathway for CO_2 fixation in *E. coli* W. In blue metabolites are indicated which are native to *E. coli* W. In green genes are indicated which were knocked out in *E. coli* W. Dark green indicates that they were knocked out before the work for this thesis was started, light green indicates that it was decided during the work to be knocked out additionally or alone. In grey additional metabolites are indicated which only occur because of the additional genes, indicated in orange.G

Chapter 4

Results and Discussion

4.1 Growth experiment

In order to proof the theory of Antonovsky et al that *E. coli* with a knockout in *pfkA, pfkB* and *zwf* is not able to grow on glucose, arabinose or xylose as sole carbon source [4] for *E. coli* W a previously prepared knockout strain (*E.coli* W Δ pfkA, Δ pfkB and Δ zwf, further called *E.coli* WKO^3) was compared in growth with wild-type *E. coli* W on minimal medium supplemented with these sugars. The results can be seen in figure 4.1. The experiment was performed twice because the first attempt did not show the expected results. As expected *E.coli* W wild-type did grow very well in the first 24 hours on all three sugars in both attempts, but the growth did slow down afterwards probably because of low sugar concentrations. *E. coli* WKO^3 did not grow significantly at first in both attempts but after a lag-phase it reached nearly the same OD600 in all cultures like the wild-type. The duration of the lag-phase in the first experiment can only be estimated but seems to conform with the 2 days observed in the second experiment. These results are not conclusive with the theoretical simulation [4], but proof that *E. coli* W is very stress resistant as claimed in literature [34] [35].

Since the results were not satisfying, the experiment was repeated with a lower OD600: DeLisa minimal-medium supplemented with 5g/l glucose, arabinose or xylose was inoculated directly by transferring one colony from an agarplate with a pipette-tip into the medium. The results of this experiment are shown in figure 4.2. At this starting OD600 of approximately $< 0,001$ *E.coli* W wild-type is able to grow significantly better than the E.coli WKO^3 on minimal-medium. Like in the first experiment the wild-type strain did grow very fast in the first 24 hours but slowed down afterwards probably caused by the low sugar concentration. The knockout strain *E. coli* WKO^3 did not grow on all three sugars. The culture of *E. coli* WKO^3 containing arabinose was contaminated by unidentified bacteria, which were observed in the microscope, so the OD600 did rise between third and seventh day of growth, but this was not investigated further.

At first this results were interpreted as success and the transformations of the constructs into competent *E. coli* WKO^3 were performed (explained in section 4.3 Transformation more in detail). With some false positive clones further growth experiments were performed (see figures A.1-8). The purpose of this experiments was to proof that *E. coli* WKO^3 is only able to grow on minimal medium supplemented with glucose, arabinose or xylose as sole carbon source

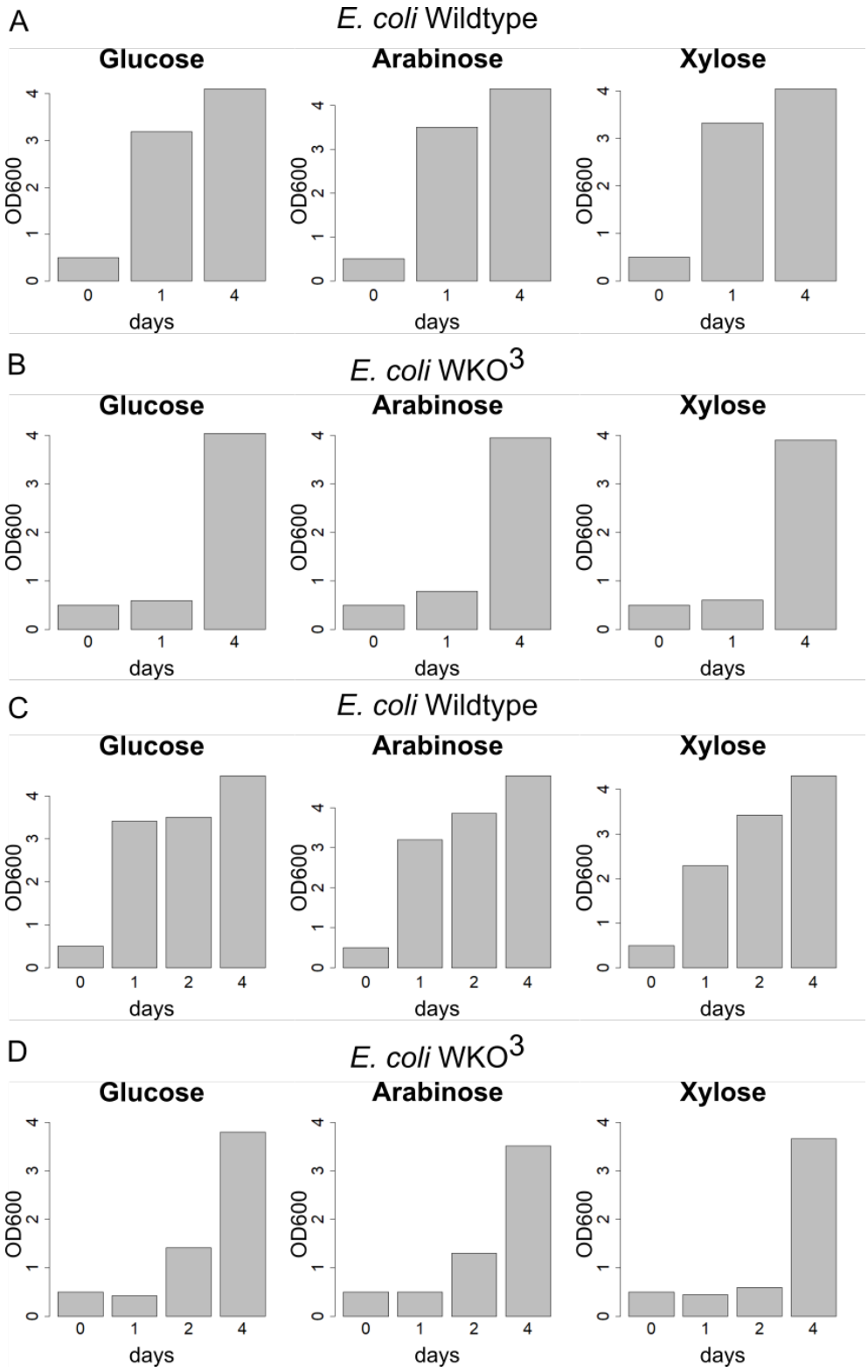

Figure 4.1: Growth of *E.coli* W in DeLisa medium supplemented with glucose, arabinose or xylose as sole carbon source with an initial OD600 of 0,5. The x-axis shows the time in days and the y-axis shows the OD600. (A) Wildtype *E.coli* W in experiment one. (B) *E.coli* WKO^3 in experiment one. (C) Wildtype *E. coli* W in experiment two. (D) *E. coli* WKO^3 in experiment two.

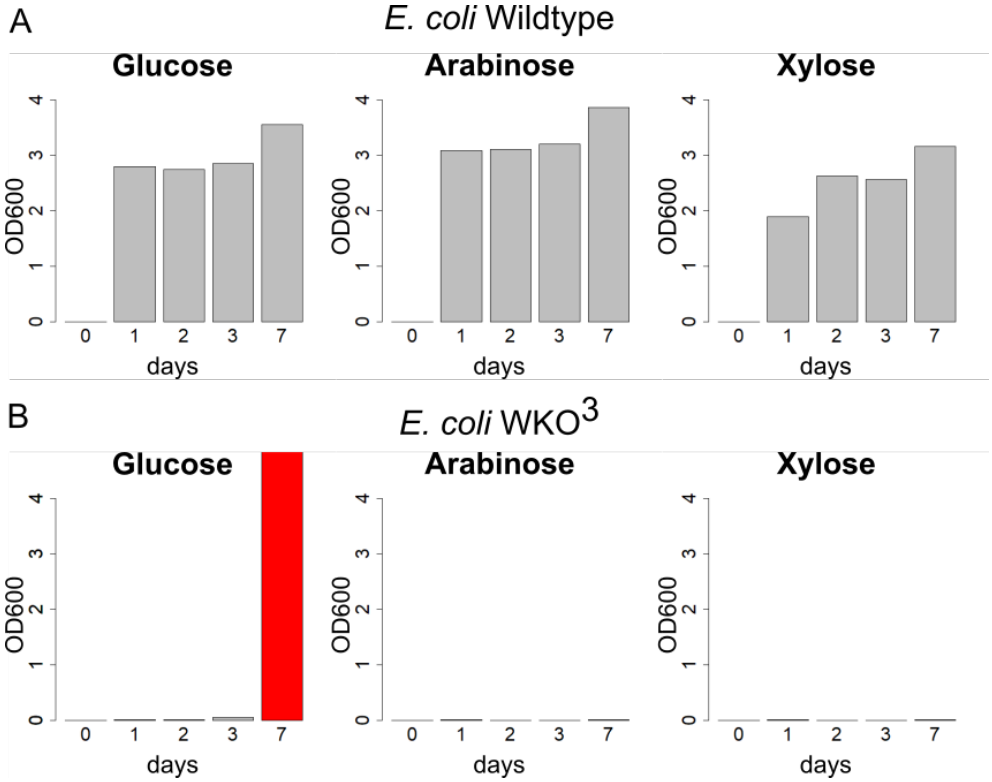

Figure 4.2: Growth of *E.coli* W in DeLisa medium with a single Carbonsource, inoculated with a single colony picked with a pipette-tip. (A) Wildtype *E.coli* W. (B) *E.coli* WKO^3. The red bar indicates a contamination detected under the microscope.

when Rubisco is able to fulfill its reaction. Since these plasmids failed to provide the required enzyme activity to fulfill a Rubisco dependent growth, this theory could not be verified. Former literature showed that *E. coli* with knockouts in *pfkA*, *pfkB* and *zwf* is not able to use glucose as sole carbon source [38]. This is conclusive with the computersimulation of Antonovsky et al [4], but does apparently not hold for *E. coli* W. The data in figure A.6 compared with other attempts using a lower initial OD600 show that as soon as the OD600 reaches 0,01 it is able to grow for at least several doublings. This is also conclusive with the results of the first growth experiments shown in figure 4.1. But as soon as the OD600 is at about 0,001 it did not necessarily show growth (like shown in figures A.3, A.4, A.5, A.7 and A.8). This could be explained by the stochiometry of the pentose-phosphate-pathway, because three pentose sugars are converted into two molecules fructose-6-phosphate and one molecule glyceraldehyde-3-phosphate [5][4]. Overall it should be impossible for *E. coli* to use pentoses as sole carbon source that way, but somehow *E. coli* W did find a way to grow under these stressful conditions, probably by adjusting the flux of some enzymes within this pathway.

Taken together the theory of forcing *E. coli* with knockouts in *pfkA*, *pfkB* and *zwf* into Rubisco dependent growth would probably work in other *E. coli* strains [4][38], but not in *E. coli* W.

4.2 *gapA* Knockout

In order to overcome the problem that *E. coli* WKO^3 is able to grow on minimal medium supplemented with glucose, arabinose or xylose we decided to knock out *gapA*. This was first proposed by Morell et al in 1992 [39] combined with the idea of expressing Prk and Rubisco in that knockout *E. coli* in order do evolve Rubisco towards a more efficient version. The idea of this approach is to totally separate the glycolysis into two parts which are then only connected over the metabolic shunt created by Prk and Rubisco expression.

The Knockout was performed with a no SCAR CRISPR/Cas9 System [40] and in three different strains: *E.coli* W, *E.coli* WKO^3 and *E.coli* WKO^4 (Δ*ldha*, Δ*adhe*, Δ*pta* and Δ*frdA*).

To perform a Knockout according to this protocol, 3 pieces of DNA are required:

4.2.1 pCas9-CR4

This plasmid contains the Cas9 nuclease to gain a double-strand-break. All three strains have been prepared previously with pCas9-CR4.

4.2.2 pKDsgRNA-*gapA*

pKD is a plasmid that consists of two parts: pKD part1 and pKD part2. At first both parts were amplified by PCR, assembled and transformed in *E.coli* TOP10. The plasmid was extracted, digested and applied on a 1% agarose gel to check it. The restriction digest was performed with BamHI and SalI, the expected fragment sizes are shown in table 6.10. Like shown in figure 4.3 A the plasmid showed the expected bands. Sequencing showed that also the sequence of the gRNA site was correct, so it was assembled in the right way. The pKD plasmid was then transformed into electro-competent *E.coli* W, *E.coli* WKO^3 and *E.coli* WKO^4, each containing the pCas9-CR4 plasmid. This transformation was checked by extracting the plasmids followed by a PCR using the PCR primer for pKD part2 amplification. The PCR products were then applied on a 1% agarose gel to check the right sizes. The expected length of the PCR products are 1652 bp for each which conforms with the bands on the gel shown in figure 4.3 B.

4.2.3 Homologue Arms Δ*gapA*

Homologue arms are required for the recombination of the target-site. Gibson Assembly was used to assemble the up- and downstream arm with a Golden-Gate Backbone 1. This plasmid was then transformed in *E.coli* TOP10, extracted, digested with BpiI and applied on a 1% agarose gel. It showed very weak bands but approximately at the right size (table 6.10). So a re-transformation of the extracted plasmid was performed, but without any success. In several other attempts of the same procedure only a band at about 1900 bp appeared, we assumed it represents the empty backbone. Following this assumption the Gibbson Assembly did not work correctly. Possible causes for this are a too low purity or concentration

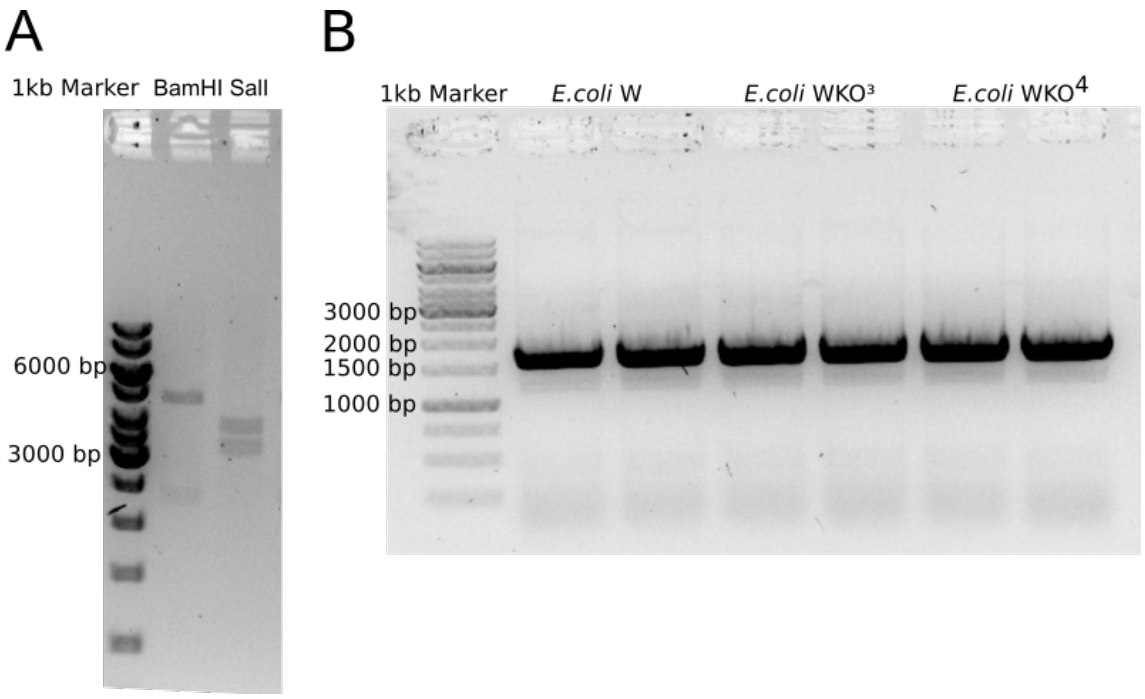

Figure 4.3: (A) 1% agarose gel of pKDsgRNA-*gapA* after isolation from *E.coli* TOP10 and restriction digest with BamHI and SalI. (B) 1% agarose gel with the PCR-product of the isolated plasmids from *E.coli* W, *E.coli* WKO^3 and *E.coli* WKO^4 using the primer for pKD part2 amplification.

of the fragments, the enzymes of the master mix are inactive or the NAD was degraded by light. In order to overcome these problems the master mix and the fragments were prepared freshly. Additionally a higher amount of DNA (0,25 pmol instead of 0,1 pmol) was used for the assembly. But also this approach did only yield false positive clones.

The next approach was to assemble the homologue arms with PCR. The two homologue arms which were used for Gibson Assembly were used as template. The forward primer used for amplification of the up-stream arm (gapA_HAFW) and the backward primer used for the amplification of the downstream arm (gapA_HABW), both without overhangs were used. The product was then applied on a 1% agarose gel and showed a band at about 1000 bp, as expected. Since during a gel purification a lot of DNA is lost the PCR product was used in three different ways for the knockout: the PCR was used directly (high concentration and low purity), purified on a 1% agarose gel followed by gel-extraction (low concentration and only DNA of interest) and only purified with the Gel-Extraction-Kit (moderate concentration and only DNA, but different species).

Since this method did not yield enough DNA to perform the knockout sufficient times until it works, a last approach was performed. The 1000 bp PCR product of the homologue arms was inserted by a Gibson Assembly into the backbone and transformed into *E. coli* TOP10. This Gibson Assembly did yield several positive clones and sequencing showed that it possesses the right sequence. This approach showed that the master mix does work, but as expected the efficiency of this method decreases with increasing number of fragments. According to the protocol (New England Biolabs) up to 5 fragments could be assembled in one

reaction, but the concentration of the fragments needs to be higher the more fragments are used and the longer these fragments are. In this case the recommended amount of DNA was used (0,1 and 0,25 pmol) but it did not yield clones containing the right plasmid in the first attempt. Maybe the assembly with three fragments would have worked if an even higher concentration of DNA was used.

4.2.4 Knockout

The knockout itself was performed after the protocoll of Reisch et al [40] with *E. coli* W, WKO^3 and WKO^4. About 500 ng of homologue arms were transformed for recombination. 25 colonies were picked from the selective agar-plates for each strain to screen for positive knockouts. A colony-PCR was performed and the products were applied on a 1% agarose gel which is shown in figure 4.4. For positive clones the product is 1000 bp long and for the wildtype gene it is 2150 bp. The kill efficiency was about 99 % but all tested clones were negative. The knockout was repeated once with freshly prepared homologue arms (again about 500 ng were used), but this did also not yield positive clones either (data not shown). And again the kill efficiency was about 99 %. This indicates that pCas9 and the gRNA did work properly and produced double-strand-breaks. Due to this it seems the problem is related to the recombination. The most probable problem is the concentration and purity of the homologue arms.

To overcome this a secound approach was performed with homologue arms in three different purities like described in the section 4.2.3 Homologue Arms Δ*gapA*. Therefore 1800 ng, 900 ng and 600 ng DNA were used of pure PCR-product, purified without gel-electrophoresis and purified with gel-electrophoresis respectively. This approach did also yield only negative clones (data not shown).

For further attempts the homologue arms should be amplified using the plasmid containing the homologue arms from *E. coli* TOP10 (mentioned in subsection 4.2.3 Homologue Arms Δ*gapA*) as template. Further a positive control, using a knockout that already worked should be performed parallel to check if the induction of λ-Red does work properly.

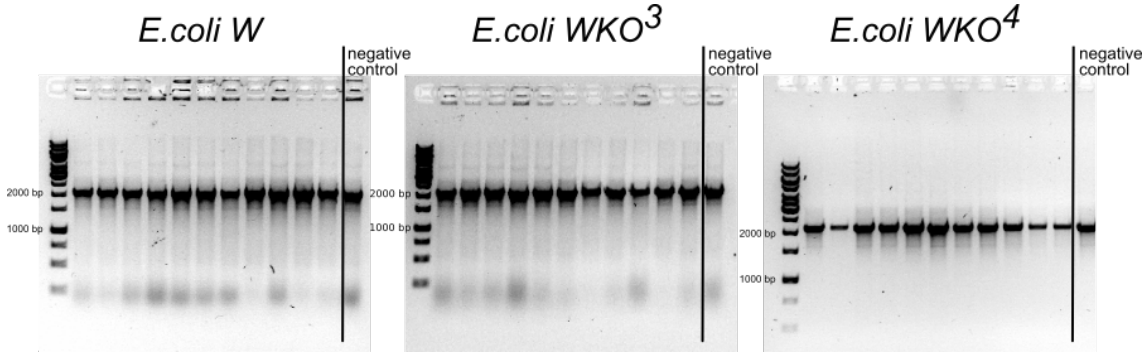

Figure 4.4: Agarose gel with PCR-products of the colony-PCR to check the knockout of *gapA* in *E. coli* W, WKO^3 and WKO^4. The marked band on the right side of each gel is the negative control. Not all checked clones are shown.

4.3 Transformation

4.3.1 Assembly of CC-Rhodo

To insert the required enzymes of the Calvin-Benson-Bassham-Cycle into *E. coli* WKO^3 two constructs were designed (shown in figure 6.1). Each of them contains a form of Rubisco, Carbonic-anhydrase and Phosphoribulokinase. CC-Selo additionally contains a chaperon. CC-Selo had been prepared previously. One Backbone 2 and the Backbone 3 Golden-Gate-Assembly of CC-Rhodo were performed during this work. *prk* was assembled with promotor 109p, terminator TT_B1001 and BB2-linker-CD in a standard Golden-Gate-Assembly. The assembly was then transformed into *E.coli* TOP10 and the plasmid was isolated and checked by restriction digest and sequencing. The plasmid of a clone with the right sequence was then used for the Backbone 3 assembly. BB3-linker-AD was assembled with BB2-*cbbM* (Rubisco), BB2-CA (Carbonic-anhydrase) and BB2-*prk* and transformed into electro-competent *E. coli* TOP10 and chemical-competent *E. coli* BL21. Only the transformation in *E. coli* BL21 did gain some clones, so the plasmids were isolated and checked by restriction digest and sequencing which proofed that the assembly yielded the correct plasmid.

4.3.2 Transformation of CC-Selo

For the first attempt of transforming CC-Selo in *E. coli* WKO^3 the standard protocol of electro-competent transformation in *E. coli* (recovery for 1h at 30° C in 1 ml SOC-medium and plate on LB-Agar supplemented with 50 mg/l kanamycin) was used. Few colonies appeared, the plasmids were isolated and tested by restriction digest. Non of these clones showed the expected bands. In order to check if something is wrong with the protocol combined with these knockout-strain a positive control with an empty BB3-linker-AD was performed. It did work on the first attempt with about the 100-fold amount of clones compared to CC-Selo and all tested clones were positive.

In order to gain more colonies, for the next attempt the cells were recovered in Erlenmeyer-flasks containing 5 ml of SOC-medium and were incubated for 1 h or 2 h at 30° C or 37° C. The amount of colonies on the selective LB agar plates increased but restriction digest revealed they were all false positive. This led to the assumption that *E.coli* WKO^3 mutates the plasmid, so it can use the kanamycine-resistance, but do not need to express the genes of interest.

Further it is known that ribulose 1,5 bisphospate, the product of Prk is toxic and needs to be converted by Rubisco into glycerate 3 phosphate as fast as possible [41][42]. Since the speed of Rubisco is strongly dependent on the amount of available CO_2 we assumed that the supply of additional CO_2 could solve this problem. 100 mM $NaHCO_3$ were added to the SOC medium and the selective LB-agar. After transformation and the analysis of some clones it was clear that this method did not work either. A possible explanation is, that the major part of CO_2 evaporated during the pH-value was adjusted to about 7,4 or while the agar for selective agar-plates was melted.

To exclude the possibility the restriction digest did not work properly it was tried to detect the plasmid by PCR amplification of the insert. Since this method did not

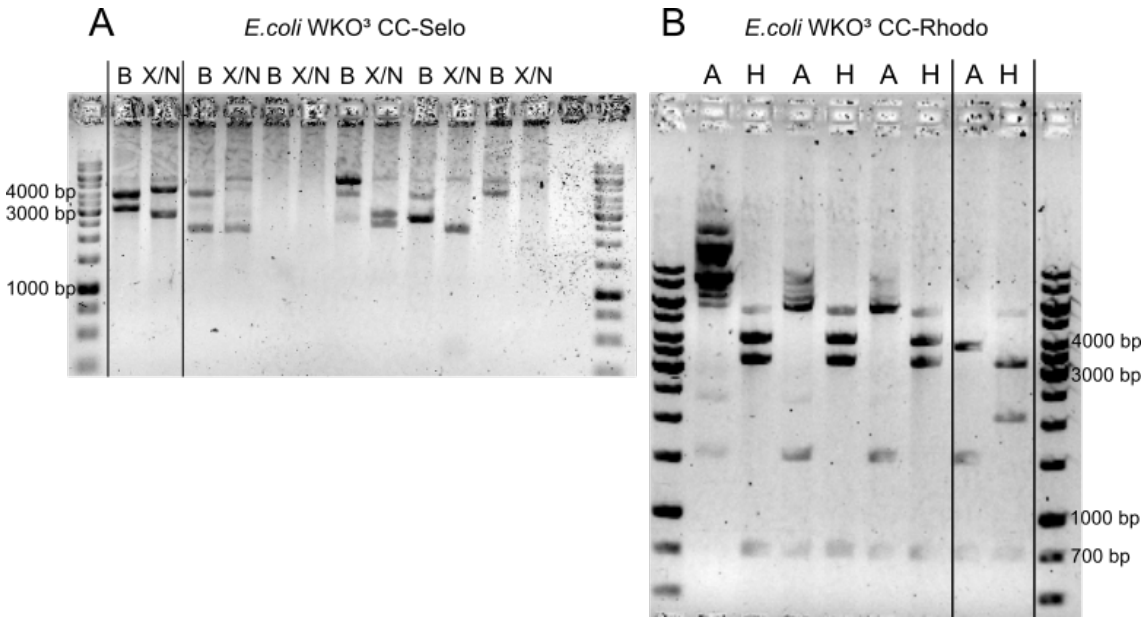

Figure 4.5: Gels from checking transformations. (A) transformation of CC-Selo into *E. coli* WKO^3. B indicates the digest with BamHI, X/N indicates a double digest with XhoI and NcoI. The first clone (marked lanes) showed the expected bands. (B) Transformations of CC-Rhodo into *E. coli* WKO^3. A indicates the restriction digest with AvrII and H indicates the restriction digest with HindIII. The last clone (marked lanes) showed the expected bands.

show the expected result, we tried to retransform the Miniprep into *E. coli* TOP10 to identify it in there but this attempt did not show any success either. These results did show clearly that the transformation itself did not work efficiently.

E. coli W possesses a different methylation-pattern than *E. coli* TOP10 or *E. coli* BL21. In theory it can be possible to improve the efficiency of a transformation by using plasmids with the same methylation-pattern like in the future host cell. So we transformed CC-Selo into *E. coli* W and extracted the plasmid with the *E. coli* W-methylation-pattern. Since we supposed that it would not work at the first time with standard-parameters, many different parameters were used parallel for this transformation. The transformations were recovered at room-temperature (10 min, 1 h, 2 h and 3 h), at 30° C (1 h, 2 h and 3 h) and at 37° C (1 h, 2 h and 3 h) in 1 ml SOC-medium. For incubation on selective agar plates the same temperature was used than for recovery. On selective agar plates some colonies appeared, surprisingly a lot of colonies appeared from those transformations which were recovered on room-temperature. Probably because they were not shocked as hard by the increasing temperature after electroporation as the other cells. But these clones did not grow well in liquid-cultures where all clones were incubated at 30° C. So we assume that a low temperature lowers also the selective pressure of antibiotics, caused by a low growth rate. In the end the best results showed those transformations which were recovered at 30° C for 1 h. Three out of six picked clones grew on liquid LB-medium and one of those showed the right bands on the agarose-gel after plasmid extraction followed by restriction digest (figure 4.5 A). But sequencing revealed a pointmutation in the promoter-sequence of *prk*.

4.3.3 Transformation of CC-Rhodo

Since it worked well with CC-Selo, CC-Rhodo was also transformed into *E. coli* W and the plasmid used for further transformations was gained by extracting it out of it. This plasmid was then transformed into *E. coli* WKO^3 and showed the right bands after Miniprep followed by restriction-digest (figure 4.5 B). But also here sequencing showed a point-mutation in the promoter-sequence of *prk*.

Taken together these two sequencing results with the fact that ribulose 1,5 bisphosphate is toxic for *E. coli* [41] probably the best way would be to down-regulate the activity of Prk. AMP is an inhibitor of Prk [43] and could also be taken up from *E. coli* [44]. So AMP was added to SOC-medium and selective-agarplates in a concentration of 5 mM for further transformations of CC-Selo and CC-Rhodo into electro-competent *E. coli* WKO^3 to lower the activity of Prk in the cells. After screening several clones, one clone with CC-Rhodo showed the right bands. Sequencing showed that there are no mutations like before. Assuming that the plasmid is stable in this host cell, a cryo was prepared without adding AMP to LB-medium for the over-night-culture. A flask-experiment to prove that *E. coli* WKO^3 with the CBB-Enzymes is able to grow better on minimal medium with glucose, arabinose or xylose as single carbon-source than without was prepared and inoculated with this cryo (see figures A.6A.7A.8, discussed in section 4.1 Growth Experiments). Since this experiment did not show a significant difference between *E. coli* WKO^3 with CC-Rhodo and with a vector-control (BB3-linker-AD), the cryo was again checked. This revealed that the plasmid was mutated again.

Since the transformation of CC-Selo in *E. coli* WKO^3 has not worked until then, some alterations of the transformation were tried. Glycerol or pyruvate were added into the SOC-medium and the selective agar plates in concentrations of 5g/l. The idea behind this is that *E. coli* WKO^3 is able to generate sufficient energy from glycerol or pyruvate and do not need to use the penthose-phosphate-pathway, so Prk does produce less ribulose 1,5 bisphosphate because of lacking substrate. Additionally another method was used to prepare chemical competent *E.coli* WKO^3. For this attempt also all supplements (glycerol, pyruvate or AMP) were added to SOC medium and selective agar-plates. All of these attempts did show some false positive clones, but none of the screened clones did contain the right plasmid.

The most successful attempt was to use AMP as inhibitor of Prk but it did not yield a functional clone in the end. This led to the awareness that the best way to solve this problem would be to control the expression of *prk* in-vivo by an inducible promoter.

4.3.4 tet-Promoter

The anhydrotetracycline-inducible promoter (tetP), also used for Cas9 induction in pCas9-CR4, was chosen to regulate *prk* activity. The plan was to amplify tetP by PCR and assemble it into a Golden-Gate-BB1.

The PCR to amplify tetP for Golden-Gate-cloning was performed using pCas9-CR4 as template. The PCR-product was then checked by application on a 1% agarose gel and showed a band between 700 bp and 1000 bp (see figure 4.6

A). Since the size of the amplified piece of DNA should be 785 bp these bands were cut out and purified. tetP was then inserted into a BB1 using a final concentration of 40 nM or 120 nM for tetP and 40 nM for BB1. These products were then transformed into electro competent *E. coli* TOP10, extracted and checked by restriction digest. The expected fragments are 785 bp and 1966 bp long, so the bands shown in figure 4.6 B resemble them. The assembly with the three-fold amount of insert was way more efficient than the assembly with equimolar amount of insert. A possible reason for that probably was the additional restriction digest to create the right overhangs for Golden-Gate-Assembly. Afterwards the DNA was purified twice using the Gel-Extraction-Kit (without application on a agarose gel) yielding 60 ng/μl but not a sufficient purity. Because of the low concentration it was decided to perform the assembly without further purification.

The plasmid from one clone of each assembly was then sent for sequencing with the result that a part of the promoter-sequence altered from the expected sequence. Since the alteration was identical, we assumed it could also occur in the template. According of lacking time I was not able to prepare sufficient amount of pCas9-CR4 to check the original sequence to include it in this work. Since the alteration is not located in the promoter sequence or the tet-repressor-gene (tetR) the tetP should work properly. This assumption is also supported by the fact that tetP does work properly in pCas9-CR4 (as shown in the chapter 4.2 gapA-Knockout).

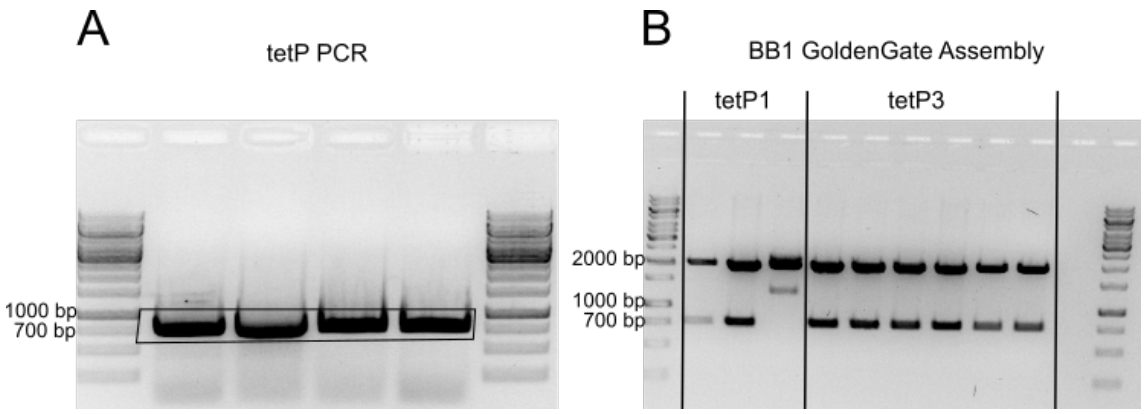

Figure 4.6: Gels to check tetP during preperation of BB1_tetP. (A) The PCR of tetP using pCas9-CR4 as template was applied on a 1% agarose gel. The marked bands were cut out for further use. (B) The BB1-Golden-Gate-Assembly was transformed into *E. coli* TOP10, isolated, digested with BpiI and applied on a 1% agarose gel. tetP1 indicates that BB1 and tetP were used equimolar for Golden Gate assembly. tetP3 indicates that tetP was used in the three-fold amount of BB1 for the Golden Gate assembly.

In order to check the function of tetP a BB2-Golden-Gate-Assembly was performed inserting *GFP* or *mCherry* regulated by tetP. These plasmids were then transformed into *E. coli* TOP10 and BL21. The idea was to check the activity of tetP in relation to the concentration of aTc by measuring the fluorescence of GFP or mCherry. The transformation was checked as usual, the gel is shown in figure 4.7 A, it yielded clones of *E. coli* BL21 with BB2 tetP GFP and BB2 tetP mCherry and *E. coli* TOP10 with BB2 tetP GFP showing the right bands (table 6.10). On

the masterplate of the transformation (figure 4.7 B) the different strains did show fluorescence, without adding aTc. To verify this, these strains were grown in LB medium, supplemented with carbenicillin and with and without the standard concentration of aTc (100 μg/l). There was no signifficant difference visible in these cultures between induced and not induced cells (data not shown). For a further verification *E. coli* BL21 containing BB2 tetP GFP were grown on LB-medium containing carbenicillin and aTC concentrations varying from 0 μg/l to 1000 μg/l. As control a strain expressing GFP with a constitutive promoter was used. The results of measured fluorescence intensity are shown in figure 4.7 C. The data show that the fluorescence intensity is not influenced by the promoter, all induced and non induced cultures as well as the control showed a fluorescence intensity within the standard deviation of the single cultures. This reveals that tetP is not repressing the promoter activity properly. For further investigation of the problem the template (pCas-CR4) should be sequenced and compared to the tetP sequence. If the sequence is right, the tetP could be inserted in the plasmids CC-Rhodo and CC-Selo to regulate the activity of *prk* followed by transformation of these plasmids into *E. coli* WKO^3. This could theoretically lead to clones containing a functionally tetP for *prk* because it adds an additional selective pressure.

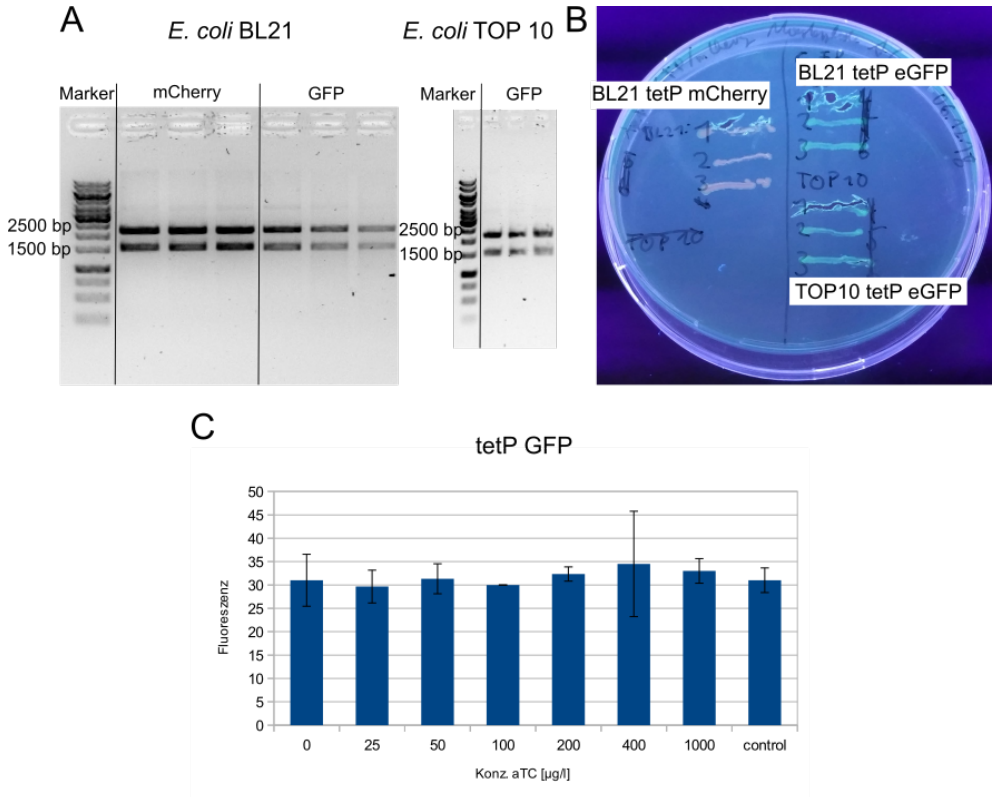

Figure 4.7: Check of tetP activity using GFP. (A) Restriction digest with BsaI of the isolated plasmids after the Golden Gate assembly and tansformation in *E. coli* BL21 and TOP10 on a 1% agarose gel. (B) Masterplate of transformation of BB2 tetP GFP and mCherry in *E. coli* BL21 and TOP10 on UV light to observe fluorescence. (C) Bar chart of fluorescence intensity of *E. coli* BL21 with BB2 tetP GFP under different aTc concentrations. As control a *E. coli* BL21 expressing GFP with a constitutive promoter was used.

Chapter 5

Outlook

When the *gapA* knockouts are prepared and CC-Selo and CC-Rhodo with tetP controlling the expression of *prk* are transformed into these knockout strains a new set of flask experiments could be performed. All *E. coli* W strains (Δ*gapA*, $WKO^3\Delta$*gapA* and $WKO^4\Delta$*gapA*) containing CC-Selo, CC-Rhodo or BB3_linker-AD as control are grown in DeLisa medium with 5 g/l xylose and 100 μg/l aTc with an initial OD600 of 0,1. Maybe the growth medium could also be supplemented with 100 mM $NaHCO_3$ to increase the CO_2 availability for Rubisco. This experiment should show that those strains expressing the CBB-enzymes are able to grow under these conditions but those without expressing these genes are not able because the *gapA* knockout cuts off the glycolysis and xylose can not be metabolised.

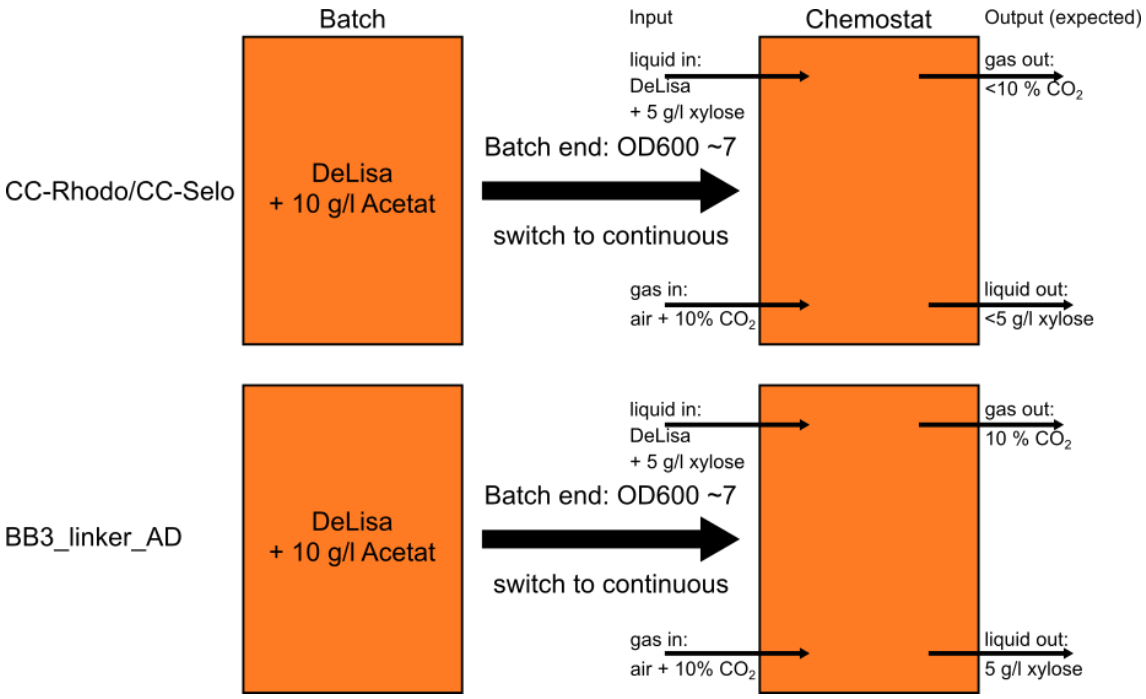

Figure 5.1: The planned fermentation of *E. coli* W Δ*gapA* with CC-Rhodo, CC-Selo or a vector control. The thick arrows indicate the switch from batch-culture to continuous-culture, the thin arrows indicate the liquid and gas input and the expected output.

When the flask experiments went as expected the strains can be grown in a fermentor. The best option for that would be a DASbox® system from Eppendorf, so four strains can be grown parallel. In figure 5.1 the overall setup for this fermentation is shown: The strains are grown in DeLisa medium containing 10 g/l acetate and 50 mg/l kanamycin until they reach a OD600 of about 7. Then the batch fermentation is switched to a continuous fermentation by feeding DeLisa medium supplemented with 5 g/l xylose, 50 mg/l kanamycin and 100 μg/l aTc to induce *prk* expression. Further 10% CO_2 should be added to the gas in, so Rubisco is able to work more efficient. The dilution rate should be chosen very low at about 0,02 to 0,05, so the strains are washed out slowly and have sufficient time to adapt to the new conditions.

It is expected that the OD600 will decrease in the beginning of the continuous fermentation for all strains. After a time of adaptation those strains expressing the CBB enzymes will grow again if all enzymes are working accurately. This will show the decreasing concentration of xylose in the harvest as well as the decreasing amount of CO_2 in the off gas. Also the cell dry weight will increase after adaptation. The control-strain should not be able do adapt to these conditions and will be washed out faster than it will be able to grow after the acetate is completely metabolized.

Additionally it can be tried to grow those strains with CC-Rhodo in anaerobic conditions. Since the photosynthesis apparatus of *Rhodospirillum rubrum* is only active under anaerobic conditions [23] Rubisco would work more efficient under these conditions.

Chapter 6

Material and Methods

6.1 Bacterial Strains and Plasmids

strain	source
Escherichia coli W DSM 1116	DSMZ
Escherichia coli BL21	New England Biolabs
Escherichia coli TOP10	BOKU
E. coli Δ*ldhA* Δ*adhE* Δ*pta* Δ*frdA* (WKO^4)	BOKU

Table 6.1: Strains used in this work

plasmid	source
BB1	BOKU
BB2	BOKU
BB3	BOKU
BB1_prk	Traxler 2017 [45]
BB2_cbbM	Traxler 2017 [45]
BB2_CA	Traxler 2017 [45]
CC-Selo	Traxler 2017 [45]
CC-Rhodo	this work
pKD_sgRNAack	lab stock
promoter	**source**
BBa_J23105 (105p)	Anderson promoter library
BBa_J23109 (109p)	Anderson promoter library
BBa_J23114 (114p)	Anderson promoter library
terminator	**source**
BBa_B1001	Anderson promoter library

Table 6.2: Plasmids used in this work

6.2 Constructs

Two different plasmids were used to insert the two variants of Rubisco into *E. coli* WKO^3. The first one is called CC-Selo and encodes Rubisco from *Synechococcus elongatus* (*rbc*) controlled by 105p. Additionally it contains a chaperon controlled by 109p to fold Rubisco correctly. In the first version *prk* was controlled by the constitutive promoter 109p, later it was planned to insert the tetP for *prk*. Carbonic Anhydrase (*icfA*) controlled by 105p was also included from *Synechococcus elongatus* as all others.

To express Rubisco (*cbbM*) from *Rhodospirillum rubrum* in *E. coli* W, a second plasmid was designed called CC-Rhodo. Rubisco and Carbonic Anhydrase were used from *Rhodospirillum rubrum* and controlled by constitutive promoter 114p. Prk was used from *Synechococcus elongatus* and at first under control of the constitutive promoter 109p, later it was planned to use tetP as promoter.

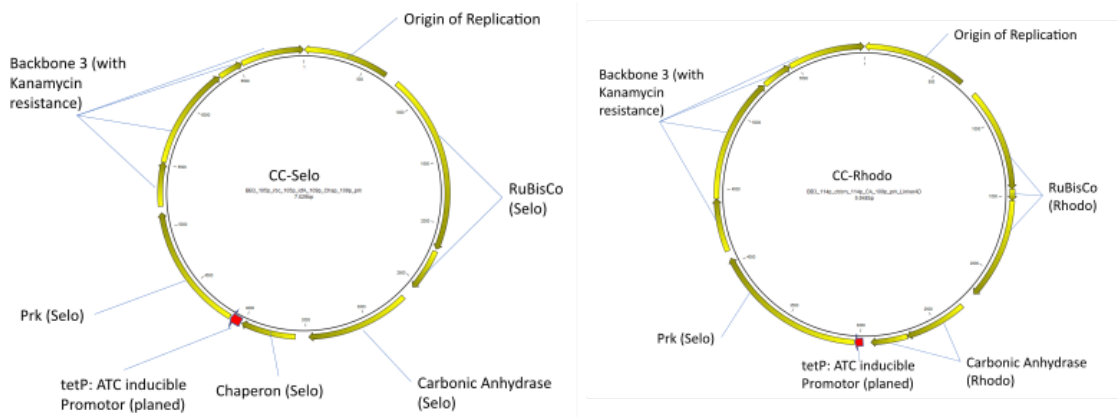

Figure 6.1: Backbone 3 plasmid constructs to insert the genes of CBB-cycle into *E. coli* WKO^3. In brackets the native host of these genes are indicated.

6.3 Growth Media

6.3.1 Lysogeny Broth (LB-Medium) and Agar

ingredient	amount
yeast extract	5 g/l
soy peptone	10 g/l
NaCl	10 g/l
agar-agar	15 g/l (for agarplates)

Table 6.3: Ingredients and concentrations of the used LB-medium and LB-agarplates.

LB medium and agar was used to grow *E. coli* cells in preculture for growth-experimnents, for cloning and plasmid propagation steps. The concentrations of the ingredients are shown in table 6.3 and the final concentrations of antibiotics

used in selective agar-plates or in cultures are shown in table 6.7. For some applications a twofold LB-medium was used, therefore the concentration of yeast extract and soy peptone were increased to 10 g/l and 20 g/l respectively.

6.3.2 DeLisa Medium

ingredient	amount
KH_2PO_4	13,3 g/l
$(NH)_4)_2HPO_4$	4 g/l
citric acid	1,7 g/l
trace elements	
$MgSO_4 * 7H_2O$	1.200 mg/l
Fe(III)citrate	100 mg/l
EDTA	8,4 mg/l
$Zn(CH_3COO)_2 * 2H_2O$	13 mg/l
$CoCl_2 * 6H_2O$	2,5 mg/l
$MnCl_2 * 4H_2O$	15 mg/l
$CuCl_2 * 2H_2O$	1,2 mg/l
H_3BO_3	3 mg/l
$NaMoO_4 * 2H_2O$	2,5 mg/l

Table 6.4: Ingredients and concentrations of the used DeLisa medium. The carbonsource is not mentioned

Defined DeLisa medium was used for growth of *E. coli* W under restricted conditions. The final concentrations are shown in table 6.4, trace elements were mixed and sterile-filtrated separately. The carbon-source was also prepared and autoclaved seperately. As carbon-source Glucose, Arabinose or Xylose were used in different concentrations. In some experiments $NaHCO_3$ and adenosine diphosphate were added with a concentration of 1 M and 5 mM respectively.

6.3.3 M9 Medium

M9 defined medium was used for one growth experiment of *E. coli* W instead of DeLisa medium. Final concentrations are showen in table 6.5, as carbon-source xylose was used in a concentration of 5 g/l.

6.3.4 SOC Medium

SOC medium was used after transformations for regeneration of *E. coli* cells. Final concentrations are shown in table 6.6. The mineral stock was prepared and sterilized separately (100 x concentrated) and added after autoclavation. For the optimization of transformations in some cases supplements were added to SOC medium: AMP, pyruvate, Glycerol or $NaHCO_3$ (for final concentrations see table 6.8).

ingredient	amount
Na_2HPO_4	33,7 mM
KH_2PO_4	22 mM
NaCl	8,55 mM
NH_4Cl	9,35 mM
$MgSO_4$	1mM
$CaCl_2$	0,3 mM
biotin	1 μg/l
thiamin	1 mug/l
trace elements	
EDTA	50 mg/l
$FeCl_3 * 6H_2O$	8,3 mg/l
$ZnCl_2$	0,84 mg/l
$CuCl_2 * 2H_2O$	0,13 g/l
$CoCl_2 * 2H_2O$	0,1 mg/l
$MnCl_2 * 4H_2O$	0,016 mg/l
H_3BO_3	0,1 mg/l

Table 6.5: Ingredients and concentrations of the used M9 medium. The carbon-source is not mentioned

ingredient	final concentration
Tryptone	20 g/l
yeast extract	5 g/l
Glucose	3,6 g/l
mineral stock	
NaCl	0,5 g/l
KCl	0,19 g/l
$MgCl_2$	1 g/l
$MgSO_4$	2,5 g/l

Table 6.6: Ingredients and and concentrations of SOC medium.

6.4 Growth Experiments

Strains for growth experiments were streaked out on a LB agar plates containing the appropriate antibiotics to select for the contained plasmids (concentrations shown in table 6.7) and incubated over night at 37° C. From these agar-plates a single colony was picked with a pipette-tip to inoculate whether LB-medium as preculture or directly the used defined medium. Precultures were incubated at 37° C over night, pelleted (4000 g, 10 min, 4° C) and washed twice with 0,9 % (w/v) NaCl and used to inoculate the defined medium with the appropriate OD600. The cultures were grown in shaking flasks containing 100 ml medium and serum flasks containing 20 ml (so the gas phase can be changed) incubated at 37° C while shaking at 200 rpm. For experiments with serum flasks gas phase was changed directly before the incubation started to 10 % or 30 % CO_2 mixed with air. The growth was observed by the measurement of the optical density at 600 nm (OD600) with a photometer. In some cases 10 μl of the sample were analyzed

antibiotic	final concentration
kanamycin	50 mg/l
carbenicillin	50 mg/l / 100 mg/l
chloramphenicol	34 mg/l
anhydrotetracycline	100 μg/l

Table 6.7: Concentrations of antibiotics in the final media. Carbenicillin was used with 50 mg/l in selective agar-plates and 100 mg/l in liquid cultures.

supplement	final concentration
AMP	5 mM
$NaHCO_3$	100 mM
glucose	5 g/l
arabinose	5 g/l
xylose	5 g/l
pyruvate	5 g/l
glycerol	5 g/l

Table 6.8: Supplements and carbon sources added to growth media in these concentrations.

under the microscope to check the culture for contamination.

6.5 Transformation

6.5.1 Electro Competent Transformation

Preperation of Electro Competent Cells with 1 mM HEPES Buffer

For preparing electro competent *E. coli* cells, the appropriate strain was streaked out on LB agar and incubated over night. One colony of this agar plate was then picked and used to inoculate a liquid culture. This culture was then grown at 37° C until a OD600 of 0,6 - 0,8 and cooled on ice immediately after the incubation. The cells were kept on ice as much as possible from now on. All centrifugation steps were performed at 4800 g for 10 min at 4° C.

The culture was washed three times by centrifugation, discarding the supernatant and resuspending the pellet in 150 ml ice cold 1 mM HEPES buffer (pH 8,0). The suspension was then centrifuged again, the supernatant discarded and the pellet was resuspended in 50 ml ice cold 10 % Glycerol. The suspension was then centrifuged again, the supernatant was discarded and the pellet was resolved in a minimal amount of 10 % glycerol and 100 μl aliquots were transferred in 1,5 ml centrifuge tubes and freezed in liquid nitrogen. These aliquots were then stored at -80° C.

Preperation of Electro Competent Cells with Glycerol/Mannitol

The culture was prepared the same way as for electro competent *E. coli* with 1 mM HEPES buffer. The centrifugation steps were also performed at 4800 g for

10 min and at 4° C and the cells were kept on ice as much as possible.

The pellet was washed two times by centrifugation, discarding the supernatant and resuspending it in 40 ml Glycerol/Mannitol (20 % and 1,5 % respectively). Afterwards it was centrifuged another time, the supernatant was discarded and the pellet was resuspended in 500 μl Glycerol/Mannitol. Afterwards 100 μl aliquots of the cell suspension were transferred in 1,5 ml centrifuge tubes and freezed in liquid nitrogen. These aliquots were then stored at -80° C.

Electro Competent Transformation in *E. coli*

The electro competent *E.coli* cells were thawed on ice for about 15 min. Then 1 μl (for extracted plasmids) up to 5 μl (for assembled plasmids) DNA solution were mixed with 50 μl competent cells in an electroporation cuvette. The electroporation was performed with following parameters: 2500 V, 25 μF and 200 Ω. Afterwards the cell suspension was transferred into 1 ml SOC medium with room temperature and regenerated, normally at 30° C for one hour, but also other conditions were tested. After regeneration the cells were plated on LB agar containing appropriate antibiotics.

6.5.2 Chemical Competent Transformation

Preperation of Chemical Competen *E. coli*

The cultures for preparing chemical competent cells were prepared as for electro competent cells and were also grown until an OD600 of 0,6 - 0,8. The centrifugation steps were also performed at 4800 g for 10 min and at 4° C and the cells were kept on ice as much as possible.

The culture was centrifuged, the supernatant discarded and the pellet was resuspended in about the half volume of ice cold, filter sterilized TFB1-buffer (ingredients are listed in table 6.9), centrifuged again and the supernatant was discarded. The pellet was resolved in 1 ml of TFB2-buffer (ingredients are listed in table 6.9), 50 μl aliquots were prepared in 1,5 ml centrifuge tubes, shock frozen in liquid nitrogen and stored at -80° C.

Chemical Competent Transformation in *E. coli*

For transfering a plasmid in chemical competent cells, the cells were first thawed on ice for about 10 min. 1 μl to 5 μl of the plasmid were added to the cells and those were chilled on ice for further 30 s. Then the cells were heat shocked at 42° C for 90 s and chilled on ice for 5 min. Then 1 ml SOC medium were added and the cells were regenerated normally 1 h at 30° C, but also other conditions were used. After regeneration the cells were plated on selective LB agar plates with the appropriate antibiotics.

6.5.3 Regeneration after Transformation

For both, electro competent and chemical competent transformation, different conditions were used to regenerate the cells after transformation in order to opti-

ingredient	final concentration
TFB1	
potassium acetate	30 mM
$CaCl_2$	10 mM
$MnCl_2$	50 mM
RbCl	100 mM
glycerol	15 %
acetic acid	for adjusting pH to 5,8
TFB2	
MOPS	100 mM
$CaCl_2$	75 mM
RbCl	10 mM
glycerol	15 %
KOH	for adjusting pH to 6,5

Table 6.9: Ingredients and concentrations for buffer TFP1 and TFB2 used for preperation of chemical competent cells.

mize the efficiency. Additional to the standard temperature of 30° C room temperature and 37° C were used. The time was varied from 10 min up to 3 h. Further some additional ingredients were added to the SOC medium during regeneration, those are listed in table 6.8. In some cases the cells were regenerated in small shaking flasks containing 5 ml SOC medium.

6.5.4 Screening for right Plasmids after Transformation

After transformation it is necessary to check if the right plasmid was transformed into the cells. Therefore the plasmid was extracted (like described in section "Plasmid Extraction"). The standard method was to digest it with restriction enzymes and apply it on a 1 % agarose gel (described in sections "Restriction Digest" and "Agarose Gel Electrophoresis"). In some cases the insert of the plasmid was amplified by PCR using appropriate primers and applied on a agarose gel.

6.6 Plasmid Extraction

In order to extract plasmids the GeneJET Miniprep Kit from Thermo Scientific was used. An appropriate colony was picked from an agar plate and about 2 ml of LB medium supplemented with the appropriate antibiotics were inoculated with it. This culture was then incubated at 37° C, with shaking at 200 rpm over night. All centrifugation steps were performed at maximum rpm. Then the culture was transferred into 2 ml centrifuge tubes and centrifuged for 2 min. The supernatant was then discarded and the pellet was resuspended in 250 μl cooled Resuspensionbuffer. 250 μl Lysisbuffer were added and the suspension was inverted 6 times. Thereafter 350 μl Neutralisationbuffer were added and the suspension

was inverted another 6 times followed by centrifugation for 10 min. The supernatant was then transfered into spin columns and these were centrifuged for 1 min. The column was washed 2 times by adding 500 μl Washingsolution followed by centrifugation for 1 min. The column was then centrifuged for 1 min to get rid of the remaining washing solution and transfered afterwards into a new 1,5 ml centrifugation tube. The plasmid was then eluted by the addition of 10 μl to 25 μl 10 mM TRIS.

6.7 Restriction Digest

In order to screen for positive clones after transformation or to prepare DNA pieces for several methods restriction digests were performed. Therefore 0,25 μl restriction enzyme and 2 μl buffer were mixed with the sample and filled to 20 μl with 10 mM TRIS. Depending on the concentration of the plasmid a amount of 4 μl up to 10 μl was used. The reaction was then incubated for at least 1 h at 37° C.

DNA	enzymes	fragments
CC-Selo	BamHI	3066 bp / 3971 bp
CC-Selo	NcoI/XhoI	2765 bp / 4272 bp
CC-Selo	EcoRI/SalI	2720 bp / 4317 bp
CC-Rhodo	AvrII	751 bp / 1510 bp / 3699 bp
CC-Rhodo	HindIII	751 bp / 2050 bp / 3159 bp
BB2_prk	BamHI/SalI	599 bp / 2691 bp
pKDsgRNA-gapA	BamHI	2329 bp / 4655 bp
pKDsgRNA-gapA	SalI	3185 bp / 3799 bp
BB1_gapA_HAs	BpiI	999 bp / 1934 bp
BB1_gapA_HAs	EcoRI	1025 bp / 1908 bp
BB1_tetP	BpiI	773 bp / 1926 bp
BB2_GFP	BsaI	1540 bp / 2179 bp
BB2_mCherry	BsaI	1540 bp / 2179 bp

Table 6.10: Tested plasmids and used restriction digests in this work.

6.8 Agarose Gel Electrophoresis

For identifying DNA fragments according to their size or to seperate one species of DNA from others agarose gel electrophoresis was used. Therefore agarose was mixed with TAE buffer to a final concentration of 1 %, heated in the microwave until all the agarose was dissolved and poured into a gel chamber. A 1kb Gene ruler from Thermo Scientific was used as marker. The samples were mixed with the 6x Purple Loading Dye from New England Biolabs and loaded on the gel. According to the application different amounts of sample were loaded ranging from 10 μl to 50 μl.

6.9 Sequencing

Plasmids were mixed with the appropriate primers and sent for Sanger sequencing (Microsynth AG, Switzerland).

6.10 Preperation of Cryo Cultures

For storing bacterial strains for longer time cryo cultures were prepared. Therefore the strain was grown on liquid LB medium over night and 1 ml of this culture was then mixed with 500 μl 75 % glycerol in cryo vials and stored at -80° C.

6.11 DNA purification and Gel Extraction

In order to purify one species of DNA a gel extraction was performed with GeneJET Gel extraction Kit from Thermo Scientific. Therefore the appropriate band of a gel electrophoresis was cut out, mixed with about the same amount of binding buffer and melted by heating it to 55° C. Then it was filtered with the spin columns by centrifugation for 1 min at max rpm. The spin column was then washed twice with 700 μl and 350 μl respecively with wash buffer and centrifugation for 1 min at max rpm. In order to get rid of all the washing buffer the spin column was centrifuged another 10 min at max rpm. The DNA was then eluted with 20 μl 10 mM TRIS which was preheated to 70° C into a fresh 1,5 ml centrifuge tube by centrifugation for 1 min at max rpm.

The GeneJET gel extraction kit was also used to purify DNA without the separation on an agarose gel. Therefore the solution was mixed with the same volume of binding buffer and it was performed as described above.

6.12 PCR

ingredient	amount
Q5-Polymerase	0,5 μl
5x Q5 Reaction Buffer	10 μl
dNTPs (10 μM)	2 μl
10 μM FW primer	2,5 μl
10 μM BW primer	2,5 μl
10 mM TRIS	31,5 μl
template	1 μl

Table 6.11: Master mix for PCR

For performing a PCR a mastermix was prepared like shown in table 6.11. The program showed beneath was used, the extension time (at 72° C) was adjusted according to the length of the amplified DNA piece: 30 s per 1000 bp.

$$\frac{98°C}{120s}\left[\frac{98°C}{10s}\middle|\frac{63°C}{30s}\middle|\frac{72°C}{xxs}\right]^{35x}\frac{72°C}{10min}\middle|\frac{10°C}{\infty}$$

6.13 Plasmid Assembly

6.13.1 Golden Gate Assembly

ingredient	amount
Cut Smart buffer	2 μl
ATP (20 mM)	2 μl
restriction enzyme	2 μl
T4 Ligase (1:10 dilution)	2,5 μl
10 mM TRIS	ad 20 μl

Table 6.12: Mix for a Golden Gate assembly, without DNA fragments.

Program on Thermocycler used for GoldenGate assembly:

$$\left[\frac{37°C}{5min}\middle|\frac{16°C}{2,5min}\right]^{45x}\frac{37°C}{30min}\middle|\frac{50°C}{5min}\middle|\frac{80°C}{10min}$$

BB1 Assembly

The gene of interest was amplified by PCR using primer which add BsaI recognition sites and the required fusion sites. Then the BB1 Golden Gate assembly was performed using BB1 with the appropriate fusion site and the insert with a final concentration of 40 nM and the mix showed in table 6.12. BsaI was used as restriction enzyme. The assembly itself was then performed in a termocycler. Afterwards the assembly was was transformed whether into chemical competent *E. coli* BL21 or electro competent *E. coli* TOP10 and checked by restriction digest and sequencing. Kanamycin was used for selection after transformation.

BB2 Assembly

To add a promoter and a terminator to the gene of interest a BB2 assembly was performed. Therefore all three BB1 containing the appropriate inserts and fusion sites and an empty BB2 with the appropriate linker and fusion sites with a final concentration of 40 nM each were mixed with the master-mix showed in table 6.12. BpiI was used as restriction enzyme. The assembly itself was then performed in a thermocycler. Afterwards the assembly was transformed whether into chemical competent *E. coli* BL21 or electro competent *E. coli* TOP10 and checked by restriction digest and sequencing. Carbenicillin was used for selection after transformation.

BB3 Assembly

In order to combine several genes on one plasmid a BB3 assembly was performed. Therefore BB2 containing the genes of interest and the appropriate linker were mixed with the master-mix shown in talbe 6.12 and a BB3 with the appropriate linker. All plasmids were used in a final concentration of 40 nM. BsaI was used as restriction enzyme. The assembly itself was then performed in a thermocycler. Afterwards the assembly was transformed whether into chemical competent *E. coli* BL21 or electro competent *E. coli* TOP10 and checked by restriction digest and sequencing. Kanamycin was used for selection after transformation.

6.14 Gibson Assembly

Gibson assemblies were performed after Gibson et al [46]. The DNA fragments of interest were amplified by PCR using primer that generate appropriate overhangs. These fragments were then applied on a agarose gel, cut out and purified by gel extraction. The purified DNA fragments were then mixed in 10 mM TRIS with a final volume of 5 μl and a equimolar concentration between 0,1 and 0,5 pmol. This was then mixed with 5 μl master mix (described in table 6.13) and incubated at 50° C for 1 h. Afterwards it was transformed whether in chemical competent *E. coli* BL21 or electro competent *E. coli* TOP10. The transformation was then checked by restriction digest and sequencing.

ingredient	amount
5X ISO buffer	2 μl
T5 exonuclease (1 U/μl)	6,4 μl
Q5 polymerase (2 U/μl	2 μl
Taq ligase (40 U/μl)	16 μl
dH_2O	ad 80 μl
5X ISO buffer	
PEG-8000	0,15 g
1 M TRIS-HCl (pH 7,5)	300 μl
2 M $MgCl_2$	15 μl
100 mM dGTP	6 μl
100 mM dATP	6 μl
100 mM dTTP	6 μl
100 mM dCTP	6 μl
1 M DTT	30 μl
100 mM NAD	30 μl
dH_2O	ad 600 μl

Table 6.13: Master-mix for Gibson assembly.

6.15 Knockout

To perform a knockout the no-SCAR System was used [40]. In order to perform this protokoll three plasmids are requred: pCas9-CR4, pKD-gRNA and homologue arms.

6.15.1 pCas9-CR4

This plasmid contains the nuclease Cas9 to generate double-strand breaks. pCas9-CR4 was transformed into the required strains for other projects and cryos were prepared, so it was possible to use these cryos also for the *gapA* knockout.

6.15.2 pKDsgRNA-xxx

pKDsgRNA-xxx does consist out of two parts. pKD part I is used for all knockouts and encodes the recombinase λ-Red, controlled by a araBAD promoter for the recombination of the double-strand-break generated by Cas9. pKD part II has to be adapted for each knockout seperately because it encodes the small guide RNA (sgRNA) which guides Cas9 to the right site to generate the double-strand-break. These two fragments were amplified by PCR. part1_FW and part1_BW were used to amplify pKD part I, using pKD_sgRNAack as template, the product should be 5363 bp long. The forward primer (gapA_PAM040_part2FW) for part II was designed so it adds the 20 bp guide RNA sequence to the PCR product, pkd_part2_BW was used as backward primer and pKD_sgRNAack as template, the product should be 1652 bp long.

The PCR reactions were then applied on a 1 % agarose gel, the appropriate bands were cut out and purified. These two parts were then assembled by Golden Gate using BpiI as restriction enzyme. The assembly was then transformed in chemically competent *E. coli* BL21 and selected with LB-agar plates containing carbenicillin. Six clones were checked by restriction digest for the right plasmid and one of the positive clones was then sent for sequencing using PAM_seq as primer.

6.15.3 Homologue Arms

Homologue arms are a 1000 bp long DNA fragment, normally amplified from a BB1 plasmid which is generated by a Gibson Assembly of the up-stream-arm, the down-stream-arm and a BB1. It is used to recombine the genomic DNA where Cas9 did generate a double-strand-break, by excluding the knocked out gene. At first both arms were amplified by PCR using primer which add overhangs for Gibson-Assembly (HAup_gapA_FW and HAup_gapA_BW for the up-stream-arm and HAdown_gapA_FW and HAdown_gapA_BW for the down-stream-arm), genomic DNA from *E. coli* W was used as template, the product was 500 bp long each. In order to add overhangs for Gibson-Assembly also the BB1 was amplified by PCR, using BB1_FW_gapA and BB1_BW_gapA as primer and BB1_p109 as template, the product was 2796 bp long. All three pieces of DNA were applied on a 1 % agarose gel, the appropriate bands were cut out and purified.

The BB1 and homologue arms were then assembled in a Gibson Assembly, transformed into electro competent *E. coli* TOP10 and chemically competent *E. coli* BL21 and selected on LB agar plates containing kanamycin. Six clones of each transformation were checked by restriction digested with BpiI and EcoRI separately.

Since this method did not yield the right plasmid, another method was used to generate homologue arms. The up-stream-arm and the down-stream-arm were both used in the same PCR reaction as template and gapA_HAFW and gapA_HABW as primer, the product was 1000 bp long and was used in three different purities for the knockout. The PCR product was used without any purification, the PCR reaction was purified with the gel extraction kit without application on a gel and the PCR reaction was applied on a 1 % agarose gel, the appropriate band was cut out and purified with the gel extraction kit.

Later a third method was used, where the PCR product from the previous method was used as template for a PCR, using HAup_gapA_FW and HAdown_gapA_BW as primer to add overhangs for Gibson Assembly to it. Then it was inserted by Gibson Assembly into the same BB1 which was used in the first method, transformed into chemically competent *E. coli* BL21 and selected on LB agar plates containing kanamycin and incubated at $30°$C over night. Six colonies were checked for the right plasmid by restriction digest using BpiI and EcoRI separately.

6.15.4 Knockout

Cells containing pCas9-CR4 were streaked out on LB agar containing chloramphenicol and made electro competent using Glycerol/Mannitol and pKD was transformed into these cells (explained more in detail in section electro competent transformation) and selected on LB agar containing chloramphenicol and carbenicillin. The transformation was checked by plasmid extraction followed by PCR using gapA_PAM040_part2_FW and pKD_part2_BW as primer. This PCR reaction was then applied on a 1 % agarose gel to check for the right band at 1652 bp and for positive clones a cryo was prepared.

The cells containing pCas9-CR4 and pKD were streaked out on a new LB agar plate containing chloramphenicol and carbenicillin. 22 ml liquid LB medium containing the same antibiotics were then inoculated with a single colony of this agar plate and incubated at $30°$ C until a OD600 of 0,4 was reached. In order to induce the araBAD promotor of λ-Red, a 7 g/l agarose stock was added to a final concentration of 0,35 % (w/v) and the incubation was proceeded for 15 min at $37°$ C. After the incubation the culture was immediately cooled on ice for several minutes. The culture was then washed two times with 20 ml washing solution (20 % (v/v) Glycerol, 1,5 % (w/v) Mannitol) by centrifugation at 4800 g for 10 min at $4°$ C and resuspending the pellet. Afterwards the cell suspension was centrifuged again and resuspended in 150 μl of washing solution. This cell suspension was then mixed in electroporation cuvettes with different amounts of homologue arms and electroporated with the same parameters used for electro competent transformation. Afterwards the cells were transfered in 1 ml SOC medium and regenerated for 1 h at $30°$ C. A negative control was performed without the addition of homologue arms. 100 μl of the cell suspension $10^{-1}, 10^{-2}$ and 10^{-3} diluted was plated on LB agar plates containing chloramphenicol, carbenicillin and aTc. For

estimating the kill efficiency the highest dilution was plated on LB agar with chloramphenicol and carbenicillin. The agar plates were packed into aluminium foil to protect aTc from light and incubated at 30° C over night.

Colony PCR

In order to screen the appearing colonies, a colony PCR (cPCR) was performed using gapAFW_cPCR_W and gapABW_sPCR_W as primer. 25 colonies of each knockout and one colony of the negative control were picked, transfered into 10 μl 10 mM TRIS, cooked an 95° C for 5 min and used as template for a PCR. A mastermix was prepared (see table 6.14), 1 μl template was added and the PCR was performed in a thermocycler with these parameters:

$$\frac{98°C}{120s}\left[\frac{98°C}{10s}\middle|\frac{62°C}{30s}\middle|\frac{72°C}{60s}\right]^{35x}\frac{72°C}{10min}\middle|\frac{10°C}{\infty}$$

Afterwards the cPCR was applied on a 1 % agarose gel to check for positive knockouts. The expected band for a positive clone is at 1000 bp.

ingredient	amount
Taq-Polymerase	0,0625 μl
Taq-buffer	1,25 μl
dNTPs	0,25 μl
10 μM FW primer	0,25 μl
10 μM BW primer	0,25 μl
10 mM TRIS	9,4375 μl
template	1 μl

Table 6.14: Master mix for colony PCR

6.16 Testing the function of tet Promoter

In order to test the tetP a BB2 Golden Gate assembly was performed, using GFP or mCherry as insert. This assembly was then transformed into *E. coli* BL21 and *E. coli* TOP10. These strains were then grown on LB agar and in liquid LB medium each with and without aTc. These cultures were then checked under UV light for fluorescence.

Additionally *E. coli* BL21 containing the plasmid with GFP was grown on different aTc concentrations in liquid LB medium over night: 0 μg/l, 25 μg/l, 50 μg/l, 100 μg/l, 200 μg/l, 400 μg/l and 1000 μg/l. As control an *E. coli* BL21 expressing GFP with a constitutive promoter was used. These cultures were then diluted to an OD600 of 1 and the fluorescence was measured in a plate reader with three technical replicates using the parameters indicated in table 6.15.

Excitation Wavelength	488 nm
Emission Wavelength	510 nm
Excitation Bandwidth	9 nm
Emission Bandwidth	20 nm
Gain	20 (Manual)
Number of Flashes	25
Integration Time	20 μs
Lag Time	0 μs
Settle Time	0 ms

Table 6.15: Parameters for the measuring of fluorescence for checking the function of tetP.

Primer Name	Sequence (5'-3')
part1_FW	GATCGAAGACGCcctgcatcgatttattatgacaac
part1_BW	GATCGAAGACGCgtgctcagtatctctatcactg
gapA_PAM040_part2FW	GATCGAAGACGCgcacAACACTCCGATGTTCGTTAAgttttagagctagaaatagcaa gttaaaataag
pkd_part2_BW	GATCGAAGACGCcaggtggcacttttcg
HAup_gapA_FW	ATATGCGGCCTCGAATTCGAAGACGCCATGACTTGGTTTGGGAATGAAAC
HAup_gapA_BW	GCGACCGAAGTCGCTCTTTTTAGATCACAGTTAGTGAATAAAAGGTTGCCTG
HAdown_gapA_FW	TAATTTTACAGGCAACCTTTTATTCACTAACTGTGATCTAAAAAGAGCGAC
HAdown_gapA_BW	AAGATCACGCGCGAATTCGAAGACGCAAGCGGAAGTGCGCTAACAG
gapA_HAFW	ACTTGGTTTGGGAATGAAAC
gapA_HABW	GGAAGTGCGCTAACAG
BB1_FW_gapA	CGCACGACTTTACGCTGTTAGCGCACTTCCGCTTGCGTCTTCGAATTC
BB1_BW_gapA	ATGCCCCGCTGTTTCATTCCCAAACCAAGTCATGGCGTCTTCGAATTC
gapAFW_cPCR_W	CACTCACCGGTTCGTAG
gapABW_sPCR_W	CACGTTAAAGTAGGTATGCAG
FS1_tet_FW	GAAGACGCGGAGttaagacccactttcacatttaag
FS2_tet_BW	GAAGACGCCATGagatcctttctcctctttagatc
PAM_seq	attcatgcaaggaaactacc

Table 6.16: List of primer used in this work.

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Appendix A

Appendix

plasmid	sugar	NaHCO3	abbreviation	0 h	2:20 h	19 h	25 h
CC-Selo	glucose	+	sg+	< 0,001	0,137	0,117	0,088
CC-Selo	arabinose	+	sa+	< 0,001	0,063	0,185	0,084
CC-Selo	xylose	+	sx+	< 0,001	0,208	0,241	0,15
CC-Selo	glucose	-	sg-	< 0,001	0,002	0,004	0,005
CC-Selo	arabinose	-	sa-	< 0,001	0,001	0,001	0,002
CC-Selo	xylose	-	sx-	< 0,001	0,001	0,002	0,002
BB3-AD	glucose	+	bg+	< 0,001	0,103	0,038	0,077
BB3-AD	arabinose	+	ba+	< 0,001	0,056	0,079	0,107
BB3-AD	xylose	+	bx+	< 0,001	0,045	0,037	0,071
BB3-AD	glucose	-	bg-	< 0,001	0,001	0,01	0,015
BB3-AD	arabinose	-	ba-	< 0,001	0,001	0,002	0,002
BB3-AD	xylose	-	bx-	< 0,001	0,001	0,002	0,003

inoculation: pipette tip
CC-Selo showed a point mutation in *prk* after sequencing.

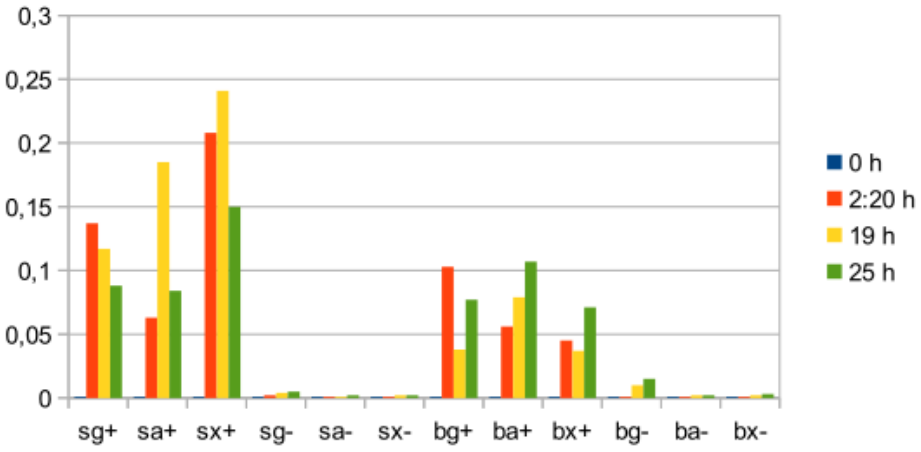

Figure A.1: Growth of *E. coli* WKO^3 CC-Selo (with a point mutation in *prk*) on DeLisa minimal medium supplemented with 5 g/l glucose, arabinose or xylose and with or without $NaHCO_3$ and 50 mg/l kanamycin compared with *E. coli* WKO^3 BB3-AD (vector control). Induction was performed with a pipette tip, so the expected starting OD600 is $< 0,001$.

plasmid	sugar	NaHCO3	abbreviation	0 h	1:40 h	3:40 h	22 h	28 h
CC-Selo	glucose	+	sg+	< 0,001	0,14	0,129	0,129	0,406
CC-Selo	arabinose	+	sa+	< 0,001	0,171	0,156	0,172	0,525
CC-Selo	xylose	+	sx+	< 0,001	0,124	0,117	0,113	0,39
CC-Selo	glucose	-	sg-	< 0,001	0,006	0,009	0,012	0,057
CC-Selo	arabinose	-	sa-	< 0,001	0,002	0,008	0,013	0,06
CC-Selo	xylose	-	sx-	< 0,001	0,003	0,024	0,019	0,018
BB3-AD	glucose	+	bg+	< 0,001	0,056	0,055	0,06	0,48
BB3-AD	arabinose	+	ba+	< 0,001	0,074	0,049	0,081	0,416
BB3-AD	xylose	+	bx+	< 0,001	0,02	0,021	0,029	0,3
BB3-AD	glucose	-	bg-	< 0,001	0,009	0,014	0,056	0,18
BB3-AD	arabinose	-	ba-	< 0,001	0,042	0,019	0,041	0,127
BB3-AD	xylose	-	bx-	< 0,001	0,025	0,008	0,029	0,046

inoculation: pipette tip
CC-Selo showed a point mutation in *prk* after sequencing.

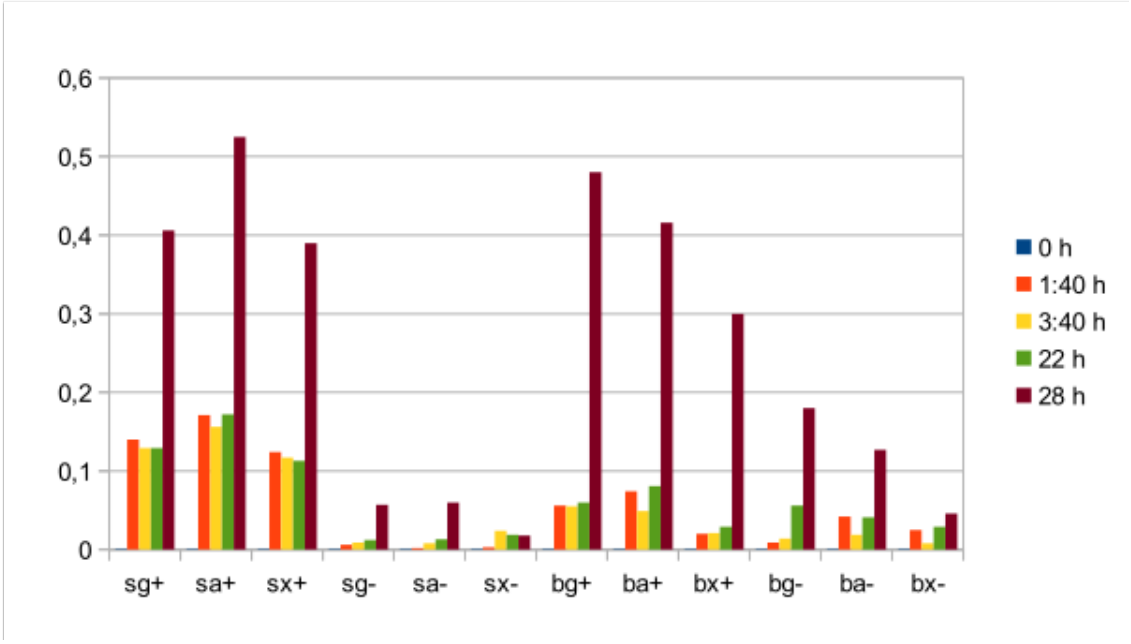

Figure A.2: Growth of *E. coli* WKO^3 CC-Selo (with a point mutation in *prk*) on DeLisa minimal medium supplemented with 5 g/l glucose, arabinose or xylose and with or without $NaHCO_3$ and 50 mg/l kanamycin compared with *E. coli* WKO^3 BB3-AD (vector control). Induction was performed with a pipette tip, so the expected starting OD600 is $< 0,001$.

plasmid	sugar	CO2	abbreviation	0 h	2 h	4:30 h	21 h	26 h	2 d	4 d	7 d
CC-Selo	glucose	+	sg+	< 0,001	0,045	0,029	0,029	0,022	0,074	0,109	0,189
CC-Selo	arabinose	+	sa+	< 0,001	0,003	0,044	0,033	0,038	0,069	0,364	0,464
CC-Selo	xylose	+	sx+	< 0,001	0,033	0,040	0,036	0,031	0,038	0,059	0,496
CC-Selo	glucose	-	sg-	< 0,001	0,011	0,111	0,074	0,026	0,157	0,138	0,409
BB3-AD	glucose	+	bg+	< 0,001	0,098	0,039	0,048	0,059	0,111	0,960	1,501
BB3-AD	arabinose	+	ba+	< 0,001	0,007	0,056	0,040	0,043	0,160	0,602	0,637
BB3-AD	xylose	+	bx+	< 0,001	0,000	0,007	0,019	0,011	0,015	0,027	0,029
BB3-AD	glucose	-	bg-	< 0,001	0,009	0,021	0,106	0,066	0,131	0,662	0,875

inoculation: pipette tip
CC-Selo showed a point mutation in *prk* after sequencing.

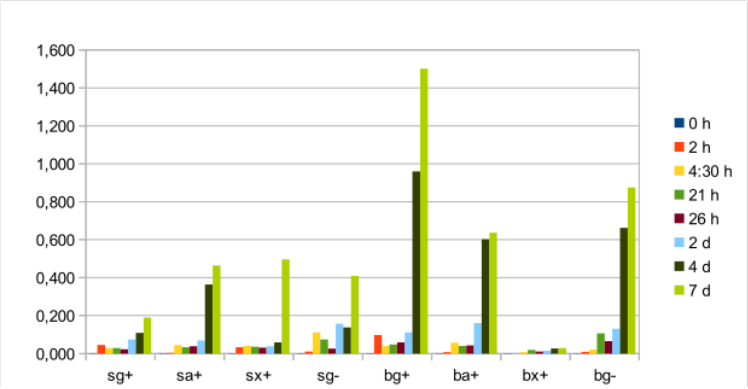

Figure A.3: Growth of *E. coli* WKO^3 CC-Selo (with a point mutation in *prk*) on DeLisa minimal medium supplemented with 5 g/l glucose, arabinose or xylose with 30% CO_2 and 50 mg/l kanamycin compared with *E. coli* WKO^3 BB3-AD (vector control) in serum flasks. A control without CO_2 was used. Induction was performed with a pipette tip, so the expected starting OD600 is $< 0,001$.

plasmid	sugar	CO2	abbreviation	0 d	1 d	2 d	3 d	4 d	5 d
CC-Selo	glucose	+	sg+	< 0,001	0,029	0,058	0,068	0,079	0,107
CC-Selo	arabinose	+	sa+	< 0,001	0,037	0,108	0,287	0,038	0,069
CC-Selo	xylose	+	sx+	< 0,001	0,033	0,040	0,036	0,031	0,038
CC-Selo	glucose	-	sg-	< 0,001	0,011	0,111	0,074	0,026	0,157
BB3-AD	glucose	+	bg+	< 0,001	0,098	0,039	0,048	0,059	0,111
BB3-AD	arabinose	+	ba+	< 0,001	0,007	0,056	0,040	0,043	0,160
BB3-AD	xylose	+	bx+	< 0,001	0,000	0,007	0,019	0,011	0,015
BB3-AD	glucose	-	bg-	< 0,001	0,009	0,021	0,106	0,066	0,131

inoculation: pipette tip
CC-Selo showed a point mutation in *prk* after sequencing.

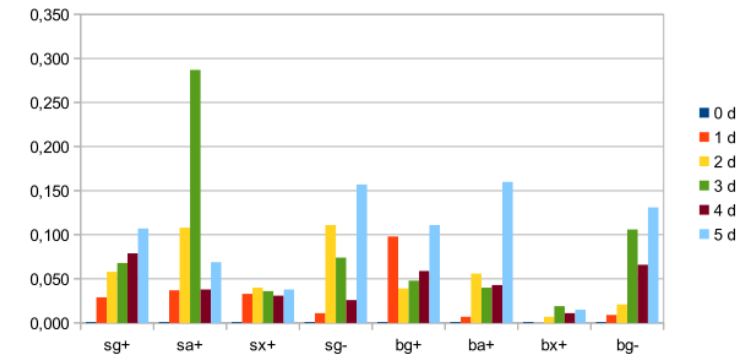

Figure A.4: Growth of *E. coli* WKO^3 CC-Selo (with a point mutation in *prk*) on DeLisa minimal medium supplemented with 5 g/l glucose, arabinose or xylose with 30% CO_2 and 50 mg/l kanamycin compared with *E. coli* WKO^3 BB3-AD (vector control) in serum flasks. A control without CO_2 was used. Induction was performed with a pipette tip, so the expected starting OD600 is $< 0,001$.

plasmid	sugar	CO2	abbreviation	0 d	3 d	4 d	10 d
CC-Selo	xylose	+	sx+	< 0,001	0,012	0,039	0,963
CC-Selo	xylose	+	sx+	< 0,001	0,032	0,081	0,550
CC-Selo	xylose	-	sx-	< 0,001	0,013	0,041	0,727
CC-Selo	xylose	-	sx-	< 0,001	0,019	0,049	0,747
BB3-AD	xylose	+	bx+	< 0,001	0,144	0,707	0,992
BB3-AD	xylose	+	bx+	< 0,001	0,132	0,653	0,870
BB3-AD	xylose	-	bx-	< 0,001	0,058	0,297	1,119
BB3-AD	xylose	-	bx-	< 0,001	0,073	0,377	1,180

inoculation: pipette tip
CC-Selo showed a point mutation in *prk* after sequencing.
Medium: M9 with 5 g/l Xylose

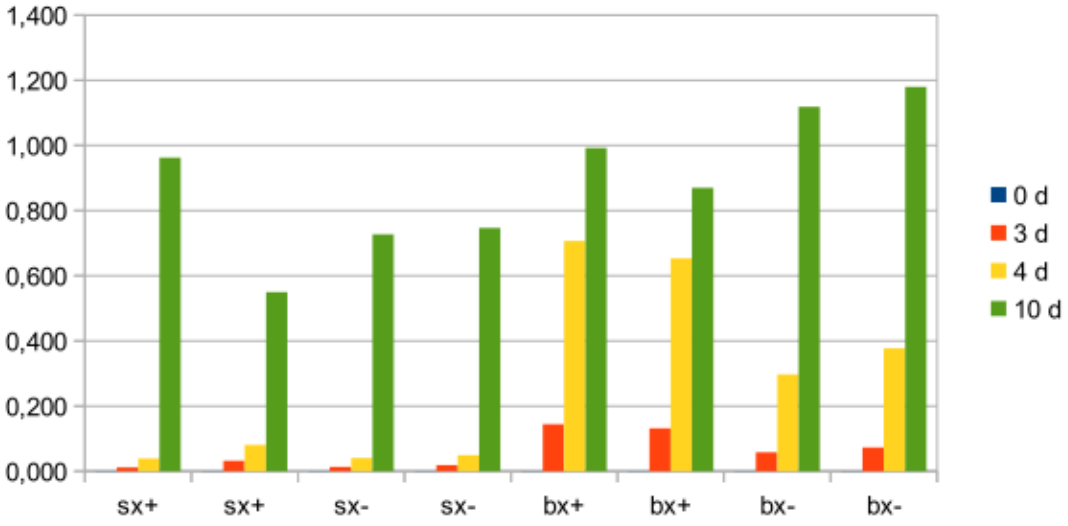

Figure A.5: Growth of *E. coli* WKO^3 CC-Selo (with a point mutation in *prk*) on M9 minimal medium supplemented with 5 g/l xylose, 10% CO_2 and 50 mg/l kanamycin compared with *E. coli* WKO^3 BB3-AD (vector control) in serum flasks. A control without CO_2 was used. Induction was performed with a pipette tip, so the expected starting OD600 is $< 0,001$.

plasmid	sugar	CO2	AMP	abbreviation	0 d	1 d	2 d
CC-Rhodo	xylose	+	+	rca1	0,01	0,045	1,603
CC-Rhodo	xylose	+	+	rca2	0,01	0,044	1,598
CC-Rhodo	xylose	+	+	rca3	0,01	0,043	1,599
CC-Rhodo	xylose	+	-	rc1	0,01	0,046	1,601
CC-Rhodo	xylose	+	-	rc2	0,01	0,045	1,604
CC-Rhodo	xylose	+	-	rc3	0,01	0,043	1,587
CC-Rhodo	xylose	-	+	ra1	0,01	0,04	1,65
CC-Rhodo	xylose	-	+	ra2	0,01	0,036	1,696
CC-Rhodo	xylose	-	+	ra3	0,01	0,039	1,647
CC-Rhodo	xylose	-	-	r1	0,01	0,046	1,629
CC-Rhodo	xylose	-	-	r2	0,01	0,037	1,628
CC-Rhodo	xylose	-	-	r3	0,01	0,039	1,642
BB3-AD	xylose	+	+	bca1	0,01	0,024	1,616
BB3-AD	xylose	+	+	bca2	0,01	0,03	1,606
BB3-AD	xylose	+	+	bca3	0,01	0,032	1,663
BB3-AD	xylose	+	-	bc1	0,01	0,01	1,591
BB3-AD	xylose	+	-	bc2	0,01	0,014	1,558
BB3-AD	xylose	+	-	bc3	0,01	0,012	1,565
BB3-AD	xylose	-	+	ba1	0,01	0,026	1,677
BB3-AD	xylose	-	+	ba2	0,01	0,022	1,642
BB3-AD	xylose	-	+	ba3	0,01	0,024	1,661
BB3-AD	xylose	-	-	b1	0,01	0,01	1,651
BB3-AD	xylose	-	-	b2	0,01	0,006	1,648
BB3-AD	xylose	-	-	b3	0,01	0,012	1,676

inoculation: OD600: 0,01
CC-Rhodo was unstable.

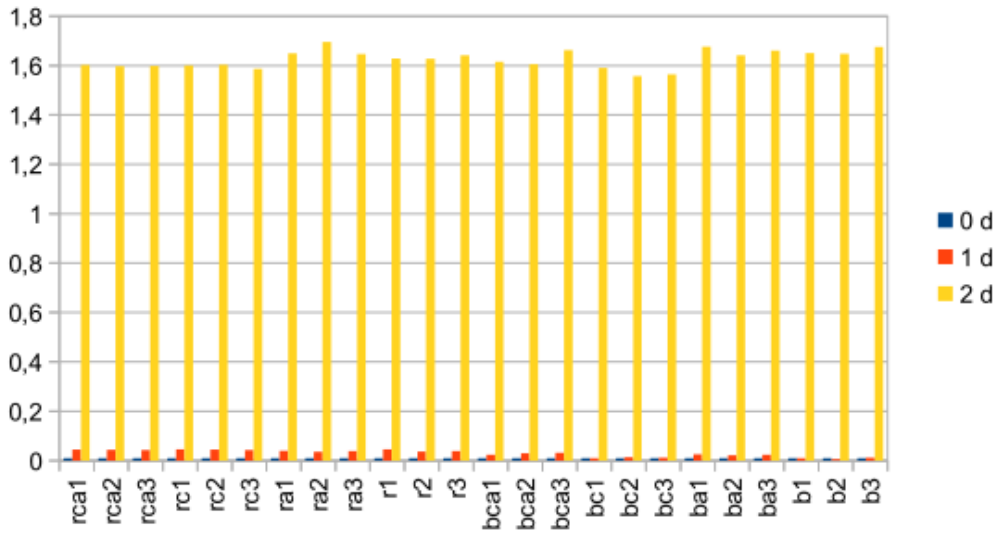

Figure A.6: Growth of *E. coli* WKO^3 CC-Rhodo (the plasmid was not stable) on DeLisa minimal medium supplemented with 5 g/l xylose and 50 mg/l kanamycin with and without 10% CO_2 and 5 mM AMP compared with *E. coli* WKO^3 BB3-AD (vector control) in serum flasks. Three biological replicates were prepared with an initial OD600 of 0,01

plasmid	sugar	CO2	AMP	abbreviation	0 d	1 d	2 d	3 d	6 d	7 d	8 d
CC-Rhodo	arabinose	+	+	rca1	0,001	0,003	0,003	0,003	0,004	0,007	0,010
CC-Rhodo	arabinose	+	+	rca2	0,001	0,003	0,004	0,004	0,006	0,009	0,009
CC-Rhodo	arabinose	+	+	rca3	0,001	0,006	0,006	0,006	0,006	0,010	0,011
CC-Rhodo	arabinose	+	-	rc1	0,001	0,003	0,004	0,003	0,003	0,006	0,009
CC-Rhodo	arabinose	+	-	rc2	0,001	0,005	0,013	0,006	0,006	0,009	0,011
CC-Rhodo	arabinose	+	-	rc3	0,001	0,002	0,005	0,005	0,041	0,399	1,210
CC-Rhodo	arabinose	-	+	ra1	0,001	0,005	0,004	0,004	0,004	0,008	0,014
CC-Rhodo	arabinose	-	+	ra2	0,001	0,004	0,007	0,011	0,102	0,948	1,131
CC-Rhodo	arabinose	-	+	ra3	0,001	0,003	0,005	0,004	0,004	0,007	0,012
CC-Rhodo	arabinose	-	-	r1	0,001	0,007	0,007	0,013	0,414	1,117	1,112
CC-Rhodo	arabinose	-	-	r2	0,001	0,005	0,004	0,004	0,005	0,008	0,009
CC-Rhodo	arabinose	-	-	r3	0,001	0,005	0,004	0,004	0,004	0,009	0,013
BB3-AD	arabinose	+	+	bca1	0,001	0,004	0,002	0,003	0,006	0,008	0,011
BB3-AD	arabinose	+	+	bca2	0,001	0,001	0,002	0,002	0,005	0,011	0,016
BB3-AD	arabinose	+	+	bca3	0,001	0,001	0,002	0,002	0,006	0,009	0,016
BB3-AD	arabinose	+	-	bc1	0,001	0,003	0,003	0,002	0,004	0,010	0,014
BB3-AD	arabinose	+	-	bc2	0,001	0,003	0,002	0,003	0,004	0,012	0,028
BB3-AD	arabinose	+	-	bc3	0,001	0,002	0,002	0,003	0,006	0,011	0,013
BB3-AD	arabinose	-	+	ba1	0,001	0,002	0,003	0,003	0,009	0,014	0,072
BB3-AD	arabinose	-	+	ba2	0,001	0,004	0,003	0,003	0,006	0,006	0,016
BB3-AD	arabinose	-	+	ba3	0,001	0,002	0,003	0,003	0,011	0,034	0,307
BB3-AD	arabinose	-	-	b1	0,001	0,002	0,002	0,003	0,005	0,017	0,256
BB3-AD	arabinose	-	-	b2	0,001	0,003	0,003	0,004	0,005	0,009	0,017
BB3-AD	arabinose	-	-	b3	0,001	0,004	0,002	0,004	0,004	0,011	0,091

inoculation: OD600=0,001
CC-Rhodo was unstable.

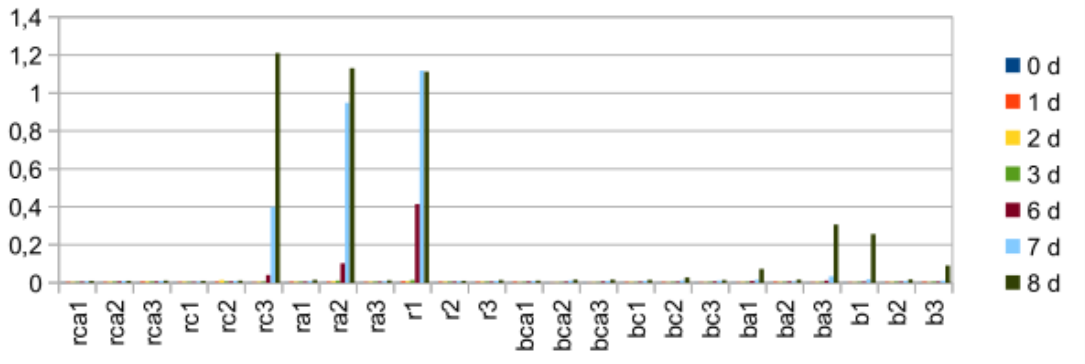

Figure A.7: Growth of *E. coli* WKO^3 CC-Rhodo (the plasmid was not stable) on DeLisa minimal medium supplemented with 5 g/l arabinose and 50 mg/l kanamycin with and without 10% CO_2 and 5 mM AMP compared with *E. coli* WKO^3 BB3-AD (vector control) in serum flasks. Three biological replicates were prepared with an initial OD600 of 0,001

plasmid	sugar	CO2	AMP	abbreviation	0 d	2 d	5 d	6 d
CC-Rhodo	xylose	+	+	rca1	0,001	0,000	0,000	0,000
CC-Rhodo	xylose	+	+	rca2	0,001	0,000	0,000	0,000
CC-Rhodo	xylose	+	+	rca3	0,001	0,000	0,000	0,000
CC-Rhodo	xylose	+	-	rc1	0,001	0,000	0,000	0,000
CC-Rhodo	xylose	+	-	rc2	0,001	0,000	0,000	0,000
CC-Rhodo	xylose	+	-	rc3	0,001	0,000	0,000	0,000
CC-Rhodo	xylose	-	+	ra1	0,001	0,000	0,000	0,000
CC-Rhodo	xylose	-	+	ra2	0,001	0,000	0,000	0,000
CC-Rhodo	xylose	-	+	ra3	0,001	0,000	0,000	0,000
CC-Rhodo	xylose	-	-	r1	0,001	0,000	0,000	0,000
CC-Rhodo	xylose	-	-	r2	0,001	0,000	0,000	0,000
CC-Rhodo	xylose	-	-	r3	0,001	0,000	0,000	0,000
BB3-AD	xylose	+	+	bca1	0,001	0,000	0,000	0,000
BB3-AD	xylose	+	+	bca2	0,001	0,000	0,000	0,000
BB3-AD	xylose	+	+	bca3	0,001	0,000	0,000	0,000
BB3-AD	xylose	+	-	bc1	0,001	0,000	0,000	0,000
BB3-AD	xylose	+	-	bc2	0,001	0,000	0,000	0,000
BB3-AD	xylose	+	-	bc3	0,001	0,000	0,000	0,000
BB3-AD	xylose	-	+	ba1	0,001	0,000	0,000	0,000
BB3-AD	xylose	-	+	ba2	0,001	0,000	0,000	0,000
BB3-AD	xylose	-	+	ba3	0,001	0,000	0,000	0,000
BB3-AD	xylose	-	-	b1	0,001	0,000	0,000	0,000
BB3-AD	xylose	-	-	b2	0,001	0,000	0,000	0,000
BB3-AD	xylose	-	-	b3	0,001	0,000	0,000	0,000

inoculation: OD600=0,001
CC-Rhodo was unstable.

Figure A.8: Growth of *E. coli* WKO^3 CC-Rhodo (the plasmid was not stable) on DeLisa minimal medium supplemented with 10 g/l arabinose and 50 mg/l kanamycin with or without 10% CO_2 and 5 mM AMP compared with *E. coli* WKO^3 BB3-AD (vector control) in serum flasks. Three biological replicates were prepared with an initial OD600 of 0,001

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A.1 Zusammenfassung

Steigende CO_2 Emissionen sind eines der größten Probleme unserer modernen Zeit. Die beste momentan bekannte Möglichkeit diese zu vermindern sind Erneuerbare Energien. Da autotroph lebende Organismen wie Pflanzen und Algen langsam wachsen und komplexe Bioreaktoren benötigen, wurde erfolgreich versucht ob *E. coli* CO_2 fixieren kann wenn er Rubisco exprimiert. In dieser Arbeit wurde versucht diese Methode in *E. coli* W zu übertragen, da dieser wesentlich stressresistenter ist.

Zuerst wurden Wachstums Experimente mit *E. coli* W Δ *pfkA*, Δ *pfkB* und Δ *zwf* (WKO^3) durchgeführt. Diese Knockouts seperieren den oxidativen Pentose Phosphat Weg von der Glykolyse und sollen mit Rubisco und Phosphoribulokinase einen neuen Stoffwechselweg bilden. Die Wachstums Experimente zeigten, dass diese Knockouts nicht ausreichen um *E. coli* W in ein Rubisco abhängiges Wachstum zu zwingen. Daher wurde entschieden, dass zusätzlich das Gen *gapA* ausgeschaltet werden soll.

Der ursprüngliche Plan ein Plasmid, das Rubisco, Phosphoribulokinase und Carboanhydrase codiert in *E. coli* WKO^3 zu transformieren funktionierte nicht, da das Plasmid in diesem Stamm nicht stabil war. Ein fast gelungener Versuch der Transformation wies eine Punktmutation in *prk* auf. Das führt zu der Vermutung, dass Ribulose 1,5 Bisphosphat, das als giftig für die Zellen gilt und das Produkt der Phosphoribulokinase ist, diese Probleme verursachte. Um dieses Problem zu lösen wurde versucht einen Promoter der durch Anhydrotetrazyklin induziert wird (tetP) zu integrieren um die Expression von *prk* zu steuern.

Am Ende konnte weder der Knockout in *gapA* noch das Integrieren des tetP um *prk* zu steuern in der vorgegebenen Zeit realisiert werden.