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Chapter 6

Genetic studies towards a cellulose producing strain of *Synechocystis* sp. PCC 6803

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Abstract

The search for renewable energy sources that can replace liquid fuel has generated a wide variety of biofuel producing strategies. One such strategy focusses on the direct production of fuel compounds by genetically modified cyanobacteria. Production of small carbon/hydrogen-rich molecules has so far been the standard for this approach. Although such compounds can be directly used as biofuel, they are difficult to separate from the growth medium. Production of extracellular, insoluble high-energy compounds, such as cellulose, could circumvent the problem of harvesting. In this study the genes encoding cellulose synthases from *Gluconacetobacter xylinus* and *Crinallium epipsammum* were introduced into *Synechocystis* sp. PCC 6803. Additionally, glycogen metabolism was re-directed towards cellulose production by replacing the native ADP-glucose pyrophosphorylase, with the UDP-glucose pyrophosphorylase from *G. xylinus* or *C. epipsammum*. ADP-glucose is the precursor for glycogen synthesis, while UDP-glucose is the precursor for cellulose synthesis. *Synechocystis* single and double transformants failed to produce quantifiable amounts of cellulose, however a partial rescue of the phenotype of the ADP-glucose pyrophosphorylase knockout was observed in double transformants. Also, an increase in the fluorescence signal from cells treated with the cellulose-binding fluorescent dye Calcofluor White was observed in *Synechocystis* strains containing a heterologously expressed cellulose synthase.

Keywords:

Synechocystis sp. PCC 6803, cyanobacteria, cellulose, biofuel, genetic modification

Introduction

The energy demand of human society is covered mainly by the oxidation of carbon-based fossil fuels, a process that leads to the release of CO₂ into the atmosphere. Levels of atmospheric CO₂ have been rising steadily since the late nineteenth century with a strong acceleration starting in the mid-twentieth century. Meanwhile, worldwide energy consumption is still on the rise, while crude oil reserves are becoming depleted [287,288]. To meet the world's energy demand and to prevent a further rise in atmospheric CO₂ levels a more sustainable alternative to fossil fuels is required. Renewable energy sources such as wind- hydro- and solar-electricity will play a major role in this [288], but they cannot replace liquid fuels in specific applications like in aviation. To this end a great deal of effort has been put into the production of sustainable biofuels, both by scientists, governments and commercial companies [289,290]. Currently, biofuels produced from biomass contribute about 10 % of the world's energy demand [291]. Three so-called generations of biofuels are currently available; (i) biofuels from the edible part of plant biomass, (ii) biofuels from non-edible plant biomass and (iii) biodiesel derived from photosynthetic microorganisms [288]. The first generation of biofuels production systems suffer from several major drawbacks since they compete with food supply (i), require large amounts of arable land and water for irrigation, and leave a huge amount of bio-waste [222]. Because third generation biofuels do not share these drawbacks, they have received a great deal of attention in recent years and great strides in large-scale production of these biofuels have been made [29,168,288,292]. Within the third generation of biofuel production systems three variations can be identified; (i) production of natural strains of cyanobacteria, micro- and macro-algae for biomass, often as a food or feed source [293-295] or as a feed stock for fermentation [27,296], (ii) trans-esterification of the lipid fraction of (mostly) green algae [297,298] and (iii) genetic modification of (mostly) cyanobacteria for the direct conversion of CO₂ into biofuel compounds [16-18]. Cyanobacteria are genetically more accessible and have a higher photosynthetic efficiency [222] than green algae and plants, making them more suitable for the latter approach, which is also known as the 'photanol approach' [4]. Most of these studies have focused on the production of small carbon/hydrogen-rich molecules that accumulate in the growth medium outside of the cell (e.g. ethanol and butanol) [16,299]. This bypasses the internal storage capacity of the cells but it complicates

the harvesting process because most compounds remain dissolved in a large amount of water, resulting in dilute and difficult to separate biofuel solutions. Production of highly volatile or insoluble products could circumvent this problem. A good candidate for an insoluble product is cellulose.

Cellulose is a β -(1,4) glucan composed entirely of D-glucose molecules and it is a major component of plant biomass. Combined with considerable amounts of lignin this plant cellulose is difficult to process, highly resistant to degradation and hardly suitable for biofuel production. However, cellulose is also produced by various chemotrophic bacteria, cyanobacteria and algae [22,24]. The cellulose in these organisms is not associated with lignin and therefore much easier to process. One of the first cyanobacteria that was identified as a cellulose producer is *Crinalium epipsammum*, a biofilm forming filamentous cyanobacterium that, presumably, produces non-crystalline cellulose as a part of its thick cell wall [23]. By optimization of the physiological conditions *C. epipsammum* can produce cellulose to up to 40 % of its dry weight [Chapter 5]. The chemotrophic bacterium *Gluconacetobacter xylinus*, one of the most studied cellulose-producing bacteria, produces up to 50 % of its dry weight as extracellular crystalline cellulose, which can lead to formation of a thick pellicle in static cultures [22]. The General Introduction of this thesis gives a more detailed description of biofuels and cellulose production in bacteria.

In this study we introduced the cellulose synthase genes from *G. xylinus* and *C. epipsammum* into the genetically modifiable cyanobacterial model organism *Synechocystis* sp. PCC 6803 (henceforth *Synechocystis*). *Synechocystis* is one of the best-characterized cyanobacteria, it is naturally transformable [300] and its genome has been sequenced [301], annotated and is publicly available at CyanoBase [302]. The genes encoding the catalytically active core of the cellulose synthase of *G. xylinus*, *bcsAB* [55], have already been successfully introduced into *Synechococcus* PCC 7942 [25] and for this study these two genes were also selected. Much less is known about the genetic make-up of *C. epipsammum*, in part because this organism was only recently sequenced [258]. The gene with the highest homology to known cellulose synthase genes was selected and named *ccsA* (for: cyanobacterial cellulose synthase A); significantly, no homologs of *bcsB* could be identified in the genome of *C. epipsammum*. All cloning procedures were carried out using the “BioBrick” strategy [303].

The major carbon and energy reserve of *Synechocystis* is glycogen, a branched glucose polymer consisting of linear chains of glucose residues with further chains branching off every 8 to 12 glucose molecules. The glucose monomers are linked linearly by α -(1,4) glycosidic bonds and at branch-points via α -(1,6) glycosidic bonds. Glycogen is synthesized from ADP-glucose, while cellulose is synthesized from UDP-glucose. So knocking out the pathway of photosynthetically fixed carbon to glycogen could be a good approach to increase cellulose production [29]. In addition to the cellulose synthase genes we also introduced the UDP-glucose pyrophosphorylase (UGPase) genes from *G. xylinus* (*ugpG*) and *C. epipsammum* (*ugpC*, annotated as UGPase and closest homolog to *ugpG* in the *C. epipsammum* genome) into the genome of *Synechocystis* so that they replace the native, deleted, ADP-glucose pyrophosphorylase (AGPase) gene (*glgC*, *slr1176*).

Results

Construction of cellulose synthase (*cs*) and UGPase (*ugp*)-carrying *Synechocystis* strains

The *bcsA* and *bcsB* genes of *G. xylinus* were introduced into (the genome of) *Synechocystis* as two separate operons, each with their own promoter and terminator (*bcsAB*), as well as in the form of a single operon (*bcsAB1op*). The catalytic subunit A of this cellulose synthase was also introduced separately (as *bcsA*). The cellulose synthase from *C. epipsammum* has only one subunit, which has been introduced in *Synechocystis* as *ccsA*. All gene constructs were placed under the control of the very strong *Trc* promoter. *Ugp* genes were introduced both with a $P_{trc}(ugp)$ and without (*ugpNT – no Trc*), placing the gene under the control of the native *glgC* promoter in the latter condition. The BioBrick BBa_B0014 transcriptional terminator was placed downstream of each gene, or operon, of interest [304]. All genes were codon-optimized for *Synechocystis* and synthesized by Genscript. Further cloning steps were conducted using the 'BioBrick' cloning strategy [303]. All artificial gene constructs under P_{trc} control were introduced into *Synechocystis* on a self-replicating plasmid via conjugation. Additionally, all genes were cloned into an integration vector, to achieve stable chromosomal incorporation (Fig. 6.1). For the *ugp* genes chromosomal incorporation was combined with the deletion of the native AGPase gene *glgC* because the *ugp* genes were targeted to the *glgC* gene site in *Synechocystis*, to fully replace the *glgC*

open reading frame. The cellulose synthase genes were introduced into the neutral docking site *slr0168* of *Synechocystis*.

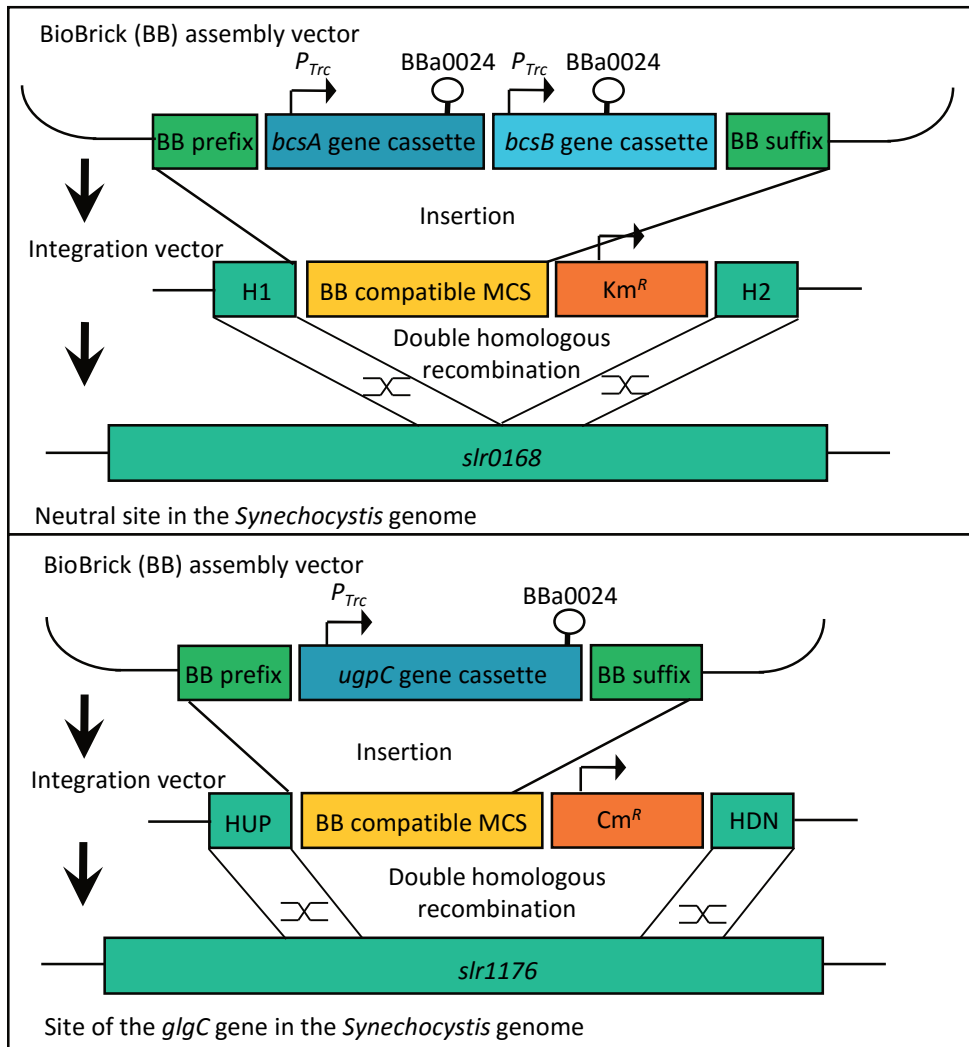


Figure 6.1. Schematic representation of the integration strategy of *bcsAB* into the neutral docking site *slr0168* and of *ugpC* in the *slr1176* (*glgC*) site of the genome of *Synechocystis*.

Both conjugal transfer and chromosomal integration of the *bcs* gene constructs has been successful (Fig 6.2A,B), in contrast, however to the introduction of the *ccsA* gene from *C. epipsammum*. Introduction of the *ugp* genes into the *glgC* site was not possible in the wild-type background. For the generation of *ugpC*-only mutants (i.e. mutants without a cellulose synthase gene), a $\Delta glgC$ mutant containing a kanamycin resistance cassette, was

used instead. In this way a strain containing the *ugpC* gene under control of the native *glgC* promoter (*ugpCnT*) was generated (Fig. 6.2C). Higher transformation frequencies were obtained when the *ugp* gene was introduced into strains that already contained a (heterologous) cellulose synthase; all *ugp* constructs could be introduced in strains with this genetic background. However, genome segregation in these strains was very slow and in many cases still incomplete at the end of this study (Fig 6.2B,D). Table 6.1 provides an overview of the *Synechocystis* strains generated in this study, together with the nomenclature that we have used and their genome-segregation status at the end of the study.

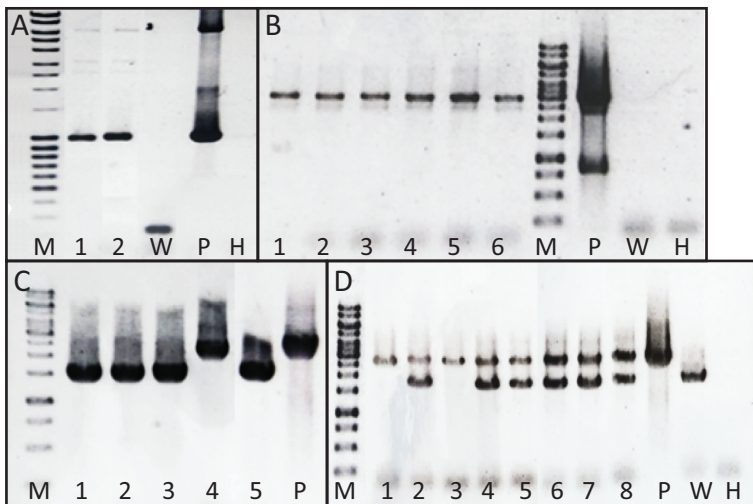


Figure 6.2. PCR analysis of partially- and fully segregated single- and double *Synechocystis* mutants. M, DNA marker; W, wild type; P, corresponding plasmid; H, H₂O. A. fully segregated *bcsAB* genes; 1, *bcsAB*; 2, *bcsAB1op*. B. Successful introduction of self-replicating plasmids containing *bcs* gene cassettes in the wild type and the *ugpCnT* strain; 1, *pVZ-bcsAB*; 2, *pVZ-bcsAB1op*; 3, *pVZ-bcsA*; 4, *vABCnT*; 5, *v1opCnT*; 6, *vACnT*. C. successful and unsuccessful introduction of *ugp* genes in the Δ *glgC* strain; 1, *ugpG*; 2, *ugpC*; 3, *ugpGnT*; 4, *ugpCnT*; 5, Δ *glgC*. D. partial segregation of *ugp* genes in *bcs* containing *Synechocystis* strains; 1, *ABCnT*; 2, *ABC*; 3, *1opC*; 4, *AC*; 5, *vAGnT*; 6, *vABGnT*; 7, *v1opGnT*; 8, *1opG*.

	1. <i>bcsAB</i>	2. <i>bcsAB-1op</i>	3. <i>bcsA</i>	4. <i>pVZ-bcsAB</i>	5. <i>pVZ-bcsAB1op</i>	6. <i>pVZ-bcsA</i>
7. <i>ugpG</i>	13. <i>ABG</i>	17. <i>1opG</i>	21. <i>AG</i>	25. <i>vABG</i>	29. <i>v1opG</i>	33. <i>vAG</i>
8. <i>ugpC</i>	14. <i>ABC</i>	18. <i>1opC</i>	22. <i>AC</i>	26. <i>vABC</i>	30. <i>v1opC</i>	34. <i>vAC</i>
9. <i>ugp-GnT</i>	15. <i>ABGnT</i>	19. <i>1opGnT</i>	23. <i>AGnT</i>	27. <i>vAB-GnT</i>	31. <i>v1op-GnT</i>	35. <i>vAGnT</i>
10. <i>ugp-CnT</i>	16. <i>ABCnT</i>	20. <i>1opCnT</i>	24. <i>ACnT</i>	28. <i>vAB-CnT</i>	32. <i>v1op-CnT</i>	36. <i>vACnT</i>
11. <i>pVZ-ugpG</i>	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
12. <i>pVZ-ugpC</i>	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Table 6.1. Matrix representation of the genetic status of all the *Synechocystis* single- and double mutants generated in this study. White, fully segregated; light grey, *ugp* genes not fully segregated; dark grey, strains that could not be constructed.

Fluorescence based assay for identification of cellulose-producing *Synechocystis* strains

Single- and double mutants either fully segregated or not, were first characterized based on their growth characteristics. Then, the presence of cellulose was analyzed with two methods: using the enzymatic assay described in Chapter 5, and by staining with Calcofluor White (CFW). The enzymatic assay used to quantify cellulose in *C. epipsammum* proved not sensitive enough to detect cellulose in any of the recombinant *Synechocystis* strains and therefore the CFW staining was used as the main assay to determine the presence of cellulose in this study. CFW is a fluorescent dye that binds β 1,3- and β 1,4-linked polysaccharides like cellulose and chitin [305]. It is used commercially as a whitening agent for paper and textile. In microbiological research it is used in clinical mycology and in parasitology to stain the cell walls of a variety of fungi, such as *Aspergillus* sp., *Candida albicans* and *Saccharomyces cerevisiae* [282]. Fungal cell walls are stained very rapidly with CFW and typical protocols indicate that only a few minutes of incubation suffices to saturate the CFW binding [282]. This proved to be different for cyanobacteria: For these Gram-negative bacteria considerably longer incubation times are required (Fig. 6.3A). The CFW assay was tested and optimized for *Crinallium epipsammum* cells and a protocol was developed: Cyanobacterial cells are mixed 1:1 (v/v) with a 0.1 mg ml⁻¹ solution of CFW (10 times more dilute than described in [282]) and incubated in the dark for 2 to 3 hours. The use of higher concentrations of CFW increases staining only slightly and leads to

significantly higher background signals (data not shown). After staining, the cells are washed once with fresh medium to minimize the background fluorescence due to unbound CFW. The cells are then immobilized by placing them on a thin layer of a solid 1 % (w/v) agar substrate. Microscope slides and cover slips are cleaned prior to use with a wet, non-cellulose containing cloth, to minimize the amount of brightly-stained contamination, such as paper dust. Samples are analyzed by fluorescence microscopy using the 365/420-470 excitation/emission filter set for CFW fluorescence. CFW absorbs in the range of 300 to 400 nm, with a maximum at 347 nm, and emits fluorescence maximally at 420 nm [282]. Unfortunately, CFW fluorescence bleaches rapidly under the conditions used: Already after 30 seconds up to 75% of the initially observed fluorescence signal in *C. epipsammum* samples is lost (Fig. 6.3B). To ensure a reproducible signal, image focusing was carried out using the cells' natural fluorescence with a 550-580/590-650 excitation/emission filter set and a fixed exposure time in the camera setting, so that pictures could be taken immediately after exposure to the excitation light. The natural fluorescence of the cells was also used as a live stain to ensure that only living/viable cells were analyzed. For further detail see Materials & Methods.

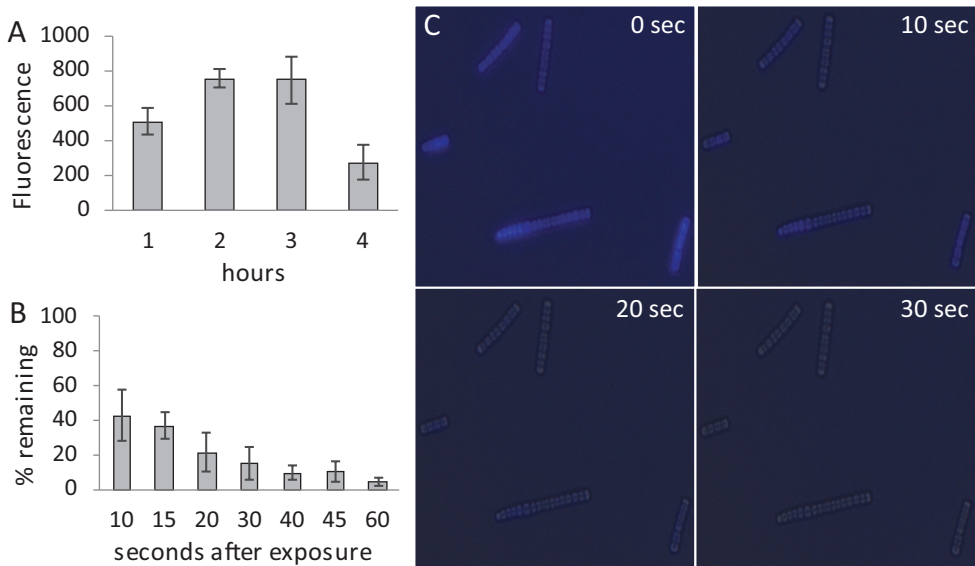


Figure 6.3. Development of a fluorescence (Calcofluor White; CFW) based assay for cellulose synthesis in *Synechocystis* sp. PCC 6803. A; Amount of blue fluorescence observed per cell after various times of incubation with CFW. B and C; Loss of CFW fluorescence over time during exposure to excitation light. Data in A and B was acquired by ImageJ analysis of 3 individual images and ≥ 3 cells per image. Fluorescence intensity was calculated as the corrected total cell fluorescence (CTCF) = integrated pixel density of a filament - (area of a filament * background mean pixel density) as described on <http://sciencetechblog.com/2011/05/24/measuring-cell-fluorescence-using-imagej/>.

Physiological characterization of *bcs* and *ugp* containing *Synechocystis* strains

Single- and double mutants were grown in BG-11 mineral medium complemented with 25 mM NaHCO_3 under ambient air and illuminated with $30 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ white fluorescent light at 30°C in a shaking incubator at 120 rpm. Growth characteristics of single mutants containing cellulose synthase genes, or the replicative plasmid *pVZ-ugpC*, are indistinguishable from WT (Fig. 6.4A), while the mutant containing *ugpCnT* grows at a similar rate as the ΔglgC knockout (Table 6.2), but with a shorter lag phase. Double mutants on the other hand, that lack the *glgC* gene or are at least compromised in its expression by partial segregation, show growth characteristics that are somewhere in between those of the WT and the *ugpCnT/\Delta\text{glgC}* strain. The presence of cellulose was not detected in any of the transformants and transconjugants, using the enzymatic cellulose assay described in Chapter 5 (data not shown). However, the Calcofluor White assay showed slightly increased binding of CFW to transformants containing cellulose synthase genes, and even stronger binding in transformants containing both a *bcs* and a *ugp* gene (Fig. 6.5).

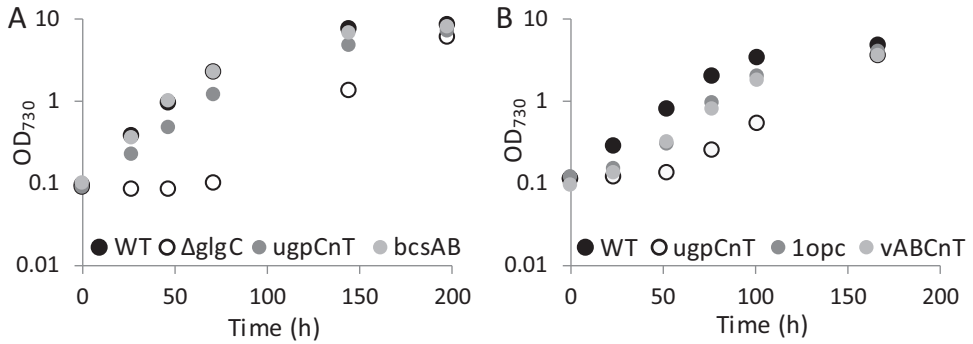


Figure 6.4. Growth curves of *Synechocystis* WT, the $\Delta glgC$ mutant, and representative single (A) and double (B) mutants generated in this study.

	μ -exp (% of WT)	μ -exp (% of WT)	μ -exp (% of WT)	μ -exp (% of WT)
<i>ΔglgC</i>	69	<i>ABg</i> 95	<i>Ag</i> 94	<i>v1opg</i> 61
<i>bcsAB</i>	93	<i>ABc</i> 88	<i>Ac</i> 66	<i>v1opc</i> 63
<i>bcsAB1op</i>	106	<i>ABgnT</i> 58	<i>AgnT</i> 100	<i>v1opgn</i> 105 <i>T</i>
<i>bcsA</i>	97	<i>ABCnT</i> 105	<i>AcnT</i> 78	<i>v1opc nT</i> 93
<i>UGPc nT</i>	82	<i>1opg</i> 91	<i>vABg</i> 79	<i>vAg</i> 76
<i>pVZ-bcsAB</i>	103	<i>1opc</i> 106	<i>vABc</i> 89	<i>vAc</i> 79
<i>pVZ-bcsAB1op</i>	97	<i>1opgnT</i> 93	<i>vABGnT</i> 81	<i>vAgnT</i> 85
<i>pVZ-bcsA</i>	109	<i>1opc nT</i> 84	<i>vABCnT</i> 95	<i>vAcnT</i> 93
<i>pVZ-UGPc</i>	107			

Table 6.2. Growth rate of transgenic *Synechocystis* strains in the exponential growth phase as percentage of the WT exponential growth rate derived from experiments as shown in figure 6.5. Data was derived from biological triplicates; the standard deviation was less than 10 %.

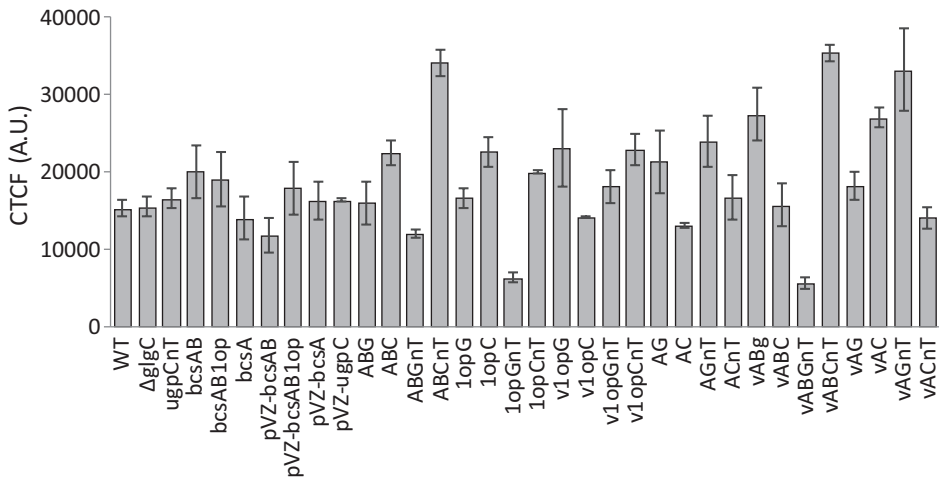


Figure 6.5. Fluorescence intensity of Calcofluor White-stained *Synechocystis* cells, derived from photographs of cells taken at the end of the growth experiments depicted in figure 6.4. The fluorescence intensity was calculated as described in Materials & Methods (see also Fig. 6.3). The black line indicates the average level of fluorescence emission from wild-type cells. No fluorescence was detected from non-stained cells (data not shown).

Application of stress conditions to increase cellulose production

To increase substrate availability for cellulose synthesis, in follow-up experiments the segregated double mutants (*i.e.* containing a cellulose synthase and a UGPase) were grown on a medium containing added glucose (5 mM), and were subjected to nitrogen deprivation. The $\Delta glgC$ mutant accumulates large amounts of the metabolic intermediates pyruvate and α -keto-glutarate. Because of this, the strain cannot grow in the presence of glucose [306], and it is impaired in its nitrogen deprivation response, resulting in a non-bleaching phenotype (further detail is given in the Discussion section). The fully segregated double mutants, however, have an alternative pathway to process the excess metabolic intermediates via cellulose production, which may repress the $\Delta glgC$ phenotype.

After an extended lag phase the *1opC* and *ABCnT* mutants (Table 6.1) started to grow in the presence of glucose. But, using the enzymatic assay no cellulose was detected and also no significant change in CFW binding was observed (data not shown). Upon nitrogen starvation the *ugp* containing mutants show a bleaching response that lies somewhere in between the WT and the *glgC* knockout (Fig 6.6A,B). This implies that at least the *ugp* gene is expressed and that the UDP-glucose pyrophosphorylase enzyme managed to funnel a part of the excess intermediates into cellulose synthesis or other pathways.

Unfortunately, no cellulose was detected in cells from these cultures. In this experiment we also determined the amount of glycogen per cell, to ensure that no glycogen is produced in the $\Delta glgC$ strains. Figure 6.6C shows that the *ABCnT*, *vABC* and *1opC* strains still produced some glycogen. These were strains in which the *ugp* gene was introduced into *Synechocystis* containing a cellulose synthase gene and the *glgC* gene. Segregation was difficult in the strains that were generated this way and although the control PCR (Fig. 6.6D) does not show any visible WT bands for these strains (note that the kanamycin box is almost the same size as the *glgC* gene; 1212 vs 1320 bp), the *glgC* gene may not have been fully deleted, which would explain the residual production of glycogen.

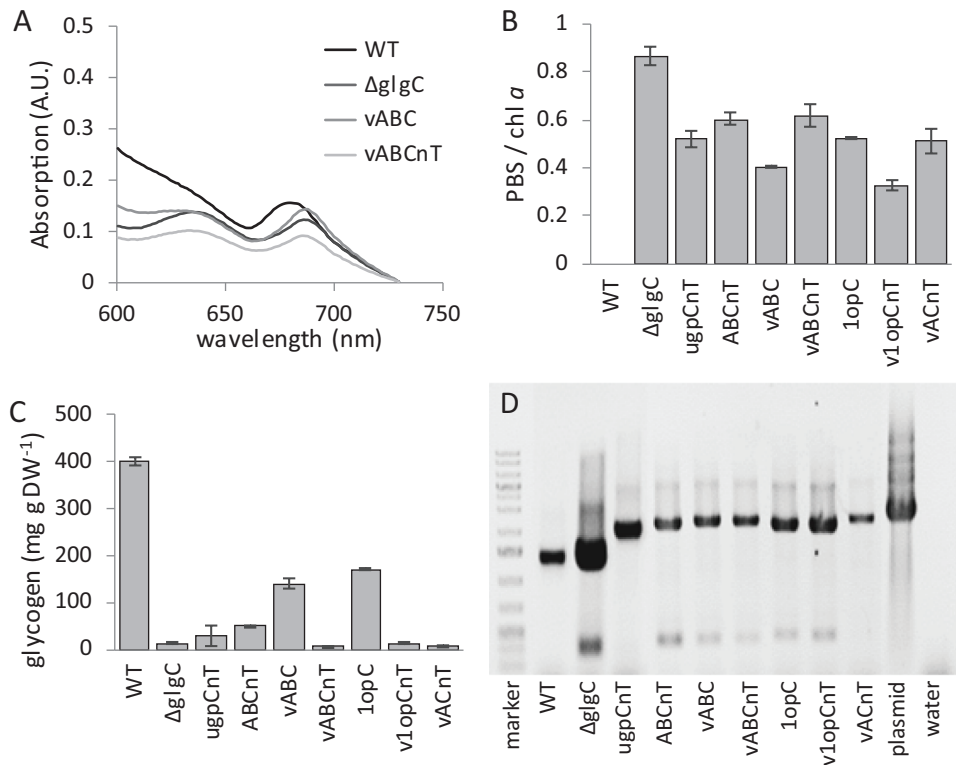


Figure 6.6. Nitrogen-deprivation induced chlorosis response of wild type *Synechocystis* and some of the fully segregated cellulose producing mutant strains after 96 hour incubation. A, Absorption spectra of representatives of the different strains studied, showing phycobilisome (630 nm) and chl *a* (680 nm) absorption peaks. B, Ratio of phycobilisome (PBS) and chl *a* absorbance of all strains studied, based on the absorption spectra shown in panel A. Peak area was determined using the peak fitting add-in for excel from <http://www.chem.qmul.ac.uk> on the raw data. C, glycogen content of the cells after nitrogen deprivation. D, gel electrophoresis image of DNA fragments generated by PCR with

specific glgC flanking primers to test possible reversion of the segregation process. Error bars show the range of biological duplicates.

Discussion

Genes for cellulose synthesis were introduced into *Synechocystis* PCC 6803 and correct chromosomal integration was confirmed by PCR analysis and sequencing of the PCR products. However, none of the transgenic strains displayed cellulose levels above the detection limit of the quantitative enzymatic assay for cellulose used in this study and under any of the conditions tested. The enzymatic assay, as described in Chapter 5, suffers from high levels of background signal in the glucose quantitation assay. The cellulose that was potentially produced by *Synechocystis* was insufficient to exceed this background signal. To localize the small amount of cellulose that nevertheless might be formed, we used the fluorescent dye Calcofluor White (CFW), that binds β 1,3- and β 1,4-linked polysaccharides like cellulose [282,305]. CFW bound to both transgenic- and to WT *Synechocystis* cells, possibly because the extracellular matrix of *Synechocystis* contains polysaccharides that are similar to cellulose. *Synechocystis* contains cellulose synthase like genes and X-ray diffraction patterns of the EPS of *Synechocystis* ATCC 27184 indicated the presence of a crystalline product similar to cellulose [24]. However, *Synechocystis* strains that contain cellulose synthase genes showed in general higher levels of fluorescence than strains that did not contain these genes, indicating that there may have been some (additional) production of extracellular cellulose. A similar approach to cellulose detection and quantitation in cyanobacteria can be found in the recent literature [25-28,41], where cellulose is always quantified using the cellulose hydrolysis - glucose quantitation assay, and cellulose is localized by either CFW staining [28,41] and/or immunogold labeling [25,28]. In Zhao et al. [28] both cellulose quantitation and CFW staining are used. A crude comparison of the CFW figure provided in this article (Fig. 6) with our own CFW images, using the comparative chl *a* fluorescence signal as a modulator, shows that the CFW fluorescence intensity reported in this chapter indicates a cellulose content of 2 % of dry weight on average for the strains that contain a cellulose synthase, with a maximum nearly 4 %. The presumably α -specific signal observed in the strains that do not contain a cellulose synthase

indicates the presence of a cellulose resembling polysaccharide at 1.7 % of dry weight, assuming a similar CFW-binding capacity of this latter polymer.

Without ADP-glucose synthesis (due to *glgC* deletion), and the resulting impairment of glycogen production, the carbon metabolism of *Synechocystis* is perturbed. As a consequence, pyruvate and α -keto-glutarate, the end products of glycolysis and of the incomplete TCA cycle, respectively, accumulate to high levels in the cell, disrupting certain cellular signaling pathways [306,307]. These compounds can accumulate to such high levels that they leak into the extracellular medium [306]. Because of the profound effect of deleting the AGPase gene (*glgC*), the resulting deletion mutant has a lower growth rate than WT and often suffers from a long lag phase in batch growth experiments. Additionally, this mutant is sensitive to addition of glucose to the medium and cannot grow in its presence. This mutant is also defective in its nitrogen stress response [306]. Replacing the *glgC* gene with a gene encoding a UGPase, to increase substrate levels for cellulose production, proved impossible in the WT *Synechocystis* strain. Attempts to incorporate the *ugp* gene were successful in a Δ *glgC* strain, but only for the *ugp* gene from *C. epipsammum* when it was expressed under the control of its native promoter (*ugpCnT*). This strain had similar growth characteristics as the Δ *glgC* mutant, but without the extensive lag phase. However, in the *Synechocystis* strains already containing cellulose synthase genes, incorporation of all *ugp* gene constructs was possible in a WT background and in some cases full segregation eventually could be demonstrated. These double mutants grew slower than the WT but faster than the Δ *glgC* and Δ *glgC/ugpCnT* strains. This implies that the combination of UGPase and cellulose synthase partially rescues the Δ *glgC* phenotype, most likely by redirecting part of the carbon molecules that were destined for glycogen synthesis into cellulose, thus potentially lowering the accumulation of pyruvate and α -keto-glutarate. Under nitrogen starvation conditions we see a similar trend: WT *Synechocystis* uses its phycobilisome antennae for nitrogen supply, and consequently turns yellow due to chlorosis, while the Δ *glgC* strain retains its antennae and its color. The fully segregated *bcs(AB)/ugp* strains show an intermediate response, with intermediate PBS/chl *a* ratios, again implying a partial rescue of the chlorosis phenotype. The fully segregated double mutant strains were also cultivated in the presence of glucose. After a long lag phase only

the *1opC* and *ABCnT* mutants displayed growth, but still no cellulose could be detected with the enzymatic assay.

The cellulose synthase enzyme of *Acetobacter xylinum* is activated by binding to the second messenger compound cyclic diguanylic acid (c-di-GMP) [35]. In bacteria c-di-GMP is involved in regulation of cell surface-associated traits [74], such as motility [308], cell aggregation [309] and biofilm formation [310]. More information on the role of c-di-GMP can be found in the General Introduction of this thesis. In cyanobacteria c-di-GMP levels can be influenced by light via cyanobacteriochromes [308,309,311] and in *Synechocystis* blue light can increase c-di-GMP levels to inhibit phototaxis towards blue light [308]. To test whether cellulose synthase can be activated with light in *Synechocystis*, single and double mutants were grown predominantly blue light (40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ 435 nm blue LED light + 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ 650 nm red LED light), but no change in the growth characteristics or cellulose content was observed, compared to white light conditions, for any of the strains tested (data not shown).

Hence, we are left with several indications that the introduced cellulose synthases and UGPases do have an effect on the physiology of *Synechocystis* but it was not possible to demonstrate the presence of cellulose with the enzymatic assay quantitatively. In cellulose-producing organisms cellulose synthases and auxiliary proteins form highly ordered membrane-bound structures on the plasma membrane, known as terminal complexes (TCs) [28,32,34]. These TCs facilitate production and protrusion of the individual cellulose fibrils through and out of the cell membrane [312,313] and the organized placement of the TCs in the membrane is necessary for mutual interaction and crystallization of the fibrils [22,32]. A more elaborate description of cellulose synthesis is given in the General Introduction of this thesis. WT *Synechocystis* does not produce cellulose, nor does it contain cellulose synthase genes. It does possess some cellulose synthase-like genes, and other β -glucosyl transferase genes, but there is insufficient homology to identify the latter as actual cellulose synthase genes [24]. This implies that *Synechocystis* presumably will not possess the auxiliary proteins required to form TCs nor the regulation required to form structured arrays of TCs on the plasma membrane. Additionally, *Synechocystis* PCC 6714 possesses a highly cross-linked and thick cell wall, resembling the cell wall of Gram-positive bacteria [314]. Hence, although the *Synechocystis* mutants described in this study may be able to make cellulose

fibrils, they may be of insufficient length and in insufficient quantity to be identifiable as cellulose, or the fibrils may remain in the periplasmic space or so closely associated with the cell wall that they cannot be reached by cellulase enzymes.

In recent years, several studies on (enhanced) cellulose production in cyanobacteria have been published [25-28,41]. Three of these studies describe introduction of cellulose synthase genes into *Synechococcus* (PCC 7942 and PCC 7002) [25,27,28]. *Synechococcus* contains cellulose synthase genes endogenously [24,28] and should therefore have the machinery to properly produce and export cellulose fibrils. In the initial study in *Synechococcus* only the *bcsAB* genes from *G. xylinus* were introduced into *Synechococcus* PCC 7942 [25] and cellulose production was confirmed and quantified. This is similar to the work described in this study. In *Synechococcus* 1.1 mg cellulose g⁻¹ wet weight could be produced, which was about 4-fold higher than the level of cellulose produced in the corresponding wild type strain [25]. Production of cellulose in *Synechococcus* was subsequently enhanced by introduction of an additional bicarbonate transport system [27] and, independently, by introduction of most of the cellulose synthase operon of *G. xylinus*, including the *bcsC* and *bcsD* genes, involved in cellulose fibril extrusion and crystallization [34,59], in combination with deletion of the native cellulose synthase gene [28]. In this latter study cellulose levels were observed of up to 13 % of the cells' dry weight under hyposaline conditions. With this cellulose content this transgenic *Synechococcus* strain is similar to *C. epipsammum*, which produces around 10 % (w/w) cellulose under non-stress conditions [Chapter 5].

Introduction of additional genes involved in cellulose synthesis into *Synechocystis* could potentially induce (enzyme-assay detectable levels of) cellulose synthesis and may even lead to overproduction, especially in a Δ *glgC* background. In *Synechococcus* the high production levels reported in [28] could only be achieved after the native cellulose synthase was removed, demonstrating that a lack of regulation of the cellulose synthesis process may in this case be beneficial to increase production levels. Furthermore, induction of c-di-GMP production by illumination of the cells with blue light, or diguanylate cyclase over-expression, could aid in further increasing cellulose levels. Also general physiological optimization of the cell in terms of enhancing photosynthetic efficiency [216,315] and/or carbon fixation [27,316] can contribute to this. Of course all these suggestions can also be

applied to *Synechococcus*, an organism in which transgenic cellulose synthesis has already been demonstrated.

The only remaining question is: Can such a heavily modified organism outcompete natural strains of cyanobacteria for cellulose production? In *C. epipsammum* cellulose production can be increased to around 30 % of dry its weight by manipulation of the culture conditions [Chapter 5] and with further manipulations, this number may further increase. The above-mentioned genetic modifications and manipulations of the physiological conditions may be able to increase cellulose production in *Synechococcus* to the same, or even above, the level found in *C. epipsammum*. However, these manipulations may not outweigh the (legislative) benefit of working with a natural strain since both *Synechococcus* sp. and *C. epipsammum* are suitable for mass cultivation. *Synechococcus* does have significantly higher growth rates and there is more genetic and physiological background information available for this organism. But for large-scale production, strain selection will have to depend largely on what is economically and legally more favorable, e.g. a natural or a transgenic strain.

Materials and Methods

Strains and growth conditions.

All cloning procedures were carried out in *Escherichia coli* XL-1 (blue) (Stratagene), grown in liquid LB medium at 37°C in a shaking incubator at 200 rpm or on solidified LB plates containing 1 % (w/v) agar. When appropriate, media were supplemented with antibiotics needed for propagation of a specific plasmid(s) or marker(s). Concentrations of antibiotics used alone or in combination were 50 µg ml⁻¹ kanamycin, 35 µg ml⁻¹ chloramphenicol, and 100 µg ml⁻¹ ampicillin. *Synechocystis* sp. strain PCC 6803 (a glucose-tolerant derivative, obtained from D. Bhaya, University of Stanford, Stanford, CA) was routinely cultivated in BG-11 medium [163] (Cyanobacteria BG-11 freshwater solution; Sigma) at 30°C in a shaking incubator at 120 rpm (Innova 43; New Brunswick Scientific) under constant 15 W cool fluorescent white-light illumination (30 µmol photons m⁻² s⁻¹) (F15T8-PL/AQ; General Electric). Cultures were supplemented with 25 mM NaHCO₃ (Sigma). Antibiotic-resistant mutant strains were grown in medium containing 50 µg ml⁻¹ kanamycin and/or 15 µg ml⁻¹

chloramphenicol. For plates, BG-11 medium was supplemented with 1 % (w/v) agar, 5 mM glucose, 0.3% (w/v) sodium thiosulfate [317], and 50 µg/ml kanamycin and/or 35 µg ml⁻¹ chloramphenicol (for resistant strains only). Concentrated stocks of cells were stored at -80°C in BG-11 medium supplemented with 5 % (v/v) dimethyl sulfoxide. For growth experiments cells from stationary phase pre-cultures were diluted to an optical density at 730 nm (OD₇₃₀) of 0.1 at the start of the experiment. Growth was monitored by recording the OD₇₃₀ (Lightwave II spectrophotometer; Biochrom).

Construction of gene cassettes.

Genes of interest were codon optimized according to [318] and at Genscript. The synthetic gene construct was designed to include BioBrick-compatible restriction sites that were used for further standard cloning procedures [303,319]. The sequence of the promoter P_{trc} was taken from [320], P_{trc} has been successfully used in *Synechocystis* [321]. The transcriptional terminator used also was a BioBrick (BBa_B0014), which was obtained from the Registry of Standard Biological Parts (<http://partsregistry.org>). Restriction enzymes and T4 DNA ligase were obtained from Fermentas. The ZR Plasmid Miniprep-Classic kit (Epigenetics) was used for plasmid preparation, according to the manufacturer's instructions. DNA fragments were purified using the MSB Spin PCRapace kit (Invitex) according to the manufacturer's instructions. The plasmids used and constructed in this study are listed in Table S6.1.

Transformation and conjugation of *Synechocystis*.

Natural transformation of *Synechocystis* was performed as described in [17,322,323]. Cellulose synthase encoding genes were paired with a cassette conferring kanamycin resistance and incorporated into the neutral site *slr0168*. UGPase-encoding genes were paired with a cassette conferring chloramphenicol resistance and inserted in place of the *glgC* (*slr1176*) reading frame. After full segregation was achieved, mutants were verified regarding the correct incorporation of the genes of interest with colony PCR, employing gene specific primers in the flanking regions (Table S6.2) and via sequencing of the PCR product (Macrogen Europe, The Netherlands) with dedicated primers (Table S6.2). Conjugation to *Synechocystis* was performed essentially as described in [18,324]. All conjugation plasmids conferred kanamycin resistance. The plasmids and strains that were constructed for this study are listed in Table S6.1.

Physiological characterization

WT and mutant strains of *Synechocystis* were inoculated from pre-cultures to a final $OD_{730} = 0.1$ into BG-11 medium with 25 mM $NaHCO_3$ and appropriate antibiotics (50 $\mu g\ ml^{-1}$ kanamycine / 17.5 $\mu g\ ml^{-1}$ chloramphenicol) and grown as described above. Growth (via OD_{730}) was monitored for 9-10 days and at the final time point samples for cellulose quantitation and Calcofluor White staining were taken. For nitrogen starvation assays pre-cultures with an OD_{730} of around 0.5 were washed and resuspended in BG-11 medium without nitrate and incubated for 4 days under the conditions described above. After nitrogen deprivation treatment the glycogen- and cellulose content were determined, room temperature UV/Vis spectra were recorded on a SpectroStar Nano UV/Vis spectrometer, and 1 μl of cell concentrate (cells resuspended at $OD_{730} = 100$) was used for PCR analysis to analyze/exclude WT reversion and loss of the *ugp* insert.

Calcofluor White (CFW) staining and analysis

A 30 μl cell sample was taken at the end of the growth experiments and mixed with 30 μl of a CFW working stock (0.1 mg ml^{-1} CFW in water). This mixture was incubated for 2-3 hours at room temperature in the dark, followed by centrifugation (3 min, 2,350 g). Pellets were washed once in fresh BG-11 plus 10 mM TES-KOH pH = 8, and re-suspended in 10 μl of the same medium. The washed cells were placed on a thin layer of 1 % (w/v) agar in BG-11 on a microscope slide. A cover slip was placed on top of the sample and fixed. The samples were analyzed with an Axiovert 40 CFL (Zeiss) microscope, equipped with an AxioCam MRc (Zeiss) camera. Pictures were taken using the Axiovision 4 software. For CFW the 365/420-470 excitation/emission filter set was used and for the cellular auto-fluorescence the 550-580/590-650 excitation/emission filter set was used. Corrected total cell fluorescence (CTCF) was determined using ImageJ, according to the protocol described on <http://sciencetechblog.com/2011/05/24/measuring-cell-fluorescence-using-imagej/>. In short, CTCF is the amount of fluorescence emitted by the cell - (the area of the fluorescent cell * the mean intensity of the background). Of each *Synechocystis* strain 3-4 photos and at least 3 cells per photo were analyzed.

Cellulose and glycogen quantification

For cellulose quantitation 15 ml of a culture was harvested by centrifugation (10 min, 3,500 x g). To the pellet 1 ml of sodium acetate buffer (20 mM, pH = 5.0) was added and the cell suspension was transferred to a 2 ml Eppendorf tube. As a control, samples containing only sodium acetate buffer (20 mM, pH 5.0) were also prepared. 5 µl celluclast suspension (Sigma) was added to the samples and controls. All tubes were incubated for 24 hours on a rotating platform at 50 °C. All tubes were spun down (5 min, 6,000 x g) and the glucose concentration in the supernatant was determined using an NADP⁺-dependent enzymatic glucose assay kit (Megazyme).

For glycogen quantitation 5 ml of a culture was harvested by centrifugation (10 min, 3,500 x g). Cells were resuspended in 1 ml of sodium acetate buffer (20 mM, pH = 5.0) and opened by bead beating with intervals for cooling. Amyloglucosidase (Boehringer Mannheim) was added to a final concentration of 1 mg ml⁻¹ and samples were incubated overnight at 55 °C on a rotating platform. Samples were spun down (5 min, 6,000 x g) and the glucose concentration in the supernatant was determined using an NADP⁺-dependent enzymatic glucose assay kit (Megazyme).

Supplemental Material

Strain and plasmid	Description	Origin
<i>E. coli</i> XL-1 (blue)	Cloning host strain	Stratagene
<i>E. coli</i> J53 (pRP4)	Conjugation helper strain	Wilde A. (Giessen)
<i>Synechocystis</i> sp. PCC6803	Wild type, glucose tolerant, natural transformable	Bhaya D. (Stanford)
<i>Syn</i> SAW011	<i>Synechocystis</i> sp. PCC6803 <i>Δslr1176::Km^r</i>	van der Woude et al. (2014)
pVZ321	Mobilizing and self-replicating plasmid for <i>Synechocystis</i>	Zinchenko et al. (1999)
pHKH001	Integration vector disrupting <i>slr0168</i> in the <i>Synechocystis</i> genome with kanamycin cassette	Angermayr et al. (2012)
pBSK+_HUPHDNCmBB	Integration vector deleting <i>slr1176</i> in the <i>Synechocystis</i> genome with chloramphenicol cassette	van der Woude et al. (2014)
pUC57 <i>bcsAB</i>	Codon optimized <i>bcsAB</i> of <i>Gluconacetobacter xylinus</i> ATCC 53582	This study
pUC57 <i>ccsA</i>	Codon optimized <i>ccsA</i> of <i>Crinallium epipsammum</i> PCC 9333	This study
pUC57 <i>ugpG</i>	Codon optimized <i>ugpG</i> of <i>Gluconacetobacter xylinus</i> ATCC 53582	This study
pUC57 <i>ugpC</i>	Codon optimized <i>ugpC</i> of <i>Crinallium epipsammum</i> PCC 9333	This study
pVZ321- <i>bcsAB</i>	self-replicating plasmid with <i>P_{trc}::bcsA::P_{trc}::bcsB::Km^r</i> <i>bcsAB</i> of <i>G. xylinus</i>	This study
pVZ321- <i>bcsAB1op</i>	self-replicating plasmid with <i>P_{trc}::bcsA::bcsB::Km^r</i> <i>bcsAB</i> of <i>G. xylinus</i>	This study
pVZ321- <i>bcsA</i>	self-replicating plasmid with <i>P_{trc}::bcsA::Km^r</i> <i>bcsA</i> of <i>G. xylinus</i>	This study
pVZ321- <i>ccsA</i>	self-replicating plasmid with <i>P_{trc}::ccsA::Km^r</i> <i>ccsA</i> of <i>C. epipsammum</i>	This study
pVZ321- <i>ugpG</i>	self-replicating plasmid with <i>P_{trc}::ugpG::Km^r</i> <i>ugpG</i> of <i>G. xylinus</i>	This study
pVZ321- <i>ugpC</i>	self-replicating plasmid with <i>P_{trc}::ugpC::Km^r</i> <i>ugpC</i> of <i>C. epipsammum</i>	This study
pHKH- <i>bcsAB</i>	Integration vector with <i>G. xylinus bcsAB</i> cassette, with the genes in separate operons	This study
pHKH- <i>bcsAB1op</i>	Integration vector with <i>G. xylinus bcsAB</i>	This study

	cassette, with both genes in one operon	
pHKH- <i>bcsA</i>	Integration vector with <i>G. xylinus bcsA</i> cassette	This study
pHKH- <i>ccsA</i>	Integration vector with <i>C. epipsammum ccsA</i> cassette	This study
pBSK- <i>upgG</i>	Integration vector with <i>G. xylinus upgG</i> cassette	This study
pBSK- <i>upgGnT</i>	Integration vector with <i>G. xylinus upgG</i> cassette without a promoter sequence	This study
pBSK- <i>upgC</i>	Integration vector with <i>C. epipsammum upgC</i> cassette	This study
pBSK- <i>upgCnT</i>	Integration vector with <i>C. epipsammum upgC</i> cassette without a promoter sequence	This study
<i>Syn</i> RMS001	<i>Synechocystis</i> sp. PCC6803 <i>P_{trc}::bcsA::Ptrc::bcsB::Km^r</i> <i>bcsAB</i> of <i>G. xylinus</i>	This study
<i>Syn</i> RMS002	<i>Synechocystis</i> sp. PCC6803 <i>P_{trc}::bcsA::bcsB::Km^r</i> <i>bcsAB</i> of <i>G. xylinus</i>	This study
<i>Syn</i> RMS003	<i>Synechocystis</i> sp. PCC6803 <i>Ptrc::bcsA::Kmr</i> <i>bcsA</i> of <i>G. xylinus</i>	This study
<i>Syn</i> RMS004	<i>Synechocystis</i> sp. PCC6803 pVZ321- <i>bcsAB</i>	This study
<i>Syn</i> RMS005	<i>Synechocystis</i> sp. PCC6803 pVZ321- <i>bcsAB1op</i>	This study
<i>Syn</i> RMS006	<i>Synechocystis</i> sp. PCC6803 pVZ321- <i>bcsA</i>	This study
<i>Syn</i> RMS010	<i>Synechocystis</i> sp. PCC6803 <i>Δslr1176::upgC::Cm^r</i> <i>upg</i> of <i>C. epipsammum</i>	This study
<i>Syn</i> RMS012	<i>Synechocystis</i> sp. PCC6803 pVZ321- <i>upgC</i>	This study
<i>Syn</i> RMS013	<i>Synechocystis</i> sp. PCC6803 <i>P_{trc}::bcsA::Ptrc::bcsB::Km^r - P_{trc}::upgG::Cm^r</i> <i>bcsAB</i> and <i>upgG</i> of <i>G. xylinus</i>	This study
<i>Syn</i> RMS014	<i>Synechocystis</i> sp. PCC6803 <i>P_{trc}::bcsA::Ptrc::bcsB::Km^r - P_{trc}::upgC::Cm^r</i> <i>bcsAB</i> of <i>G. xylinus</i> , <i>upgC</i> of <i>C. epipsammum</i>	This study
<i>Syn</i> RMS015	<i>Synechocystis</i> sp. PCC6803 <i>P_{trc}::bcsA::Ptrc::bcsB::Km^r - upgG::Cm^r</i> <i>bcsAB</i> and <i>upgG</i> of <i>G. xylinus</i>	This study
<i>Syn</i> RMS016	<i>Synechocystis</i> sp. PCC6803 <i>P_{trc}::bcsA::Ptrc::bcsB::Km^r - Δslr1176::upgC::Cm^r</i>	This study

	<i>bcsAB</i> of <i>G. xylinus</i> , <i>ugpC</i> of <i>C. epipsammum</i>	
<i>Syn</i> RMS017	<i>Synechocystis</i> sp. PCC6803 <i>P_{trc}::bcsA::bcsB::Km^r - P_{trc}::ugpG::Cm^r</i> <i>bcsAB</i> and <i>ugpG</i> of <i>G. xylinus</i>	This study
<i>Syn</i> RMS018	<i>Synechocystis</i> sp. PCC6803 <i>P_{trc}::bcsA::bcsB::Km^r -</i> <i>Δslr1176::P_{trc}::ugpC::Cm^r</i> <i>bcsAB</i> of <i>G. xylinus</i> , <i>ugpC</i> of <i>C. epipsammum</i>	This study
<i>Syn</i> RMS019	<i>Synechocystis</i> sp. PCC6803 <i>P_{trc}::bcsA::bcsB::Km^r - ugpgG::Cm^r</i> <i>bcsAB</i> and <i>ugpG</i> of <i>G. xylinus</i>	This study
<i>Syn</i> RMS020	<i>Synechocystis</i> sp. PCC6803 <i>P_{trc}::bcsA::bcsB::Km^r - ugpgC::Cm^r</i> <i>bcsAB</i> of <i>G. xylinus</i> , <i>ugpC</i> of <i>C. epipsammum</i>	This study
<i>Syn</i> RMS021	<i>Synechocystis</i> sp. PCC6803 <i>P_{trc}::bcsA::Km^r - P_{trc}::ugpgG::Cm^r</i> <i>bcsA</i> and <i>ugpG</i> of <i>G. xylinus</i>	This study
<i>Syn</i> RMS022	<i>Synechocystis</i> sp. PCC6803 <i>P_{trc}::bcsA::Km^r - P_{trc}::ugpgC::Cm^r</i> <i>bcsA</i> of <i>G. xylinus</i> , <i>ugpC</i> of <i>C. epipsammum</i>	This study
<i>Syn</i> RMS023	<i>Synechocystis</i> sp. PCC6803 <i>P_{trc}::bcsA::Km^r - ugpgG::Cm^r</i> <i>bcsA</i> and <i>ugpG</i> of <i>G. xylinus</i>	This study
<i>Syn</i> RMS024	<i>Synechocystis</i> sp. PCC6803 <i>P_{trc}::bcsA::Km^r - ugpgC::Cm^r</i> <i>bcsA</i> of <i>G. xylinus</i> , <i>ugpC</i> of <i>C. epipsammum</i>	This study
<i>Syn</i> RMS025	<i>Synechocystis</i> sp. PCC6803 pVZ321- <i>bcsAB</i> - <i>Δslr1176::P_{trc}::ugpgG::Cm^r</i> <i>ugpG</i> of <i>G. xylinus</i>	This study
<i>Syn</i> RMS026	<i>Synechocystis</i> sp. PCC6803 pVZ321- <i>bcsAB</i> - <i>Δslr1176::P_{trc}::ugpgC::Cm^r</i> <i>ugpC</i> of <i>C. epipsammum</i>	This study
<i>Syn</i> RMS027	<i>Synechocystis</i> sp. PCC6803 pVZ321- <i>bcsAB</i> - <i>Δslr1176::ugpgG::Cm^r</i> <i>ugpG</i> of <i>G. xylinus</i>	This study
<i>Syn</i> RMS028	<i>Synechocystis</i> sp. PCC6803 pVZ321- <i>bcsAB</i> - <i>Δslr1176::ugpgC::Cm^r</i> <i>ugpC</i> of <i>C. epipsammum</i>	This study
<i>Syn</i> RMS029	<i>Synechocystis</i> sp. PCC6803 pVZ321- <i>bcsAB1op</i> - <i>P_{trc}::ugpgG::Cm^r</i> <i>ugpG</i> of <i>G. xylinus</i>	This study
<i>Syn</i> RMS030	<i>Synechocystis</i> sp. PCC6803 pVZ321- <i>bcsAB1op</i> - <i>P_{trc}::ugpgC::Cm^r</i> <i>ugpC</i> of <i>C. epipsammum</i>	This study

<i>Syn</i> RMS031	<i>Synechocystis</i> sp. PCC6803 pVZ321- <i>bcsAB1op</i> - <i>ugpG::Cm^r</i> <i>ugpG</i> of <i>G. xylinus</i>	This study
<i>Syn</i> RMS032	<i>Synechocystis</i> sp. PCC6803 pVZ321- <i>bcsAB1op</i> - Δ <i>slr1176::ugpC::Cm^r</i> <i>ugpC</i> of <i>C. epipsammum</i>	This study
<i>Syn</i> RMS033	<i>Synechocystis</i> sp. PCC6803 pVZ321- <i>bcsA</i> - $P_{trc}::ugpG::Cmr$ <i>ugpG</i> of <i>G. xylinus</i>	This study
<i>Syn</i> RMS034	<i>Synechocystis</i> sp. PCC6803 pVZ321- <i>bcsA</i> - $P_{trc}::ugpC::Cmr$ <i>ugpC</i> of <i>C. epipsammum</i>	This study
<i>Syn</i> RMS035	<i>Synechocystis</i> sp. PCC6803 pVZ321- <i>bcsA</i> - <i>ugpG::Cm^r</i> <i>ugpG</i> of <i>G. xylinus</i>	This study
<i>Syn</i> RMS036	<i>Synechocystis</i> sp. PCC6803 pVZ321- <i>bcsA</i> - Δ <i>slr1176::ugpC::Cm^r</i> <i>ugpC</i> of <i>C. epipsammum</i>	This study

Table S6.1. Strains and plasmids used in this study

Name	Sequence
pVZ321 seq s	TGGAGAAAAAATCACTG
pVZ321 seq as	GCTGCGCAGGGCTTTATT
Kan_control_R	TCAGCAACACCTTCTTCACG
H1_seg_F	TGTCGCCGCTAAGTTAGA
H2_seg_R	CTGTGGGTAGTAACTGGC
KanF_back_SEQ	TCCGTTGAATATGGCTC
HUP-150_F	GACAACCTCAGCTCCGAATCCCAAC
HUP+140_R	AAGAATGCCCAGGCCGTCCAAT
CAM_out_R	GCCGATCAACGTCTCATTTT
bcsA_F	GCCGATGGTTCCATTATTGTG
bcsB_R	CTTTCAAGGTGTAGTTGTGC
TRC_F	CATCCGGCTCGTATAATGTGTG
Bba_R	TATAAACGCAGAAAGGCCAC

Table S6.2. Primers used in this study