Generation of synthetic shuttle vectors enabling modular genetic engineering of cyanobacteria

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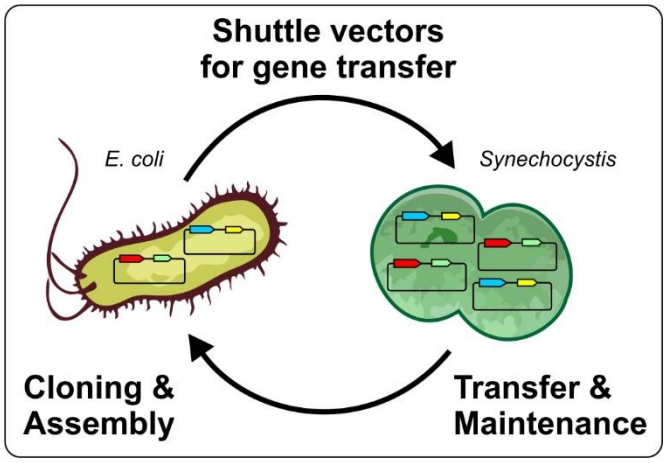
Abstract

Cyanobacteria have raised great interest in biotechnology due to their potential for a sustainable, photosynthesis-driven production of fuels and value-added chemicals. This has led to a concomitant development of molecular tools to engineer the metabolism of those organisms. In this regard, however, even cyanobacterial model strains lag behind compared to their heterotrophic counterparts. For instance, replicative shuttle vectors that allow gene transfer independent of recombination into host DNA are still scarce. Here, we introduce the pSOMA shuttle vector series comprising ten synthetic plasmids for comprehensive genetic engineering of *Synechocystis* sp. PCC 6803. The series is based on the small endogenous plasmids pCA2.4 and pCB2.4 each combined with a replicon from *E. coli*, different selection markers as well as features facilitating molecular cloning and the insulated introduction of gene expression cassettes. We made use of genes encoding green fluorescent protein (GFP) and a Baeyer-Villiger monooxygenase (BVMO) to demonstrate functional gene expression from the pSOMA plasmids *in vivo*. Moreover, we demonstrate the expression of distinct heterologous genes from individual plasmids maintained in the same strain and thereby confirmed compatibility between the two pSOMA sub-series as well as with derivatives of the broad-host-range plasmid RSF1010. We also show that gene transfer into the filamentous model strain *Anabaena* sp. PCC 7120 is generally possible, which is encouraging to further explore the range of cyanobacterial host species that could be engineered via pSOMA plasmids. Altogether, the pSOMA shuttle vector series displays an attractive alternative to existing plasmid series and thus meets the current demand for the introduction of complex genetic setups and to perform extensive metabolic engineering of cyanobacteria.

Keywords: synthetic biology, shuttle vectors, cyanobacteria, (photo)-biotechnology, genetic engineering, molecular tools

Graphical Table of Content



Introduction

Cyanobacteria are a monophyletic but extraordinary diverse group of phototrophic bacteria.[1] They are the only prokaryotes able to perform oxygenic photosynthesis, a process that makes use of solar energy to oxidize water, which thereby produces dioxygen and protons. The obtained electrons are used to drive an autotrophic metabolism, i.e. to fix atmospheric CO_2 into organic carbon molecules and biomass. Accordingly, cyanobacteria have a huge environmental impact as key players in global biogeochemical cycles.[2] Due to their photosynthetic lifestyle, cyanobacteria have also drawn huge interest as promising biotechnological hosts, as they could be exploited for a sustainable and CO_2-neutral production of fine chemicals or fuels.[3–8]

However, the biotechnological utilization of cyanobacteria requires the concomitant development of molecular tools for metabolic engineering. Several cyanobacteria have been used as microbial chassis, whereby the most prominent example is the unicellular model strain *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*). Meanwhile, an increasing number of biological parts and building blocks, i.e. BioBricks,[9] and other standardized genetic elements have become available. These include, for instance, a modular cloning system[10] to assemble and introduce genes as well as promoters, optimized ribosome binding sites, various transcription terminators or regulatory RNAs to control their expression.[11–14] Nevertheless, the current molecular toolset and the metabolic engineering capacity of *Synechocystis* as well as other cyanobacteria are still somewhat limited.

Some cyanobacteria, including *Synechocystis*, are naturally competent, enabling the uptake of exogenous DNA and to integrate it into their genome via homologous recombination.[15] Although neutral chromosomal sites for the integration of heterologous genes devoid of pleiotropic effects are available,[16] the method is impaired by chromosome polyploidy,[17] which requires time-consuming genetic segregation. Moreover, the number of neutral sites is inevitably limited, which requires additional alternatives to introduce genes. In this regard, replicative plasmids could be used. These extrachromosomal, circular DNA elements are autonomously maintained, either based on the endogenous replication machinery or in a host-independent manner, i.e. using factors encoded on the plasmids itself. However, plasmids that are commonly used for cloning in *Escherichia coli* (hereafter *E. coli*) are usually not suitable for cyanobacteria. Their origins of replication are not supported by the intrinsic replication machinery and hence, cannot be maintained as extrachromosomal element.[16] In contrast, the broad-host-range shuttle vector RSF1010[18,19] belongs to the IncQ plasmids and encodes its own replication factors for host-independent maintenance in a variety of gram-negative bacteria,[20] including several cyanobacterial strains.[21,22] In 1990, the earliest utilization of a RSF1010 derivative in

Synechocystis was reported.[23] Since then, replicative plasmids based on RSF1010 have been extensively used in cyanobacteria. Thereby, several optimization and size reduction steps resulted in different plasmid series, including e.g., pVZ,[24] pPMQAK1,[25] pSHDY,[26] and the pSEVAx5n series.[27,28] Additionally, an alternative broad-host-range vector that is instead based on RK2[29] has recently been shown to replicate in *Synechocystis* as well.[10] However, the limited availability of different, i.e. compatible replicative plasmids impedes comprehensive genetic engineering of cyanobacteria and thus, to fully exploit their biotechnological potential.

To overcome this problem, a number of chimeric shuttle vectors have been generated that exhibit two fused replicons to enable maintenance and cloning in *E. coli* as well as extrachromosomal replication in certain cyanobacteria. This comprises for instance pDC1 from *Nostoc* sp. MAC PCC 8009,[30] pDU1 from *Nostoc* sp. PCC 7524,[31] pFDA from *Fremyella diplosiphon*,[32] and pANS (also called pUH24) from *Synechococcus elongatus* PCC 7942.[33] However, none of these have been described to be maintained in *Synechocystis* cells. *Synechocystis* harbors seven extrachromosomal elements[34] subdivided into four large plasmids, ranging from about 44 to 120 kilobase pairs (kb),[35] and three small ones: pCA2.4 (2.4 kb),[36] pCB2.4 (2.4 kb),[37] and pCC5.2 (5.2 kb).[38] These small plasmids were predicted to replicate via rolling-circle amplification[36–38] and have already been investigated concerning copy numbers[39,40] as well as potential open reading frames.[41] Furthermore, initial attempts using synthetic derivatives of pCA2.4,[42] pCB2.4,[42] and pCC5.2[41,43] indicated stable maintenance and exhibited reporter gene expression in *Synechocystis.* However, to implement complex genetic setups, a prerequisite for extensive metabolic engineering, entire series of compatible plasmids would be advantageous.

In this study, we introduce the pSOMA shuttle vector series to expand the available molecular toolset to genetically engineer cyanobacteria. This library of ten individual plasmids is based on chimeric fusions of the two smallest endogenous plasmids from *Synechocystis*, either pCA2.4 or pCB2.4, as well as the pSC101 replicon originating from *E. coli*.[44] It further features multiple selection markers and allows customized cloning in *E. coli* as well as the transfer of insulated gene expression cassettes into *Synechocystis*. Moreover, we demonstrate compatibility of the two subseries with each other and further shuttle vectors, i.e. RSF1010-based replicative plasmids in *Synechocystis*. As demonstrated here, the pSOMA series enables the introduction and maintenance of distinct heterologous genes by individual plasmids and hence, provides further options for extensive and flexible metabolic engineering of *Synechocystis*.

Results

Generation of the pSOMA shuttle vector series

Among the seven endogenous plasmids of *Synechocystis*, especially the three small ones pCA2.4,[36] pCB2.4,[37] and pCC5.2[38] display attractive targets for genetic modifications due to their higher copy numbers relative to the chromosome or larger plasmids like pSYSM.[39,40] Moreover, their small size of 2.4-5.2 kb in principle allows molecular cloning and thus, synthetic combination with other elements *in vitro*. Therefore, we made use of pCA2.4 and pCB2.4 to generate a synthetic shuttle vector series that can be utilized in both *E. coli* as well as *Synechocystis*. Each replicon, i.e. the sequence harboring a vegetative origin of replication as well as two (pCA2.4) or three (pCB2.4) open reading frames (ORFs), whose products (potentially) enable replication, was fused to the pSC101 backbone[45] that is feasible for the maintenance in *E. coli.* The overall design of the pSOMA shuttle vector series is shown in **Figure 1**.

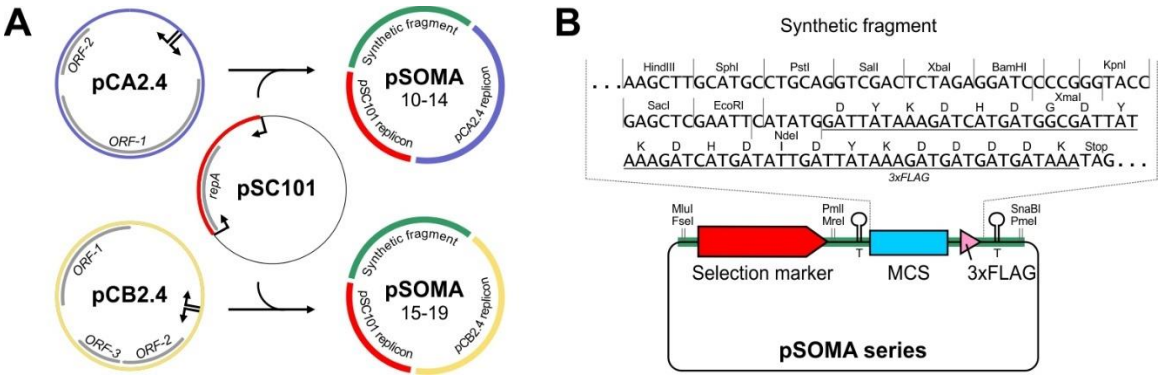


Figure 1. Generation and structure of the pSOMA shuttle vector series. (A) Different replicons were amplified by PCR and fused to each other in the illustrated order (arrows indicate primer binding sites). The replicon of pSC101 for maintenance in *E. coli* was fused to the entire sequences from either pCA2.4 or pCB2.4 to enable replication in *Synechocystis.* Moreover, a synthetic fragment harboring one of five antibiotic resistance genes (selection marker) and further features was added. (B) For easy-to-use cloning, all plasmids feature a multiple cloning site (MCS, sequence and restriction sites are highlighted). Genes can be cloned in frame with a sequence encoding a 3xFLAG-tag, which in turn allows detection of recombinant fusion proteins via a specific antibody (sequence underlined). The MCS is flanked by transcriptional terminators (T) to avoid read-through from inserted units.

In a previous report by Liu and Pakrasi (2018),[42] the usability of pCA2.4 and pCB2.4 as basis for synthetic plasmids has already been demonstrated by combining them individually with pUC118[46] or pSC101 replicons. The two generated shuttle vectors pCA-UC118 and pCB-SC101 harbor a chloramphenicol and streptomycin resistance cartridge, respectively. In addition to that, the pSOMA library is especially aiming for modularity and compatibility and hence, consists of two subseries, each exhibiting five different selection markers. The pSOMA vectors were further

equipped with a multiple cloning site (hereafter MCS) accommodating distinct restriction endonuclease sites as well as a sequence encoding a 3xFLAG-tag, which potentially allows fusion with coding sequences and finally the synthesis of easily detectable, i.e. tagged proteins (**Figure 1B**). To avoid undesired read through from the gene of interest that is potentially inserted later on, the MCS module was flanked by *oop* transcription terminators,[47] which has been applied in *Synechocystis* before.[48,49] Moreover, the surrounding unique restriction sites enable modular exchange of selection markers and the MCS module between all derivatives of the pSOMA series, which substantially increases cloning flexibility from a user perspective. Detailed features of the individual plasmids are given in **Table 1**.

Table 1. Plasmids generated or used in this study. Feature description: Amp^R = ampicillin resistance, Cm^R = chloramphenicol resistance, Gen^R = gentamicin resistance, Km^R/Neo^R = kanamycin/neomycin resistance, $Strep^R$/$Spec^R$ = streptomycin/spectinomycin resistance, Tet^R = tetracycline resistance, MCS = multiple cloning site, P_{J23101} = BioBrick BBa_J23101 synthetic promoter,[9] P_{nrsB} = *nrsB* promoter from *Synechocystis*,[50] *atpE* 5'UTR = untranslated region upstream of *E. coli atpE* gene,[51] RBS* = synthetic ribosome binding site,[52] *bvmo* = Baeyer-Villiger monooxygenase gene from *Acidovorax* sp. CHX100,[53] *sfgfp* = superfolder green fluorescent protein gene,[54] 3xFLAG-tag = sequence coding for triple FLAG protein tag, Step-tag = sequence coding for streptavidin protein tag, BBa_B0015 = BioBrick BBa_B0015 double transcription terminator,[9] T_{oop} = *oop* transcription terminator[47]

Plasmid	Features	Replicons	References
pSOMA10	Km^R/Neo^R, MCS, 3xFLAG-tag	pSC101, pCA2.4	This study
pSOMA11	Cm^R, MCS, 3xFLAG-tag, T_{oop}	pSC101, pCA2.4	This study
pSOMA12	Gen^R, MCS, 3xFLAG-tag, T_{oop}	pSC101, pCA2.4	This study
pSOMA13	$Strep^R$/$Spec^R$, 3xFLAG-tag, T_{oop}	pSC101, pCA2.4	This study
pSOMA14	Tet^R, MCS, 3xFLAG-tag, T_{oop}	pSC101, pCA2.4	This study
pSOMA15	Km^R/Neo^R, MCS, 3xFLAG-tag, T_{oop}	pSC101, pCB2.4	This study
pSOMA16	Cm^R, MCS, 3xFLAG-tag, T_{oop}	pSC101, pCB2.4	This study
pSOMA17	Gen^R, MCS, 3xFLAG-tag, T_{oop}	pSC101, pCB2.4	This study
pSOMA18	$Strep^R$/$Spec^R$, MCS, 3xFLAG-tag, T_{oop}	pSC101, pCB2.4	This study
pSOMA19	Tet^R, MCS, 3xFLAG-tag, T_{oop}	pSC101, pCB2.4	This study
pPMQAK1-*bvmo*	Amp^R, Km^R, P_{nrsB}, RBS*, *bvmo*, Strep-tag, BBa_B0015	RSF1010	Ref. [55]
pSOMA10-*bvmo*	P_{nrsB}, RBS*, *bvmo*, Strep-tag, BBa_B0015	pSOMA10	This study
pSEVA351	Cm^R, MCS	RSF1010	Ref. [28]
pSEVA351-*sfgfp*	P_{J23101}, *atpE* 5'UTR, *sfgfp*, BBa_B0015	pSEVA351	This study
pSOMA10-*sfgfp*	P_{J23101}, *atpE* 5'UTR, *sfgfp*, BBa_B0015	pSOMA10	This study
pSOMA11-*sfgfp*	P_{J23101}, *atpE* 5'UTR, *sfgfp*, BBa_B0015	pSOMA11	This study
pSOMA15-*sfgfp*	P_{J23101}, *atpE* 5'UTR, *sfgfp*, BBa_B0015	pSOMA15	This study
pSOMA16-*sfgfp*	P_{J23101}, *atpE* 5'UTR, *sfgfp*, BBa_B0015	pSOMA16	This study

The pSOMA derivatives can be used as shuttle vectors due to the maintenance in *E. coli* and *Synechocystis*

The successful cloning already proved the maintenance of the pSOMA plasmids in *E. coli*. However, to verify their usability as shuttle vectors, i.e. their replication and maintenance in both

E. coli and *Synechocystis,* we performed a transformation-isolation-analysis circuit with selected plasmids (**Figure 2A**). This included isolation of the plasmids from *E. coli* (step 1), which were then used to transform *Synechocystis* via electroporation (step 2). In general, colony forming units (CFUs) were obtained on selective agar plates after transformation of *Synechocystis* with pSOMA10-13 and pSOMA15-17. In case of pSOMA14 and pSOMA19, harboring a tetracycline resistance cartridge, a green cell lawn instead of single colonies was obtained, likely due to the light-sensitivity of this antibiotic. These plasmids were therefore excluded from the following experiments. Several *Synechocystis* colonies were randomly checked by colony PCR, which confirmed the presence of the recombinant plasmids (**Supplemental Figure S1**). However, it should be noted that for each pSOMA plasmid only low transformation efficiencies, i.e. 4 - 600 CFU μg_{DNA}^{-1} were achieved. In comparison, RSF1010 derivatives, such as pSEVA351, yielded by average around ten times more colonies from 180 up to 2,500 CFU μg_{DNA}^{-1}.

After initial selection, plasmids (including also the endogenous ones) were isolated from monoclonal *Synechocystis* cultures harboring pSOMA10, pSOMA11, or pSOMA15 (step 3) and *E. coli* cells were transformed using these mixtures (step 4). Again, colonies formed by cells resistant against the corresponding antibiotics were selected and plasmids re-isolated (step 5), followed by control digestion (step 6). Finally, a comparative digestion of the initially obtained pSOMA plasmid and the one which has been transferred into *Synechocystis* and subsequently back into *E. coli* was performed (**Figure 2B**). This result, in combination with Sanger sequencing (not shown) ultimately proved that both molecules were identical. Thus, the pSOMA plasmids can indeed be maintained as extrachromosomal elements and facilitate gene transfer into *Synechocystis* independent from recombination into the host DNA. Moreover, they allow consecutive transfer of genetic information between *E. coli* and *Synechocystis* without any observed sequence deviations or decisive structural changes, making them valuable molecular tools.

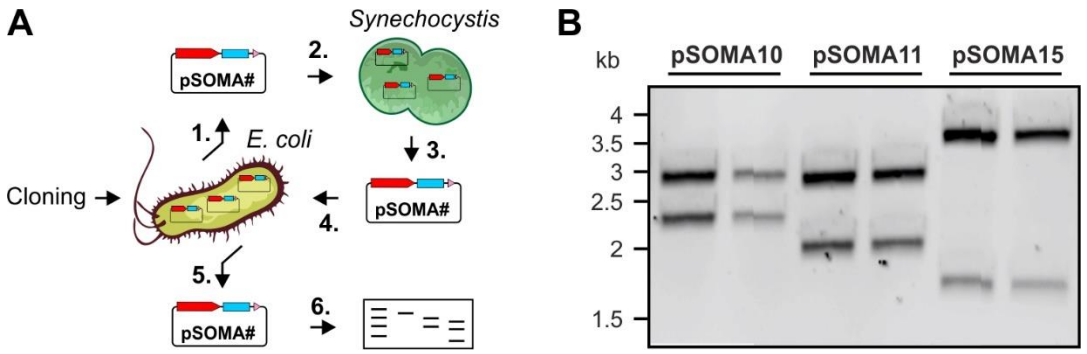


Figure 2. The pSOMA plasmids can be utilized as shuttle vectors. (A) Verification circuit of consecutive transformation of and plasmid isolation from *E. coli* and *Synechocystis* to confirm the pSOMA series as true shuttle vector system for genetic engineering of two different species. *In vitro* assembly of DNA vector parts was followed by (1.) selection and plasmid isolation from *E. coli*, (2.) transformation of *Synechocystis*, (3.) selection and plasmid preparation from *Synechocystis*, (4.) transformation of *E. coli* with plasmid mixture, (5.) selection and plasmid isolation form *E. coli*, and finally (6.) control digestion and Sanger sequencing. (B) Representative agarose gel showing restriction analyses of pSOMA10, pSOMA11, and pSOMA15 of both the initially obtained version (step 1) and after passing through the entire circuit (step 5). In each case, the left lane represents the plasmid after step 1, the right lane the same plasmid but after step 5, i.e. after it has already been maintained in *Synechocystis*. Enzymes used: PstNI and KpnI for pSOMA10 and pSOMA11, BcuI and KpnI for pSOMA15.

Utilizing the pSOMA shuttle vectors as molecular tool for genetic engineering of *Synechocystis*

To further demonstrate the applicability of the pSOMA shuttle vectors to engineer cyanobacteria, we introduced heterologous genes and analyzed their products in *Synechocystis*. First, we introduced the *sfgfp* gene encoding the superfolder green fluorescent protein[54] (hereafter GFP) into pSOMA11 and pSOMA16 that are based on two different replicons, pCA2.4 and pCB2.4, respectively. The pSOMA series does not contain promoters to drive the expression of inserted genes by default, preserving flexibility for future applications. However, favored promoters can easily be interconnected to the gene of interest beforehand, e.g. by a fusion PCR approach, to subsequently introduce the synthetic fragment into the pSOMA plasmids via classical assembly procedures (**Figure 3A**). Accordingly, the *sfgfp* coding sequence was fused to the artificial BioBrick promoter BBa_J23101[9] (hereafter P_{J23101}) that was found to promote high and constitutive expression in *Synechocystis*[56] and *Synechococcus elongatus* PCC 7942[57] (**Figure 3A**). Indeed, a specific GFP signal could be observed in cells of two recombinant *Synechocystis* strains harboring the plasmids pSOMA11-*sfgfp* or pSOMA16-*sfgfp*. Via fluorescence microscopy these strains could clearly be distinguished from cells accommodating the respective empty vectors (**Figure 3B**).

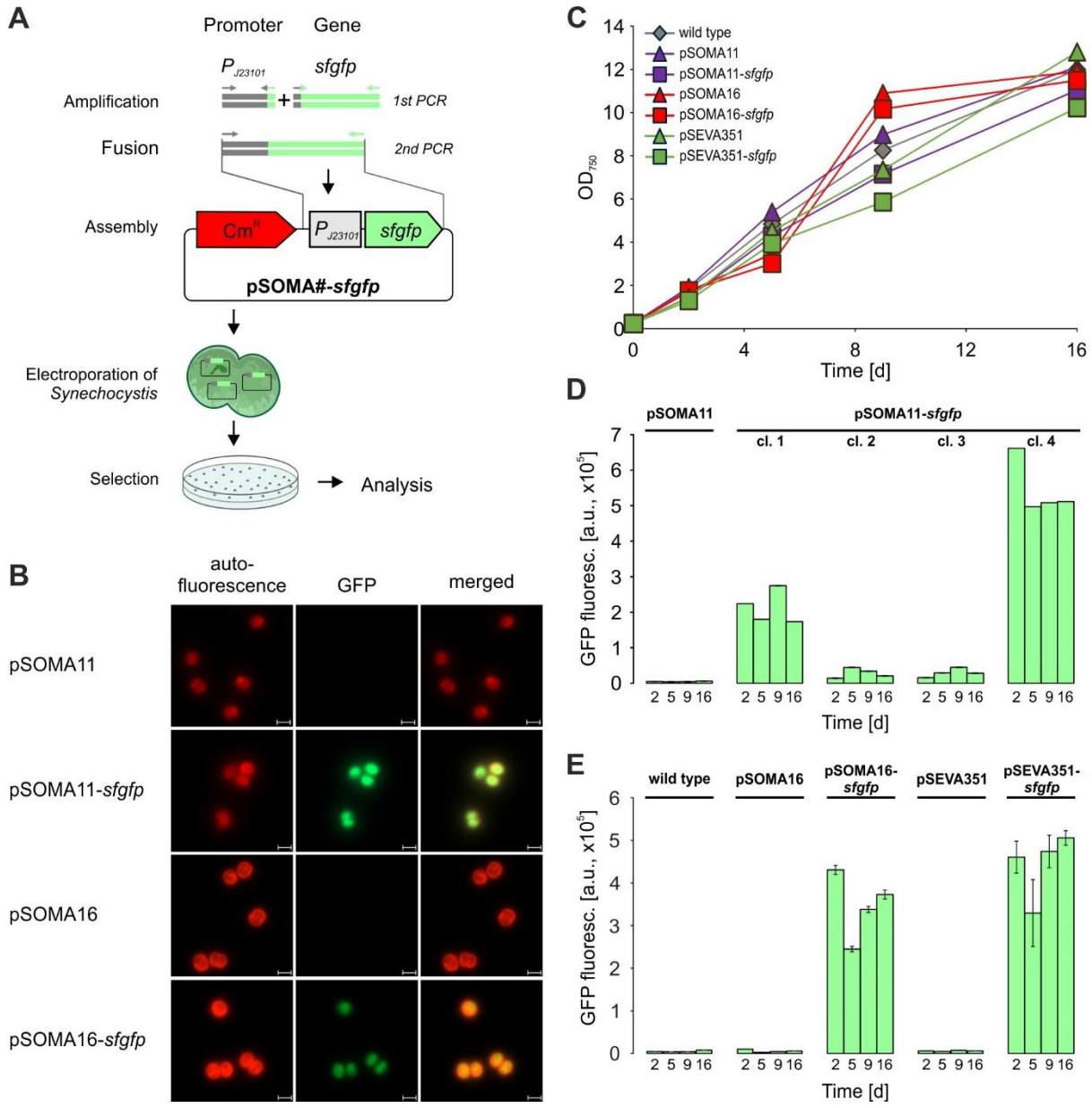


Figure 3. Expression of a reporter gene from pSOMA11 and pSOMA16. (A) Cloning strategy for the genetic constructs. A gene encoding the superfolder green fluorescent protein (*sfgfp*) was fused to a synthetic minimal promoter (BioBrick BBa_J23101, = P_{J23101}) that was found to mediate constitutive and high expression in *Synechocystis*.[56] The construct was inserted into respective pSOMA variants conferring chloramphenicol resistance (Cm^R), particularly pSOMA11 (pCA2.4 backbone) and pSOMA16 (pCB2.4 backbone). As control, the same *sfgfp* expression cassette was also inserted into the RSF1010 derivative pSEVA351. (B) Fluorescence microscopy pictures of *Synechocystis* cells expressing *sfgfp* from pSOMA11 or pSOMA16. Autofluorescence and GFP were visualized separately or as merged pictures (scale bar 2 µm). (C) Cell growth as a measure of OD_{750}. The wild type parental strain lacks a synthetic plasmid and was cultivated without chloramphenicol. (D, E) Spectrofluorimetrical quantification of GFP fluorescence *in vivo*. Shown are GFP signals normalized to OD_{750} (arbitrary units, a.u.) obtained from *Synechocystis* transformed with either pSOMA11-*sfgfp* (D), pSOMA16-*sfgfp* (E), pSEVA351-*sfgfp* (E), or the respective empty vectors without *sfgfp*, as well as the parental strain (wild type). Panel D shows individual data obtained from independent clones (cl.1-4), each measured in three technical replicates. In panel E, data are the mean ±SD of at least three biological replicates, i.e. clones.

To evaluate the stability of the genetic setup, the GFP fluorescence signal was quantitatively monitored over a period of 16 days. For a comparison with widely used RSF1010 plasmids, the same P_{J23101}*::sfgfp* cassette was also inserted into pSEVA351,[28] giving pSEVA351-*sfgfp*, which was also introduced into *Synechocystis.* In general, maintenance of the pSOMA plasmids did not impair cellular fitness as displayed by similar growth behavior of the recombinant strains under selection pressure compared to the wild type cultivated without antibiotics (**Figure 3C**). A GFP signal considerably exceeding fluorescence of control strains could continuously be detected in the same time range, consistent with a constitutive expression of the *sfgfp* gene (**Figure 3D, E**). However, it should be noted that individual transformants harboring pSOMA11-*sfgfp* showed high biological variation, whereas strains harboring pSOMA16-*sfgfp* and pSEVA351-*sfgfp* behaved more homogenous (**Figure 3D, E**). Furthermore, the maximum relative fluorescence intensities for strains carrying pSOMA11-*sfgfp* or pSOMA16-*sfgfp* were comparable to those harboring pSEVA351-*sfgfp*.

In addition to GFP fluorescence, we illustrate the utilization of pSOMA shuttle vectors to implement heterologous metabolic pathways and hence, to design cyanobacterial photobiocatalysts. Here we made use of previously demonstrated concepts of biotransformations using electrons derived from photosynthesis.[55,58] In particular, we introduced the gene encoding Baeyer-Villiger monooxygenase (hereafter BVMO) from *Acidovorax* sp. CHX100 into *Synechocystis*. This cyclohexanone monooxygenase is $NADP^+$/NADPH-dependent and uses molecular oxygen (O_2) as co-factor to oxidize cyclic ketones to lactones or esters while forming water as by-product.[59] BVMO is part of a multistep process for the reaction cascade transforming cyclohexane to the value-added chemical ε-caprolactone, in which BVMO catalyzes the conversion of cyclohexanone to ε-caprolactone (**Figure 4A**). The product ε-caprolactone is a chemical commodity and can be used as precursor for the production of adipic acid and its derivatives, e.g. nylon polymers.[60] To achieve *bvmo* expression from pSOMA plasmids in *Synechocystis*, a previously assembled construct[55] was amplified and transferred into pSOMA10. Accordingly, the resulting plasmid pSOMA10-*bvmo* contained the *bvmo* gene in fusion with a Strep-tag and the nickel ion (Ni^{2+})-dependent *nrsB* promoter (**Figure 4B**). The latter is native to *Synechocystis*[61] and shows tight repression as well as a high dynamic range in this strain.[50] After transformation, the recombinant *Synechocystis* strains carrying either pSOMA10 or pSOMA10-*bvmo* were analyzed for sufficient *bvmo* expression and *in vivo* activity of the corresponding enzyme. First, soluble proteins were separated by SDS-PAGE, whereby a Ni^{2+}-inducible *bvmo* expression was already indicated by a discrete protein band at ~60 kDa that did not appear in the lane representing the control strain with empty vector. Furthermore, the Strep-tagged fusion protein was immunologically detected via

Western Blot and the obtained signal corresponds to the band that became visible in the SDS gels (**Figure 4C**). Consistently, specific BVMO activity, i.e. ε-caprolactone formation from added cyclohexane, could only be detected in presence of Ni^{2+}, whereas background signals without inducer and a control strain lacking the *bvmo* gene were negligible (**Figure 4D**). As a positive control, we made use of the previously generated plasmid pPMQAK1-*bvmo*[55] that harbors the same gene cassette but is instead based on the broad-host-range vector RSF1010. Thereby, a maximum of ~44 U g_{CDW}^{-1} was observed for cells carrying pSOMA10-*bvmo*, exhibiting ~90% productivity relative to the positive control (~49 U g_{CDW}^{-1}; **Figure 4E**).

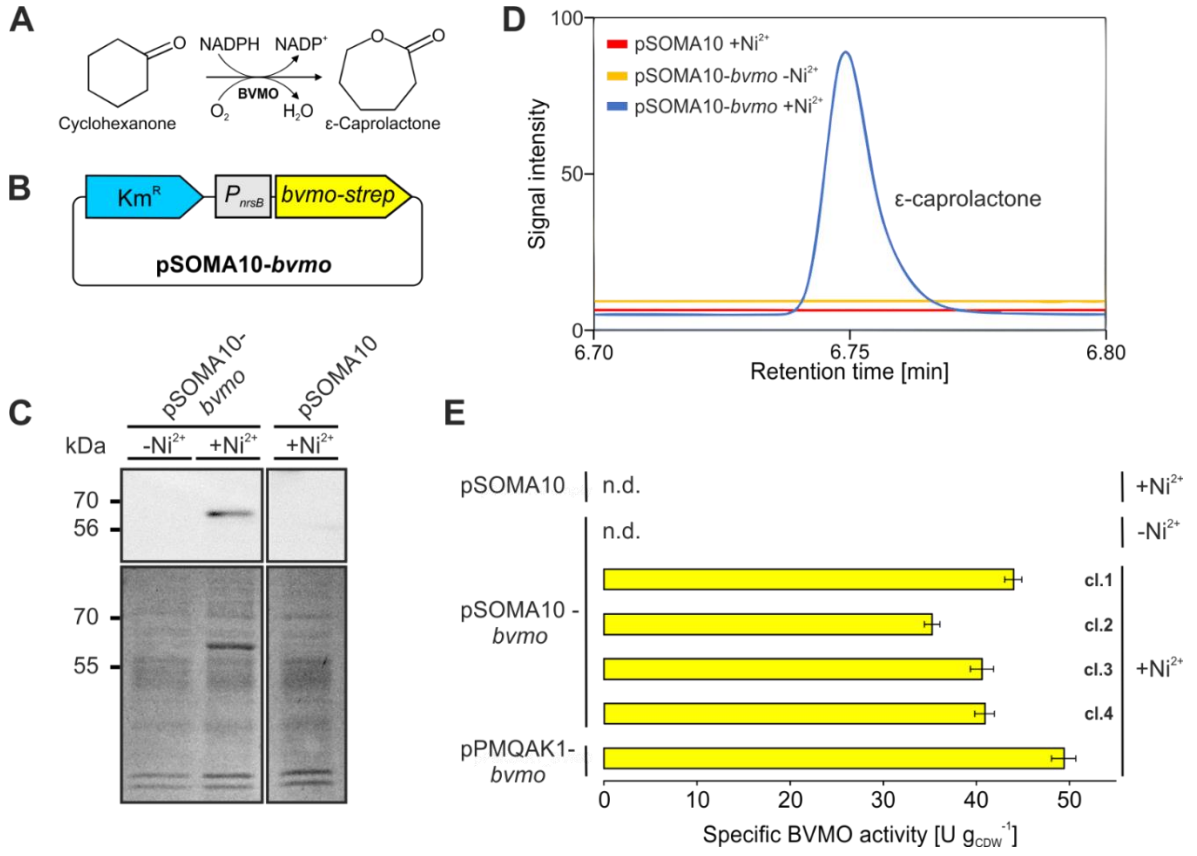


Figure 4. Implementation of a heterologous enzyme reaction in *Synechocystis* using pSOMA10. (A) Reaction catalyzed by Baeyer-Villiger monooxygenase (BVMO) from *Acidovorax* sp. CHX100, which performs the oxygen- and NADPH-dependent biotransformation of cyclohexanone to ε-caprolactone.[60] (B) Schematic illustration of the genetic constructs. The *bvmo* gene encoding a C-terminally Strep-tagged fusion protein was inserted into pSOMA10. Ni^{2+} serves as inducer for the *nrsB* promoter (P_{nrsB}).[50] (C) Western Blot for the detection of a BVMO-Strep fusion protein (~60.2 kDa). Soluble proteins separated by SDS-PAGE are shown as loading control (lower panel). (D) Representative gas chromatograms for *Synechocystis* extracts revealing ε-caprolactone product formation from cyclohexanone via BVMO-driven biotransformation. (E) Specific BVMO whole-cell activity of four *Synechocystis* clones accommodating pSOMA10-*bvmo* (cl.1-4) or indicated controls (n.d. = not detectable). Cyclohexanone conversion units (in µmol min^{-1}) were normalized to cell dry weight (CDW). Data are the mean ± SD of four technical replicates.

The two pSOMA subseries allow combination with each other and further compatible plasmids

Next, we introduced two compatible plasmids with different origins of replication and selection markers into one cell lineage of *Synechocystis* to investigate, whether the pSOMA shuttle vectors can be used in combination with other plasmids, such as the widely used RSF1010 derivatives (**Figure 5A**). Therefore, we subsequently transformed our obtained *Synechocystis* strain carrying pSOMA10-*bvmo* either with pSEVA351[28] or pSEVA351-*sfgfp,* which harbored the same P_{J23101}*::sfgfp* cassette as used before (**Figure 3**). This strategy permitted us to analyze the presence of the two distinct plasmids by measuring the GFP signal and BVMO activity. As expected, specific GFP fluorescence as well as ε-caprolactone production were detected again, yet, both in a single strain (**Figure 5B, C**). Analogously, we introduced representatives of the pCB2.4-based pSOMA subseries, namely pSOMA16 or pSOMA16-*sfgfp* into the same *Synechocystis* host strain that already carried pSOMA10-*bvmo*. Consistently, this strain also showed both activities, illustrating the possibility of combining both pSOMA subseries based on pCA2.4 or pCB2.4 (**Figure 5D, E**).

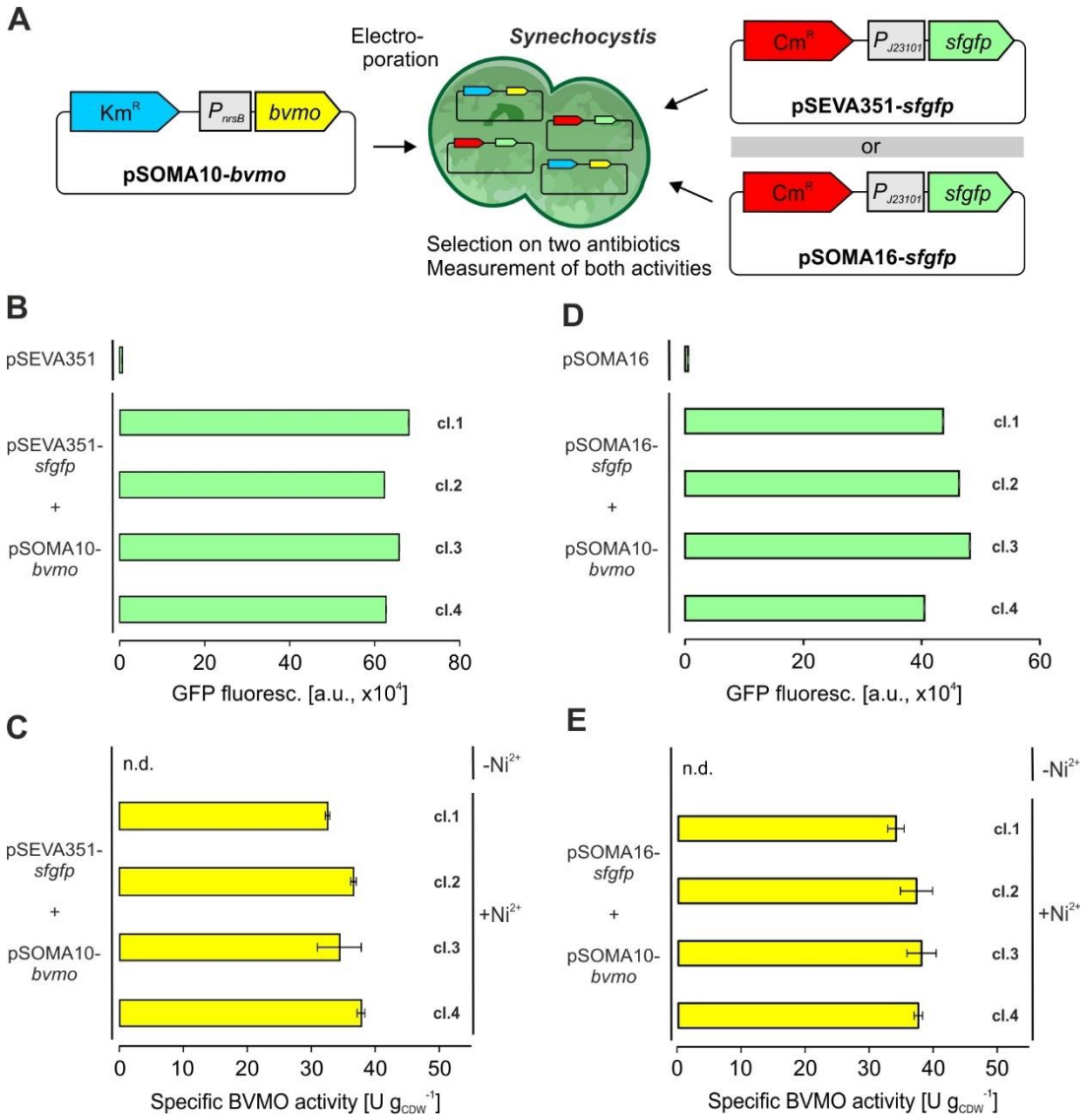


Figure 5. Expression of distinct heterologous genes from individual shuttle vectors. (A) Generated *Synechocystis* strain with two replicative plasmids. Wild type cells were transformed with the pCA2.4 derivative pSOMA10-*bvmo* mediating kanamycin resistance (Km^R) as well as either the RSF1010-based pSEVA351-*sfgfp* or the pCB2.4-derived pSOMA16-*sfgfp* (plus respective empty vector without *sfgfp*) giving chloramphenicol resistance (Cm^R). (B, D) Spectrofluorimetrical quantification of GFP fluorescence of cells carrying the given plasmid combination. Data are the mean ±SD of three technical replicates. (C, E) Specific BVMO whole-cell activity of the same strains. Data are the mean ± SD of four technical replicates.

The pSOMA shuttle vectors show a limited host range

As the application in different species might be of interest, we tested if the pSOMA plasmids could also be transferred into other cyanobacteria. These comprised the filamentous strains *Anabaena* sp. PCC 7120 and *Trichormus* (previously known as *Anabaena*) *variabilis* ATCC 29413, as well as two fast-growing strains that have attracted attention as potential production systems: *Synechococcus elongatus* UTEX 2973 and the recently discovered marine strain *Synechococcus*

sp. PCC 11901. In particular, pSOMA10 and pSOMA15, as well as respective derivatives harboring the *sfgfp* reporter gene, pSOMA10-*sfgfp* and pSOMA15-*sfgfp*, were utilized. For *T. variabilis*, *Synechococcus elongatus* UTEX 2973, and *Synechococcus* sp. PCC 11901 no CFUs could be obtained after transformation via electroporation. However, several colonies were gained on selective plates in case of the filamentous model strain *Anabaena* sp. PCC 7120 following transformation with pSOMA10-*sfgfp*. Remarkably, reporter gene expression could also be illustrated by fluorescence microscopy, whereby several independent filaments that clearly show GFP fluorescence were observed (**Figure 6**). In contrast, a similar pattern was not detected in any of the wild type filaments. Moreover, GFP fluorescence was distributed over various individual cells of the same filament. This indicates that the respective pSOMA plasmid was maintained in all these cells, due to the fact that the reporter protein cannot diffuse through the septal junctions.[62] However, it should be noted that the fluorescence intensity between different filaments varied and even a variation between cells within the same filament could be observed, which necessitates further characterization and optimization. Nonetheless, with this proof of principle we suggest that the host range of pSOMA shuttle vectors based on the pCA2.4 replicon can be extended to at least one more cyanobacterium in addition to *Synechocystis.*

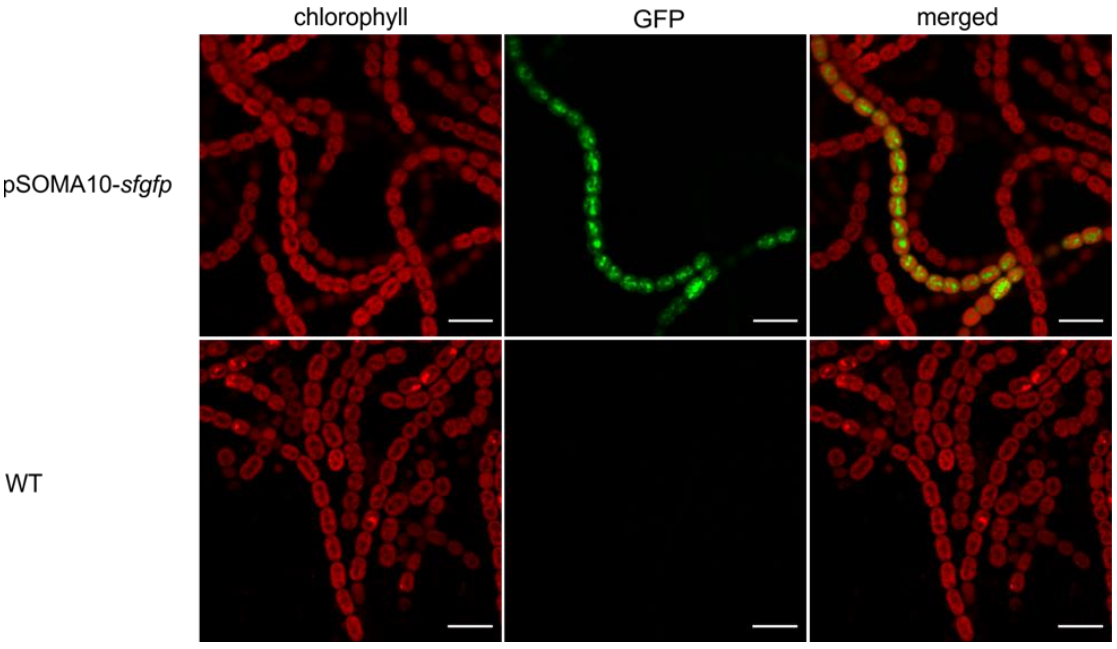


Figure 6. Representative fluorescence microscopy pictures of *Anabaena* sp. PCC 7120 wild type (WT) and cells harboring pSOMA10-*sfgfp*. It should be noted that none of the WT filaments showed a similar GFP fluorescence pattern as observed for various individual filaments harboring pSOMA10-*sfgfp*. Samples were excited at 488 nm and fluorescence emission detected separately for chlorophyll (red) and GFP (green). Both channels together are shown in the merged images (scale bar 10 µm).

Discussion

Due to the restricted number of replicative plasmids, integration of (heterologous) genes into endogenous DNA is still the predominant strategy for metabolic engineering of *Synechocystis.* Accordingly, this has been accompanied by the development of recombination and selection strategies as well as the search for suitable integration sites.[16,40,63] However, genetic segregation in a polyploid organism, such as *Synechocystis*, is a laborious procedure and all the more time-consuming when carried out sequentially, even if neutral sites are targeted. Moreover, targeting endogenous plasmids via homologous recombination directly is accompanied by the same problems and appears to be even more difficult compared to chromosomal sites.[40,64,65] For instance, when targeting pCC5.2, even after several attempts and multiple rounds of selection wild type alleles were still detectable, i.e. only partially segregated mutant strains were obtained.[40,64] In addition, once a recombinant strain was generated by homologous recombination, it cannot be easily altered, e.g. a selection marker cannot be changed without implementing genetic modifications within the host DNA. Therefore, this strategy has a rather low flexibility, in particular for extensive engineering. The latter might be required to implement complex genetic scenarios such as for sophisticated metabolic pathways and/or heterologous enzyme complexes which also include various maturation factors.

To overcome these problems, we designed a chimeric shuttle vector series to expand the molecular toolset of cyanobacteria, in particular *Synechocystis*. As the limited number of replicative plasmids currently presents a bottleneck for the introduction of genes independent from homologous recombination, similar studies were conducted previously.[41–43] An overview of so far reported synthetic shuttle vectors derived from small endogenous plasmids of *Synechocystis*, including this study, is given in **Table 2**. For example, Jin et al. (2018) generated the pCC5.2-derived shuttle vector pSCBe that was fused to the replicon of pMB1[66] from *E. coli*. Their study confirmed heterologous expression of a reporter gene from pSCBe in *Synechocystis* as well as stable maintenance of the plasmid for at least 50 generations even without antibiotic selection. Moreover, similar to our study, stable co-existence of pSCBe with an RSF1010-based plasmid was reported.[41] The principle usability of pCA2.4 and pCB2.4 to generate synthetic shuttle vectors for *Synechocystis* has also been demonstrated previously.[42] Albeit the two obtained plasmids pCA-UC118 and pCB-SC101 could generally be used as expression platforms for heterologous genes, several aspects might require optimization to finally enable user-friendly cloning and a flexible combination of customized plasmids with genetic setups that might already exist in a particular lab. Therefore, our study expands these initial attempts by introducing a whole library of replicative plasmids based on pCA2.4- and pCB2.4. It comprises a broad spectrum of selection

markers as well as suitable cloning features, such as a MCS and a 3XFLAG-tag for the subsequent synthesis of proteins that can easily be detected. Moreover, it is known that efficient promoters can interfere with plasmid replication and diminish expression of plasmid-specified genes.[67] Therefore, the MCS module was additionally flanked by transcription terminators that prevent transcriptional read-through and hence, shield the potentially inserted gene expression cassettes to ensure plasmid stability and support high gene expression.

Table 2: **Overview of reported chimeric shuttle vectors for *Synechocystis*.** All plasmids were derived from small endogenous plasmids pCA2.4, pCB2.4, and pCC5.2 (or ORFb minimal replicon), which were individually fused to a replicon from *E. coli*. Feature description: ori = origin of replication, Cm^R = chloramphenicol resistance, Gen^R = gentamicin resistance, Km^R = kanamycin resistance, $Strep^R/Spec^R$ = streptomycin/spectinomycin resistance, oriT = origin of transfer for conjugation, MCS = multiple cloning site

Plasmid	**S*ynechocystis* replicon**	**Further relevant features**	**Plasmid introduction**	**Application**	**Reference**
pCA-UC118	pCA2.4	pUC118 ori, Cm^R, fluorescent reporter expression cassette	transformation based on natural competence	shuttle vector, reporter verification, compatibility analysis	Ref. [42]
pSOMA10-13	pCA2.4	pSC101 ori, $Km^R/Cm^R/Gen^R/Strep^R/Spec^R$, MCS, 3xFLAG-tag, flanking transcription terminators	electroporation	shuttle vector	This study
pCB-SC101	pCB2.4	pSC101 ori, $Strep^R/Spec^R$, fluorescent reporter expression cassette	transformation based on natural competence	shuttle vector, reporter verification, compatibility analysis	Ref. [42]
pSOMA15-18	pCB2.4	pSC101 ori, $Km^R/Cm^R/Gen^R/Strep^R/Spec^R$, MCS, 3xFLAG-tag, flanking transcription terminators	electroporation	shuttle vector, compatibility analysis	This study
pSCB	pCC5.2 (ORFb)	pMB1 ori, $Strep^R/Spec^R$, oriT, MCS	conjugation	shuttle vector	Ref. [41]
pSCB-YFP	pCC5.2 (ORFb)	pMB1 ori, $Strep^R/Spec^R$, oriT, MCS, fluorescent	conjugation	shuttle vector, reporter verification, stability assay, compatibility analysis	Ref. [41]

		reporter expression cassette			
pSCBe	pCC5.2 (ORFb)	pMB1 ori, $Strep^R$/$Spec^R$, oriT, MCS, expression cassette (P_{trc1O}, FLAG-tag, His-tag)	conjugation	shuttle/expression vector	Ref. [41]
pCCM1-FbFP	pCC5.2	pMB1 ori, $Strep^R$/$Spec^R$, fluorescent reporter expression cassette, encoded single guide RNA targeting native pCC5.2	transformation based on natural competence	shuttle vector, reporter verification, simultaneous curing of native pCC5.2 via CRISPR/Cas9	Ref. [43]

Albeit the mechanisms of transformation based on natural competence are not experimentally verified for *Synechocystis*, the anticipated process likely results in the linearization of supplied plasmids,[68] which are then taken up as single-stranded DNA.[15,69] Consequently, the linearized, single-stranded DNA needs to be repaired to maintain a replicative plasmid within the cell. This, however, might interfere with the overall transformation procedure and/or raise the possibility of recombination events, e.g. into the endogenous plasmids pCA2.4, pCB2.4, or pCC5.2. Therefore, the pSOMA series should be introduced into cells via one-step transformation by electroporation. This approach has been proven to work well in case of replicative plasmids for *Synechocystis* while maintaining their structural integrity[27] and it also spares the need for time-consuming microbial segregation to obtain axenic cultures following conjugation (triparental mating).[70] Previous studies in fact reported the successful transformation with synthetic shuttle vectors via natural competence (see **Table 2**). However, re-isolation of these plasmids followed by molecular investigation had not been performed yet. Here, we demonstrate that the pSOMA vectors can be re-isolated from recombinant cells without showing any variation from the plasmid that was initially supplied for transformation. Thus, they serve as true shuttle vectors and could be used as standard tools for DNA exchange between the molecular "work horse" *E. coli* and *Synechocystis*.

The introduction of multiple plasmids into one cell increases flexibility of genetic combinations and hence, supports customized engineering. The maintenance of several shuttle vectors in one cell lineage and compatibility with the broad-host-range plasmid RSF1010 have already been indicated for *Synechocystis*.[41,42] However, the simultaneous expression of distinct heterologous genes and the determination of their associated activities from two different plasmids have not

been demonstrated. To the best of our knowledge, this is shown here for the first time in a cyanobacterium by confirming BVMO activity (gene maintained on pSOMA10) and GFP fluorescence (gene provided either by pSOMA16 or an RSF1010 derivative, see **Figure 5**). Likewise, other combinations, e.g. also with pCC5.2 derivatives, or even more than two compatible plasmids might be possible. Such coexistence facilitates the insertion of multiple genes or pathway clusters of large size, which may be difficult to assemble and to be maintained on just one plasmid or to be even recombined into the host DNA.

However, genetic engineering via pSOMA plasmids still requires further optimization, as we achieved only low and fluctuating transformation efficiencies for *Synechocystis*. This may be caused by the electroporation procedure itself. The competition with the native endogenous plasmids and their overall regulation, e.g. abundance control or the differential expression of the encoded genes with unknown function, also present possible hurdles. For example, the copy number of pCA2.4 and pCB2.4 was shown to vary depending on the growth phase and nutritional conditions in *Synechocystis*.[39] To avoid this problem, a *Synechocystis* host strain cured from the native counterparts could be used in the future. This should in principle be possible, as both endogenous plasmids were reported to be dispensable under standard phototrophic growth conditions.[36–38,43] Simultaneous curing of the native plasmids by a CRISPR/Cas approach could be another option, as already described for a pCC5.2-based shuttle vector.[43] The eliminated competition could also enhance gene expression levels while reducing the observed variation in GFP fluorescence between individual clones, which might be due to different pSOMA plasmid copy numbers. Controversially, individual clones harboring a similar pSOMA plasmid based on pCA2.4 but instead the gene encoding BVMO showed rather low variation. This contrast points towards the influence of the respective reporter gene and its expression driven either by a constitutive or inducible promoter, rather than a general pCA2.4-related effect. Nevertheless, variation between different transformants and gene expression levels obtained from various derivatives of the pSOMA series are worthwhile to become evaluated in prospective studies. Another factor to be considered in the future is plasmid stability. Therefore, maintenance should be investigated without the unpleasant supplementation of antibiotics after initial selection, as already shown for RSF1010[27,40] and pCC5.2 derivatives.[41]

Cyanobacterial plasmids typically show a rather narrow host range and only replicate in closely related strains, e.g. plasmid pMA4 isolated from the thermophilic strain *Synechococcus* sp. MA4 could be introduced into *Synechococcus* sp. MA19.[71] However, shuttle vectors originating from the endogenous plasmid pANS of *Synechococcus elongatus* PCC 7942 were successfully used to transform phylogenetically distant *Anabaena* sp. PCC 7120, whereas it was not possible to

introduce them into *Leptolyngbya* BL0902, *Synechocystis* WHSYN, and *Synechocystis*.[33] Moreover, derivatives of pDU1 from *Nostoc* sp. PCC 7524 could be maintained in a number of rather distantly related strains, including *Anabaena* sp. PCC 7120 and *Anabaena* sp. M-131[31]; *Fischerella muscicola* sp. PCC 7414 and *Chlorogloeopsis fritschii* sp. PCC 6912[72]; *Fischerella thermalis* (also known as *Mastigocladus laminosus*) SAG 4.84[73], *Chroococcidiopsis* spp. CCMEE 029, 057, and 123[74]; as well as *Oscillatoria* MKU 277.[75] Similar to the mentioned studies, we investigated the cyanobacterial host range of pSOMA plasmids. Therefore, four different cyanobacterial species other than *Synechocystis* were tested, whereby transformants were obtained for *Anabaena* sp. PCC 7120 carrying the pCA2.4 derivative pSOMA10-*sfgfp* (**Figure 6**). Therefore, it is likely that at least pCA2.4-based vectors could in principle be used for the transformation of filamentous strains related to *Anabaena*. Interestingly, *Anabaena* species seem to have relaxed requirements for supported heterologous plasmids, even from phylogenetically distant strains, as they are able to maintain plasmids based on pANS[33] and pDU1,[31] besides the acceptance of pCA2.4 derivatives reported in this study. However, it should be noted that the transformation efficiency was again rather low, similar to the observations made for *Synechocystis*. Therefore, in case of the other tested strains we cannot finally conclude if pSOMA plasmid transfer and maintenance is indeed not possible or the transformation efficiency was simply too low. Nevertheless, the results are promising for the development of novel cyanobacterial shuttle vectors that have a broader host range.

Material and Methods

Strains and culture conditions

E. coli strain DH5α was grown at 30-37°C in liquid LB medium shaking at 180-200 rpm or LB agar plates. The medium was supplemented with 35 µg ml^{-1} chloramphenicol, 10 µg ml^{-1} gentamicin, 50 µg ml^{-1} kanamycin, 25 µg ml^{-1} streptomycin, or 10 µg ml^{-1} tetracycline when necessary. The RepA protein of pSC101 is temperature-sensitive,[76] which is why the incubation of *E. coli* exhibiting pSOMA was performed at 30°C in order to maintain the plasmid.

Cyanobacterial freshwater strains *Synechocystis* (sp. PCC 6803) and *Synechococcus elongatus* UTEX 2973 were cultivated in BG11 liquid medium, containing 16 µM Na_2EDTA (yBG11),[77] or on BG11[78] agar plates, both buffered with 10-50 mM HEPES to pH 7.2. The marine strain *Synechococcus* sp. PCC 11901 was cultivated with a modified AD7 liquid or solid medium (MAD) containing 96 mM $NaNO_3$, 240 µM $FeCl_3$, 1.2 mM KH_2PO_4, 18 g l^{-1} NaCl, 0.6 g l^{-1} KCl, and 3 pM cobalamin (vitamin B_{12}).[79] Filamentous cyanobacteria, *Anabaena* sp. PCC 7120 and *Trichormus variabilis* ATCC 29413 were grown in BG11 liquid media or on solid BG11 agar plates[78] buffered

with 10 mM TES to pH 8.2. The media were supplemented with 2-10 µg ml^{-1} chloramphenicol, 2-10 µg ml^{-1} gentamicin, 10-50 µg ml^{-1} kanamycin, or 4-20 µg ml^{-1} streptomycin as needed. Conditions of growth were set to 28-30°C, ambient CO_2, 25-50 µmol photons m^{-2} s^{-1}, 120-200 rpm for shake flasks permitting gas exchange, and 75% humidity.

Construction of plasmids

The pSOMA plasmids were assembled using PCR-generated DNA fragments. PCR fragments were obtained using Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific) and interconnected via overlap extension PCR utilizing the same enzyme or Gibson Assembly[80] according to the manufacturer's instructions. 5' extensions were added to the primers to create homologous overhang sequences of the antibiotic resistance cartridges and the two replicons. All primers for the amplification of PCR products and their respective templates are given in **Supplemental Table S1**. The MCS module containing two flanking *oop* transcription terminators,[47,48] 11 unique restriction sites (HindIII, SphI, PstI, SalI, XbaI, BamHI, XmaI, KpnI, SacI, EcoRI, and NdeI), as well as a 3xFLAG-tag was synthesized by Eurofins Genomics. The generated plasmids are given in **Table 1**. Control digestion was performed using FastDigest™ restriction endonucleases (Thermo Scientific). Purification of PCR products, plasmids, and genomic DNA was performed using the respective kits NucleoSpin™ Gel & PCR Clean-up, NucleoSpin Plasmid QuickPure™ (MARCHEREY-NAGEL), and peqGOLD Bacterial DNA (PEQLAB) according to the manufacturer's instructions. Verification of plasmids was done by control digestion by different FastDigest™ restriction enzymes or colony PCR using DreamTaq™ DNA Polymerase (Thermo Scientific) or GoTaq® DNA Polymerase (Promega). *In silico* work was performed using the software Geneious (Biomatters).

Expression cassettes to be inserted into individual pSOMA plasmids were generated beforehand including promoters, ribosome binding sites and the gene of interest. The plasmid pSEVA351-*sfgfp* was assembled by first amplifying two linear fragments with homologous overhangs suitable for insertion into PstI cut pIGA[81]: BioBrick BBa_B0015[9,25] and the reporter gene *sfgfp*[54,82] with additionally added 5' primer extension containing the BioBrick BBa_J23101 (P_{J23101})[9,56] promoter (with C→G substitution at 3' end) and a KpnI restriction site, yielding the intermediate plasmid pIGA_Ribo. The *E. coli atpE* 5' untranslated region[51] was amplified with homologous overhangs allowing subsequent introduction between promoter and gene through restriction at the interconnecting KpnI site, creating the intermediate plasmid pIGA_Ribo atpE. Promoter, *atpE* 5' untranslated region, and gene are thereby fused by KpnI scars (5'-TAC-3' and 5'-GTACC-3', respectively) and downstream of *sfgfp* is a BioBrick scar.[9] The whole P_{J23101}*::sfgfp* cassette was afterwards amplified to comprise homologous ends for insertion into KpnI-linearized pSEVA351.[28]

The same P_{J23101}::*sfgfp* reporter construct was equipped with homologous sequences for insertion into pSOMA11 and pSOMA16 cut by KpnI. The P_{nrsB}::*bvmo* gene expression unit; consisting of P_{nrsb},[50] RBS*,[52] *bvmo*,[53] linker 5'- AGCGCT-3', Strep-tag II, and BioBrick BBa_B0015; was obtained by PCR amplification from the template pEERM3_BVMO[55] with suitable homologous overhangs for the subsequent insertion into KpnI-linearized pSOMA10. The complete DNA sequences of the pSOMA plasmids are provided as genbank files in the supplementary material.

Generation of recombinant cyanobacterial strains

Synechocystis sp. PCC 6803, *Synechococcus elongatus* UTEX 2973, and *Synechococcus* sp. PCC 11901 cells were made electro-competent, transformed via electroporation, and selected as described elsewhere.[83] Briefly, cultivation was performed as described above until logarithmic growth phase of an optical density at λ=750 nm (OD_{750}) from ~0.5-1. Afterwards, cells were harvested and the resulting pellet washed three times with ice-cold HEPES (1 mM, pH 7.5) and prepared as 60 µl aliquots. For transformation via electroporation, 100-500 ng of plasmid DNA were added to an aliquot of electro-competent cells. An electroporation pulse was performed in respective cuvettes by applying 2.5 kV for 5 ms (12.5 kV cm^{-1}) in an Eporator® 4309 (Eppendorf). Afterwards, cells were resuspended in 1 ml respective media, i.e. yBG11 for freshwater strains or MAD for the marine strain *Synechococcus* sp. PCC 11901, and added to fresh liquid medium for 24 h shaking standard incubation without antibiotics and subsequently collected to be spread on selective agar plates containing appropriate antibiotics with concentration as given above. Colonies appeared within ~10 days after further cultivation at 25-50 µmol photons m^{-2} s^{-1} and 30°C. Transformants were checked for plasmid presence via colony PCR using suitable primers listed in **Supplemental Table S1**. For *Anabaena* sp. PCC 7120 and *T. variabilis* electroporation was performed as described by Thiel and Poo (1989)[84] with minor modifications. pSOMA plasmids were first transferred to *E. coli* strain HB101[pRL623], which encodes three methylases protecting the plasmid from the native restriction enzymes AvaI, II and III in *Anabaena* sp. PCC 7120.[85] The plasmids were isolated from *E. coli* using the Qiagen Plasmid Midi kit and adjusted to a DNA concentration of ~ 1 µg ml^{-1}. Cyanobacteria were grown as described above until they reached OD_{750} of 0.5-1. Cells were than collected by centrifugation at 5,000 x *g* for 5 min and washed once in TE buffer (2 mM Tricine, 2 mM EDTA pH 8.0) and twice in distilled water. Electroporation was done in 40 µl aliquots containing 20 µg chlorophyll using a MicroPulser (Biorad). After addition of 10 µg plasmid DNA, the mixture was kept for 2 min on ice before applying a pulse of 6 kV cm^{-1} for 5 ms. Cells were resuspended in 1 ml yBG11 and incubated for 48 h at 10-20 µmol photons m^{-2} s^{-1} and 28°C before spreading on an Immobilon membrane (HATF, Millipore) on a selective BG11 agar plate supplemented with 15 µg ml^{-1} neomycin. Once colonies appeared, they were transferred to fresh BG11 agar plates with 15 µg ml^{-1} neomycin.

GFP fluorescence determination

The superfolder green fluorescent protein[54] (referred to as GFP) quantitative measurement was performed with adapted settings as described previously.[86] *Synechocystis* pre-cultures, which harbored plasmids that encode GFP or their particular empty vectors, were grown in yBG11 medium supplemented with 10 µg ml^{-1} chloramphenicol as given above and diluted to an initial OD_{750} of ~0.25 for main cultures. Cells were then cultivated for 16 days and throughout this period, samples were taken and diluted to an OD_{750} of ~0.25 with yBG11 medium in a final volume of 1,200 µl. Samples were then transferred into an opaque black flat microtiter 96-well-plate (Nunc) as technical triplicates (each 200 µl), followed by fluorescence measurements at excitation/emission wavelengths of 485 nm/535 nm, respectively, using an Infinite 200 PRO microplate reader (Tecan, gain: 124, integration time: 2000, excitation bandwidth: 9 nm, emission bandwidth: 20 nm, z-position: 2000 µm, 25 flashes). Furthermore, an identical technical triplicate of each sample was taken to measure the absorption at λ=750 nm in a transparent flat microtiter 96-well-plate (Nunc), also using the Infinite 200 PRO microplate reader (bandwidth: 9 nm, 25 flashes). Technical triplicates were combined as means and the blank of the yBG11 medium background was subtracted. The fluorescence intensities were normalized by division through respective OD_{750}. For confocal fluorescence microscopy analysis of *Synechocystis*, cells were taken from an exponentially growing culture, cultivated as described above, and visualized by using the Zeiss AxioObserver.Z1/7 microscope (Zeiss) together with the Plan-Apochromat 100x/1.40 Oil Ph 3 M27 objective lens (Zeiss), filters 474-528 as well as 650-4095, and Colibri.2 Illumination System (Zeiss). Fluorescence was detected using filter set 38, BP 470/40, FT 495, BP 525/50, for GFP at excitation/emission wavelengths 488/509 nm and autofluorescence at 599/625 nm. *Anabaena* sp. PCC 7120 cells were visualized with a confocal laser scanning microscope (CLSM) SP8 (Leica) using a 63x/ 1.4 HCPL APO CS2 objective. Samples were excited at 488 nm and emission detected from 500-540 nm for GFP and 670-720 nm for chlorophyll. Images were analyzed using Fiji.[87]

Expression analyses

Synechocystis strains containing pSOMA10 or pSOMA10-*bvmo* were cultivated as described for whole-cell biotransformation assays[55] and samples of 750 µl with an OD_{750} ~20 taken ~24 h after 10 µM $NiSO_4$ was added (except control -Ni^{2+}). Cell disruption was performed buffered in TBS (100 mM Tris, 150 mM NaCl, 1 mM PMSF protease inhibitor, pH 7.5) using a Precellys® Evolution homogenizer (Bertin) equipped with a Cryolys® cooling system (Bertin). Therefore, cell suspensions were transferred to 2 ml Precellys® tubes (Bertin) together with a mixture (0.09-0.15 mm, 0.17-0.18 mm, and 0.5 mm diameter) of glass beads (Sartorius™) and disruption performed for 4x30 s at 10.000 rpm with 30 s breaks in between cooled with liquid N_2 to ~0-4°C. Supernatants

of soluble extract were separated by centrifugation and collected for protein concentration determination using Bradford Dye Reagent (Thermo Scientific) according to manufacturer's instructions. Samples of ~10 µg soluble protein were separated by SDS-PAGE (10% acrylamide separation gel) and transferred to 0.45 µm pore size nitrocellulose membranes (GVS). Membranes were subsequently treated and hybridized with *Strep*-tactin® horse radish peroxidase conjugate (iba) and exposed to WesternSure® PREMIUM Chemiluminescent Substrate (LI-COR) to detect chemiluminescence using a FluorChem FC3 System (ProteinSimple), according to manufacturer's instructions.

Whole-cell biotransformation assays

Specific BVMO (cyclohexanone monooxygenase, EC 1.14.13.22) activity determination using the recombinant enzyme from *Acidovorax* sp. CHX100[53] and gas chromatography (GC) analysis of product formation were performed as described elsewhere.[55] Briefly, different *Synechocystis* strains were cultivated at standard conditions until reaching an OD_{750} of ~1. Expression of *bvmo* was induced using 10 µM $NiSO_4$ 24 h prior to the biotransformation. Therefore, 1 ml cell suspension was adjusted to a cell dry weight (CDW) of 1 g_{CDW} l^{-1} using a correlation factor of 0.225 g_{CDW} l^{-1} for OD_{750}=1 as determined previously.[88] These samples were transferred into 10 ml Pyrex® tubes (Pyrex®) and equilibrated at 30°C, 150 µmol photons m^{-2} s^{-1}, and 200 rpm 10 min before the assay. The assay was started by the supplementation of 3 mM cyclohexanone substrate and stopped after 30 min by adding an equal volume of diethyl ether with 0.2 mM n-decane as internal standard. Samples for GC were taken in technical duplicates. One unit (U) is defined as the production of 1 µmol ε-caprolactone min^{-1} normalized to g_{CDW} l^{-1}.

Supporting Information

Verification of different pSOMA plasmids in recombinant *Synechocystis* strains, table of used oligonucleotides (SI File 1); GenBank files of the individual pSOMA plasmids (SI File 2)

Author contributions

S.K. designed the study. F.O., N.A.S., and S.K. generated the plasmids and performed experiments. A.T., J.T., and B.B. contributed to BVMO activity determination by supplying non-published DNA constructs and experimental expertise for biotransformation assays. D.J.N. and J.G.H. provided the experimental data for *Anabaena* sp. PCC 7120. F.O. & S.K. wrote the manuscript with contributions from all co-authors.

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Conflicts of Interest: None.

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