PCP
PLANT & CELL PHYSIOLOGY

Overcoming Intrinsic Restriction Enzyme Barriers Enhances Transformation Efficiency in *Arthrospira platensis* C1

Wattana Jeamton[1], Sudarat Dulsawat[1], Morakot Tanticharoen[1], Avigad Vonshak[2] and Supapon Cheevadhanarak[1,3,*]

[1]Pilot Plant Development and Training Institute, King Mongkut's University of Technology Thonburi (Bang Khun Thian), Bangkok 10150, Thailand
[2]The Jacob Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Sede Boqer campus 84990, Israel
[3]School of Bioresources and Technology, King Mongkut's University of Technology Thonburi (Bang Khun Thian), Bangkok 10150, Thailand
*Corresponding author: E-mail, supapon.che@kmutt.ac.th; Fax, +66-2452-3455.
(Received February 15, 2016; Accepted January 23, 2017)

Regular Paper

The development of a reliable genetic transformation system for *Arthrospira platensis* has been a long-term goal, mainly for those trying either to improve its performance in large-scale cultivation systems or to enhance its value as food and feed additives. However, so far, most of the attempts to develop such a transformation system have had limited success. In this study, an efficient and stable transformation system for *A. platensis* C1 was successfully developed. Based on electroporation and transposon techniques, exogenous DNA could be transferred to and stably maintained in the *A. platensis* C1 genome. Most strains of *Arthrospira* possess strong restriction barriers, hampering the development of a gene transfer system for this group of cyanobacteria. By using a type I restriction inhibitor and liposomes to protect the DNA from nuclease digestion, the transformation efficiency was significantly improved. The transformants were able to grow on a selective medium for more than eight passages, and the transformed DNA could be detected from the stable transformants. We propose that the intrinsic endonuclease enzymes, particularly the type I restriction enzyme, in *A. platensis* C1 play an important role in the transformation efficiency of this industrial important cyanobacterium.

Keywords: *Arthrospira platensis* • Cyanobacteria • Nuclease • Transformation efficiency • Liposome.

Abbreviations: GFP, green fluorescent protein; PC, phycocyanin.

Introduction

The increased interest in the cyanobacterium *Arthrospira platensis*, also known as '*Spirulina*', arises from its value as one of a few microalgae that is currently mass produced at a large scale for a variety of products in food and feed industries (Richmond 1986, Chaumont 1993, Belay 1997, Eriksen 2008, Barzegari 2014). This successful mass production has indicated its potential use as a cell factory for the production of heterologous proteins and other high value compounds, rather than only being used as food or feed supplements for humans and animals owing to its high nutritional value (Khan et al. 2005, Teas and Irhimeh 2012). Nevertheless, one of the major obstacles in further development and exploitation of *A. platensis* to its full potential is the lack of a transformation system, limiting the ability to improve its biotechnological capacity by genetic modification as well as to explore its complex metabolic pathways and regulatory networks.

The development of a stable and efficient transformation system for *A. platensis* has been attempted by many research groups with very limited success. To identify the factors contributing to the prevention of exogenous DNA uptake into the genome of *A. platensis*, recently the genome sequences of *A. platensis* (Ling et al. 2007, Fujisawa et al. 2010, Janssen et al. 2010, Cheevadhanarak et al. 2012), including the collection of proteome (Hongsthong et al. 2008, Hongsthong et al. 2009, Kurdrid et al. 2011) and transcriptome data (Panyakampol et al. 2015) of *A. platensis* C1 have been established. These data and information are highly valuable not only for functional genomic studies and genome engineering, but also for developing a reliable gene transfer system for *A. platensis*.

It has been well documented that the digestion of transforming DNA by host nucleases, especially endonucleases, is a major barrier for the successful DNA transfer in cyanobacteria and other organisms (Duyvesteyn et al. 1983, Porter 1986, Koksharova and Wolk 2002). Preventing transforming DNA from digestion by nucleases, such as by DNA methylation (Thiel and Poo 1989, Moser et al. 1993, Elhai et al. 1997) and the use of a nuclease-deficient host (Iwai et al. 2004, Dong et al. 2010, Bian and Li 2011), has been reported to increase the efficiency of gene transfer, provided that the host is transformable. Indeed, the first research group that achieved a gene transfer system in a filamentous cyanobacterium developed a specific shuttle vector and transformation procedure to overcome the problem (Wolk et al. 1984). However, the genome sequences of *Arthrospira* spp. revealed a large number of restriction–modification (RM) systems (Zhao et al. 2006, Ling et al. 2007, Fujisawa et al. 2010, Janssen et al. 2010, Cheevadhanarak et al. 2012). This makes it highly impractical to protect its DNA from these enzymes using the aforementioned approaches.

A liposome is a vesicle particle that has been extensively used for drug and gene delivery systems (Mahato et al. 1997, Balazs and Godbey 2011). The formation of the DNA and

Plant Cell Physiol. 58(4): 822–830 (2017) doi:10.1093/pcp/pcx016, Advance Access publication on 1 February 2017, available online at www.pcp.oxfordjournals.org

liposome complex not only has facilitated gene delivery but this structure also protects entrapped DNA from exonuclease and endonuclease in the cytoplasm of the host cell (Rizzo et al. 1983, Houk et al. 1999, Xu et al. 1999, Elouahabi and Ruysschaert 2005, Oliveira et al. 2009), which has consequently increased the gene transfer efficiency.

Orc is the bacteriophage T7 protein that possesses inhibitory activity for the type I RM enzymes. This protein was reported to protect the target DNA from digestion by various type I restriction enzymes. The Orc protein mimics the structure of the target DNA and competes with it to bind the active sites of the restriction enzymes, thereby protecting the unmodified DNA from degradation (Bandyopadhyay et al. 1985, Walkinshaw et al. 2002, Zavil'gel'skiĭ and Rastorguev, 2009). The transformation efficiency of a number of bacteria has been improved substantially by applying a commercial Orc protein, TypeOne™ Restriction Inhibitor (Epicentre, USA), to the transformation protocol (Dubarry et al. 2010, Matsumoto and Igo 2010).

The Tn5 transposon is the element whose transposition mechanism has been extensively studied for chromosomal integration (Reznikoff 1993, Reznikoff et al. 1999, Steiniger-White et al. 2004) due to its rapid cut and paste mechanism accomplished by the function of the transposase enzyme only. This element was used effectively in many studies to create stable mutant strains in cyanobacteria (McCarren and Brahamsha 2005, Tolonen et al. 2006, Taton et al. 2012) and was also reported for introducing DNA into the genome of *A. platensis* strain C1 (Kawata et al. 2004). Unfortunately, no single stable transformant clone could be isolated from the mixed culture of the transformed cells.

The strong host promoter that controls the expression of the selectable marker gene is also one of the keys to achieving a successful transformation system. Previously, we reported the ability of the phycocyanin (PC) promoter of *A. platensis* C1 to control the expression of the reporter green fluorescent protein (GFP) in heterologous hosts (Jeamton 2011). Thus, it may be expected to be a useful tool in the development of a transformation system in *Arthrospira*.

Another tool used in the attempt to deliver foreign DNA into a diverse number of cyanobacteria is electroporation. This procedure has advantages over other methods since it requires only exogenous DNA and washed host cells. This method was previously applied in an attempt to develop a transformation system for *Arthrospira* sp. (Toyomizu et al. 2001). However, the transformant cells did not seem to last for a long time period and, as such, the procedure was not developed further.

In this study, we have tried to take all the above-mentioned limitations into account and, using a detailed systematic approach, to develop an efficient tool for DNA transfer into the *A. platensis* genome, especially by overcoming the DNA digestion barrier. The electroporation and Tn5 transposition systems were chosen for introducing foreign DNA into the genome of *A. platensis* C1. We demonstrate that an efficient system was achieved when a type I restriction inhibitor and cationic liposomes were applied for digestion protection purposes. The stable antibiotic resistance transformants were obtained from the activity of the PC promoter. The DNA transfer system from this study has the potential to become a powerful tool for better understanding the unique characteristics as well as for genome engineering of this industrially important cyanobacterium.

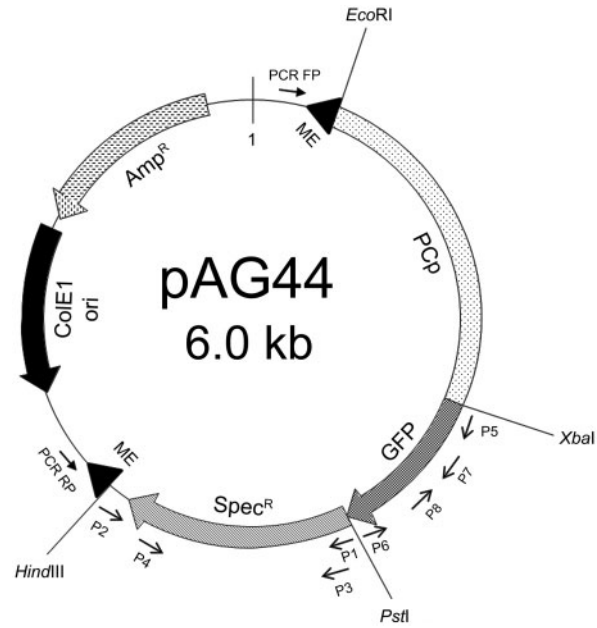

Fig. 1 Schematic diagram of plasmid pAG44 carrying the transposon cassette of the chimeric operon of the GFP and spectinomycin resistance (Spec^R) genes under the control of the PC promoter (PCp). The arrows represent the primers used in this study.

Results

Design and validation of the plasmid pAG44 and transposon cassette to be used in the transformation

The plasmid pAG44, harboring the transposon cassette containing a chimeric operon of GFP and spectinomycin resistance genes under the control of the PC promoter, was constructed as described in the Materials and Methods and is illustrated in **Fig. 1**. This operon was designed to allow the selection of transformants with resistance to spectinomycin and simultaneously to observe the production of the GFP reporter protein in the putative transformed cells of *A. platensis* C1. To verify the expression of this operon, *Escherichia coli* cells, harboring the plasmid pAG44, were cultured on agar medium containing spectinomycin, and the green fluorescent signal was clearly observed in these cells (data not shown). These results demonstrate that this chimeric operon was functional and that the PC promoter of *A. platensis* C1 could drive the expression of both the spectinomycin resistance gene and the GFP gene in *E. coli*.

Identification and growth observation of *A. platensis* C1 transformants

The next step in the construction of the transformation system was to choose the Tn5 transposon as the tool for integrating

Table 1 Components of transformation reactions and their transformation efficiency

Reaction	Transposome complex[a]	Type I inhibitor[b]	DOTAP[c]	Transformation efficiency, cfu μg^{-1} DNA
1	−	−	−	0
2	+	−	−	3.4×10^5
3	+	+	−	$>6.2 \times 10^5$
4	+	−	+	$>1.2 \times 10^6$
5	+	+	+	$>1.5 \times 10^6$

[a]A 1 µl aliquot of transposome complex was applied (+) or not applied (−) in the transformation reaction.
[b]A 1 µl aliquot of 5 µg μl^{-1} type I inhibitor was applied (+) or not applied (−) in the transformation reaction.
[c]A 1 µl aliquot of 1 µg μl^{-1} DOTAP was applied (+) or not applied (−) in the transformation reaction.

the transformed DNA into the genome of *A. platensis* C1. For transformation, to avoid the DNA digestion by type I restriction enzymes, we employed a type I restriction inhibitor, and to prevent enzyme type IV restriction, we PCR amplified the transposon cassette instead of amplifying the plasmid in *E. coli* to prevent the DNA from being exposed to DNA methylation. To minimize the exposure of the transformed DNA to type II and type III restriction enzymes, the transformed DNA was delivered to *A. platensis* C1 by encapsulation with liposomes. As such, five transformation reactions were performed, as shown in **Table 1**, in which the type I restriction inhibitor and liposomes were applied to prevent the restriction of the DNA with the aim to improve the transformation efficiency further. After electroporation, the transformed cells were plated on a spectinomycin-containing medium, and their growth was monitored under the microscope. Distinct differences in growth of the transformants could be observed within the first 4 d of the incubation between those of reactions 2, 3, 4 and 5 and the host cells of the control reaction 1 (**Fig. 2**; Supplementary Figs. S1–S5). It can be clearly seen that the surviving transformants on the selection plates had long and curled trichomes, while almost all the trichomes from the control reaction were broken and died on the spectinomycin-selective medium. After 8 d of growth on the selection plates, colonies of the transformants were visible with the naked eye, and under the microscope, their trichomes were longer and tightly curled with darker blue-green color than those of the 4-day-old transformants. It was also observed that the longer the period of incubation, the longer were the trichomes, giving rise to the compact spiral colonies, particularly the transformants from reactions 4 and 5, in which liposomes were added to the transformation reaction. The transformation reaction 2, which contained only the transposome complex, yielded the lowest number of transformed colonies, while more colonies were obtained from the transformation reactions in which either the type I restriction inhibitor or liposomes was involved in combination with the transformation complex. However, it was notable that the efficiency of colony formation significantly increased when both type I restriction inhibitor and liposomes were combined in the transformation reaction, as shown in **Table 1**.

Stability of transformants on a spectinomycin-selective medium

After 1 month of growth on the spectinomycin-selective medium, 48 independent transformant colonies obtained from each transformation reaction were randomly selected and transferred to a microplate containing new broth medium supplemented with spectinomycin at the same concentration as the original selective medium. Most of the selected colonies grew well, and a dense green suspension was observed after 1 month of incubation (**Fig. 3**). Cultures from each well were transferred into 5 ml of a new selective medium at least once a month to gain stable transformants. Some of the transformants grew poorly and gradually died, eventually leaving about 50% of the original transferred colonies that could continue growing well after more than eight passages. Stable transformants were selected to test for their ability to resist higher spectinomycin concentrations, and thus they were transferred to medium containing 1.0 and 2.0 µg ml^{-1} spectinomycin as compared with the original concentration of 0.5 µg ml^{-1}. However, we were not able to rescue any transformants that were challenged by spectinomycin at higher concentrations (data not shown), and thus continued to work with those grown on the medium with 0.5 µg ml^{-1} spectinomycin. Four transformants that could grow well on 0.5 µg ml^{-1} spectinomycin-containing medium were scaled up to 100 ml cell cultures for further verification of the presence of the transformed DNA in their genomes.

PCR analysis of the transformed DNA in the transformant genomes

In order to verify that the ability to grow on the spectinomycin-selective medium of the transformants was the result of the transformed DNA that integrated into their genomes, PCR amplification of the corresponding transformed DNA was performed using the genomic DNA of four transformants and that of the wild type. As depicted in **Fig. 4**, all expected PCR products of 0.7 and 1.0 kb fragments, representing GFP and the spectinomycin resistance genes residing in the transposon cassette, could be amplified from the genomes of the transformants, and there was no detectable product from the wild type. This result reveals that the transformants indeed received the GFP and spectinomycin resistance genes, and the survival of these transformants was attributable to the expression of the spectinomycin resistance gene.

Detection of the integrated spectinomycin resistance gene in transformant genomes

A Southern blot analysis was performed to confirm that the transposition facilitated by the trans-supplemented

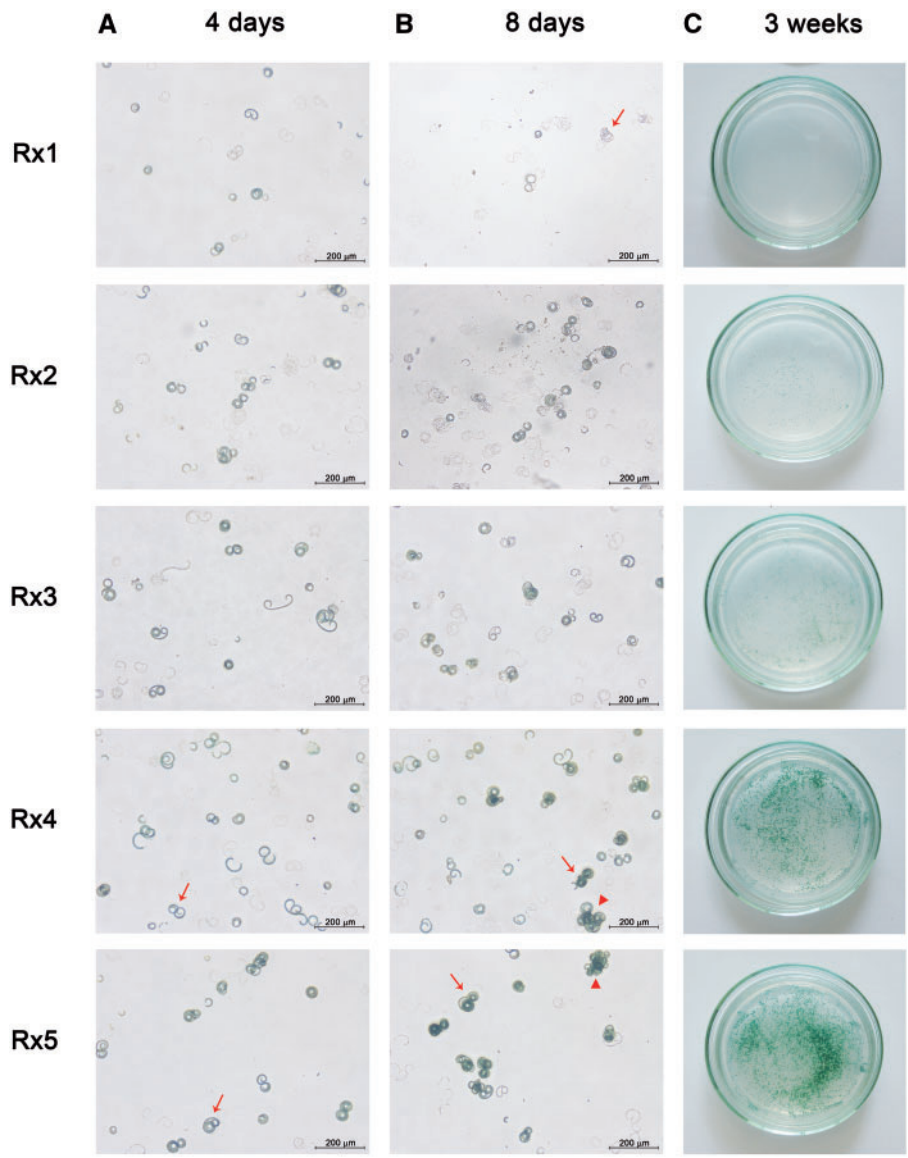

Fig. 2 Observation of the transformant growth obtained from each of the transformation reactions (Rx1–Rx5) under a microscope after identification on the selective medium for 4 and 8 d, and visualization of these transformant colonies on the selection plate after 3 weeks. The arrow in Rx1 (B) depicts the debris of the dead untransformed cell. The arrows in Rx4 (A) and Rx5 (A) indicate short trichomes. The arrows in Rx4 (B) and Rx5 (B) indicate aggregated longer trichomes. The triangles in Rx4 (B) and Rx5 (B) indicate the forming colonies. An enlarged image of this figure can be found in Supplementary Figs. S1–S5.

transposase enzyme could mobilize and integrate the transposon cassette into the genomes of the transformants. The GFP and the spectinomycin resistance genes, which are the elements of the transposon cassette, were used as probes for the detection of this mobility. As shown in **Fig. 4**, both GFP and spectinomycin resistance gene probes hybridize to the same DNA fragment on the Southern blot and gave rise to a single hybridized band from the genome of transformants T1, T2 and T3, while there are two from transformant T4. This result demonstrated that the GFP and spectinomycin genes were transposed and co-integrated into the genomes of transformants T1, T2 and T3 at only one locus, whereas multiple integrations occurred in transformant T4.

Discussion

It has been well known that restriction enzymes are the major barrier for the transformation in cyanobacteria. The information retrieved from the *A. platensis* C1 genome revealed that there are four types of restriction enzymes, of which type II is the most abundant, followed by type I, type IV and type III (Cheevadhanarak et al. 2012; see also Supplementary Table S1). Thus, in the current work, we have tried to combine the methodologies used in the past with more effective tools to reduce or prevent the foreign DNA from digestion by numerous endogenous nucleases, thus enabling the insertion and expression of the transformed DNA in *A. platensis* C1. As postulated, the digestion of transformed DNA within *A. platensis* C1 is the

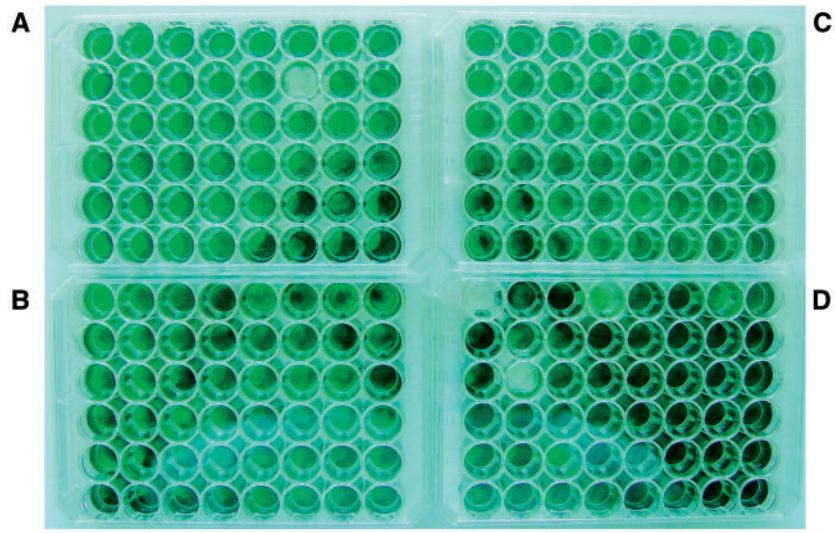

Fig. 3 The culture of transformants (1 month old) in spectinomycin-containing medium after transfer from the selection plates. (A) Transformants from transformation reaction 2; (B) transformants from transformation reaction 3; (C) transformants from transformation reaction 4; (D) transformants from transformation reaction 5.

major barrier for efficient transformation. Indeed, we found that the number of colony-forming transformants was improved when the type I restriction inhibitor was included in the transformation reaction. In the cyanobacterium *Thermosynechococcus elongatus* BP-1, the transformation efficiency was significantly higher in the mutant in which a putative type I restriction endonuclease gene was disrupted than in the wild type (Iwai et al. 2004). This event supported our results in that the type I restriction enzyme is one of the transformation barriers in *A. platensis* C1, and that use of the type I restriction inhibitor could improve the transformation efficiency. In addition to the type I restriction inhibitor, using liposomes was another approach that we employed to address the high nuclease activity from the intrinsic diverse nucleases. Unlike the type I restriction inhibitor that is specific to only type I restriction endonucleases, liposomes encapsulate and protect DNA from any nucleases, including exonucleases and endonucleases (Rizzo et al. 1983, Houk et al. 1999, Xu et al. 1999, Elouahabi and Ruysschaert 2005, Oliveira et al. 2009). In addition, encapsulating DNA with cationic liposomes could assist in the binding of the encapsulated DNA to the cell surface of the *A. platensis* C1 competent cells that has an overall negative charge like Gram-negative bacteria. This facilitated the DNA in entering into the *A. platensis* C1 competent cells by electroporation. As demonstrated in **Fig. 2** and **Table 1**, the addition of liposomes in the transformation reaction (reaction 4) significantly increased the transformation efficiency by 2-fold compared with that using the type I restriction inhibitor (reaction 3). However, the efficiency of colony formation remarkably increased when both the type I restriction enzyme inhibitor and liposomes were applied to the transformation reaction. Therefore, to obtain a high transformation frequency, we suggested that the combination of the type I restriction inhibitor and liposomes be applied to the transformation system for *A. platensis* C1.

From the results of the PCR and Southern blot analysis, we demonstrated that the stability of the transformants to resist the antibiotic spectinomycin in more than eight passages was the result of the expression of the spectinomycin resistance gene in the transposon cassette that transformed and integrated into the transformant genome. We also attempted to monitor the green fluorescent signal from the transformant cells under a fluorescent microscope. Unfortunately, we could not detect a fluorescent signal from any transformant, although this gene could be detected by PCR and Southern blot analysis in the genome of these transformants. This might be due to the interference from the autofluoresence of Chl and other pigments in the transformant cells. Franklin et al. (2002) reported that the absorption of incident light directed to the GFP by the Chl and other pigments in the chloroplast of *Chlamydomonas reinhardtii* might limit the ability to detect the green fluorescent signal in the transformed chloroplasts, while it was simply observed in the nucleus of the transformant cells in which the GFP gene was placed. In addition, these pigments might reabsorb some of the light emitted from the GFP of the transformed chloroplasts. We postulated that the low expression of GFP is the main cause of the undetectable green fluorescent signal in the *A. platensis* C1 transformant. This is consistent with the observation that the transformants could grow on a medium containing spectinomycin at the selective concentration; however, they could not survive when the spectinomycin concentration was increased. As the GFP gene was constructed as a chimeric operon preceding the spectinomycin resistance gene under the common functional PC promoter, the strength of the expression of both genes should be similar since they were co-expressed as a polycistronic mRNA. The low activity of the PC promoter to control the expression of the chimeric operon of the GFP and spectinomycin resistance genes is also another possible reason, due to the competition for limited transcription or translation factors with the endogenous PC

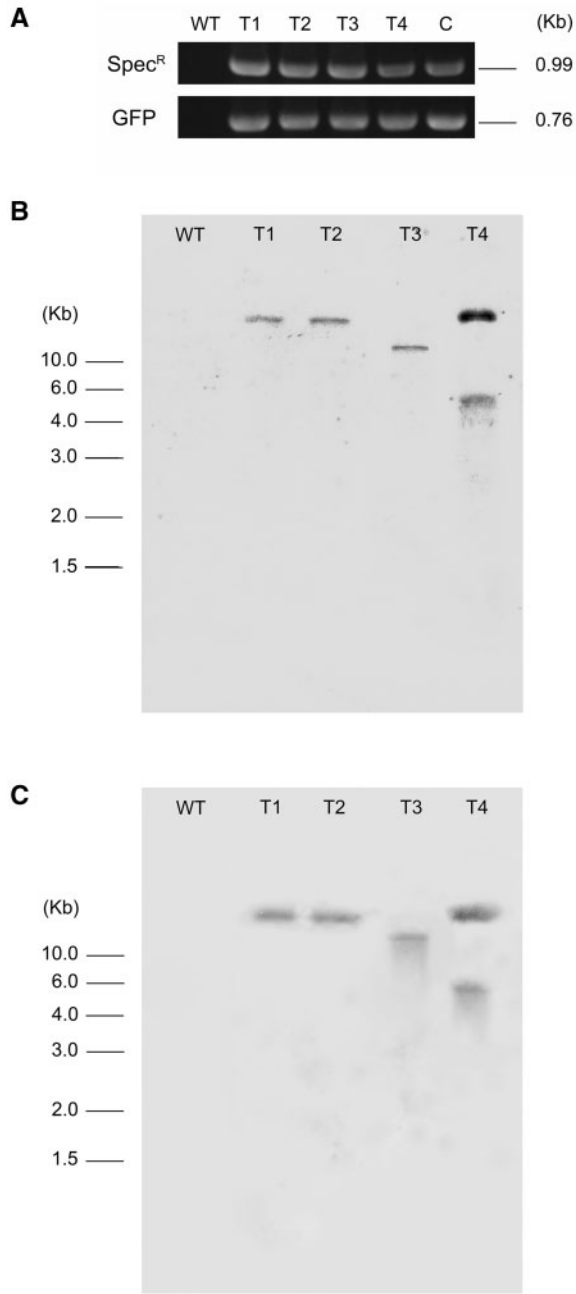

Fig. 4 Detection of transformed DNA in *A. platensis* C1 transformant genomes. (A) PCR analysis of genomic DNA from the wild type (WT) and four transformants (T1–T4) using two primer sets corresponding to the spectinomycin resistance gene ($Spec^R$) and the GFP gene (GFP). The plasmid pAG44 was used as a control (C) reaction. (B) Southern blot analysis of genomic DNA from the wild type (WT) and four transformants (T1–T4). The amplified spectinomycin resistance gene from plasmid pAG44 was used as a probe. (C) Southern blot analysis of genomic DNA from the wild type (WT) and four transformants (T1–T4). The amplified GFP gene from plasmid pAG44 was used as a probe.

gene that is normally expressed at high levels in this organism (Nomsawai et al. 1999). This hypothesis is supported by the study of Manuell et al. (2007), which demonstrated that the expression of mammalian protein in the *Chlamydomonas* chloroplast under the control of the chloroplast *psbA* promoter is low if the endogenous *psbA* gene remains in the host. They found increased mammalian protein accumulation when the endogenous chloroplast *psbA* gene was eliminated. They also showed that reintroducing the endogenous *psbA* gene along with its own promoter resulted in a greater reduction in the mammalian protein accumulation, whereas reintroducing the *psbA* gene controlled by the *psbD* promoter had little impact on the expression of mammalian protein. From these results, they concluded that the competition with the endogenous *psbA* gene is the dominant factor in the expression of heterologous mammalian protein in the *Chlamydomonas* chloroplast. Moreover, the low expression of the GFP and spectinomycin resistance genes might be due to the incomplete segregation of the chromosomes in the transformants, as in many cases the average copy number of the chromosome in cyanobacteria is more than one copy per genome (Camsund and Lindblad 2014, Ramey et al. 2015, Watanabe et al. 2015). Thus, the strategies to promote complete chromosomal segregation and genetic stability for full utilization can be achieved by using a slow growing culture that will reduce the copy number of the genome, as suggested by Vonshak and Richmond (1981).

We would like to add that during the prolonged period of maintaining the transformed cell, there was not one case in which loss of resistance to the antibiotic observed resulted from a random mutation. In all cases in which loss of resistance was observed, it was associated with the loss of the of spectinomycin resistance gene from the genomes of transformants. Based on our results, we conclude that the digestion of transformed DNA within the cytoplasm is the major barrier to gene transfer in *A. platensis* C1. The protection of transforming DNA from nuclease digestion with the simple techniques developed in this study allowed us to obtain an efficient and stable transformation system for this organism, although the expression of the transformed gene in the transformants was lower than expected.

Materials and Methods

Construction of the integrative transposon vector pAG44

First, the promoterless GFP, and spectinomycin resistance genes, derived from the plasmid pGFPM2, a gift from Professor Dr, James W. Golden (Texas A&M University, College Station, TX, USA; Cormack et al. 1996), and pSTV1, a gift from Professor Dr. Miklós Szekeres (Institute of Plant Biology, Biological Research Centre, Hungarian Academy of Sciences, Hungary), were assembled as a chimeric operon under the PC promoter, using pMG241 (Jeamton et al. 2011) as a backbone plasmid and subsequently the chimeric operon was subcloned into the pMOD™-2-<MCS> transposon construction vector (Epicentre). The PC promoter was chosen to drive the chimeric operon because this promoter is well known as a strong promoter and is used for driving heterologous genes in cyanobacteria (Sode et al. 1998, Wang et al. 2012, Zhou et al. 2012). Also the PC promoter of *Arthrospira* C1 was already functionally shown to be expressed well in the heterologous host bacterium *E. coli* and the cyanobacterium *Synechococcus elongatus* PCC7942 (Jeamton 2011).

The 1.3 kb fragment containing a promoterless spectinomycin resistance gene and its 3′ terminator was derived from the plasmid pSTV1 by PCR

Table 2 Primers used in this study

Primer	Sequence (5′→3′)
P1	ctgcagGGAGAGATAACATGCGCTCACGCAACTG
P2	aagcttGATGTTGCGATTACTTCG
P3	TCACGCAACTGGTCCAGAAC
P4	CGACTACCTTGGTGATCTCG
P5	gatttctagaTTTAAGAAGGAGATATACATATGA GTAAAGGAGAAG
P6	aactgcagGTCTGGACATTTATTTGTATAGTTCATCCATGCC
P7	TTCCATGGCCAACACTTGTC
P8	TGGTCTGCTAGTTGAACGCT
PCRFP	ATTCAGGCTGCGCAACTGT
PCRRP	GTCAGTGAGCGAGGAAGCGGAAG

Additional nucleotides with restriction sites are indicated by lower case letters.

amplification. The PCR was carried out with two oligonucleotide primers, P1 and P2 (**Table 2**). The amplified fragment was restricted with the restriction enzymes *Pst*I and *Hin*dIII, and then replaced the *E. coli* rrnBT1 terminator of the pMG241 plasmid at corresponding sites, resulting in the pAG42 plasmid harboring the chimeric operon comprising GFP and spectinomycin resistance genes, consecutively, preceded by the PC promoter (as shown in Supplementary Fig. S6). The resulting chimeric operon was then restricted from the pAG42 plasmid with *Eco*RI and *Hin*dIII, and subsequently cloned between the mosaic end (ME) sequences of the pMOD™-2-<MCS> transposon construction vector at the respective sites (Epicentre). This resultant plasmid was designated as plasmid pAG44 (**Fig. 1**). *Escherichia coli* DH5α was used for plasmid propagation, and all DNA manipulation was carried out as described by Sambrook et al. (1989). To investigate the expression of the chimeric operon in the transposon cassette, the *E. coli* cells harboring plasmid pAG44 was cultured in LB medium containing 50 μg ml^{-1} spectinomycin. Then the overnight-cultured cells were visualized under an epifluorescent microscope BX60 (Olympus).

Preparation of the transposome complex and *A. platensis* C1 transformation

The fragments of the transposon expression cassette region were amplified from the plasmid pAG44 with the pMOD™-<MCS> forward PCR primer or the PCRFP and reverse PCR primers or PCRRP (**Table 2**) using the KOD Xtreme™ Hot Start DNA polymerase (Novagen). The amplified fragments were purified using the Gel/PCR DNA fragment extraction kit (Geneaid), and the concentration was adjusted to 100 ng μl^{-1} in TE buffer. A 2 μl aliquot of purified fragments was mixed with 4 μl of EZ-Tn5 Hyper transposase (Epicentre) and 2 μl of 100% glycerol. The reaction mixture was incubated for 30 min at room temperature to form the transposome complex. Then 1 μl of the transposome complex was used alone for designated transformation reactions (**Table 1**) or combined with 1 μl of either 1 μg μl^{-1} DOTAP Liposomal Transfection Reagent (Roche) or 5 μg μl^{-1} Type One Inhibitor (Epicentre), or a combination of both. When DOTAP was to be used, it was mixed with the transposome complex for 15 min at room temperature, followed by the type I inhibitor as the final step, if it was required.

For electroporation, cells of *A. platensis* C1, obtained from Professor Avigad Vonshak (Algal Biotechnology, Ben-Gurion University of Negev, Israel), were grown in Zarrouk's medium (Zarrouk, 1966) at 35°C under incandescent lamps (100 μmol photons m^{-2} s^{-1}) with constant shaking at 150 r.p.m. When the OD_{560} of the culture reached 0.4, the cells were washed twice in an ice-cold buffer of 1.0 mM HEPES, pH 7.2 and suspended in the same buffer at a concentration of 7×10^6 cells ml^{-1}. A 40 μl aliquot of *A. platensis* C1 competent cells was added to each reaction mix, and subsequently transferred into a 2 mm electroporation cuvette. These reactions were incubated on ice for 10 min and then submitted to electric field pulses, applied by the Gene Pulser Xcell™ System (Bio-Rad). Electroporation conditions were a single pulse of 200 Ω, 0.8 kV and 25 μF at 5 ms. Next, 960 μl of a modified Zarrouk's medium containing $NaHCO_3$ (5 g l^{-1}) was immediately added into each electroporated cell reaction, and the cell culture was transferred to incubation at 30°C for 2 d under dim light (5 μmol photons m^{-2} s^{-1}). The transformants were selected by plating 100 μl aliquots of cell culture on 0.8% Zarrouk's agar medium containing 0.5 μg μl^{-1} spectinomycin and incubated under dim light. Transformant growth was periodically observed under a microscope and compared with the control reaction of electroporated *A. platensis* C1 competent cells without any additional transformation component. The single colonies of transformant from each transformation reaction were taken at random and cultured in 1 ml of Zarrouk's medium containing 0.5 μg μl^{-1} spectinomycin in 48-hole microplates for at least 1 month. Then the cultures were scaled up to 5 ml in the same medium and transferred to a new medium at least once a month to test for the resistance stability.

Calculation of transformation efficiency

To calculate the transformation efficiency, the number of the colonies of the transformants on the transformation plate was first counted with the aid of a grid. The total number of transformants per transformation plate was then calculated by multiplying the average number of colonies per grid section by the total number of grid sections of one plate. The number of the colonies of transformant per microgram of transformed DNA was then defined as the transformation efficiency.

PCR analysis

The genomic DNA was isolated from the wild-type and transformant cells using the Wizard Genomic DNA Purification Kit (Promega). The PCRs were performed on 500 ng of each isolated DNA using KOD Xtreme™ Hot Start DNA polymerase with two respective primer pairs (**Fig. 1**). These primer pairs were P3 and P4 for the spectinomycin resistance gene, and P5 and P6 for the GFP gene residing in the transposon cassette.

Southern blot analysis

A 5 μg aliquot of genomic DNA from each transformant was digested with the enzyme *Eco*RV and separated in a 1.0% agarose gel. The DNA was transferred onto a Hybond-N Nylon membrane (GE Healthcare) by capillary blotting. Hybridization of the integrated spectinomycin resistance and GFP genes in the transformant genomic DNA was detected by the AlkPhos Direct Labeling and Detection System (GE Healthcare), according to the manufacturer's instructions. The PCR fragments corresponding to the spectinomycin resistance and GFP gene, amplified from the plasmid pAG44 by primers P3 and P4, and primers P7 and P8, respectively, were used as probes. Both spectinomycin resistance and GFP gene probes do not contain an *Eco*RV restriction site.

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the King Mongkut's University of Technology, Thonburi, Thailand [grant No. 136939 and 150398].

Acknowledgments

We thank Professor Dr. James W. Golden (Texas A&M University, College Station, TX, USA) for providing plasmid pGFPM2 and Professor Dr Miklós Szekeres (Institute of Plant Biology, Biological Research Centre, Hungarian Academy of Sciences, Hungary) for providing the plasmid pSTV1. We also thank Dr. Yoshikazu Kawata [National Institute of Advanced Industrial Science and Technology (AIST), Osaka, Japan] for

kindly offering suggestions. Finally, we thank Eliza Stefaniw and Craig Butler for proofreading the manuscript.

Disclosures

The authors have no conflict to declare.

References

Balazs, D.A. and Godbey, W. (2011) Liposomes for use in gene delivery. *J. Drug Deliv.* 2011: 326497.

Bandyopadhyay, P.K., Studier, F.W., Hamilton, D.L. and Yuan, R. (1985) Inhibition of the type I restriction–modification enzymes EcoB and EcoK by the gene 0.3 protein of bacteriophage T7. *J. Mol. Biol.* 182: 567–578.

Barzegari, A., Saeedi, N., Zarredar, H., Barar, J. and Omidi, Y. (2014) The search for a promising cell factory system for production of edible vaccine. *Hum. Vaccin. Immunother.* 10: 2497–2502.

Belay, A. (1997) Mass culture of *Spirulina* outdoors—the Earthrise Farms experience. *In Spirulina platensis* (*Arthrospira*): Physiology, Cell Biology and Biotechnology. Edited by Vonshak, A. pp. 131–158. Taylor and Francis Press, London.

Bian, J. and Li, C. (2011) Disruption of a type II endonuclease (TDE0911) enables *Treponema denticola* ATCC 35405 to accept an unmethylated shuttle vector. *Appl. Environ. Microbiol.* 77: 4573–4578.

Camsund, D. and Lindblad, P. (2014) Engineered transcriptional systems for cyanobacterial biotechnology. *Front. Bioeng. Biotechnol.* 2: 40.

Chaumont, D. (1993) Biotehnology of algal biomass production: a review of systems for outdoor mass culture. *J. Appl. Phycol.* 5: 593–604.

Cheevadhanarak, S., Paithoonrangsarid, K., Prommeenate, P., Kaewngam, W., Musigkain, A., Tragoonrung, S., et al. (2012) Draft genome sequence of *Arthrospira platensis* C1 (PCC9438). *Stand. Genomic Sci.* 6: 43–53.

Cormack, B.P., Valdivia, R.H. and Falkow, S. (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 173: 33–38.

Dong, H., Zhang, Y., Dai, Z. and Li, Y. (2010) Engineering *Clostridium* strain to accept unmethylated DNA. *PLoS One* 52: e9038.

Dubarry, N., Du, W., Lane, D. and Pasta, F. (2010) Improved electrotransformation and decreased antibiotic resistance of the cystic fibrosis pathogen *Burkholderia cenocepacia* strain J2315. *Appl. Environ. Microbiol.* 76: 1095–1102.

Duyvesteyn, M.G.C., Korsnize, J., de Waard, A., Vonshak, A. and Wolk, C.P. (1983) Sequence-specific endonucleases in strains of *Anabaena* and *Nostoc*. *Arch. Microbiol.* 134: 276–281.

Elhai, J., Vepritskiy, A., Muro-Pastor, A.M., Flores, E. and Wolk, C.P. (1997) Reduction of conjugal transfer efficiency by three restriction activities of *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* 179: 1998–2005.

Elouahabi, A. and Ruysschaert, J.M. (2005) Formation and intracellular trafficking of lipoplexes and polyplexes. *Mol. Ther.* 11: 336–347.

Eriksen, N.T. (2008) Production of phycocyanin—a pigment with applications in biology, biotechnology, foods and medicine. *Appl. Microbiol. Biotechnol.* 80: 1–14.

Franklin, S., Ngo, B., Efuet, E. and Mayfield, S.P. (2002) Development of a GFP reporter gene for *Chlamydomonas reinhardtii* chloroplast. *Plant J.* 30: 733–744.

Fujisawa, T., Narikawa, R., Okamoto, S., Ehira, S., Yoshimura, H., Suzuki, I., et al. (2010) Genomic structure of an economically important cyanobacterium, *Arthrospira* (*Spirulina*) *platensis* NIES-39. *DNA Res.* 17: 85–103.

Hongsthong, A., Sirijuntarut, M., Prommeenate, P., Lertladaluck, K., Porkaew, K., Cheevadhanarak, S., et al. (2008) Proteome analysis at the subcellular level of the cyanobacterium *Spirulina platensis* in response to low-temperature stress conditions. *FEMS Microbiol, Lett.* 288: 92–101.

Hongsthong, A., Sirijuntarut, M., Yutthanasirikul, R., Senachak, J., Kurdrid, P., Cheevadhanarak, S., et al. (2009) Subcellular proteomic characterization of the high-temperature stress response of the cyanobacterium *Spirulina platensis*. *Proteome Sci.* 7: 33.

Houk, B.E., Hochhaus, G. and Hughes, J.A. (1999) Kinetic modeling of plasmid DNA degradation in rat plasma. *AAPS PharmSci.* 1: E9.

Iwai, M., Katoh, H., Katayama, M. and Ikeuchi, M. (2004) Improved genetic transformation of the thermophilic cyanobacterium, *Thermosynechococcus elongatus* BP-1. *Plant Cell Physiol.* 45: 171–175.

Janssen, P.J., Morin, N., Mergeay, M., Leroy, B., Wattiez, R., Vallaeys, T., et al. (2010) Genome sequence of the edible cyanobacterium *Arthrospira* sp. PCC 8005. *J. Bacteriol.* 192: 2465–2466.

Jeamton, W., Dulsawat, S., Laoteng, K., Tanticharoen, M. and Cheevadhanarak, S. (2011) Phycocyanin promoter of *Spirulina platensis* controlling heterologous expression in cyanobacteria. *J. Appl. Phycol.* 23: 83–88.

Kawata, Y., Yano, S., Kojima, H. and Toyomizu, M. (2004) Transformation of *Spirulina platensis* strain C1 (*Arthrospira* sp. PCC9438) with Tn5 transposase–transposon DNA–cation liposome complex. *Mar. Biotechnol.* 6: 355–363.

Khan, Z., Bhadouria, P. and Bisen, P.S. (2005) Nutritional and therapeutic potential of *Spirulina*. *Curr. Pharm. Biotechnol.* 6: 373–379.

Koksharova, O.A. and Wolk, C.P. (2002) Genetic tools for cyanobacteria. *Appl. Microbiol. Biotechnol.* 58: 123–137.

Kurdrid, P., Senachak, J., Sirijuntarut, M., Yutthanasirikul, R., Phuengcharoen, P., Jeamton, W., et al. (2011) Comparative analysis of the *Spirulina platensis* subcellular proteome in response to low- and high-temperature stresses: uncovering cross-talk of signaling components. *Proteome Sci.* 9: 39.

Ling, N., Mao, Y., Zhang, X., Mo, Z., Wang, G. and Liu, W. (2007) Sequence analysis of *Arthrospira maxima* based on fosmid library. *J. Appl. Phycol.* 19: 333–346.

Mahato, R.I., Rolland, A. and Tomlinson, E. (1997) Cationic lipid-based gene delivery systems: pharmaceutical perspectives. *Pharm. Res.* 14: 853–859.

Manuell, A.L., Beligni, M.V., Elder, J.H., Siefker, D.T., Tran, M., Weber, A., et al. (2007) Robust expression of a bioactive mammalian protein in *Chlamydomonas* chloroplast. *Plant Biotechnol. J.* 5: 402–412.

Matsumoto, A. and Igo, M.M. (2010) Species-specific type II restriction–modification system of *Xylella fastidiosa* Temecula1. *Appl. Environ. Microbiol.* 76: 4092–4095.

McCarren, J. and Brahamsha, B. (2005) Transposon mutagenesis in a marine *Synechococcus* strain: isolation of swimming motility mutants. *J. Bacteriol.* 187: 4457–4462.

Moser, D.P., Zarka, D. and Kallas, T. (1993) Characterization of a restriction barrier and electrotransformation of the cyanobacterium *Nostoc* PCC 7121. *Arch. Microbiol.* 160: 229–37.

Nomsawai, P., Tanseau de Marsac, N., Thomas, J.C., Tanticharoen, M. and Cheevadhanark, S. (1999) Light regulation of phycocyanin and gene expression in *Spirulina platensis* C1 (*Arthrospira* sp. PCC9438). *Plant Cell Physiol.* 40: 1194–1202.

Oliveira, A.C., Ferraz, M.P., Monteiro, F.J. and Simões, S. (2009) Cationic liposome–DNA complexes as gene delivery vectors: development and behaviour towards bone-like cells. *Acta Biomater.* 5: 2142–2151.

Panyakampol, J., Cheevadhanarak, S., Sutheeworapong, S., Chaijaruwanich, J., Senachak, J., Siangdung, W., et al. (2015) Physiological and transcriptional responses to high temperature in *Arthrospira* (*Spirulina*) *platensis* C1. *Plant Cell Physiol.* 56: 481–496.

Porter, R.D. (1986) Transformation in cyanobacteria. *Crit. Rev. Microbiol.* 13: 111–132.

Ramey, C.J., Barón-Sola, Á., Aucoin, H.R. and Boyle, N.R. (2015) Genome engineering in cyanobacteria: where we are and where we need to go. *ACS Synth. Biol.* 4: 1186–1196.

Reznikoff, W.S. (1993) The Tn5 transposon. *Annu Rev. Microbiol.* 47: 945–963.

Reznikoff, W.S., Bhasin, A., Davies, D.R., Goryshin, I.Y., Mahnke, L.A., Naumann, T., et al. (1999) Tn5: a molecular window on transposition. *Biochem. Biophys. Res. Commun.* 266: 729–734.

Richmond, A. (1986) Outdoor mass culture of microalgae. *In* Handbook of Microalgal Mass Culture. Edited by Richmond, A. pp. 285–330. CRC Press, Boca Raton, FL.

Rizzo, W.B., Schulman, J.D. and Mukherjee, A.B. (1983) Liposome-mediated transfer of simian virus 40 DNA and minichromosome into mammalian cells. *J. Gen. Virol.* 64: 911–919.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sode, K., Yamamoto, Y., and Hatano, N. (1998) Construction of a marine cyanobacterial strain with increased heavy metal ion tolerance by introducing exogenic metallothionein gene. *J. Mar. Biotechnol.* 6: 174–177.

Steiniger-White, M., Rayment, I. and Reznikoff, W.S. (2004) Structure/function insights into Tn5 transposition. *Curr. Opin. Struct. Biol.* 14: 50–57.

Taton, A., Lis, E., Adin, D.M., Dong, G., Cookson, S., Kay, S.A., et al. (2012) Gene transfer in *Leptolyngbya* sp. strain BL0902, a cyanobacterium suitable for production of biomass and bioproducts. *PLoS One* 7: e30901.

Teas, J. and Irhimeh, M.R. (2012) Dietary algae and HIV/AIDS: proof of concept clinical data. *J. Appl. Phycol.* 24: 575–582.

Tolonen, A.C., Liszt, G.B., and Hess, W.R. (2006) Genetic manipulation of *Prochlorococcus* strain MIT9313: green fluorescent protein expression from an RSF1010 plasmid and Tn5 transposition. *Appl. Environ. Microbiol.* 72: 7607–7613.

Thiel, T. and Poo, H. (1989) Transformation of a filamentous cyanobacterium by electroporation. *J. Bacteriol.* 171: 5743–5746.

Toyomizu, M., Suzuki, K., Kawata, Y., Kojima, H. and Akiba, Y. (2001) Effective transformation of the cyanobacterium *Spirulina platensis* using electroporation. *J. Appl. Phycol.* 13: 209–214.

Vonshak, A. and Richmond, A. (1981) Genome multiplication as related to the growth rate in blue-green algae *Anacystis nidulans*. *Plant Cell Physiol.* 22: 1367–1373.

Walkinshaw, M.D., Taylor, P., Sturrock, S.S., Atanasiu, C., Berge, T., Henderson, R.M., et al. (2002) Structure of Ocr from bacteriophage T7, a protein that mimics B-form DNA. *Mol. Cell* 9: 187–194.

Wang, B., Wang, J., Zhang, W., and Meldrum, D.R. (2012) Application of synthetic biology in cyanobacteria and algae. *Front. Microbiol.* 3: 344.

Watanabe, S., Ohbayashi, R., Kanesaki, Y., Saito, N., Chibazakura, T., Soga, T., et al. (2015) Intensive DNA replication and metabolism during the lag phase in cyanobacteria. *PLoS One*; 10: e0136800.

Wolk, C.P., Vonshak, A., Kehoe, P. and Elhai, J. (1984) Construction of shuttle vectors capable of conjugative transfer from *Escherichia coli* to nitrogen-fixing filamentous cyanobacteria. *Proc. Natl. Acad. Sci. USA* 81: 1561–1565.

Xu, Y., Hui, S.W., Frederik, P. and Szoka, F.C., Jr. (1999) Physicochemical characterization and purification of cationic lipoplexes. *Biophys. J.* 77: 341–353.

Zarrouk, C. (1966) Contribution à l' étude d' une cyanophycée. Influence de divers' facteurs physiques et chimiques sur la croissance et la photosynthèse de *Spirulina maxima*. Ph.D. Thesis, Université de Paris, Paris.

Zavil'gel'skiĭ, G.B. and Rastorguev, S.M. (2009) Antirestriction proteins ArdA and Ocr as effective inhibitors of the type I restriction–modification enzymes. *Mol. Biol.* 43: 264–273.

Zhao, F., Zhang, X., Liang, C., Wu, J., Bao, Q. and Qin, S. (2006) Genome-wide analysis of restriction–modification system in unicellular and filamentous cyanobacteria. *Physiol. Genomics* 24: 181–90.

Zhou, J., Zhang, H., Zhang, Y., Li, Y. and Ma, Y. (2012) Designing and creating a modularized synthetic pathway in cyanobacterium *Synechocystis* enables production of acetone from carbon dioxide. *Metab, Eng.* 14: 394–400.