Biotechnology Research and Innovation (2017) 1, 123–128

Biotechnology Research & Innovation

http://www.journals.elsevier.com/biotechnology-research-and-innovation/

SHORT COMMUNICATION

Improved genetic transformation of *Synechococcus elongatus* PCC 7942 using linear DNA fragments in association with a DNase inhibitor

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Received 21 August 2017; accepted 28 September 2017
Available online 16 October 2017

KEYWORDS
Cyanobacteria;
EDTA;
Exonuclease;
PCR products

Abstract The genetic manipulation in many cyanobacterial strains is challenging yet. Thus, the development of new transformation protocols is desirable to facilitate the genetic engineering in cyanobacteria. Transformations using linear fragments yielded by PCR have advantages such as: less laborious methodology, faster procedure, low cost and unnecessary cloning steps. However, some strains presence extracellular nucleases, which reduce the efficiency in obtaining transformants. In this study, we demonstrate an improved protocol for genetic transformation in *Synechococcus elongatus* PCC 7942 using linear fragments employing EDTA-mediated inhibition of DNases. To conduct the transformation, linear PCR products containing the spectinomycin antibiotic resistance gene were employed. As result, 40 mM EDTA treatment increased the number of transformants obtained by eightfold in comparison to the conventional protocol using plasmid DNA. Thus, the application of exonuclease inhibitors can be considered a relevant improvement to manipulate cyanobacteria in a more efficient, faster way and as a low-cost alternative. This protocol must be helpful for other strains of cyanobacteria.

Introduction

Cyanobacteria is a miscellaneous and successful group of bacteria defined by their ability to perform oxygenic photosynthesis. It produces a large variety of secondary metabolites with potential to be used as pharmaceuticals, aquaculture products, chemicals, nutritional supplements or energy sources, either in its natural form or as chemical derivatives (Burja, Dhamwichukorn, & Wright, 2003; Nunnery, Mevers, & Gerwick, 2010). The pathway for the synthesis of several metabolites has been discovered due to increasing number of sequencing of cyanobacterial

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https://doi.org/10.1016/j.biori.2017.09.001

genomes. Until now, more than 150 genomes of cyanobacteria are already available (https://www.ncbi.nlm.nih.gov/). Consequently, it is possible to improve the metabolites production employing genetically manipulated cyanobacteria. However, according to Stucken, Koch, and Dagan (2013) the establishment of transformation protocols in many cyanobacterial strains is challenging. Thus, the development of new transformation protocols is desirable to facilitate the genetic engineering in cyanobacteria.

Currently, three procedures are available for introduction of foreign DNA into cyanobacteria: natural transformation, electroporation, and conjugation (Vioque, 2007). The transformation successful in cyanobacteria is dependent of species since they have different physical and biochemical barriers against to foreign DNA insertion in their genome (Stucken et al., 2013). Furthermore, the transformation depends on the length, form and concentration of the foreign DNA used during the genetic manipulation (Nagarajan, Winter, Eaton-Rye, & Burnap, 2011). Usually integrative and replicative vectors are employed to carry target sequences in transformation. Integrative plasmids incorporate the heterologous gene into genomic DNA by homologous recombination (Heidorn et al., 2011). On the other hand, replicative plasmids allow fast introduction of heterologous genes and are capable of self-replicating in the cell (Wang et al., 2013). Both types of plasmids have been developed for cyanobacteria. For the integration into *Synechococcus elongatus* PCC 7942 genome, three neutral sites were already identified where homologous recombination can occur without adverse effects (Clerico, Ditty, & Golden, 2007).

It has already been demonstrated that is possible to use linear DNA fragments for genetic manipulation in *Synechocystis* sp. (Kufryk, Sachet, Schmetterer, & Vermaas, 2002; Nagarajan et al., 2011). Transformations using linear PCR products have many advantages, such as less laborious methodology, faster, low cost procedure and unnecessary cloning steps. However, some cyanobacterial strains produce extracellular nucleases, which reduce the efficiency in obtaining transformants (Stucken et al., 2013). Therefore, here we report by the first time an improved protocol for genetic transformation in *Synechococcus elongatus* PCC 7942 using linear fragments employing EDTA-mediated inhibition of DNases.

Material and methods

Strain and culture conditions

Synechococcus elongatus PCC 7942 strain was obtained from GeneArt® *Synechococcus* Engineering kit (Invitrogen). Cyanobacteria strain was grown in BG-11 medium at 34 °C under continuous illumination with white fluorescence lamps.

Linear PCR products

Linear fragments containing the spectinomycin resistance gene were obtained by PCR of pSyn_1 plasmid (Invitrogen). These fragments have the homologous sequence to the neutral site of *S. elongatus*, flanking the spectinomycin resistance gene (Fig. 1A). When transformed by PCR products containing an antibiotic resistance cassette and neutral site sequences, a double homologous recombination event occurs between the neutral site vector and the *S. elongatus* chromosome.

The PCR was carried out employing specific primers: (LINEAR-FOR: 5′-CGGGTTTCGCCACCTCTGACTTG-3′) and (LINERAR-REV: 5′-GCAAGCAGCAGATTACGCGCAGA-3′) using the Platinum Taq DNA Polymerase kit (Invitrogen), following the protocol: 94 °C/2 min, followed by 35 cycles of 94 °C/30 s, 60 °C/30 s, 72 °C/3 min and 30 s, and final extension of 72 °C/10 min. The products obtained were analyzed under agarose gel electrophoresis 1% and purified using Illustra GFX PCR DNA kit (GE HealthCare), following the manufacturer protocol. The fragments with 1400 bp were quantified in Qubit fluorimeter (Invitrogen) using Quant-iT dsDNA HS Assay kit (Invitrogen), following manufacturer protocol.

Cyanobacteria transformation

The linear fragments were inserted in *S. elongatus* PCC 7942, following the protocol described in Gene Synechococcus Engineering kit (Invitrogen). The pSyn_1 circular plasmid was employed as control. As negative control, samples of *S. elongatus* were incubated without exogenous DNA. After incubation, cells were plated in solid media, with BG-11 media, 1.5% agar and spectinomycin antibiotic (10 mg/mL). The negative control (without fragment) was plated in media with and without antibiotic.

Confirmation of integration

To confirm the integration in *S. elongatus* genome, a PCR employing specific primers was performed (SynREC-FOR: 5′-TGCTGCGTAACATCGTTGCTGCT-3′ and SynREC-REV: 5′-ATGTGATCGGAACCCTGAGCCGT-3′). If the fragment was integrated in the *S. elongatus* genome, one fragment of approximately 1400 bp would be amplified (Fig. 1B). In this sense, colonies of transgenic and non-transgenic (control) of *S. elongatus* were selected and transferred to plastic tubes with 10 μL of UltraPure™ DNase/RNase-Free Distilled water (Invitrogen). The genomic DNA was extracted of all samples by heating at 95 °C during 5 min. The PCRs were performed using Platinum Taq DNA Polymerase kit (Invitrogen). PCR was taken under amplification conditions of: 94 °C/2 min, 35 cycles of 94 °C/30 s, 58 °C/30 s, 72 °C/1 min and 30 s, and final extension of 72 °C/5 min. After PCR, samples were analyzed under agarose gel electrophoresis 1%.

Identification of DNase activity

For the identification of DNase activity, 15 mL of stationary-phase culture was centrifuged, washed with nuclease-free water, resuspended in BG-11 media and sonicated for 2 min (30 s on; 30 s off). Cell pellet (cell debris) and supernatant (cell lysate) were collected by centrifugation (11,000 × *g*/5 min). DNase activity was assayed by incubation of: (i) cell debris re-suspended in nuclease-free water and; (ii) cell lysate with 200 ng of pSyn-1 plasmid

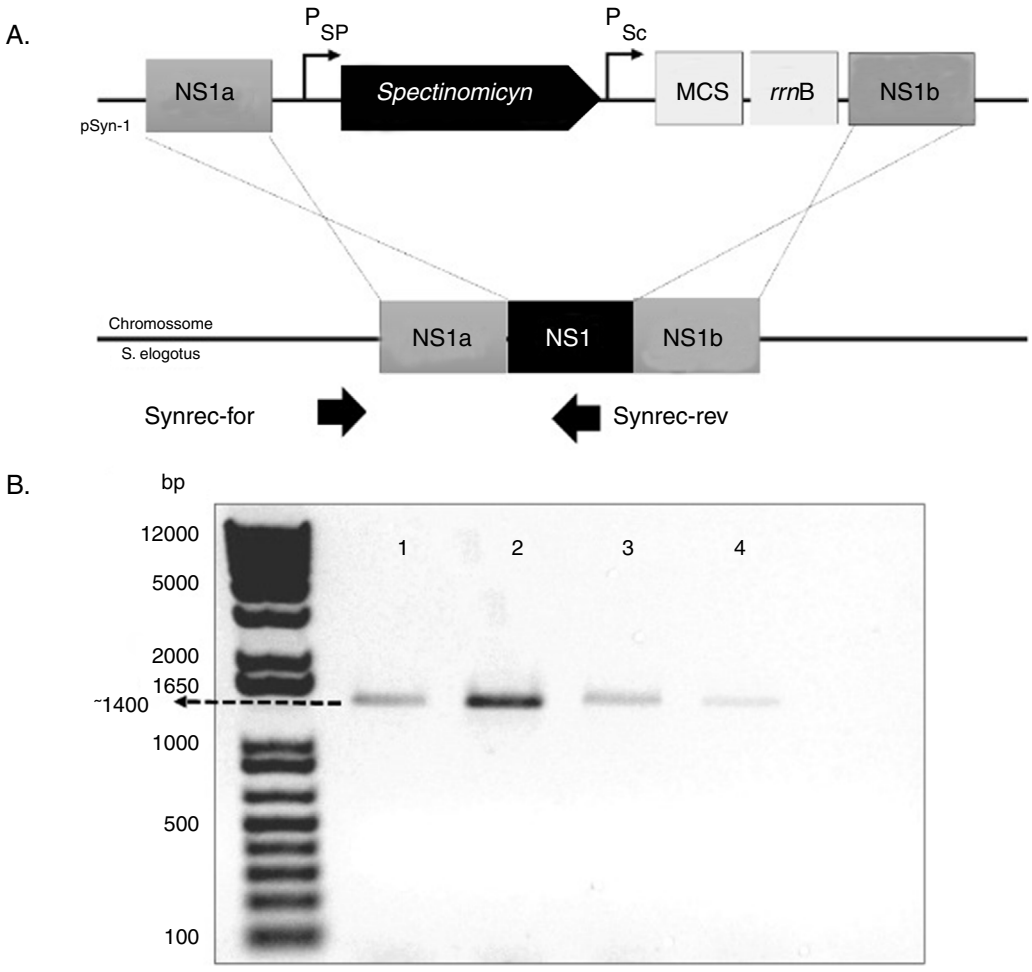

Figure 1 Linear fragment map and confirmation of spectinomycin gene integration at neutral site 1 of *Synechococcus elongatus* PCC 7942 by PCR. (A) Scheme of pSyn_1 plasmid linearized by PCR. The fragment produced contains the spectinomycin gene flanked by the neutral site 1 (NS1), which allows homologous recombination in *S. elongatus* genome. (B) Confirmation of spectinomycin gene integration at NS1 of *S. elongatus* by PCR. Genomic DNA was extracted from four selected transformants in media containing spectinomycin antibiotic and PCR was conducted employing SynREC-FOR primer, which anneals at region 5′, flanking the NS1, and SynREC-REV primer that anneals in spectinomycin gene, producing a fragment with approximately 1400 bp. Psp, spectinomycin promoter; Psc, constitutive promoter; MCS, multiple cloning site; *rrn*B, transcription termination region.

and linear fragment at 37 °C/30 min. Immediately after incubation, the samples were submitted under agarose gel electrophoresis 1% to evaluate the extent of degradation caused by the DNase.

DNase-inhibitor activity of EDTA

DNase-inhibitor activity of EDTA was analyzed under PCR linear fragments with cell lysate of *S. elongatus* obtained as previously described. For this, 200 ng of DNA was incubated with 200 μL of cell lysate at 34 °C for 0 h, 1 h, 2 h, 3 h and 4 h with 40 mM EDTA (final concentration) and without EDTA (control). This EDTA concentration was used since it was efficient to inhibit the DNase activity in other studies (Carballada & Esponda, 2001; Lanes, Sampaio, & Marins, 2009). Immediately after incubation, the samples were stored in −80 °C and then submitted to agarose gel electrophoresis 1% to evaluate the extent of DNase degradation.

Efficiency test of EDTA at transformation

The EDTA efficiency as DNase inhibitor was analyzed transforming the *S. elongatus* PCC 7942 under 40 mM of EDTA and without EDTA (control). The cyanobacteria were transformed with the linear DNA produced by PCR containing the resistance gene to spectinomycin antibiotic, employing the protocol previously described. The total number of transformants was counted on the plates containing antibiotic spectinomycin medium. Four independent transformations were performed for each treatment (with and without EDTA). Confirmation of spectinomycin gene integration at NS1 of *S. elongatus* was analyzed by PCR. The genomic DNA was extracted by heating at 95 °C during 5 min from ten transformants selected in each plate and the PCR was conducted employing the primers SynREC-FOR and SynREC-REV, producing a fragment with approximately 1400 bp, employing the protocol previously

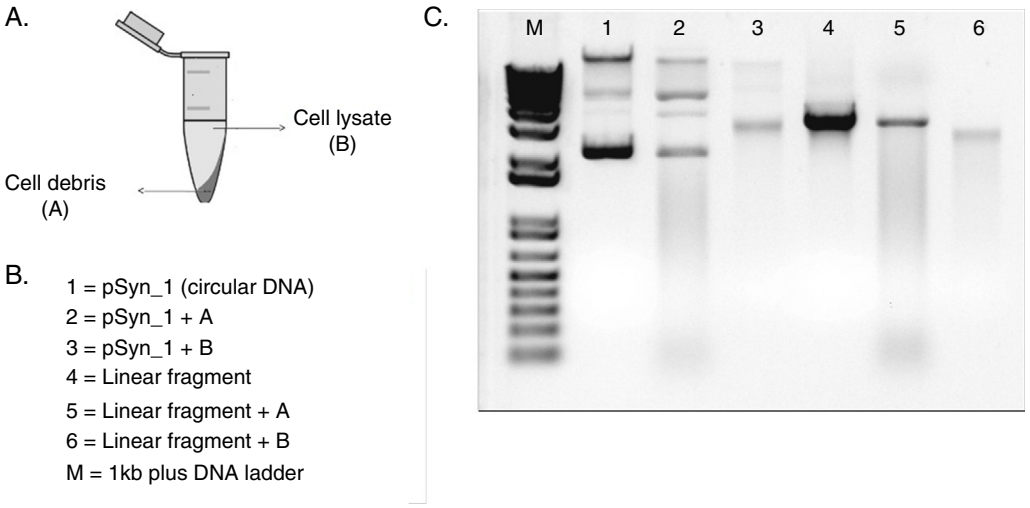

Figure 2 Identification of DNase activity in *Synechococcus elongatus* PCC 7942 strain. (A) Scheme of the extract where activity was evaluated: (A) cellular remains from culture submitted to sonication and, posteriorly, centrifugation; (B) supernatant. (B) DNase activity analyzed under agarose gel electrophoresis 1%. M, 1KB plus ladder; lanes 1: circular DNA (pSyn_1); 2: circular DNA incubated with cell debris (A); 3: circular DNA incubated with cell lysate (B); 4: pSyn_1 linearized by PCR, 5: linear DNA incubated with cell debris (A) and 6: linear DNA incubated with cell lysate (B).

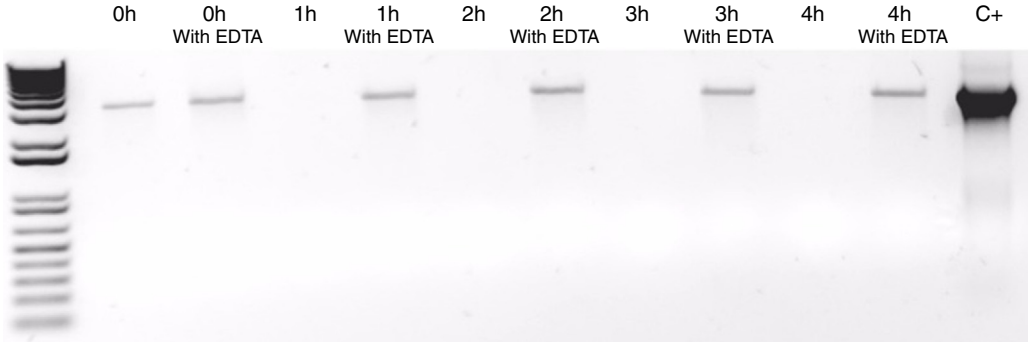

Figure 3 EDTA effect on DNase activity of *Synechococcus elongatus* PCC 7942 lysate cell. DNase activity analyzed under agarose gel electrophoresis 1% over five times (0 h, 1 h, 2 h, 3 h and 4 h of incubation); 200 ng of linear DNA was incubated with 200 μL of cell lysate at 34 °C with 40 mM EDTA and without EDTA. C+: DNA non-incubated (control).

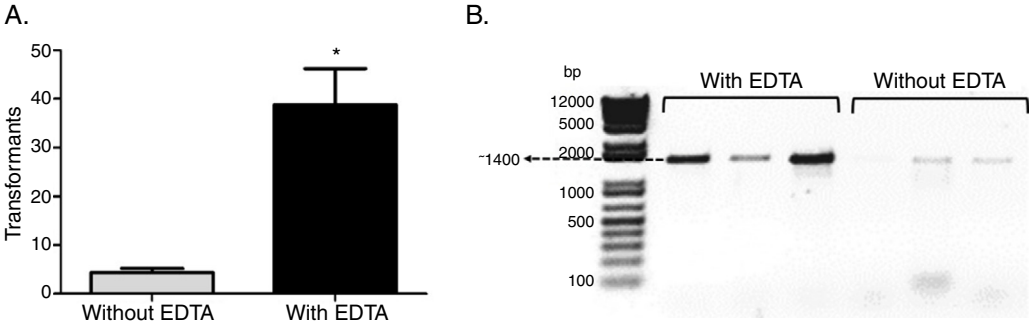

Figure 4 EDTA transformation with linear fragments and confirmation of integration. (A) Number of transformants of *Synechocossus elongatus* PCC 7942 obtained by transformation with linear fragments under PCR, containing the resistance gene to spectinomycin antibiotic. The transformation was made with (black bar) and without EDTA (gray bar). (B) Confirmation of spectinomycin gene integration at NS1 of *S. elongatus* by PCR. The genomic DNA was extracted from three transformants selected in media with spectinomycin antibiotic and the PCR was conducted employing primer. SynREC-FOR and SynREC-REV, producing a fragment with approximately 1400 bp. Average number of transformants ± standard deviation. *Significant differences, $p < 0.05$.

described. After PCR, samples were analyzed under 1% agarose gel electrophoresis. For the construction of Fig. 4B only one colony of each plate was submitted to electrophoresis.

Statistical analysis

The number of transformants obtained with and without EDTA was compared using paired *t*-tests. The significant difference was inferred when $p < 0.05$.

Results and discussion

In this study, *Synechococcus elongatus* PCC 7942 cells were transformed with pSyn_1 circular plasmid and the linear fragment produced by PCR, containing neutral site for homologous recombination and the spectinomycin antibiotic resistance gene. Firstly, only cyanobacteria transformed with circular plasmid grow in media with spectinomycin antibiotic. The integration of pSyn_1 circular plasmid, at neutral site 1, was confirmed by PCR, where part of the spectinomycin resistant gene was amplified and part of the strain genomic DNA (Fig. 1B). The lack of transformants with linear DNA led us to inquire if would be possible the presence of DNases in cells of *Synechococcus elongatus*, as described for some cyanobacteria (Stucken et al., 2013).

A search in cyanobacteria database CyanoBase (http://genome.microbedb.jp/CyanoBase) revealed the presence of five exonucleases in *S. elongatus* genome (Synpcc7942_0251, Synpcc7942_0517, Synpcc7942_0678, Synpcc7942_1886, Synpcc7942_2563). These enzymes hydrolyze phosphodiester link in extremities 3′ and 5′, digesting DNA sequences with free extremities. Herein we confirm the presence of DNases, when incubate circular or linear DNA with the lysate and cell debris (Fig. 2). DNase activity was detected mainly in cellular lysate, where there was fragment degradation in just 30 min of incubation (Fig. 2C). In addition, results of transformation pointed out that circular DNA is not so vulnerable to exonucleases, while linear DNA fragments seems to be quickly degraded by exonucleases since no positive colony was observed in the plates. Transfection protocols in mammal cells have also shown that circular DNA is more efficient than linear DNA due to presence of exonucleases (McLenachan, Sarsero, & Ioannou, 2007; Von Groll, Levin, Barbosa, & Ravazzolo, 2006).

With respect to exonuclease presence, Kufryk et al. (2002) verified in *Synechocystis* sp. PCC 6803 that the deletion of 5′–3′ RecJ exonuclease increased transformation efficiency in approximately 100×, regardless of the DNA employed (linear or circular). Another search in CyanoBase confirmed the presence of RecJ exonuclease and probably one more endonuclease in the genome of *Synechocystis* sp. PCC 6803. As previously cited, *Synechococcus elongatus* PCC 7942 exhibit in its genome sequences that codify for five exonucleases, performing higher protection against linear exogenous DNA strand. Therefore, It is possible that cleaving of linear DNA fragments by exonucleases had been the mainly reason for the inefficiency of the method applied.

In order to decrease DNase activity, we employed a DNase inhibitor, ethylenediamine tetraacetic acid (EDTA). The EDTA is an organic chelating that removes free divalent cations (Ca^{2+} and Mg^{2+}) from media, which are essential to enzymes activities. Other studies have already employed EDTA with the same objective and obtained promising results of nucleation activity inhibition in flounder semen (*Paralichthys orbignyanus*) (Lanes et al., 2009), human blood samples (Barra et al., 2015) and cyanobacteria *Spirula platensis* (Cao, Xu, Qiu, & Li, 1999).

In this study, the exonuclease activity inhibition under EDTA was firstly analyzed by incubation of the linear fragment in different incubation periods (Fig. 3). After 1 h, it was possible to detect almost complete degradation of the fragment, while that incubated under EDTA remained intact. Therefore, genetic manipulation of *S. elongatus* was conducted using linear DNA and 40 mM EDTA. Integration of linear fragments of spectinomycin gene at neutral site 1 of *S. elongatus* was confirmed, and those treated with EDTA had an increase of eight times in the number of transformants obtained (Fig. 4A). These results indicate that linear DNA integration is higher than circular DNA, when exonucleases are inactive.

Synechococcus elongatus PCC 7942 transformation with linear DNA is innovative for this strain. This method performs advantages regarding to traditional transformation methods, mainly with respect to methodological applicability and efficiency in transformants acquisition. Therefore, employment of exonuclease inhibitors as EDTA, allows manipulating cyanobacteria strains with linear DNA fragments in a faster and more efficient way.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This work received financial support from the Foundation for Research of the State of Rio Grande do Sul (FAPERGS No. 2257-2551/14-3) and DVA was a research fellow from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and FAPERGS (DOCFIX Proc. No. 1635-2551/13-1).

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