Advances in Microbiology, 2013, 3, 69-79
http://dx.doi.org/10.4236/aim.2013.36A009 Published Online October 2013 (http://www.scirp.org/journal/aim)

Substitution of Nitrite Reductase of *Thermosynechococcus elongatus* BP-1 by the Homologous Gene of *Phormidium laminosum*

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Received August 27, 2013; revised September 17, 2013; accepted September 23, 2013

ABSTRACT

Even though the nitrate assimilation operon has been extensively studied in *Phormidium laminosum*, some aspects still remain unclear. The genetic manipulation of this cyanobacterium is problematic that hinders the elucidation of further aspects of nitrogen metabolism. To circumvent this, *Thermosynechococcus elongatus* BP-1 was selected as a surrogate host and its *nirA* gene was substituted by the homologous gene of *P. laminosum*. This process, based on Long Flanking Homology Polymerase Chain Reaction and the natural competence of *T. elongatus* BP-1, required an intermediate *T. elongatus* BP-1 Δ*nirA*::*kat* mutant, which carries a gene encoding a thermostable kanamycin nucleotidyl transferase in place of *nirA_Te*. In the presence of *nirA_Pl*, *nirA* defective mutants of *T. elongatus* BP-1 recovered the ability to grow with nitrate as the sole nitrogen source, and showed a phenotype similar to that observed in wild-type cells. The procedure could be useful to substitute other genes from *T. elongatus* BP-1 with the homologues from *P. laminosum* in order to study this particular operon. Furthermore, it may be used as a general tool to explore phenotypic changes due to the exchange of a single gene between cyanobacteria.

Keywords: Thermophilic Cyanobacteria; Long Flanking Homology Polymerase Chain Reaction; Nitrite Reductase; Gene Transference

1. Introduction

Nitrate assimilation in cyanobacteria involves three main steps. Firstly, the anion entry inside the cell mediated by Nrt, the active ABC (ATP-Binding-Cassette) transporter [1-3]. Then, the intracellular nitrate is reduced to ammonium by two consecutive reactions catalyzed by nitrate reductase (NarB) and nitrite reductase (NirA). Finally, the resulting ammonium is incorporated into *L*-glutamate through the glutamine synthetase-glutamate synthase cycle (for reviews see: [4-6]).

In previous papers, we reported the purification and characterization of the proteins responsible of the uptake and reduction of nitrate and nitrite in the thermophilic cyanobacterium *P. laminosum* [7-10]. As in other cyanobacteria, in *P. laminosum* these proteins (NrtA, NrtB, NrtC, NrtD, NarB and NirA) are encoded by genes that are clustered together into an operon that presents the sequence *nirA-nrtA-nrtB-nrtC-nrtD-narB* [11]. Even though nitrogen assimilation has been studied in a large number of cyanobacteria, the structure and function of the proteins codified by the genes of this operon have been scarcely studied and poorly understood. The phylogenetic relationship among NirA from some cyanobacteria is shown in supplementary **Figure S1**.

In *P. laminosum*, although some advances have been made [7-11], the complete understanding of the functionality of this operon seems difficult without using genetic manipulation tools. These methods, frequently applied to analyze genomes and found out the particular roles of some proteins, could come in handy to continue investigating the roles and interactions of these proteins in *P. laminosum*. However, when considering the genetic manipulation of cyanobacteria, some strains present disadvantages over others. For instance, the genetic modification of *P. laminosum* is difficult mainly due to its filamentous character and because its genome has not been sequenced so far. Several transformation strategies have been developed and applied in some cyanobacteria, es-

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pecially in those whose genomes have been sequenced [12-17], as is the case of *T. elongatus* BP-1. This unicellular, rod-shaped, non-N_2-fixing cyanobacterium, originally isolated from a hot spring in Beppu, Japan [18], seems to have branched very close to the evolution of cyanobacteria. One of the most distinctive characteristics of *T. elongatus* BP-1 is its thermophilic character (showing an optimum growth temperature of approximately 55˚C). Furthermore, the genome of *T. elongatus* BP-1 has been sequenced [19] making this cyanobacterium a suitable candidate for genetic studies [20,21]. Moreover, *T. elongatus* BP-1 presents a remarkable natural competence. This property, found in some prokaryotes [22-25], allows the incorporation of naked DNA into their genome without resorting to techniques such as electroporation or conjugation (for reviews see: [26-30]). Formerly, the DNA fragments engineered for gene substitution were constructed by using PCR, restriction enzymes and ligases. As PCR has become a more common technique, methods to synthesize these fragments by using exclusively PCR and thus avoiding restriction enzymes are being established [31-34]. Such is the case of Long Flanking Homology Polymerase Chain Reaction (LFH-PCR). This technique, initially applied in *Saccharomyces cerevisiae* (denoted as Short Flanking Homology-Polymerase Chain Reaction (SFH-PCR) [35-38], was afterwards adapted to be used in many other microorganisms [13,31-34,39]. The synthesized DNA fragment, called "conversion cassette", consists of a marker gene flanked by the upstream and downstream regions of the target gene to be substituted. The capacity of the "conversion cassette" to originate the desired modifications, avoiding the alteration of any other region of the genome, relies on the process of homologous recombination [40-42].

With the goal of integrating the previous knowledge and experience acquired in nitrogen metabolism in *P. laminosum* and to be able to perform genetic transformation strategies, we have established a method that allows the substitution of a target gene of *T. elongatus* BP-1 with a homologous from *P. laminosum*. In this paper, we apply this process to obtain recombinant *T. elongatus* BP-1 cells carrying a substitution of nitrite reductase (*nirA_Te*) with the homologous from *P. laminosum* (*nirA_Pl*) and characterize their phenotype at different temperatures.

2. Materials and Methods

2.1. Materials

Oligonucleotides (**Table 1**) were obtained from Bonsaitech (Madrid, Spain) and dNTPmix from GE Healthcare (Uppsala, Sweden). *Pfu* polymerase and BioTaq polymerase were obtained from Biotools B&M Labs (Madrid, Spain) and from Bioline (London, United Kingdom), respectively. All other chemicals were of analytical or molecular biology grade.

2.2. Strains and Growth Conditions

The thermophilic, unicellular, non-diazotrophic cyanobacterium *Thermosynechococcus elongatus* BP-1 (NIES-2133) was obtained from Prof. M. Rögner (University of Bochum, Germany). Cells were grown photoautotrophically in 250 ml Erlenmeyer flasks containing 100 ml of sterile mineral medium BG_{11} [43]. Flasks were continu-

Table 1. Oligonucleotides used for LFH-PCR and for checking transformation (TC).

Primer	TC	DNA target	Sequence (5'-3')
FU1	Yes	*nirA_Te*	GCACCTGTCCTAATCCCTTGAAT
RU1	No	*nirA_Te*, *kat*	**GGTCCATTCATATGCCTCAC**AATGGGTCAGTTGAACATC
FD1	No	*nirA_Te*, *kat*	**CGCATACCATTTTGAACGG**CCGGTATTGACTGTATC
RD1	Yes	*nirA_Te*	CGACTGTCGGTGCTGATATTCAG
FM1	No	*kat*	GATGTTCAACTGACCCATT**GTGAGGCATATGAATGGACC**
RM1	No	*kat*	GATACAGTCAATACCGGG**CCGTTCAAAATGGTATGCG**
FU2	Yes	*kat*	GCACCTGTCCTAATCCCTTGAAT
RU2	No	*kat*, *nirA_Pl*	GCTGGAACGGTACTGGTCATAATGGGTCAGTTGAACATC
FD2	No	*kat*, *nirA_Pl*	TTGGCGCAAGACCGAAATGACCCGGTATTGACTGTATC
RD2	Yes	*kat*	CGACTGTCGGTGCTGATATTCAG
FM2	No	*nirA_Pl*	GATGTTCAACTGACCCATTATGACCAGACCGTTCCAGC
RM2	No	*nirA_Pl*	GATACAGTCAATACCGGGTCATTTCGGTCTTGCGCCAA
FnirA_Pl	Yes	*nirA_Pl*	ATGACCAGTACCGTTCC
RnirA_Pl	Yes	*nirA_Pl*	TCATTTCGGTCTTGCGC

Boldface and underlined text indicate homology to *kat*, or to *nirA* from *P. laminosum*, respectively. F, forward; R, reverse; TC, transformation check.

ously shaked in orbital incubators (Kuhner Shaker X, Basel, Switzerland) at 140 - 180 rpm. Light intensity (10 - 50 $\mu E \cdot m^{-2} \cdot s^{-1}$) and temperature (45˚C or 55˚C) were fixed depending on the type of assay. The air atmosphere inside the incubator was enriched with 1% (v/v) CO_2. Nitrogen-free BG_{11} culture medium (denoted here as $BG11_0$) was used supplemented with the indicated nitrogen source in each case. Thus, when *T. elongatus* BP-1 cells were cultured with nitrate as the sole nitrogen source, $BG11_0$ medium was supplemented with 17 mM $NaNO_3$ (denoted as BG11 medium) before autoclaving. When cells were cultured with ammonium, the sterile $BG11_0$ medium was supplemented with 2 mM $(NH4)_2SO_4$ using a concentrated stock solution of this salt previously sterilized by filtration (0.2 µm Millex®-FG filter, Millipore Co., Bedford, MA, USA). When agar plates of solid medium were required, $BG11_0$ medium and 1.5% (w/v) agar in ultrapure water (MilliQ, Millipore Co., Bedford, MA, USA) were autoclaved separately and mixed. In this moment, kanamycin (when indicated) and the necessary nitrogen source were added. The nitrogen source was added at the same final concentration used in liquid culture. Due to the high temperature used to grow cells of *T. elongatus* BP-1, in order to prevent desiccation, a thick layer of BG11-agar was solidified in the plates. Moreover, plates were incubated in a humid atmosphere.

The thermophilic, filamentous, non-diazotrophic cyanobacterium *Phormidium laminosum* strain OH-1-p.Cl_1 (renamed as *Geitlerinema* sp., PCC 8501) was grown axenically at 45˚C in 250 ml Erlenmeyer flasks containing 100 ml of sterile mineral medium D [44] supplemented with 0.5 $g \cdot l^{-1}$ $NaHCO_3$ as an additional carbon source. Cultures were grown with continuous illumination at a light intensity of 100 $E \cdot m^{-2} \cdot s^{-1}$.

Escherichia coli NovaBlue Single™ cells were used to propagate the different pT7Blue vectors used. Cells were grown in LB medium with IPTG, X-gal, carbenicillin and tetracycline according to the instructions indicated in the Perfectly Blunt Cloning Kit (Novagen, Darmstadt, Germany).

2.3. Genomic DNA Purification

The genomic DNA of cyanobacterial cells was extracted and purified using Nucleo Spin Tissue kit (Machery Nagel, Düren, Germany). A previous step was included, in which cells were incubated at 37˚C for 1 h with 50 mM Tris-HCl, pH 8.0, supplemented with 50 mM EDTA, 1% (v/v) Triton X-100, 20 $mg \cdot ml^{-1}$ lysozyme and 0.3 $mg \cdot ml^{-1}$ RNase [45]. The obtained DNA was stored at −20˚C.

2.4. Long Flanking Homology-Polymerase Chain Reaction

Oligonucleotides used to amplify genomic DNA of *T. elongatus* BP-1 (**Table 1**) were designed considering sequences from Cyanobase (http://genome.kazusa.or.jp/cyanobase). The sequence of *P. laminosum* nitrite reductase gene (*nirA_Pl*) was obtained from GenBank ID: Z19598.1. In all reactions 1 U of *Pfu* was used, and the amplification mixture (50 µl) contained 0.2 mM of each dNTP, 5 µl of (10X) PCR buffer, 2 mM $MgCl_2$, 1 µM of each oligonucleotide and the corresponding template.

To obtain the conversion cassette for *nirA_Te* replacement with *kat* (C1), four different PCRs were carried out (**Figure 1**). In the first, primers FU1 and RU1 were used to amplify the upstream fragment U1. In the

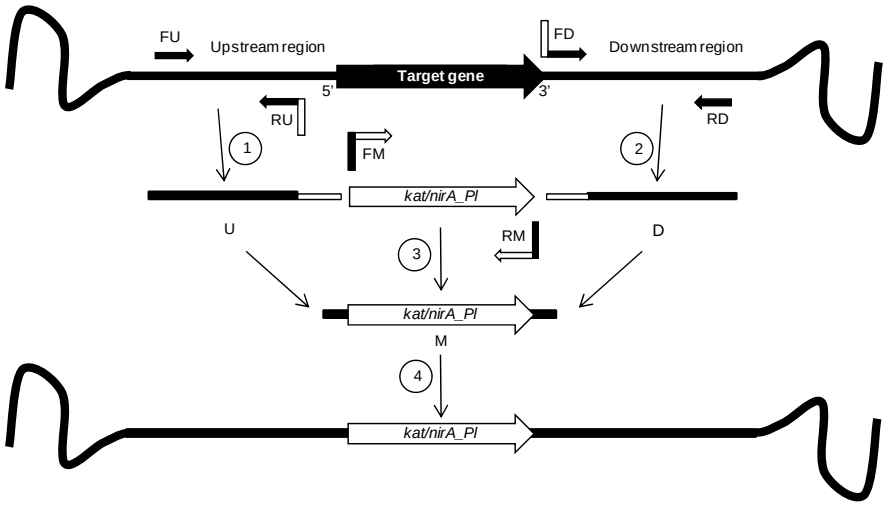

Figure 1. Schematic diagram of the LFH-PCR used. Small arrows indicate primers (see Table 1); *kat*, encodes a thermostable kanamycin resistance gene; *nirA_Pl* encodes nitrite reductase from *P. laminosum*. U, upstream region; M, marker gene; D, downstream region. Straight and curved lines indicate linear and genomic DNA, respectively. Circled numbers 1, 2, 3 and 4 indicate the four different PCR performed.

second, the downstream fragment D1 was obtained, using primers FD1 and RD1. In both PCRs 10 ng of *T. elongatus* BP-1 genomic DNA was used as a template under the same conditions: heating to 94˚C for 2 min; 30 cycles of denaturation at 94˚C for 30 s, annealing at 58˚C for 1 min, extension at 72˚C for 2 min, and finally an additional extension at 72˚C for 10 min. A third PCR allowed the amplification of M1 using primers FM1 and RM1. In this reaction, plasmid pKT1 was used as a template for *kat* with the following conditions: 2 min denaturation at 94˚C followed by 30 cycles of denaturation at 94˚C for 30 s, annealing at 62˚C for 1 min, extension at 72˚C for 2 min, and finally an additional extension at 72˚C for 10 min.

To prepare the conversion cassette to replace *kat* with *nirA_Pl* (C2), four different PCRs were carried out. In the first, primers FU2 and RU2 were used to amplify the upstream fragment U2. In the second, downstream fragment D2 was obtained, using primers FD2 and RD2. In both PCRs, 10 µl of *T. elongatus* BP-1 *nirA*/*kat*$^+$ genomic DNA was used as a template, under the same conditions used to amplify U1 and D1. A third PCR allowed the amplification of M2 using FM2 and RM2 oligonucleotides. In this reaction 10 ng of *P. laminosum* genomic DNA was used as a template, under the following conditions: 2 min denaturation at 94˚C followed by 30 cycles of denaturation at 94˚C for 30 s, annealing at 63˚C for 1 min, extension at 72˚C for 4 min, and finally an additional extension at 72˚C for 10 min. After gel extraction, purification and concentration of fragments U2, M2 and D2 from agarose gels, a fourth PCR was performed using approximately 200 ng U2, 400 ng M2 and 200 ng D2. PCR conditions were as follows: heating to 94˚C for 2 min; 30 cycles of 94˚C for 30 s, 60˚C for 1 min and 72˚C for 8 min; followed by a final extension at 72˚C for 10 min.

2.5. DNA Cloning and Plasmid Purification

When circular DNA was used, the fragments corresponding to the conversion cassettes were extracted from agarose gels using DNA Gel Extraction Kit (Millipore Co, Mildford, MA, USA) and cloned into pT7Blue vector using Perfectly Blunt Cloning Kit. Finally, plasmid DNA was purified with Qiaprep® Spin Miniprep Kit (Qiagen, Hilden, Germany) when used for sequencing, or with NucleoBond®Xtra Midi Plus Kit (Macherey Nagel, Düren, Germany) when used to perform *T. elongatus* BP-1 transformation assays.

2.6. *T. elongatus* BP-1 Transformation Assays

Conversion cassette C1, pT7Blue-C1 and pT7Blue-C2 were concentrated (to 1.5 $\mu g \cdot \mu l^{-1}$) and used for transformation of exponentially growing, *T. elongatus* BP-1. Transformation assays were performed according to (Iwai *et al*., 2004), with some modifications. To obtain *T. elongatus* BP-1 *nirA*$^-$/*kat*$^+$ cells, 6 µg of C1 or different amounts (from 6 µg to 50 µg) of pT7Blue-C1 were used. In this case, selection was carried out on solid $BG11_0$ medium supplemented with ammonium sulphate ($BG11_0$AS) and with 40 $\mu g \cdot ml^{-1}$ kanamycin. To insert *nirA* from *P. laminosum* in substitution of the *T. elongatus* BP-1 gene, different amounts (from 6 µg to 50 µg) of pT7Blue-C2 were used, and selection was performed in solid BG11 medium.

2.7. Analytical Methods

Growth of *T. elongatus* BP-1 was determined by measuring the turbidity of the cultures at 730 nm in a DU®800 UV/Vis spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA). DNA concentration and purity was determined by measuring its absorbance at 260 nm and 280 nm, or using agarose gels by comparison to a known concentration of DNA markers. To perform agarose gel electrophoresis of DNA, 1% (w/v) agarose in Tris-acetate-EDTA (TAE) was used. Gels contained 5 µl of SYBR® Safe DNA Stain (Invitrogen, Carlsbad, CA, USA). DNA was supplemented with 0.25% (w/v) bromophenol blue and 30% (v/v) glycerol loading buffer. In all gels, 5 µl of DNA HyperLadder I (Bioline, Randolph, MA, USA) were also loaded. Electrophoresis was carried out using a horizontal Mini-Sub Cell GT (BioRad, Hercules, PA, USA). An image analyzer GenoSmart (VWR International, Darmstadt, Germany) was used to visualize the bands. Recovery of DNA fragments from agarose gels was carried out with DNA Gel Extraction Kit (Millipore Co., Milford, MA, USA).

To screen transformants, colonies were picked from the corresponding plates, and colony PCR was performed. To lyse cells, 50 µl of buffer containing 1% (v/v) Triton X-100, 20 mM Tris-HCl, pH 8.0 and 500 mM EDTA, was added to each colony.

Samples were heated at 95˚C for 10 min and then centrifuged (3000 × g, 10 min). Finally, 4 µl of the supernatant was used as a template to perform PCR verifications with the corresponding oligonucleotides.

3. Results

The process followed to obtain *T. elongatus* BP-1 cells carrying *nirA_Pl* consisted of two differentiated steps. In a first stage, *nirA_Te* was eliminated from *T. elongatus* BP-1 and substituted by *kat*, a gene coding for a thermostable kanamycin nucleotidyl transferase variant from *Staphylococcus aureus* [46]. In the second step, the resulting cells (Δ*nirA*::*kat* mutant) were transformed with *nirA_Pl*, which was inserted in the same locus. To obtain both types of cells a constructed DNA fragment (conver-

sion cassette) is used to target a particular gene or sequence without promoting any other modification within of *T. elongatus* BP-1 genome. Construction of such conversion cassettes was achieved using LFH-PCR (**Figure 1**) and was followed by transformation of *T. elongatus* by natural competence. Results obtained during each step were as follows.

3.1. Construction of *T. elongatus* BP-1 Δ*nirA*::*kat* Mutant

To obtain this first cell type, the conversion cassette C1, targeted to replace *nirA* by *kat* without promoting any other modification within the genome of *T. elongatus* BP-1, was constructed. For this purpose, fragments U1, M1 and D1 were amplified in three different PCRs (**Figure 2(a)**) and assembled by a fourth PCR. The length of U1 and D1 is critical for transformation efficiency and it was determined from similar studies carried out in *Synechocystis* [15-17]. Thus, C1 consisted of the *kat* selection marker, flanked by the upstream and downstream regions *nirA*, U1 (508 bp) and R1 (536 bp). The correct assembly of the C1 conversion cassette was confirmed by agarose electrophoresis (**Figure 2(b)**) and sequencing.

Taking into account that there is only a single copy of *nirA* in the genome of *T. elongatus* BP-1, and that the expression of this gene is completely required by cells to grow in media with nitrate or nitrite as the sole nitrogen source, $BG11_0$ medium supplemented with ammonium ($BG11_0$AS) was used to grow Δ*nirA* mutants.

Initially, *T. elongatus* BP-1 cells were transformed with linear C1, the resulting mutants were selected in $BG11_0$AS plates supplemented with kanamycin and, finally, colony PCR was performed to verify the genotype of the transformants. To force the complete segregation of the genome, colonies were re-streaked in $BG11_0$AS

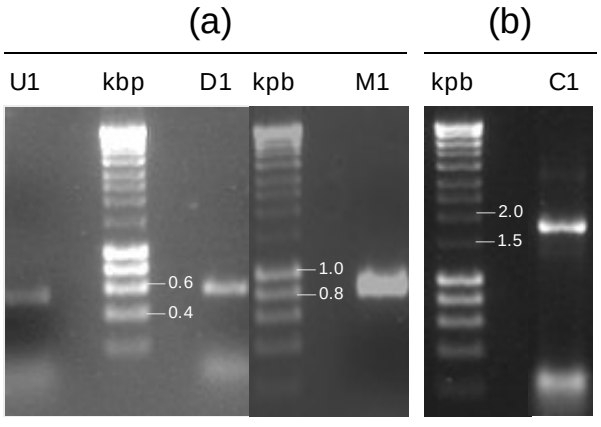

Figure 2. Analysis in agarose gels of (a) the PCR synthesized fragments to construct C1, and (b) the resulting C1 conversion cassette. U1, upstream fragment; D1, downstream fragment; M1, fragment containing the marker gene (*kat*).

plates with increasing kanamycin concentrations and analyzed by PCR. After 10 days, several colonies were able to grow in the presence of kanamycin, indicating that cells had acquired *kat*. PCR analysis, using FU1 and RD1 oligonucleotides, was done to ascertain the correct location of the insert and to guarantee the absence of *nirA* in the genome. These primers annealed at both ends of C1, and amplification by PCR indicated whether *nirA* (1536 bp) had been or not replaced by *kat* (approx. 800 bp). The PCR analysis by agarose electrophoresis shown in **Figure 3(A.a)** compares wild-type (lane WT) and transformed cells (lane T). In the case of transformants, two bands can be detected. This result suggested that transformation was incomplete and merodiploid cells, containing both *nirA* in some copies of their genome and *kat* in others, were obtained. Since *T. elongatus* BP-1 cells contain various copies of its whole genome [19], care has to be taken to guarantee that all copies of the target gene were replaced by gene conversion. Merodiploidity evidenced by PCR was confirmed phenotypically as cells were able to grow both in $BG11_0$AS medium supplemented with kanamycin and in medium with nitrate (BG11 medium) without kanamycin (**Figure 3B.c**).

The obtained result was not completely satisfactory because *nirA* should be removed from all genome copies of *T. elongatus* BP-1 before complementing cells with *nirA* of *P. laminosum*. To improve transformation efficiency, cells were transformed with circular DNA. In order to perform the transformation assay with circular DNA, C1 was cloned into pT7Blue, and after transformation and selection, the mutants were genotypically analyzed. Wild-type (**Figure 3(A.b)**, lane WT) and transformed cells (**Figure 3(A.b)**, lanes T1, T2 and T3) were analyzed. In this case, the absence of a 2500 bp product in T1, T2 and T3 (**Figure 3(A.b)**) confirmed the total replacement of all copies of *nirA_Te* in the transformants. Moreover, transformation was verified phenotypically by assessing cell growth in medium with nitrate as the sole nitrogen source. In this case, mutants grew in medium $BG11_0$AS supplemented with kanamycin, but were unable to grow with nitrate as the sole nitrogen source (BG11 medium) (**Figure 3(B.d)**).

3.2. Incorporation of *P. laminosum nirA* Gene into the Chromosome of *T. elongatus* BP-1 Δ*nirA*::*kat* Mutant

To construct this cell type, previously obtained *T. elongatus* BP-1 Δ*nirA*::*kat* mutants were transformed with pT7Blue-C2 circular DNA. To construct the conversion cassette C2, fragments U2, M2 and D2 were amplified in three different PCRs (**Figure 4(a)**) and assembled by a fourth PCR. U2 and D2 were the same regions amplified when designing C1. In this occasion, C2 consisted of

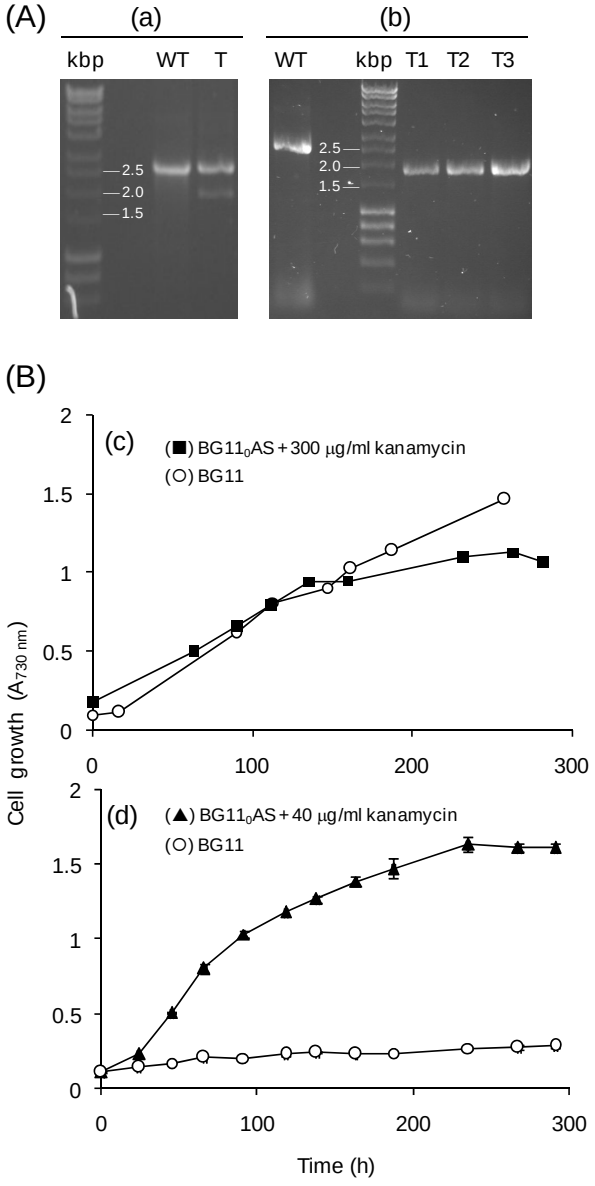

Figure 3. Mutant analysis of *T. elongatus* BP-1. (A) Genotypic assessment of cells transformed with the conversion cassettes C1 (a) and pt7Blue-C1 (b). Oligonucleotides used were FD1 and RD1, and the templates for PCR were genomic DNA extracted from wild type (WT) or transformed (T) colonies. In panel (b), T1, T2 and T3 correspond to transformants grown in BG11$_0$AS medium supplemented with 40, 100 and 300 μg·ml^{-1} kanamycin, respectively. (B) Phenotypic assessment of cells transformed with the conversion cassettes C1 (c) and pt7Blue-C1 (d). Cell growth was assessed in BG11 and BG11$_0$AS media supplemented with 40 or 300 μg·ml^{-1} kanamycin. Each point in curves represents mean values (±standard deviations) of data obtained from two independent cultures.

nirA_Pl as the marker gene, flanked by the upstream and downstream regions of kat in *T. elongatus* BP-1 Δ*nirA*::*kat* mutants. The C2 construct was confirmed by agarose electrophoresis (**Figure 4(b)**), sequenced and

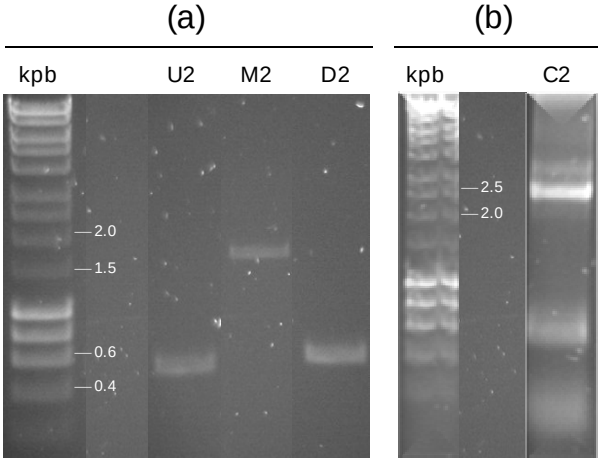

Figure 4. Analysis in agarose gels of (a) the PCR synthesized fragments to construct C2, and (b) the resulting C2 conversion cassette. U2, upstream fragment; M2, fragment containing *nirA_Pl*; D2, downstream fragment.

finally cloned to create pT7Blue-C2. This construct was used to transform *T. elongatus* BP-1 Δ*nirA*::*kat* mutants. Once transformed, selection was carried out in BG11 plates. Colonies were picked and analysis by PCR was performed using FU2 and RD2.

The presence of 2500 bp products confirmed that cells had incorporated nirA from *P. laminosum* (**Figure 5(A.a)**).

Furthermore, genotypic verification was also done with primers annealing at both ends of *nirA* from *P. laminosum* (*FnirA_Pl* and *RnirA_Pl*) to guarantee the identity of the insertion. Transformation was checked (**Figure 5(A.b)**) confirming that cells had incorporated *nirA* instead of *kat*. However, when transferred to liquid medium after several attempts, mutants were unable to grow optimally in BG11 medium. In order to improve the genotype of the transgenic cells, the assay was repeated lowering the temperature used for transformation and cell growth. After repeating transformation at 48˚C, a higher number of transformants was obtained, whose genotype was confirmed with FU2, RD2 (**Figure 5(B.a)**) and *FnirA_Pl*, *RnirA_Pl* (**Figure 5(B.b)**). After an initial incubation in BG11$_0$AS medium to facilitate growth, cells were transferred to BG11 medium where optimum growth was observed (**Figures 6(a)** and **(b)**).

3.3. Nitrate Assimilation of *T. elongatus* BP-1 Δ*nirA*::*kat* Mutants Complemented with *P. laminosum nirA*

Finally, to determine whether *nirA_Pl* could completely restore the capacity of complemented cells to grow with nitrate and also to determine whether growth was affected by the presence of *nirA* (from *T. elongatus* BP-1 or from *P. laminosum*), the growth of *P. laminosum* cells and *T. elongatus* BP-1, both wild-type and recombinant cells carrying *nirA* from *P. laminosum*, was assessed at

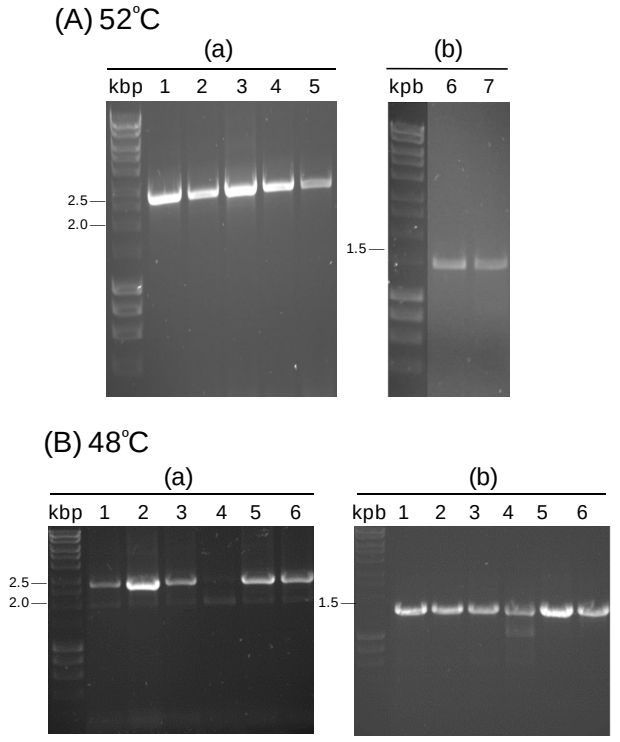

Figure 5. Agarose gel analysis of PCR performed with DNA extracted from several colonies of *T. elongatus* BP-1 transformed at (A) 52˚C and (B) 48˚C. Transformation was checked with primers (a) FU2 and RD2, and (b) *FnirA_Pl* and *RnirA_Pl*.

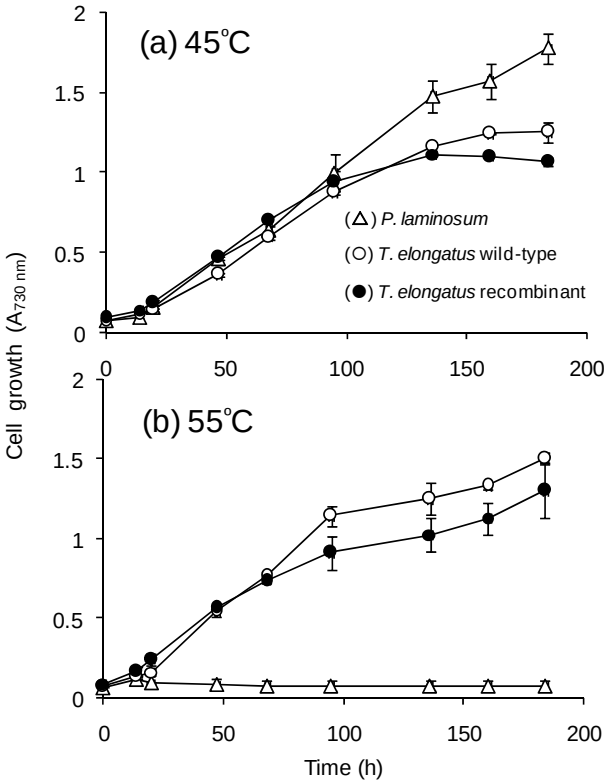

Figure 6. Cell growth in BG11 medium at (a) 45˚C, and (b) 55˚C of *P. laminosum* and wild-type and recombinant *T. elongatus* BP-1. Each point in curves represents mean values (±standard deviations) of data obtained from two independent cultures.

45˚C (**Figure 6(a)**) and 55˚C (**Figure 6(b)**). These temperatures were determined as optimum to grow cells of *P. laminosum* and *T. elongatus* BP-1, respectively. As expected, *P. laminosum* was unable to grow at 55˚C and grew optimally at 45˚C. In case of *T. elongatus* BP-1, although slight differences were observed between wild-type and chimeric cells carrying *P. laminosum nirA*, this effect was more noticeable at 55˚C than at 45˚C.

4. Discussion

Globally, studies related to bacterial transformation are targeted to eliminate, disrupt or insert a particular gene or group of genes within their genome or plasmids. In all cases, marker genes are needed for this purpose. These markers, which allow selection of transformed cells, usually code for proteins that provide a selective advantage to its host. In this study, the process followed to obtain transgenic cells (*i.e.*, *T. elongatus* BP-1 cells bearing the *nirA* gene from *P. laminosum*) is a combination of all the above-mentioned strategies. Although direct substitution of *nirA_Te* with *nirA_Pl* may have been feasible, it might have been difficult to distinguish both cell types phenotypically due to the fact that the proteins codified by both genes have the same catalytic activity.

One of the requirements to carry out the transformation is to have a marker gene that allows selecting transformants successfully. Therefore, the construction of the first cell type (*i.e.*, *T. elongatus* BP-1 Δ*nirA*::*kat*) required a marker gene stable enough under the culture conditions of *T. elongatus* BP-1 (55˚C under constant illumination). Although many selection markers are available for the transformation of mesophilic microorganisms, few are suitable for the transformation of thermophilic cyanobacteria. The use of a gene coding for a thermostable kanamycin nucleotidyl transferase (*kat*) allowed the selection of mutants at high temperature in $BG11_0$AS plates supplemented with kanamycin.

Verification of mutants transformed with linear C1 (**Figure 3(A.a)**) indicated that this initial attempt failed, giving place to merodiploid cells bearing *kat* in some copies of their genome and *nirA* in others. Incomplete gene deletion or substitution usually stems from the deleted gene (in this case *nirA*) being essential. Nevertheless, this is not the case because cells cultured using ammonium as nitrogen source do not need to express nitrite reductase to grow. When working with merodiploid microorganisms, re-streaking colonies in medium containing increasing concentration of antibiotic usually promotes the genome segregation and complete substitu-

tion of the target gene by the antibiotic resistance marker [13]. However, re-streaking of colonies in the presence of increasing concentrations of kanamycin did not improve genome segregation. Other authors have reported the same problem when transforming cells with linear DNA [32]. One explanation could be based on some DNA uptake sequences found in other bacteria. Some authors have reported the importance of DNA uptake sequences (DUS) [29,47] for efficient transformation. The fact that the lack of these sequences would have limited the entrance of C1 to the inside of *T. elongatus* BP-1 seems improbable, as other researchers have reported transformation in this cyanobacterium independently of the DNA sequence used [20,21]. In the present work, the best explanation for this result might be the lower transformation efficiency of *T. elongatus* BP-1 cells compared to some other bacteria and cyanobacteria. The quantity of C1 used may not have been enough for the complete substitution of *nirA* by *kat* gene, and/or this linear DNA might have been digested by nucleases, resulting in reduced transformation efficiency [48].

In the case of the cyanobacterium *Synechocystis* sp. PCC6803, where transformation has been extensively evaluated, some authors [15-17] reported that efficiency is higher when circular DNA was used in the conversion cassette. In the case of *T. elongatus* BP-1, colony PCR (**Figure 3(A.b)**) and growth assessment (**Figure 3(B.d)**) confirmed that gene substitution was complete, strongly suggesting that transformation with circular DNA is more efficient than with its linear counterpart.

T. elongatus BP-1 Δ*nirA*::*kat* mutant grown in $BG11_0$AS medium was transformed with pt7Blue-C2. Due to the low integration efficiency experienced with linear cassette C1, cells were transformed with pT7Blue-C2 and not with linear C2. As reported in the Results section, the first attempts to complement *T. elongatus* BP-1 Δ*nirA*::*kat* mutants were unsuccessful. Even though mutant analysis by PCR indicated that cells had incorporated *nirA* effectively replacing *kat* (**Figure 5(A.a)**), amplification with *FnirA_Pl* and *RnirA_Pl* was only detected in two colonies (**Figure 5(A.b)**). Furthermore, the obtained colonies were few and yellowish, presenting a color similar to that observed when cells were subjected to nitrogen starvation or under other situations related to metabolic stress. It is important to highlight that these cells have been transferred from an ammonium to a nitrate containing medium. Ammonium is the easiest assimilable source of inorganic nitrogen because it does not require further reduction and can be taken up by cells through either active transport or diffusion mechanisms [49-51]. However, the uptake of nitrate and/or nitrite from the external medium by cells requires the expression of a number of proteins involved in their transport to the cytoplasm and further reduction to ammonium. Consequently, when a nitrogen source is shifted to a less favorable one, cells need some time to synthesize or balance the levels of the required newly synthesized proteins. However, even after allowing extra acclimatization, transformants showed a phenotype typical of cells unable to grow adequately with nitrate.

Although data are insufficient to confirm it, the results point towards the differences in *nirA* thermostability between *P. laminosum* and *T. elongatus* BP-1. Considering that *P. laminosum* (which shows optimal growth at 45˚C) presents a lower thermophilicity compared to *T. elongatus* BP-1, the temperature of 52˚C used to select and cultivate mutants may have been too high for the expression or correct function of *P. laminosum nirA*. This explanation could be reinforced by previous data obtained in our laboratory of the nitrite reductase activity from wild-type *P. laminosum* [7]. These results indicated that the purified *nirA* was completely thermostable *in vitro* at temperatures up to 40˚C, but its activity decreased rapidly at higher temperatures, although 45˚C was estimated as the optimum temperature for cell growth under laboratory conditions.

To discard temperature as a factor compromising the correct expression of *nirA* in *P. laminosum*, the transformation assay was repeated lowering the temperature to 48˚C. After selecting some colonies from BG11 plates and growing them at moderate light intensity (initially at 48˚C in liquid $BG11_0$AS to increase cell density, and then in BG11 medium), some colonies were able to grow in BG11 medium (*i.e.*, showing a normal phenotype), and unable to grow in medium supplemented with kanamycin.

Finally, when comparing wild-type and transgenic *T. elongatus* BP-1 growth curves (**Figure 6**), even though there were no significant differences, these were greater at 55˚C (**Figure 6(b)**) than at 45˚C (**Figure 6(a)**), probably due to differences in thermostability between both nitrite reductases.

5. Conclusion

In conclusion, we have constructed transgenic cells of *T. elongatus* BP-1 in which *nirA* was substituted by the homologous gene of *P. laminosum*. These recombinant cells can grow in BG11 medium, indicating that the *P. laminosum nirA* gene was expressed in an active form in cells of *T. elongatus* BP-1. Slight differences between the growth of wild-type and transgenic cells of *T. elongatus* BP-1 were observed, probably due to the different thermostability of NirA of both cyanobacteria. The followed process to construct transgenic cells of *T. elongatus* BP-1 was mainly based on the LFH-PCR technique and the remarkable natural competence showed by this cyanobacterium. Moreover, the thermostable kanamycin nucleotidyl transferase-encoding *kat* gene has also been very helpful in the intermediate stage of the process. This

procedure can be applied in the future to transfer other genes present in *nirA-permease-narB* operon from *P. laminosum* to *T. elongatus* BP-1 and would be a useful tool to explore changes in phenotype due to the exchange of a single gene between cyanobacteria.

5. Acknowledgements

This work was supported by the University of the Basque Country (UPV/EHU) (Grants GIU07/55 and GIU11/25). We are grateful to Prof. J. Berenguer and Dr. A. Hidalgo (Autonomous University of Madrid, Spain) for providing us with the pKT1 plasmid carrying the *kat* gene. A strain of *T. elongatus* BP-1 cells was kindly provided by Prof. M. Rögner (University of Bochum, Germany). MB was the recipient of a scholarship from the UPV/EHU.

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Supplement

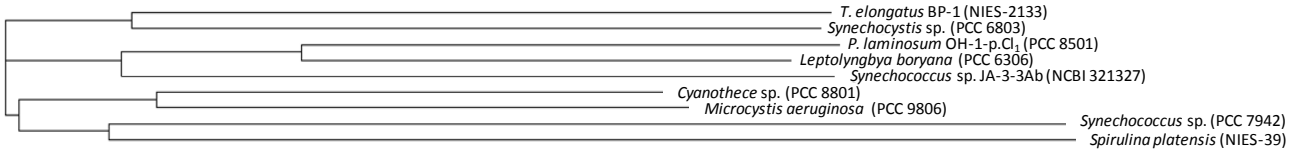

Figure S1. Phylogenetic tree reflecting nitrite reductase protein (NirA) of some cyanobacteria from Chroococcales (*T. elongatus* BP-1, *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7942, *Synechococcus* sp. JA-3-3Ab, *Cyanothece* sp. PCC 8801, *Microcystis aeruginosa* PCC 9806) and Oscillatoriales (*P. laminosum* OH-1-p clone1, *Leptolyngya boryana, Spirulina platensis* NIES-39) order. Sequences were obtained from NCBI and alignment was performed with clustalW2 (EMBL-EBI).

