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UNIVERSIDADE DE COIMBRA

Design of synthetic oxygen consuming devices (OCDs) and their characterization in the cyanobacterium *Synechocystis* sp. PCC 6803: The F_1F_0 ATP synthase.

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímca, realizada sob a orientação científica da Doutora Paula Tamagnini (Universidade do Porto), da Doutora Catarina Pacheco (Universidade do Porto) e da Doutora Paula Veríssimo (Universidade de Coimbra).

Sérgio Ferreira
2012

"Success is going from failure to failure without loss of enthusiasm."

-Winston Churchill

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Contents

Abstract **9**

Resumo **11**

1 Introduction **13**

2 Materials and Methods **21**

2.1 Organisms and standard growth conditions **23**
2.2 Construction of the vectors **23**
2.2.1 Site directed mutagenesis (SDM) **23**
2.2.2 Plasmid DNA isolation and mutation screening of the mutations **25**
2.2.3 Insertion of the selection marker **26**
2.3 *Synechocystis* transformation **27**
2.4 Confirmation of the segregation of the mutants **28**
2.5 Characterization of the mutants **28**
2.5.1 Growth curves **28**
2.5.2 Chlorophyll *a* content **29**
2.5.3 O_2 consumption assays **29**
2.5.4 ATP measurements **30**

3 Results and Discussion **31**

3.1 Vectors **33**
3.2 Generation and confirmation of the mutants **36**
3.3 Characterization of the βI179A mutant **39**

4 Conclusions and Future Perspectives **43**

5 References **47**

Abstract

Cyanobacteria are a ubiquitous group of prokaryotes that perform oxygenic photosynthesis and many are also able to fix atmospheric nitrogen. These organisms may possess several enzymes involved in hydrogen metabolism: nitrogenase(s) catalyzing the production of molecular hydrogen along with the reduction of nitrogen to ammonia, an uptake hydrogenase involved in the consumption of the hydrogen produced by the nitrogenase, and a bidirectional hydrogenase with the ability to consume or produce hydrogen. This makes cyanobacteria good candidates for photobiological hydrogen production. Using cyanobacteria/cyanobacterial enzymes for hydrogen production faces a huge limitation which is the sensitivity of the enzymes to oxygen. One possible strategy to solve this problem is the use of oxygen consuming devices (OCDs) to create a microaerobic environment inside the cell. In this work, *Synechocystis* sp. PCC 6803 mutants, containing variants of the native ATP synthase, were generated in order to assess the feasibility of using variants of this enzyme as OCDs. The ATP synthase is the enzyme responsible for synthesizing most of the cellular ATP. It is known that the ATP/ADP ratio regulates the respiration rate; therefore impairing the activity of the ATP synthase should increase the oxygen consumption as a compensation for the decrease in the ATP/ADP ratio. The results obtained for the βI179A mutant, with a mutated β subunit of the ATP synthase, show a significant increase in oxygen consumption compared to the wild type, suggesting that this variant of the ATP synthase might be a good OCD. Other variants of the ATP synthase were generated in this work but have not yet been characterized.

Keywords: Cyanobacteria; ATP synthase; oxygen consuming devices; hydrogen.

Resumo

As cianobactérias são um amplo grupo de procariotas que são capazes de realizar fotossíntese com libertação de oxigénio, muitas são também capazes de fixar azoto atmosférico. Estas bactérias possuem várias enzimas envolvidas no metabolismo do hidrogénio: nitrogenase(s) que produzem hidrogénio durante a fixação de azoto atmosférico, a hidrogenase de assimilação que está envolvida no consumo do hidrogénio produzido pela nitrogenase e a hidrogenase bidireccional que tem a capacidade de consumir ou produzir hidrogénio. Estas características fazem das cianobactérias excelentes candidatas para a produção fotobiológica de hidrogénio. O uso das cianobactérias ou das suas enzimas para a produção de hidrogénio apresenta uma grande limitação que é a sensibilidade destas enzimas ao oxigénio. Uma das possíveis estratégias para a resolução deste problema é o uso de módulos de consumo de oxigénio (OCDs – *Oxygen Consuming Devices*) de forma a criar um ambiente microaeróbico no interior da célula. Neste trabalho geraram-se mutantes de *Synechocystis* sp. PCC 6803 com variantes da ATP sintase com o objectivo de testar a possibilidade de usar estas variantes da enzima como OCDs. A ATP sintase é a enzima responsável pela síntese da maior parte do ATP intracelular. Sabe-se que o rácio ATP/ADP regula a taxa respiratória. Assim diminuindo a actividade da ATP sintase deverá aumentar a taxa respiratória de forma a compensar o decréscimo do rácio ATP/ADP. Os resultados obtidos para o mutante βI179A, com uma mutação na subunidade β da ATP sintase, mostram um aumento significativo do consumo de oxigénio comparativamenta à estirpe selvagem, sugerindo que esta variante poderá ser utilizada como OCD. Durante este trabalho geraram-se mutantes com outras variantes da ATP sintase mas estes ainda não estão completamente segregados não foi possível fazer a caracterização.

Palavras chave: Cianobactérias; ATP sintase; módulos de consumo de oxigénio; hidrogénio

1 Introduction

With the growing concern over the environment and consequences of the pollution caused by the use of fossil fuels it becomes more important everyday to find cleaner fuels. One of the major candidates to substitute fossil fuels is hydrogen (Demirbas, 2012; McLellan et al, 2012), therefore it is important to devise strategies for clean, competitive and sustainable hydrogen production. One of the promising strategies is the photobiological production of hydrogen, using photosynthesis and the natural water-splitting process, in which the organisms, e.g. cyanobacteria, absorb energy from the sun and evolve H_2 directly from water without an intermediate biomass stage (Ghirardi et al, 2007).

Cyanobacteria are a ubiquitous and ancient group of photosynthetic prokaryotes that perform oxygenic photosynthesis, similar to that of algae and higher plants, and have a long evolutionary history, which is believed to extend to at least 3 500 Ma ago (Schopf, 2000). These organisms play a major role as primary producers in the Earth's carbon cycle, and it is also commonly accepted that they were the first major group of phototrophs with the ability to use water for the production of molecular oxygen, leading to the transition from the Earth's anaerobic atmosphere in the Proteozoic Eon, to its current aerobic condition (Knoll, 2008; Schopf, 2000). Cyanobacteria display a wide range of morphologies including unicellular, colonial and filamentous (Whitton B.A., 2000), and many strains are also able to fix N_2, making them prominent players in the nitrogen cycle (Knoll, 2008). These characteristics contribute for their ability to thrive in many environments ranging from fresh to salt water, soils and extreme environments such as hot springs or desertic areas.

Cyanobacteria may possess several enzymes involved in hydrogen metabolism: nitrogenase(s) catalyzing the production of molecular hydrogen along with the reduction of nitrogen to ammonia, an uptake hydrogenase involved in the consumption of the hydrogen produced by the nitrogenase, and a bidirectional hydrogenase with the ability to consume or produce hydrogen (Fig. 1). Both cyanobacterial hydrogenases are NiFe enzymes, the most common hydrogenases found in bacteria and *Archaea* (Vignais & Colbeau, 2004). The physiological function of the bidirectional hydrogenase is still not clear, however it was proposed that could function as a redox regulator in different cellular processes, e.g. as an electron valve during photosynthesis, or controlling the

excess reducing power produced during fermentation (Carrieri et al, 2011; McIntosh et al, 2011; Tamagnini et al, 2007). Although this enzyme is able to work in both ways, catalyzing the formation or consumption of hydrogen it was recently shown to have a bias towards its production (McIntosh et al, 2011).

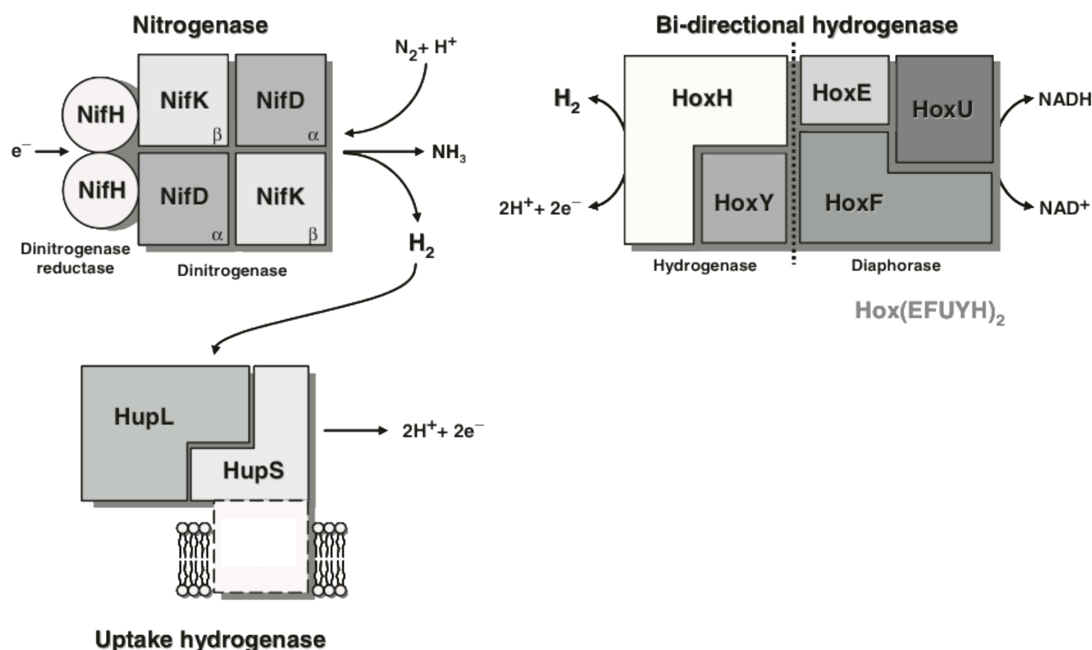

Fig. 1. Enzymes directly involved in hydrogen metabolism in cyanobacteria. While the uptake hydrogenase is present in most of the nitrogen-fixing strains, the bidirectional enzyme is present in non-N_2-fixing and N_2-fixing strains but is not a universal enzyme. The existence of a third subunit anchoring the uptake hydrogenase to the membrane is yet to be confirmed (adapted from Tamagnini et al, 2007).

Two main strategies can be used for hydrogen production in cyanobacteria: using the nitrogenase that produces H_2 as a by-product of N_2 fixation or using the bidirectional hydrogenase. These strategies face a big limitation, which is the high sensitivity of both enzymes to oxygen. There are many approaches to solve the O_2 problem, for instance, the production of oxygen tolerant enzymes (Lukey et al, 2011) or the temporal separation between light capture and hydrogen production (Melis et al, 2000). In this work a different strategy will be explored, the use of oxygen consuming devices (OCDs) to promote a microanaerobic environment within the cell. In this case, the OCD will be the native F_1F_0 ATP synthase from the unicellular cyanobacterium

Synechocystis sp. PCC 6803, that will be modified in order to increase intracellular O_2 consumption. The cyanobacterial F-type ATP synthases have not been as well studied as the ones from other organisms, but so far it is known that they share traits of the enzymes from both chloroplasts and bacteria. This is due, no doubt, to the presence of a photosynthetic apparatus and oxidative phosphorylation complexes analogous to those of higher plant chloroplast and other bacteria, respectively. The F_0 portion of the cyanobacterial enzyme is an integral membrane protein complex comprising single copies of the a, b and b' subunits and about ten copies of the c subunit. The F_1 complex is attached to the F_0 and has a subunit stoichiometry of 3α, 3β, γ, δ, ε (Bryant, 1994; von Ballmoos et al, 2009). The structure of an analogous enzyme, from *Escherichia coli,* can be observed in Fig. 2.

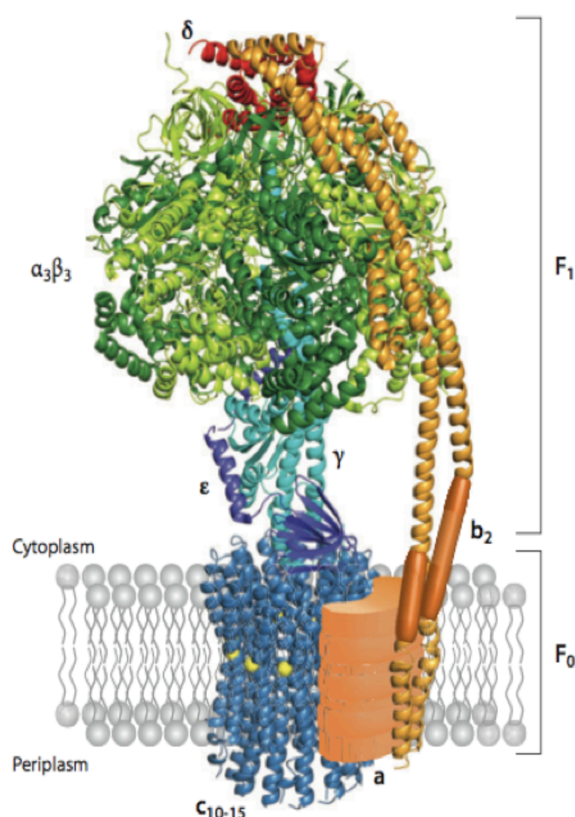

Fig. 2. F-Type ATP synthase structure from *E. coli* (von Ballmoos et al, 2009).

Generally, in cyanobacteria the subunits of the ATP synthase are encoded by two operons, *atp1* and *atp2*. The *atp1* operon in *Synechocystis* sp. PCC 6803

encodes the subunits a, c, b', b, δ, α, γ and the *atp2* operon encodes the genes for the β and ε subunits (Fig. 3) (Lill & Nelson, 1991; McCarn et al, 1988).

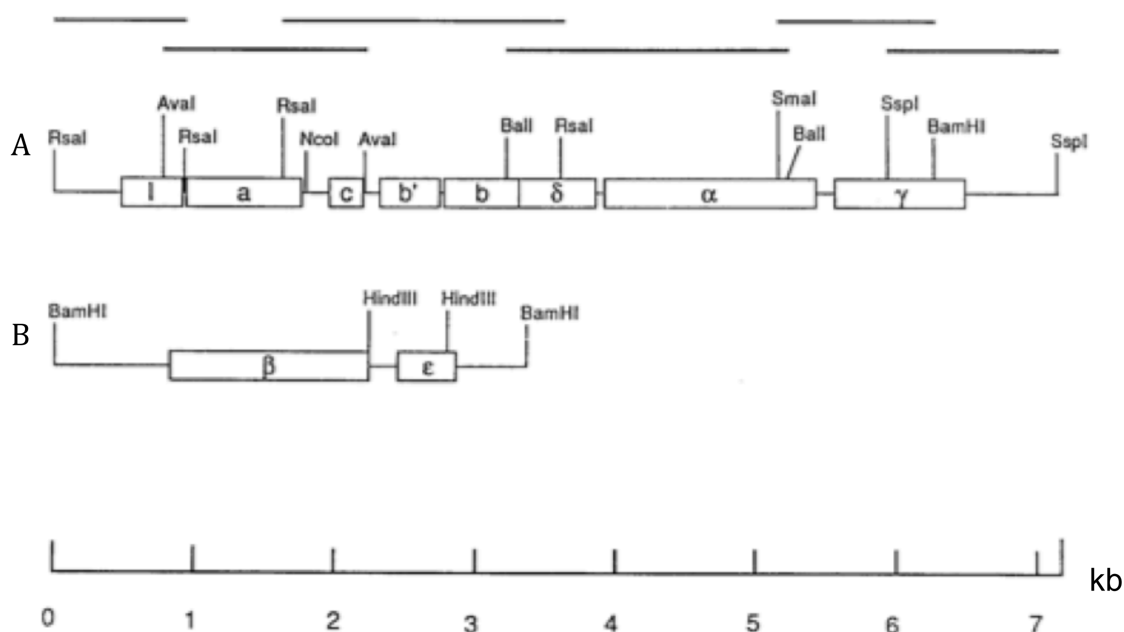

Fig. 3. *Synechocystis* sp. PCC 6803 *atp* operons. The two operons are shown schematically, A represents *atp1* and B represents *atp2,* with the coding regions for the subunits of F_0F_1 ATP-synthase (Lill & Nelson, 1991).

To produce ATP, the ATP synthase works as a miniature engine composed of two rotary motors, the F_0 and the F_1. The F_0 complex is embedded in the membrane and works as a channel, being able to harness the energy from the proton electrochemical gradient through the membrane to power the synthesis of ATP from ADP and inorganic phosphate. The synthesis of ATP takes place in the F_1 complex at a catalytic site in each β subunit. The γ and the $\gamma\varepsilon c_{10}$ (a complex formed from γ, ε, and 10 c subunits) rotate during the catalysis via a three 120 ° steps rotation creating the conformational changes necessary for ATP synthesis. The hinge domain of the β subunit plays a particularly important role in the synthesis of ATP because it is responsible for supporting the conformational

changes during catalysis and rotation (Bryant, 1994; Claggett et al, 2007; Kashiwagi et al, 2008; von Ballmoos et al, 2009).

The F-type ATP synthase is a highly conserved enzyme, although subtle changes can be observed between the enzymes found in mitochondria, bacteria and chloroplasts. An increase in complexity due to the evolution from prokaryotic to eukaryotic organisms is evident when studying the gene organization, subunit composition and regulatory mechanisms (Bryant, 1994; Claggett et al, 2007).

The ATP synthase has been widely studied, in *E. coli,* using different mutants and studying the effect of the mutation on different parameters such as growth, respiration rate and metabolic activity (Claggett et al, 2007; Kashiwagi et al, 2008; Noda et al, 2006; Rao et al, 1988). These mutations can be either on the F_0 or the F_1 complex. In this work, the effects of mutations in the F_1 complex will be analyzed, both on the hinge domain of the β subunit and in the α subunit. Mutations in the β subunit such as the replacement of the serine 174 to a phenylalanine (βSer 174 $\rightarrow$ Phe) or the isoleucine 163 for an alanine (βIle 163 $\rightarrow$ Ala) (Kashiwagi et al, 2008) cause a slower rotation of the F_1 subunit and a lower ATP production yield. Mutations in the α subunit, such as, the replacement of the serine 373 a phenylalanine (αSer 373 $\rightarrow$ Phe) originate a defective ATP synthase, which causes an uncoupling of the phosphorylation (Bakels et al, 1993; Bryant, 1994; Noda et al, 2006). The main objective for the creation of mutants with impaired ATP synthases was to decrease the ATP/ADP ratio inside the cell. It is well known that this ratio is an important controlling factor of the respiration rate, which tends to increase when the ATP/ADP ratio decreases. The control mechanism by which this process is regulated is still not clear (Noda et al, 2006).

The organism chosen for this work was *Synechocystis* sp. PCC 6803, a unicellular non-nitrogen fixing cyanobacterium. This is the most studied cyanobacterial strain, it has a medium size genome that is fully sequenced, and is naturally transformable, making it a prime candidate for biotechnological applications such as biohydrogen production.

The main goal of this work was to generate *Synechocystis* sp. PCC 6803 mutants with several variants of the ATP synthase, to modulate intracellular

oxygen levels. The mutants were evaluated in terms of growth, oxygen consumption and ATP levels. The best performing variant of the enzyme can be used in the future as an oxygen consuming device (OCD) creating an environment where efficient hydrogen production can be achieved.

2 Materials and Methods

2.1 Organisms and standard growth conditions

The unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (obtained from the Pasteur Culture Collection, Paris, France) was grown in 100 mL glass Erlenmeyer flasks containing 50 mL of BG11 media (Rippka et al, 1979). Cultures were incubated at 25 ºC under a 16 h light / 8 h dark regimen and a light intensity of 6-7 μE m^{-2} s^{-2} and orbital shaking at 80 rpm. For solid medium, BG11 was supplemented with 1.5% Noble agar (Difco), 0.3% sodium thiosulfate and 10 mM TES–KOH buffer (pH 8.2). For the selection of the mutants, BG11 medium was supplemented with kanamycin (10–200 μg ml^{-1})

E. coli DH5α, Top10 (Invitrogen) and XL10-Gold (Stratagene) were grown in LB broth at 37 ºC with orbital shaking. For solid medium, LB was supplemented with 1.5% of bacteriological European type agar (Cultimed). For the selection of mutants the LB was supplemented with kanamycin, 50 μg mL^{-1} and/or with ampicillin, 100 μg mL^{-1}.

2.2 Construction of the vectors

2.2.1 Site directed mutagenesis (SDM)

Three plasmids containing the sequences of the *atp1* (divided in two parts) and the *atp2* operons were previously designed in our laboratory. The operon sequences were synthesized and cloned in to pJ244 (DNA 2.0) originating the plasmid pJ244ATP1_Up (containing 'slr1413 + *atp1IHGFD*) pJ244 ATP1_D (containing *atpAC* + slr1411') and pJ244 ATP2 (containing *atpBE* +ymxG'). In this work the vectors pJ244 ATP1_D and pJ244 ATP2 (Fig. 4) were used to introduce the desired mutations in the ATP synthase genes. In order to facilitate the screening for successful mutations a silent mutation was also introduced changing a recognition site for a restriction enzyme (AseI or BstXI). For this purpose, the Quickchange Multi Site-Directed Mutagenesis Kit (Stratagene) was used along with the designed primers, SDM.S187F, SDM.I179A and SDM.S363F (Table 1). In this protocol the plasmids are amplified by PCR using primers containing the desired mutations and afterwards digested with DpnI in order to

remove the methylated, non-mutated, DNA. XL10-Gold ultracompetent cells were transformed using the products from the SDM according to the manufacturers instructions.

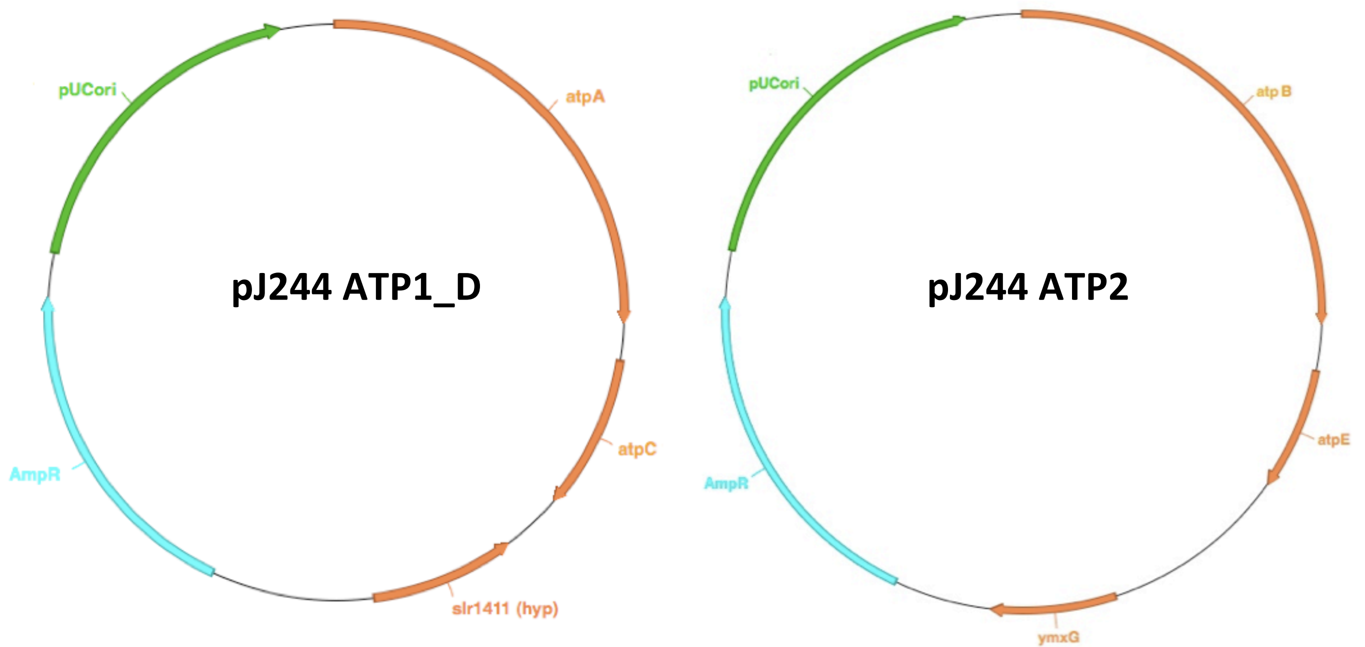

Fig. 4. Schematic representation of the plasmids used in this work.

Table 1. Primers used in this work.

Name	Sequence (5' → 3')	Application
SDM.S187F	CGCCATCCAACATGGA[1]GGTGTATTTGTATTTGGTGGCGT	SDM*
SDM.I179A	AACCGTAATCATGATGGAATTGATTAAT[2]AACATCGCCGCC CAACATGGTGGTG	
SDM.S363F	GGCTTCCGTCCTGCTATTAAT[3]GCTGGTATTTTTGTGAGCCG GGT	
Km.Fwd.SpeI	GAACTAGT[4]AGAAAGCCAGTCCGCAG	Km Resistance cassette amplification
Km.Rev.ClaI	GATCGAT[5]CCTCTAGCGAAC	
ATP1_D. F	TGTCCCTGTTGATGCGTCGTC	Mutant confirmation
ATP1_D.2R	TGAGGTAATCCCGCAGACCC	
ATP2.F	CTTTCCCCATTCACCGTCCC	
ATP2.R	GGGTTCGTTCATCTGACCGTAC	

*Nucleotides for the mutation are underlined. [1]BstXI recognition site removed; [2, 3]AseI recognition site added; [4]SpeI recognition site added; [5]ClaI recognition site added

2.2.2 Plasmid DNA isolation and mutation screening of the mutations

Colonies resulting from the transformation procedure were used to inoculate LB broth and were then incubated overnight. The plasmid DNA was isolated from the liquid cultures using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich), according to the manufacturer's instructions. After the plasmid was obtained it was digested either with AseI, BstXI or with both, according to the mutation to be tested, following the manufacturer's instructions (Fermentas).

The products of the digestions were analyzed by gel electrophoresis in 1% (w/v) agarose gels using 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA)

according to standard protocols (Sambrook J., 2001.). Ethidium bromide was added to the gels and the stained DNA was directly visualized under UV light, GeneRuler DNA Ladder Mix 100-10000 bp (Fermentas) was used as a molecular marker. The plasmids that presented the restriction pattern typical of the desired mutations were then sequenced (Stab Vida).

2.2.3 Insertion of the selection marker

Once the plasmids containing the mutated genes were obtained and confirmed by restriction by restriction analysis and sequenciation, a kanamycin resistance cassette was inserted in order to serve as a selection marker to be used for selection of the transformants of *Synechocystis* sp. PCC 6803.

The selection cassette conferring resistance to kanamycin was amplified by PCR from the plasmid pK18mobsacB (Schäfer et al, 1994) using the primers Km.Fwd.SpeI and Km.Rev.ClaI shown in Table 1. The PCR was done in reactions of 20 μL containing 30 ng of DNA template, 1 μM of each primer, 200 μM of dNTPs, 1X Taq DNA Polymerase Buffer, 2 mM $MgSO_4$ and 1 unit of Flexi GoTaq DNA polymerase (Promega). The PCR program used was: initial denaturation of 2 minutes at 95 ºC; 30 cycles of 95 ºC for 30 seconds, 52 ºC for 30 seconds, 72 ºC for 1 minute, followed by a final extension at 72 ºC for 7 minutes. The products obtained were separated by agarose gel electrophoresis as previously described and the DNA purified from agarose using the illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare), according to the manufacturer's instructions. The purified fragments and the plasmids obtained in 2.1.1 were both digested with SpeI and ClaI (Fermentas) following the manufacturer's recommendations. The digestion products were analyzed by agarose gel electrophoresis and the resistance cassette and linearized vectors were purified as previously described and then ligated with T4 DNA ligase (Promega) according to the manufacturer's instructions.

The plasmids with the kanamycin resistance cassette were cloned using Top10 ultra competent cells (Invitrogen) following the manufacturer's recommendations, plated in LB-Agar medium supplemented with 50 μg/mL of

kanamycin and 100 μg mL^{-1} of ampicillin and incubated overnight. Colonies resulting from the transformation procedure were used to inoculate LB broth supplemented with 50 μg mL^{-1} of kanamycin and 100 μg mL^{-1} of ampicilin and were then incubated overnight. In order to confirm the presence of the kanamycin resistance cassette the plasmid DNA was retrieved as previously described and then digested with SpeI and ClaI (Fermentas). The products were analyzed by agarose gel electrophoresis and the plasmids showing the expected digestion pattern were then sequenced.

2.3 *Synechocystis* transformation

Synechocystis sp. PCC 6803 was transformed based on a previously described procedure (Williams, 1988). Briefly, *Synechocystis* was grown in BG11 medium at 30 ºC, under continuous light (20 μE m^{-2} s^{-1}), until an $OD_{730} \approx 0.5$. Cells were harvested by centrifugation and resuspended in 1/10 volume of BG11. 100 μL of these cells were incubated with the purified plasmid for 5 h, in light at room temperature, with a final plasmid DNA concentration of 6-20 μg mL^{-1}. Cells were then spread onto Immobilon™-NC membranes (0.45 μm pore size, 82 mm, Millipore) resting on solid BG11 plates, incubated at 30 ºC under continuous light, and after 24 h transferred to selective plates containing the appropriate antibiotic concentration (e.g. Km 10 μg mL^{-1}). Transformants were observed after 1-2 weeks. For complete segregation, antibiotic resistant colonies were grown at increasing (antibiotic) concentrations (e.g. 25 and 50 μg mL^{-1} Km) and finally transferred in to liquid medium.

2.4 Confirmation of the segregation of the mutants

Confirmation of mutant segregation was performed by PCR. For this purpose DNA was extracted using samples of 2-5 mL of liquid culture collected and centrifuged for one minute at 14000 g, the supernatant was discarded. The cells were washed with 1 mL of sterile water and centrifuged for another minute at 14000 g discarding the supernatant. The pellet was then resuspended in 200 μL of sterile water and 1 μL of RNAse at a concentration of 20 mg mL^{-1} and 0.2 g of glass beads were added; the cells were then disrupted by two cycles of one minute of vortex followed by one minute of incubation in ice, and finally the samples were centrifuged for two minutes at 14000 g and the supernatant was collected.

The DNA was then used as template for a PCR similar to the one described on 2.2.2 using primers designed for this application shown in Table 1. The PCR products were analyzed using agarose gel electrophoresis and the desired bands were purified as previously described. The purified fragments were then digested with AseI or BstXI (Fermentas) and the results were analysed by agarose gel electrophoresis. In order to verify the sensitivity of this procedure the PCR and digestion were repeated using 30 ng of DNA from a putatively segregated mutant at an initial concentration of mixed with different dilutions of wild-type (wt) DNA (containing 2, 0.02 or 0.002 ng of DNA).

The fragments presenting the expected digestion pattern were then sequenced in order to confirm the mutations.

2.5 Characterization of the mutants

2.5.1 Growth curves

Inocula of 5 mL from the maintenance culture with an $OD_{730} \approx 2.0$ were transferred to fresh BG11 medium and incubated under standard conditions for 24 hours. Afterwards the inocula were added to sterile 100 mL Erlenmeyer flasks with 45 ml of BG11 medium. The experiments were done in triplicate and

under aseptic conditions as described by Lopo et al (2012), and the cultures were kept under the growth conditions previously described.

A sample from each culture was taken daily in order to measure the OD_{730} using a spectrophotometer (UV-Vis Mini 1240, Shimadzu).

2.5.2 Chlorophyll *a* content

To extract the chlorophyll *a*, 2 mL of cell cultures were and centrifuged at 14000 g for 5 minutes, the supernatant was discarded and the cells were resuspended in 100 µL of sterile water, 900 µL of methanol 100% (v/v) were then added. The samples were incubated for approximately 1h in darkness at room temperature. The absorbance was measured with a spectrophotometer (UV-Vis Mini 1240, Shimadzu) operating at a wavelength of 663 nm. The concentration of chlorophyll *a* was determined using the equation µg Chl. *a* mL^{-1} = $(12.7 \times Abs_{663})/2$, as previously described (Meeks & Castenholz, 1971).

2.5.3 O_2 consumption assays

The O_2 consumption was determined by measuring total O_2 consumed in the dark for a given period of time using a Clark type oxygen electrode (Oxygraph; Hansatech).

For this test 2 mL of a culture at Chl. concentrations of 3 and 6 µg mL^{-1} were transferred to the electrode reaction chamber. The sample was agitated for 2 minutes in the dark, and the decrease in dissolved oxygen was then recorded. Moderate stirring (100 rpm) of the sample was necessary in order to exchange the liquid layer next to the electrode, thereby minimizing the influence of the oxygen consumed by the electrode (Bhargava et al, 2008; Jensen & Michelsen, 1992). The O_2 consumption was expressed as nmol of O_2 per mL per min..

2.5.4 ATP measurements

A 2 mL culture sample was collected and centrifuged for 10 minutes at 14000 g, the media was then discarded and the pellet was resuspended in 1 mL of sterile water. The cells were disrupted by vortex mixing for 30 seconds with glass beads, this was repeated for a total of three times, with 30 seconds pauses in-between. The homogenate was passed through a syringe-driven filter with 0.22 μm pores (Millipore, Bedford, MA) to separate the soluble fraction from the suspension. The ATP in the soluble fraction was then determined using a luminometer (MicroLumat LB 96 P, EG&G Berthold), and luciferase-luciferin reagent (ENITEN™; Promega). According to the manufacturer's recommendations 50 μL of ENLITEN™ reagent were added to 150 μL of the soluble fraction previously obtained and the chemoluminescence was measured over 1 second. A standard ATP (Sigma Chemical Co.) curve was generated as previously described by (Allakhverdiev et al, 2005). The ATP concentration was calculated using graphic interpolation and expressed as mol of ATP per mg of Chlorophyll *a*.

3 Results and Discussion

3.1 Vectors

The first step of this work was the selection of the ATP synthase mutations to be introduced in the enzyme of *Synechocystis* sp. PCC 6803 (this work was based on data available from *E. coli* and performed by other team members, see Introduction and Table 2).

Table 2. Mutations to be introduced to *Synechocystis* sp. PCC 6803

Designation	**Target gene (encoding)**	**Mutated aminoacid in *Synechocystis* sp. PCC 6803**	**Reference**
αS363F	*atpA* (α subunit)	Serine 363 to Phenylalanine	Noda et al, 2006
βI179A	*atpB* (β subunit)	Isoleucine 179 to Alanine	(Kashiwagi et al, 2008)
βS187F	*atpB* (β subunit)	Serine 187 to Phenylalanine	(Kashiwagi et al, 2008)
βI179A + βS187F	*atpB* (β subunit)	Isoleucine 179 to Alanine + Serine 187 to Phenylalanine	(Kashiwagi et al, 2008)

The genes encoding the subunits of the ATP snthase listed in Table 2 were previously inserted into the respective plasmids (Fig. 4, Materials and Methods), then mutated by site directed mutagenesis. Subsequently a selection marker (kanamycin resistance cassette) was inserted, originating the plasmids represented in Fig. 5.

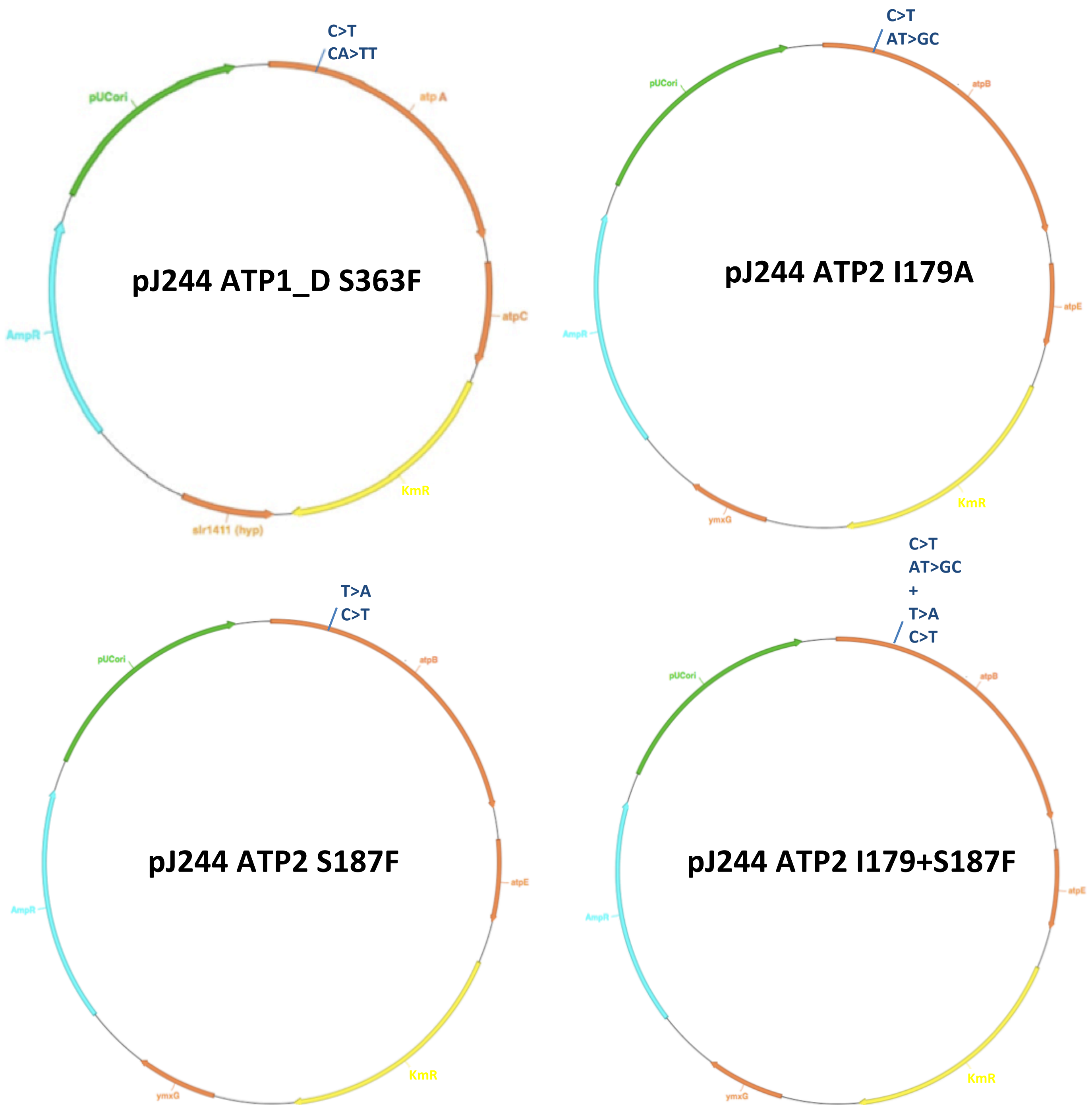

Fig. 5. Schematic representation of the plasmids used in this work, after site directed mutagenesis of the genes encoding the subunits of the ATP synthase from *Synechocystis* sp. PCC 6803 (mutated nucleotides shown in dark blue) and the insertion of the kanamycin resistance marker.

The successful mutations were confirmed by restriction analysis, since the mutations introduced also lead to changes in restriction sites, and therefore in restriction patterns (Fig. 6; Fig. 7).

All the the restriction patterns were the expected ones, confirming that the mutations were successful.

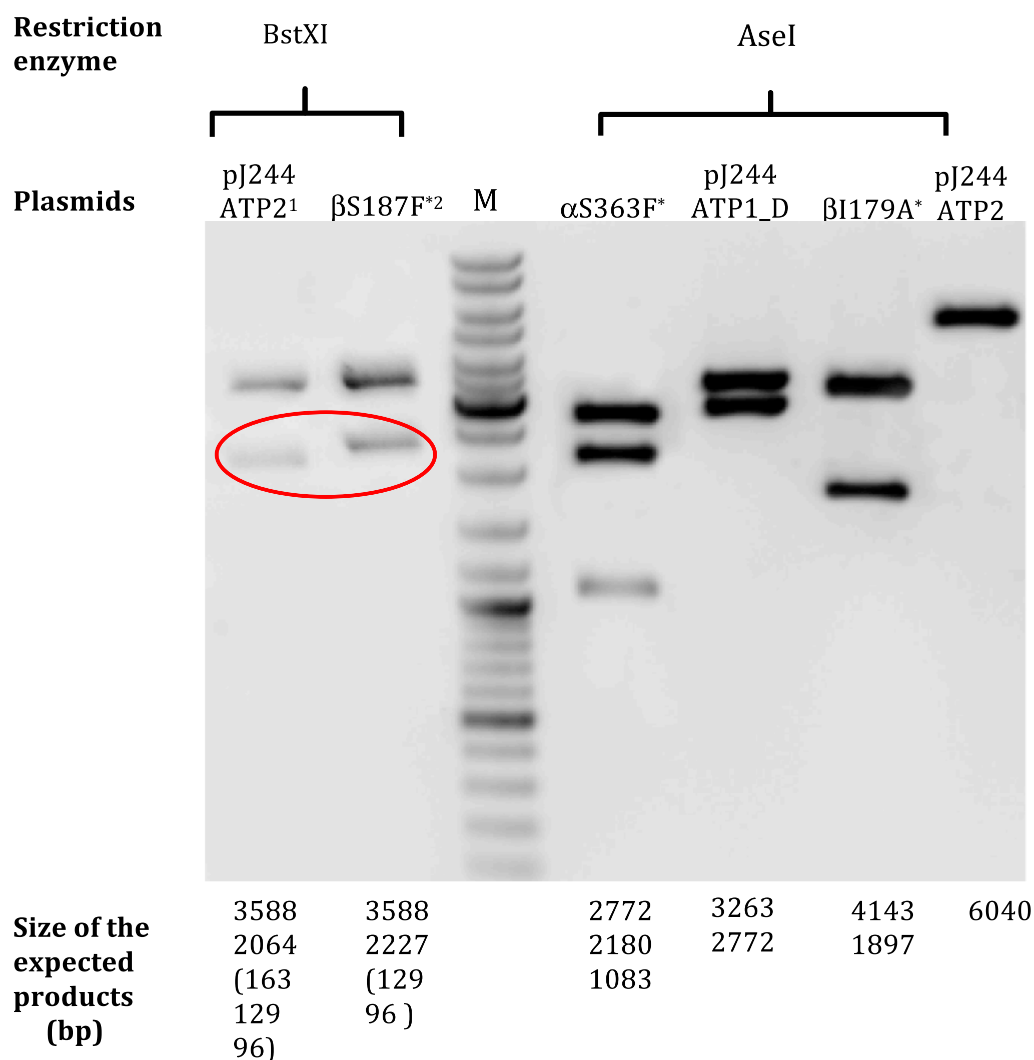

Fig. 6. Confirmation of the mutations αS363F, βI179A and βS187F by restriction analysis. The plasmids were digested with the endonuclease AseI or with BstXI.
[1,2] The smaller bands are not visible (in brackets) and the bands where the difference is visible are highlighted with a red elipse.
*Modified Plasmids.

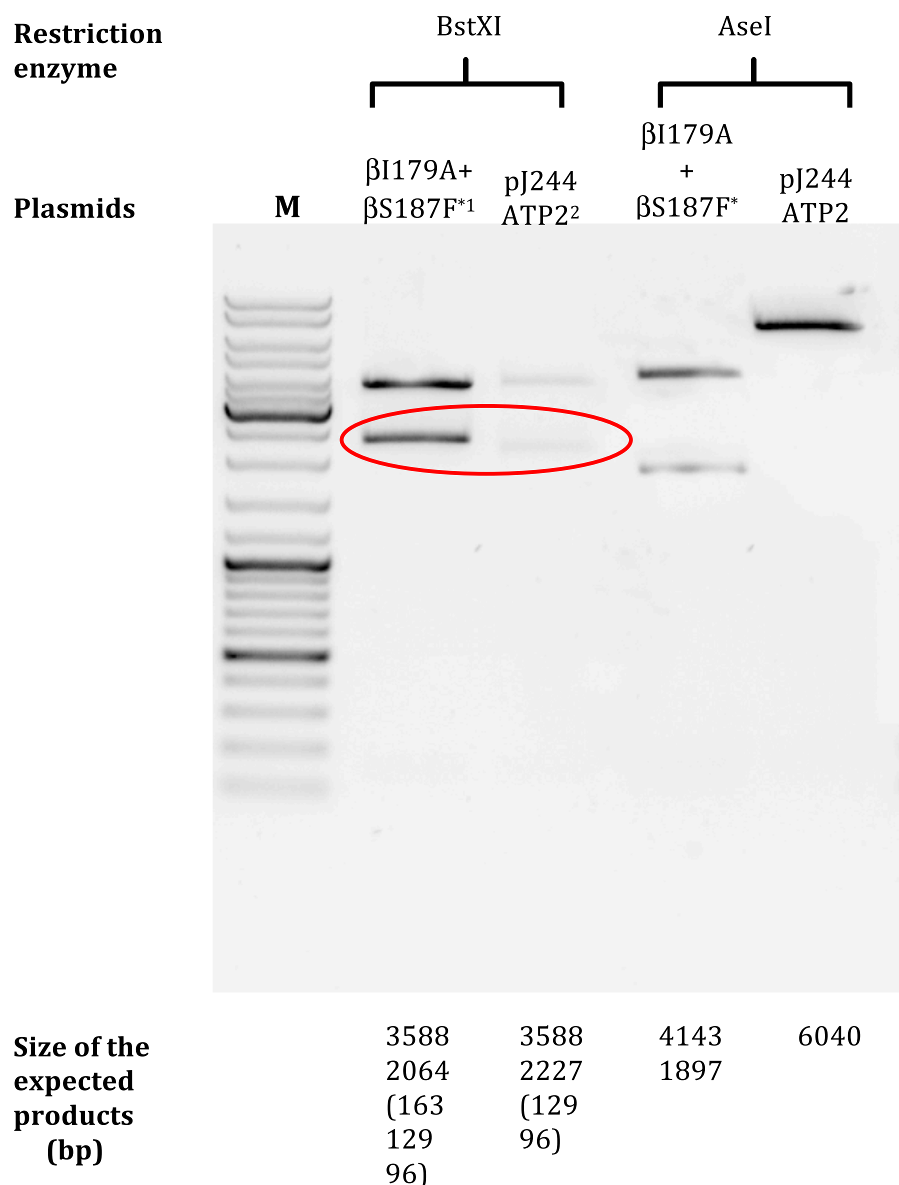

Fig. 7. Confirmation of the mutation βI179A+βS187F by restriction analysis. The plasmids were digested with the endonuclease AseI or with BstXI.
[1,2] The smaller bands are not visible (in brackets) and the bands where the difference is visible are highlighted with a red elipse.
*Modified Plasmids.

3.2 Generation and confirmation of the mutants

The four plasmids containing the mutated genes were then used to transform *Synechocystis* sp. PCC 6803. Since cyanobacteria in general, and *Synechocystis* sp. PCC 6803 in particular, contain several copies of the chromosome within a single cell (Griese et al, 2011) it is necessary to confirm

that all the copies are mutated (that the mutants are fully segregated). For this purpose, a fragment within the mutated gene was amplified by PCR using the primers ATP1_D. F and ATP1_D.R (Table 1) for the αS363F mutation and the primers ATP2.F and ATP2.R (Table 1) for the βI179A mutation. Subsequently, the PCR products were digested with AseI and analysed by agarose gel electrophoresis (Fig. 8).

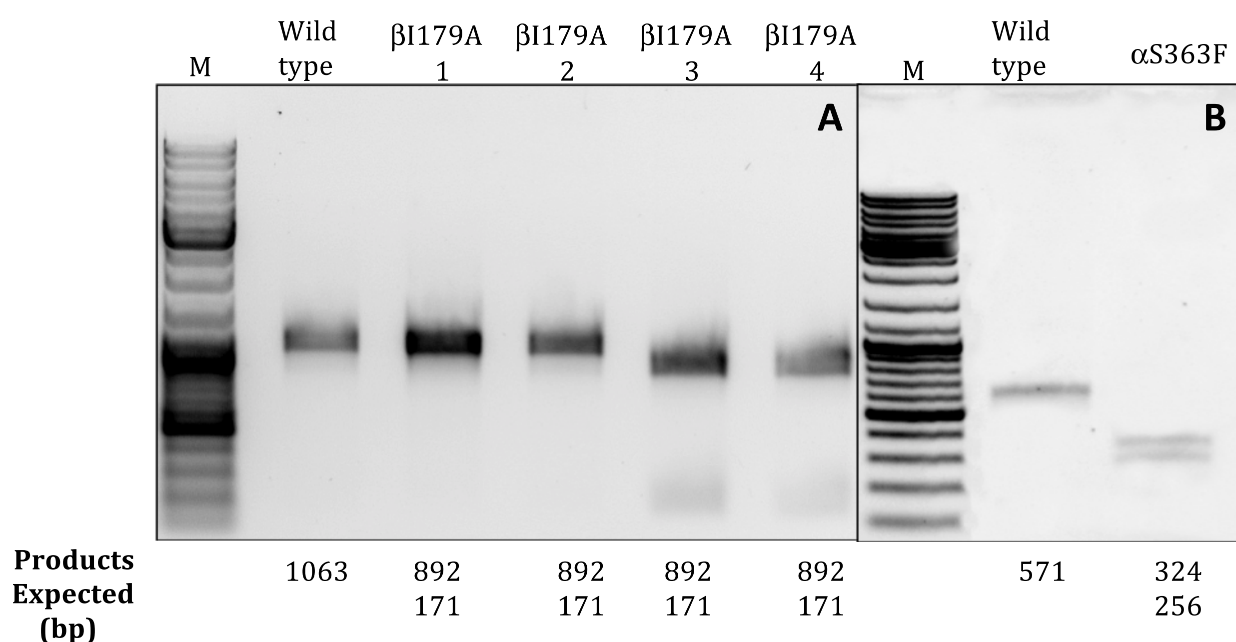

Fig. 8. Confirmation of the segregation of *Synechocystis* sp. PCC 6803 βI179A (A) and αS363F (B) mutants by PCR amplification of a fragment of the *atpB* (A) and *atpA*(B) genes and restriction analysis, using the endonuclease AseI. 1-4: Four different clones of the mutant βI179A

179A clones 3 and 4 and the αS363F clone presented the expected restriction patterns, suggesting complete segregation of these mutants. To discard false positive results, a second PCR was done using DNA extracted from the mutants mixed with different dilutions of the wild-type DNA (until 1:1000) to verify if low concentrations of the wild-type DNA could be detected. In Fig. 9 are displayed the results for the αS363F mutant, and similar results were obtained for the βI179A mutant.

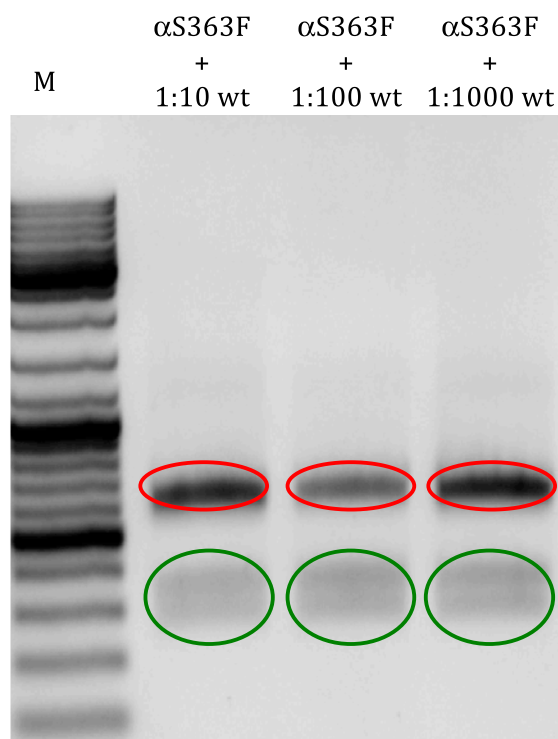

Fig. 9. Validation of the confirmation of the mutant segregation by PCR amplification followed by digestion with AseI of mixtures of different dilutions of *Synechocystis* sp. PCC 6803 wild type DNA, containing 2, 0.2 or 0.002 ng of DNA, with 30 ng of DNA from the αS363F mutant.
Bands from mutant DNA highlighted with a green elipse (see also Fig. 6 B). Bands from wild-type DNA highlited with a red elipse.

These results show that this procedure is sensitive to very low concentrations of DNA, even with a 1:1000 dilution (0.002 ng of DNA) we can see both the band characteristic of the wild type and the two bands characteristic of the mutant. In order to further verify these results, DNA samples from the two βI179A clones and the αS363F clone were sequenced. The sequencing results confirmed the successful mutation of both the βI179A clones, but revealed that the αS363F mutant had the restriction site altered but not the mutation corresponding to the amino acid alteration. This mutant was, therefore, discarded. The remaining mutants (βS187F and βI179A+βS187F) are still segregating.

3.3 Characterization of the βI179A mutant

After confirmation of the βI179A mutant segregation we proceeded to its characterization. The first parameter to be evaluated was growth (Fig. 10).

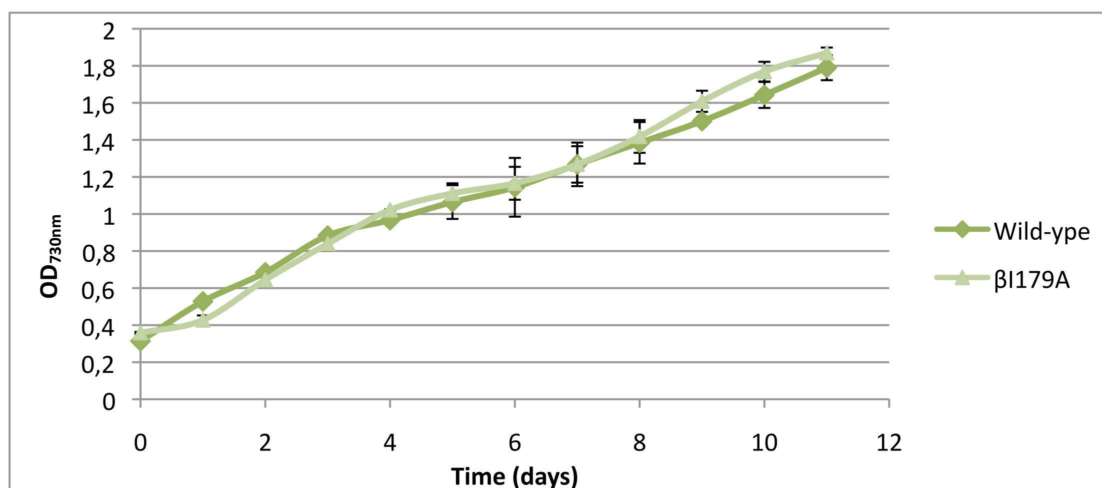

Fig. 10. Growth curve of *Synechocystis* sp. PCC 6803 wild type and its βI179A mutant. Grown at 25 °C with a 16 h light (6-7 μE m^{-2} s^{-1}) / 8 h dark regimen.

Analysing the growth curves we can verify that the wild type and the mutant present similar growth curves, suggesting that the mutation does not have a pronounced effect at least under the conditions tested.

To further characterize the mutant the oxygen consumption was assayed using a Clark-type oxygen electrode (Fig. 11). Analysing these results it is possible to observe that the βI179A mutant presents an increase of 110% in oxygen consumption when compared to the wild type.

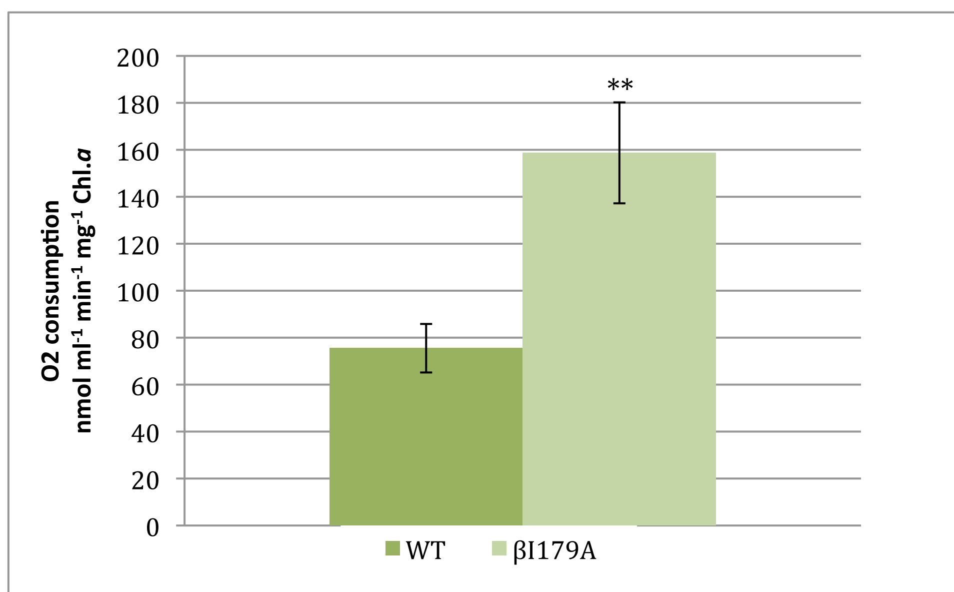

Fig. 11. Oxygen consumption by cell suspensions of *Synechocystis* sp. PCC 6803 wild-type and its βI179A mutant measured using a Clark-type electrode.
** (P=0.0033)

Moreover, the ATP levels were measured using a luminometry based procedure, and using an ATP standard curve obtained by measuring the luminescence of different ATP solutions with known concentrations. The results of the ATP quantification are presented in Fig. 12.

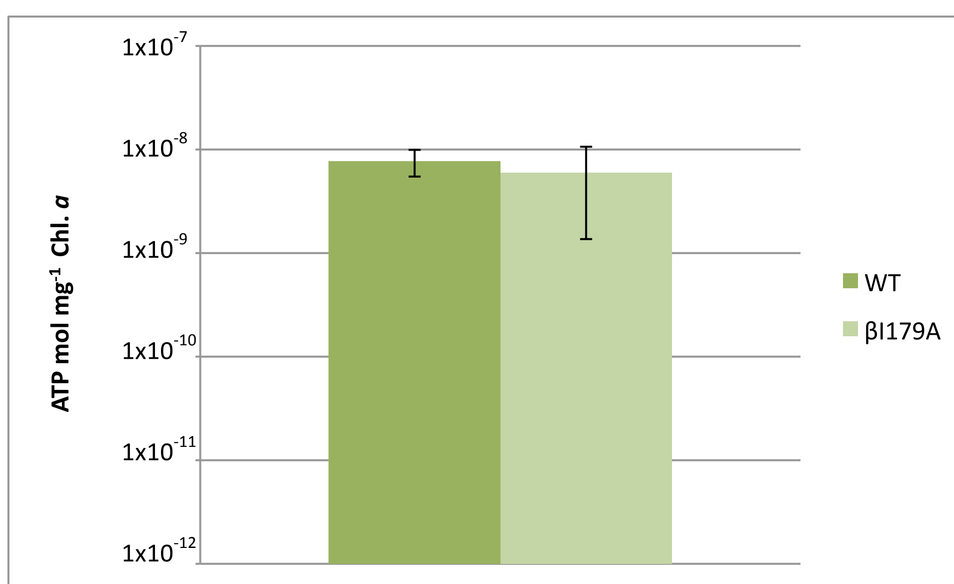

Fig. 12. Luminometric ATP quantification of *Synechocystis* sp. PCC 6803 wild type and its βI179A mutant.

Synechocystis sp. PCC 6803 wild type and its βI179A mutant do not present significant differences in ATP levels, suggesting that the inserted mutation is not reducing substantially the overall ATP production.

Previous work with *E. coli,* with a mutation analogous to βI179A (Iko et al, 2001; Kashiwagi et al, 2008) showed a decrease of ATP synthase activity to 14%. The enzyme activity was not tested in this work, but the results obtained for the βI179A mutant show an increased O_2 consumption. This increase suggests that the metabolic rate has increased to compensate the decrease in ATP/ADP ratio caused by the impaired ATP synthase activity. Further testing is necessary to confirm this hypothesis.

4 Conclusions and Future Perspectives

The aim of this work was to generate *Synechocystis* sp. PCC 6803 mutants containing variants of the native ATP synthase, with the main goal of modulating intracellular oxygen levels. For this purpose, four plasmids, containing the genes encoding the α and β subunits of the ATP synthase and mutated by directed site mutagenesis were constructed. These plasmids were then used to generate *Synechocystis* sp. PCC 6803 mutants. Only one mutant, on the β subunit, was completely segregated and therefore characterized. The results obtained for the βI179A mutant showed a significant increase in oxygen consumption (110% more compared to the wild type) and no differences in growth and ATP levels. These results are extremely promising, since the objective of an oxygen consuming device (OCD) is exactly to increase the oxygen consumption without disrupting other cellular functions. These results also indicate that the mutated ATP synthase might function as an efficient OCD.

It is important to further characterize the βI179A mutant and to proceed the work with the other mutants. For the βI179A mutant one should also consider to analyse growth under different conditions e.g. continuous light, and mixotrophic conditions. The ATP synthase activity should be measured, as well as hydrogen evolution.

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