

PetG and PetN, but not PetL, are essential subunits of the cytochrome *b₆f* complex from *Synechocystis* PCC 6803

Dirk Schneider^{a,b}, Thomas Volkmer^a, Matthias Rögner^{b,*}

^a Institut für Biochemie und Molekularbiologie, Albert-Ludwigs-Universität Freiburg, Stefan-Meier-Strasse 19, 79104 Freiburg, Germany

^b Lehrstuhl für Biochemie der Pflanzen, Ruhr-Universität Bochum, Universitätsstrasse 150, 44801 Bochum, Germany

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Abstract

The cytochrome *b₆f* complex consists of four large core subunits and an additional four low molecular weight subunits, the function of which is elusive thus far. Here we sought to determine whether small subunits PetG, PetL, and PetN are essential for a cyanobacterial cytochrome *b₆f* complex. We found that only PetL is dispensable, whereas PetG and PetN appear to be essential. Possible roles of the small cytochrome *b₆f* complex subunits are discussed, and observations from our study are compared with previous findings.

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1. Introduction

Several membrane-localized transport processes and enzymatic reactions are fulfilled by large transmembrane protein complexes, often composed of several subunits. Interestingly, in several membrane protein complexes, small subunits can be found, the functions of which are as yet largely unknown. These small subunits usually have molecular masses of less than 10 kD and most of the amino acids are hydrophobic. The polypeptide chains span the lipid bilayer with a single transmembrane α -helix and the soluble domains are usually very small. Small, single-span transmembrane subunits can, for example, be found in photosystem 2 [3,27], photosystem I [9], the cytochrome *bc₁* complex [8] and cytochrome *c* oxidase [31]. The physiological function of some of these subunits has been elucidated in recent years by mutational analysis, and it has generally been found that the small subunits are important for stability and assembly of the protein complexes. Although this functional classification is usually

not as comprehensive as desired, it demonstrates that recruitment of small single-span subunits by large transmembrane protein complexes is of structural importance.

Small, single-span transmembrane proteins are also subunits of the cytochrome *b₆f* complex, which exists in chloroplasts from green algae and plants, as well as in cyanobacteria. In cyanobacteria the cytochrome *b₆f* complex is an essential part of both the respiratory and the photosynthetic electron transport chain [15,18,19]. The complex oxidizes plastoquinol and subsequently transfers electrons to a soluble electron carrier, cytochrome *c₆* or plastocyanine. The cytochrome *b₆f* complex consists of four major subunits: cytochrome *f* (PetA, 32 kD), cytochrome *b₆* (PetB, 24 kD), the Rieske iron–sulfur protein (PetC, 19 kD) and subunit IV (PetD, 17 kD) [10]. Interestingly, in some cyanobacteria, a family of several Rieske genes exists which appears to code for proteins with different functions [22–24]. Besides the four major subunits for which homologous proteins can be found in cytochrome *bc₁* complexes, an additional four small polypeptides are subunits of the cytochrome *b₆f* complex [5,6,20,30]. PetG, PetL, PetM, and PetN have molecular masses of less than 10 kD, and each polypeptide spans the membrane once in an α -helical conformation [12,29,33].

* Corresponding author.

E-mail address: matthias.roegner@rub.de (M. Rögner).

The exact function of these additional subunits is not yet clear. While deletion of the genes encoding PetG or PetN in green algae or higher plants has caused dramatic defects [1,5], deletion of the *petM* gene in a cyanobacterium and of the *petL* gene in *Chlamydomonas reinhardtii*, resulted only in moderate phenotypes [21,30]. Besides these four small subunits in green algae, at least one additional subunit (PetO) exists which is involved in regulation of state transitions [7]. Nevertheless, the four large and the four small subunits seem to form the core, which is common to all cytochrome *b₆f* complexes in chloroplasts and cyanobacteria.

In contrast to chloroplasts, it is still unknown how deletions of *petG*, *petL*, and *petN* affect cyanobacteria. In the absence of a cytochrome *bc₁* complex, the cytochrome *b₆f* complex is essential in cyanobacteria, and any mutation within genes which are required for its activity will result in a lethal phenotype [14]. Also, cyanobacteria have multiple genome copies and any attempt to replace or delete an essential gene will result in a not completely segregated merodiploid strain. In such a strain, only some of the multiple genome copies are replaced, while in several genome copies of the wild-type (wt) gene remain preserved. Some cyanobacteria can be genetically modified, and analyses of the role of the small cytochrome *b₆f* complex subunits can be accomplished more easily in cyanobacteria than in chloroplasts.

In the present study we examined the role of the small cytochrome *b₆f* complex subunits PetG, PetL and PetN in the cyanobacterium *Synechocystis* sp. PCC 6803. Based on deletion analysis, we show that protein PetL is not essential in *Synechocystis*, whereas the PetG and PetN subunits appear to be essential under the chosen experimental conditions. Possible roles of the small cytochrome *b₆f* complex subunits in cyanobacteria are discussed and the observations of this study are compared to earlier observations made in chloroplasts.

2. Materials and methods

2.1. Growth conditions

A glucose-tolerant *Synechocystis* PCC 6803 strain was used in this study. *Synechocystis* PCC 6803 wt and mutant strains were grown at 30 °C in BG11 media [16] under cool-white fluorescent illumination at a light intensity of 40 $\mu\text{E m}^{-2} \text{s}^{-1}$. Growth of the wt and mutant *Synechocystis* strains was monitored at 730 nm (OD_{730}) under photoautotrophic, photomixotrophic (+10 mM glucose) or photoheterotrophic (+10 mM glucose, +10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)) conditions.

Escherichia coli strain DH5 α , which was used for plasmid propagation, was grown in LB broth or agar supplemented as needed with 100 $\mu\text{g/ml}$ ampicillin and 30 $\mu\text{g/ml}$ chloramphenicol.

2.2. DNA techniques

Molecular cloning was carried out using standard protocols [17]. Enzymes used for PCR and cloning were obtained from

MBI Fermentas. PCR was carried out using a BioRad thermocycler. DNA sequencing was performed by MWG Biotech (Ebersberg, Germany). Genomic *Synechocystis* DNA was prepared as described recently [13].

For deletion of *petG*, the gene and flanking regions were amplified by PCR using the primer 5'*petG* (5'-cgttctagatgttg-gacaatcaaaaccgagca-3') and 3'*petG* (5'-taactcgagaagcccaat tctgtt-3'). The resulting DNA fragment of 1650 bp was restriction-digested with *Xba*I and *Xho*I. These restriction sites have been introduced by the primers. The DNA fragment was subsequently ligated to the equally restriction-digested plasmid pBluescript II SK (Stratagene). A *cat* cassette coding for chloramphenicol acetyl transferase was introduced into a single *Pst*I site located within the *petG* gene. The resulting plasmid was used for transformation of *Synechocystis* cells.

The *petL* gene was amplified by PCR with flanking regions using the primer 5'*petL* (5'-caataattgccgttgccgcatc-3') and 3'*petL* (5'-gaagcatacttagccgcgtag-3'). The resulting 1900 bp fragment was directly ligated to the GC-T-Vector (Genecraft). A 60 bp fragment was removed from the *petL* gene using *Bst*EII and *Hpa*I, the resulting sticky ends were polished with T4 DNA polymerase and a *cat* cassette was subsequently ligated to the plasmid.

For the deletion of *petN* the upstream and downstream regions of the gene were amplified individually by PCR. The *petN* upstream region was amplified using the primer 5'*petNus* (5'-tttcggatccttagtcagggtgtttggc-3') and 3'*petNus* (5'-acatgtcatatgagtctttttgtaataacgc-3'). The resulting 950 bp fragment was restriction-digested with *Bam*HI and *Nde*I and ligated to the equally restriction-digested plasmid pRSET6a [25]. An 820 bp long DNA region 3' of the *petN* gene was amplified by PCR using the primer 5'*petNds* (5'-atattcatatggaagcaactc tactcct-3') and 3'*petNds* (5'-atctctagactttgtgttttcggctaa-3'). The DNA fragment was restriction-digested with *Nde*I and *Xba*I and ligated to the equally restriction-digested plasmid which already contained the *petN* upstream region. A *cat* cassette was introduced into a single *Nco*I site prior to transformation of the plasmid into *Synechocystis* cells.

Transformation of *Synechocystis* with plasmids was carried out as described in Ref. [34]. Since *Synechocystis* contains several identical genome copies, numerous rounds of selection on BG11 plates with increasing amounts of chloramphenicol (5–50 $\mu\text{g/ml}$) are necessary to obtain fully segregated mutants. Individual *Synechocystis* clones were tested by PCR for complete segregation.

2.3. Mutant characterization

Oxygen evolution rates were measured at 30 °C using a Clark-type oxygen electrode under actinic light (600 $\mu\text{E m}^{-2} \text{s}^{-1}$). *Synechocystis* cells were harvested in the mid-log phase and diluted to a chlorophyll concentration of 20 $\mu\text{g/ml}$ in BG11 medium. Oxygen evolution was measured after addition of 0.5 mM 2,6-dichloro-*p*-benzoquinone (DCBQ) or of 10 mM NaHCO_3 .

77 K fluorescence emission spectra were recorded in an AMINCO fluorimeter [21]. Cells were diluted in BG11

medium to a chlorophyll concentration of 5 µg/ml and frozen in liquid nitrogen. Monochromators were set to a slit width of 4 nm.

3. Results and discussion

In order to test whether the *petG* (*smr0010*), *petL* (*ssl3803*) and *petN* (*sml0004*) genes which code for three of the four small cytochrome *b₆f* complex subunits are essential in *Synechocystis*, we disrupted the individual genes by insertional mutagenesis (Fig. 1A).

Analysis of the genomic organization of *petG* and *petL* revealed that these genes are presumably not organized in an operon. In the case of *petG*, the adjacent genes are transcribed from the opposite DNA strand. 5' of *petL*, the adjacent gene, is also transcribed in the opposite direction, whereas 3' of *petL*, the closest gene, is located more than 230 bp away, and sequence analysis using the genome atlas program (www.cbs.dtu/services/genomeatlas) indicated that each gene has its own promoter. In addition, for both genes, no gene cluster, which is conserved in cyanobacteria, was predicted, which would have been a strong indication of an operon organization. Furthermore, since *petL* was deleted without significant effects (see below), the gene was definitely not organized in a vital operon.

To delete *petG* and *petL* we replaced parts of the genes by a *cat* cassette, which codes for the chloramphenicol acetyl transferase. Resulting transformants were grown on BG11 plates containing 5 mM glucose [16] and increasing concentrations of chloramphenicol (0, 10, 20, 50 µg/ml).

Close to the 3' end of *petN* an open reading frame is predicted (*sgl0002*) which codes for a hypothetical protein with high similarity to an Na⁺/H⁺ antiporter [11]. Since we could not exclude a potential operon between *petN* and *sgl0002*, we decided to completely delete the *petN* gene and to fuse

sgl0002 to the promoter/operator region of *petN* (Fig. 1A). Also, this strain was grown on BG11 plates with increasing concentrations of chloramphenicol. After several rounds of striking individual clones of the transformants on BG11 plates with 50 µg/ml chloramphenicol, individual colonies were grown for several generations in BG11 medium in the absence of antibiotics, and thereafter, genomic DNA was isolated. To test for complete segregation, genomic DNA from the individual clones was used in a PCR to amplify the genomic region, which carries the wt *pet* gene and/or the resistance marker (see Section 2 and Fig. 1A). While we were not able to completely delete either *petG* or *petN* in *Synechocystis*, a completely segregated Δ *petL* mutant was isolated. After using genomic DNA from wt *Synechocystis* as a template in a PCR, a fragment of 1900 bp was amplified which corresponded to the *petL* gene including part of the up- and downstream regions. When genomic DNA from a Δ *petL* strain was used in the PCR, a single DNA fragment with a size of about 3000 bp was amplified, which corresponded to the disrupted *petL* gene region containing the *cat* cassette (Fig. 1B). The wt fragment of 1900 bp was no longer amplified, demonstrating that the wt gene had been replaced in all *Synechocystis* genome copies.

The observation that the *petG* and *petN* genes could not be completely deleted suggests that the encoded polypeptides are essential for the structure and/or function of the cytochrome *b₆f* complex in *Synechocystis*. This is in good agreement with observations made after deletion of *petG* and *petN* in algae and higher plants [1,5]. Deletion of *petG* in the green alga *C. reinhardtii* resulted in a highly reduced level of the major cytochrome *b₆f* complex subunits within the thylakoid membrane, and the strain was unable to grow photosynthetically [1]. These observations indicate that PetG is absolutely vital for the assembly and stability of the complex (or both). Consequently, deletion of this essential cytochrome *b₆f* complex subunit was also not possible in *Synechocystis*, which

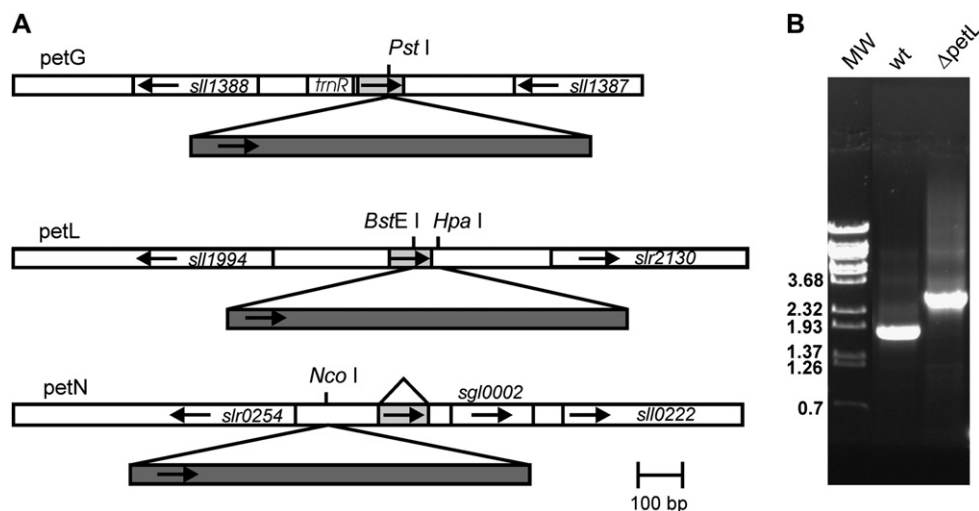


Fig. 1. (A) Physical organization of gene regions around individual *pet* genes. The indicated restriction sites have been used to replace part of the *pet* genes (light gray) by a *cat* cassette (dark gray), which codes for the chloramphenicol acetyl transferase. The arrows describe the direction of transcription of the genes. (B) PCR analysis of the Δ *petL* strain in comparison to the wt. Genomic DNA was used as a template and the genomic region around the *petL* gene was amplified by PCR using the primers 5'*petL* and 3'*petL*. MW, molecular weight marker.

suggests that the PetG subunit has an essential function in cyanobacteria as well.

Deletion of the *petN* gene in tobacco resulted in loss of a functional cytochrome *b₆f* complex and it has been reported that the resulting $\Delta petN$ plant was unable to grow photosynthetically. The amount of photosystems 1 and 2 seemed to be at wt level in the mutant plant, whereas re-oxidation of the PQ pool was highly reduced and the cytochrome *b₆f* complex subunit cytochrome *f* was reduced to almost undetectable amounts in this mutant [5]. In summary, the PetN subunit appears to be absolutely essential for cytochrome *b₆f* complex function in chloroplasts as well as in cyanobacteria.

Deletion of *petL* in *C. reinhardtii* resulted in reduced accumulation of other cytochrome *b₆f* complex subunits in the thylakoid membrane; moreover, the overall stability of the cytochrome *b₆f* complex appeared to be impaired in the mutant strain. Nevertheless, although electron transfer reactions within the cytochrome *b₆f* complex were slowed down after deletion of the *petL* gene, the mutant strain was still able to grow photoautotrophically [30].

Interestingly, deletion of *petL* resulted in a completely segregated *Synechocystis* strain (Fig. 1B). To test whether the deletions resulted in any observable phenotype, we characterized the mutant strain. The mutant strain grew with about the same doubling time as the wt under the tested growth conditions (see Section 2). To test for a potential effect of deletion on the function and amount of the two photosystems, we measured the whole cell fluorescence emission spectra at 77 K, by which the relative amounts of the two photosystems can be determined. However, we were not able to observe significant differences between the mutant and wt strain. In addition, we measured oxygen evolution of the $\Delta petL$ mutant strains in the presence of phenyl-para-benzoquinone or HCO_3^- as electron acceptors. In comparison to the wt, the $\Delta petL$ strain displayed slightly reduced chlorophyll content (about $85 \pm 10\%$ of the wt level per cell) and the oxygen evolution rates were slightly reduced (about $80 \pm 18\%$ of the wt). Whether these small changes are of any significance should be explored in future experiments.

The experimental results indicate that, while PetG and PetN appear to be essential subunits of the cyanobacterial cytochrome *b₆f* complex, PetL (this study) and PetM [21] are dispensable.

Recently, the 3D-structures of the cytochrome *b₆f* complex from the cyanobacterium *Mastigocladus laminosus*, as well as from the green alga *C. reinhardtii*, have been resolved [12,29], and in these structures all four small subunits are located on the opposite side of the cytochrome *b₆f* complex dimer interface.

Surprisingly, three of the four small subunits (PetG, PetL, and PetN) were localized differently in the two structures [12,29], which is most likely due to difficulties in localizing the small subunits in the structures. In the *Chlamydomonas* structure, PetM and PetL were positioned peripherally and PetG and PetN deeper inside the structure (compare with Fig. 2). In contrast, in the *Mastigocladus* structure, PetM and PetN were attributed to the peripheral locations, whereas

PetG and PetL were attributed to locations deeper inside the structure. The observation that PetG and PetN, but not PetL and PetM, are essential subunits argues for a peripheral location of PetL and PetM, which is in agreement with the attribution of these small subunits to the *Chlamydomonas* structure. If PetG and PetN are located more deeply inside the structure and interact with various other subunits, they are likely to be of greater structural importance than the small subunits located outside. This assumption is supported by the deletional analysis, described in Ref. [21] and here, which demonstrate that the genes encoding PetL and PetM can be disrupted in *Synechocystis*. Similarly, deletion of the *petL* gene resulted in only moderate defects in *Chlamydomonas* and no observable phenotype in tobacco [4,30]. Unfortunately, deletion of the *petM* gene in green algae or higher plants has not yet been reported.

The four small cytochrome *b₆f* complex subunits form a kind of transmembrane four-helix bundle, which has no direct counterpart in the structure of the cytochrome *bc₁* complex. The cytochrome *bc₁* and the cytochrome *b₆f* complexes are homologous in their structure and function and both complexes most likely evolved from an ancient cytochrome *bc* complex [26,28]. The small subunits fill a space in the cytochrome *b₆f* complex, which forms a large groove in the cytochrome *bc₁* complex structure. In the cytochrome *bc₁*

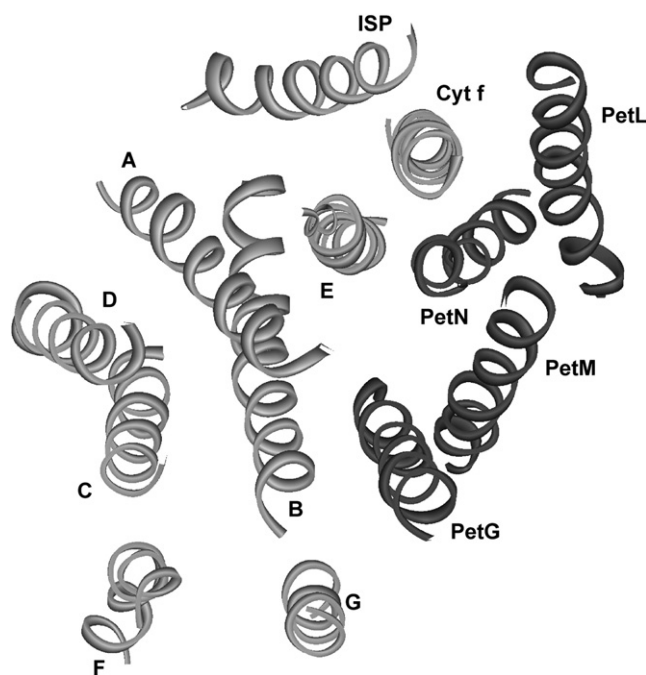


Fig. 2. Structure of the cytochrome *b₆f* complex from the cyanobacterium *Mastigocladus laminosus*. The structure was built using pdb file 1UM3 and only the transmembrane part of one monomer within a dimer is shown. Transmembrane helices are named according to the cytochrome *bc₁* complex terminology: A, B, C, and D for cytochrome *b₆*; E, F, and G for subunit IV; Cyt f, cytochrome f; ISP, Rieske iron–sulfur protein. Positions of the small subunits PetG, PetL, PetM, and PetN are indicated. The locations of the small subunits were attributed differently than described in the original citation [12], but in agreement with the cytochrome *b₆f* complex structure from *C. reinhardtii* [29]. For details and arguments see the text.

complex this groove is partially filled with lipids, and it is likely that these lipids are structurally important for the assembly and stability of the complex. Small, single-span transmembrane helices could have been recruited during evolution to stabilize larger transmembrane protein complexes when lipids could not fulfill these requirements, because of the structures and properties of either the available lipids or the proteins. In the cavity, which is filled with the four small subunits in the cytochrome *b₆f* complex structure, a carotene molecule is bound and this cofactor seems to be essential for the cytochrome *b₆f* complex function [32,35]. Therefore, the stabilizing function of the lipids in the cytochrome *bc₁* complex presumably had to be taken over by special peptides in the cytochrome *b₆f* complex, which were able to bind the carotenoid cofactor as well. It is noteworthy that in the photosystem 2 reaction center carotenoids are bound by small transmembrane subunits [3].

Without a doubt, more detailed analyses are needed to define the structural and functional role of the four small subunits in the cytochrome *b₆f* complex, and at this stage one can only hypothesize as to their possible functions. It remains puzzling, however, that essentially no phenotype has been observed after disruption of the *petL* gene in *Synechocystis* (this study) and in tobacco [4], whereas the deletion of *petL* caused defects in *C. reinhardtii*. While a stabilizing function of PetL has been proposed [2], it cannot be excluded that in the absence of PetL the cytochrome *b₆f* complexes of *Synechocystis* and tobacco are destabilized, as observed in *Chlamydomonas*.

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