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PetC1 Is the Major Rieske Iron-Sulfur Protein in the Cytochrome b_6f Complex of *Synechocystis* sp. PCC 6803*

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Many of the completely sequenced cyanobacterial genomes contain a gene family that encodes for putative Rieske iron-sulfur proteins. The Rieske protein is one of the large subunits of the cytochrome *bc*-type complexes involved in respiratory and photosynthetic electron transfer. In contrast to all other subunits of this complex that are encoded by single genes, the genome of the cyanobacterium *Synechocystis* PCC 6803 contains three *petC* genes, all encoding potential Rieske subunits. Most interestingly, any of the *petC* genes can be deleted individually without altering the *Synechocystis* phenotype dramatically. In contrast, double deletion experiments revealed that *petC1* and *petC2* cannot be deleted in combination, whereas *petC3* can be deleted together with any of the other two *petC* genes. Further results suggest a different physiological function for each of the Rieske proteins. Whereas PetC2 can partly replace the dominating Rieske isoform PetC1, PetC3 is unable to functionally replace either PetC1 or PetC2 and may have a special function involving a special donor with a lower redox potential than plastoquinone. A predominant role of PetC1, which is (partly) different from PetC2, is suggested by the mutational analysis and a detailed characterization of the electron transfer reactions in the mutant strains.

In 1997 the complete DNA sequence of the mesophilic cyanobacterium *Synechocystis* PCC 6803 was published (1). About 3200 open reading frames were identified within the genome, and for about 55% of them a function was inferred by similarity and homology searches, whereas the function of the remaining open reading frames remained unclear (2). Although computational analysis indicated the occurrence of many gene families, the function of these is elusive so far. The best investigated example at present is the D1 protein of photosystem II, which is encoded by three different *psbA* genes (3, 4). It was shown that all three genes encode functional D1 proteins, although only *psbD1* and *psbD2* are expressed at a significant level. Similarly, three potential *petC* genes are present encoding for the Rieske subunit of the cytochrome b_6f complex (5).

The Rieske protein is an essential subunit of all cytochrome *bc* complexes, where it is involved in quinol binding and electron transfer within the complex. In addition to the Rieske protein, cytochrome bc_1 complexes contain the cytochrome *b* subunit carrying two *b*-type hemes and a *c*-type cytochrome with one covalently bound heme. Although in the cytochrome b_6f complex the *b*-type cytochrome is split into the two subunits cytochrome b_6 and subunit IV (6), the overall structure is conserved, and both complexes can transfer electrons from a quinol to the *c*-type cytochrome (7). The structures of the cytochrome bc_1 complexes as well as of the cytochrome b_6f complex have been determined by x-ray crystallography recently (8–10). Although the sequence similarities between the Rieske proteins from both complexes are quite low (11), structural analysis of the water-soluble parts of these proteins revealed a highly conserved three-dimensional structure (11, 12).

In the genomic sequence of the cyanobacterium *Synechocystis* PCC 6803, no genes encoding subunits of a cytochrome bc_1 complex were found, *i.e.* the cytochrome b_6f complex is an essential component of both the respiratory and the photosynthetic electron transfer chain. In general, the thylakoid membrane of cyanobacteria harbors a mixed photosynthetic and respiratory electron transport chain (13) with the plastoquinone pool receiving electrons from both photosystem II and from respiratory dehydrogenases; these are then transferred via the cytochrome b_6f complex and soluble carriers (plastocyanin or cytochrome c_6) to photosystem I and the terminal respiratory oxidase. Due to this central function of the cytochrome b_6f complex, it is impossible to delete crucial subunits of this complex (14). Within the cyanobacterial cytochrome b_6f complex only for the Rieske subunit a gene family has been found. Besides *Synechocystis*, which contains three potentials Rieske genes, multiple Rieske genes have also been shown to exist in other cyanobacteria like *Synechococcus* PCC 7002 and *Anabaena* PCC 7120. However, in contrast to the *psbA* gene family, up to now no specific physiological function is known for the *petC* gene family encoding for the Rieske subunit.

In this study the function of the three *petC* genes in *Synechocystis* PCC 6803 was investigated by deletional mutagenesis of *petC* genes and by characterization of the generated mutants. The presented results show that all *petC* genes can be deleted individually without altering the *Synechocystis* phenotype dramatically. Although *petC1* and *petC2* can be deleted individually, it was not possible to delete the genes in combination in *Synechocystis* wild type (wt).[1] This indicates that *petC2* and *petC1* have at least partly overlapping functions and that *petC2* can complement for the *petC1* deletion. Further detailed characterization of the Δ*petC1* strain suggested that the function of *petC1* can only to some degree be substituted by *petC2*, and the deletion of *petC1* leads to a partial loss of the cytochrome b_6f complex function.

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[1] The abbreviations used are: wt, wild type; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; E, einstein; DBMIB, 2,5-dibromomethylisopropyl-*p*-benzoquinone.

MATERIALS AND METHODS

Growth Conditions—*Synechocystis* PCC 6803 wt and mutant strains were grown at 30 °C in BG11 media (15) under cool-white fluorescent illumination at a light intensity of 40 μE m^{-2} s^{-1}.

Transformation of *Synechocystis* with plasmids was carried out as described previously (16). Because *Synechocystis* contains several identical genome copies, numerous rounds of selection on BG11 plates with increasing amounts of spectinomycin, kanamycin, or chloramphenicol (5–100 μg/ml) were necessary to obtain fully segregated mutants. For completeness of the segregation, individual clones were tested by PCR.

Growth of the wt and mutant strains was monitored at 730 nm (OD_{730}) under photoautotrophic, photomixothrophic (+10 mM glucose), or photoheterotrophic conditions (+10 mM glucose, +10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)).

Escherichia coli strain DH5α, which was used for plasmid propagation, was grown in LB broth or agar supplemented as needed with 100 μg/ml ampicillin, 30 μg/ml kanamycin, 30 μg/ml chloramphenicol, or 30 μg/ml spectinomycin, respectively.

DNA Techniques—Molecular cloning was carried out by using standard techniques according to Ref. 17. Enzymes used for PCR and cloning were obtained from MBI Fermentas. PCR was carried out using a Bio-Rad Thermocycler. DNA sequencing was performed by MWG Biotec (Ebersberg, Germany). For PCR, genomic DNA of *Synechocystis* PCC 6803 was prepared as described recently (18), and inactivation of the various *petC* genes in *Synechocystis* PCC 6803 was done following the protocol in Ref. 16.

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

Spectroscopic Methods—77 K fluorescence emission spectra were recorded in an Aminco fluorimeter with a chlorophyll concentration of 5 μg/ml (19). Cells were diluted by BG11 medium and frozen in liquid nitrogen. Monochromators were set to a slit width of 4 nm.

The yield of the variable chlorophyll fluorescence (F_V) from photosystem II depends on the redox state of Q_A, the primary quinone acceptor, and, indirectly, also on the state of the plastoquinone pool, which thus can be monitored by using chlorophyll fluorescence measurements (20). Light-induced changes of chlorophyll fluorescence were measured at 686 nm in a laboratory-built set up at room temperature (23 °C), using a 1 × 1-cm cuvette with a 2-ml sample volume. Cells were incubated for 2 min in the dark at a chlorophyll concentration of 2.5 μM in BG-11 medium, with or without further additions, as indicated in the figure legends. After the dark incubation, the cells were illuminated with blue light (center wavelength of 445 nm, intensity of 205 μE m^{-2} s^{-1}). Illumination was controlled by an electronic photoshutter; the duration of the light pulse was 250 ms or 1 s, respectively, as indicated. The level of minimum fluorescence, F_o, which corresponds to the oxidized state of Q_A, was determined from the initial fast phase of fluorescence rise, which was concomitant with shutter opening. After addition of 10 μM of the photosystem II inhibitor DCMU and another 2 min of dark incubation, each sample was illuminated again to determine the maximum fluorescence F_{max}, which corresponds to the reduced state of Q_A. The variable fluorescence was calculated as $F_V = F_{max} - F_o$.

RESULTS

Insertional Deletion of the Three Putative petC Genes—The three open reading frames of *Synechocystis* PCC 6803 encoding putative Rieske proteins were assigned *petC1* (*sll1316*), *petC2* (*slr1185*), and *petC3* (*sll1182*) (5, 21) with the numbers given in parentheses showing the deposited name in the Cyano data base (22).

The gene encoding the PetC1 protein was amplified with flanking regions by PCR and cloned into the plasmid pUC18 (New England Biolabs, Beverly, MA) after restriction of the PCR product using restriction sites that had been introduced by the primers. A 260-bp fragment from the *petC1* encoding and upstream region was replaced by a chloramphenicol resistance cassette, which was amplified by PCR without a transcription termination signal (Fig. 1). By this method the promoter of the chloramphenicol acetyltransferase (*cat*) cassette can be used for the transcription of *petA* encoding for the cytochrome

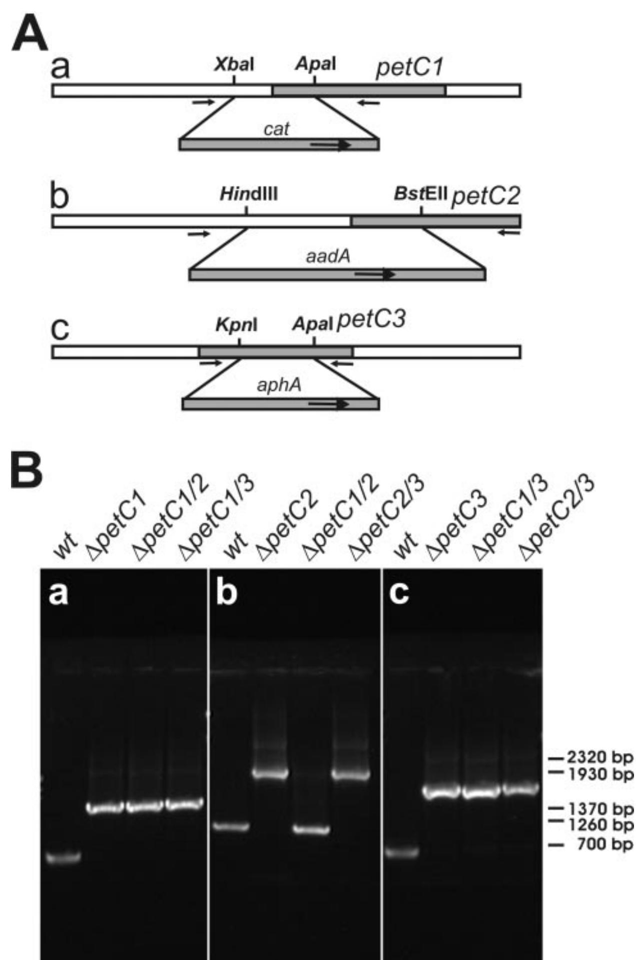

FIG. 1. *A*, strategy for the deletion of *petC1* (*a*), *petC2* (*b*), and *petC3* (*c*) in the genome of *Synechocystis* PCC 6803. *cat*, chloramphenicol resistance; *aad*A, spectinomycin resistance; *aphA*, kanamycin resistance. *Arrows* in the *gray boxes* representing the antibiotic resistance cassettes indicate the orientation of the respective cassette. *Small arrows* indicate the positions where the primers used for PCR anneal at the genomic DNA. Restriction sides used for the insertional mutagenesis of the *petC* genes are indicated. *B*, PCR analysis of genomic DNA from wt and mutants. A DNA fragment containing the *petC* gene was amplified from genomic *Synechocystis* DNA of the wt and mutant strains as indicated. The size of the wt *petC* fragments (*petC1*, 543 bp; *petC2*, 537 bp; *petC3*, 402 bp) is shown in *lanes 1*, *5*, and *9*. *a*, the region containing the *petC1* gene was amplified; *b*, the region containing *petC2* was amplified; and *c*, the *petC3* gene was amplified. The introduction of the resistant cassettes increased the size of any of the PCR fragments. The results are described in detail in the text.

f subunit in *Synechocystis*. A wild type *Synechocystis* PCC 6803 strain was used for transformation of the deletion construct, and transformants were grown on plates containing increasing amounts of chloramphenicol to select for complete segregation and to obtain a homozygous genotype.

For the inactivation of *petC2*, the gene was amplified by PCR with flanking regions, and after ligation of the PCR product to the plasmid pBluescript II SK (MBI Fermentas, St. Leon-Rot, Germany), a 640-bp fragment was replaced by a spectinomycin resistance cassette from pHP45Ω (23). Wild type, Δ*petC1*, and Δ*petC3* strains of *Synechocystis* PCC 6803 were transformed with this construct to yield the appropriate single or double deletion strains.

For deletion of *petC3*, the gene was amplified by PCR with flanking regions and cloned into the plasmid pASK-IBA4 (IBA GmbH, Goettingen, Germany). To inactivate the *petC3* gene, a 215-bp DNA fragment was replaced by a kanamycin resistance cassette obtained from the plasmid pBSL14 (24). The resulting

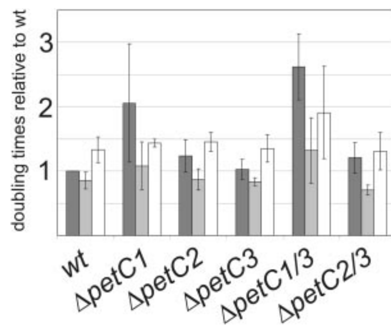

FIG. 2. **Growth rates (doubling times relative to wt) of the mutant *Synechocystis* PCC 6803 strains under photoautotrophic, mixotrophic, and photoheterotrophic growth conditions.** *Error bars* indicate the S.D. of 3–6 independent measurements. As internal control, the wt strain grown under photoautotrophic conditions was always used, and the doubling time of that strain was set as 100% (*1*) in each measurement.

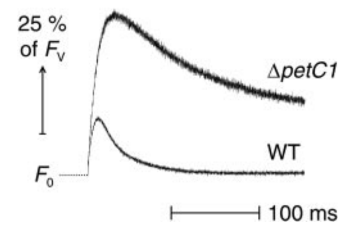

FIG. 3. **Characterization of light-induced plastoquinol oxidation in wt and the Δ*petC1* mutant.** Cells were preincubated with 1 mM of the oxidase inhibitor KCN for 2 min in the dark in order to obtain a reduced state of the plastoquinol pool. Fluorescence induction curves were recorded during the subsequent actinic illumination with blue light (250 ms). Each curve represents an averaged signal from four different samples; the signal/noise ratio is lower for the mutant cells, which is caused by a lowered photosystem II content in the Δ*petC1* strain (see also Fig. 6). The level of F_{max} was determined by illumination in the presence of DCMU. Chlorophyll *a* concentration was 2.5 μg/ml.

construct was transformed into the wt, the Δ*petC1*, and the Δ*petC2* strain to obtain the single and double deletion strain. For complete segregation of the Δ*petC3* cells, mutants were selected on BG11 plates containing increasing amounts of kanamycin.

PCR was used to test whether the various strains were homozygous for the deletion of the different *petC* genes (Fig. 1). Although the PCR fragments of the DNA regions containing the wt gene were less than 1000 bp in size, the introduction of the respective resistant cassettes increased the size of these PCR fragments by more than 500 bp (Fig. 1). Fig. 1 also shows that the individual deletion of all three *petC* genes is possible. In neither case was a fragment corresponding to the size of the wt DNA fragment amplified. The simultaneous deletion of *petC2* with *petC3* was possible as well as the deletion of *petC1* in combination with *petC3*. All attempts to delete *petC2* completely in the Δ*petC1* strain failed (Fig. 1) as well as attempts to delete *petC1* in the Δ*petC2* strain. These results indicate an overlapping function of these two genes, whereas the function of *petC3* does not seem to overlap directly with the possible roles of the other two *petC* genes.

Effect of the petC Deletions on Cell Growth—To test whether the deletions have any effect on cell viability under different growth conditions, growth rates of the completely segregated mutant strains relative to the wt were measured under photoautotrophic, mixotrophic, and photoheterotrophic growth conditions, respectively.

As can be seen in Fig. 2, strains carrying the single deletions Δ*petC2* or Δ*petC3,* as well as the Δ*petC2/3* double deletion do not alter significantly in their growth properties under any of the tested growth conditions when compared with wt cells; this suggests a nonessential function of PetC2 and PetC3 under the chosen growth conditions. Although the Δ*petC1* strain shows similar growth rates as the wt strain under photomixotrophic, and photoheterotrophic conditions (Fig. 2) the growth rate is slowed down under photoautotrophic conditions. It should be noted that growth of this mutant in the presence of chloramphenicol, *i.e.* the antibiotic used for obtaining a completely segregated deletion strain, resulted in highly impaired growth under photoheterotrophic conditions (data not shown); this indicates a considerable stress of the Δ*petC1* strain caused by the gene deletion. Most interestingly, although the single deletion of *petC3* did not cause any observable growth defect and the single deletion of *petC1* had a somewhat stronger impact on the growth properties, the combination of both in the double deletion strain Δ*petC1/3* resulted in a distinctly increased doubling time under all tested growth conditions when compared with the wt strain.

Chlorophyll Fluorescence as a Probe for the Reduction and Reoxidation of the PQ Pool—To examine the effect of the *petC1* deletion on the redox activity of the cytochrome b_6f complex, the photooxidation of the PQ pool by photosystem I was monitored by measuring the chlorophyll fluorescence of photosystem II, which is dependent on the redox state of Q_A and thus indirectly reflects the redox state of the PQ pool. In order to obtain a reduced PQ pool in darkness (due to PQ reduction by respiratory dehydrogenases), wt and Δ*petC1* cells were incubated with 1 mM KCN, which blocks the reoxidation by the terminal oxidases. During a subsequent illumination of wt cells, the yield of variable fluorescence (F_V) increases quickly at the onset of illumination but returns to a low level after a short lag phase due to the fast photooxidation of PQH_2 by photosystem I (Fig. 3). In contrast, the reoxidation of PQH_2 is drastically slowed down in the Δ*petC1* cells, and the transient increase of F_V is much stronger with about 60% of the maximum yield of F_V. This is about three times higher than in wt and indicates a decreased rate of electron transport between the photosystems, indicating a severely impaired activity of the cytochrome b_6f complex in Δ*petC1* cells.

In addition to the cytochrome b_6f complex, plastoquinol can also be oxidized by a cytochrome *bd*-type oxidase in *Synechocystis* PCC 6803 (20, 25–27). If PQH_2 oxidation could only occur via the cytochrome b_6f complex, illumination of the cyanobacterial cells in the presence of DBMIB, a cytochrome b_6f complex inhibitor, should result in a completely reduced PQ pool. For this reason, the partial quenching of F_V in the presence of saturating concentrations of DBMIB, as shown in Fig. 4*A*, is evidence for such an alternative route of PQH_2 oxidation (20). On average, photoheterotrophically grown wt cells (Fig. 4*A*) show a quenching of 28 ± 10% (n = 10) of the total yield of F_V. Δ*petC1* cells show a similar effect (Fig. 4*B*), but here a significantly higher amount of F_V quenching (52 ± 3%, n = 4) is observed, indicating a distinctly higher oxidation rate of the PQ pool via the cytochrome *bd*-type complex in this mutant. As a control, the cells were illuminated in the presence of both DBMIB and KCN. In this case, a rapid reduction of the PQ pool during illumination is expected, as neither respiratory nor light-dependent oxidation of plastoquinol is possible. Indeed, this effect could be observed for both wt (Fig. 4*C*) and mutant cells (Fig. 4*D*).

Identical measurements have also been performed with the Δ*petC2* and Δ*petC3* strains; however, there are no significant differences in comparison to wt. F_V quenching in the presence of DBMIB (conditions as in Fig. 4, *A* and *B*) was 18 ± 1% (n = 4) in Δ*petC2* and 19 ± 7% (n = 4) in Δ*petC3* (not shown).

Photosynthetic Activity—In order to test the photosynthetic

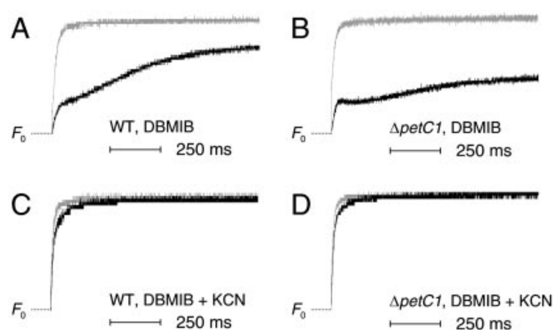

FIG. 4. **Fluorescence induction curves in the presence of the inhibitors DBMIB and KCN.** Each panel shows a pair of curves, of which the *lower curve* was recorded in the presence of the additions as indicated. The *upper curve* is the reference signal and shows the full yield of F_V obtained from a second illumination of the same sample after addition of 10 μM DCMU. Before illumination (1 s of blue light), the cells (2.5 μg of chlorophyll/ml) have been preincubated for 2 min in the dark in the presence of 50 μM DBMIB (*A* and *C*) or 50 μM DBMIB + 1 mM KCN (*B* and *D*), respectively.

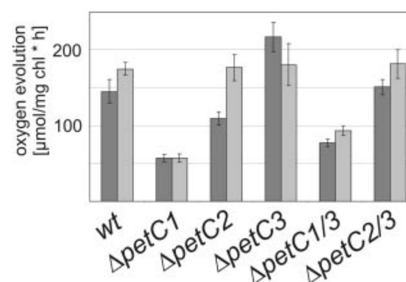

FIG. 5. **Oxygen evolution measurements of wt and mutant *Synechocystis* cells.** Cells were grown photoautotrophically, and oxygen evolution was measured in the presence of phenyl-*p*-benzoquinone as electron acceptor at photosystem II (*dark gray column*) or in the presence of HCO_3^- (*light gray column*) to measure the electron transfer throughout the entire photosynthetic electron transfer chain. *Error bars* indicate the standard deviation of five measurements.

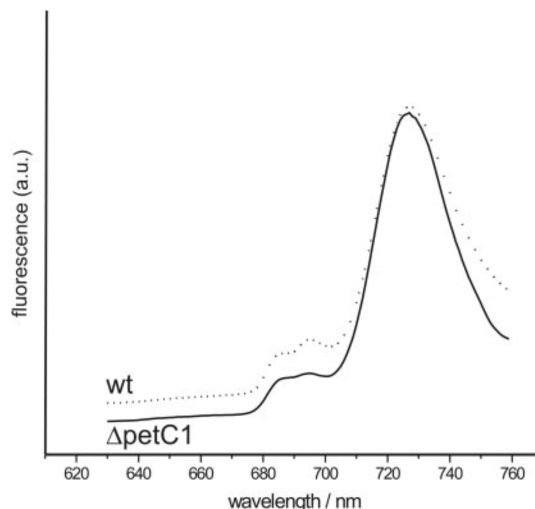

FIG. 6. **77 K fluorescence emission spectra of whole *Synechocystis* PCC 6803 wt (—), and Δ*petC1*, cells (···).** Cells were diluted with BG11 to a final concentration of 5 μg of chlorophyll/ml. Chlorophylls were excited at 435 nm, and spectra were normalized to the photosystem I emission peak at 725 nm.

activities of the generated deletion strains, the rate of oxygen evolution was determined for all Δ*petC* strains using phenyl-*p*-benzoquinone as electron acceptor at photosystem II. These measurements, which were normalized to the total chlorophyll concentration, indicate an ~60% reduced amount of active photosystem II in the Δ*petC1* and Δ*petC1/3* strains in comparison with wt (Fig. 5). Photosystem II activity in the other mutant strains was also impaired, although to a lesser extent as in the case of the Δ*petC1* strain. Using HCO_3^- as electron acceptor allows the determination of the activity of the whole electron transport chain. With this acceptor the Δ*petC1* mutant cells showed only 40% of the activity of wt cells (175 ± 5 *versus* 57 ± 9% μmol O_2/(mg of chlorophyll·h)). The strain carrying the double deletion Δ*petC1/3* showed similar photosystem II activity as the Δ*petC1* strain as can be seen in Fig. 5. In contrast, all other mutant strains, *i.e.* Δ*petC2*, Δ*petC3*, and Δ*petC2/3*, were similar active as the wt.

To study further the reason for the decreased oxygen evolution rate in the Δ*petC1* strain, wt and Δ*petC1* cells were analyzed by 77 K fluorescence spectroscopy. The relative content of photosystem I and II in the thylakoid membrane of cyanobacteria can be determined from their fluorescence emission spectra at 77 K. Upon chlorophyll excitation at 435 nm, photosystem II shows a characteristic emission spectrum with maxima at 695 and 685 nm, and photosystem I shows a characteristic emission maxima at 725 nm. The emission spectra shown in Fig. 6 indicate an increased photosystem I content relative to photosystem II in the Δ*petC1* mutants, whereas the photosystem I to photosystem II ration in the Δ*petC2* and Δ*petC3* mutants was identical to wt (data not shown). A decreased photosystem II content rather than an increased photosystem I content in the Δ*petC1* strain is supported by the observation that the concentration of chlorophyll per cell (cell density measured as OD_{730}) is decreased by more than 7% in the Δ*petC1* mutant strain.

In summary, the oxygen evolution measurements as well as the 77 K fluorescence measurements indicate a reduced photosystem II content in the Δ*petC1* strain.

DISCUSSION

Presence of Multiple Genes for Rieske Proteins—To investigate the specific role of three potential Rieske proteins in *Synechocystis* PCC 6803, their genes have been deleted individually and in combination. In general, the deletion of subunits of the cyanobacterial cytochrome b_6f complex is difficult because this complex has a central role for both the photosynthetic and the respiratory electron transport chain of cyanobacteria (14). Up to now, only the successful deletion of the gene encoding the small subunit PetM was reported (19), which, however, does not seem to have an essential function. Here we show that the complete deletion of each of the three *petC* genes alone is possible. These results are in contrast to a recent report (28) on deletion of the *petC1* gene in the cyanobacterium *Synechococcus* PCC 7002. In this study, deletion of *petC1* did not lead to a viable Δ*petC1* segregant, and the authors concluded that *petC2* and *petC3* are silent with respect to a possible function in the cytochrome b_6f complex. However, the authors did not perform deletions of the two additional *petC* genes, and potentially overlapping function of any of the *petC* genes was not studied. In addition, it is possible that the function of the two additional *petC* genes is less important in *Synechococcus* than in *Synechocystis* making a deletion of *petC1* impossible in *Synechococcus*.

Our attempts to delete *petC1* in *Synechocystis* completely, using a resistance cassette with a transcription termination signal, also failed, most likely because of an interruption of the *petA* transcription. Only the use of a resistant cassette without a transcription termination signal resulted in a successful deletion of *petC1*. Most likely in this case the *cat* promoter is also used for the transcription of the *petA* gene. The deletion experiments also indicate that *petC2* does not have any special function in the *Synechocystis* cytochrome b_6f complex. Although the single deletion of *petC2* did not result in a signifi-

cantly altered phenotype, the Δ*petC2/3* double deletion strain was also unaffected in the stoichiometry or the activity of the photosynthetic protein complexes and in the growth rate (doubling time) of the strain. Although the single deletion of *petC3* did not result in significantly altered growth properties of the mutant strain, the results obtained with the Δ*petC1/3* double deletion strain indicate that *petC3* shares some function with *petC1*. Because the double deletion was possible, the functions of these two genes seem to be different and not replaceable as in the case of *petC1* and *petC2*. Whereas the phenotype of the Δ*petC1/3* mutant was basically identical to the Δ*petC1* strain, the combined deletion of *petC1* and *petC2* turned out to be impossible. From these data we conclude that the Rieske protein PetC2 can indeed replace the Rieske protein PetC1 in the cytochrome b_6f complex to some extent. This is consistent with the relative high sequence similarity and especially with the similar midpoint potential of these two proteins (5). Also the fact that the *petC2* gene is functionally expressed in *Synechocystis* supports the view of various co-existing Rieske proteins in the cytochrome b_6f complex of this organism (5). Because it was already shown that *petC3* is also functionally expressed in *Synechocystis* and that the encoded protein is associated with the cytochrome b_6f complex, although to rather low extent (5), the results presented in this study indicate a function of PetC3 that is different from the function of PetC1. We showed recently (5) that the *petC3* gene encodes for a truncated Rieske protein with a highly lowered midpoint potential. The observation of this study that PetC3 seems to have some function *in vivo* together with the observed highly lowered midpoint potential argue for the existence of different cytochrome b_6f complexes in *Synechocystis* as has been suggested.

PetC1 Is the Main Rieske Protein in Synechocystis PCC 6803—As the cytochrome b_6f complex is a central component of the energy-transducing system of *Synechocystis* PCC 6803, it seemed unlikely that the deletion of an essential subunit such as the Rieske protein would be possible. However, our results show that the *petC1* gene can be deleted and that its function can at least partly be replaced by another *petC* gene. Nevertheless, in contrast to the Δ*petC2* and Δ*petC3* strains, the Δ*petC1* mutant strain showed some new characteristics when compared with the wt, indicating an important and specific role of the *petC1* gene. The electron transfer through the cytochrome b_6f complex was still possible in this mutant, although the activity of the complex seemed to be impaired, and chlorophyll fluorescence measurements showed a significant reduction of the PQ reoxidation rate by the cytochrome b_6f complex (Fig. 3). In addition, an increased activity of the cytochrome *bd*-type complex was indicated in the Δ*petC1* mutant strain (Fig. 4) by fluorescence measurements. *Synechocystis* PCC 6803 contains three different respiratory terminal oxidases (25, 27). Two of them are homologous to the mitochondrial cytochrome *c* oxidase, and the third one, the cytochrome *bd*-type complex, is a quinol oxidase that can function in parallel to the cytochrome b_6f complex. Its function in thylakoid membranes of *Synechocystis* PCC 6803 has been elucidated recently (20). It was shown that the activity of the cytochrome *bd*-type complex increases in particular when the rate of plastoquinol oxidation via the cytochrome b_6f complex appears to be insufficient. The changes in the electron transport system as well as the decreased photosystem II content of the Δ*petC1* mutant strain can be interpreted as adjustments in response to the partially defective cytochrome b_6f complex and more reduced PQ pool. On the other hand, the fact that photosystem I seems to be assembled at wild-type levels (as judged from the 77 K fluorescent spectra) and that photosynthetic electron transfer through the whole electron transport chain is still operative indicates rather strongly that the cytochrome b_6f complex is still active. Finally, the diminished growth of the Δ*petC1* strains under photoautotrophic conditions is in agreement with the observation that the photosystem II content is lowered in this mutant resulting in less provided energy by the photosynthetic light reaction.

But why does the Δ*petC1* strain show characteristics indicating a less active cytochrome b_6f complex? The presented mutational data indicate that *petC*2 can partly compensate for the *petC1* deletion, although the activity of the complex is lowered to some degree. In two recent studies the electron transfer activity of the cytochrome b_6f complex Rieske protein with mutations in the flexible hinge region was analyzed. This glycine-rich region connects the transmembrane domain with the soluble domain, and it was shown in the cytochrome bc_1 complex that the activity of the complex is very sensitive to perturbations of the sequence and structure of that hinge region (29–34). Most interestingly, changes in the flexible regions of the cytochrome b_6f complex Rieske proteins did not alter the complex function as dramatically as observed with the cytochrome bc_1 complex (28, 35). Nevertheless, even in these studies changes were observed indicating an important role of this region for proper function of the Rieske protein. In contrast to PetC1 and other Rieske proteins, PetC2 contains one glutamine and one lysine residue in the hinge region that can highly diminish the flexibility of that region, and electrostatic interactions to other subunits are possibly favored. Because the electron transfer within the cytochrome *bc* complex Rieske proteins is accomplished by a movement of the soluble Rieske domain (10, 36), the observed differences in the amino acid sequence in the hinge region could cause less flexibility resulting in impaired electron transfer by domain movement.

petC Gene Families in Cyanobacteria—A recent genome-wide expression analysis in the cyanobacterium *Anabaena* sp. PCC 7120 showed the functional expression of multiple *petC* genes (37). The aim of that study was to investigate the up-regulation of genes after nitrogen deprivation, and the authors could show that the two putative Rieske genes *all0606* and *all1512*, which encode for proteins with a high degree of homology to *Synechocystis* PetC3 and PetC2 proteins, respectively, were up-regulated more that 5.0-fold under the chosen growth conditions. Because *petC1*, which is cotranscribed with *petA* in cyanobacteria (38), is the mainly transcribed *petC* gene, these results indicate the functional expression of three Rieske proteins both in *Anabaena* and in *Synechocystis*.

Although in many of the cyanobacterial genomes, such as in *Synechococcus* PCC 7002 and *Anabaena* PCC 7120, a *petC* gene family can be found, only the main *petC* gene, which is cotranscribed with *petA,* is present in the genomes of the thermophilic cyanobacterium *Thermosynechococcus elongatus* (39, 40) and of the mesophilic cyanobacterium *Gloeobacter violaceus* PCC 7421 (41). Therefore, the occurrence of a *petC* gene family seems to be common but not obligatory in cyanobacteria. This is in good agreement with our observation that the additional *petC* copies *petC2* and *petC3* can be deleted without any significant effect. In higher plants, the functional expression of two almost identical chloroplast Rieske proteins is also reported (14). However, the similar expression pattern of the two genes and their high degree of sequence identity argue rather for the simultaneous expression of two *petC* genes encoded by both parental chromosomes. In this case no special function of the different Rieske proteins is expected.

As outlined in the Introduction, *petC* is not the only gene for which a gene multiplication has been found in *Synechocystis* PCC 6803. Best characterized is *psbA* encoding the D1 subunit of photosystem II with three gene copies. In principle, the

expression of all three genes leads to a functional D1 protein (4), although under standard growth conditions more than 90% of the total *psbA* transcripts originate from the *psbA2* gene and the remaining part from the *psbA3* gene. In contrast to the *petC* genes, the *psbA2/3* genes share 99% nucleotide identity and encode identical proteins. The function of these various D1 proteins is not yet completely clear although increased synthesis of *psbA3* has been observed under stress conditions (42). As all three Rieske proteins in *Synechocystis* are sufficiently different (5), various properties of the resulting proteins can also be expected in their physiological context, although the physiological functions of the *petC* gene family are elusive so far.

In conclusion, our results show that PetC1 has a predominant function in the cytochrome b_6f complex of *Synechocystis* PCC 6803, which can partly be taken over by PetC2. Although PetC3 also seems to have a specific function, the protein cannot replace PetC1 or PetC2 and may only operate in parallel to one of the two other Rieske proteins. Based on the presented data, it can be speculated that under normal physiological conditions PetC1 is the dominant Rieske isoform that can, under certain conditions, be replaced by PetC2, although at the expense of activity loss or decreasing cytochrome b_6f complex activity. Independent of PetC1 and PetC2, PetC3 may have a specific physiological function within a small community of cytochrome b_6f complexes, which is especially indicated by its much more negative redox potential. Although difficult due to its small amount in the membrane, this special role of PetC3 is still to be elucidated.

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