Ssr2998 of *Synechocystis* sp. PCC 6803 Is Involved in Regulation of Cyanobacterial Electron Transport and Associated with the Cytochrome b_6f Complex[*]

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To analyze the function of a protein encoded by the open reading frame *ssr2998* in *Synechocystis* sp. PCC 6803, the corresponding gene was disrupted, and the generated mutant strain was analyzed. Loss of the 7.2-kDa protein severely reduced the growth of *Synechocystis*, especially under high light conditions, and appeared to impair the function of the cytochrome b_6f complex. This resulted in slower electron donation to cytochrome f and photosystem 1 and, concomitantly, over-reduction of the plastoquinone pool, which in turn had an impact on the photosystem 1 to photosystem 2 stoichiometry and state transition. Furthermore, a 7.2-kDa protein, encoded by the open reading frame *ssr2998*, was co-isolated with the cytochrome b_6f complex from the cyanobacterium *Synechocystis* sp. PCC 6803. *ssr2998* seems to be structurally and functionally associated with the cytochrome b_6f complex from *Synechocystis*, and the protein could be involved in regulation of electron transfer processes in *Synechocystis* sp. PCC 6803.

Up to now, 36 different cyanobacterial genomes have been completely sequenced, and the genomic information is available or will be available soon. The first cyanobacterium, the genome of which has been completely sequenced and released in 1996, was the mesophilic cyanobacterium *Synechocystis* sp. PCC 6803 (1). In this genome almost 3200 potential protein encoding open reading frames (ORFs)[2] have originally been identified and based on the available DNA sequences from *Synechocystis* and other organisms, as well as on homology searches; about 1700 of the *Synechocystis* genes have been annotated as ORFs coding for hypothetical proteins with unknown function. Because many of these hypothetical proteins are conserved within cyanobacteria or even show significant sequence homologies to proteins from

other bacteria or plants, it is reasonable to assume that many of these proteins have important physiological functions, which have to be revealed.

Two strategies have been applied in the recent years to elucidate possible biological functions of hypothetical proteins encoded by ORFs in *Synechocystis*. In the first approach an ORF of interest has been interrupted or deleted by genetic methods. The observation, whether an ORF can be deleted or not, indicates whether the encoded gene product is essential for *Synechocystis* or not (2). A subsequent analysis of a constructed mutant strain can give further insights on the possible function of a hypothetical protein, and many examples can be found in the literature. The CyanoMutant data base tries to compile information available about the generation of *Synechocystis* mutants and phenotypic characterizations (3).

In contrast to a directed mutagenesis approach, the gene products encoded by nonannotated ORFs from *Synechocystis* have been identified in several proteomic approaches (4–13). Although such a global analysis answers the question of whether an ORF is expressed and encodes for a physiological relevant protein, the identification of a protein alone does not allow us to draw any conclusion about a probable function of the protein. However, a first hint on the function of a protein can be gained if a hypothetical protein is co-purified with a protein or protein complexes, as it has *e.g.* recently been described for photosystem 2 (PS 2) from *Synechocystis*. After very mild solubilization of PS 2 from *Synechocystis*, several proteins have been co-purified with the complex, and a function of these newly identified proteins for the activity or assembly of PS 2 has been proposed (14).

To analyze the potential *in vivo* function of a small polypeptide encoded by the ORF *ssr2998*, we combine the genetic approach with protein purification. The ORF *ssr2998*, which is highly conserved in cyanobacteria, has been interrupted in *Synechocystis*, and the generated mutant strain was analyzed to reveal the physiological function of Ssr2998. The observed regulatory role of this protein for the cytochrome b_6f complex activity, its purification with this complex, and the fact that a gene encoding a homologous protein can be found in all cyanobacterial genomes sequenced so far suggest that Ssr2998 is functionally associated with, at least, the cytochrome b_6f complex in cyanobacteria.

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[2] The abbreviations used are: ORF, open reading frame; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; PPBQ, *para*-phenyl-benzoquinone; wt, wild type; PS, photosystem.

EXPERIMENTAL PROCEDURES

Molecular Methods—Molecular methods have been performed in *Escherichia coli* strain DH5α according to standard protocols (15). Genomic DNA of *Synechocystis* sp. PCC 6803 was prepared by harvesting cells from an agar plate and resuspending them in 100 μl of 1 mM Tris/HCl, 0.1 mM EDTA, pH 8.0. After boiling the cell suspension for 3 min and one centrifugation step, the supernatant contained genomic DNA and was directly used for PCR.

Construction of a Δssr2998 Mutant—A DNA fragment containing the *ssr2998* ORF and 700-bp upstream and downstream region was amplified by PCR using the following primers: 5′ primer, 5′-gtcaaagcttccacttgggagggtattaa-3′; and 3′ primer, 5′-cttgaattctcagagtggagaggttacc-3′. The resulting 1.2-kb fragment was restriction digested with EcoRI and HindIII and ligated to the EcoRI/HindIII restriction digested pUC18 vector. The resulting plasmid pSsr2998 contains a unique SacI site in the ORF *ssr2998*. To interrupt the ORF *ssr2998*, the plasmid pSsr2998 was linearized via SacI and ligated to a kanamycin-resistant cassette (*ntpII*) taken from pBSL15 (16).

For transformation of *Synechocystis* sp. PCC 6803, a mixotrophic grown culture with an OD_{750} of about 0.5 was adjusted to OD_{750} of 2.5. From this cell suspension, 200 μl was mixed with 5 μg of pSsr2998::K_m and incubated for 30 h in a culture tube at 30 °C at 50 μE/m²s light without shaking. The cells were then plated on BG11 agar supplemented with 10 μg/ml kanamycin. Single colonies were subsequently plated on BG11 medium with increasing concentrations of kanamycin. Because *Synechocystis* contains multiple identical genome copies, the obtained mutants were checked for complete segregation. Genomic DNA of cells grown for various rounds in BG11 medium without added antibiotics was prepared as described above, and segregation was checked by PCR.

Culture Conditions for Cyanobacterial Strains—The cells were cultivated as shaking culture in Erlenmeyer flasks or in U-tubes aerated with 2% CO_2 at 30 °C. The cultures were inoculated with precultures of $OD_{750} = 1$ (± 0.2) and started at $OD_{750} = 0.05$. Illumination was done with white fluorescence light of 20 μE/m²s (low light) or 80 μE/m²s (high light). For photoautotrophic growth, BG11 medium was used that was supplemented by 10 mM glucose for mixotrophic and further by 10 μM 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU; Diuron) for photoheterotrophic growths conditions. Three independent cultures of each analyzed strain were measured 3-fold.

Oxygen Evolution and Consumption—Oxygen evolution and consumption were measured in 1 ml of cell suspension (OD_{750} of ~1, about 5 μg/ml chlorophyll) using a fiber-optic oxygen meter (PreSens, Regensburg, Germany). The cells were cultivated photoautotrophically under aeration at 20 μE/m²s light. Three independent cultures of each analyzed strain were measured three times. Photosynthetic rates were determined in the presence of 15 mM $NaHCO_3$ and PS 2 activity in the presence of 0.5 mM *para*-phenyl-benzoquinone (PPBQ). Respiratory oxygen consumption was recorded in the dark, and photosynthetic oxygen evolution was recorded at 1000 μE/m²s light. Photosynthetic rate and PS 2 activity were determined as a difference of the net oxygen evolution in light and oxygen consumption in the dark. PS 1 activity was measured as oxygen consumption in the presence of 20 μM DCMU, 2 mM NaN_3, 200 μM 2,3,5,6-tetramethyl-1,4-phenylenediamine, 5 mM sodium ascorbate, and 200 μM methyl viologen. A base line was recorded in dark and light. Oxygen consumption caused by the Mehler reaction was determined as corrected oxygen consumption in the light by oxygen consumption in the dark. The chlorophyll content of the individual strains was determined as described in Ref. 17 using methanol as solvent for extraction.

Low Temperature Fluorescence Emission Spectroscopy—Low temperature fluorescence emission spectra were measured using an Aminco-Bowman Series 2 luminescence spectrometer (SLM Spectronic Instruments) with cell suspensions of 5 μg/ml chlorophyll. After 10 min of dark adaptation, the samples were frozen in liquid nitrogen (18) with two individual clones of each strain being analyzed. Excitation and emission were carried out with a bandwidth of 4 nm. Upon phycobilisome excitation at 580 nm and chlorophyll excitation at 440 nm, fluorescence emission spectra were recorded in the range of 630–750 nm.

Measurement of Phycobilisome Movements ("State Transitions")—State transitions of wild type (wt) and mutant cells were analyzed by low temperature fluorescence emission spectra after excitation of the phycobilisomes at 580 nm (see above). The samples were prepared in different ways to induce different states prior freezing. For adjusting the cells to State 2 prior to freezing, the samples were incubated in the dark for 10 min. State 1 was induced by a 3-min exposure to strong far red light (200 μE/m²s) after a 10-min dark incubation (19, 20). Light of 700 nm was generated by using an Omega BP700 filter. Alternatively to light, 150 μM PPBQ was used to induce State 1 (21).

Cytochrome f and P₇₀₀ Absorption Kinetics—Cytochrome *f* redox kinetic measurements were performed on a home-built photometer, as previously described (22). The cells were suspended to a final concentration of 12.5 μg/ml chlorophyll in BG11 medium. Kinetics induced by 50-ms saturating flashes (655 nm, LED cone; Walz GmbH, Effeltrich, Germany) were determined at 551, 556, and 561 nm under continuous far red background illumination as in Ref. 23. Kinetics of four different samples and 32 (2 times 16) individual traces of each sample was averaged (repetition frequency, 1 Hz). The cytochrome *f* kinetics were calculated as a difference of the kinetics measured at 556 nm minus the average of the kinetics at 551 and 561 nm (23). The resulting cytochrome *f* kinetics was smoothed by a Fourier correction, which allows subtracting the periodic noise and a subsequent Gaussian-weighted smoothing. P_{700} kinetics (single trace) was measured by a dual PAM-100 measuring system (Walz GmbH) with a chlorophyll concentration of 50 μg/ml. The redox kinetics of cytochrome *f* and P_{700} were fitted with single exponential functions.

Purification of the Cytochrome b₆f Complex—The cytochrome b_6f complex was isolated from a photosystem 1 less strain of *Synechocystis* sp. PCC 6803 as described in detail recently (24). Membranes of the cyanobacterium *Synechocystis* were prepared as described in Ref. 25, and membrane proteins were extracted from thylakoids by extraction with 1% (w/v) *n*-dodecyl-β-D-maltoside for 30 min at room temperature. After centrifugation at 200,000 × *g* (4 °C, 40 min), the cyto-
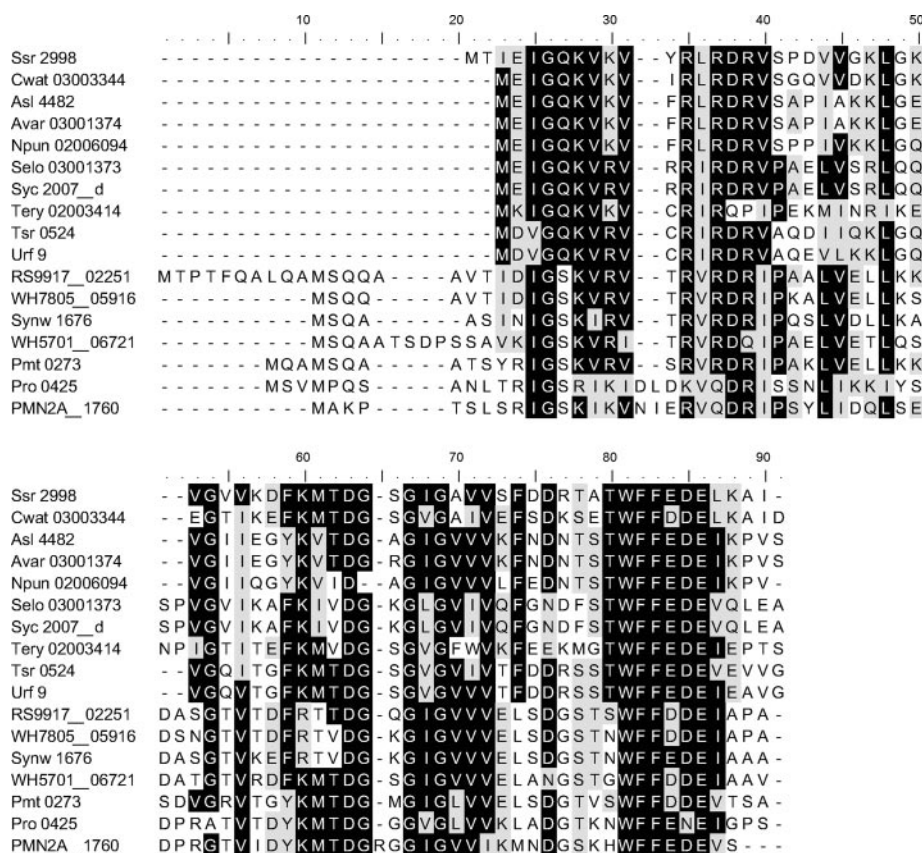
Δssr2998 Affects Cytochrome b₆f Activity

FIGURE 1. **Alignment of the protein sequence Ssr2998 from *Synechocystis* sp. PCC 6803 with homologous proteins encoded in other cyanobacterial genomes.** Identical amino acids are shown in *black*, and conservatively exchanged amino acids are in *gray*. *Ssr 2998, Synechocystis* sp. PCC 6803; *Cwat 03003344, Crocosphaera watsonii; Asl 4482, Nostoc* sp. PCC 7120; *Avar 03001374, Anabaena variabilis* ATCC 29413; *Npun 02006094, Nostoc punctiforme* PCC 7942; *Selo 03001373, Synechococcus elongatus* PCC 7942; *Syc 2007_d, Synechococcus elongatus* PCC 6301; *Tery 02003414, Trichodesmium erythraeum* IMS101; *Tsr 0524, Thermosynechococcus elongatus* BP-1; *Urf 9, Synechococcus* sp. PCC 6716; *RS9917_02251, Synechococcus* sp. RS9917; *WH7805_05916, Synechococcus* sp. WH 7805; *Synw 1676, Synechococcus* sp. WH 1676; *WH5701_06721, Synechococcus* sp. WH 5701; *Pmt 0273, Prochlorococcus marinus* MIT 9313; *Pro 0425, Prochlorococcus marinus* SS 120; *PMN2A_1760, Prochlorococcus marinus* NATL2A.
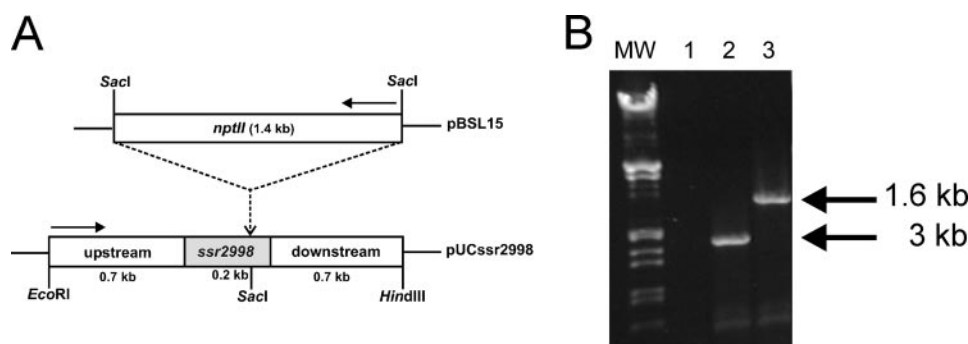

FIGURE 2. **Disruption of *ssr2998* in *Synechocystis* sp. PCC 6803.** *A*, strategy for disruption of *ssr2998* in the genome of *Synechocystis* sp. PCC 6803. The *nptII* gene confers resistance to kanamycin. The *arrows* indicate the direction of the *nptII* and the *ssr2998* genes. *B*, PCR analysis of genomic DNA from wt and Δssr2998 cells. Segregation of the Δssr2998 mutant was controlled by agarose gel electrophoresis of PCR products. *Lane 1*, negative control without template DNA; *lane 2*, PCR product with wt DNA as template; *lane 3*, PCR product with Δssr2998 DNA as template. *MW*, molecular mass standard.

the cytochrome b_6f complex eluted at a concentration of about 1 M ammonium sulfate. After concentration and desalting of the cytochrome b_6f complex containing fractions, the complex was further purified on an anion exchange column (Uno Q6; Bio-Rad).

RESULTS

Ssr2998 Codes for a 7.2-kDa Protein Conserved in Cyanobacteria—Several cyanobacterial genomes have been completely sequenced in the recent years, and in all genomes a high number of potential ORFs has been identified that code for proteins with unknown functions. The ORF *ssr2998* from *Synechocystis* sp. PCC 6803 encodes a small hypothetical protein with unknown function. The protein has a calculated mass of 7.2 kDa, and no known protein domain or structural motif is predicted by computational methods. Based on the amino acid sequence, *ssr2998* does not code for a transmembrane protein but rather for a soluble one. Interestingly, the encoded protein is highly conserved in cyanobacteria (Fig. 1), whereas no homologous proteins can be found in higher plants or green algae. Because the protein is conserved in cyanobacteria, it is reasonable to speculate that it has a physiological function important for cyanobacterial cells.

Construction of a Synechocystis Δssr2998 Strain—To investigate the function of the protein encoded by the *Synechocystis* ORF *ssr2998*, a mutant strain was constructed, in which the ORF is interrupted by a kanamycin resistance cassette (Fig. 2*A*). Because *Synechocystis* contains multiple identical genome copies, complete segregation of the mutant strain was confirmed by PCR. After several rounds of selection, the cells were grown in medium without antibiotics, and genomic DNA was prepared and subsequently used as a

chrome b_6f complex was purified from the supernatant by two high performance liquid chromatography steps. In the first step the membrane extract was loaded onto a hydrophobic interaction column (POROS 20BU; Applied Biosystems, Foster City, CA). Upon applying a decreasing ammonium sulfate gradient,

template for PCR. When genomic DNA from a *Synechocystis* wt strain was used as a template, a fragment of 1.6 kb was amplified, corresponding to the size of the *ssr2998* gene and parts of the upstream and downstream sequences (Fig. 2*B*). In contrast, no fragment corresponding to the size of the wt *ssr2998* gene

was amplified by PCR when genomic DNA from *Synechocystis* Δ*ssr2998* was used as a template. The exclusively observed fragment of 3 kb corresponds in size to the wt fragment (1.6 kb) with the integrated resistance cassette (1.4 kb). These observations prove that the ORF *ssr2998* can be completely deleted, and the encoded protein has therefore no essential function in *Synechocystis* under the chosen growth conditions.

Disruption of ssr2998 Influences Growth of Synechocystis—To analyze potential effects of the gene disruption on cellular processes, growth rates of wt and Δ*ssr2998 Synechocystis* cells were determined under photoautotrophic, mixotrophic, and photoheterotrophic growth conditions, as well as under two different light conditions (Table 1). Although wt and Δ*ssr2998* cells show similar growth rates under photoautotrophic conditions at low light (20 μE/m^{2}s), the mutant strain showed a decreased growth rate or no growth under mixotrophic and photoheterotrophic conditions. This effect was more severe

under high light than under low light conditions. Additionally, photoautotrophic growth of the mutant strain was not increased as much as in the wt strain under increasing light conditions. These observations indicated that Ssr2998 is involved in light dependent electron transfer reactions of *Synechocystis*. Because growth of the mutant strain depended on the light conditions, the photosynthetic electron transport rates of the mutant strain was analyzed and compared with the wt strain.

Activity and Stoichiometry of Photosynthetic Complexes—The photosynthetic rates of *Synechocystis* cells were determined by measuring oxygen evolution in the presence of HCO_3^-, which allows determination of the activity of the whole electron transfer chain. Activity of PS 2 alone was measured as oxygen evolution in the presence of excess PPBQ. As the electron acceptor PPBQ replaces plastoquinone, PS 2 activity is independent of the redox state of the plastoquinone pool and of other, downstream localized components of the photosynthetic electron transport chain. Furthermore, PS 1 activity was determined by oxygen consumption caused by the Mehler reaction (26).

As shown in Table 2, the photosynthetic rate of the mutant strain was found to be reduced by about 18% in comparison with the wt strain, when measured with HCO_3^- as electron acceptor. To test whether this effect was caused by a decreased PS 2 content, oxygen evolution of PS 2 was measured with PPBQ as electron acceptor and also with this acceptor a reduction of oxygen evolution of about 24% was observed (Table 2). These results suggested that reduction of the PS 2 activity was responsible for the reduced photosynthetic rates in the Δ*ssr2998* mutant strain. In contrast, the activity of PS 1 was determined to be slightly increased relative to the wt strain as measured by oxygen consumption caused by the Mehler reaction (see above).

To investigate whether the individual activity or the overall quantity of PS 2 had changed, the relative amount of the photosystems and the phycobilisomes was determined by low temperature fluorescence emission spectroscopy (Fig. 3). After excitation of chlorophylls at 440 nm, fluorescence emission spectra indicated an increased PS 1/PS 2 ratio in the Δ*ssr2998* mutant. Normalization of these emission spectra to the PS 2 peak at 695 nm (27) indicated a relative increase of PS 1 (with a fluorescence maximum at 725 nm) in the mutant strain. In contrast, upon excitation of the phycobilisomes at 580 nm, no significant differences between the emission spectra of wt and mutant cells have been observed. So, why is the PS 2 content reduced in *Synechocystis* cells after disruption of *ssr2998*? One possible explanation was that PS 2 synthesis was down-regulated initiated by an over-reduced PQ pool.

Disruption of ssr2998 Influences Energy Transfer between Phycobilisomes and Photosystems—An over-reduced PQ pool could be also a rea-
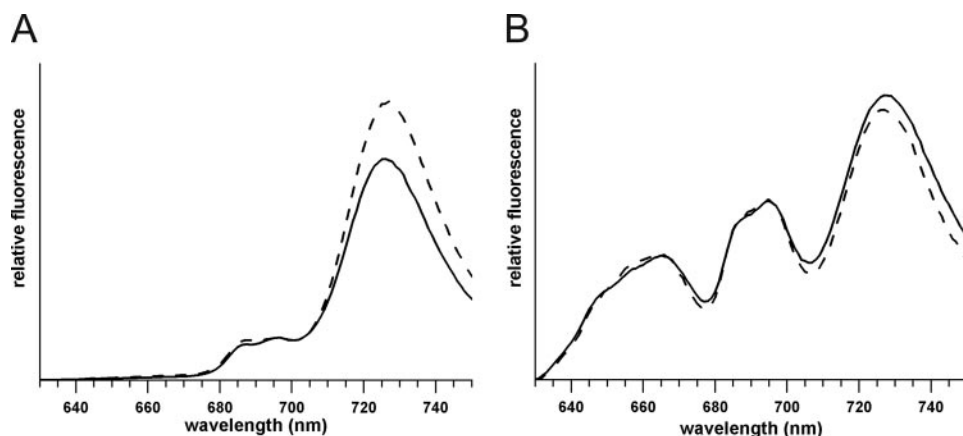
TABLE 1

Growth rates of wt and Δ*ssr2998 Synechocystis* cells grown in the presence of 2% CO$_2$ at 30 °C and two different light intensities

A dash indicates no growth observed. The results were obtained by 3-fold measurements using three independent cultures.

Growth condition	Light intensity	Doubling time/h		Mutant doubling
		wt	Δ*ssr2998*	
	μE/m^{2}s			time (% of wt)
Photo-autotrophic	20	21.1 ± 1.2	22.3 ± 0.8	106
	80	4.3 ± 0.2	8.7 ± 1.2	202
Mixotrophic	20	10.6 ± 0.7	—	—
	80	4.2 ± 0.2	—	—
Photo-heterotrophic	20	13.2 ± 0.3	19.6 ± 0.9	148
	80	13.3 ± 1.0	—	—

TABLE 2

Oxygen evolution and consumption of wt and Δ*ssr2998* mutant grown with 5% CO$_2$ at 30 °C and 20 μE/m^{2}s

The results were obtained by 3-fold measurements using three independent cultures.

	Wt	Δ*ssr2998*	Δ*ssr2998*
	μmol/mg Chl/h	μmol/mg Chl/h	% of wt
Photosynthetic rate	71 ± 20	58 ± 15	82
PS 2 activity	62 ± 14	47 ± 3	76
PS 1 activity	−1356 ± 63	−1537 ± 166	113

FIGURE 3. **Fluorescence emission spectra recorded with *Synechocystis* wt (*solid line*) and Δ*ssr2998* mutant (*dashed line*) cells after incubation of the cells in the dark.** The spectra were recorded at 77 K after chlorophyll excitation at 440 nm (*A*) with spectra being normalized to the PS 2 emission peak at 695 nm. The spectra recorded after excitation of phycobilisomes (*B*) were normalized to the emission at 650 nm.
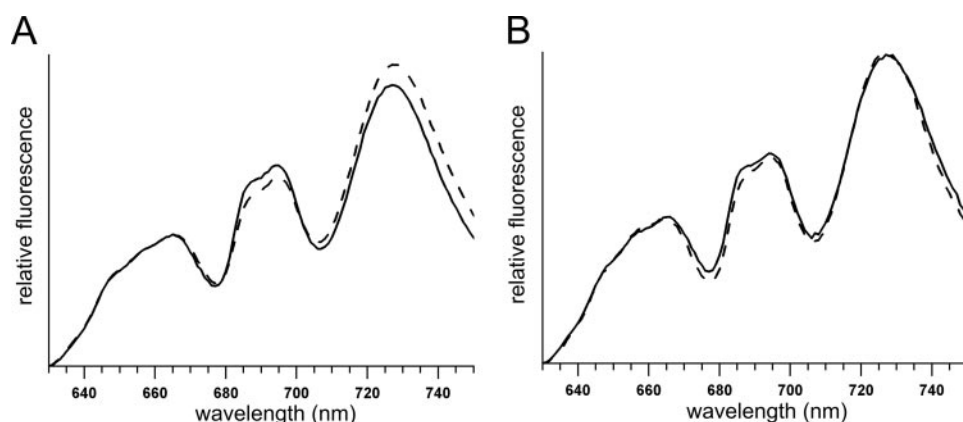
FIGURE 4. **Light induced changes in energy coupling between phycobilisomes and photosystems.** 77 K spectra (λ_{excit} = 580 nm) of wt (*A*) and the Δ*ssr2998 strain* (*B*) recorded after different preincubation are shown. The cells were either incubated for 10 min in the dark (*dashed line*) or exposed to far red light (700 nm at 200 μE/m²s) for 3 min after the dark incubation (*solid line*). The spectra are normalized to the emission peak at 650 nm.
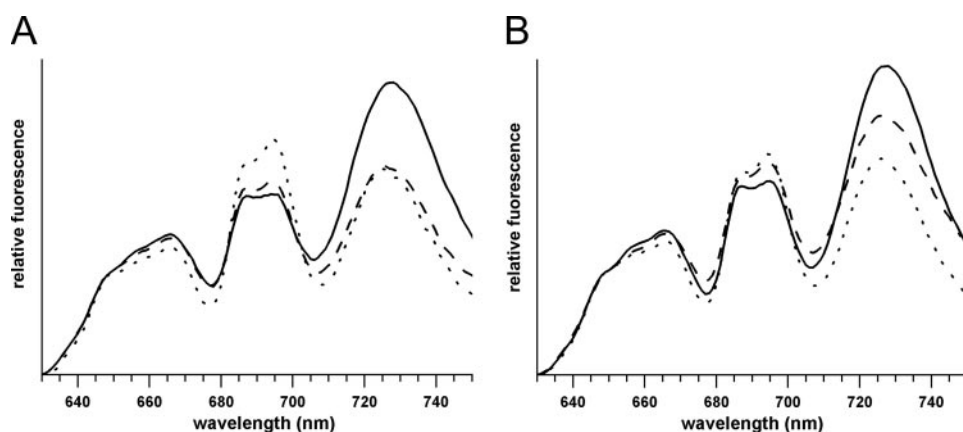

FIGURE 5. **Energy transfer from phycobilisomes to photosystems in the presence of PPBQ.** 77 K fluorescence emission spectra (λ_{excit} = 580 nm) of wt (*A*) and the Δ*ssr2998* mutant cells (*B*) are shown. The samples were incubated with (*dashed line*) or without (*solid line*) 150 μM PPBQ for 10 min in the dark. Thereafter, dark-adapted samples with PPBQ were illuminated (*dotted line*) by far red light (700 nm at 200 μE/m²s). The spectra were normalized to the emission peak at 650 nm.

son for missing adaptation capability of the mutant cells to certain growth parameters, especially to elevated light regime (Table 1). Cyanobacterial cells respond to changes in the light conditions by altering the light absorption properties of the two photosystems (28–30). One important way to adapt to changes in light is to change coupling of the soluble light harvesting proteins (phycobilisomes) to the two photosystems (31, 32). This process is regulated by the redox state of the PQ pool and called state transition. To investigate whether disruption of *ssr2998* has an impact on the ability of the cells to react on changing light conditions, capability for performing state transition was tested either by illumination and/or chemical oxidation of the PQ pool. Fluorescence emission of whole cells was measured at 77 K after excitation of phycobilisomes at 580 nm. During transition from State 2 to State 1 PS 1 fluorescence emission at 725 nm should decrease, whereas PS 2 emission at 695 nm should increase (33, 34).

Fig. 4 shows fluorescence emission spectra, which were measured after preincubating cells in dark (State 2) and in far red light (State 1), respectively. In wt cells (Fig. 4*A*), the PS 1 emission at 725 nm decreases, whereas the PS 2 emission at 695

nm increases upon excitation of the phycobilisomes. These changes are caused by an increased binding of phycobilisomes to PS 2, and a decreased coupling to PS 1, which results in increased energy transfer to the PS 2 reaction center and decreased energy transfer to PS 1, respectively. In contrast, Δ*ssr2998* cells showed no changes of the fluorescence emission at 695 and 725 nm after exposure of the cell cultures to the different light conditions (Fig. 4*B*). This observation suggested that the light quality does not influence binding of phycobilisomes to PS 2 and/or PS 1 in the mutant strain. This could have been caused either by a misregulation of phycobilisome movement or by a shift in the redox state of the PQ pool. Contrary to higher plants, in cyanobacteria the PQ pool is partially reduced in darkness (35) because of light-independent alternative electron transport routes (36). This reduction level is usually decreased by light because of the high PS 1:PS 2 ratio and the resulted high oxidative force of PS 1 (35). Over-reduction of the PQ pool or a diminished capacity at its oxidation could be explained by the absence of the state transition effect in the mutant after light induction. To test this possibility, we have investigated the energy transfer from phycobili-

somes to PS 1 and PS 2 in the Δ*ssr2998* strain in the presence of PPBQ, which decreases the reduction level of the PQ pool and thereby induces State 1 chemically. As shown in Fig. 5 both wt (Fig. 5*A*) and the Δ*ssr2998* mutant (Fig. 5*B*) performed a fluorescence decrease at 725 nm and an increase at 695 nm in the presence of PPBQ. The effect was more pronounced when PS 1 was selectively excited by far red light illumination. All of these data indicate that increased energy transfer from phycobilisomes to PS 2 involving binding and/or movement of phycobilisomes is not inhibited in the mutant. In other words, the absence of state transition under normal conditions can be explained by the existence of an over-reduced PQ pool in the mutant.

The PQ Pool Is Over-reduced in the Δssr2998 Mutant Because of the Impaired Electron Transport through the Cytochrome b₆f Complex—Disruption of *ssr2998* resulted in decreased content of active PS 2 and absence of state transition. Presumably, both phenomena were consequences of an over-reduced plastoquinone pool. The activity of PS 1 was, however, not impaired. These observations could either been explained by an involvement of Ssr2998 in biogenesis and/or stabilization of PS 2, or
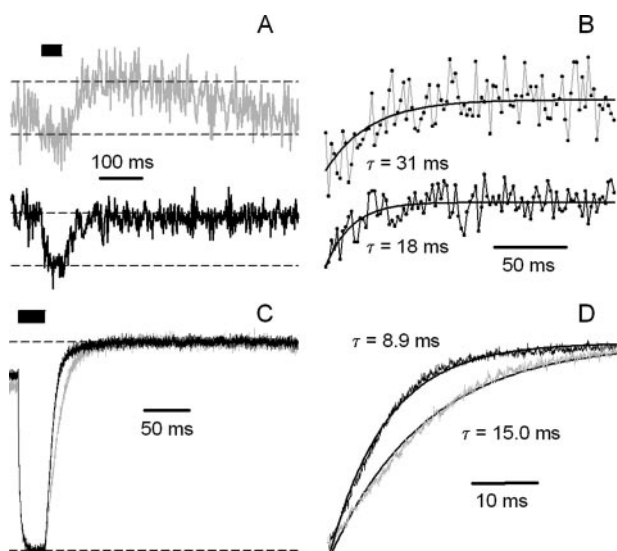
FIGURE 6. **Reduction kinetics of cytochrome f (A and B) and P$_{700}$ (C and D) after a saturating flash.** The *black rectangles* above the curves show the duration of the flash. The *lower* and *upper dashed lines* in *A* and *C* indicate the completely oxidized and reduced components, respectively. *B* and *D* show the results of the fit of the experimental curves. For details see "Experimental Procedures."

FIGURE 7. **SDS-gel of the cytochrome b$_6$f complex at various stages of its purification.** Protein standard (*lane 1*), membrane extract (*lane 2*), cytochrome b$_6$f complex after first column (hydrophobic interaction chromatography, *lane 3*) and second column (ion exchange chromatography, *lane 4*). The cytochrome b$_6$f complex was purified as described in Wenk *et al.* (24).

more likely, the protein acts in between PS 2 and PS 1. Electrons provided by PS 2 are transferred to PS 1 via the cytochrome b$_6$f complex and plastocyanine (or cytochrome c$_6$). It has recently been shown that mutations within the cytochrome b$_6$f complex can result in an highly reduced PQ pool (23, 37).

Direct kinetic measurements on cytochrome *f* and the primary PS 1 electron donor P$_{700}$ were performed to investigate the electron transport through the cytochrome b$_6$f complex. As shown in Fig. 6 (*A* and *B*), the reduction kinetics of cytochrome *f* after a saturating flash was significantly (1.7 times) slower for the mutant cells. The calculated time constants (τ) were 18 and 31 ms in the wt and mutant strain, respectively. Also, there was a considerable difference in the steady-state level of the oxidized cytochrome *f* population induced by continuous far red illumination (Fig. 6*A*). In contrast to wt cells, where apparently all of the cytochrome *f* was reduced before the flash, about 50% of the cytochrome was reduced in the mutant (Fig. 6*A*). This indicated that the far red background light induced a significant preoxidation of the high potential chain in the mutant, which could be explained by a slower electron flow to cytochrome *f* from the PQ pool. Both effects showed a diminished capability in reduction of cytochrome *f*. These data are in good agreement with the P$_{700}$ kinetics parameters obtained by identical experimental set-ups; the reduction kinetics in the Δssr2998 mutant was about 1.7 times slower than in the wt. In this case, the corresponding time constants were 8.9 and 15.0 ms, respectively (Fig. 6*B*). The fact that the P$_{700}$ reduction was faster than that of cytochrome *f* is due to the redox potential difference of the P$_{700+}$/P$_{700}$ and cytochrome *f$^+$*/cytochrome *f* couples (P$_{700}$ reduced first).

Ssr2998 Can Be Co-purified with the Cytochrome b$_6$f Complex—The impaired electron transport through the cytochrome b$_6$f complex in the absence of Ssr2998 suggested that Ssr2998 is an integral part or an interaction partner of the cyto-
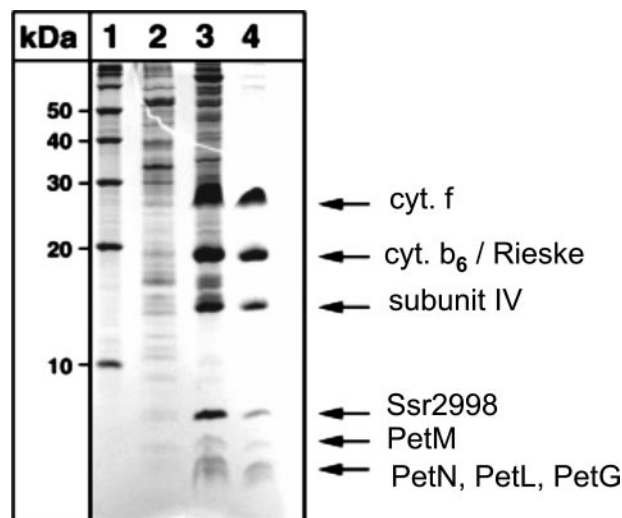
chrome b$_6$f complex. To test whether Ssr2998 can bind to the cytochrome b$_6$f complex, we have isolated this complex from *Synechocystis* membranes. After isolation and purification of the cytochrome b$_6$f complex, all known subunits of this complex were identified by immunological analysis as well as by N-terminal sequencing. Besides the known subunits, an additional protein of 7.2 kDa was co-isolated with the complex (Fig. 7). N-terminal sequencing of this protein yielded the first 9 amino acids (TIEIGQKVK), which could be attributed to the N terminus of Ssr2998 after post-translational removal of the N-terminal methionine residue. Because the isolated cytochrome b$_6$f complex from *Synechocystis* was highly pure and virtually no protein contamination was present, the observation that the protein was co-isolated with the complex suggests that the protein is functionally associated with the cytochrome b$_6$f complex *in vivo*.

To test whether disruption of *ssr2998* resulted in a decreased content of the cytochrome b$_6$f complex in the mutant strain, Western analyses were performed using whole cells of *Synechocystis* wt and Δ*ssr2998*, and the amount of the four cytochrome b$_6$f complex core subunits was determined. As can be seen in Fig. 8, the absence of Ssr2998 does not cause a decrease in the content of cytochrome *f*, cytochrome b$_6$, subunit IV, or the major Rieske iron-sulfur protein (PetC1). In conclusion, these observations indicated that Ssr2998 is bound to the cytochrome b$_6$f complex but is not directly involved in assembly or stabilization of the complex.

DISCUSSION

Ssr2998 Is Associated with the Cytochrome b$_6$f Complex—In the present study we investigated the function of a hypothetical protein encoded by the ORF *ssr2998* in *Synechocystis* sp. PCC 6803. The ORF *ssr2998* encodes a soluble 7.2-kDa protein, and no structural domains or motives have been detected by computational methods. Nevertheless, the protein is highly conserved in cyanobacteria (Fig. 1), and it is therefore reasonable to
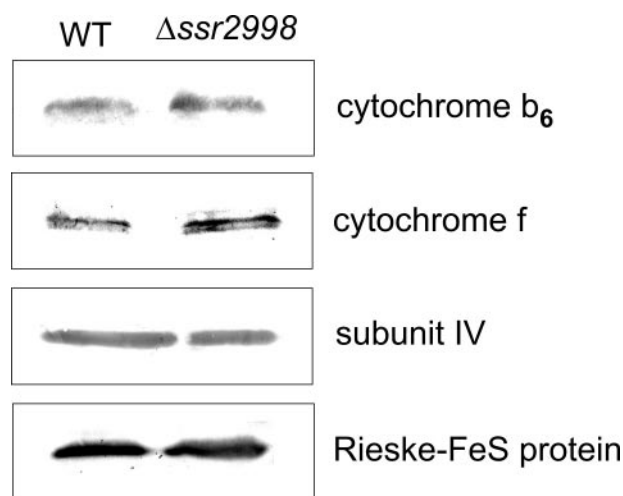
Δssr2998 Affects Cytochrome b₆f Activity

FIGURE 8. **Western blot analysis of cytochrome b_6f complex core proteins revealed a similar level of these proteins in wt and Δ*ssr2998* *Synechocystis* cells.**

conclude that it has a physiological function. The data presented in this study indicate that Ssr2998 is associated with the cytochrome b_6f complex, and disruption of *ssr2998* impairs cytochrome b_6f complex function but not its assembly.

Besides the four core subunits, *i.e.* PetA–D, the cytochrome b_6f complex of the cyanobacterium *Synechocystis* sp. PCC 6803 contains the four small subunits PetG, PetL, PetM, and PetN, and, as shown in this contribution, a 7.2-kDa protein is also associated with the complex. Because no other proteins were co-purified with the complex, the ORF *ssr2998* most likely encodes a protein that is specifically associated with the complex, rather than a purification artifact. This conclusion is further supported by the observed physiological consequences after *ssr2998* disruption.

Why this protein has never been reported to be associated with the cytochrome b_6f complex so far cannot simply be answered. Possibly, the protein is only loosely associated with the cytochrome b_6f complex and easily lost in the course of purification unless this is done with very soft methods. This assumption is supported by the fact that Ssr2998 is predicted to be soluble on the basis of its amino acid sequence. Several previously undetected proteins have been reported recently to be co-purified with other membrane proteins, especially if milder solubilization and purification procedures have been used. A prominent example is the PS 2 complex from *Synechocystis*, which contains more subunits when purified by an attached His tag via an affinity column (14).

Physiological Functions of Ssr2998—The function of Ssr2998 was elucidated by the construction of a *Synechocystis* Δ*ssr2998* disruption strain. Because cyanobacteria do not contain cytochrome bc₁ complexes, deletion of cytochrome b_6f complex subunits in *Synechocystis* sp. PCC 6803 is only possible if the function of the whole complex is not impaired, *i.e.* if the subunit is not essential for the overall function of the complex (2). This has previously been shown for the small subunit PetM as well as for Rieske iron-sulfur proteins (PetC1–3), the corresponding genes of which have been deleted in *Synechocystis* (23, 37, 38). Also the ORF *ssr2998* can be completely disrupted in *Synechocystis*, which suggests that the encoded protein is not essential

for the survival of *Synechocystis* under moderate culture conditions. Because a highly active dimeric cytochrome b_6f complex has been isolated from the thermophilic cyanobacterium *Mastigocladus laminosus* and this complex does not contain a Ssr2998 homologous protein (39), Ssr2998 is apparently not needed for structural and functional integrity of the cyanobacterial complex. However, this observation does not exclude a regulatory role of the protein, which can only be investigated *in vivo*, and the results obtained with the Δ*ssr2998* strain indeed indicate a physiological role of Ssr2998.

When compared with the wt, a decreased rate of photosynthetic electron transfer was observed as well as an equivalent decrease of PS 2 activity in thylakoid membranes. In contrast, the content of PS 1 remains almost unchanged within the range of error. Although the electron transfer reactions downstream of the cytochrome b_6f complex are not affected by the *ssr2998* disruption, effects on the amount and activity of PS 2 are evident. This dynamic in the stoichiometry of photosynthetic membrane proteins is in agreement with observations that the amount of PS 1 in *Synechocystis* sp. PCC 6803 thylakoid membranes remains stable, whereas the amount of PS 2 varies according to the change of external parameters (40).

The growth rate of the Δ*ssr2998* *Synechocystis* strain under higher light conditions was drastically decreased in comparison with the wt strain. More dramatically, when the mutant strain was grown in medium containing glucose, it was not viable anymore (Table 1). However, if PS 2 was blocked by the inhibitor DCMU, the Δ*ssr2998* strain survived under (mixotrophic) low light conditions. Therefore, reduction of the PQ pool by both PS 2 and dehydrogenases appeared to highly overload the PQ pool, which severely affected cell growth or even resulted in cell death. These observations further suggest that an impaired re-oxidation of PQ by the cytochrome b_6f complex is somehow impaired in the mutant strain. Based on the difference in the cytochrome f kinetics, the site of this impairment can be localized between the bound PQ and cytochrome f.

Dysfunction of the cytochrome b_6f complex causes a highly reduced PQ pool, which results in the observed decrease of functional PS 2 population. In addition, the highly reduced PQ pool also influences the energy transfer from phycobilisomes to the photosynthetic reaction centers. In contrast to wt cells, where phycobilisomes are mainly bound to PS 1 under State 2 conditions (induced by dark incubation) and to PS 2 under State 1 conditions (induced by far red light or an oxidized PQ pool) (31, 32, 41), movement of phycobilisomes has not been observed in the Δ*ssr2998* strain. Independently of the light quality, phycobilisomes remained tightly bound to PS 1. Because movement of phycobilisomes could be re-established by increasing the pool of oxidized quinones with addition of PPBQ in the dark, the observed difference is most likely indirectly caused by the highly reduced PQ pool, rather than directly by the absence of Ssr2998 in the mutant strain.

Functional Homologous in Higher Plants—Although a data base search did not reveal a Ssr2998 analogous protein encoded in genomes of higher plants or algae, the existence of a new cytochrome b_6f complex subunit has recently been reported for *Chlamydomonas reinhardtii*. PetO is a soluble protein of about 15 kDa that undergoes reversible phosphorylation during

state transitions (42), and it has been suggested that the protein is involved in regulation of state transitions. Because no homologs of Ssr2998 exist in chloroplasts, the protein could have a function similar to PetO in green algae and higher plants. Two different proteins may have developed because the cyanobacterial protein is involved in regulation of phycobilisome movement, whereas its counterpart regulates intrinsic light-harvesting complexes. The results reported here might stimulate further experiments to find out whether these two subunits have equivalent functions in regulating photosynthetic electron transport.

In conclusion, we could show that the gene product of the ORF *ssr2998* from the cyanobacterium *Synechocystis* sp. PCC 6803 has an important but not essential function. Disruption of this gene effected electron transfer through the cytochrome b_6f complex. Although the amount of cytochrome b_6f complexes was not altered in the mutant strain, electron transfer through the complex appeared to be affected. As a result, the reduction rate of cytochrome f and P_{700} was decreased, whereas the PQ pool was highly reduced, which further caused a decrease of functional PS 2 complexes. Furthermore, a strong impact on movement of the phycobilisomes has been observed. The observation that no homologous proteins can be found in green algae and higher plants suggests a specific function for cyanobacteria. Future studies have to prove whether Ssr2998 is a *bona fide* cytochrome b_6f subunit that can be named PetP.

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