Native isolation of the CcsB protein from *Synechocystis* sp. PCC 6803 involved in cytochrome *f* maturation in cyanobacteria and plastids

M. TICHÝ

Laboratory of Photosynthesis, Institute of Microbiology, Academy of Sciences, 379 81 Třeboň, Czech Republic and Institute of Physical Biology, University of South Bohemia, 373 33 Nové Hrady, Czech Republic

Abstract

The last step for biosynthesis of *c* type cytochromes, indispensable for photosynthesis in cyanobacteria and plants, involves heme transport across the membrane and its covalent attachment to the apoprotein. In cyanobacteria, heme attachment occurs in the thylakoid lumen and probably also in the periplasm and requires at least four proteins, believed to be organized in intrinsic membrane protein complex. To allow isolation and identification of such complex, CcsB protein was tagged with 6xHis tag on its N terminus and expressed under the strong *psbAII* promoter in the cyanobacterium *Synechocystis* sp. PCC 6803. Similarly, CcsA protein was tagged with FLAG tag under the control of the same promoter. Although expression of both proteins under strong cyanobacterial promoter did not increase steady state contents of the CcsB protein, the fusion tags did not influence properties of the CcsB and CcsA proteins and the resulting mutants had the same phenotype as the wild type. Protein fraction containing CcsBHis protein was partially isolated from the solubilised membranes under native conditions.

Additional key words: doubling time; mutants; photosystem 2; polyacrylamide gel electrophoresis; thylakoids; whole chain electron transfer.

Introduction

C-type cytochromes are indispensable components of photosynthetic electron transport chains in cyanobacteria and chloroplasts. They are distinguished from other cytochromes by the covalent bond of the heme group to the conserved CXXCH motif of the apoprotein. This bond is formed in a process called cytochrome (cyt) *c* maturation (Kranz *et al.* 1998, Page *et al.* 1998). *C*-type cytochromes are functioning in the thylakoid lumen of cyanobacteria and chloroplasts, in the periplasm of bacteria, and in the intermembrane space of mitochondria, always across the membrane from the site of heme and protein biosynthesis. Therefore, cyt *c* maturation is a complex process involving heme transport, apocytochrome processing, and reduction and heme attachment to the apoprotein. Interestingly, genetic analyses revealed that three distinct systems of cyt *c* maturation evolved among various organisms (reviewed in Kranz *et al.* 1998, Page *et al.* 1998, Xie and Merchant 1998, Thöny-Meyer 2002).

In yeast and animal mitochondria (maturation system III, Kranz *et al.* 1998) only single type of protein, called cyt *c* lyase, for soluble cyt *c* (Dumont *et al.* 1987) and cyt c_1 lyase for membrane bound cyt c_1 (Zollner *et al.* 1992) is required for the heme attachment.

In system I represented by Gram-negative bacteria about ten genes, whose products are involved in assembly of both soluble and membrane-associated *c*-type cytochromes, were identified (reviewed in Fabianek *et al.* 2000, Thöny-Meyer 2002). In *Escherichia coli* eight genes required for cyt *c* maturation are organised in one operon (Thöny-Meyer *et al.* 1995) and their protein products are believed to be organised in intrinsic membrane protein complex, in which the different steps of the maturation pathway could be easily co-ordinated (Thöny-Meyer 2002).

Functionally similar maturation complex is expected to be present in system II found in cyanobacteria, chloroplasts, and some bacteria (Tichy and Vermaas 1999, Hamel *et al.* 2003), although sequence similarity between systems II and III is limited to one tryptophan-rich motif and thioredoxin motif. Also in system II such complex

will be also involved in heme transport, apocytochrome binding and reduction, and heme insertion. System II was discovered in the green alga *Chlamydomonas reinhardtii* by screening for non-photosynthetic mutants deficient in cyt b_6/f (Howe and Merchant 1992). Genetic analysis identified that at least four nuclear loci and one plastid locus are required for cyt c maturation in this organism (Xie *et al.* 1998). Two of these loci were identified (Xie and Merchant 1996, Inoue *et al.* 1997). Plastid *ccsA* (cytochrome *c* synthesis) gene codes for membrane protein with the tryptophan-rich motif. The second protein is CcsB (Ccs1 in *Chlamydomonas*). CcsB is also intrinsic membrane protein with no sequence homology to proteins with known function (Dreyfuss *et al.* 2003). Recently, we suggested that CcsB may be an apocytochrome chaperone, as the N terminal Δ(M1-A24) CcsB mutant accumulated high amounts of the unprocessed apocytochrome *f* in the membrane (Tichy and Vermaas 1999). The CcsA and CcsB proteins were suggested to function together in a complex, as inactivation of either of the two genes led to deficiency in both proteins (Kranz

et al. 1998, Xie *et al.* 1998, Hamel *et al.* 2003). Moreover, in *Helicobacter pylori*, CcsA and CcsB homologues appear to be encoded by a single open reading frame (Goldman and Kranz 1998). Also in *Chlamydomonas*, CcsB homologue Ccs1 was shown by native electrophoresis to be a part of a ~200 kDa membrane complex (Hamel *et al.* 2003).

Another two components of the cyt *c* maturation machinery in system II were identified in *Bacillus subtilis*, *Bordetella pertussis,* and *Arabidopsis thaliana* (Schiött *et al.* 1997, Beckett *et al.* 2000, Lennartz *et al.* 2001). One is a membrane, thioredoxine-like protein ResA/HCF164; the other is CcdA. These two proteins may function in transmembrane thiol metabolism and reduction of apocytochrome before heme attachment.

As most of the data about proteins involved in cyt *c* maturation in system II are based on mutant screening and targeted gene inactivation, I tried different approach to isolate the putative cyt *c* maturation complex from the cyanobacterium *Synechocystis* 6803 and characterise individual proteins forming the complex.

Materials and methods

Growth: Wild type and mutants of *Synechocystis* sp. PCC 6803 were grown in liquid BG-11 medium (Rippka *et al.* 1979) at 30 °C at 40 μmol m^{-2} s^{-1} on a rotary shaker. Strains were grown with 5 mM glucose, unless photoautotrophic conditions are indicated. Solid media were supplemented with 1.5 % (m/v) agar, 0.3 % (m/v) sodium thiosulfate, and 10 mM TES-NaOH buffer, pH 8.2. For anaerobic growth, the strains were grown in a 1 % CO_2, 99 % N_2 atmosphere.

Mutant construction: *CcsBHis* strain was constructed expressing CcsB protein containing 6xHis protein tag on its N terminus, under the strong *psbAII* promoter. The *CcsB* gene was amplified by PCR using the mix of *Taq* and *Pfu* DNA polymerases (*Fermentas*) and gene specific primers with artificially generated restriction sites for *Nde*I and *Bam*HI and with the forward primer containing additional 6 codons for histidine. After restriction, the PCR fragments were cloned into *Nde*I and *Bam*HI sites of the pSBA2 plasmid (Lagarde *et al.* 2000) containing the upstream and downstream regions of the *Synechocystis* 6803 *psbAII* gene. Interestingly, after *E. coli* transformation I was unable to obtain any plasmid with *ccsB* gene properly inserted. This indicated that *psbAII* promoter is functional in *E. coli* and that the intrinsic membrane CcsB protein is toxic for *E. coli*. As an alternative, I amplified the whole *PsbAII* region containing the *ccsB* gene from the ligation mix by PCR and transformed the *psbAII-KS* strain, where the whole *psbAII* gene is replaced by kanamycin-resistance cartridge (*KmR*) and by *sacB* gene coding for levan sucrase yielding the *psbAII-KS* strain sucrose sensitive (Fig. 1*A*) (Lagarde *et al.* 2000). After transformation, the *Synechocystis* cells were

grown in BG-11 medium for 4 d. Transformants were then plated and selected for growth in the presence of 5 % (m/v) sucrose. Sucrose-resistant colonies were also checked for kanamycin sensitivity.

Segregation of the resulting *CcsBHis* strain was checked by PCR (not shown). Sequencing of the *psbAII* region of the *CcsBHis* strain confirmed that *ccsBHis* gene has been correctly inserted under the control of the *Synechocystis* 6803 *psbAII* promoter. Wild type copy of the *ccsB* gene in the *CcsBHis* strain was deleted using the Δ(M1-I244) construct (Tichy and Vermaas 1999) yielding the *CcsBHis/CcsB⁻* strain (Fig. 1*B*).

Identical procedure was used to generate *CcsAFLAG* strain, expressing CcsA protein containing FLAG protein tag (*Sigma*) on its N terminus, also under the *psbAII* promoter. Wild type copy of the *ccsA* gene in the *CcsAFLAG* strain was deleted using *ccsA* interruption construct with kanamycin resistance marker from the pUC4K at the *Bam* HI site at position 813 of the coding region of *ccsA* (Tichy and Vermaas 1999) yielding the *CcsAFLAG/CcsA⁻* strain.

Oxygen evolution was measured as described in Tichy and Vermaas (1998) using a Clark-type electrode in the presence of 1 mM $K_3Fe(CN)_6$ and 0.1 mM dimethyl-*p*-benzoquinone (DMBQ) for photosystem (PS) 2 activity, or 10 mM $NaHCO_3$ for whole chain electron transport.

Preparation of cell extracts and thylakoid membranes, and SDS-PAGE: Cell extracts and thylakoid membranes were isolated from exponentially grown cells (OD_{730} 0.6–0.8). Cell pellets were re-suspended in thylakoid buffer (1/100 of the original culture volume)

containing 50 mM MES/NaOH, pH 6.0, 5 mM MgCl$_2$, 5 mM CaCl$_2$, and 10 % glycerol (v/v). Cell suspensions (0.7 cm^3) were transferred to 2 cm^3 Eppendorf tubes, mixed with the same volume of glass beads (100-200 μm diameter), and kept on ice. The cells were broken in a *MiniBeadBeater* by 3 breaking cycles (30-s shaking followed by a 3-min cooling on ice). After centrifugation at 2 000×*g* for 1 min to remove unbroken cells and cell debris, the supernatant was centrifuged in the centrifuge at 30 000×*g* for 10 min at 4 °C. The thylakoids were resuspended in thylakoid buffer to chlorophyll concentration 250 g m^{-3} and dodecyl maltoside was added to final concentration 0.6 %. After 10-min incubation and another centrifugation the supernatant was used further. For protein fractionation by ammonium sulphate the dodecyl maltoside extract was precipitated by 1.1 M ammonium sulphate (from 3 M stock solution) incubated on ice for 10 min and centrifuged at 30 000×*g* for 10 min at 4 °C.

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) without urea. Using Tris-Tricine system, 10 % acrylamide gel was used for separation, 4 % acrylamide gel for stacking. Samples were solubilised in sample buffer at room temperature for 15 min before loading on the gel. 30–50 μg protein was loaded per lane. To estimate the protein concentration, the cell extract or thylakoid sample (5 mm^3) was incubated in 2 % SDS at 37 °C for 15 min. The total protein content was measured using Bradford protein assay reagents with bovine serum albumine as a standard. Relative yield of the CcsB protein was estimated from Western blots of serial dilutions of samples before and after ammonium sulfate fractionation.

Western blotting: After electrophoresis, proteins were transferred to *PVDF* membrane (*Hybond-P, Amersham Biosciences*) using wet blotting system. Blotted membranes were subjected to immunoblot analysis using the CcsB antibody (1 : 10 000 dilution) or *polyHistine* antibody (*Sigma H 1029*, 1 : 3 000 dilution). Primary antibody was detected with alkaline phosphatase-conjugated secondary antibody. CcsB antibody was a gift from Andreas Seidler (Bochum University, Germany).

Results

Generation of the *CcsBHis and CcsAFLAG* strains: *CcsBHis* strain was constructed to express CcsB protein containing 6xHis protein tag on its N terminus, under the *psbAII* promoter. Sequencing of the *psbAII* region confirmed proper insertion of the *ccsB* gene and in frame position of the 6xHis tag. Functionality of the CcsBHis protein in cyt *c* maturation was confirmed by successful deletion of the wild type copy of the *ccsB* gene using the Δ(M1-I244) construct yielding *CcsBHis/CcsB$^-$* strain

(Fig. 1*B*) (Tichy and Vermaas 1999). Expression of the CcsBHis protein was confirmed by detection by immunobloting using polyHistidine antibody (Fig. 2). PolyHistidine antibody specifically recognised a 50 kDa protein band in the region expected for the CcsB protein (458 amino acid residues). This band was assigned to CcsBHis protein as it was missing in the wild type (not shown) and as the same band was labelled by CcsB antibody (Fig. 2). Both antibodies (Fig. 2) also detected
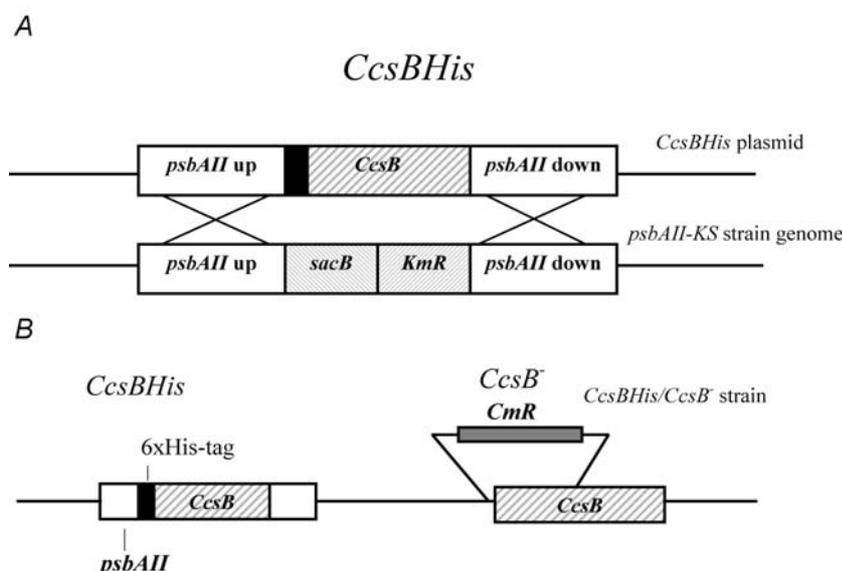

Fig. 1. Construction of the *CcsBHis/CcsB$^-$* strain. (*A*) Construction of the *CcsBHis* strain by replacement of the *sacB* gene, encoding a levan sucrase, and *KmR* gene conferring kanamycin resistance. (*B*) Inactivation of the wild type copy of the *CcsB* gene in the *CcsBHis* strain by chloramphenicol resistance marker.

Table 1. Growth rates and photosynthetic electron transport in wild type and protein fusion tag mutants. Means of 3 experiments, reproducible within 15 % of the average rate.

Strain	Oxygen evolution [μmol(O_2) kg^{-1}(Chl) s^{-1}]		Doubling time [h]
	PS2 ($H_2O \rightarrow$ DMBQ)	Whole chain ($H_2O \rightarrow CO_2$)	
Wild type	139	131	13
CcsBHis/CcsB$^-$	125	119	14
CcsAFLAG/CcsA$^-$	117	119	15

CcsBHis protein expressed in *E. coli* with slightly higher molecular mass. CcsB antibody was used in further experiments because it was about 5 times more sensitive than polyHistidine antibody.
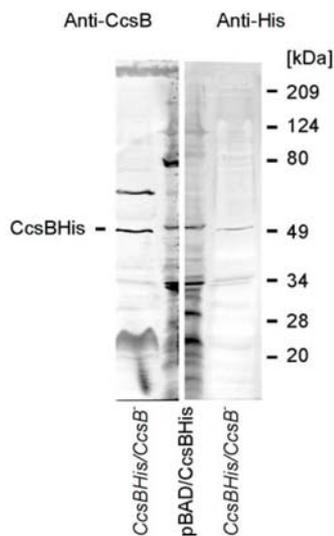

Fig. 2. Detection of the CcsBHis protein using CcsB antibody (*left*) and polyHistidine antibody (*right*). CcsBHis protein expressed in *E. coli* (pBAD/CcsBHis plasmid) (*middle*) was detected using both antibodies. Note slightly slower mobility of the *E. coli* protein caused by N-terminal extension. Enriched protein fraction from the *CcsBHis/CcsB*$^-$ strain precipitated by 1.1 M ammonium sulfate was used for electrophoresis as no CcsBHis band was detected in crude dodecyl maltoside extract by polyHistidine antibody. 30 μg of protein per lane was loaded on a polyacrylamide gel.
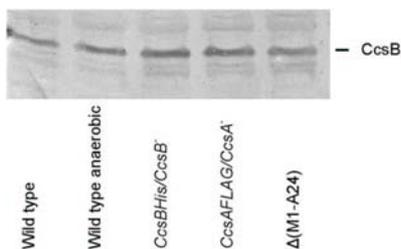

Fig. 3. Accumulation of CcsB protein in various mutants. Dodecyl maltoside fraction of thylakoid membranes was tested for accumulation of CcsB protein by immunoblot analysis using CcsB antibody. 50 μg of protein per lane was loaded on a polyacrylamide gel. Δ(M1–A24) mutant was grown anaerobically, wild type both aerobically and anaerobically.

Similarly, *CcsAFLAG* strain was generated, expressing CcsA protein containing FLAG protein tag on its N terminus, again under the *psbAII* promoter. Proper insertion of the FLAG tag and functionality of the CcsAFLAG protein was confirmed by sequencing and by easy inactivation of the wild type copy of the *ccsA* gene. ANTI-FLAG antibody was tested to detect the CcsAFLAG protein with no success. Apparently, sensitivity of this antibody is too low to detect contents of the CcsA protein present in *Synechocystis* cells.

Characterisation of tagged mutants: Both *CcsBHis/CcsB*$^-$ and *CcsAFLAG/CcsA*$^-$ strains exhibited normal photoautotrophic growth with oxygen evolution rates comparable to the wild type (Table 1). This indicates that cyt *c* maturation pathway was not significantly influenced by tagging of CcsB and CcsA proteins. Accumulation of CcsB protein in thylakoid membranes from several strains grown under aerobic or anaerobic conditions was almost constant, possibly only slightly increased *CcsBHis/CcsB*$^-$ and *CcsAFLAG/CcsA*$^-$ strains (Fig. 3). Even in the Δ(M1-A24) strain expressing truncated CcsB protein, leading to impaired apocyt *f* processing and heme attachment, the CcsB protein level was unchanged (Fig. 3).
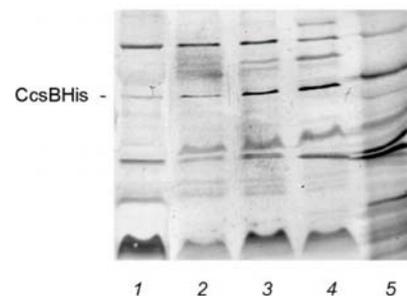

Fig. 4. Fractionation of dodecyl maltoside extract by ammonium sulphate and CcsBHis enrichment. Accumulation of CcsBHis protein in ammonium sulphate fractions was tested by CcsB antibody. *1*: Dodecyl maltoside extract of thylakoid membranes isolated from *CcsBHis/CcsB*$^-$ strain. *2*, *3*, and *4*: Protein fractions precipitated by 0.9 M, 1.1 M, and 1.3 M ammonium sulphate, respectively. *5*: pBAD/CcsBHis control plasmid. 30 μg of protein per lane was loaded on polyacrylamide gel (50 μg for lane *4*).

CcsB protein enrichment by ammonium sulfate fractionation: CssBHis detection using the polyHistidine antibody and CcsAFLAG detection using the ANTI-

FLAG antibody was not successful when dodecyl malto-side extract of membrane fraction was directly used for electrophoresis and blotting. Therefore I used fractio-nation by ammonium sulphate to enrich the extract for CcsB protein. CcsB protein precipitated at low ammo-nium sulphate concentrations, before precipitation of major complexes PS1 and PS2. Results of such precipi-tation are shown in Fig. 4. Increased concentration of ammonium sulphate increased total yield of the CcsBHis

Discussion

Isolation of the putative cyt *c* maturation complex: Isolation of membrane complexes followed by isolation and identification of individual protein sub-units is fre-quently used to determine protein composition of such complexes. Recently, the protein tagging technique, that is a common approach for purification of over-expressed proteins by affinity chromatography usually in *E. coli*, has been extended for isolation of protein complexes. In photosynthesis research most of the work was devoted to construction and purification of His-tagged PS2 com-plexes (*e.g.* Sugiura and Inoue 1999). In these reports, the His-tag was engineered into one of the polypeptides in the native host and such modification thereby permits isolation of the multi-protein complex. Using this ap-proach, several new sub-units of PS2 were identified (Kashino *et al.* 2002). I used the same approach to isolate the putative cyt *c* maturation complex with the help of His and FLAG tagged proteins. Existence of such com-plex is highly probable as CcsB homologue was shown by native electrophoresis to be a part of a ~200 kDa membrane complex (Hamel *et al.* 2003). It is expected that isolation of the cyt *c* maturation complex will be more complicated than in case of PS2 as a much lower abundance of maturation complex is expected.

Both CcsBHis and CcsAFLAG proteins were expres-sed in *Synechocystis* 6803 under the strong cyanobacterial *psbAII* promoter in attempt to increase CcsA and CcsB accumulation in membranes. Accumulation of the CcsB protein was only slightly increased in *CcsBHis/CcsB⁻* and *CcsAFLAG/CcsA⁻* strains indicating that CcsB accumu-lation is controlled by other factors than by transcript levels. On the other hand, successful deletion of the wild type copy of the *CcsB* gene in the *CcsBHis/CcsB⁻* back-ground and deletion of the *CcsA* gene *CcsAFLAG/CcsA⁻* background demonstrated that CcsBHis and CcsAFLAG are functioning in cyt *c* maturation as both deletions are lethal in wild type background (Tichy and Vermaas 1999). Moreover, both *CcsBHis/CcsB⁻* and *CcsAFLAG/CcsA⁻* strains exhibited normal photoautotrophic growth with oxygen evolution rates comparable to the wild type, indicating that cyt *c* maturation pathway was not signifi-cantly influenced by tagging of CcsB and CcsA proteins.

As expected, accumulation of the CcsB protein and probably also contents of the CcsA protein were very low and partial purification was necessary to detect CcsBHis

protein, but also increased amount of contaminating pro-teins (Fig. 4, line *4*). Concentration of 1.1 M ammonium sulphate was chosen for further isolations. With this con-centration approximately 20 % of the CcsBHis protein but only 4 % of the total protein was precipitated result-ing in enrichment factor of five. In this fraction CcsBHis protein was easily detectable by polyHistine antibody (Fig. 2).

by polyHistidine antibody. Protein fractionation by am-monium sulphate was suitable for this purpose, as CcsB protein precipitated at low ammonium sulphate concen-trations with most other proteins remaining still in the supernatant. The precipitated fraction was 5 times en-riched in the CcsBHis protein. This fraction will be fur-ther used for native isolation of the maturation complex by Ni-affinity chromatography followed by identification of individual proteins by 2D electrophoresis.

CcsB protein as an apocyt *f* chaperone: Another reason why I followed CcsB accumulation in several mutants was our hypothesis that CcsB protein may serve as apo-cytochrome chaperone. This hypothesis was based on the fact that N-terminally truncated Δ(M1-A24) *CcsB* strain accumulated large amounts of pre-apocyt *f* in thylakoid membrane (Tichy and Vermaas 1999). This unusual stability of pre-apocyt *f* in Δ(M1-A24) mutant was ex-plained by possible protection of the apoprotein from processing, heme attachment, as well as degradation by the altered CcsB protein. This would mean that pre-apo-cyt *f* accumulating in the Δ(M1-A24) mutant is associated with CcsB and that CcsB will accumulate to high levels in this mutant. I did not observe increased CcsB accu-mulation in the Δ(M1-A24) mutant (Fig. 3). This indi-cated that the accumulation of pre-apocyt *f* in thylakoid membrane was probably caused by increased pre-apocyt *f* expression in response to shortage of holocyt *f*. Although our hypothesis was wrong, this fact does not exclude that CcsB serves as cyt *f* chaperone.

Δ(M1-A24) mutant also needed anaerobic conditions to grow (Tichy and Vermaas 1999). It was suggested that oxygen sensitivity of this mutant is caused by the fact that truncated CcsB is unable to protect apocytocrome or heme against oxidation under aerobic conditions as a potential functional analog of CcsB in system I accumu-lated to 20-fold-higher levels upon transfer from anaero-bic to aerobic conditions (Gabbert *et al.* 1997). Appar-ently, this was not the case of the CssB protein as its accumulation was the same for the wild type cells grown aerobically or anaerobically and for Δ(M1-A24) mutant grown anaerobically (Fig. 3).

Finally, I believe that the use of tagged proteins to iso-late the putative cyt *c* maturation complex is a promising approach to identify its individual protein components.

587

References

Beckett, C.S., Loughman, J.A., Karberg, K.A., Donato, G.M., Goldman, W.E., Kranz, R.G.: Four genes are required for the system II cytochrome *c* biogenesis pathway in *Bordetella pertussis*, a unique bacterial model. – Mol. Microbiol. **38**: 465-481, 2000.

Dreyfuss, B.W., Hamel, P.P., Nakamoto, S.S., Merchant, S.: Functional analysis of a divergent system II protein, Ccs1, involved in *c*-type cytochrome biogenesis. – J. biol. Chem. **278**: 2604-2613, 2003.

Dumont, M.E., Ernst, J.F., Hampsey, D.M., Sherman, F.: Identification and sequence of the gene encoding cytochrome *c* heme lyase in the yeast *Saccharomyces cerevisiae*. – EMBO J. **6**: 235-241, 1987.

Fabianek, R.A., Hennecke, H., Thony-Meyer, L.: Periplasmic protein thiol:disulfide oxidoreductases of *Escherichia coli*. – FEMS Microbiol. Rev. **24**: 303-316, 2000.

Gabbert, K.K., Goldman, B.S., Kranz, R.G.: Differential levels of specific cytochrome *c* biogenesis proteins in response to oxygen: Analysis of the *ccl* operon in *Rhodobacter capsulatus*. – J. Bacteriol. **179**: 5422-5428, 1997.

Goldman, B.S., Kranz, R.G.: Evolution and horizontal transfer of an entire biosynthetic pathway for cytochrome *c* biogenesis: *Helicobacter*, *Deinococcus*, *Archae* and more. – Mol. Microbiol. **27**: 871-874, 1998.

Hamel, P.P., Dreyfuss, B.W., Xie, Z., Gabilly, S.T., Merchant, S.: Essential histidine and tryptophan residues in CcsA, a system II polytopic cytochrome *c* biogenesis protein. – J. biol. Chem. **278**: 2593-2603, 2003.

Howe, G., Merchant, S.: The biosynthesis of membrane and soluble plastidic *c*-type cytochromes of *Chlamydomonas reinhardtii* is dependent on multiple common gene products. – EMBO J. **11**: 2789-2801, 1992.

Inoue, K., Dreyfuss, B.W., Kindle, K.L., Stern, D.B., Merchant, S., Sodeinde, O.A.: Ccs1, a nuclear gene required for the post-translational assembly of chloroplast *c*-type cytochromes. – J. biol. Chem. **272**: 31747-31754, 1997.

Kashino, Y., Lauber, W.M., Carroll, J.A., Wang, Q., Whitmarsh, J., Satoh, K., Pakrasi, H.B.: Proteomic analysis of a highly active photosystem II preparation from the cyanobacterium *Synechocystis* sp. PCC 6803 reveals the presence of novel polypeptides. – Biochemistry **41**: 8004-8012, 2002.

Kranz, R., Lill, R., Goldman, B., Bonnard, G., Merchant, S.: Molecular mechanisms of cytochrome *c* biogenesis: three distinct systems. – Mol. Microbiol. **29**: 383-396, 1998.

Lagarde, D., Beuf, L., Vermaas, W.: Increased production of zeaxanthin and other pigments by application of genetic engineering techniques to *Synechocystis* sp. strain PCC 6803. – Appl. environ. Microbiol. **66**: 64-72, 2000.

Lennartz, K., Plücken, H., Seidler, A., Westhoff, P., Bechtold, N., Meierhoff, K.: *HCF164* encodes a thioredoxin-like protein involved in the biogenesis of the cytochrome *b₆f* complex in *Arabidopsis*. – Plant Cell **13**: 2539-2551, 2001.

Page, M.D., Sambongi, Y., Ferguson, S.J.: Contrasting routes of *c*-type cytochrome assembly in mitochondria, chloroplasts and bacteria. – Trends biochem. Sci. **23**: 103-108, 1998.

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., Stanier, R.Y.: Generic assignments, strain histories and properties of pure cultures of cyanobacteria. – J. gen. Microbiol. **111**: 1-61, 1979.

Schiött, T., Thorne-Holst, M., Hederstedt, L.: *Bacillus subtilis* CcdA- defective mutants are blocked in a late step of cytochrome *c* biogenesis. – J. Bacteriol. **179**: 4523-4529, 1997.

Sugiura, M., Inoue, Y.: Highly purified thermo-stable oxygen-evolving photosystem II core complex from the thermophilic cyanobacterium *Synechococcus elongatus* having His-tagged CP43. – Plant Cell Physiol. **40**: 1219-1231, 1999

Thöny-Meyer, L.: Cytochrome *c* maturation: a complex pathway for a simple task? – Biochem. Soc. Trans. **30**: 633-638, 2002.

Thöny-Meyer, L., Fischer, F., Künzler, P., Ritz, D., Hennecke, H.: *Escherichia coli* genes required for cytochrome *c* maturation. – J. Bacteriol. **177**: 4321-4326, 1995.

Tichy, M., Vermaas, W.: Functional analysis of combinatorial mutants altered in a conserved region in loop E of the CP47 protein in *Synechocystis* sp. PCC 6803. – Biochemistry **37**: 1523-1531, 1999.

Xie, Z., Merchant, S.: The plastid-encoded *ccsA* gene is required for heme attachment to chloroplast *c*-type cytochromes. – J. biol. Chem. **271**: 4632-4639, 1996.

Xie, Z., Merchant, S.: A novel pathway for cytochromes *c* biogenesis in chloroplasts. – Biochim. biophys. Acta **1365**: 309-318, 1998.

Xie, Z., Culler, D., Dreyfuss, B.W., Kuras, R., Wollman, F.-A., Girard-Bascou, J., Merchant, S.: Genetic analysis of chloroplast *c*-type cytochrome assembly in *Chlamydomonas reinhardtii*: One chloroplast locus and at least four nuclear loci are required for heme attachment. – Genetics **148**: 681-692, 1998.

Zollner, A., Rodel, G., Haid, A.: Molecular cloning and characterization of the *Saccharomyces cerevisiae cyt2* gene encoding cytochrome c_1 heme lyase. – Eur. J. Biochem. **207**: 1093-1100, 1992.