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Respiratorische Terminale Oxidasen im Cyanobakterium *Synechococcus* sp. Stamm PCC7942

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Respiratory Terminal Oxidases of the Cyanobacterium *Synechococcus* sp. Strain PCC7942

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Chapter 1

Introduction

1.1 Cyanobacteria

Cyanobacteria are the most important living beings in the history of life on this planet. They are responsible for the origin of the oxygenic atmosphere and by that for the first snowball earth event 2.3 - 2.2 Ga ago [1]. By releasing dioxygen, cyanobacteria probably were responsible for the extinction of many precambrian prokaryotes for which oxygen was toxic [2]. They are the only prokaryotes possessing both PSI and PSII, like higher plants. According to the endosymbiotic theory ancient cyanobacteria are thought to be the progenitors of chloroplasts [3] and facilitated the evolution of higher plants. By generating dioxygen, cyanobacteria released a molecule with a high redox potential and gave living cells the possibility to obtain energy through aerobic respiration - the main energy generating process for all animals and fungi. According to the conversion hypothesis [4], aerobic respiration developed in photosynthetic oxygenic bacteria (cyanobacteria) and every kind of respiring bacterium descended from these bacteria. All in all life on earth would not nearly be the way it is today without cyanobacteria.

By definition, cyanobacteria are oxygenic photosynthetic prokaryotes possessing two photosystems (PSI and PSII) and using H_2O as reductant in photosynthesis [5].

Cyanobacterium *UCYN-A* is the only exception of this definition found until now. It lacks photosystem II and therefore does not generate O_2 [6].

All known cyanobacteria are able to live photoautotrophically, in addition some facultative photo- or chemo-heterotrophic cyanobacteria exist.

Cyanobacteria are a monophyletic group comprising all known oxygenic photosynthetic prokaryotes. A few cyanobacteria contain no intracellular membrane (*Gloeobacter*). Most cyanobacteria contain an intracellular membrane that contains PSI and PSII. These cyanobacteria are subdivided into 5 sections [5, 7] based on morphological traits. Only section IV and section V which are comprised of heterocyst forming cyanobacteria are a monophyletic group together.

- Section I: Unicellular cyanobacteria that reproduce by equal binary fission or by budding. Examples are *Synechocystis* PCC6803 or *Synechococcus* PCC7942.
- Section II: Unicellular cyanobacteria that reproduce either exclusively, or at some stages of their life cycle by the formation of small, spherical cells, called baeocytes, through multiple fission. An example is *Pleurocapsa* PCC7319.
- Section III: Filamentous cyanobacteria reproducing by binary fission in one plane and producing only vegetative cells. Examples are *Arthrospira* (*ex Spirulina*) PCC7345 or *Oscillatoria* PCC7515.
- Section IV: Filamentous cyanobacteria reproducing by binary fission in one plane and capable of cell differentiation (heterocysts (all), akinetes or hormogonia (some)). Examples are *Nostoc* (*ex Anabaena*) PCC7120 or *Anabaena* PCC7122.
- Section V: Filamentous cyanobacteria capable of cell differentiation and dividing in multiple planes resulting in true branching. Examples are *Fischerella* PCC7115 or *Chlorogloeopsis* PCC6912.

1.2 Cyanobacterial Genetics

The genome sizes of cyanobacteria with available total sequences vary between 1.641.879 bp (*Prochlorococcus marinus* MIT 9301, 1949 ORFs, no plasmids) and 9.059.191 bp (*Nostoc punctiforme* ATCC 29133, 6794 ORFs, 5 plasmids, chromosome size 8.234.322 bp with 6191 ORFs). The genome size of *Synechococcus* sp. PCC7942 (hereafter PCC7942) is 2.742.269 bp with 2715 ORFs. PCC7942 possesses one multicopy chromosome with a size of 2.695.903 bp with 2665 ORFs [8] and two plasmids, pANL (also called pUH25) with a size of 46.366 bp and 50 ORFs [9] and pANS (also called pUH24) with 7835 bp and 36 ORFs [10].

Many cyanobacteria are amenable to genetic manipulation. Effective methods used are transformation (see below), conjugation [11] and electroporation [12]. During this work transformation was used for mutating cyanobacteria.

The first cyanobacterium that was shown to be naturally competent for transformation was *Synechococcus* sp. PCC7943 (formerly known as *Anacystis nidulans* 602) in 1970 [13]. Some years later also PCC7942 (formerly known as *Anacystis nidulans* R2) [14], PCC6803 and other cyanobacteria were shown to be transformable [15]. Experiments characterizing the transformation process in PCC7942 in detail were made in the 1980´s [16, 17, 18].

At least some cyanobacteria possess multiple copies of their chromosome. For *Anabaena cylindrica* polyploidy was already estimated in the 70´s [19]. R. Simon calculated with the DNA mass per vegetative cell and the measured molecular weight of the chromosome that *Anabaena cylindrica* contains about 25 chromosome copies during the late logarithmic growth phase. Later, copy numbers for PCC6803 were published [20, 21]. Chromosome copy numbers in PCC7942 were determined in 1996 by Mori using flow cytometry [22] and in 2011 by Griese using real-time PCR [21]. These works estimate PCC7942 to have about 3-5 chromosome copies per cell. The chromosome copy number was also determined for some other strains and until now far more strains seem to be oligoploid or polyploid than monoploid [21].

Oligoploidy or polyploidy affect the genetic manipulation procedures with cyanobacteria. The successful insertion of a DNA fragment into the cyanobacterial chromosome does not imply that every chromosome is mutated and the cell is homozygous for this mutation. Hence, it is necessary to check the cells for homozygosity as described in 3.10.2, page 49. In transformation experiments with cyanobacteria it was shown, that essentially only one DNA molecule is taken up by one transformed cell (Schmetterer, unpublished). Before cell division the chromosomes are replicated and segregate to the daughter cells in a still unknown way. As daughter cells without mutated alleles will die on selective media, mutated alleles will accumulate after some cell cycles. If the mutated gene is not essential, it is likely to obtain homozygous mutants after some time.

1.3 Cyanobacterial Bioenergetics

Cyanobacteria possess 3 different membranes, the outer membrane, the cytoplasmic or cell membrane (hereafter CM) and the intracellular or thylakoid membrane(s) (hereafter ICM). *Gloeobacter* sp. is the only known exception, possessing no ICM. The CM and the ICM are the bioenergetically active membranes: The ICM harbours the two photosystems (exception *UCYN-A* with only PSI) and one or more respiratory e^--transport chains. In general, the CM contains only one or more respiratory e^--transport chains but no photosystem. In addition to generating energy through oxygenic photosynthesis cyanobacteria respire in the dark. Endogenous respiration uses glycogen accumulated during photosynthesis. Some cyanobacteria are able to respire using exogenous substrates like glucose, sucrose or fructose. Some cyanobacteria are capable of anoxygenic photosynthesis, using sulfide as e^- donor for the reduction of CO_2 [5]. Also less important energy sources exist, like oxidation of H_2 (often produced by cyanobacteria themselves while fixing nitrogen) by bidirectional hydrogenases.

1.3.1 Photosynthesis and Respiration

Photosynthesis and respiration are the main energy generating pathways of cyanobacteria. Electrons are transferred from one enzyme to the next enzyme with a higher redox potential in the chain. In some of these enzymes protons are pumped across the membrane to generate a chemiosmotic potential. In the energy conserving step, this proton gradient is used to generate ATP.

In cyanobacteria photosynthesis and respiration occur in the same compartment and, in case of the ICM, take place even in the same membrane. Some components, like the cytochrome b_6f complex are shared by both systems (figure 1.1). Hence, it is necessary to understand respiration *and* photosynthesis to be able to efficiently exploit cyanobacteria as energy source.

1.3.2 Respiratory Terminal Oxidases

All known cyanobacterial respiratory terminal oxidases (RTOs) are membrane bound proteins catalysing the reaction

$$O_2 + 4H^+ + 4e^- \rightarrow 2\,H_2O$$

using dioxygen as terminal electron acceptor. In cyanobacteria there are at least seven different types of RTOs that can be grouped into three protein families:

1. homologs of mitochondrial cytochrome *c* oxidases:
    - aa_3-type cytochrome *c* oxidases (Cox)
    - 3 types of alternate respiratory terminal oxidases (ARTOs)
    - cbb_3-type cytochrome *c* oxidases (Cbb_3)

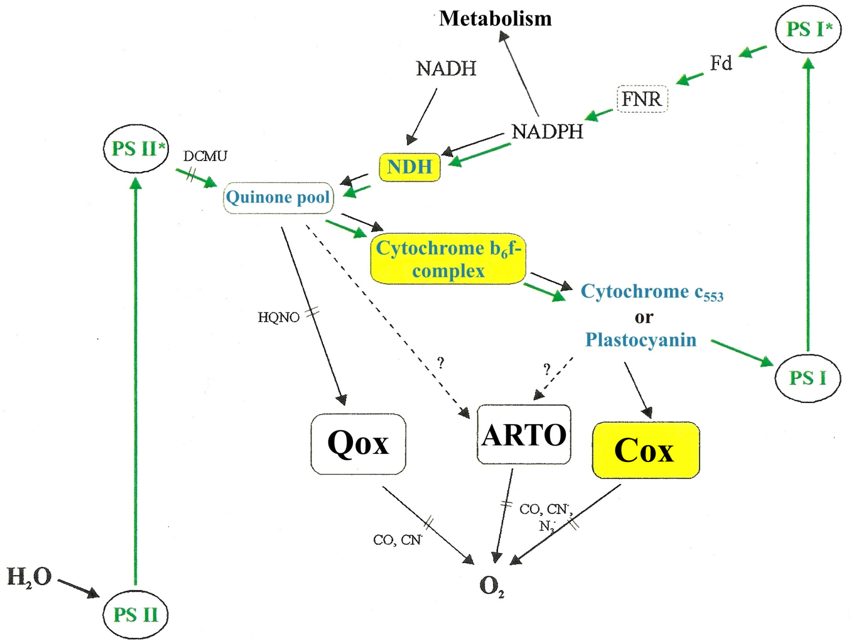

Metabolism
PS I*
Fd
NADH
FNR
NADPH
PS II*
DCMU
NDH
Quinone pool
Cytochrome b6f-complex
Cytochrome c553
or
Plastocyanin
HQNO
?
?
PS I
Qox
ARTO
Cox
CO, CN⁻, N3⁻
CO, CN⁻
H2O
O2
PS II

Figure 1.1: Schematic model of photosynthetic and respiratory electron transport in *Synechocystis* PCC6803. Green arrows: photosynthetic e^- transport, black arrows: respiratory e^- transport, frames: membrane proteins or membrane associated components, frames highlighted in yellow: proton pumps, blue font: proteins of photosynthesis *and* respiration, NDH: NAD(P)H dehydrogenase, FNR: ferrodoxin $NADP^+$ oxidoreductase, Fd: ferrodoxin, DCMU (3–(3,4–dichlorophenyl)–1,1–dimethylurea), HQNO (2–n–heptyl-4–hydroxyquinoline N-oxide), CO, CN^-, N_3^-: inhibitors of electron transport

2. homologs of the *E. coli* cytochrome *bd* type quinol oxidase (Qox)
3. homologs of the plastidic terminal oxidases (Ptox)

The homologs of mitochondrial cytochrome *c* oxidases belong to the haem copper oxidases, Cox and Cbb_3 use cytochrome *c* as electron donor while the donor(s) for ARTOs is (are) not yet known [23]. Qox and Ptox use quinol as electron donor [23, 24]. All RTOs except Ptox are sensitive to cyanide.

In PCC7942 three RTOs can be found through genome analysis. These RTOs are an aa_3-type cytochrome *c* oxidase, a cbb_3-type cytochrome *c* oxidase and a *bd*-type quinol oxidase, which are further described in the next 3 sections.

1.3.3 aa_3-Type Cytochrome *c* Oxidase

The cyanobacterial Cox enzyme consists of 3 subunits, encoded by the genes *coxA* (encoding the major subunit), *coxB* and *coxC*, localized on the chromosome in the order *coxBAC* (see figure 1.2). These 3 subunits are homologous to the subunits I, II and III of the mitochondrial cytochrome *c* oxidase, which in contrast contains up to 13 subunits. The name "aa_3" refers to the two haems in subunit I. The first "a" refers to a low spin haem, the electron donor to the binuclear center, composed of a high spin haem "a_3" and a copper ion [25].

Cox is very common in cyanobacteria and *Trichodesmium erythraeum* IMS101 is the only totally sequenced cyanobacterium without a *cox* locus (Schmetterer, unpublished). All subunits show high sequence similarity to each other in different cyanobacteria. Results of a protein BLAST of the Cox subunits of PCC7942 against *Synechocystis* PCC6803 are given in table 1.1.

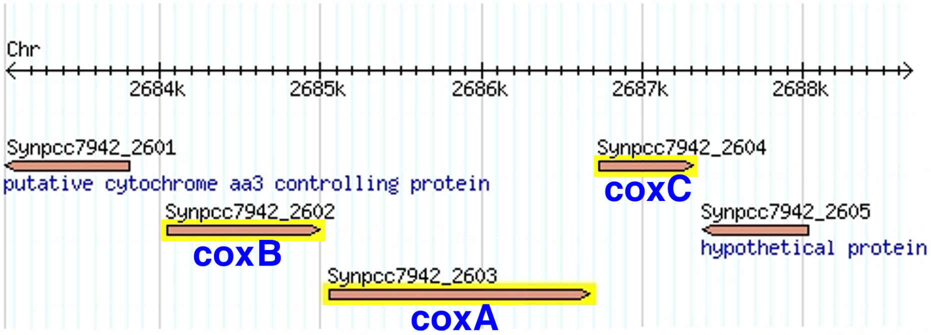

Chr
2684k
2685k
2686k
2687k
2688k
Synpcc7942_2601
putative cytochrome aa3 controlling protein
Synpcc7942_2604
coxC
Synpcc7942_2602
Synpcc7942_2605
hypothetical protein
coxB
Synpcc7942_2603
coxA

Figure 1.2: *cox* locus of PCC7942. *coxB* from 2684044 to 2684991 (=948 bp), *coxA* from 2685051 to 2686676 (=1626 bp) and *coxC* from 2686728 to 2687315 (=588 bp). Image taken and modified from Cyanobase [8]. Base pair counting is according to Cyanobase.

Additionally, the Cox subunits are very similar to the ARTO subunits (for example present in *Synechocystis* PCC6803) but can be distinguished by two conserved amino acid motifs [23].

Subunit CoxA is also very similar to the major subunit CcoN of the cbb$_3$-type cytochrome *c* oxidase of PCC7942 (see table 1.2, page 13).

Blasted Protein	E value
CoxA	0.000
CoxB	3e-98
CoxC	2e-75

Table 1.1: BLASTx [26] of the three Cox subunits from PCC7942 against *Synechocystis* PCC6803. The E value (Expect value) is a parameter for the number of hits that can be expected when aligning sequences by chance. The lower this value, the smaller is the probability that the base pairs matched by chance and the higher is the significance of the matches.

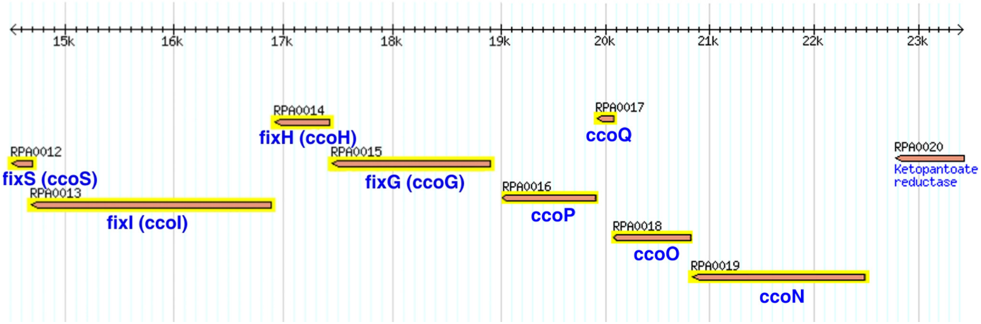


Figure 1.3: cbb_3 locus of *Rhodopseudomonas palustris*. Image taken and modified from Cyanobase [8].

1.3.4 cbb_3-Type Cytochrome c Oxidase

cbb_3-type cytochrome c oxidases are well characterized in purple bacteria. Since the total sequences of *Synechococcus* PCC6301 and PCC7942 are available, it is known, that there exist some genes homologous to cbb_3 of purple bacteria in at least these two cyanobacteria. Until this work nothing was known about the proteins encoded by these genes in cyanobacteria - not even whether the genes encode an active enzyme. Although in cyanobacteria nothing is known about the haems bound to the protein, it will be called Cbb_3 hereafter.

In purple bacteria like *Rhodopseudomonas palustris* the Cbb_3 enzyme consists of 4 subunits, encoded by the genes *ccoNOPQ*. This operon is found close to the *ccoGHIS* gene cluster. CcoNOPQ are thought to be the structural subunits of the enzyme, while the CcoGHIS subunits are necessary for the assembly of the Cbb_3 oxidase [27]. The gene map of this locus is shown in figure 1.3. CcoN is the major subunit containing the low spin haem b and the reaction center with the high spin haem b_3 and a copper ion. CcoO and CcoP are thought to transfer electrons from cytochrome c (hereafter cyt c) to CcoN. Little is known about the function of CcoQ but it may interact with expression of photosynthesis related genes.

Cbb_3 knockout experiments were made in different purple bacteria with different results. In *Bradyrhizobium japonicum* $ccoQ^-$ and $ccoP^-$ knockout mutants still showed Cbb_3 oxidase activity [28]. In *Rhodobacter capsulatus* Cbb_3 needs CcoP to be assembled to an active complex [29].

Since more and more total sequences of prokaryotes are available, it was shown that cbb_3-type cytochrome c oxidases are not restricted to Proteobacteria. Genes homologous to cbb_3 genes were found throughout the different phyla of eubacteria with only some exceptions. In archaea, no *cbb*$_3$ genes were found. Genomes containing *cbb*$_3$ genes, always contain *ccoN* and *ccoO*. In many proteobacteria the whole *ccoNOPQ* cluster was found, while in other bacteria *ccoQ* or *ccoP* or *ccoQ* and *ccoP* were missing [30].

In cyanobacteria *cbb*$_3$ genes are found only in *Synechococcus* PCC6301 and PCC7942. *ccoN* and *ccoO* are the only genes homologous to the *ccoNOPQ* cluster of purple bacteria. Also *ccoGHIS* is missing. The *cbb*$_3$ locus of PCC7942 is shown in figure 1.4.

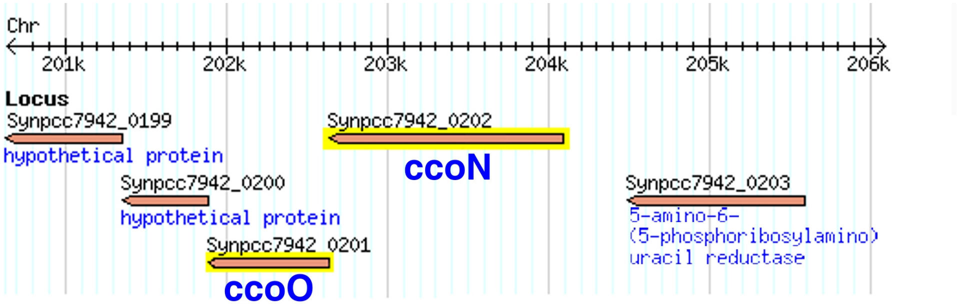

Chr
201k
202k
203k
204k
205k
206k
Locus
Synpcc7942_0199
hypothetical protein
Synpcc7942_0200
hypothetical protein
Synpcc7942_0201
ccoO
Synpcc7942_0202
ccoN
Synpcc7942_0203
5-amino-6-(5-phosphoribosylamino) uracil reductase

Figure 1.4: *cbb*$_3$ locus of PCC7942. *ccoN* from 202638 to 204089 (=1452 bp) and *ccoO* from 201889 to 202641 (=753 bp). Image taken and modified from Cyanobase [8]. Base pair counting is according to Cyanobase.

Directly downstream of the *ccoNO* locus, two genes (*synpcc7942_0199* and *synpcc7942_0200*, hereafter *0199* and *0200*) encoding putative transmembrane proteins exist. Whether these two genes belong to a *cbb*$_3$ cluster in cyanobacteria is not known. A protein BLAST with 0200 and 0199 in cyanobacteria led to the following 6 results:

- The genes *0200* and *0199* are wide-spread in cyanobacteria. They are localized on the genome always together and in the order *0200* followed by *0199*. The genes are overlapping (up to 4 bp) or are separated only by a few base pairs.

- In all strains (with available total sequence) containing one or more ARTO1 or ARTO3, *0200-0199* is always found directly upstream of ARTO1 or ARTO3. The ORFs of the ARTOs and *0200-0199* are always oriented in the same direction. *Synechocystis* PCC6803 is an exception to this observation. It contains genes for ARTO1 which are not clustered. Here *0200-0199* is found downstream from genes encoding an NADH dehydrogenase.

- In *Gloeobacter violaceus* PCC7421: *0200-0199* is found directly upstream of two genes encoding subunits I and II of a possible ARTO without the CAELC motif. A gene encoding subunit III beloning to this ARTO locus is not present in the genome.

- In Cyanothece this locus consists of 3 genes: two *0200* genes separated by 321 base pairs followed by *0199*, 4 base pairs overlapping with the second *0200* gene.
- *0200-0199* is never found close to genes encoding Cox (distinguished from ARTO1 and ARTO3 by the CAELC motif in subunit CoxB)
- *0200-0199* is not present in cyanobacteria without ARTO1 or ARTO3 with 2 exceptions: In *Synechococcus* PCC6301 and PCC7942 *0200-0199* is found directly downstream of *ccoNO* and upstream of a gene encoding an NADH dehydrogenase.

As *0200-0199* is always close to a sequence encoding proteins involved in energy conversion it is reasonable to assume that it is related to the Cbb$_3$ enzyme of *Synechococcus* PCC6301 and PCC7942 in some way.

A protein BLAST (table 1.2) considering genes for Cbb$_3$ in *R. palustris* and PCC7942 led to the following results: CcoN and CcoO of PCC7942 are highly similar to the corresponding protein of *R. palustris*. CcoN is also similar to CoxA while no significant similarity of CcoO to another protein of PCC7942 was found. No significant similarities for *R. palustris* CcoP and CcoQ were found in PCC7942. Also no significant similarities for 0199 and

Blasted		most significant alignment		E
Protein	Organism	in	Protein	value
CcoN	*R. palustris*	PCC7942	CcoN	1e-50
CcoN	PCC7942	PCC7942	CoxA	2e-10
CcoO	*R. palustris*	PCC7942	CcoO	3e-20
CcoO	PCC7942	PCC7942	hypothetical protein 0880	0.001
CcoP	*R. palustris*	PCC7942	Cytochrome c_6	8e-08
CcoQ	*R. palustris*	PCC7942	HAD family hydrolase	0.001
0200	PCC7942	*R. palustris*	Pyrophosphatase	0.001
0199	PCC7942	*R. palustris*	NADH dehydrogenase M	0.005
0200	PCC7942	PCC7942	0199	0.001
0199	PCC7942	PCC7942	0200	5e-20

Table 1.2: BLASTx [26] (protein BLAST) of proteins belonging or probably belonging to *cbb*$_3$-type cytochrome *c* oxidases. Only the two organisms PCC7942 and *R. palustris* were considered.

0200 were found in *R. palustris*. Within PCC7942, 0199 and 0200 are found to be significantly similar to each other.

1.3.5 *bd*-Type Quinol Oxidases

The cyanobacterial Qox enzyme consists of 2 subunits, encoded by *cydA* and *cydB* (figure 1.5). The protein sequence is highly similar to the *E. coli* cytochrome *bd* quinol oxidase, while no significant homologies of *cydAB* to *coxBAC* or *ccoNO* are found. It is not known whether cyanobacterial Qox really is a cytochrome *bd* oxidase as haem *d* was not detected in cyanobacteria until now [23].

Qox oxidises the quinone pool and is thought to act as an electron valve to prevent overreduction of the quinone pool through photosynthesis. It was shown that Qox is located in the ICM and its activity is strongly depending on culture conditions - both observations are compatible with Qox functioning as an electron valve [31]. Additional localisation of Qox in the CM cannot be excluded. Qox is also reducing O_2 in the dark [32] and therefore its function does not seem to be limited to an electron valve.

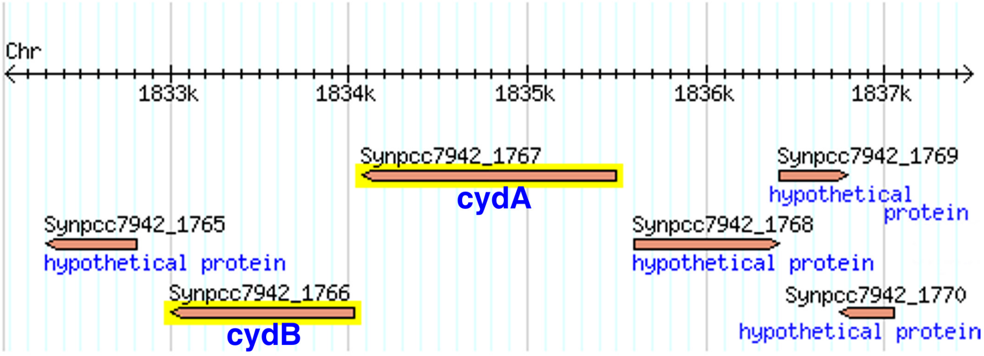

Chr
1833k
1834k
1835k
1836k
1837k
Synpcc7942_1767
cydA
Synpcc7942_1769
hypothetical protein
Synpcc7942_1765
hypothetical protein
Synpcc7942_1768
hypothetical protein
Synpcc7942_1766
cydB
Synpcc7942_1770
hypothetical protein

Figure 1.5: *qox* locus of PCC7942. *cydA* from 1834078 to 1835490 (=1413 bp) and *cydB* from 1832298 to 1834029 (=1032 bp). Image taken and modified from Cyanobase [8]. Base pair counting is according to Cyanobase.

1.4 Definition of Task

Recently genes with high sequence similarity to subunits of cbb_3-type cytochrome *c* oxidase from purple bacteria were found in *Synechococcus* PCC6301 and PCC7942. Until now no experiments on the RTOs of these strains were done with this background knowledge. As PCC7942 is amenable to genetic manipulation and a homozygous $ccoN^-$ mutant was already available, this strain was chosen for further experiments.

The main questions were:

- Is Cbb_3 an active cytochrome *c* oxidase in PCC7942 although some subunits present in purple bacteria are missing?
- Can differences in distribution or activity of the two cytochrome *c* oxidases of PCC7942 be found in the CM and the ICM?

Two mutant strains of PCC7942 had to be created: one cox^- mutant for cytochrome *c* oxidase activity tests and one qox^- mutant to show, whether all RTOs are dispensible in PCC7942. Knockout plasmids had to be constructed, and PCC7942 had to be transformed with these plasmids. After transformation, it had to be validated that the mutant strains were homozygous.

The membranes of the two mutant strains (PCC7942cox^- and PCC7942$ccoN^-$) and the PCC7942 wild type had to be separated by sugar gradient density centrifugation. The proteins of the two separated membranes of the 3 strains had to be tested by dual wavelenght spectroscopy for their cytochrome *c* oxidation activity.

Chapter 2

Materials

2.1 Bacterial Strains

E. coli Strains

- DH5α
  Genotype: F^-, *end*A1, *hsd*R17 (r_k^-, m_k^+), *sup*E44, *thi*-1, λ^-, *rec*A1, *gyr*A96, *rel*A1, Φ80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F)U169 [33].
  This strain was used for all cloning experiments during the construction of the plasmids.

Cyanobacterial Strains

- *Synechococcus* sp. PCC7942
  This strain was formerly known as *Anacystis nidulans* R2. It is a unicellular cyanobacterium reproducing by binary fission. Hence it belongs to Section I of Rippka´s groups of cyanobacteria [7]. PCC7942 is closely related to the type strain *Synechococcus* sp. PCC6301 [5]. A main feature of this bacterium is its capability for natural transformation, one of the reasons why it was chosen for my experiments. The wild type strain of this bacterium was provided by Dr. Susan Golden. The geographical origin of PCC7942 is unknown [34].

- *Synechococcus* sp. PCC7942*ccoN*$^-$
  This mutant was provided by Dr. Susan Golden. It has a chloramphenicol resistance cassette inserted in *ccoN*, the gene encoding the major subunit of the *cbb*$_3$-type respiratory terminal oxidase. The cassette was inserted with GeneJumper™ Primer Insertion Kit (Invitrogen™) after base pair 381 from gene start (figure 2.1). The mutant originates from a project during which the majority of the PCC7942 ORFs were

mutated to examine the cyanobacterial circadian clock [35]. More information about this (insert number: 3F2-A6) and other inserts made in Dr. Golden´s laboratory are found on the Cyanobike homepage [36].

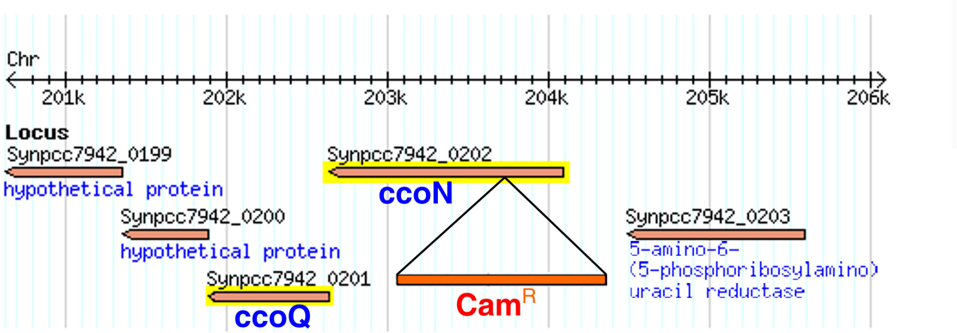

Chr
201k
202k
203k
204k
205k
206k
Locus
Synpcc7942_0199
hypothetical protein
Synpcc7942_0202
ccoN
Synpcc7942_0200
hypothetical protein
Synpcc7942_0203
5-amino-6-
(5-phosphoribosylamino)
uracil reductase
Synpcc7942_0201
ccoQ
Cam^R

Figure 2.1: cbb_3 locus of PCC7942$ccoN^-$. The chloramphenicol resistance cassette was inserted after base pair 381 from gene start. Image taken and modified from Cyanobase [8]. Base pair counting is according to Cyanobase.

- *Synechococcus* sp. PCC7942cox^-
  This mutant was created during this work. In this deletion mutant a spectinomycin resistance cassette replaces the whole gene encoding major subunit CoxA as well as parts of the genes for subunits CoxB and CoxC (figure 2.2).

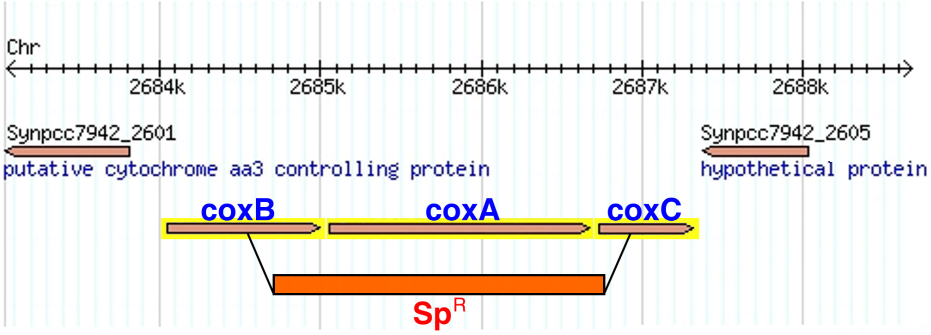

Chr
2684k
2685k
2686k
2687k
2688k
Synpcc7942_2601
putative cytochrome aa3 controlling protein
Synpcc7942_2605
hypothetical protein
coxB
coxA
coxC
Sp^R

Figure 2.2: *cox* locus of PCC7942cox^-. The spectinomycin resistance cassette was inserted after base pair 466 from *coxB* gene start and reaches until 394 base pairs before *coxC* gene end. Image taken and modified from Cyanobase [8]. Base pair counting is according to Cyanobase.

- *Synechococcus* sp. PCC7942qox^{-}
  This mutant was also created during this work. In this deletion mutant a kanamycin resistance cassette replaces parts of the genes encoding subunits CydA and CydB of PCC7942 Qox.

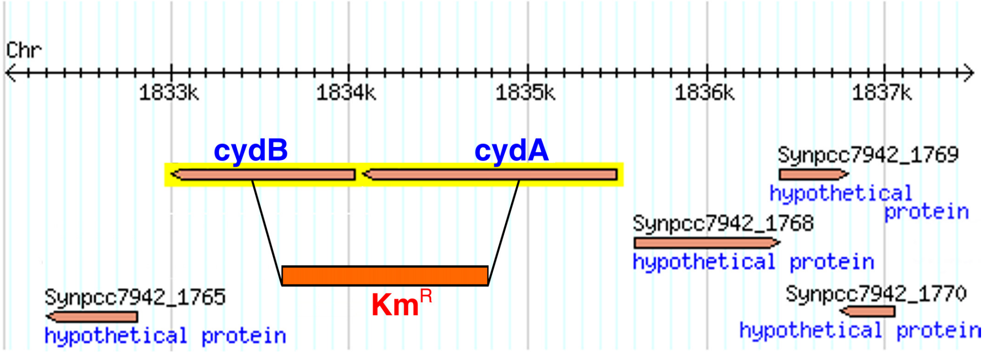

Chr
1833k
1834k
1835k
1836k
1837k
cydB
cydA
Synpcc7942_1769
hypothetical protein
Synpcc7942_1768
hypothetical protein
Km^{R}
Synpcc7942_1765
hypothetical protein
Synpcc7942_1770
hypothetical protein

Figure 2.3: *qox* locus of PCC7942qox^{-}. The kanamycin resistance cassette was inserted after base pair 550 from *cydA* gene start and reaches until 452 base pairs before *cydB* gene end. Image taken and modified from Cyanobase [8]. Base pair counting is according to Cyanobase.

2.2 Plasmids

- pUC18 [37]
  This plasmid was used as basis for the creation of the plasmids pHBUV9, pHBUV10, pHBUV11 and pHBUB12 (see below). The multiple cloning site is located within the *lacZ* gene. Thereby it is possible to use blue/white screening for selecting transformants in the first cloning step (see 3.11, page 51). It contains a replicon for *E. coli*, but not for cyanobacteria.

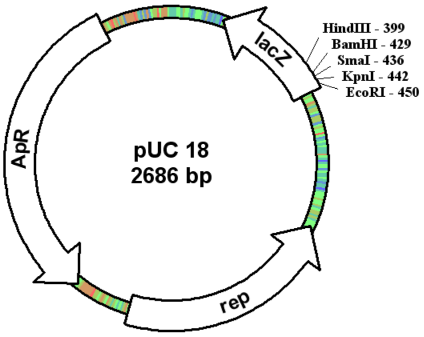

HindIII - 399
BamHI - 429
SmaI - 436
KpnI - 442
EcoRI - 450
lacZ
ApR
pUC 18
2686 bp
rep

Figure 2.4: Plasmid pUC18. ApR = ampicillin resistance cassette; rep = replicon; lacZ = *lacZ* gene including a multiple cloning site (the restriction sites used in my experiments are marked: *Hind*III, *Bam*HI, *Sma*I, *Kpn*I and *Eco*RI.)

- pRL446
  (consisting of S.A1, L.EHE1 and C.K2, nomenclature of Elhai and Wolk 1988 [38])
  This plasmid has a length of 3813 bp. It carries a kanamycin resistance cassette with a length of 1139 bp, flanked by two *Bam*HI restriction sites. It was used to cut out the resistance cassette to create pHBUV10 and pHBUV12 (page 20).

- pRL463
  (pUC18/19 containing L.HEH1 and C.S3, nomenclature of Elhai and Wolk 1988 [38])
  This plasmid has a length of 4754 bp. It carries a streptomycin / spectinomycin resistance cassette with a length of 2056 bp, flanked by two *Bam*HI restriction sites. It was used to cut out the resistance cassette to create pHBUV9 and pHBUV11 (page 20).

- pHBUV9 & pHBUV11 (fig. 4.3, page 65)
  These two plasmids were constructed during this work (page 61) to generate the cox^- knockout mutants. It carries a spectinomycin resistance cassette. The fragments CS1 (*cox* sequence 1) and CS2 (*cox* sequence 2) surround the antibiotic resistance cassette. CS1 is identical to the *Synechococcus* sp. PCC7942 chromosomal region from base pair 2683582 to base pair 2684548 (according to the base pair counting of Cyanobase [8], see figure 1.2). It has a length of 966 bp and overlaps subunit B of the *cox* locus. CS2 is identical to the region from bp 2686929 to 2687790, has a length of 870 bp and overlaps subunit C of the *cox* locus. Hence, a knockout mutant after double recombination with one of these plasmids lacks parts of the genes encoding the subunits CoxB and CoxC as well as the whole gene encoding subunit CoxA. The plasmid with the spectinomycin resistance cassette ORF parallel to the ORF of the *cox* sequences in the CS fragments was called pHBUV9, while the plasmid with the resistance cassette antiparallel was called pHBUV11.

- pHBUV10 & pHBUV12 (fig. 4.5, page 69)
  These two plasmids were constructed during this work (page 67) to generate the qox^- knockout mutants. They carry a kanamycin resistance cassette and fragments identical to parts of the PCC7942 *qox* sequence (QS1 and QS2). QS1 is identical to the region from base pair 1832528 to base pair 1833450 bp (=922 bp) and overlaps *cydB* of the *qox* locus. QS2 contains the region from base pair 1834940 to 1835784 (=844 bp) and overlaps *cydA* of the *qox* locus. A knockout mutant

created with these plasmids lacks large parts of *cydA* and *cydB* of the *qox* locus. The plasmid with the kanamycin resistance cassette ORF parallel to the ORF of the *cox* sequences in the CS fragments was called pHBUV10, while the plasmid with the resistance cassette antiparallel was called pHBUV12.

- Plasmids pHBUV1–pHBUV4, pHBUV7 and pHBUV8 were intermediates during construction of pHBUV9–pHBUV12 and are characterized in table 4.1, page 61 and table 4.3, page 68.

2.3 PCR-Primers

- Primers for fragment CS1:
  Primer 7942cox1
  `5´-CTC GGT ACC AAC GTG CCA CAA CCC ACT AC-3´`
  Primer 7942cox2
  `5´-AAT GGA TCC GGC AGA GCC TCT GTA GG-3´`

- Primers for fragment CS2:
  Primer 7942cox3
  `5´-TGA GGA TCC AGC GAT TAA CAC AAC CAT C-3´`
  Primer 7942cox4
  `5´-AAA TCT AAG CTT AGT CAG TTA TGG TTG GG-3´`

- Primers for fragment QS1:
  Primer 7942qox1
  `5´-GAG GAA TTC AAG GGA GTT GCT G-3´`
  Primer 7942qox2
  `5´-TTA GGA TCC AGA CCG CTA AAC TCG-3´`

- Primers for fragment QS2:
  Primer 7942qox3
  `5´-TTT GGA TCC GGA AGT AGT CCA GTA C-3´`
  Primer 7942qox4
  `5´-AAC AGA AAG CTT CTG CTG CCG TTA CAA GGC-3´`

- Primers for checking homozygosity of the cox^- mutant:
  Primer cox7942-7
  5´-GTA AAC CCA ACG ATG TCA CTG ACG-3´
  Primer cox7942-8
  5´-AGT AGT CCT ACG AGG ACG TGT AG-3´

- Primers for checking homozygosity of the qox^- mutant:
  Primer qox5
  5´-AAT GGT GGC GAG TTT AGC GGT-3´
  Primer qox6
  5´-GAC GGG CAT TTT CAG GTA CTG GAC-3´

- Primers for checking homozygosity of Dr. Golden´s cox^- mutant:
  Primer 7942cox1
  5´-GCG ATT CAC TCC AGG ACT CC-3´
  Primer 7942cox2
  5´-CAC CAC TAC CCG CTG GCA CG-3´

2.4 Solutions

Bradford reagent:

		100 ml:	
116,8 µM	Coomassie - Brilliant Blue G	10 mg	Coomassie - Brilliant Blue G
5 % v/v	EtOH	5 ml	EtOH p.a.
dissolve the dye completely			
8.5 % v/v	H_3PO_4	10 ml	phosphoric acid (85 % v/v)
			H_2O to 100 ml

filtrate through filter paper and store at 4 °C in a light protected bottle for a maximum of 4 weeks

CsCl Gradient Centrifugation solutions:

Filling solution:

		100 ml:	
5.94 M	CsCl	100 g	CsCl
2.18 mM	ethidium bromide	8.6 ml	ethidium bromide (10 mg×ml^{-1})
			H_2O to 100 ml

Extraction solution:

≈ 100 ml:	
20 ml	TE
	add CsCl until saturation (≈ 25g)
50 ml	isopropyl alcohol

mix firmly and wait until 3 phases have developed; the phase on top is isopropyl alcohol saturated with H_2O and CsCl and is used for the plasmid preparation

HEN:

		1000 ml:	
20 mM	HEPES *	4.77 g	HEPES
10 mM	EDTA	3.72 g	EDTA
5 mM	NaCl	0.29 g	NaCl

add NaOH to adjust buffer to pH 7.4

* HEPES: 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid

IH-I solution:

		200 ml:	
50 mM	Glucose	10 ml	Glucose (1M)
25 mM	Tris HCl pH 8.0	5 ml	Tris HCl pH 8.0 (1M)
10 mM	EDTA pH 8.0	4 ml	EDTA pH 8.0 (0.5M)
		181 ml	H_2O

glucose must be autoclaved separately; store at 4 °C;
add 4 mg/ml lysozyme just before use

IH-II solution:

		200 ml:	
1 % w/v	SDS	10 ml	SDS (20 % w/v)
0.2 N	NaOH	20 ml	NaOH (2M) (prepared with autoclaved H_2O)
		170 ml	autoclaved H_2O

filtrate through a 0.2 µm filter

IH-III solution:

		200 ml:	
3 M	Potassium acetate	120 ml	Potassium acetate (5M)
11,5 % v/v	Glacial acetic acid	23 ml	Glacial acetic acid
		57 ml	H_2O

autoclave

Phenol:
Phenol was distilled at 160 °C after purchse to remove oxidation products and stored at -20 °C in 100 ml aliquots under nitrogen gas.

The aliquots were melted when needed at 68 °C in a water bath, 8-hydroxyquinoline was added to a final concentration of 0.1 % w/v. The solution was extracted several times with 100 ml of 1 M Tris (pH 8.0) in a separatory funnel until the pH of the phenolic phase was >7.6. Then the phenol was extracted once with 100 ml 0.1 M Tris (pH 8.0) + 0.2 ml β-mercaptoethanol.

This solution was overlayed with 0.1 M Tris (pH 8.0) and stored in a bottle wrapped in aluminium foil at 4 °C. This solution was also used to prepare phenol-chloroform and phenol-chloroform-isoamyl alcohol solutions.

RNase I solution:

		110 ml:	
1 % w/v	RNase I	1 g	RNase I
0.01 M	Sodium acetate	100 ml	Sodium acetate (0.01M)

dissolve and heat to 100 °C for 15 min. Let cool down to room temperature and add

100 mM	Tris HCl (pH 7.4)	10 ml	Tris HCl (1M, pH 7.4)

aliquot 1 ml into microcentrifuge tubes and store at -20 °C for up to years; dilute 100× with TE before use

STE:

		200 ml:	
10 mM	Tris HCl pH 8.0	2 ml	Tris HCl pH 8.0 (1M)
1 mM	EDTA pH 8.0	0.4 ml	EDTA pH 8.0 (0.5M)
100 mM	NaCl	4 ml	NaCl (5M)
			H_2O to 200 ml

autoclave

STET:

		100 ml:	
8 % w/v	Sucrose	8 g	Sucrose
10 mM	Tris HCl pH 8.0	1 ml	Tris HCl pH 8.0 (1M)
50 mM	EDTA pH 8.0	10 ml	EDTA pH 8.0 (0.5M)
			H_2O to 100 ml

autoclave, then add

5 % v/v	Triton X-100	5 ml	Triton X-100

TAE 50×:

		1000 ml:	
2 M	Tris	242 g	Tris
1 M	Acetic Acid	57.1 ml	Acetic Acid
50 mM	EDTA pH 8.0	100 ml	EDTA pH 8.0 (0.5M)
			H_2O to 1000 ml

dilute the buffer 50× before use

TE:

		100 ml:	
10 mM	Tris HCl pH 8.0	1 ml	Tris HCl pH 8.0 (1M)
1 mM	EDTA pH 8.0	0.2 ml	EDTA pH 8.0 (0.5M)
			H_2O to 100 ml

autoclave

Chapter 3

Methods

3.1 Cultivation of ***Synechococcus*** sp. PCC7942

For optimal growth *Synechococcus* sp. PCC7942 needs different media, depending on whether it is cultured on solid medium on plates (3.1.1), in 50 ml liquid medium in Erlenmeyer flasks in a shaker (3.1.2) or in liquid medium in 10 liter bottles gassed with CO_2 enriched air (3.1.3). The basis of all these media is BG-11, a medium which was introduced by Rosemarie Rippka et al. [7]. It consists of four stock solutions and the trace metal mix A5, which are added to distilled water in ratios given below:

Stock I (1:1000):	$K_2HPO_4 \cdot 3\ H_2O$	40	g/l
Stock II (1:100):	$MgSO_4 \cdot 7\ H_2O$	7.5	g/l
	$CaCl_2 \cdot 2\ H_2O$	4.75	g/l
	$NaNO_3$	150	g/l
Stock III (1:1000):	$Na_2MgEDTA$	1	g/l
	$FeNH_4$ citrate	6	g/l
	Citric acid	6	g/l
Stock IV (1:1000):	Na_2CO_3	20	g/l
Trace metal mix A5 (1:1000):	H_3BO_3	2.86	g/l
	MoO_3	0.232	g/l
	$MnCl_2 \cdot 4\ H_2O$	1.81	g/l
	$ZnSO_4 \cdot 7\ H_2O$	0.222	g/l
	$CuSO_4 \cdot 5\ H_2O$	0.079	g/l
	$Co(NO_3)_2 \cdot 6\ H_2O$	0.0494	g/l

After adding all solutions to the distilled water, the BG-11 medium is autoclaved.

3.1.1 Growth on solid medium (BG-11-S-Agar)

Good growth on solid agar medium can be achieved by adding 1 mM sodium thiosulfate ($Na_2S_2O_3$) to BG-11-Agar (1.5 % Difco-Agar) [18]. A stock solution of 100 mM $Na_2S_2O_3$ has to be prepared and sterilized by passage through a 0.22 µm nitrocellulose filter. 300 µl of this solution are added to 30 ml of autoclaved BG-11-Agar and dispensed on a petri dish.

The cyanobacteria can be streaked by using an inoculation loop (with platinum wire) or with a Drigalski spatula made of glass. Both ways are adequate to grow single cell colonies on the plate. To obtain a bacterial lawn, the Drigalski spatula is preferred.

- Single cell colonies:
  Using an inoculation loop one must pick some cells (growing on another plate with solid medium, in a liquid culture, or from a batch of frozen bacteria) with a sterilized loop and spread them on a new plate. By distributing the streaked bacteria there will be some single cell colonies in most cases.
  Using a Drigalski spatula one must have about 100 µl of bacteria suspension which is pipetted onto the plate and then streaked with the spatula. A first suspension may come directly from a liquid culture or from cells from another plate or a frozen batch suspended in liquid media. It is important to make a serial dilution and to plate different dilutions on different plates to be sure to have one plate with the right dilution to obtain single cell colonies.

- Bacterial lawn:
  Bacterial lawns are sometimes helpful to start a liquid culture because there is a chance that cultures inoculated with a single cell colony do not start to grow. Therefore it is important that the lawn originates from one single cell colony. One large colony (about 1 mm diameter) is taken with an inoculation loop and suspended in about 100 µl of liquid media. The suspension is pipetted onto the plate and streaked with a Drigalski spatula.

After the bacteria have been streaked, plates are incubated in an illuminated climate cabinet. In order to prevent drying of the plates, they are put into a transparent plastic box, which has been sterilized with alcohol. The cabinet used had a temperature of 32 °C, a humidity of 80 % and a light flux of 40 to 50 $\mu mol \cdot s^{-1} \cdot m^{-2}$ in the upper level and about 20 $\mu mol \cdot s^{-1} \cdot m^{-2}$ in the lower level. The light was emitted from two Sylvania “Grolux” and two Philips “Cool White” fluorescent lamps. With these settings it took about one week to see first cyanobacterial colonies and about two weeks to obtain large colonies. Bacteria of single cell colonies were viable for about 4 weeks in the cabinet. Bacteria in a lawn turned yellow as early as after two weeks.

3.1.2 Liquid 50 ml culture (BG-11)

For 50 ml cultures BG-11 medium works quite well. 50 ml of BG-11 are filled into 100 ml Erlenmeyer flasks. The flasks are closed with cotton and aluminium foil before they are autoclaved. The cultures can be inoculated with a single cell colony or with a larger amount of cells taken from a bacterial lawn, originating from a single cell colony.

These cells were incubated in a Heraeus-Vötsch climate chamber at 32 °C, 60 % humidity, and 0.3 % CO_2 on a New Brunswick shaker with 110 rpm. The light flux varied between 10 and 11 $\mu\text{mol} \cdot s^{-1} \cdot m^{-2}$. Light was emitted from several Sylvania "Grolux" and Philips "Cool White" fluorescent lamps lightening the whole chamber and two 60 W Osram sodium vapor lamps, placed directly above the shaker. It takes about 2 weeks to have a culture with an OD_{730} of 0.1 grown to a culture with an OD_{730} of 3. The maximal OD_{730} reached under these conditions was about 5. After 4 to 6 weeks of growth the cultures started to turn yellow and died.

3.1.3 Liquid 10 l culture (BG-11-OK)

BG-11 has a low buffering capacity and when it is used for cultures that are gassed with air/CO_2 the pH-value is very unstable. Therefore a slightly modified BG-11 medium (called BG-11-OK) was used. To obtain 10 l of BG-11-OK, 100 ml BG-11 Stock Solution II and 10 ml BG-11 Stock Solution III were added to 8890 ml distilled water in a 10 l Schott bottle with a magnetic stir bar. BG-11 Stock Solutions I and IV were replaced by the Kratz & Myers [39] carbonate stock solution (Na_2CO_3 10 g/l, $NaHCO_3$ 82 g/l) and the phosphate stock solution ($K_2HPO_4 \,.\, 3\ H_2O$ 131 g/l). 100 ml of each solution were added to 800 ml distilled water. Both solutions were autoclaved and united in the 10 l bottle. The bottle was closed with a sterile rubber stopper including

(1) a pipette reaching nearly to the bottom of the bottle for gassing the culture with CO_2 enriched air, filtered through a 0.22 µm nitrocellulose filter,
(2) a short piping with a valve for pressure relief and
(3) a tube reaching into the culture, closed on the bottom end. The tube was filled with water and a thermometer was inserted to measure the approximate temperature of the culture.

The culture was inoculated with a dense 50 ml liquid culture (OD_{730} 3-5), placed on a magnetic stirrer and connected to the CO_2-air pump. The light was up-regulated slowly.

The 10 l cultures were kept on a magnetic stirrer in the climate chamber. At the beginning of the growth, only light from the ceiling lightening was used. After 2 days a 60 W Osram sodium vapor lamp was switched on additionally, shining directly on the bottle at a distance of 20 to 30 cm. Every two days an additional lamp was switched on, so that by the 9th day 4 lamps were shining on the culture. The cells were harvested on the 11th day with an OD_{730} of approximately 1. Because of the lamps, the culture grew warmer with time. To prevent this, a fan was used to cool down the culture and keep it stable at 32 °C.

3.1.4 Usage of Antibiotics

When using antibiotics for cultures grown on solid media, the antibiotics (dissolved in water) are pipetted onto the middle of the petri dish. The liquid agar medium has to be cooled down to approximately 45 °C before it is mixed with the antibiotic, as most antibiotics are unstable at higher temperatures. The agar and the antibiotics are mixed gently. If a streptomycin / spectinomycin resistance cassette was used, spectinomycin was used to prepare the selective medium for PCC7942.

Antibiotic	**Final concentration (µg/ml)**	**Source of resistance cassette**
Chloramphenicol	7.5	GeneJumper™
Spectinomycin	40	pRL463
Kanamycin	40	pRL446

Table 3.1: Antibiotics used to select transformants of *Synechococcus* sp. PCC7942.

3.1.5 Storage of *Synechococcus* sp. PCC7942

PCC7942 will die very quickly (about 1 or 2 days), if it is stored in a refrigerator at 4 °C. To keep the bacteria alive for some weeks, the easiest way is to store petri dishes at room temperature at a shady but not dark place. To store cyanobacteria for years, it is best to freeze them:

- Transfer a 50 ml culture into a 50 ml Falcon tube and centrifuge it for 10 min at room temperature and 4000 g.

- Resuspend the cell pellet in 1 ml of growth media and transfer it into a sterile 2 ml Nunc cryo tube™.
- Add 300 µl fresh DMSO (dimethyl sulfoxide) and mix with a vortex shaker.
- Freeze the vial in liquid nitrogen and store at -80 °C.

3.2 Cultivation of *E. coli*

The most common medium for *E. coli* is the LB medium. (According to the inventor of this medium, Giuseppe Bertani, there are a lot of incorrect interpretations of the acronym LB like Luria broth, Lennox broth or Luria-Bertani medium. He intended LB to stand for "lysogeny broth" [40].)

Yeast extract	5	g/l
Tryptone	10	g/l
NaCl	10	g/l

These ingredients are weighed in a bottle and the appropriate amount of distilled water is added before autoclaving. The autoclaved bottles can be stored at room temperature.

3.2.1 Growth on solid medium (LA)

E. coli can easily be cultivated on LA medium, consisting of LB medium plus 1.5 % agar. When cultivating *E. coli* on solid media, the purpose is to obtain single cell colonies. In contrast to *Synechococcus* sp. PCC7942 just a few *E. coli* cells are required to start a liquid culture of any size described below. Therefore there is no need for a bacteria lawn when working with *E. coli*. To obtain single cell colonies it is possible to use an inoculation loop or to work with serial dilutions and a Drigalski spatula as described for growing PCC7942 on solid medium (page 28). The plates are incubated over night at 37 °C in the dark. Colonies are visible after 6 to 8 hours of incubation.

3.2.2 Liquid mini culture (3 - 5 ml LB)

Cultures with volumes from 3 to 5 ml are sufficient for plasmid minipreps (page 35), the Rapid Boiling Method (page 33), inoculation of larger cultures (see below) or freezing *E. coli* (page 32). 3 to 5 ml of autoclaved LB medium are filled into a sterile glass test tube with a metal cap. The tube is inoculated with some cells from a single cell colony picked from a plate with an inoculation loop. The tubes are incubated in a shaker at 37 °C and

200 rpm over night. During this procedure, the tubes have to be in a slightly angular position to maximize the surface of the medium and optimize the gas exchange.

3.2.3 Liquid cultures (50 - 1000 ml LB)

To gain bigger amounts of plasmids midipreps (50 ml cultures), maxipreps (500 - 1000 ml cultures) (page 36) or Caesium Chloride Plasmid Preparations (page 37) are useful. 100 ml Erlenmeyer flasks are used for midipreps. For 500 - 1000 ml cultures the LB medium is prepared and autoclaved in Fernbach flasks. Regardless of whether Erlenmeyer or Fernbach flasks are used, the flasks have to be closed with a piece of cotton wool and aluminium foil before they are autoclaved. After inoculation with a mini culture or a single cell colony the flasks are incubated on a shaker at 37 °C and 200 rpm over night.

3.2.4 Usage of Antibiotics

The preparation of selective media is as described for PCC7942 (page 30). The concentrations used for selecting *E. coli* are different and are shown in Table 3.2.

Antibiotic	**Final concentration (µg/ml)**	**Source of resistance cassette**
Ampicillin	50	pUC18
Streptomycin	50	pRL463
Kanamycin	50	pRL446

Table 3.2: Antibiotics used to select *E. coli* harbouring plasmids with resistance cassettes.

3.2.5 Storage of *E. coli*

E. coli can easily be stored at 4 °C on petri dishes for up to several weeks. Liquid cultures can be stored just for some days at 4 °C. Also for *E. coli* storage for years is possible by freezing:

- Mix 1 ml of an *E. coli* liquid mini culture with 150 µl glycerol (80 % v/v glycerol, 20 % v/v H_2O, autoclaved) in a 2 ml Nunc cryo tubes™.
- Freeze the vial in liquid nitrogen and store at -80 °C.

3.3 Preparation of Plasmids

There are different methods to prepare plasmids. The methods consume different amounts of time and yield different quantities of plasmid with different purities. After every method it is useful to run a DNA agarose gel to determine the approximate yield.

3.3.1 Rapid Boiling Method (Holmes and Quigley)

The Rapid Boiling Method [41] is a fast and relatively clean method for the preparation of plasmids in small amounts. In most cases the plasmids are pure enough for further uses like restriction or electroporation. Sometimes it is useful to make a phenol extraction (as during the Ish-Horowicz preparation, see page 35ff.) as well. The procedure (the original was slightly modified) is described here:

- Harvest 3 ml of cells: Fill a 1.5 ml microcentrifuge tube with 1.5 ml of an *E. coli* culture harbouring the plasmid, centrifuge the tube for 1 min at 12000 rpm and room temperature, discard the supernatant and repeat the step to pellet 3ml of cells altogether.
- Resuspend the pellet in 350 µl STET (page 25) by pipetting up and down.
- Add 25 µl fresh lysozyme solution (10 mg/ml).
- Heat the tube in boiling water for 50 s, cool down afterwards immediately on ice.
- Centrifuge the microcentrifuge tube for 15 min at 12000 rpm and 4 °C.
- Remove the gelatinous pellet with a sterile toothpick.
- Add 400 µl isopropyl alcohol for precipitation of the DNA.
- Centrifuge the microcentrifuge tube for 15 min at 12000 rpm and 4 °C.
- Discard the supernatant and wash the pellet with 1 ml of 70 % v/v ethanol.
- Discard the supernatant, centrifuge for a few seconds and remove the supernatant with a pipettor.
- Dry the pellet in a vacuum desiccator at 20 mbar.
- Dissolve the pellet in 20 µl TE (page 25).

3.3.2 JAT-Prep

The origin of the JAT-Prep Method is not well-known. It was published in the internet by B. Babb who suggests that it comes from Prof. J. A. Thomson of the University of Cape Town [42]. It is a very fast method to test whether an *E. coli* colony contains a plasmid with an insert. It does not need over night growth of a mini culture but it yields a very impure product. No further uses are possible except for gel electrophoresis. This method is very helpful when a lot of colonies have to be tested, like in cases where neither blue-white screening nor antibiotics can be used for selecting colonies:

- Transfer parts of colonies into a microcentrifuge tube with 0.6 ml LB (including antibiotics when appropriate).
- Incubate with shaking at 37 °C for more than 4 hours.
- Centrifuge tubes at 14000 rpm for 1 min.
- Discard the supernatant and resuspend the pellet in 60 µl STE (see page 25).
- Add 60 µl phenol-chloroform-isoamyl alcohol (25:24:1, v/v).
- Vortex for 15 s.
- Centrifuge at 14000 rpm for 5 min.
- Transfer the upper aqueous phase to a fresh tube and use 10 µl for an agarose gel electrophoresis.

cccDNA of known size that can be compared with the sample DNA (like the plasmid before the last cloning step) is used as a reference. As it is not possible to test the DNA prepared with the JAT-Prep method with restriction enzymes, the DNA of colonies, shown to be promising by JAT-Prep, are also prepared with another method and cut with restriction enzymes afterwards.

3.3.3 Ish-Horowicz Mini-Preparation

For the Ish-Horowicz Miniprep [43], IH-Solutions I and III (page 23) can be stored at 4 °C for prolonged periods. IH-II solution must be prepared freshly or not be older than two weeks as the yield of plasmid decreases otherwise.

- Harvest cells as described in 3.3.1.
- Resuspend the pellet in 100 µl IH-I solution (including 4 mg/ml lysozyme) and incubate for 5 min at room temperature.
- Add 200 µl IH-II solution, invert the microcentrifuge tube several times and incubate on ice for 5 minutes.
- Add 150 µl IH-III solution, invert several times and incubate on ice for 5 minutes.
- Centrifuge the tube for 5 min at 14000 rpm and 4 °C; transfer the supernatant into a new microcentrifuge tube.
- Phenol extraction:
  - Add 0.5 ml phenol (see 2.4, page 24), vortex the tube and centrifuge it for 3 min at 14000 rpm and 4 °C; transfer the supernatant and the interphase to a new tube and repeat this step.
  - Add 0.5 ml chloroform : isoamyl alcohol (24:1 v/v), vortex and centrifuge for 1 min at 14000 rpm and 4 °C; transfer the supernatant to a new tube.
  - Add 0.5 ml chloroform, vortex and centrifuge for 1 min at 14000 rpm and 4 °C; transfer the supernatant to a new tube.
- Add 2.5 volumes of ethanol (-20 °C) and centrifuge for 15 min at 14000 rpm and 4 °C.
- Discard the supernatant and wash the pellet with 1 ml of 70 % v/v ethanol.
- Discard the supernatant, centrifuge for a few seconds and remove the supernatant with a pipettor.
- Dry the pellet in a vacuum desiccator at 20 mbar.
- Dissolve the pellet in 20 µl TE (page 25).

3.3.4 Plasmid Maxi-Preparation

The plasmid maxi-preparation described here is an extension of the Ish-Horowicz plasmid preparation [43]. The protocol was passed to Georg Schmetterer by Peter Wolk and then adjusted to fit for our laboratory. This protocol is written for starting with a 1000 ml culture of *E. coli* but can also be adjusted for preparations with smaller culture volumes.

- Harvest the cells by centrifugation in two 500 ml centrifuge bottles in a JA-10 rotor for 10 min at 6000 rpm and room temperature;
- Discard the supernatant and completely resuspend the pellet in the remaining medium by vortexing.
- Add 10 ml of IH-I solution per 500 ml bottle and pool the suspensions in one bottle.
- Add 5 mg$\times ml^{-1}$ lysozyme and incubate for 5 min at room temperatures.
- Add 40 ml of IH-II solution, mix gently by inverting the bottle; cool on ice for 5 min.
- Add 30 ml of cold IH-III solution, mix gently until a white precipitate forms and cool on ice for 5 min.
- Mix again for a short time and centrifuge in a JA-10 rotor for 20 min at 6000 rpm and room temperature.
- Transfer the supernatant into a 150 ml Corex tube and measure the volume.
- Add 0.6 volumes isopropyl alcohol; incubate for 5 min at room temperature for precipitation.
- Centrifuge with a rubber adapter in a JA-14 rotor for 15 min at 7000 rpm and room temperature.
- Discard the supernatant and wash the pellet two times with 70 % v/v ethanol (-20 °C).
- Dry the pellet in a vacuum desiccator at 20 mbar; dissolve the pellet in 5 ml TE on ice.
- Add 0.5 volumes ammonium acetate (7.5M in TE) and incubate for 20 min on ice to precipitate further proteins.

- Centrifuge in a JA-14 rotor for 20 min at 7000 rpm and 4 °C.
- Transfer the supernatant into a 25 ml Corex tube and measure the volume.
- Add 2.5 volumes of ethanol (-20 °C) and incubate for 15 min or over night at -20 °C.
- Centrifuge in a JS-13.1 rotor for 20 min at 8750 rpm and 4°C or in a JA-20 rotor at 8000 rpm.
- Discard the supernatant and dry the pellet in a vacuum desiccator at 20 mbar.
- Dissolve the pellet in 2.5 ml NaCl solution (50 mM in TE) and add 2.5 µl RNase I solution (2.4, page 24)
- Transfer the solution in 5 microcentrifuge tubes (500 µl per tube).
- Perform phenol extractions for each tube as described in 3.3.3 on page 35.
- Add 0.1 volumes sodium acetate (3M).
- Add 2.5 volumes of ethanol (-20 °C) and precipitate the DNA for 15 min at -20 °C.
- Centrifuge for 15 min at 14000 rpm and 4 °C; discard the supernatant and wash the pellet with 70 % v/v ethanol (-20 °C).
- Discard the supernatant, centrifuge for a few seconds and remove the supernatant with a pipettor.
- Dry the pellet in a vacuum desiccator at 20 mbar.
- Dissolve the pellet in 20 µl TE.

3.3.5 CsCl Gradient Centrifugation

The CsCl Gradient Centrifugation Plasmid Preparation yields a big amount of very clean DNA that can be stored for years at 4 °C. It is worth to be performed with *E. coli* cultures with volumes from 500 to 1000 ml. The first steps are made with the Ish-Horowicz solutions and are performed as described in 3.3.4 to the point of the first precipitation of the DNA with isopropyl alcohol, washing and then drying the pellet. Proceed as described below:

- Dissolve the pellet in 10 ml of TE.
- Add 10 g CsCl and 860 µl ethidium bromide solution (10 mg$\times ml^{-1}$).
- Inject the solution into a Beckman Quick-Seal-Tube (polyallomer, 39 ml, 25 × 89 mm) with a syringe.
- Fill up the tube with filling solution (page 23) until only a little air bubble with a diameter of about 1 cm is left.
- Tare the tubes so the weight is not differing more than 10 mg. Do this by pulling out solution with cellulose cloth and take care that the neck of the tube is dry.
- Seal the tube with a proper metal sealing cap: put the cap on the neck of the tube and press it down slowly with the preheated sealer. Check for tightness of the tube by turning it around and applying some pressure.
- Centrifuge in a VTI-50 rotor for 15 to 20 h at 48000 rpm and 16 °C; the brake is turned off or at minimal force.
- Carefully remove the rotor from the centrifuge and the tube from the rotor without disrupting the gradient and place the tube onto a rack near a UV lamp (366 nm).
- Puncture the tube at the top with an 18-gauge needle to provide a vent; turn on the UV lamp, attach some tape and puncture the tube at the level of the lower band (the upper band is chromosomal DNA) with an 18-gauge needle (bevelled side up) placed on a syringe and slowly draw off the DNA.
- Transfer the DNA to a 15 ml Kimax tube
- Extract the solution 3 times to remove the ethidium bromide:
  - Add 5 ml of the isopropyl alcohol phase of the extraction solution (page 23) and shake well.
  - Remove the upper phase (if after the third extraction the upper phase is not yet colorless, add a further extraction step).
- Transfer the DNA solution to a Corex tube (for volumes smaller than 1.6 ml use a 25 ml tube, for larger volumes use a 150 ml tube).

- Add 4 volumes of H_2O and 10 volumes of ethanol (-20 °C) and precipitate the DNA for 15 min at -20 °C.
- Centrifuge in a JS-13.1 rotor for 20 min at 8750 rpm and 4 °C or in a JA-20 rotor at 8000 rpm.
- Discard the supernatant and wash the pellet with 12 ml of 70 % v/v ethanol.
- Dry the pellet in a vacuum desiccator at 20 mbar.
- Dissolve the pellet in 100 µl TE (page 25).

3.4 Preparation of Cyanobacterial DNA

The DNA preparation described here is based on a method developed for the filamentous cyanobacteria *Nostoc* sp. PCC7120 [44, 45] and modified by Georg Schmetterer for single-celled cyanobacteria [46]. This method yields DNA consisting of chromosomal DNA and plasmids:

- Grow cells as described in 3.1.2 (page 29).
- Transfer the cell suspension to a 50 ml Falcon tube and centrifuge for 10 min at room temperature and 4000 g.
- Discard the supernatant and resuspend the pellet in a final volume of 1.5 ml in a microcentrifuge tube and centrifuge for 10 min at 14000 rpm and room temperature.
- Discard the supernatant and resuspend the pellet by vortexing in 400 µl TE.
- Add 150 µl sterile glass beads;

  Preparation of the glass beads:

  - Wash glass beads (212 - 300 µm in diameter) with half-concentrated nitric acid.
  - Wash with dH_2O until the washing liquid has a neutral pH.
  - Dry at 100 °C.
  - Heat sterilize for 2 hours at 180 °C.
- Add 20 µl of 10 % w/v SDS.

- Add 450 µl phenol-chloroform (1:1, v/v).
- Repeat 10 times:
  - Vortex the tube for 1 min.
  - Cool the tube on ice for 1 min.
- Centrifuge the tube for 15 min at 14000 rpm and 4 °C; transfer the supernatant to a new microcentrifuge tube.
- Phenol extraction:
  - Add 0.4 ml phenol, vortex the tube and centrifuge for 3 min at 14000 rpm and 4 °C; transfer the supernatant and the interphase to a new tube and repeat this step.
  - Add 0.4 ml phenol-chloroform (1:1, v/v), vortex the tube and centrifuge for 3 min at 14000 rpm and 4 °C; transfer the supernatant and the interphase to a new tube and repeat this step.
  - Add 0.4 ml chloroform-isoamyl alcohol (24:1, v/v), vortex and centrifuge for 1 min at 14000 rpm and 4 °C; transfer the supernatant to a new tube.
  - Add 0.4 ml chloroform, vortex and centrifuge for 1 min at 14000 rpm and 4 °C; transfer the supernatant to a new tube.
- Add 0.1 volumes sodium acetate (3M).
- Add 2.5 volumes of ethanol (-20 °C) and precipitate the DNA for 15 min at -20 °C.
- Centrifuge for 15 min at 14000 rpm and 4 °C; discard the supernatant and wash the pellet with 70 % v/v ethanol (-20 °C).
- Discard the supernatant, centrifuge for a few seconds and remove the supernatant with a pipettor.
- Dry the pellet in a vacuum desiccator at 20 mbar.
- Dissolve the pellet in 50 µl TE.

3.5 Polymerase Chain Reaction

Polymerase chain reaction was used to amplify the DNA fragments (CS1, CS2, QS1 and QS2) needed for the knockout plasmids and to validate the homozygosity of the created mutants. Cyanobacterial chromosomal DNAs (wild type or mutant) were used as templates. To amplify the sequences for cloning *Pfu* polymerase from Fermentas was used. To amplify the fragments for validation of homozygosity *Taq* polymerase from New England Biolabs (NEB) was used. The primers used are described in 2.3, page 21. The polymerase buffers were supplied with the polymerases, dNTPs came from NEB. The polymerase chain reactions were set up as described below:

	42	µl	dH_2O
	5	µl	Polymerase Buffer (10×)
	1	µl	chromosomal DNA
2 ×	0.5	µl	respective Primers
	0.5	µl	dNTPs 10mM
	0.5	µl	(*Taq* or *Pfu*) Polymerase

The reactions were performed in a Perkin Elmer GeneAmp PCR System 2400 with the following settings:

		5 min	94 °C
30–40 Cycles	Denaturation:	30 s	94 °C
	Annealing:	40 s	50–54 °C
	Elongation:	80*–150* s	72 °C
		7 min	72 °C
		∞	4 °C

Which annealing temperature between 50 and 54 °C was used depended on the melting temperature of the primers. x* is a setting variant of the PCR cycler in which the elongation time is x seconds in the first cycle and is extended by 1 s per cycle.

After the polymerase chain reaction the DNA was stored at 4 °C for up to several days. When the PCR was performed for validating homozygosity, a minigel was prepared and loaded with 5–10 µl of the PCR product. When the PCR was performed to create fragments for cloning, a maxigel was prepared, the whole PCR product was loaded and a gel extraction was performed after the gel electrophoresis.

3.6 Restriction Digest

In a restriction digest DNA is cut by one or more restriction enzymes at a sequence characteristic for the particular enzyme.

It is important that the DNA is relatively clean, so it sometimes helps to perform a phenol extraction (page 35) with the DNA when the restriction enzymes do not cut. In the protocols, supplied by the manufacturers of the restriction enzymes, BSA (bovine serum albumin) is sometimes added. For this work BSA was always added without any problems. The amounts of DNA used for restrictions in this work were between 0.5 and 3 µg. The total volume of a reaction was 20 µl. This is a generalized approach for a restriction digest:

x µl DNA (e.g. 1 µg)
2 µl BSA 10×
2 µl Restriction Buffer 10×
2 µl RNase I solution (2.4, page 24)
y µl Restriction Enzyme(s) (2 u/µg DNA)
(fill up to 20 µl with H_2O)

When a double digest was performed (digestion with two different enzymes), sometimes it was possible to use one buffer for both enzymes, although one of the enzymes was not in its optimal buffer. For finding the best buffer for a double digest the program "Double Digest Finder" on the New England Biolabs homepage was used. If no single buffer is adequate for a double digest it may be possible to digest with one enzyme in the less concentrated buffer first. Then chemicals are added to obtain the concentrations of the second buffer and finally, the second enzyme for digestion is added. If this is not possible either, the buffer will have to be changed completely between the two reactions. To change the buffer, the DNA was extracted with the same volume of chloroform, precipitated with 0.1 volumes sodium acetate (3M) and 2.5 volumes ethanol and dissolved in a small volume of dH_2O. For restrictions performed during this work the incubation time was 2 h. After a restriction digest there were performed either:

- an agarose gel electrophoresis (3.7, page 43) to check the DNA lengths,
- an agarose gel electrophoresis followed by a gel extraction (3.8, page 45) to isolate a fragment or a vector for cloning,
- or a ligation of the cut vector with a fragment (3.9, page 46).

3.7 Agarose Gel Electrophoresis

Agarose gel electrophoresis is used to separate, visualize, identify or purify DNA. The process can be subdivided into 3 parts:

3.7.1 Preparation of an Agarose Gel

Agarose is a polysaccharide obtained from the red algae *Gelidium*. It is a white powder melting at 90-95 °C and gelatinising at 30–45 °C, depending on the agarose concentration. Different agarose concentrations in the electrophoresis buffer TAE (2.4, page 25) are appropriate for efficient separation of DNA of different size (see Table 3.3). The expected fragment lengths were considered before preparation of the gel.

Agarose Concentration % w/v	**Length of DNA Fragments (kb)**
0.3	5–60
0.6	1–20
0.7	0.8–10
0.9	0.5–7
1.2	0.4–6
1.5	0.2–3
2.0	0.1–2

Table 3.3: Concentrations of agarose in the gel needed for efficient separation of DNA fragments of different sizes [47].

Minigels have a volume of 30–40 ml and each slot holds a volume of maximal 15 µl of sample; they were used mainly for controlling DNA lengths after restriction digests or quantification of DNA after preparations.

Maxigels have a volume of 300–350 ml and each slot holds volumes of 20-50 µl, depending on the teeth size of the comb used. Because of the bigger volume of the slots they were used for DNA purification by gel electrophoresis and gel extraction.

Preparation of the gel:

- Pour the appropriate amount of TAE 1× buffer (see above) into a flask with the double capacity of the buffer volume. Add the correct amount of agarose to obtain a gel with an agarose concentration suitable for the expected DNA fragment lengths (see Table 3.3).

- Heat the slurry with a microwave oven until the agarose has dissolved. Let the agarose solution cool down to about 60 °C and pour into a sealed gel mould with an inserted comb.

- Let the solution cool down until the gel is solidified, place the mould with the gel in an electrophoresis chamber and fill the chamber with TAE buffer until the buffer covers the gel to a depth of about 1 mm and remove the comb.

3.7.2 Loading and Running a Gel

The next step was to prepare the DNA marker or ladder and the sample(s) for loading. Some markers / ladders are ready-to-use and were loaded into the slot directly. Otherwise the marker was treated the same way as the samples.

Before loading, the DNA was mixed with loading dye solutions that contain xylene cyanol and bromophenol blue to colour the samples, and glycerol or sucrose to increase the density of the samples. They are concentrated 6× or 10×, hence one-sixth or one-tenth, respectively, of the total volume to be loaded was added. To avoid overloading the gel with DNA, not more than 100 ng of DNA were loaded into a minigel and 500 ng into a maxigel. A typical loading sample mixture for a minigel is shown below:

12.5	µl	dH_2O
1.5	µl	10× loading dye
1.0	µl	DNA (100 ng/µl)

The ingredients were mixed on a piece of Parafilm and pipetted into a slot of the gel. A DNA marker or ladder was always loaded on the gel additionally as a positive control and to help determining the lengths of the sample fragments.

The electrophoresis chamber was closed and power was applied. The voltage to be used depends on the distance between the two electrodes of the chamber. It has to be 5-20 V/cm when running a minigel and 1-5 V/cm when running a maxigel to obtain sharp bands [47].

3.7.3 Staining and Photographing a Gel

There are two ways of staining an agarose gel. Either the gel can be stained by adding ethidium bromide solution [10 mg/ml] to a final concentration of 0.5 µg/ml after melting the agarose or the gel is incubated in ethidium bromide solution after the run. When ethidium bromide is present in the gel during the run it reduces the mobility of the DNA and the bands are not as sharp as if the gel is stained after the run.

For this work the gels were mostly stained after electrophoresis. The gel was incubated in a glass dish with H_2O and 0.5 µg/ml ethidium bromide for 20 minutes or longer. Afterwards the gel was incubated in H_2O to wash away the ethidium bromide not bound to DNA to reduce background fluorescence.

The DNA bands were examined and documented with a UV transilluminator and Polaroid MP4 camera equipped with a UV filter. The UV transilluminator has an emission maximum at 302 nm. This wavelength is absorbed by ethidium bromide bound to DNA. The light emitted by the dye / DNA complex has a wavelength of 590 nm and is photographed by the camera [47].

3.8 Gel Extraction

Gel electrophoresis followed by gel extraction is a DNA purifying step. It can be used to remove proteins, small molecules, or unwanted DNA or RNA after PCR, restriction digests or plasmid preparations.

The first step in gel extraction was to perform a gel electrophoresis as described in 3.7. The gel pieces containing the desired DNA were cut out from the rest of the gel with a razor blade while illuminating with UV radiation. These pieces were weighed and then extracted with a gel extraction kit. For this work the Invisorb® Spin DNA Extraction Kit was used. The kit is made for extracting DNA with lengths between 80 an 80000 base pairs from gel slices up to 300 mg.

- Transfer the gel slice into a weighed microcentrifuge tube and determine the gel weight.

- Add 500 µl Gel Solubilizer S to gel slices up to 150 mg and 1 ml to gel slices up to 300 mg.

- Incubate the tube in a water bath at 50 °C for about 10 min (or until the gel is solubilized).

- Add 250 µl (500 µl) Binding Enhancer and vortex for a few seconds; transfer 800 µl of the solution to a Spin Filter placed in a 2 ml Receiver Tube. Centrifuge at 10000 rpm for 1 min in an Eppendorf centrifuge and discard the filtrate. If the volume of the solution is larger than 800 µl, the Receiver Tube has to be centrifuged again with the rest of the solution.

- Pipette 500 µl Washing Buffer onto the Spin Filter and centrifuge at 12000 rpm for 30 s. Discard the filtrate and repeat this washing step.
- Remove the Spin Filter from the 2 ml Receiver Tube and place it in a 1.5 ml Receiver Tube. Pipette 20 µl Elution Buffer or autoclaved H_2O directly onto the surface of the filter and incubate at room temperature for 5 to 10 min. Centrifuge at 12000 rpm for 1 min. The eluate can be directly used for further reactions.

3.9 Ligation of DNA Fragments

Ligation reactions were used for inserting sequences, identical to parts of the cyanobacterial chromosome, or antibiotic cassettes into the vector pUC18 or plasmids based on pUC18. Some essentials for ligations are: firstly, to have a small reaction volume (10 µl) and secondly, to add fragment and vector at appropriate ratios. There are two main kinds of ligations:

- sticky-end ligations and
- blunt-end ligations.

Sticky ends are a result of a previous restriction digest of the fragment and vector, performed with restriction enzymes leaving short overhangs.

Blunt ends result from a restriction digest with enzymes leaving no overhangs or when the fragments are created in a PCR with a polymerase, leaving blunt ends, like *Pfu* DNA polymerase [47, 48].

A typical mixture for a ligation reaction is:

x µl	Vector (about 30 fmol or 25–100 ng)
y µl	Fragment (y has to be calculated)
1 µl	T4 DNA Ligase
1 µl	T4 DNA Ligase Buffer (10×)
	H_2O to 10 µl

The ligation mixtures were incubated at 16 °C for 16 h.

The volume of fragment DNA solution was calculated: In a reaction with sticky ends the molar ratio of vector to fragment should be 1 : 1–3, in a reaction with blunt ends 1 : 3–5. 1 bp has a molecular weight of 660 g/mol. For example: the molecular weight of pUC18 (2686 bp) is 1772.76 kg/mol. When pUC18 and CS1 (see 2.2) were ligated, about 65 ng (37 fmol) pUC18 were used. CS1 (966bp) has 637.56 kg/mol, hence, 37 fmol have a mass of 23.6 ng. Hence, for a blunt-end ligation reaction between pUC18 and CS1 about 65 ng pUC18 and 70 ng CS1 were used to achieve a molar ratio of 1 : 3.

A ligation of a vector and a fragment both digested with two different restriction enzymes yielding incompatible sticky ends is called “directional cloning”. After this reaction all closed plasmids should contain equally oriented inserts, nevertheless sometimes unexpected products arise. If one restriction enzyme yielding sticky ends, or one or more restriction enzymes yielding blunt ends are used, an insert will have two possible orientations and additionally the vector will be able to recircularize without insert. Using DNA produced with *Pfu* polymerase also leads to this result. Therefore, a control restriction digest has to be performed after transformation and preparation of the plasmids.

3.10 Transformation

The uptake of free DNA by a bacterium is called transformation. This uptake can be performed by a naturally competent bacterium or it has to be forced by chemical or physical means. Often special treatment of the cells before setting up the transformation increases the number of transformants (see 3.10.1, page 47). In the experiments of this work the DNA to be taken up were plasmids. The uptake of DNA becomes useful when the DNA (or a part of the DNA) is manifested. Manifestation can happen by means of integration into the bacterial chromosome or into a bacterial plasmid or by replication of the transformed plasmid.

3.10.1 Electroporation of *E. coli* DH5α

E. coli DH5α is not naturally competent. The method of choice in my experiments to force the bacteria into taking up DNA was electroporation. It yields high numbers of transformants mostly. It is necessary to prepare the cells for the transformation first.

Preparation of electro-competent DH5α cells

The preparation of the electro-competent cells performed was based on a protocol specially written for *E. coli* DH5α [49]. It is possible to prepare many tubes, ready for electroporation, that can be stored at -80 °C.

- Grow 5 ml of *E. coli* DH5α as described in 3.2.2, page 31.

- Inoculate 500 ml of LB in a Fernbach flask (autoclaved) with the overnight culture. Incubate the cells in a shaker at 200 rpm and 37 °C.

- Check the optical density at 600 nm regularly and harvest the cells when they reach an OD_{600} between 0.5 and 0.75 (mid-log phase).
- Transfer the cells to two precooled sterile 250 ml centrifuge bottles and chill on ice for 15 min.
- Centrifuge the bottles in a JA-14 rotor for 10 min at 6000 rpm and 4 °C.
- Discard the supernatant and resuspend the pellets in 2 x 50 ml sterile dH_2O (4 °C); centrifuge for 10 min at 6000 rpm and 4 °C and repeat this washing step once.
- Discard the supernatant and resuspend the pellets in 2 x 20 ml cold sterile 10 % v/v glycerol.
- Pool the pellets in one 250 ml centrifuge bottle and centrifuge for 10 min at 6000 rpm at 4 °C.
- Discard the supernatant and resuspend the cells in 1 ml cold sterile 10 % v/v glycerol.
- Transfer 75 µl cells into microcentrifuge tubes, freeze in liquid nitrogen and store at -80 °C.

The frozen cells can be stored at -70 °C - -80 °C for 6 month without loss of transformation efficiency [50]. If some of the cells need to be used immediately the freezing step can be omitted and the cells are kept on ice until used.

Electroporation

For the experiments of this work a Bio-Rad Gene Pulser set at 2.5 kV, 25 µF and 200 Ω was used. The electroporation cuvettes and the cuvette slide were precooled at -20 °C as the transformation efficiency drops as much as 100-fold if carried out at room temperature [50]. The cuvettes were new or were washed well with water and sterilized with ethanol after the last use.

- Take the cells from -80 °C and let them thaw on ice.
- Add 1-20 ng of DNA in a volume of 1-2 µl and incubate for 5 min on ice.
- Transfer 60 µl cell suspension to the bottom of sterile precooled electroporation cuvette.

- Dry the outside of the cuvette and the cuvette slide, place the cuvette into the cuvette slide and insert into the cuvette holder. Deliver an electric pulse to the cells with the two red knobs. The time constant shown on the display should be around 4.7 ms.

- Add 1 ml of SOC-medium [51] (room temperature) as quickly as possible to the cells.

SOC-medium:	Yeast extract	5 g/l
	Tryptone	20 g/l
	NaCl	0.5 g/l

  - Add 900 ml dH_2O, adjust the pH to 7.5 with KOH.
  - Fill up to 970 ml with dH_2O and autoclave.
  - Add 5 ml of 1M MgCl and 5 ml of 1M $MgSO_4$ solution (autoclaved).
  - Add 20 ml of autoclaved 1M glucose solution.

- Transfer the cell suspension to a sterile glass test tube with a metal cap and incubate for one hour at 200 rpm and 37 °C in an *E. coli* shaker.

- Transfer the cells to a microcentrifuge tube and concentrate the cells by centrifugation so the volume is 100 µl.

- Streak the cells on a plate with antibiotics (and Xgal if blue-white screening is performed, see 3.11, page 51).

- Incubate the plate at 37 °C over night.

The next day, the plate can be checked for colonies. The colonies are tested whether they took up the desired DNA by the Rapid Boiling Method (3.3.1, page 33) or JAT-Prep (3.3.2, page 34).

3.10.2 Transformation of *Synechococcus* sp. PCC7942

PCC7942 is naturally competent for transformation. Some parameters influence the efficiency of the transformation like incubation time of bacteria with plasmids, incubation in light or dark, amount of DNA or time of culturing before adding antibiotics [17, 18]. Hence the strict observance of the protocol is important to obtain a high number of transformants. The following protocol works for 10 transformations:

- Transfer 30 ml of a dense culture to a 50 ml Falcon tube and centrifuge it for 10 min at room temperature and 4000 g.

- Discard the supernatant, resuspend the pellet in 15 ml of 10 mM NaCl and centrifuge the tube again.

- Discard the supernatant and resuspend the pellet in 3 ml BG11.
- Transfer 300 µl of cell suspension to a 1.5 ml microcentrifuge tube and add 10 ng - 1 µg DNA (in 1-20 µl of TE).
- Wrap the tubes in aluminium foil for dark incubation in a shaker at 120 rpm and 30 °C for 4 to 16 hours.
- Spread 100-150 µl of the cell suspension on a plate with 40 ml BG-11-S-Agar and incubate in light at 32 °C for 4-6 hours.
- Add 400 µl of 100× concentrated antibiotic stock solution under the agar slab. The final concentration of the medium has to be as described on page 30).

Transformed colonies appear after 4-7 days of incubation in the light at 30 °C. The colonies are restreaked on fresh agar plates (including antibiotic) to get sufficient cells to inoculate a liquid culture (including antibiotic). As PCC7942 possesses multiple identical chromosomes it is necessary to test the transformants for integration of the fragment and homozygosity two times:

After growing the cells in BG11 including antibiotic(s) the chromosomal DNA is prepared (see 3.4, page 39) and controlled using a polymerase chain reaction (see 3.5, page 41) with primers for a homozygosity check. The PCR and the subsequent gel electrophoresis show whether the antibiotic cassette was inserted at the desired position in the genome and whether the cells possibly are homozygous. If there is a much higher number of mutant alleles than wild type alleles, the PCR maybe is not able to amplify the wild type DNA sufficiently to get a visible band on a gel. Therefore, the homozygosity is checked further. The cells are cultured for 2 or more cycles in 50 ml liquid media without selective agent. One cycle begins with inoculating 50 ml BG11 (with cells from a plate or 3 ml dense liquid culture), the cells are cultured for about 2 weeks until a dense cell suspension is obtained. With 3 ml of this culture the next 50 ml BG11 are inoculated. In absence of selective pressure, cells containing wild type alleles have no disadvantage over cells containing predominantly mutated alleles. The percentage of wild type alleles in the chromosome pool can rise. DNA preparation followed by PCR and gel electrophoresis are made again to show homozygosity of the mutated cyanobacteria.

3.11 Blue-White Screening

Blue-white screening is a colony screening method based on the complementation of a partially deleted *lacZ* gene on the bacterial chromosome with a fragment containing the deleted part, present on the transformed plasmid. The part present on the plasmid is called α-fragment while the part present on the chromosome is called ω-fragment; the event is called α-complementation. For *E. coli* strains with this *lacZ* mutation, like strain DH5α, the term "*lacZ*ΔM15" is used (see 2.1, page 17). A plasmid suitable for blue-white screening is pUC18 (see 2.2, page 19). It contains the α-fragment of the *lacZ* gene and the *lac* promoter with a multiple cloning site inframe inbetween.

lacZ is the first of three genes of the *lac*-operon *lacZYA*. The gene product of *lacZ* is the enzyme β-galactosidase which hydrolyses β-galactosides into the monosaccharides. When X-gal (5–bromo–4–chloro–3–indolyl–β–D–galactopyranoside) is the substrate of β-galactosidase, the products are galactose and a blue insoluble dye which colours the whole colony.

If DNA is inserted into the multiple cloning site of the plasmid the α-fragment is no longer synthesized. *E. coli* cells without any plasmid die because of antibiotic on the plate, cells with a pUC18 derivative without an insert form blue colonies while colonies with a plasmid with an insert form white colonies [52, 47]. The following protocol is written for blue-white screening of *E. coli* DH5α, transformed with a ligation mixture of pUC18 and a suitable insert in the *lacZ* gene:

- Prepare a plate with LA and 50 µg/ml ampicillin.
- Spread 40 µl X-gal (stock solution 20 mg/ml) with a Drigalski spatula on the plate.
- Spread transformed *E. coli* DH5α cells on the plate.
- Incubate over night at 37 °C.

The next morning blue and white colonies can be visible. If the blue colour is hard to see, incubation at 4 °C for some hours may intensify the blue colour [48]. Some of the white colonies are then checked for containing the correct plasmid. This is done by plasmid preparation, restriction and gel electrophoresis of the restricted fragments to verify that the fragments are of expected length.

3.12 Membrane preparation from PCC7942

During membrane preparation the CM and the ICM were separated and concentrated. The method used is based on a publication of Omata and Murata [53] and was upscaled and modified for working with a zonal rotor.

3.12.1 Harvesting the Cells

- Prepare a 10 l culture, as described in 3.1.3 (page 29);
- Harvest the cells by centrifugation in 500 ml centrifugation bottles at 8000 rpm and room temperature for 10 min with a JA-10 rotor. Discard the supernatant and repeat the centrifugation step until all cells are pelleted.
- Perform a washing step by resuspending the pellets in HEN (2.4, page 23) and centrifuging the bottles at 8000 rpm and room temperature for 10 min.
- Discard the supernatant, resuspend the pellets in some ml of HEN and pool the pellets in 2 bottles. Fill up the bottles with HEN and centrifuge these bottles again.
- Discard the supernatant, resuspend the pellets in some ml of HEN and pool the pellets in one bottle. Fill up the bottle with HEN and centrifuge the bottle.
- Discard the supernatant, resuspend the pellet and fill up with HEN to a weight of 300 g; add 75 g sucrose (= 20 % w/w) and withdraw 400 µl of cell suspension for measuring the cell concentration with hematocrit tubes (see below).
- Add 752 mg lysozyme (= 0.2 % w/w).
- Incubate in a shaker at 100 rpm and 35 °C for 1 h 15 min.

3.12.2 Measuring the Cell Concentration

To measure the cell concentration hematocrit tubes were used. These tubes are normally used to measure the cell concentration of blood cells but can also be used to measure cyanobacterial cell concentrations.

- Fill the capillaries of two tubes with H_2O and add 200 µl cell suspension.

- Place the hematocrit tubes into two 50 ml centrifuge tubes and centrifuge at 4000 g and room temperature for 15 min in a swinging bucket rotor.

- Read the volume of cells on the scale of the tube and multiply it with 5 to get µl cells/ml.

3.12.3 Cell Disruption with a French Pressure Cell

After shaking the cells for 1 h 15 min a 1.5 ml sample was transferred to a microcentrifuge tube to test whether the lysozyme incubation was sufficient. The microcentrifuge tube was centrifuged for 1 minute at 14000 rpm. If the supernatant was bluish, the cells were ready for further processing; otherwise the cells were incubated for another 15 min and tested again.

After taking the cells from the shaker, the bottle was centrifuged at 8000 rpm and room temperature for 10 min. The bluish supernatant was discarded. The pellet was resuspended in cold HEN (4 °C) to obtain a cell density of 80 to 100 µl cells / ml suspension, the optimal concentration for disruption in a 40 ml French Pressure Cell.

- Add 0.0075 % w/w solid DNase I.

- Add PMSF to a final concentration of 1 mM; (PMSF is dissolved in isopropyl alcohol at a concentration of 100 mM).

- Chill the sample on ice.

Before running the French Pressure Cell, all the air was removed by rinsing the device with cold HEN several times.

The cell suspension was treated with a pressure of 680 psi (= 46.88 bar) and extruded from the cylinder very slowly (about 1 drop per second) to have the best disruption effect. Both, the cell suspension and the membrane suspension, were chilled on ice during the operation.

After finishing the cell disruption, the French Pressure Cell was rinsed several times with H_2O and then disassembled. All parts that have been in contact with the cell suspension were cleaned very well with H_2O before they were reassembled.

The membrane suspension was centrifuged at 8000 rpm and 4 °C for 10 min to pellet not disrupted cells and bigger cell debris. The supernatant was transferred to a weighed Erlenmeyer flask to measure the weight of the membrane suspension.

3.12.4 Separation of CM and ICM

The separation of the CM and the ICM was done by a density gradient centrifugation. Sucrose was added to the membrane suspension to a concentration of 42 % (w/w). Additionally, 6 sucrose solutions were prepared: 10 %, 30 %, 35 %, 42 %, 50 % (500 ml each) and 60 % (2 l) sucrose in HEN (w/w). All these solutions were at 4 °C when used.

The solutions were successively pumped into a Beckman Ti-15 Zonal Rotor, rotating with 2000 rpm in an open Beckman Ultracentrifuge, beginning with the 10 % and ending with the 50 % solution. 340 ml of each solution were pumped into the rotor. For the 42 % sucrose solution the membrane suspension was pumped first and then 42 % sucrose / HEN was pumped to give a total volume of 340 ml. For the pumping procedure the filling assembly was put onto the rotor.

When filling the rotor was complete, the filling assembly was taken off and the rotor was sealed with the sealing stopper. The centrifuge was closed and the run was started with a setting of 31000 rpm and 4 °C for 48 h. During the run, each cell component migrated into the sugar solution with the same density. As a result, the gradient contained the various main cell components in the different densities as described in figure 3.1.

After the 31000 rpm run was stopped the centrifuge slowed down to 2000 rpm and was opened. The sealing stopper was removed, the filling assembly positioned on the rotor and a 60 % sucrose solution was pumped into the rotor. The 60 % sucrose solution displaced the different solutions in the rotor which were collected as 10 ml fractions in test tubes. The spectra of these fractions (4.3.1, page 72) were screened for chlorophyll *a* content to determine which fractions were processed further as CM or ICM.

3.12.5 Concentrating the Membranes

The chosen fractions were concentrated by ultracentrifugation. The CM fractions were diluted twofold, the ICM fractions were diluted threefold with HEPES buffer (20 mM HEPES, pH 7.4). 8 Beckman 26.3 ml polycarbonate ultracentrifuge bottles were filled with membrane suspension and centrifuged in a Ti-60 or Ti-70 rotor at 60000 rpm and 4 °C for 50 minutes. The supernatant was discarded and the bottles refilled with membrane suspension. The centrifugation was repeated until the membranes of all chosen fractions were pelleted. The 8 CM pellets were resuspended in 2–3 ml HEPES buffer by vortexing and united in one bottle. Similarly the 8 ICM pellets were transferred to one bottle. Both membrane suspensions were centrifuged at 60000 rpm and 4 °C for 50 min. The supernatant was discarded and the

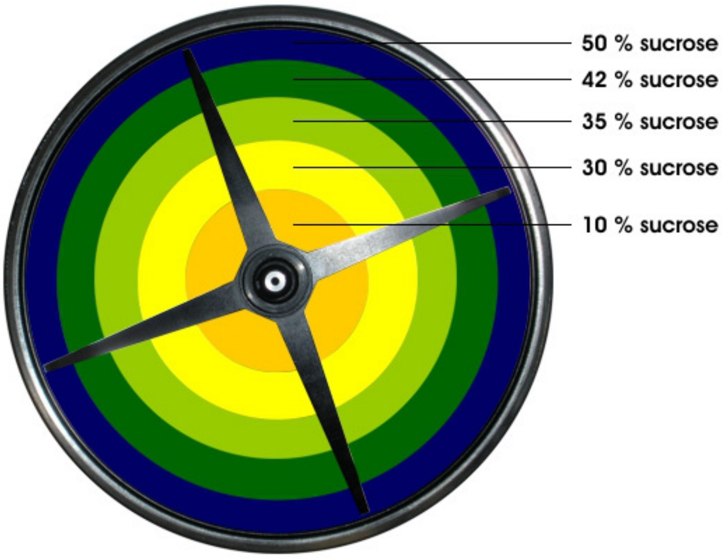


Figure 3.1: Zonal Rotor with sugar gradient. The different densities are shown in colours similar to the real colours of the fractions obtained through the centrifugation. The main (colouring) components of the fractions are: 10 % sucrose: CM with carotenoids, 30 % sucrose: CM, 35 % sucrose: CM and ICM (with chlorophyll *a*), 42 % sucrose: ICM (with chlorophyll *a*), 50 % sucrose: ICM (with chlorophyll *a* and phycocyanin).

membranes resuspended in 2–3 ml HEPES buffer by vortexing and using a Potter Elvehjem Homogenizer.

3.13 Measuring the Protein Concentration

The protein concentration of the membrane suspension was measured using a Bradford (Coomassie Blue) protein assay [54]. Coomassie Blue binds to protein, interacting with the basic groups, with the absorbance maximum of the dye shifting from 465 to 595 nm. The main amino acid acting as reaction partner is arginine, while also histidine, lysine, tyrosine, tryptophan and phenylalanine residues react with Coomassie Blue to a lesser extent [55, 56].

To perform the assay, the Bradford reagent (2.4, page 22) was prepared and a standard curve with BSA in different concentrations was made. The measurement was performed as described below:

- Warm up the spectrophotometer for at least 15 min before use.
- Mix the following solutions in microcentrifuge tubes or directly in polystyrene cuvettes and take the time.

	H_2O [µl]	**protein solution [µl]**	**reagent [µl]**
Standard curve:	50	0	950
	30	20 [100 µg/ml BSA]	950
	0	50 [100 µg/ml BSA]	950
	40	10 [1 mg/ml BSA]	950
	35	15 [1 mg/ml BSA]	950
	30	20 [1 mg/ml BSA]	950
	25	25 [1 mg/ml BSA]	950
Sample:	50 - x	x	950

- After dark incubation for 15–30 min measure the absorbance at 595 nm; the time from mixing to measuring has to be the same for all BSA samples and the membrane sample(s).
- Compare the membrane sample to the standard curve to calculate the protein concentration.

3.14 Reducing Cytochrome *c*

For the measurements of the membrane activity reduced equine heart cytochrome *c* (hereafter cyt *c*) from Sigma Aldrich was used, which is in partly oxidised and partly reduced state. Hence, it had to be further reduced with ascorbic acid and then desalted with a Bio-Rad Econo-Pac® 10DG Column. Thereafter, the degree of reduction was measured.

3.14.1 Reduction

30 mg (2.42 nmol) cyt *c* were dissolved in 1 ml HEPES buffer (20 mM). 5 mg ascorbic acid (approximately tenfold molar excess) were added to ensure total reduction of the cyt *c*.

3.14.2 Desalting

For desalting the protocol of Bio-Rad Econo-Pac® 10DG Columns was used and slightly modified:

- Remove the upper cap of the column and discard the excess buffer.
- Add 20 ml of HEPES-NaCl buffer (20 mM HEPES, 50 mM NaCl) to the column, remove the bottom tip and drain the buffer until its surface reaches the top frit;

- Add the cyt *c* solution (1 ml) to the column and allow it to enter the part of the column beneath the top frit.
- Add 2 ml of HEPES-NaCl buffer to the column and allow it to enter the part of the column beneath the top frit.
- Add 8 ml of HEPES-NaCl buffer and collect 1 ml fractions from the column.

The darkest red fractions were used further on.

3.14.3 Measuring Cyt $c_{\mathbf{red}}$ Amount

Measurement of the reduction state of cyt *c* is based on an absorption peak for reduced cyt *c* at 550 nm (figure 3.2). To calculate the percentage of reduced cyt *c* following steps were made:

- Dilute 15 µl of the desalted cyt *c* solution in 1485 µl HEPES buffer.
- Measure A_{550} of this diluted cyt *c* solution (= E).
- Reduce the measured sample with a little solid ascorbic acid and measure A_{550} again; repeat this step until A_{550} does not increase anymore (highest $A_{550} = E_{(red)}$).
- Add a little solid potassium ferricyanide ($K_3[Fe(CN)_6]$) to oxidise the sample and measure A_{550}; repeat this step until A_{550} does not decrease anymore (lowest $A_{550} = E_{(ox)}$).

The percentage of reduced cyt *c* is:

$$a_{(red)} = \frac{E - E_{(ox)}}{E_{(red)} - E_{(ox)}} \cdot 100$$

The percentage of reduced cyt *c* had to be known in order to determine whether enough reduced cyt *c* for the membrane activity measurements (4.3.3, page 73) were available. If the cyt *c* vial was used for more than 1 day its degree of reduction was measured again. A value between 90 and 99 is optimal. In case a value of 100 was calculated, the possibility that the ascorbic acid was not sufficiently removed was high and the cyt *c* solution was desalted again.

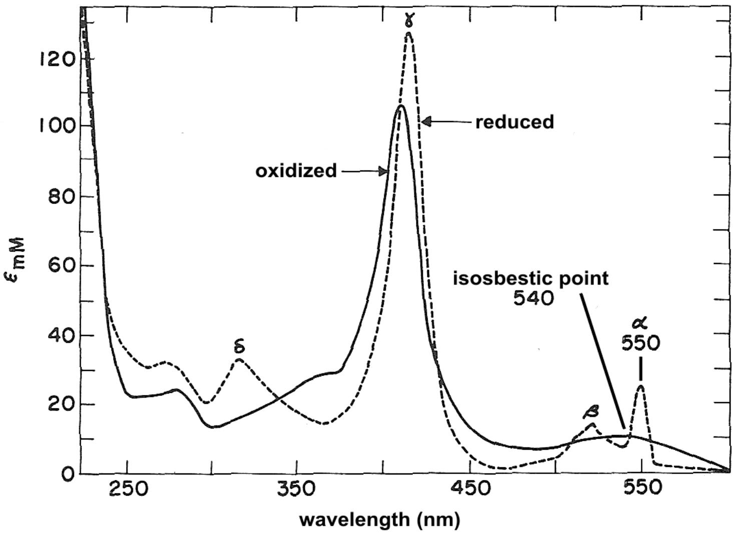


Figure 3.2: Absorbance spectra of reduced and oxidised equine heart cyt *c*. The graph was taken and modified from Margoliash and Walasek [57].

3.15 Cytochrome *c* Oxidase Activity Assay

The cytochrome *c* oxidase activity of the membranes is measured by dual wavelength spectroscopy using the peak of reduced cyt *c* at 550 nm and an isosbestic point (absorption of cyt $c_{(\mathrm{red})}$ equals absorption of cyt $c_{(\mathrm{ox})}$) at 540 nm (figure 3.2).

The spectrophotometer measures $\mathrm{A}_{(550)}$ and $\mathrm{A}_{(540)}$ alternately and reads out $\mathrm{A}_{(550)}$ - $\mathrm{A}_{(540)}$ versus time. With the data a graph is drawn with time (min) on the x-axis and $\mathrm{A}_{(550-540)}$ on the y-axis (4.3.3, figures 4.9 – 4.14, pages 76 – 81). With this graph ΔA/min is calculated.

According to Beer–Lambert´s law

$$A = c \cdot l \cdot \varepsilon$$

where A is the absorption, c is the molar concentration of the absorbing species, l is the width of the cuvette (the distance the light has to travel through the sample) and ε is the differential extinction coefficient for cyt c:

$$\begin{aligned}\varepsilon_{red-ox} &= (\varepsilon_{550} - \varepsilon_{540})_{red} - (\varepsilon_{550} - \varepsilon_{540})_{ox} \\ &= (27.9 - 8.8) - (9.1 - 10.2)\ \mathrm{mM^{-1}cm^{-1}} = 19.9\ \mathrm{mM^{-1}\ cm^{-1}}\end{aligned}$$

The ε values for reduced and oxidised cyt c at 540 and 550 nm were published by Margoliash [58]. With $l = 1$ cm and $\varepsilon = 19.9$ mM^{-1} cm^{-1} it is possible to rearrange the formula to:

$$\Delta c = \Delta A_{(550\text{-}540)} \cdot 50.3\ \mu\mathrm{M}$$

In the experiments of this work Δc is the decrease of the concentration of cyt c_{red}.

The specific activity is defined as:

$$\text{specific activity} = \text{nmol cyt } c_{\mathrm{red}\rightarrow\mathrm{ox}} \cdot \mathrm{min}^{-1} \cdot \mathrm{mg}^{-1}_{(protein)}$$

Using Beer–Lambert´s law and the data from the spectrophotometric analysis, the specific activity is calculated:

$$\text{specific activity} = \frac{\Delta A \cdot 50.3\ \mathrm{nm}_{(\text{cyt } c_{\mathrm{red}\rightarrow\mathrm{ox}})} \cdot 10^{-3} \cdot \mathrm{RV}}{\mathrm{min} \cdot \mathrm{mg}_{(protein)}} \tag{3.1}$$

RV is the reaction volume in the cuvette and has to be entered in the formula in µl.

To measure ΔA/min, quartz cuvettes with stir bars were used. The zero line was adjusted with HEPES buffer. Then cyt *c* solution was added and the absorptions were measured for a short time to show that ΔA was constant. The membrane suspension was added and ΔA was measured for some minutes to obtain a reliable ΔA/min value. To show that cytochrome *c* oxidases accounted for the measured activity, the oxidation of cyt *c* was stopped with KCN which inhibits cytochrome *c* oxidases.

For the measurements a Varian Cary 500 UV-VIS NIR spectrophotometer with the following settings was used:

- User Collect: Read(550) - Read(540)
- Block Temperature: 30 °C
- Spectral Bandwidth (SBW): 1 nm
- Start (min): 0
- Stop (min) : 30
- Time Factor: 1

Chapter 4

Experiments and Results

4.1 Construction of PCC7942cox^-

Prior to this work we received a PCC7942cox^- mutant from Dr. S. Golden. This mutant was made during a circadian clock project with the aim of constructing single knockout mutants for each ORF in PCC7942. The mutant was an insertion mutant with the insertion number 8B10-C10 [36] created with the GeneJumper™ Primer Insertion Kit (Invitrogen™). It contained a chloramphenicol resistance cassette inserted into the *coxA* locus. After testing the strain it turned out not to be homozygous (figure 4.1). Hence, we decided to construct another PCC7942cox^- mutant with a Sm/Sp^R cassette inserted by double recombination into the *coxBAC* locus (see 2.1, page 18 and figure 2.2, page 18).

4.1.1 Construction of plasmids pHBUV9 and pHBUV11

To construct the cox^- knockout plasmids pHBUV9 and pHBUV11 three intermediate plasmids had to be created first. An overview of the plasmids is given in table 4.1.

Plasmid	**Inserts**
pHBUV1	CS1
pHBUV3	CS2
pHBUV7	CS1 + CS2
pHBUV9	CS1 + $\overrightarrow{Sm/Sp^R}$ + CS2
pHBUV11	CS1 + $\overleftarrow{Sm/Sp^R}$ + CS2

Table 4.1: List of the constructed *cox* knockout plasmids and their inserts

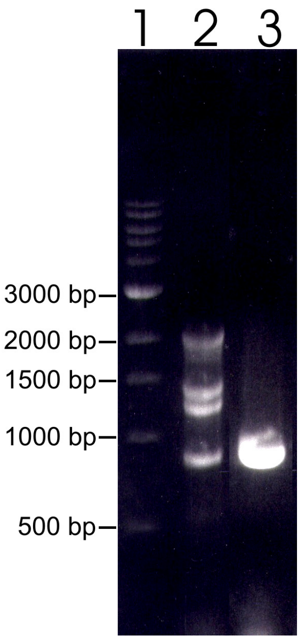


Figure 4.1: Heterozygosity of Dr. Golden´s PCC7942cox^- mutant shown by PCR. Amplification of the *cox* locus using different DNAs as substrate and the primers 7942cox1 and 7942cox2 (see 2.3, page 22): lane 1: 1 kb DNA Ladder (NEB), 2: PCC7942cox^-, 3: PCC7942 wild type. wild type band: 866 bp, mutant band: 2185 bp

Construction of plasmids pHBUV1 and pHBUV3

The fragments CS1 and CS2 are identical to sequences of the cyanobacterial genome of PCC7942. They were needed to initiate double recombination of the plasmids pHBUV9 and pHBUV11 to produce the cox^- knockout mutants. Further information about these fragments is given in 2.2, page 20. These fragments were created by polymerase chain reactions using the primer pairs listed in 2.3, page 21 and *Pfu* polymerase, yielding fragments with blunt ends. The DNA template was prepared from PCC7942 wild type as described in 3.4, page 39.

The polymerase chain reaction was set up as described in 3.5, page 41, with an annealing temperature of 50 °C and an elongation time of 120* seconds.

pUC18 was cut with *Sma*I, a restriction enzyme producing blunt ends. Its recognition site is `5´...CCCGGG...3´` which is found in the multiple cloning site of pUC18. The restriction was performed as described in 3.6, page 42, using 3 µg of plasmid, *Sma*I and buffer Tango™ from Fermentas. The restriction was performed at 25 °C and stopped after 2 hours by heat inactivation at 65 °C for 20 min.

The ligations were performed as described in 3.9, page 46. The fragments CS1 and CS2 were ligated into pUC18 using a molar ratio of plasmid to fragment of about 1 : 3. The reactions were performed at 16 °C for 16 hours.

E. coli was transformed with the ligation mix using electroporation as described in 3.10.1, page 48. The electroporated cell suspensions were streaked on plates containing ampicillin and Xgal. Blue-white screening was used the next day to find transformed colonies with inserts (3.11, page 51).

Several white colonies were examined further by restriction digest. As *Sma*I yields blunt ends, CS1 and CS2 had two possibilities to insert into pUC18. Two colonies containing plasmids with CS1 and CS2 ligated into pUC18 with the part of the *coxB* ORF parallel to the *lacZ* ORF were propagated further. These orientations had the best distributions of restriction sites for the further steps. The plasmid containing CS1 in this orientation was called pHBUV1, the plasmid containing CS2 in this orientation was called pHBUV3.

Construction of plasmid pHBUV7

pHBUV3 was sequentially digested with *Bam*HI (recognition site `5´...GGATCC...3´`) and *Hin*dIII (recognition site `5´...AAGCTT...3´`) yielding a CS2 fragment with one *Bam*HI and one *Hin*dIII end. The polylinker region of pHBUV1 was cut with *Bam*HI and *Hin*dIII adjacent to CS1.

The first restriction digest was made with *Hin*dIII and NEB Buffer 2. After 2 hours incubation at 37 °C the reaction was stopped by heat inactivation at 65 °C for 20 min. 2 µl of 50 mM NaCl solution were added to obtain the molar concentrations of NEB Buffer 3, *Bam*HI was added and the sample was incubated for another 2 hours at 37 °C.

pHBUV1 and CS2 were purified by gel electrophoresis and gel extraction. The ligation was performed as described before.

The ligation mixture was electroporated into *E. coli* DH5α and the cell suspension was streaked on plates containing ampicillin. As neither antibiotics nor blue-white screening could be used to select transformants containing pHBUV7, an enlarged screening for colonies with plasmids was done with JAT-Prep (3.3.2, page 34). Several of the promising colonies were propagated further to prepare the plasmids with the Rapid Boiling Method. They were checked with restriction digests and cells which contained the desired plasmid were frozen and used for plasmid maxi preparation.

Inserting the (Sm/Sp^{R}) cassette into pHBUV7

E. coli strains with pRL463 (containing the Sm/Sp^{R} cassette, 2.2, page 20) and pHBUV7, were cultured in 500 ml LB medium each. The plasmids were purified by plasmid maxi preparations.

Both plasmids were cut with the restriction enzyme *Bam*HI. The restriction digests were incubated at 37 °C for 2 hours. The antibiotic resistance cassette and the linearized pHBUV7 were purified by gel electrophoresis followed by gel extraction and were then ligated.

As pHBUV7 and the cassette were cut with only one enzyme (*Bam*HI), the antibiotic resistance cassette was ligated into the plasmid in two different directions. Depending on the orientation of the cassette the plasmids were called pHBUV9 or pHBUV11 (see 2.2, page 20 for definitions of the plasmids).

The ligation mixture was electroporated into *E. coli* DH5α and the cell suspension was streaked on a plate containing ampicillin and streptomycin.

Validation of pHBUV9 and pHBUV11

Some colonies that grew on streptomycin were picked and cultured for preparation of the plasmids by the Rapid Boiling Method and for freezing if appropriate. The plasmids were tested with 3 different restriction enzymes to verify that they had the expected inserts (figure 4.2).

The gel showed that the plasmids were cut into fragments with expected lengths (table 4.2) and that the colonies harboured the desired plasmids. The fragment lengths were calculated with the online program NEBCUTTER [59] from NEB.

	*Bam*HI	*Alw*NI	*Xmn*I
pHBUV9:	4484, 2056	4487, 2053	3359, 3181
pHBUV11:	4484, 2056	3496, 3044	4523, 2017

Table 4.2: Restriction fragment lengths of pHBUV9 and pHBUV11(in bp).

Maps of pHBUV9 and pHBUV11 are shown in figure 4.3. *E. coli* containing the desired plasmids were frozen and used for plasmid preparations with CsCl Gradient Centrifugation (3.3.5, page 37) to obtain plasmids as pure as possible for the transformation of PCC7942.

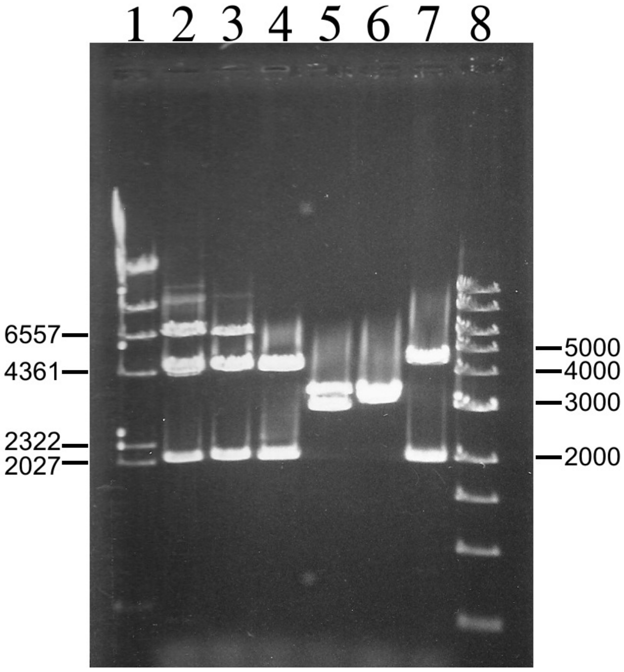
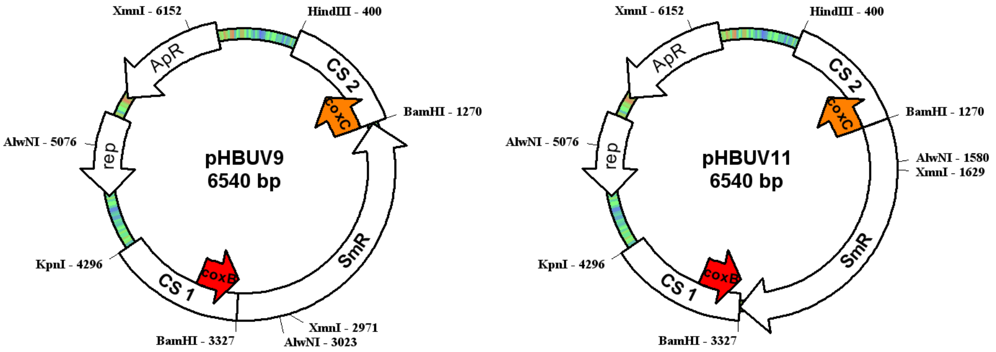

1 2 3 4 5 6 7 8
6557
4361
2322
2027
5000
4000
3000
2000

Figure 4.2: Restriction of pHBUV9 and pHBUV11.
lane 1: Marker Lambda×*Hind*III, 2: pHBUV9×*Bam*HI, 3: pHBUV11×*Bam*HI, 4: pHBUV9×*Alw*NI, 5: pHBUV11×*Alw*NI, 6: pHBUV9×*Xmn*I, 7: pHBUV11×*Xmn*I, 8: 100 bp DNA Ladder (NEB).


XmnI - 6152
HindIII - 400
ApR
CS 2
coxC
BamHI - 1270
AlwNI - 5076
rep
pHBUV9
6540 bp
SmR
KpnI - 4296
CS 1
coxB
XmnI - 2971
AlwNI - 3023
BamHI - 3327
XmnI - 6152
HindIII - 400
ApR
CS 2
coxC
BamHI - 1270
AlwNI - 5076
rep
pHBUV11
6540 bp
AlwNI - 1580
XmnI - 1629
SmR
KpnI - 4296
CS 1
coxB
BamHI - 3327

(a) (b)

Figure 4.3: Plasmids pHBUV9 and pHBUV11. CS = *cox* sequence; *coxB* and *coxC* are parts of the open reading frames of the *coxBAC* locus; SmR = streptomycin resistance cassette; ApR = ampicillin resistance cassette; rep = replicon.

4.1.2 Transformation of PCC7942 and Homozygosity Check

To obtain the cox^- knockout mutants the PCC7942 wild type strain was transformed with plasmids pHBUV9 and pHBUV11. Several transformation experiments were performed with a PCC7942 wild type strain obtained from the Pasteur Culture Collection of Cyanobacteria in Paris. None of these experiments resulted in successfully transformed colonies. Another PCC7942 wild type strain was obtained from Dr. S. Golden and the transformation of this strain worked at the first attempt. The transformations with both strains were performed as described in 3.10.2, page 49. While not a single cell of the Pasteur Culture Collection strain was transformed, more than 1000 colonies were visible on each plate with selective media one week after transformation of PCC7942 cells from Dr. S. Golden´s lab. Obviously the vial obtained from the Pasteur Culture Collection contained a PCC7942 strain with a mutation preventing transformation or homologous recombination.

The cells transformed with pHBUV9 were called PCC7942$cox^-{}_f$, the cells transformed with pHBUV11 were called PCC7942$cox^-{}_r$.

Some of the colonies that grew on the selective media were cultured further in liquid BG11 medium containing spectinomycin. After obtaining dense 50 ml cultures, cells were used to check whether they had integrated the antibiotic resistance cassettes into the desired sequences of the chromosome. DNA preparations (3.4, page 39) were performed and the DNAs were used for polymerase chain reactions (3.5, page 41). For validating the homozygosity of the cox^- mutants the primers cox7942-7 and cox7942-8 were used in a reaction with an annealing temperature of 51 °C and an elongation time of 150* s. For both strains only mutated chromosomes were detected. To corroborate this result another PCR was performed with DNA from cells after 3 growth cycles without selective agent (3.10.2, page 49). The results of the second PCR are shown in figure 4.4. The expected fragment lengths were 2913 bp for the wild type and 2597 bp for the mutant PCR product.

A result obtained from this experiment was, that it is possible to create homozygous cox^- knockout mutants of PCC7942. Hence, the Cox enzyme is not essential for PCC7942.

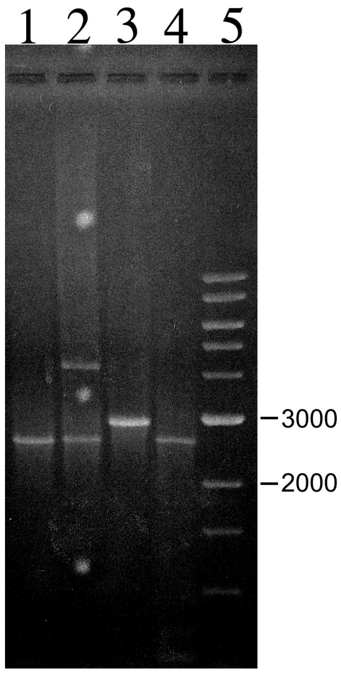


Figure 4.4: Homozygosity of the *cox*$^-$ mutants shown by PCR.
Amplification of the *cox* locus using different DNAs as substrate and the primers cox7942-7 and cox7942-8: lane 1: PCC7942*cox*^-_f, 2: pHBUV9, 3: PCC7942 wild type, 4: PCC7942*cox*^-_r, 5: 1 kb DNA Ladder (NEB).

4.2 Construction of PCC7942*qox*$^-$

As it was shown that it is possible to obtain homozygous knockouts of Cox (this work) and Cbb$_3$ (obtained from Dr. S. Golden) it was interesting whether also the third respiratory terminal oxidase Qox is dispensible in PCC7942. A *qox*$^-$ mutant was constructed with a Km^R cassette inserted by double recombination into the *cydAB* locus encoding the Qox enzyme. The kanamycin resistance cassette was chosen to obtain 3 mutants of 3 different respiratory terminal oxidases with 3 different resistances in the end. So it will be possible to construct double and triple mutants based on these single mutants in the future.

4.2.1 Construction of plasmids pHBUV10 and pHBUV12

To construct the *qox*$^-$ knockout plasmids pHBUV10 and pHBUV12 three intermediate plasmids had to be created first. An overview of the plasmids is given in table 4.3.

Plasmid	Inserts
pHBUV2	QS1
pHBUV4	QS2
pHBUV8	QS1 + QS2
pHBUV10	QS1 + $\overrightarrow{Km^R}$ + QS2
pHBUV12	QS1 + $\overleftarrow{Km^R}$ + QS2

Table 4.3: List of the constructed *qox* knockout plasmids and their inserts

Construction of plasmids pHBUV2 and pHBUV4

The fragments QS1 and QS2 are identical to sequences of the cyanobacterial genome of PCC7942. They were needed to initiate double recombination of the plasmids pHBUV10 and pHBUV12 to produce the qox^- knockout mutants. Further information about these fragments is given in 2.2, page 20. The fragments were created by polymerase chain reactions (primers qox1 – qox4, annealing temperature 50 °C, elongation time 120* s) and ligated into pUC18, cut with *Sma*I. Restriction and ligation were performed as described for the construction of pHBUV1 and pHBUV3 (4.1.1, page 62). The ligation mix was electroporated into *E.coli*. Screening for transformed colonies was performed with ampicillin and Xgal. The isolated plasmids from white colonies were verified by restriction digest with *Pst*I (cutting in pUC18 and QS1) and *Kpn*I (cutting in pUC18 and QS2). A plasmid with the *cydB* ORF of QS1 ligated antiparallel to the *lacZ* ORF was called pHBUV2. A plasmid with the *cydA* ORF of QS2 parallel to the *lacZ* ORF was called pHBUV4.

Construction of plasmid pHBUV8

Fragment QS2 was excised from pHBUV4 with *Bam*HI and *Hin*dIII. The polylinker region of pHBUV2 was cut with *Bam*HI and *Hin*dIII adjacent to QS1. This digest was performed the same way, as described for pHBUV7 (4.1.1, page 63).

QS2 was ligated into pHBUV2, the ligation mixture was electroporated into *E. coli* DH5α and the cell suspension was streaked on plates containing ampicillin. Screening was performed with JAT-Prep and the Rapid Boiling Method. Cells which contained the desired plasmid were frozen and used for plasmid maxi preparation.

Inserting the (Km^R) cassette into pHBUV8

E. coli strains with pRL446 (containing the Km^R cassette, 2.2, page 20) and pHBUV8, were cultured in 500 ml LB medium each. The plasmids were purified by plasmid maxi preparations.

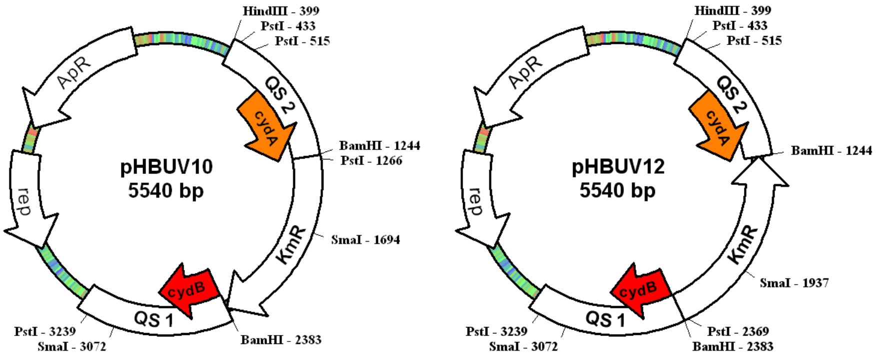
Both plasmids were cut with the restriction enzyme *Bam*HI. The antibiotic resistance cassette and the linearized pHBUV8 were purified by gel electrophoresis followed by gel extraction. The cassette was ligated into the plasmid and depending on the orientation of the cassette the plasmids were called pHBUV10 or pHBUV12 (see 2.2, page 20 for definitions of the plasmids).

The ligation mixture was electroporated into *E. coli* DH5α and the cell suspension was streaked on a plate containing ampicillin and kanamycin.

Validation of pHBUV10 and pHBUV12

Some colonies that grew on kanamycin were picked and cultured for plasmid preparation by the Rapid Boiling Method and for freezing if appropriate. The plasmids were tested with 3 different restriction enzymes to verify that they had the expected inserts (figure 4.6).

Maps of pHBUV10 and pHBUV12 are shown in figure 4.5. *E. coli* containing the desired plasmids were frozen and used for plasmid preparations with CsCl Gradient Centrifugation (3.3.5, page 37) to obtain plasmids as pure as possible for the transformation of PCC7942.


HindIII - 399
PstI - 433
PstI - 515
ApR
QS 2
cydA
pHBUV10
5540 bp
BamHI - 1244
PstI - 1266
rep
KmR
SmaI - 1694
cydB
QS 1
PstI - 3239
SmaI - 3072
BamHI - 2383
HindIII - 399
PstI - 433
PstI - 515
ApR
QS 2
cydA
pHBUV12
5540 bp
BamHI - 1244
rep
KmR
SmaI - 1937
cydB
QS 1
PstI - 3239
SmaI - 3072
PstI - 2369
BamHI - 2383

(a) (b)

Figure 4.5: Plasmids pHBUV10 and pHBUV12. QS = *qox* sequence; *cydA* and *cydB* are parts of the open reading frames of the *qox* locus; KmR = kanamycin resistance cassette; ApR = ampicillin resistance cassette; rep = replicon.

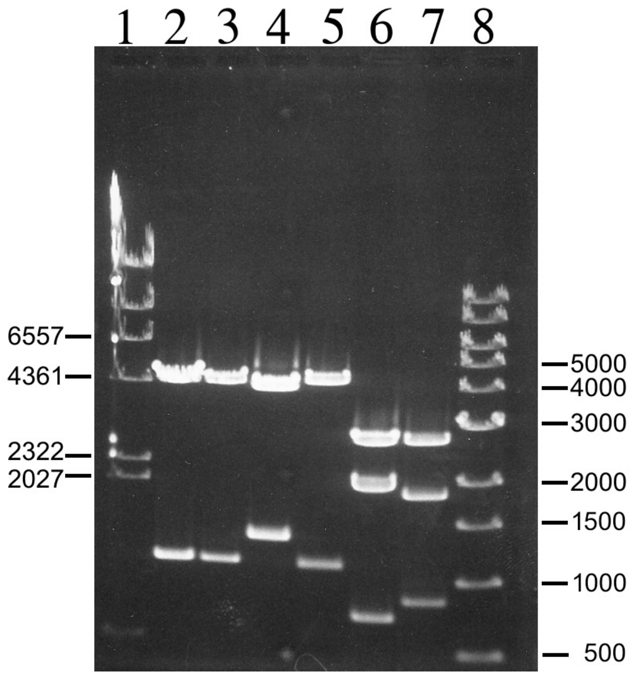


Figure 4.6: Restriction of pHBUV10 and pHBUV12.
lane 1: Marker Lambda×*Hin*dIII, 2: pHBUV10×*Bam*HI, 3: pHBUV12×*Bam*HI, 4: pHBUV10×*Sma*I, 5: pHBUV12×*Sma*I, 6: pHBUV10×*Pst*I, 7: pHBUV12×*Pst*I, 8: 100 bp DNA Ladder (NEB).

The gel showed that the plasmids were cut into fragments with expected lengths (table 4.4) and that the colonies harboured the desired plasmids.

	*Bam*HI	*Sma*I	*Pst*I
pHBUV10:	4401, 1139	4162, 1378	2734, 1973, 751, (82)
pHBUV12:	4401, 1139	4405, 1135	2734, 1854, 870, (82)

Table 4.4: Restriction fragment lengths of pHBUV10 and pHBUV12 (in bp).

4.2.2 Transformation of PCC7942 and Homozygosity Check

To obtain the *qox*$^-$ knockout mutants the PCC7942 wild type strain (from Dr. S. Golden´s lab) was transformed with the plasmids pHBUV10 and pHBUV12 as described in 3.10.2, page 49. After one week more than 1000 colonies were visible. The transformation efficiency was similar for all 4 plas-

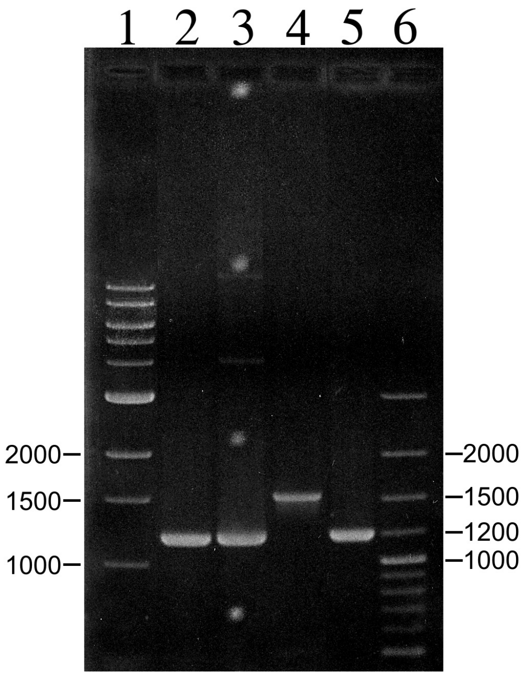


Figure 4.7: Homozygosity of the qox^- mutants shown by PCR.
Amplification of the *qox* locus using different DNAs as substrate and the primers qox5 and qox6: lane 1: 1 kb DNA Ladder (NEB), 2: PCC7942qox^{-}_{f}, 3: pHBUV10, 4: PCC7942 wild type, 5: PCC7942qox^{-}_{r}, 6: 100 bp DNA Ladder (NEB).

mids (pHBUV9 – pHBUV12) used.

The cells transformed with pHBUV10 were called PCC7942qox^{-}_{f}, the cells transformed with pHBUV12 were called PCC7942qox^{-}_{r}.

Some of the colonies that grew on kanamycin were cultured further in liquid medium containing kanamycin. DNA preparations (3.4, page 39) were performed and the DNAs were used for polymerase chain reactions (3.5, page 41). For validating the homozygosity of the qox^- mutants the primers qox5 and qox6 were used in a reaction with an annealing temperature of 54 °C and an elongation time of 80* s. This, and another PCR performed with DNA from cells after 3 growth cycles in liquid media without selective agent (see 3.10.2, page 49) proved homozygosity of the mutated cyanobacteria. The results of the second PCR are shown in figure 4.7. The expected fragment lengths were 1551 bp for the wild type and 1199 bp for the mutant PCR product.

The success in creating homozygous qox^- mutants also showed that *all* respiratory terminal oxidases are dispensible in PCC7942.

After proving the homozygosity, the mutated cyanobacterial strains were frozen and stored at -80 °C (3.1.5, page 30). Additionally the PCC7942$cox^-{}_f$ mutant was cultivated further in liquid cultures for membrane preparations and cytochrome *c* oxidase activity tests.

4.3 Cytochrome *c* Oxidase Activities

After the molecular biological part of this work 3 different strains of PCC7942 were available for measurements of cyt *c* oxidase activities. PCC7942 wild type, PCC7942$cox^-{}_f$ and PCC7942$ccoN^-$ were tested for potential cyt *c* oxidase activity in both of their bioenergetic membrane systems, the CM and the ICM.

The PCC7942 wild type and the two mutant strains were cultured in 10 l bottles (3.1.3, page 29). The membranes were separated by sugar gradient centrifugation as described in 3.12, page 52. Before the membrane fractions were concentrated by ultracentrifugation, the sugar concentration and the spectra of every tenth fraction were measured.

4.3.1 Spectra of the Membrane Fractions

The spectra of the membrane fractions were used to consider which fractions were processed further as CM or ICM. Those fractions which did not have any significant absorption at 673 nm (peak for chlorophyll *a* in HEN) were processed as CM, while those fractions with a high absorption at 673 nm and a sugar content between 42 and 47 % w/v were processed as ICM. The absorption spectra were measured with a Varian Cary 500 UV-VIS NIR spectrophotometer in a range of 250 nm to 800 nm. As the results for wild type and mutants were very similar, only spectra of the wild type are shown in figure 4.8.

In the fractions with higher sugar concentrations the chlorophyll *a* peak (673 nm) and the protein peak (270 nm) are higher due to large amounts of the photosystem proteins and chlorophyll *a* in the ICM. In addition chlorophyll *a* contributes to the peak at 435 nm but this peak cannot be used to distinguish CM and ICM as also carotenoids absorb in this region. The absorption at 673 nm was not significant until fraction 31. Hence only fractions from 1 – 30 were further processed as CM.

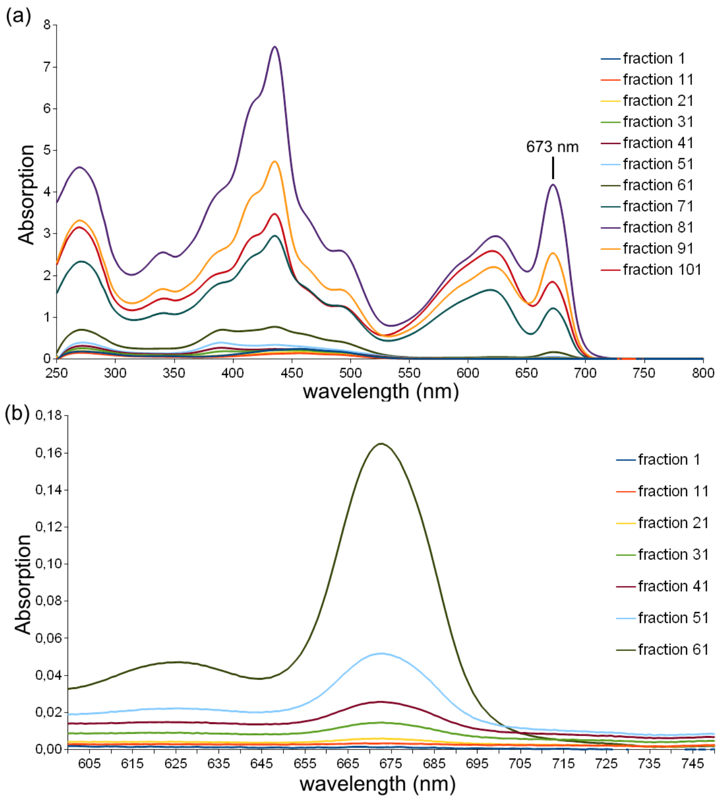


Figure 4.8: Spectra of the membrane fractions of PCC7942 wild type.
(a) spectra from 250 – 800 nm of every tenth sample from fraction 1 to fraction 101.
(b) spectra from 600 – 750 nm of every tenth sample from fraction 1 to fraction 61 to emphasise the region of the chlorophyll *a* peak

4.3.2 Membrane Protein Concentrations

The membranes were concentrated by ultracentrifugation (3.12.5, page 54) and the protein concentrations were measured with Bradford Protein Assays (3.13, page 55). The results are shown in table 4.5.

These protein amounts were the reference values to determine the cyt *c* oxidase activities (formula 3.1, page 59). The vastly higher protein content of the ICM compared to the CM is primarily due to the absence of the photosynthetic apparatus in the CM (compare figure 4.8).

4.3.3 Measurements of Cytochrome *c* Oxidation

For measuring the cyt *c* oxidation, HEPES buffer, reduced cyt *c*, membrane suspension and KCN (if necessary) were added as described in 3.8, page 59. The amounts of protein used for oxidising cyt *c* and the total reaction volumes

Sample	**Protein Concentration [µg/ml]**
CM wt	449
CM *cox*$^-$	761
CM *ccoN*$^-$	759
ICM wt	10514
ICM *cox*$^-$	10554
ICM *ccoN*$^-$	15770

Table 4.5: Results of the Bradford Protein Assay.

can be found in the cyt *c* oxidation diagrams (figures 4.9 – 4.14).

The spectrophotometer measured the $A_{(550-540)}$ (y-axis) versus time (x-axis) and listed these data. The complete measured data were exported to LibreOffice 3.4.5 spreadsheets and converted to graphs (hereafter called complete data diagrams, figures 4.9(a)– 4.14(a)). For the calculation of ΔA / min only the linear range following the addition of membranes until the flattening of the curve was used. For that reason further graphs (linear range diagrams) were made with these linear ranges during the cyt *c* oxidation. The slopes (ΔA / min) were then calculated from these linear range diagrams (figures 4.9(b)– 4.14(b)).

All complete data diagrams contain a first abrupt increase in $A_{(550-540)}$, which is due to the addition of reduced cyt *c*. This increase is followed by a horizontal zone where $\Delta A_{(550-540)}$ has to be zero to show that no oxidative agent is present in the sample. After some minutes the membrane suspension was added. This addition becomes apparent as an abrupt decrease in $A_{(550-540)}$. Then the line either slopes down slowly which means, that cyt *c* is oxidised or becomes horizontal again, which means that cyt *c* is not oxidised. If the line was not horizontal at this point, KCN was added after some minutes. This KCN addition leads to a horizontal line in the graph, if the oxidation of cyt *c* is due to respiratory terminal oxidases.

The diagrams for the wild type (4.9 and 4.12) show a decrease of $A_{(550-540)}$ (oxidation) after addition of membrane suspension. CM and ICM suspensions oxidise cyt *c* and the oxidation can be stopped by addition of KCN. Hence, in both membranes, CM and ICM of PCC7942, active cytochrome *c* oxidases exist.

The CM diagrams for PCC7942*cox*$^-$ (figure 4.10) show a horizontal line (no oxidation) after addition of membrane suspension. As the wild type shows activity in the CM, Cox must be the only active cytochrome *c* oxidase in the wild type CM.

The ICM diagrams for PCC7942cox^- (figure 4.13) show a decrease of $A_{(550-540)}$ after addition of membrane suspension. As Cbb3 is the only putative cytochrome *c* oxidase left in this strain, Cbb3 must be an active cytochrome *c* oxidase that is performing the whole cyt *c* oxidation in the ICM of the PCC7942cox^- mutant.

The CM diagrams for PCC7942$ccoN^-$ (figure 4.11) show a decrease of $A_{(550-540)}$ after addition of membrane suspension. This result corroborates the statement that Cox is the active cytochrome *c* oxidase in the wild type CM.

The ICM diagrams for PCC7942$ccoN^-$ (figure 4.14) show a horizontal line after addition of membrane suspension. As the ICM suspensions of the wild type and the PCC7942cox^- mutant oxidise cyt *c*, while PCC7942$ccoN^-$ ICM does not, Cbb3 must be the only active cytochrome *c* oxidase in the ICM of PCC7942.

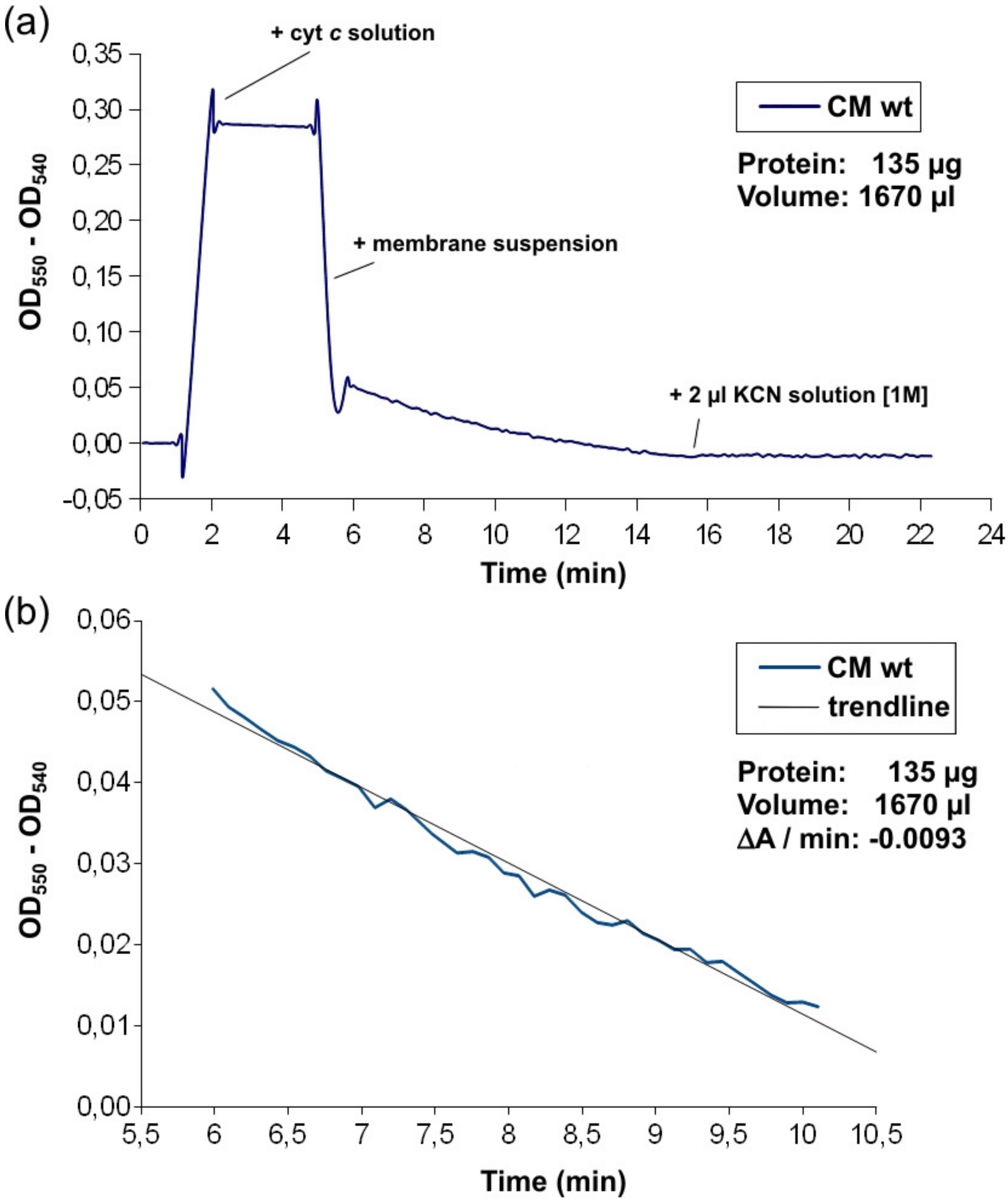


Figure 4.9: (a) Complete data diagram of cyt *c* oxidation by cytochrome *c* oxidase(s) in the CM of PCC7942 wt.
(b) Linear range diagram showing oxidation of cyt *c* by cytochrome *c* oxidase(s) in the CM of PCC7942 wt.

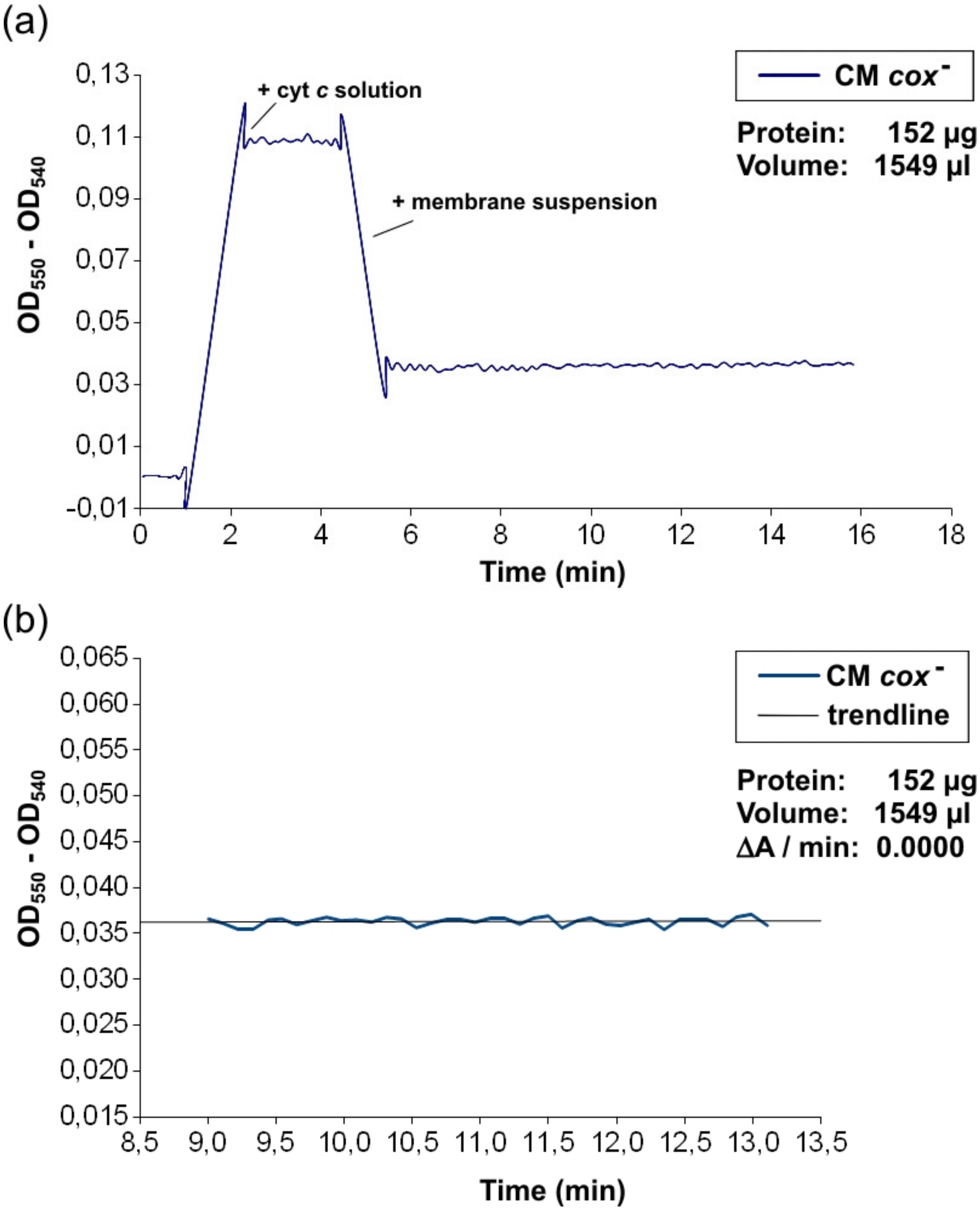


Figure 4.10: (a) Complete data diagram of cyt *c* oxidation by cytochrome *c* oxidase(s) in the CM of PCC7942cox^- mutant.
(b) Linear range diagram showing no oxidation of cyt *c* by cytochrome *c* oxidase(s) in the CM of PCC7942cox^- mutant.

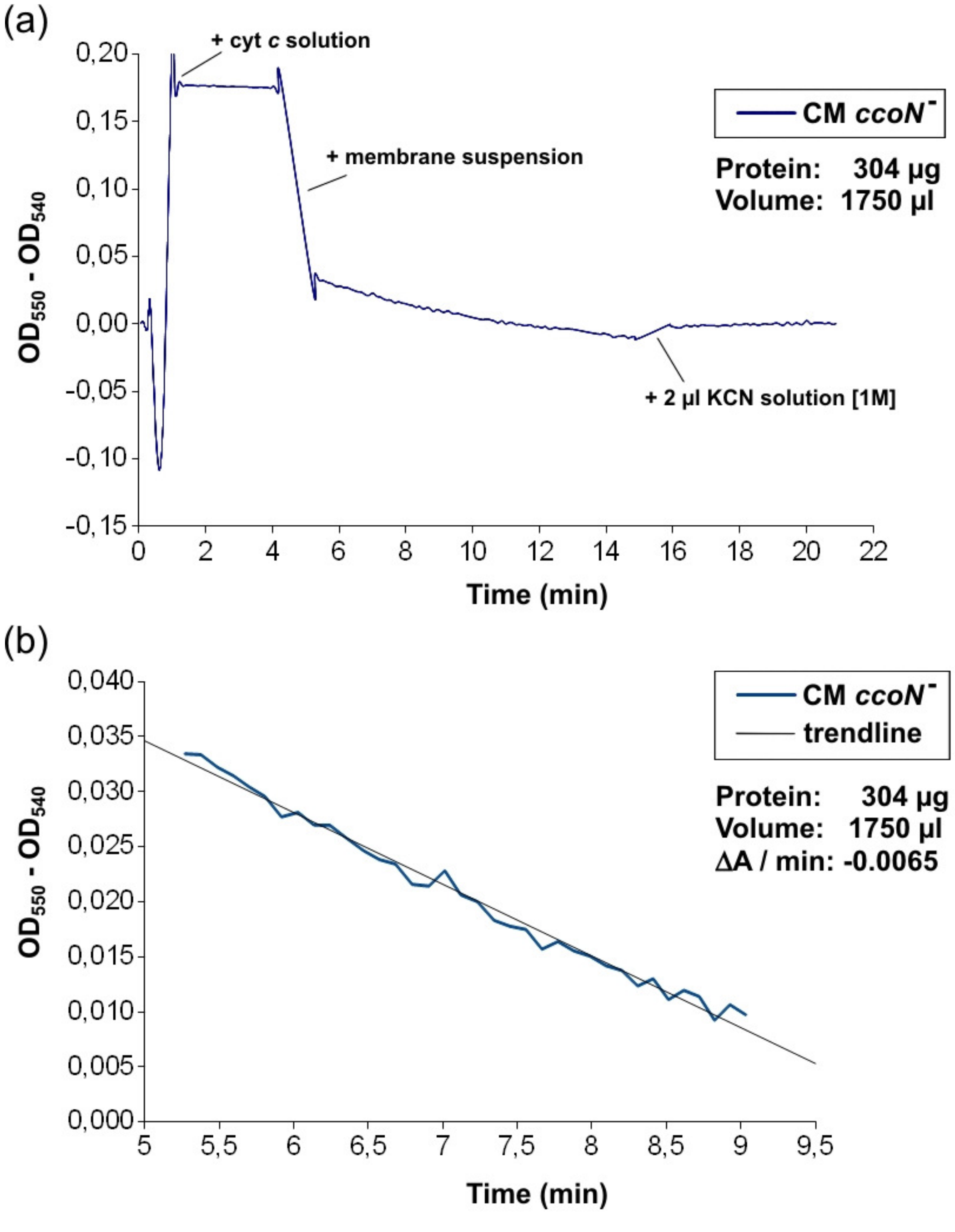


Figure 4.11: (a) Complete data diagram of cyt c oxidation by cytochrome c oxidase(s) in the CM of PCC7942$ccoN^-$ mutant.
(b) Linear range diagram showing oxidation of cyt c by cytochrome c oxidase(s) in the CM of PCC7942$ccoN^-$ mutant.

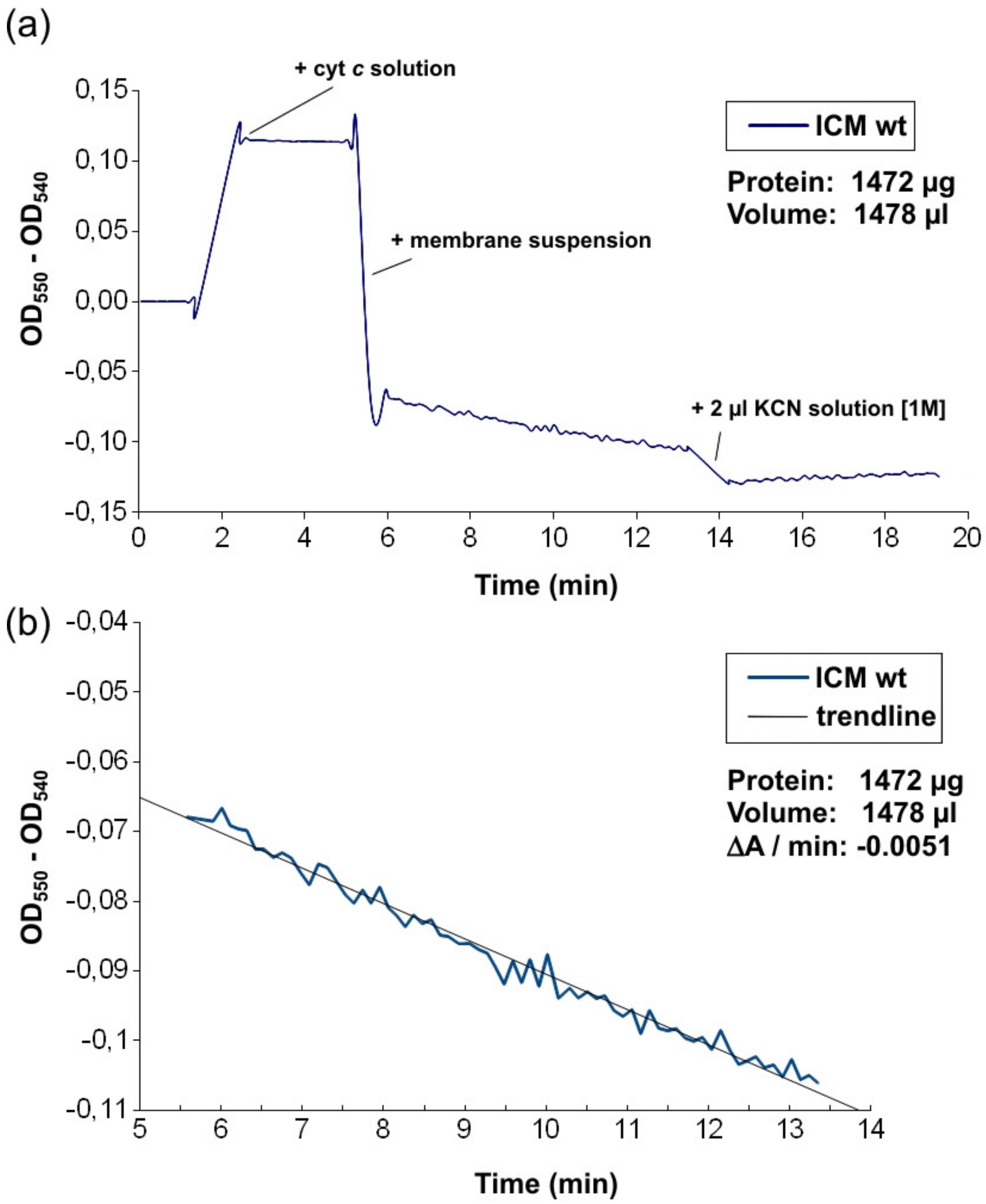


Figure 4.12: (a) Complete data diagram of cyt *c* oxidation by cytochrome *c* oxidase(s) in the ICM of PCC7942 wt.
(b) Linear range diagram showing oxidation of cyt *c* by cytochrome *c* oxidase(s) in the ICM of PCC7942 wt.

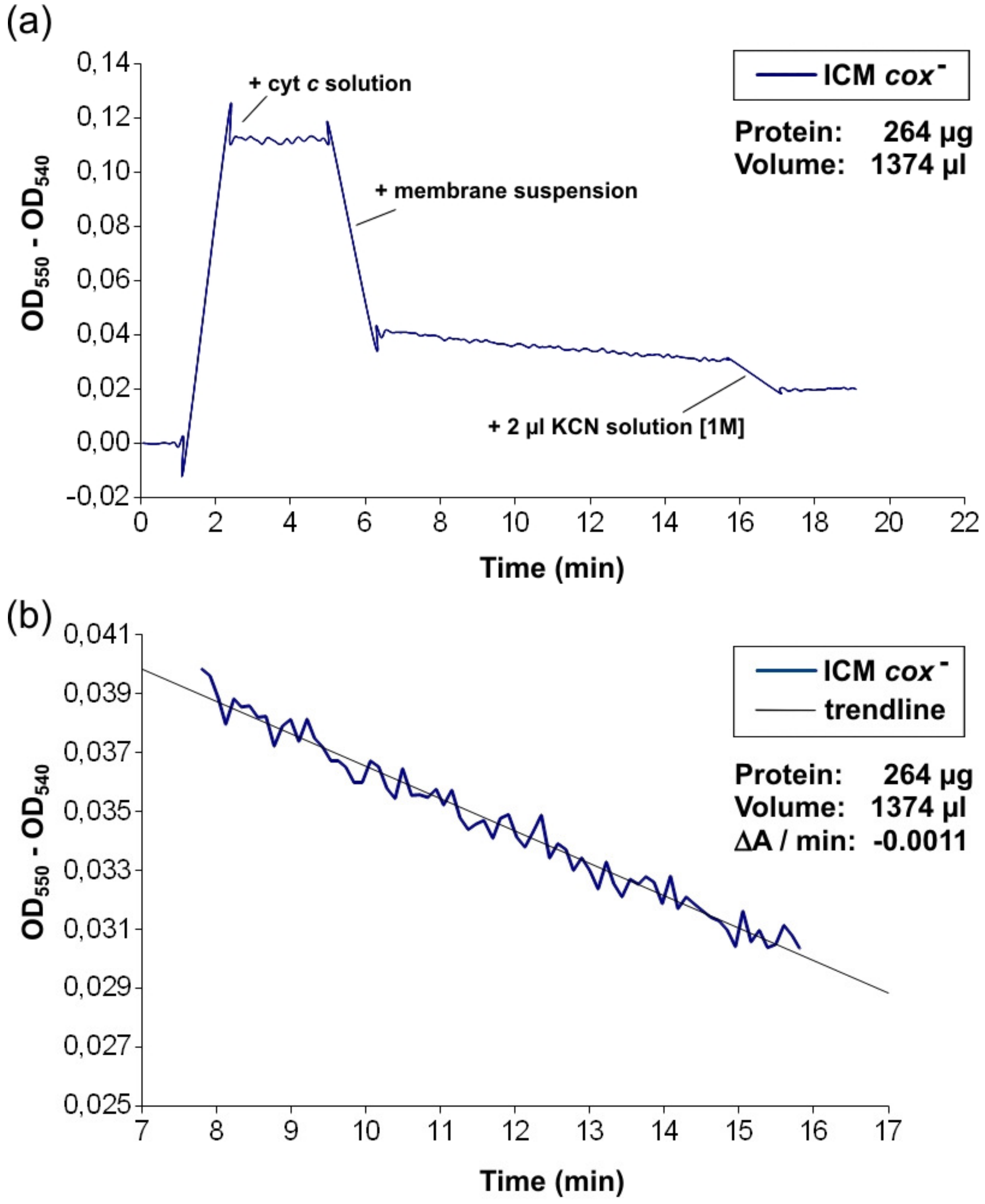


Figure 4.13: (a) Complete data diagram of cyt *c* oxidation by cytochrome *c* oxidase(s) in the ICM of PCC7942cox^- mutant.

(b) Linear range diagram showing oxidation of cyt *c* by cytochrome *c* oxidase(s) in the ICM of PCC7942cox^- mutant.

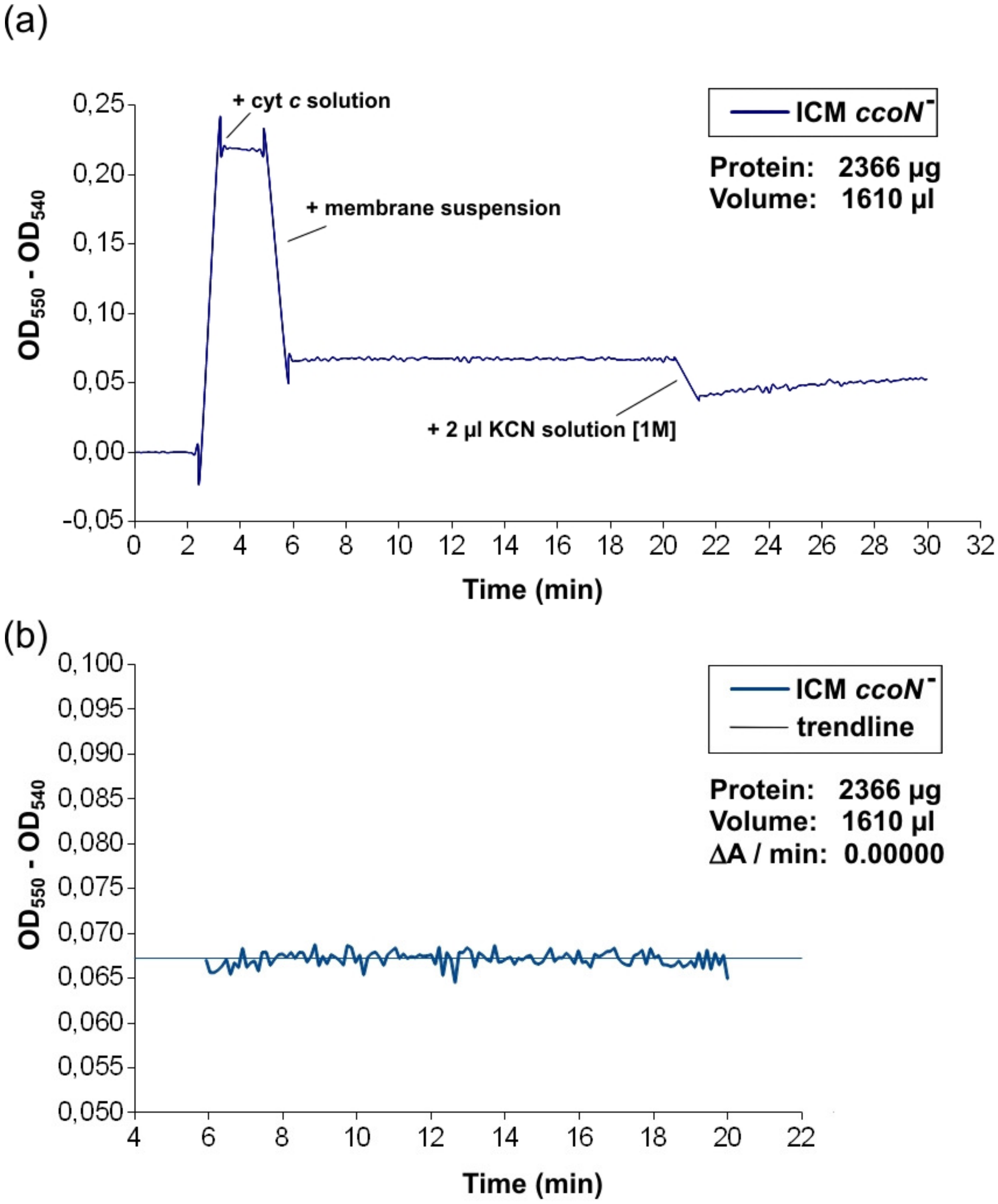


Figure 4.14: (a) Complete data diagram of cyt *c* oxidation by cytochrome *c* oxidase(s) in the ICM of PCC7942$ccoN^-$ mutant.
(b) Linear range diagram showing no oxidation of cyt *c* by cytochrome *c* oxidase(s) in the ICM of PCC7942$ccoN^-$ mutant.

The calculated ΔA / min, the reaction volumes and the amounts of protein were substituted into formula 3.1, page 59, to calculate the specific activities shown in table 4.6.

Sample	**Specific Activity** $[\mathbf{nmol}_{(\mathbf{cyt}\ c)} \cdot \mathbf{min}^{-1} \cdot \mathbf{mg}^{-1}_{(\mathbf{protein})}]$
CM wt	5,7867
CM *cox*$^-$	0,0000
CM *ccoN*$^-$	1,8821
ICM wt	0,2576
ICM *cox*$^-$	0,2880
ICM *ccoN*$^-$	0,0000

Table 4.6: Results of the cyt *c* oxidation assays.

The overall protein content of the ICM is much higher than of the CM, due to the photosynthetic proteins. As the Specific Activity is based on the overall protein content, it is not possible to compare the results of the cytochrome *c* assays of ICM and CM except for identifying activity or no activity in cyt *c* oxidation.

Chapter 5

Discussion

It is possible to knock out each of the three respiratory terminal oxidases in PCC7942 (Cox, Cbb_3 and Qox). This shows that none of the respiratory terminal oxidases is essential for PCC7942 under laboratory conditions.

Knockout mutants for each respiratory terminal oxidase present in a cyanobacterium were only created for PCC6803 [32] until now. The respiratory terminal oxidases in *Synechocystis* PCC6803 are Cox, ARTO and Qox. Viable homozygous double and triple mutants in *Synechocystis* PCC6803 were also created showing that the whole aerobic respiration is not essential for PCC6803 under laboratory conditions. In this work the knockout plasmids and the mutants created possess different antibiotic resistance cassettes to facilitate creation of double and triple knockout mutants of PCC7942 in the future.

3 conclusions are made based on the cytochrome *c* oxidase activity assays:

- cbb_3-type respiratory terminal oxidase is an active cytochrome *c* oxidase in PCC7942 although some subunits present in purple bacterium *Rhodopseudomonas palustris* are missing in this cyanobacterium.
- Cbb_3 is only present in the ICM.
- Cox is only present in the CM.

The location and activity of Qox, the third RTO of PCC7942, was not determined until now. As it is thought to act as an electron valve for the photosystems (1.3.5, page 14), the probable location is the ICM. However, the presence of Qox in the CM cannot be excluded.

The cox^- mutant shows nearly the same cytochrome *c* oxidase activity in the ICM as the wild type. Apparently the absence of the *coxBAC* locus has no or no visible effect on the activity of the Cbb_3 located in the ICM. However the *ccoN*$^-$ mutant shows only one third of the wild type cytochrome *c* oxidase activity in the CM. It is known that activities of different respiratory terminal oxidase mutants often do not add up to the activity of the wild type [23] but the reasons are not known. This question seems to be unsolvable at the moment as the reasons could be found in any stage of protein expression or metabolism. At least it can be stated that the loss of the CcoN subunit of the Cbb_3 has an effect on the activity of the Cox located in the CM.

In PCC7942 activity of Cox is found only in the CM while in *Synechocystis* sp. PCC6803 Cox is probably located only in the ICM [23]. As the protein sequences of CoxBAC in PCC7942 and CoxBAC in *Synechocystis* sp. PCC6803 are highly similar (table 1.1, page 10), the question arises, why and how the proteins are transported to two different membranes. Cyanobacteria seem to be prokaryotes with two not connected membrane systems inside the cell wall [60]. This leads to similar demands in protein targeting as in eukaryotes, that need to transport their proteins to different membrane systems within the cell, comprising the cellular compartments. Targeting sequences, as well characterized in *E. coli* or different eukaryotic model systems, could be responsible for the transport to one of the membranes. Therefore the sequence differences of the two Cox enzymes in PCC7942 and PCC6803 could contain useful information about protein targeting in cyanobacteria.

Rhodopseudomonas palustris cbb_3 oxidase consists of 4 structural subunits and additionally there are 4 genes important for enzyme maturation (1.3.4, page 11). The question arises, how it is possible for PCC7942 to possess an active Cbb_3 with only two subunits homologous to the Cbb_3 of purple bacteria. Two possible explanations for this are:

1. Cbb_3 in PCC7942 is functioning with only two subunits or
2. there are one or more still unknown subunits, not homologous to the *Rhodopseudomonas palustris* subunits, that belong to the Cbb_3 in PCC7942.

The first possibility is supported by observations that there exist bacteria which possess the *ccoNOPQ* cluster in the wild type but are able to assemble an active oxidase in *ccoP*$^-$ and *ccoQ*$^-$ mutants. For other bacteria, however, CcoP is an essential subunit to form an active complex (1.3.4, page 11). Additionally several bacteria from different phyla have genes for CcoN and CcoO but do not have genes for CcoP and CcoQ. These data are the result of genome analysis and are not corroborated by measurements of enzyme activities.

The second possibility is supported by the existence of two genes (*synpcc7942_0199* and *synpcc7942_0200*, figure 1.4, page 12) encoding putative transmembrane proteins directly downstream of the cbb_3 locus in PCC7942 and PCC6301. A protein BLAST shows these proteins to belong to the DUF2231 superfamily, predicted membrane proteins without known function [61, 26]. These proteins do not show any significant homology to CcoP or CcoQ but could belong to an alternative cbb_3 cluster in PCC7942. Therefore the construction of knockout mutants of these genes may be useful for further characterization of the Cbb_3 in PCC7942.

This work provides evidence that there exists an active Cbb_3 in PCC7942 and localises the enzyme in the ICM. Many questions concerning assembly, subunit composition or the reason for having different cytochrome *c* oxidases in one cell remain to be solved. Although more and more total sequences of cyanobacteria are available, nothing is known about their protein targeting and targeting sequences. The identification of a PCC7942 Cox enzyme, located in another membrane (CM) than the highly homologous Cox enzyme of PCC6803 (ICM), may contribute to filling the lack of knowledge about protein targeting in cyanobacteria.

Summary

By definition, cyanobacteria are prokaryotes performing oxygenic photosynthesis; they possess two photosystems (PSI and PSII) and use H_2O as reductant in photosynthesis.

In addition to photosynthesis, cyanobacteria are capable of aerobic respiration. The key enzymes of respiration are the respiratory terminal oxidases, catalysing the reaction $O_2 + 4H^+ + 4e^- \rightarrow 2\,H_2O$.

In cyanobacteria seven different types of respiratory terminal oxidases are known which can be assigned to 3 main protein families:

- homologs of mitochondrial cytochrome *c* oxidases (Cox, ARTOs and Cbb_3)
- homologs of the *E. coli* cytochrome *bd* type quinol oxidase (Qox)
- homologs of the plastidic terminal oxidases (Ptox)

cbb$_3$-type cytochrome *c* oxidases are well characterized in purple bacteria but were unknown to exist in cyanobacteria before the total genomic sequences of *Synechococcus* PCC6301 and PCC7942 were determined. These two strains are the only cyanobacteria in which genes for *cbb*$_3$-type cytochrome *c* oxidases were found. While the Cbb_3 enzyme contains 4 subunits in the purple bacterium *Rhodopseudomonas palustris*, only 2 genes (*ccoN, ccoO*) homologous to genes encoding Cbb_3 subunits in *R. palustris* are found in cyanobacteria.

The aim of this work was to determine whether Cbb_3 is an active cytochrome *c* oxidase in PCC7942 and in which membrane(s), cytoplasmic membrane (CM) and/or intracellular membrane (ICM), the 2 cytochrome *c* oxidases of this strain (Cox and Cbb_3) are active. A cbb_3^- strain was kindly provided by Dr. S. Golden. A cox^- strain was created to be tested for cytochrome *c* oxidase activity. A qox^- strain was created to have knockouts of every respiratory terminal oxidase to determine whether any of the respiratory terminal oxidases is essential for PCC7942.

Two plasmids for the cox^- knockout (Sm/Sp^R) and two plasmids for the qox^- knockout (Km^R) were constructed. The respective plasmids had the antibiotic resistance cassettes inserted in both possible orientations. PCC7942 wild type was transformed with the plasmids and transformants were selected on plates with antibiotics. After extensive tests to prove homozygosity of the mutant strains, PCC7942 wild type, cox^- and cbb_3^- were cultured in 10 l bottles. CM and ICM were separated by sugar gradient density centrifugation in a zonal rotor. The proteins of the separated membranes of the three strains were tested for their cytochrome *c* oxidase activity by dual wavelength spectroscopy.

The main results of the experiments are: Each of the 3 respiratory terminal oxidases is dispensible in PCC7942. Cbb_3 is an active cytochrome *c* oxidase in PCC7942. Cbb_3 is located only in the ICM, Cox is located only in the CM.

Zusammenfassung

Per Definition sind Cyanobakterien Prokaryoten, die zur oxygenen Photosynthese befähigt sind; sie besitzen zwei Photosysteme (PSI und PSII) und können Wasser als Elektronendonor verwenden.

Zusätzlich können Cyanobakterien auch Sauerstoff atmen. Die Schlüssel-Enzyme der Atmung, die Respiratorischen Terminalen Oxidasen, katalysieren die Reaktion $O_2 + 4H^+ + 4e^- \rightarrow 2\,H_2O$.

In Cyanobakterien gibt es mindestens sieben verschiedene Respiratorische Terminale Oxidasen, die in drei Protein–Familien eingeteilt werden:

- Homologe der mitochondrialen Cytochrom *c* Oxidasen (Cox, ARTOs, Cbb_3)
- Homologe der *bd* Chinol Oxidase aus *E. coli* (Qox)
- Homologe der plastidischen terminalen Oxidase (Ptox)

Cbb_3 Oxidasen sind in Purpurbakterien entdeckt und charakterisiert worden. Daß es Gene, homolog zu den cbb_3 Genen aus Proteobakterien, auch in Cyanobakterien gibt, ist erst seit den Totalsequenzierungen von *Synechococcus* PCC6301 und PCC7942 bekannt. Diese zwei Stämme sind die einzigen Cyanobakterien, in denen cbb_3 Gene gefunden wurden. Während Cbb_3 im Alpha Proteobakterium *Rhodopseudomonas palustris* aus 4 Untereinheiten (CcoNOPQ) besteht, gibt es in Cyanobakterien nur 2 Gene (*ccoN, ccoO*), die zu den 4 Cbb_3 codierenden Genen von *R. palustris* homolog sind.

In dieser Arbeit soll gezeigt werden, ob *ccoNO* in PCC7942 eine aktive Cytochrom *c* Oxidase codiert und in welchen Membranen, Cytoplasmamembran (CM) und/oder Thylakoidmembran (ICM), die zwei möglichen Cytochrom *c* Oxidasen von PCC7942 (Cox und Cbb_3) aktiv sind. Eine cbb_3^- Mutante wurde von Dr. S. Golden zur Verfügung gestellt. Eine cox^- Mutante wurde während dieser Arbeit für die Cytochrom *c* Oxidase Aktivitätstests hergestellt. Eine qox^- Mutante wurde hergestellt, um Mutanten aller Respiratorischen Terminalen Oxidasen zu erhalten um darauf schließen zu können, ob eine oder mehrere der Oxidasen für PCC7942 essentiell ist bzw. sind.

Es wurden Plasmide für den cox^- (Sm/Sp^R) und den qox^- Knockout (Km^R) hergestellt. Für die Knockouts wurden je 2 Plasmide hergestellt, die die Antibiotika Resistenz Kassetten in verschiedener Orientierung insertiert haben. Der PCC7942 Wildtyp wurde mit den verschiedenen Plasmiden transformiert und Transformanten wurden auf Platten mit Antibiotikum selektiert. Die Mutanten wurden auf Homozygotie getestet und dann in 10l Flaschen kultiviert. CM und ICM wurden durch Zucker-Dichtegradientenzentrifugation in einem Zonal Rotor getrennt. Die Proteine in den getrennten Membranen wurden mittels Zweistrahl-Spektrophotometrie auf ihre Cytochrom *c* Oxidase Aktivität getestet.

Die Ergebnisse der Experimente zeigten: Keine der 3 Respiratorischen Terminalen Oxidasen ist für PCC7942 essentiell. Cbb_3 ist eine aktive Cytochrom *c* Oxidase in PCC7942. Cbb_3 ist nur in der ICM, Cox nur in der CM aktiv.

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