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DISSERTATION

Titel der Dissertation

Heterotrophic growth of the cyanobacterium *Anabaena* (*Nostoc*) sp. strain PCC7120 and its dependence on a functional *cox1* locus encoding cytochrome *c* oxidase

Verfasser

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TABLE OF CONTENTS

1. Abstract

Cyanobacteria are prokaryotes that perform oxygenic photosynthesis. However, all cyanobacteria respire at dark periods. In eukaryotic phototrophs like algae and higher plants photosynthesis and respiration are separated to the different organells chloroplasts and mitochondria, while in cyanobacteria these two processes occur in the same compartment and many components are shared. The respiratory terminal oxidases (RTOs) are key enzymes because they are not involved directly in photosynthesis but transfer electrons to terminal acceptor O_2. Cyanobacterial oxidases belong to different classes.

There are the heme copper oxidases homologous to mitochondrial cytochrome *c* oxidase, the quinol oxidases homologous to cytochrome *bd* in *Escherichia coli* and homologues to the plastid terminal oxidases that are insensitive to cyanide. Nearly all cyanobacteria contain at least one heme copper oxidase of the cytochrome aa_3 type. There are three different subclasses identified:

a) The genuine cytochrome *c* oxidases, containing the characteristic motives.
b) A second class, called alternative respiratory terminal oxidase (ARTO).
c) An oxidase of type cbb_3 (first characterized in purple bacteria).

Anabaena sp. PCC7120 belongs to the unbranched filamentous cyanobacteria that can undergo cell differentiation. If bound nitrogen (nitrate, nitrite, ammonia) is absent some vegetative cells will differentiate into heterocysts with a non random distribution. PCC 7120 has five different respiratory terminal oxidases: one genuine cytochrome *c* oxidase (Cox), two ARTOs, one quinol oxidase (Qox) and one plastid terminal oxidase (Ptox). As the two ARTOs seem to be restricted to heterocysts, only 1 Cox, 1 Qox and 1 Ptox remain in vegetative cells. When *cox* is knocked out by an antibiotic resistance cassette the strain loses any cytochrome *c* oxidase activity as an assay clearly demonstrated with isolated membranes and cytochrome c_{550} isolated from horse heart. Moreover the Cox seems to be essential for chemoheterotrophic growth in the dark. Despite previous reports, which classify PCC7120 as strictly photolithoautotrophs, experiments revealed that this strain can grow heterotrophically when high amounts of fructose were added to the medium. Evidently PCC7120 has the capacity for heterotrophic growth and fructose metabolism inside its cells, however, heterotrophic growth can only be observed at very high fructose concentrations (at least 50mM) in the medium. Experiments have shown that fructose enters the cells the faster the higher the outside concentration is. Introduction of glucose carrier gene *gtr* from *Synechocystis* PCC6803 by conjugation results in a transgenic strain that can grow photoorganoheterotrophically on lower concentrations of fructose than the wild type (WT). However, glucose is toxic for PCC7120gtr^+ while the WT is tolerant towards it. The mutant from which *cox* has been deleted fails to grow chemoheterotrophically. This is analogous to experiments in *Synechocystis* PCC6803 and *Anabaena variabilis* ATCC29413, where also the genuine cytochrome *c* oxidase is essential for chemoheterotrophic growth.

2. Introduction

2.1. General Introduction

Cyanobacteria are defined as prokaryotes that perform oxygenic photosynthesis. While other phototrophic bacteria like purple bacteria and green bacteria oxidize components like H_2S, H_2, thereby creating S, SO_4^{2-}, H^+, cyanobacteria use H_2O as an electron source by oxidizing it to O_2, which is liberated. Anoxygenic phototrophs possess only one photosystem (either type I or II), whereas cyanobacteria have both. Cyanobacteria were probably the first organisms that produced molecular oxygen. According to the endosymbiosis theory (Mereschkowsky, 1905) chloroplasts and cyanobacteria have the same ancestors. As soon as liberated O_2 accumulated in higher concentrations, the organisms had to protect themselves against oxygen. Therefore it is very likely that cyanobacteria were the first organisms that developed an aerobic respiration (Broda, 1975). The O_2 enrichment of the atmosphere led to the extinction of a variety of organisms that were strictly anaerobic.

Three growth modes are known in cyanobacteria: photolithoautotrophy, photoorganoheterotrophy and chemoorganoheterotrophy.

Cyanobacteria differ in their cellular structure and their organisations. There are unicellular and filamentous organisms. Some of the filamentous strains can undergo cell differentiation. If there is a deficiency of bound nitrogen (e. g. NH_4^+ or NO_3^-) some strains have the capacity to form heterocysts. These are specialised cells capable of fixing dinitrogen. Vegetative cells differentiate at a non random distance (every 10-20 cells) to heterocysts. This differentiation is irreversible. Once formed, heterocysts can neither divide nor redifferentiate anymore.

2.2. Systematics of cyanobacteria

Initially cyanobacteria were classified as blue green algae (Cyanophyta), because of their photosynthetic nature, which was thought to be a characteristic of only algae and higher plants. Stanier and Cohen-Bazire discovered their prokaryotic nature (1977). Initially cyanobacteria were classified into five orders. and the species were named according to the binary nomenclature (Geitler, 1932). Rippka et al replaced the classical orders by sections (1979). The number of genera was reduced and the species names were abolished. Each genus summarizes one or more strains, which were numbered after the Pasteur Culture Collection PCC or the American Type Culture Collection ATCC.

Section I and II include unicellular organisms, which have the shape of cocci or bacilli. Some of them can form aggregates, which are held together by capsules.

Section I: Chroococcales

Unicellular organisms reproducing by binary fission or budding

Genera: *Gloeobacter*, *Gloeothece*, *Synechocystis*, *Synechococcus*

Gloeobacter, which is the only cyanobacterium that lacks thylakoids, is now often put into a group separated from all five sections.

Section II: Pleurocapsales

Unicellular organisms reproducing by multiple fission into 4-1000 small endospores, which are called baeocytes. However, some strains can reproduce by binary fission as well.

Genera: *Pleurocapsa*, *Mycosarcina*, *Xenococcus*, *Dermocarpa*

Section III, IV and V include filamentous cyanobacteria, which form filaments. In some strains a short filament, which is called hormogonium, can break off from the mother filament and reproduces to a new vegetative filament. The filaments of cyanobacteria from section III do not show any cell differentiation, whereas members of both section IV and V have the potential for the differentiation of some cells into heterocysts or akinetes (spores).

Section III: Oscillatoriales

Filamentous non heterocystous cyanobacteria (all dividing in one plane)

Genera: *Oscillatoria*, *Spirulina*, *Plectonema*

Section IV: Nostocales

Filamentous heterocystous cyanobacteria dividing in one plane

Genera: *Nostoc*, *Anabaena*, *Calothrix*, *Nodularia*

Section V: Stigonematales

Filamentous cyanobacteria dividing in more than one plane

Genera: *Fischerella*, *Chlorogloeopsis*

Besides these five sections there exist three genera (*Prochloron*, *Prochlorococcus*, *Prochlorothrix*), which also perform oxygenic photosynthesis but they differ in some ways from the described sections. They have chlorophyll *b* but lack phycobilisomes and their thylakoids are stacked in grana. These characteristics can

also be found in the chloroplasts of green algae (chlorophyta) and higher plants, whereas the chloroplasts of red algae (rhodophyta) share the properties of classical cyanobacteria. Because of the similarity of the three genera to chlorophyta these organisms are named prochlorophyta (as being the ancestors of chlorophyte chloroplasts), but genetic analysis revealed that they are cyanobacteria.

2.3. The different modes of growth existing in nature

Organisms can be distinguished by their metabolism according to energy source, electron source and carbon source.

2.3.1. Energy source

Energy is won by light or chemical reactions and stored as ATP molecules. When ATP is hydrolyzed, energy is released.

a) Chemotrophy

ATP is won by exergonic chemical reactions and no input of light energy is needed

b) Phototrophy

ATP is generated by the conservation of light energy. Electrons of a molecule with a relatively low reducing potential can be elevated energetically. As a consequence other molecules can be reduced, which have a higher reducing potential at normal conditions.

2.3.2. Electron source

The external electron donor (the first component in the reaction) can be an organic or an inorganic molecule

a) Lithotrophy

The electron donor is an inorganic molecule (greek *λιθος* = *lithos* means "stone") like H_2, Fe^{2+}, H_2S, S_8, $S_2O_3^{2-}$, NH_3, NO_2^-, NO_3^-

b) Organotrophy

The electron donor is an organic molecule

2.3.3. Carbon source

Carbon is the major element in organic molecules, as their backbones are carbon atoms covalently linked to each other.

a) Autotrophy

The carbon source is a molecule with only one carbon atom like CO_2, HCOOH, CH_3OH, CH_4. This means autotrophic organisms synthesize all covalent bonds between C atoms, which are characteristic of organic molecules like sugars, amino acids, fatty acids…, by themselves (greek *αυτος* = *autos* means "self").

b) Heterotrophy

The carbon source is a molecule with at least two C atoms covalently linked. So some C-C bonds can be directly used from the food.

2.3.4. Combination of the diverse modes

These characteristics can be combined in nearly all combinations. It is named in the following order: energy-/electron-/carbon-troph e.g.: photolithoautotroph. The only combinations that do not occur in nature are the two (both photo- and chemo-) combinations of organotroph and autotroph, as it would be very illogical, if an organism can synthesize all organic molecules based on a source with 1 C atom, but uses an organic molecule with at least 2 C atoms as its electron source. Nevertheless mutations in some organisms have created models for these naturally not occurring metabolisms. The other six possible combinations have already been discovered in nature. Many organisms are capable of more than one growth mode. For instance some of them are facultative heterotrophs, which means that they are capable of heterotrophy but can also grow autotrophically. Organisms that can only reproduce heterotrophically are called obligate heterotrophs.

a) Photolithoautotrophy

All photosynthesizing eukaryotes like plants and algae are photolithoautotrophs. They use light as energy-, H_2O as electron-, and CO_2 as carbon source. This is called oxygenic photosynthesis because they liberate O_2 by oxidizing H_2O. Among prokaryotes there is a greater variety within photolithoautotrophy. Cyanobacteria are the only prokaryotes that perform oxygenic photosynthesis, however there exist many organisms that perform other forms of photosynthesis. Purple sulfur bacteria or green sulfur bacteria for example oxidize H_2S to S_8, which can be further oxidized to SO_4^{2-}. Some can even use H_2 as an electron source. While H_2 or H_2S oxidizers use only one photosystem, H_2O oxidizers need two photosystems, as the reducing potential of H_2O is very much lower than that of NADP(H).

b) Photoorganoheterotrophy

Light is used as energy source, but organic molecules serve as both electron and carbon donor. Photoorganoheterotrophy exists among green nonsulfur bacteria. In

many cyanobacteria this trophy can be enforced by chemicals that inhibit photosystem II (see below).

c) Chemolithoautotrophy

Some organisms can incorporate CO_2 into organic material but unlike phototrophs they obtain their whole energy by chemical reactions. This modus is widespread among Fe^{2+} oxidizing bacteria.

d) Chemolithoheterotrophy

As an electron source H_2, H_2S, NH_3, Fe^{2+} is used, but for carbon metabolism organic molecules with C-C bonds are needed. Chemolithoheterotrophy exists among *Desulfovibrio* strains.

e) Chemoorganoheterotrophy

Organic molecules e. g. sugars serve as energy, electron and carbon source. This is the modus for many eukaryotes like animals, fungi, but even for plants in dark periods. This metabolism is also widespread among prokaryotes like *E. coli*.

f) Mixotrophy

Some organisms can use different modes of growth. For example if phototrophic organisms like the cyanobacterium *Synechocystis* PCC6803 is grown under light in a BG11 medium (according to Rippka et al, 1979) supplemented with glucose, the cells can choose between autotrophic growth, as light induces photosynthesis and incorporation of CO_2, and heterotrophy, as glucose (organic material) can be imported by the Gtr carrier and metabolized. These conditions are called mixotrophy.

Aerobic and anaerobic respiration and photosynthesis are all processes at least partially located in membranes, where a proton gradient is built up that can be used for ATP generation. If there is not enough of the terminal acceptor, the organism may perform fermentation, which is performed by soluble components, when ATP has to be quickly produced. Some organisms cannot respire, but only fermentate.

2.4. Genetics of cyanobacteria

The cyanobacterial genome has a size between 3 and 20 Mbp, especially filamentous heterocystous strains have a rather large genome. They have a multiple copy number of their chromosome (10-20 per cell) (Herdman et al 1979; Herdman et al, 1982) and a very active recombination system, which are both very protective against damage by UV radiation. Many cyanobacteria contain large plasmids (2kbp to 1Mbp). *Synechocystis* PCC6803, which was one of the first organisms that has been

totally sequenced (Kaneko et al, 1996), has seven plasmids (Cyanobase, 2011). Cyanobacteria use the standard genetic code. Some introns have been discovered within the genome. Most promotors from *E. coli* are also recognized in cyanobacteria. The origins of replicons differ, however, because except for the incQ plasmids all other plasmids that have a noncyanobacterial origin are not recognized in cyanobacteria. There are different methods of manipulating cyanobacteria.

a) Some strains like PCC6803 or *Synechococcus* sp. strain PCC7002 are naturally competent for transformation (Grigorieva and Shestakov, 1982). This means DNA has just to be added to the medium and the cells will take up the DNA. It has been shown that natural transformation is much more efficient for integrative than for replicative plasmids in PCC6803.

b) Another possibility is electroporation (Thiel and Poo, 1989).

c) Plasmids can be transferred from *E. coli* to cyanobacteria by conjugation, a mechanism that is mediated by conjugative plasmids like RP4. This method was invented by Wolk et al (1984).

d) A fourth possibility, transduction by a cyanophage, is not widely used in genetic work, because all known cyanophages are lytic.

After the introduction the DNA has to be manifested. There are two possibilities: replication and integration.

a) For replication the transferred DNA has to have its own origin that it can act as a replicon.

b) For integration a homologous sequence of about 1kbp is necessary for recombination between the transferred plasmid and the cyanobacterial chromosome so that the former will become part of the latter.

For the creation of a gain of function mutant (e. g. the transfer of *gtr* into PCC7120) a self replicating plasmid can be used because the new transgenic gene does not have to be integrated into any chromosome. The plasmid’s origin is recognized by the replication system of the new host.

Creating a knock out mutation is much more difficult. The transferred plasmid has to contain a cassette that encodes a resistance towards a special antibiotic. This cassette is flanked by two regions, which are homologous to regions in the chromosome, where the gene to be deleted is located. When double recombination (at both homologous sequences) takes place the open reading frame (ORF) of the gene will be disrupted by the antibiotic resistance cassette. For knock out mutations the plasmid vector should not have an origin that is recognized by the cyanobacterial host, to exclude a manifestation of the resistance cassette without integration into the chromosome. If the two flanking sequences are directly neighboured on the

chromosome, the result will be an insertion mutation, because nothing of the ORF is missing, it is just disrupted. For a deletion-insertion mutant there has to be a distance between the two flanking sequences on the chromosome and the resistance cassette may even replace the whole ORF. A deletion-insertion mutant is preferred, because it excludes that an excision of the cassette will lead to a reversion to the WT, as at least a part of the gene is missing.

In PCC6803 a whole integrated plasmid caused by single recombination is unstable, so double recombination will automatically occur. For selection of the mutated allele the appropriate antibiotic is used. For knock out mutations in PCC6803 transformation will be done, as this strain is naturally competent. PCC6803 does not encode for any restriction enzymes, so the foreign plasmid need not be protected by methylation. DNA is added to the medium and later the cells will be exposed to the antibiotic.

For PCC7120 conjugation is performed. An *E. coli* strain containing the two plasmids RP4 and pRL528 is mixed with another *E. coli* strain containing the plasmid which is to be transferred. RP4 (conjugative plasmid) can initialize conjugation to other bacteria. pRL528 (coconjugative plasmid) cannot mediate conjugation by itself, however, if conjugation was initialized by a conjugative plasmid, it is transferred through the conjugative junction, so both cells have a copy. pRL528 encodes a nickase that recognizes a special sequence: the "basis of motility (*bom*) site" or "*oriT* (transfer)", which is nicked and a single strand is transferred. The third plasmid does not encode a nickase, however, contains a *bom* site that is recognized by the nickase of pRL528. The strain, which contains the third plasmid, receives the other two plasmids during the first conjugation between the two *E. coli* strains. As a result some cells possess all three plasmids now. The mixture of *E. coli*s is added to PCC7120. RP4 mediates the second conjugation, which takes place from *E. coli* to PCC7120. All three plasmids are transferred into the cyanobacterial host, but neither RP4 nor pRL528 can be manifested in cyanobacteria. The third plasmid contains either an origin that is recognized by the host or homologous sequences, which allow integration into the chromosome.

Cyanobacterial cells, which are not transformed or conjugated, will die in the presence of the antibiotic. Knock-out plasmids have to perform a double recombination. As a consequence transformed or conjugated cells will survive in a medium containing the antibiotic, however, they will still show a WT phenotype because most chromosomal copies have the WT allele. When cells divide the chromosomes are randomly segregated. Daughter cells having only WT alleles will die because of the antibiotic. The chromosomes replicate before division and within a few generations some cells will accumulate mutated alleles. After some cycles there will be cells that are mutated homozygously (they do not contain a WT allele any more). These cells will divide into two totally mutated cells and so on. In its offspring the WT

allele will not reappear. Because homozygous WT cells die, the segregation will drive the cells towards more alleles with the resistance. If cells are transferred to new plates or into new liquid cultures several times it is possible to obtain a culture that is completely mutated. This has to be further proved by polymerase chain reaction (PCR). Sometimes a gene cannot be knocked out homozygously. Then the gene is essential for life (e.g. *recA* in cyanobacteria) under the chosen conditions.

For PCC7120 the construction of a homozygous knock-out mutant is much more difficult. In contrast to PCC6803 double recombination is very rare. Knock out plasmids for PCC7120 carry an antibiotic resistance cassette flanked by two homologous sequences on a vector derived from plasmid pRL278 that contains the *sacB* gene. *sacB* encodes a levansucrase, which metabolizes sucrose to levanes, which are toxic for the cells (Cai and Wolk, 1990). Besides *sacB* pRL278 contains a neomycin phosphate transferase (*npt*), an origin for *E. coli* (but not for cyanobacteria) and an *oriT* (*bom* site) that is recognized by the nickase encoded by pRL528. As a consequence one can select both for (adding kanamycin) and against (under the presence of sucrose) pRL278 within *E. coli*. pRL278 itself cannot be manifested in cyanobacteria because it lacks a corresponding origin and homologous sequences, however, the introduction of an antibiotic resistance cassette flanked by two homologous sequences of the gene that has to be deleted results in a functional knock out plasmid. In the successfully conjugated cyanobacterial cell the plasmid first has to resist the host's defence system. In contrast to PCC6803, PCC7120 codes for three restriction enzymes called *Ava*I, *Ava*II and *Ava*III (Elhai et al, 1997). To protect its own genome the strain codes for methylases, which methylate the sites that are recognized by the restriction enzymes. A methylated site will not be cut any more. pRL528 does not only code for a nickase, but also for 2 methylases: M*Ava*I methylating *Ava*I sites and M*Eco*47I methylating *Ava*II sites (*Eco*47I is an isoschizomer of *Ava*II, it cuts at the same sites, but is derived from *E. coli*). However a further problem arises, as half of the methylated *Ava*I sites are digested in $mcrB^+$ strains. Therefore $mcrB^-$ strains like HB101 and Top10 are essential for conjugation to PCC7120. While the two *E. coli* strains conjugate pRL528 is transferred into the other strain, where the methylating genes are expressed, which methylate all *Ava*I and *Ava*II sites including those of the knock out plasmid before conjugation to PCC7120. So the plasmid is already protected before entering the PCC7120 cells. First the plasmid will integrate via single recombination after the conjugation. The exconjugant has one chromosome containing both the WT allele and the resistance cassette, while all other chromosomes are normal WT. Selection with the appropriate antibiotic will accumulate chromosomes with the integrated plasmid. Occasionally a recombination will occur within the other flanking sequence. Now sucrose will be added together with the antibiotic. This selects both for integration of the cassette and for excision of the suicide vector. Once sucrose is added, all cells that have any integrated vector are eliminated. The surviving cells contain at least one resistance cassette integrated by

double recombination. Now cultivation with antibiotic will generate totally mutated cells, where sucrose is not necessary any more, because all suicide vectors are excised and cannot replicate in PCC7120.

2.5. Photosynthesis and respiration

2.5.1. Photosynthesis

During the light reaction the electrons are transferred from H_2O via photosystem II and I (PSII and PSI) to $NADP^+$ yielding NADPH. While passing the cyt b_6f complex a proton motive force is generated at the thylakoid membrane, which can be used for ATP synthesis. Both ATP and NADPH are essential in the calvin cycle (dark reaction) for fixing CO_2. For PSII the phycobilisomes serve as light harvesting complex (LHC). They contain the accessory pigments phycocyanin and allophycocyanin (and in some cyanobacterial strains also phycoerythrin). The reaction centers (RC) of both photosystems are chlorophyll *a* pairs (Deisenhofer et al, 1984). The RC of PSII has its maximal absorbance at 680nm, the one of PSI at 700nm. H_2O is oxidized by PSII and O_2 is liberated.

2.5.2. Cyanobacterial respiration (Schmetterer, 1994; Schmetterer and Pils, 2004)

Cyanobacteria are the only organisms in which oxygenic photosynthesis and aerobic respiration occur in the same compartment, contrary to eukaryotic phototrophs, where these processes are separated to chloroplasts and mitochondria. According to the conversion hypothesis (Broda, 1975) respiration was established as a consequence of the produced oxygen, as the cells had to protect themselves against oxygen. Therefore cyanobacteria must have been the first organisms developing respiration as they are exposed inside to oxygen. This protection mechanism has been evolved. As a consequence electrons derived from organic compounds, pass an electron transport chain, which generates a proton motive force that can be used for ATP synthesis, when no light is available in dark periods.

The cyanobacterial respiration chain differs from strain to strain. It is best characterized in PCC6803. This strain possesses two different respiratory chains, one in the cytoplasmic membrane and one in the intracytoplasmic membrane. The latter is closely linked to the photosynthetic electron transport chain as some components are shared by both. In the dark glycogen is phosphorylized to glucose monomers, which are catabolized primarily via the oxidative pentose phosphate cycle. Electrons are transferred to $NADP^+$ yielding NADPH, which reduces the quinone pool by means of NADH dehydrogenase. Via the cytochrome b_6f complex to cytochrome c_{553} the respiratory chain is equal to the photosynthetic electron transport chain. The b_6f

complex generates a proton gradient in the same way as it occurs in the photosynthetic electron transport chain. From cytochrome c_{553} electrons are transferred to the cytochrome *c* oxidase, which reduces O_2 to H_2O. It seems that plastocyanin can replace cytochrome c_{553} for both photosynthesis and respiration, as a cytochrome c_{553} knock out mutant can still grow chemoheterotrophically, as long as the cytochrome *c* oxidase was functional. So plastocyanin may have the ability to transfer electrons to photosystem I or to cytochrome *c* oxidase. This would mean that cytochrome *c* and plastocyanin are both electron donors for cytochrome *c* oxidase. However, the possible electron transfer between plastocyanin and cytochrome *c* oxidase has not been proven further. In the isolated cytoplasmic membrane (CM) from PCC6803 no cytochrome *c* oxidase has been identified. It is expected that in the CM electrons are transferred from cytochrome c_{553} via "alternate respiratory terminal oxidase" (ARTO), which is located in the CM, to O_2, although it is not clear, whether ARTO can oxidize cytochrome c_{553} (no in vitro activity). In the ICM there exists a quinol oxidase (qox) which can transfer electrons directly from the quinon pool to O_2, without passing the cyt b_6f complex.

2.5.2.1. Electron donors for the quinone pool

Normally when cyanobacteria live photolithoautotrophically, the quinone pool is reduced by photosystem II, however, under dark periods the main electron donor is NADPH. The latter even occurs in the light at the cyclic electron flow. Glucose is mainly degraded to CO_2 via the oxidative pentose phosphate cycle, where NADPH is produced. When *Synechococcus* PCC6301 was transferred from aerobiosis to anaerobiosis, the amount of NADPH, ADP and AMP increased, $NADP^+$ and ATP decreased, whereas NADH and NAD^+ remained unchanged. It was concluded that NADPH and not NADH was the primary electron donor for respiration (Biggins, 1969). While organic compounds are oxidized, NADPH is generated, however, it cannot be reoxidized, as the terminal acceptor O_2 is missing in anaerobic conditions. Without O_2 electrons cannot pass the respiration chain and no ATP will be generated. Both NADH and NADPH are in vitro oxidized by NAD(P)H dehydrogenases (NDH). In vivo NADH might be made from NADPH by a pyridine nucleotide transhydrogenase.

Three types of NDH are known. Type-I NDH (resembles the mitochondrial type in eukaryota) has seven subunits and is believed to be localized in both membranes (ICM and CM). Type-II NDH is probably a monomer. The third type is a ferredoxin:NADPH dehydrogenase (FNR), which can also function as a NADPH dehydrogenase. Besides NADPH succinate and H_2 can be used as electron donors for the quinon pool. Two succinate dehydrogenases have been discovered in PCC6803. For H_2 three hydrogenases are known: Nitrogenases, uptake hydrogenases and bidirectional hydrogenases. Nitrogenases produce H_2, if H^+ but not N_2 is the substrate.

2.5.2.2. Quinone pool

Stoichometrically, the quinones are the components that are most prevalent within the cell. Therefore the redox state by a cell is defined by the quinone pool. They are localized in both membranes. If the pool is over reduced (e.g. there is more PSII than cyt b_6f) it will be oxidized directly by the quinol oxidase (Qox) or by the plastid like terminal oxidase (Ptox), however the latter exists only in a few organisms and little is known yet.

2.5.2.3. Cytochrome b_6f complex

It is reduced by the quinon pool and oxidized by cytochrome *c* or plastocyanin. When electrons pass the b_6f complex, which is located in both membranes, protons are translocated and an H^+ gradient is built up. In cyanobacteria the b_6f complex is passed in both the photosynthetic and the respiratory electron chain. Cyanobacteria are believed to be the only organisms, where b_6f is also used for respiration, because in eukaryotic mitochondria it is replaced by cytochrome bc_1 complex.

2.5.2.4. Peripheral intermediate electron carriers

These factors are not localized to a special membrane, but are soluble. They transfer electrons from cytochrome b_6f complex to photosystem I or cytochrome *c* oxidase. Two types are known in cyanobacteria: cytochrome *c*, which has a heme iron cofactor, and plastocyanin, which has a copper cofactor. The usage of the two classes of electron carriers in photosynthesis (and respiration) differs among the cyanobacterial strains. Sandmann and Böger (1980) discovered that the presence of plastocyanin and cytochrome *c* was dependent on the Cu^{2+} concentration among some species. According to Sandmann (1986) there are three groups:

1) strains that have a gene only for cytochrome *c*, but not for plastocyanin
2) strains that have genes for both classes, but their expression depends on the Cu^{2+} content of the growth medium. At low concentrations only cytochrome *c*, whereas at high Cu^{2+} concentrations only plastocyanin is expressed.
3) strains that have genes for both and express cytochrome *c* constitutively, but plastocyanin only at high Cu^{2+} concentrations.

Synechocystis PCC6803 belongs to the second group, so the contribution of the intermediate electron carriers can be controlled by the Cu^{2+} concentration. Over 300nM Cu^{2+} there is nearly no cytochrome *c* and under 0.1µM there is hardly any plastocyanin (Zhang et al, 1992). When grown in BG11 medium according to Rippka et al (1979) there is a medium Cu^{2+} concentration of 0.3µM so that both plastocyanin and cytochrome *c* are present. In no cyanobacterium it was possible to knock out plastocyanin as well as cytochrome *c*. It is assumed that either of the two is sufficient for both photosynthesis and respiration. In some strains that were thought to belong to

Sandmann group 1 the gene for cytochrome *c* had been knocked out (Laudenbach et al., 1990), but later a gene for plastocyanin was discovered. At the moment PCC7002 is believed to be the only member of group 1 as no gene for plastocyanin has been discovered. Plastocyanin expression at low Cu^{2+} concentration is not completely inhibited (Pils et al, unpublished), because a cytochrome *c* knock out mutant from PCC6803 could still grow at a normal level when raised at very low Cu^{2+} concentrations (performed by Zhang et al, 1994). The same is true for cytochrome *c* expression at high Cu^{2+} concentrations.

2.5.2.5. Respiratory terminal oxidases

Cox, Qox, Ptox and ARTO are summarized as respiratory terminal oxidases (RTOs), which all reduce O_2 to H_2O. While several components like the quinone pool, cytochrome b_6f complex, cytochrome *c* and plastocyanin (the latter only assumed) are active in both photosynthetic and respiratory electron transport, RTOs are considered as the key enzymes of respiration, as they have no direct function in photosynthesis.

a) Heme copper oxidases

There exist three types of heme copper oxidases: the genuine cytochrome *c* oxidases (Cox), the alternative respiratory terminal oxidase (ARTO) and the type cbb_3 oxidase.

a1) Genuine cytochrome *c* oxidases

Except for very few strains all known cyanobacteria have at least one Cox. It is also very rare that a strain possesses more than one genuine Cox. Cytochrome *c* is oxidized either by photosystem I or by the Cox, as a cox^- (Schmetterer et al., 1994) and a PSI^- single mutant (Vermaas et al, 1994) but not a PSI^-/cox^- double mutant could be created in PCC6803 (Manna and Vermaas, 1997). The cyanobacterial Cox is assembled from three subuntits called A, B and C, which are homologous to the subunits I, II and III of mitochondrial cytochrome *c* oxidase. In mammalian mitochondria cytochrome *c* oxidase is a large protein with 13 subunits, however, only subunits I-III are encoded by the mitochondrial genome. The other subunits are encoded by chromosomes inside the nucleus. Within cyanobacteria no genes have been found that were homologous to the genes in the eukaryotic nucleus encoding mitochondrial Cox subunits IV-XIII.

In all known cyanobacteria the genes for the Cox subunits are clustered in an operon called *coxBAC* or *ctaCDE* (Howitt and Vermaas, 1998, Schmetterer et al., 2001). The oxidation of cytochrome *c* by Cox could be demonstrated by an in vitro assay. The membranes from cyanobacteria were isolated and ICM and CM were separated. The pure membranes were mixed with pre-reduced cytochrome *c* that had been isolated from horse heart. For PCC6803 it was concluded that only membranes containing a Cox could oxidize cytochrome *c*. cbb_3, which can also oxidize horse heart

cytochrome *c*, is not present in this strain. In PCC6803 Cox seems to be located exclusively in the ICM, as the CM shows no cytochrome *c* oxidase activity. In other strains like *Plectonema boryanum* PCC6306 and *Synechococcus* PCC7942, however, the CM can oxidize horse heart cytochrome *c* (Peschek et al, 1988). In many strains the capacity for chemoheterotrophic growth is dependent on the presence of a functional cytochrome *c* oxidase. This has been so far demonstrated for PCC6803 (Pils et al, 1997) and ATCC29413 (Schmetterer et al, 2001), where knock out mutations of *cox1* eliminated this capacity.

a2) Alternative respiratory terminal oxidase (ARTO)

Many cyanobacteria have more *coxBAC* clusters, which are named *cox1BAC*, *cox2BAC* and so on. The numbers indicate the homology to the subunits I, II, III of mitochondrial cytochrome *c* oxidase, with *cox1BAC* being the cluster most closely related to mitochondrial *cox*. Usually only *cox1*BAC codes for a real Cox, whereas the others code for an ARTO. Besides the homology between Cox and ARTO there are two main differences in their protein sequences (Schmetterer et al, unpublished).

1) At the Mg^{2+} binding site in subunit I
   Cox: WAHHMF
   ARTO: WVHHMF (in most ARTOs, but not universal)
2) At the Cu^{2+} binding site in subunit II
   Cox: CaElCgpyHgvM (Cu^{2+} binding motive)
   ARTO: DsqfSGaYfatm (function not yet clear)

The capital letters indicate conserved amino acids, the others are frequent, but not universal and sometimes replaced by related amino acids. A heme copper oxidase needs to have both Cox characteristics to be defined as a Cox. If one or both are missing the enzyme is viewed as an ARTO. As a conclusion there are three subclasses of ARTOs.

Type I: Cox motive neither in subunit I nor in subunit II (ARTO in both senses)
Type II: Cox motive only in subunit II
Type III: Cox motive only in subunit I

The motive of subunit I in ARTO (type III excluded) is not universal. Mostly the alanin from Cox is replaced by valin, however, it may be substituted by another amino acid. As the Mg^{2+} binding site of subunit I is essential for the transmembrane translocation of H^+, it is probably necessary for creation of a proton gradient. This might explain, why ARTOs from type I and II (most of the ARTOs) cannot support chemoheterotrophic growth. Perhaps a type III ARTO has this capacity, however, type III is very rare, therefore this has not been tested so far. The Cu^{2+} binding site is essential for acceptance of electrons from cytochrome *c*, which leads as a consequence to a positive horse heart cytochrome *c* oxidation assay. This means that both Cox and

ARTO from type II can oxidize horse heart cytochrome *c* in vitro. Therefore type II ARTOs have been classified as Cox in the past. Although the Cu^{2+} binding motive is missing in ARTOs except type II, it is replaced by another motive, which is not very similar to the Cox motive, however, it is also very conserved, as all Cox and ARTOs have one or the other sequence. The function of the ARTO motive in subunit II remains unclear, as no specifical knock out of this region has been done so far. Perhaps it is the motive required for oxidation of a yet unknown component of a respiratory chain. Despite the significances of the Mg^{2+} and Cu^{2+} binding motives in the two subunits, they are not the only differences between ARTO and Cox. A hypothetical Cox subunit II, whose Cu^{2+} binding motive is replaced by the ARTO typical sequence, is still more closely related to the Cox subunits II of all cyanobacteria and vice versa, however, the importance of the different regions within a subunit has not been analysed yet. Apart from cyanobacteria the cytochrome *bo* quinol oxidase from *E. coli* seems to be related to ARTOs (especially type I), however, ARTOs have not been demonstrated to oxidize the quinone pool.

The number of ARTOs differs very much between the diverse strains. Nearly all ARTOs are clustered in the same orientation as Cox: *coxBAC*. An important exception is *Synechocystis* PCC 6803, whose *coxB2* is located far away from *cox2AC*, which constitute an operon as expected. Although sequence analysis of ARTOs from other strains revealed that CoxB2 was the missing subunit II, it was questioned whether it was really necessary. Perhaps CoxB1 of the real Cox can assemble to a functional ARTO with CoxA2 and CoxC2. As CoxB2 does not include a Cu^{2+} binding site, the expected ARTO is from type I (no in vitro cytochrome *c* oxidase activity). If CoxB1 is really able to trimerize with CoxA2 and CoxC2, a type II ARTO (with in vitro cytochrome *c* oxidase activity) will be made, which increases the RTO variety.

The biological function of ARTO in PCC6803 remains unclear. A strain had been constructed which had no RTO except the ARTO. It respired (consumed O_2) but only at a very low level (about 1% of the WT respiration). However, this strain was able to take up 3-O-methylglucose (an analogon of glucose that cannot be metabolized) in the dark at a rate of about 50% of the WT, whereas a mutant strain, whose RTOs were all deleted, failed to take up any 3OMG. This ARTO mediated uptake was abolished if the gene for cytochrome *c* was deleted (Pils and Schmetterer, 2001). Therefore an electron chain has to exist, which involves both cytochrome *c* and ARTO, although ARTO failed to oxidize cytochrome *c* in vitro. Perhaps there has to be another component between them, or the oxidase activity of ARTO is too small to be detected, but has enough significance in vivo.

As the subunit II of ARTO was the only peptide of an RTO that has been detected in an isolated CM of PCC6803 (Huang et al, 2002), it was concluded that ARTO was localized in the CM and its activity provided enough energy for the uptake of sugars and analogons. Despite the low respiration of a strain with ARTO as the only

RTO, its contribution to total respiration in the WT may be much higher. A strain of PCC6803 which had a deleted Cox but an intact ARTO and an intact Qox (see below) respired half the rate of the WT. If HQNO or PCP was added (both are inhibitors of Qox, but not for Copper heme oxidases like Cox and ARTO) only 72% and 83% were inhibited. Thus the remaining respiration had to be due to the activity of the ARTO (Pils and Schmetterer, 2001).

PCC7120 has 3 Cox/ARTO clusters. *cox1BAC* encodes the real cytochrome *c* oxidase, whereas *cox2BAC* and *cox3BAC* code for ARTOs, Cox2 is of type II, Cox3 of type I. In filamentous PCC7120 both ARTOs seem to be exclusively located to the heterocysts, whereas the genuine Cox is active in the vegetative cells. PCC7120 grown in media containing NH_4^+ or NO_3^- showed *cox1*, but no *cox2* or *cox3* transcription. *cox1* transcription was constitutive and did not change when the strain was grown on a medium lacking combined nitrogen, which induces heterocyst formation. In that situation both *cox2* and *cox3* are enhanced. In isolated heterocysts no *cox1* was transcribed. A *cox2*$^-$/*cox3*$^-$ double mutant lost the capacity to grow diazotrophically (on di-nitrogen without bound nitrogen), whereas both single mutants were still capable of diazotrophic growth. Thus in heterocysts a main function of RTOs is to remove O_2 by reducing it to H_2O, as nitrogenase is extremely sensitive towards O_2 (Valladares et al, 2003).

Anabaena ATCC 29413 has five *cox* clusters. One (*cox1BAC*) seems to encode a genuine Cox, as its knock out abolishes the capacity for heterotrophic growth (Schmetterer et al, 2001). One (*cox2BAC*) encodes an ARTO of type II, the remaining three (*coxBAC3-5*) code for ARTOs of type I. Only *cox2BAC* was enhanced, when the strain was grown under diazotrophic conditions, however, the locus was transcribed under combined nitrogen (NO_3^-) too, but at a lower extent (Pils et al, 2004). This observation was contrary to PCC7120, where *cox2BAC*, which encodes the same RTO type (ARTO type II) will not be expressed, if the medium is supplemented with NO_3^- or NH_4^+. In ATCC 29413 the transcription of *cox3BAC* is very weak and is not enhanced, when the strain is grown under diazotrophic conditions (contrary to PCC7120).

a3) Cytochrome *cbb*$_3$ type oxidase (Schmetterer, unpublished)

Oxidases of type *cbb*$_3$ are not very widespread among cyanobacteria. It has been identified in the two *Synechococcus* sp. strains PCC7942 and PCC6301. It can oxidize horse heart cytochrome *c*. In purple bacteria it seems that *cbb*$_3$ acts as a cytochrome *c* oxidase. In cyanobacterial strain *Trichodesmium erythraeum* IMS101, where a real Cox is missing, the *cbb*$_3$ oxidase might be the most important in the respiratory chain.

b) Quinol oxidase (Qox)

It has two subunits, which are homologous to the two subunits of cytochrome *bd* quinol oxidase in *E. coli*. They are encoded by the genes *cydA* and *cydB*, which are clustered in an operon. Within cyanobacteria these genes were first discovered in PCC6803, when the complete genomic sequence was determined (Kaneko et al, 1996). Later quinol oxidases have been detected in various cyanobacterial strains. A PCC6803 mutant strain that lacks both Cox and ARTO with Qox being the only remained functional RTO respired at a similar rate than the WT. When Qox was deleted too, the triple mutant did not respire any more (Howitt and Vermaas, 1998). As no further genes were discovered with sequence similarities to subunits of RTOs, it was concluded that PCC6803 had three RTOs: 1 Cox, 1 ARTO, 1 Qox. It has been demonstrated that Qox mediates the oxidation of the quinone pool without involving the cytochrome b_6f complex (Berry et al, 2002). The components of both the respiratory and the photosynthetic electron transport chain do not exist in equal amounts. There is a dramatic over excess of the quinone pool. If the quinone pool is totally reduced, it cannot oxidize PSII or NADPH anymore. PSII* (PSII, which is activated by photons) will revert to PSII, the electrons will be transferred back to O_2, but in an uncontrolled manner. Without an oxidase enzyme reactive oxygen species like O_2^- will be created that damage the cell. Therefore Qox has to prevent an over reduction of the quinone pool, if it is not oxidized as quickly by PSI as it is reduced by PSII. Under these circumstances Qox has to transfer electrons from quinone to O_2 to ensure an appropriate redox state.

While in the WT of PCC6803 40-50% of the total respiration in the dark was inhibited after HQNO or PCP was added, the respiration of the strain with Qox as the only RTO was abolished completely after HQNO or PCP addition. So it is assumed that in the WT 40-50% of the total O_2 consumption is mediated by Qox. Both deletion and inhibition of an RTO by a specific reagent can influence the O_2 consumption rate of the other RTOs. Normally the respiration rate of PCC6803 can be enhanced when glucose (the only known organic compound that can be used by this strain for chemoheterotrophic growth) is added. If Cox and ARTO were deleted, the Qox mediated respiration was not enhanced by glucose any more. Both heme copper oxidases and quinol oxidases are inhibited by potassium cyanide (KCN).

c) Plastid terminal oxidase (Ptox)

First it was thought that in cyanobacteria there do not exist any KCN insensitive RTOs, which have been discovered in mitochondria of plants and some other eukaryotes. They were called alternative oxidases (Aox) (Vanlerberghe and McIntosh, 1997). In chloroplasts of plants a similar class of oxidases was found, the so called plastid terminal oxidases (Ptox). Both classes share an insensitivity towards KCN but they must have diverged before endosymbiosis took place. Prokaryotic orthologs of

both groups have been found (McDonald et al, 2003). In PCC7120 a *ptox* gene was identified, which was shown to be expressed. In chloroplasts the Ptox directly oxidizes plastoquinol (the quinone of chloroplasts) and seems to be important for carotinoid biosynthesis (Carol et al, 1999; Wu et al, 1999) and chlororespiration (Carol and Kuntz, 2001; Peltier and Cournac, 2002).

2.5.2.5.1. Inhibitors of the terminal oxidases

KCN

KCN inhibits heme copper oxidases as well as quinol oxidases. The only RTOs, which are known to be insensitive towards KCN are the plastid terminal oxidases (Ptox). KCN is very toxic because it inhibits the cytochrome *c* oxidase in mitochondria and blocks cellular respiration. In respiration measurements with PCC6803 KCN is added at the end of the experiment. The strain does not respire anymore, as it does not have any KCN insensitive RTOs.

HQNO (2-heptyl-4-hydroxyquinoline-N-oxide)

It inhibits specifically cytochrome *bd* type quinol oxidase in *E. coli* (Borisov, 1996). It is used for determintation of the portion of the total respiration mediated by quinol oxidases. For example if the respiration of the WT of PCC6803 is measured, 40 % is inhibited when HQNO is added. Therefore it is assumed that the Qox respiration (O_2 consumption) is 40% of the total respiration. The remaining 60% are mediated by Cox and ARTO. A PCC6803 mutant with Qox as the only RTO is completely inhibited by HQNO (Pils and Schmetterer, 2001).

PCP (pentachlorophenol)

It was shown in PCC6803 to have the same effect as HQNO (Borisov, 1996; Pils and Schmetterer, 2001).

Azide

Its effect is opposite of HQNO as it inhibits heme copper oxidases, but not Qox (Borisov, 1996).

DMBIB (2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone)

It blocks the cytochrome b_6f complex. So neither photosynthesis via photosystem I nor respiration via cytochrome *c* oxidase is possible. Electrons both from photosystem II and from NADPH will reduce the quinone pool, which can only be oxidized by the quinol oxidase (Qox), which is very important now to prevent over reduction of the quinone pool. In experiments with PCC7120, however, the respiration was enhanced by DMBIB. Apparently the quinol oxidase was activated, transferring all electrons reducing the quinone pool to O_2.

Octyl gallate

It specifically inhibits plastid terminal oxidases (perhaps the only inhibitor for them).

Unfortunately there does not exist an inhibitor that distinguishes between Cox and ARTO. So it is very difficult to determine the portion of the ARTO mediated respiration within the total respiration.

DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea)

Actually it is not an inhibitor of respiratory terminal oxidases. It binds to photosystem II and prevents the transfer of electrons to the quinone pool. As a consequence the liberation of O_2 is stopped, however, photosystem I still functions and the electrons may undergo the cyclic electron flow. In the light, when PSI is active, O_2 is neither produced nor consumed. This means that the O_2 content of the surrounding medium remains constant.

2.6. Different trophies in cyanobacteria

According to their photosynthetic nature they are designed for photolithoautotrophy. Although all cyanobacteria can respire in the dark, many cyanobacteria are obligate photolithoautotrophs. During light periods CO_2 is fixed within the calvin cycle and the produced glucose is incorporated into glycogen which is stored in the glycogen granuli. During dark periods the glycogen is phosphorylized, however, only a few strains are able to grow in the dark on organic molecules (chemoheterotrophy).

ATCC 29413 was demonstrated to grow in complete permanent darkness with fructose as a carbon source (Wolk and Shaffer, 1976). Additionally fructose enhanced photolithoautotrophic growth under diazotrophic conditions (Haurey and Spiller, 1981). *Nostoc punciforme* ATCC29133 can grow in the dark on both glucose and fructose (Summers et al, 1995). For PCC6803 the only organic substrate that can be used is glucose. However, PCC6803 grown in the dark on a medium containing glucose needs an illumination of at least five minutes per 24h to grow, which was called light-activated heterotrophic growth (LAHG, Anderson and McIntosh, 1991). Although growing in permanent darkness failed, it is thought to be a real chemoheterotrophic growth, as the same strain did not grow at 5min light per 24h, when glucose was missing. Besides the energy obtained from 5min illumination per day did not explain the growth rate when glucose was added. It was believed that the short illumination is necessary to activate a phytochrome, which can change between two forms, within a day all molecules of this phytochrome revert spontaneously to the lower energized form. So they have to be activated in a defined interval.

Although these two strains are not closely related (PCC6803: unicellular, section I; ATCC29413: filamentous, heterocystous, section IV), in both cases the capacity for chemoheterotrophic growth is strictly dependent on the presence of the genuine cytochrome *c* oxidase, as *cox1* knock-out mutations in both strains lose this

capacity. PCC7120 has one other RTO with in vitro cytochrome *c* oxidase activity, but this enzyme is now classified as ARTO, type II and does not support chemohetrotrophic growth, as a *cox1*$^{-}$/*cox2*$^{+}$ strain clearly demonstrated. While fructose is a suitable energy and carbon source for ATCC29413, it is not only useless but even toxic for PCC6803, because the cells also die when grown in the light under the presence of fructose.

Transmembrane carrier Gtr (also called glucose-fructose permease or GlcP) is responsible for both glucose and fructose uptake in PCC6803, as a mutation in *gtr* abolishes the capacity for heterotrophic growth. Normally fructose is toxic for this strain. When plating a PCC6803 culture onto agar containing fructose, a few colonies appear. Genetic analysis revealed that they all had mutations in the *gtr* gene. So Gtr is not only responsible for the capacity for heterotrophic growth, but also for the sensitivity towards fructose (Flores and Schmetterer, 1986; Joset et al, 1988; Zhang et al, 1989). Gtr can import both glucose and fructose, but with a different affinity. The toxicity of fructose can be prevented, if 10% glucose of the amount of fructose was added. It was concluded that glucose binds to Gtr with an affinity of at least 10 times compared to fructose. If there is enough glucose, it will be imported and fructose has no chance to bind to Gtr, even if there is a ten times amount of fructose (Joset et al, 1988). When the strictly photolithoautotrophic strain *Synechococcus* sp. PCC7942 was transformed with *gtr*, glucose was taken up, however, the cells died in the presence of glucose (Zhang et al, 1998). This clearly demonstrates that diverse strains differ in both transport system and metabolism.

Experiments with radiolabeled chemicals revealed that glucose but also 3-O-methylglucose (3OMG) was taken up by WT PCC6803, whereas a *gtr*$^{-}$ knock-out mutation called GS1 failed at both sugars. However, there was a difference in the uptake properties between these two substances. The amount of incorporated glucose continuously increased, whereas 3OMG first was taken up at a similar rate, but after about an hour the incorporated amount stagnated. Glucose was phosphorylated and further metabolized (e.g. incorporated into glycogen), so the inner cellular amount remained always zero and the uptake continued. On the other hand 3OMG is only taken up but cannot be further metabolized, so it remains unchanged inside the cell. Once there will be such a high concentration inside the cell that the active carrier cannot translocate any more against the gradient and the uptake of 3OMG stops.

In nature cyanobacteria can grow photolithoautotrophically in the light (all) and chemoheterotrophically in the dark (some), however, in the laboratory photohetrotrophic conditions can be generated. If DCMU (see above) is added, the transfer of electrons from PSII to the quinone pool is blocked and the noncyclic pathway is inhibited so no H_2O can be oxidized anymore. Organic compounds like glucose will be needed both as electron and as carbon sources, therefore the cells perform organoheterotrophy. However, photosystem I is still active and electrons can

undergo the cyclic electron transport pathway, thereby creating ATP while passing the cytochrome b_6f complex. As light is still used as an energy source the cells grow photoorganoheterotrophically. Photoheterotrophy can also be performed by strains that lack a genuine cytochrome *c* oxidase, because cytochrome *c* or plastocyanin can be oxidized by PSI. Like chemoheterotrophy, photoheterotrophy is also strictly dependent on the presence of a carrier molecule that imports the organic substrate.

PCC6803*gtr*$^-$ cannot grow in the presence of glucose and DCMU in the light. According to Rippka et al (1979), PCC7120 was believed to be strictly photolithoautotrophic despite the presence of 5 RTOs (1 genuine cytochrome *c* oxidase). Considering the fact that ATCC29413, which is closely related to PCC7120, can grow chemohetrotrophically dependent on fructose, the question is raised, whether the incapacity of heterotrophic growth by PCC7120 is exclusively caused by the missing of a sugar transport system, analogous to the *frtRABC* genes in ATCC29413. If PCC7120 is manipulated that it can take up for instance fructose, it will probably gain the ability of heterotrophic growth. According to this hypothesis Ungerer et al. (2008) identified an operon of three genes *frtABC*, which was under the control of *frtR*, a gene located next to the operon and encoding a repressor. In *Nostoc punctiforme* ATCC 29133 a homologous operon was identified, but not in PCC7120. It was observed that *ftrR* was very important, because an *frtR*$^-$ mutation was extremely sensitive towards fructose. It was discussed that fructose might be toxic when it is imported in an uncontrolled manner, without a repressor. Ungerer et al. (2008) introduced *frtRABC* into PCC7120 where chemoheterotrophic growth at a low rate was observed. Photoheterotrophic growth was also reported but not shown in that article. An introduction of *frtABC* without *ftrR* into PCC7120 led to a phenotype similar to that of ATCC29413*frtR*$^-$.

2.7. Aim of the doctoral thesis

PCC7120 has 5 RTOs: 1 Cox, 2 ARTOs, 1 Qox, 1Ptox. Knock out mutations already existed of the two ARTOs (*cox2* and *cox3*) (Valladares et al, 2003). Single mutants of Cox and Ptox were created to analyse their phenotypes (e.g. by Clarke electrode). We supposed that isolated membranes of a *cox1*$^-$ strain do not show any in vitro oxidase of horse heart cytochrome *c* and the the influence of specific inhibitors to the respiration rate differs from the WT.

gtr (from PCC6803), which was located on the replicative plasmid pBWUV6, was introduced into PCC7120 by conjugation. Since it was known that transfer of *frtRABC* from the closely related strain ATCC29413 resulted in facultative chemoheterotrophs, it was unclear, whether *gtr* from a more distantly related unicellular strain would have the same effect in PCC7120. No plasmids occurring naturally in PCC6803 can replicate in PCC7120 and vice versa. The sequences for

promoters may also differ. Plasmid pBWUV6 that was conjugated into PCC7120 contains the whole ORF and upstream regulatory sequences. It was not sure, whether it would be recognized in the new host. Experiments with labelled glucose or 3OMG reveal, whether the transgenic PCC7120*gtr*$^{+}$ strain can take up sugars (the gene has to be expressed and its product has to act as expected).

Two mutant strains of PCC6803 have been created, which have ARTO as the only active RTO, BC2 and BC3. In BC2 all Qox genes (*cydA* and *cydB*) and two subunits of Cox (*coxA1* and *coxC1*, sometimes also called *ctaDI* and *ctaEI*) are deleted, whereas in BC3 all Qox (*cydA* and *cydB*) and all Cox (*coxB1*, *coxA1* and *coxC1*) genes are deleted. The *cox1* locus (2 genes in BC2, 3 genes in BC3) has been knocked out by a neomycin phosphate transferase, the *cyd* locus by an erythromycin resistance cassette. In comparison to the WT BC2 and BC3 had a respiration rate of 1%, but took up half the amount of 3OMG. These two strains do not differ in their phenotype since the intact CoxB1 is not sufficient to trimerize to a functional Cox when assembling with 2 ARTO subunits, so in both cases ARTO is the only active RTO.

To analyse the role of CoxB2 for the assembled ARTO *coxB2* was knocked out in both strains. Now the hypothesis was tested whether CoxB1 and CoxA2C2 could trimerize to a functional ARTO. If *coxB2* is deleted in a BC2 strain, CoxB1 will still be present to form an ARTO, however, in strain BC3 this is not possible. If BC2*coxB2*$^{-}$ still takes up 3OMG, a trimerization between 1 Cox and 2 ARTO subunits will be realistic. The same experiment has to be performed with BC3*coxB2*$^{-}$ as a further check. If this strain also takes up 3OMG, it will indicate that CoxB2, whose ORF was located far away, is not necessary for a functional ARTO at all. If no uptake occurs at any experiment, the fuction of the ARTO can be concluded as being strictly dependant on the presence of CoxB2.

3. Materials and Methods

3.1. Strains

Cyanobacterial strains

- *Synechocystis* PCC6803 (Rippka et al, 1979; sequenced by Kaneko et al, 1996): WT, which includes the *gtr* gene, which was used for further cloning.
- *Synechocystis* PCC6803GS1: deletion of *gtr* (*sll0771* according to Cyanobase, 2011) by a neomycinphosphate transferase (Schmetterer, 1990)
- *Synechocystis* PCC6803ABC (Howitt and Vermaas, 1998): all known RTOs in this strain have been knocked out
- *Synechocystis* PCC6803BC2 (Howitt and Vermaas, 1998) both subunits of Qox and 2 subunits of Cox (A and C) are deleted, but *coxB1*(*sll0813* according to Cyanobase, 2011) was left intact. Nevertheless ARTO is the only remained active RTO
- *Synechocystis* PCC6803BC3 (Pils and Schmetterer, 2001) both Qox and all 3 subunits of Cox have been deleted. The three ORFs encoding the ARTO are the only intact genes for RTO subunits.
- *Synechocystis* PCC6803BC2*coxB2*$^-$direct (this thesis)
- *Synechocystis* PCC6803BC2*coxB2*$^-$inverse (this thesis)
- *Synechocystis* PCC6803BC3*coxB2*$^-$direct (this thesis)
- *Synechocystis* PCC6803BC3*coxB2*$^-$inverse (this thesis)
- *Anabaena* PCC7120 (Rippka et al, 1979; sequenced by Kaneko et al, 2001)
- *Anabaena* PCC7120*cox1*$^-$: a part of *coxA1* was deleted and replaced by a neomycinphosphate transferase, which had been inserted in the same direction (constructed during this thesis)
- *Anabaena* PCC7120(pBWUV6) = PCC7120*gtr*$^+$: contains plasmid pBWUV6 that includes the *gtr* from PCC6803 (constructed during this thesis)
- *Anabaena* PCC7120*ptox*$^-$: *ptox* was interrupted by a gentamicin resistance cassette, which had been inserted in the same direction.

***E. coli* strains**

- ***Escherichia coli* HB101** (Boyer and Roulland-Dussoix, 1969)
  F$^-$ mcrB mrr hsdS20(r_B^- m_B^-) recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20(Sm^R) glnV44 λ^-
- ***Escherichia coli* JM109** (Yanisch-Perron, 1985)
  endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB$^+$ Δ(lac-proAB) e14- [F' traD36 proAB$^+$ lacI^q lacZΔM15] hsdR17($r_K^-$$m_K^+$)

- ***Eschericha coli* TOP10**
  F^- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Sm^R) endA1 λ^-
- ***Escherichia coli* DH5α** (Woodcock, 1989)
  F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(r_K^- m_K^+), λ^-

3.2. Plasmids

pRL278 (Black et al, 1993) contains a neomycin phosphate transferase for positive selection and a *sacB* gene for negative selection if sucrose is added.

pBWUV1 (Wurzinger, diploma thesis): a portion of 7120 WT genome including the whole *coxA1* and part of *coxB1* and *coxC1* open reading frames had been amplified by specific degenerated primers named 7120coxB and 7120coxC (see section 3.4.1), which bind inside the *cox1B* and *cox1C* ORF.

On the 5' ends of both primers an *Xba*I site had been attached and two bases 5' next to it, to ensure that the PCR fragement can be cut on both sides. PCR was done with isolated DNA from PCC7120WT as template. The PCR product was purified by a 0.7% agarose gel. Fragment with 2448bp was cut out and purified. Then it was digested with *Xba*I. pRL278, which contains three *Xba*I sites, was also digested with *Xba*I. Both the digested PCR product and the digested pRL278 were purified by a 0.7% agarose gel. The PCR product of 2438bp and the longest pRL278 fragment of 5461bp were cut out, purified and ligated.

pBR328 (Soberon et al, 1980, Prentki et al 1981) contains resistances against chloramphenicol and tetracycline.

pBR328B was digested (linearized) with *Bam*HI and treated with mung bean nuclease to remove overhanging ssDNA. After that it was religated. The new plasmid kept resistance against chloramphenicol but lost resistance towards tetracycline (*Bam*HI site is localized within cassette) (Wurzinger, diploma thesis).

pBWUV2 (Wurzinger, diploma thesis): An *Asi*SI-*Sgr*AI fragment was cut out from the middle of the *coxA1* ORF on pBWUV1 and replaced by a chloramphenicol resistance cassette. pBR328B, which contained a chloramphenicol resistance cassette, had been linearized with *Ava*I and served as template for the PCR performed with the primers Cm5 and Cm3 (see section 3.4.2). Cm5 contained a *Pvu*I site, whereas Cm3 contained an *Age*I site.

The PCR was performed with the following program:

5min	94°C
30s	94°C
30s	41°C
1min 40s	72°C
7min	72°C

After the PCR the product was purified by a 0.7% gel, cut out (at 1076bp) and extracted. Then it was digested with *Pvu*I, extracted by chloroform, and precipitated with ethanol. After that it was digested with *Age*I. pBWUV1 was digested with *Asi*SI. The reaction mix was extracted with chloroform and precipitated with ethanol. Afterwards it was digested with *Sgr*AI. Both double digested samples were purified by a 0.7% agarose gel and fragments at 7681bp (pBWUV1) and 933bp (CmR) were cut out, extracted and ligated.

pBWUV3 (Wurzinger, diploma thesis) This plasmid was constructed for pBWUV2, from which the neomycin resistance was deleted. pBWUV2 was digested with *Bam*HI and *Eag*I. Then it was extracted with chloroform and precipitated with ethanol. After that it was treated with mung bean nuclease, which removed the overhanging ends. Finally it was purified on a 0.7% agarose gel, cut out (about 7200bp) and ligated (recirculated without the neomycin fragment).

pRStUV3 (constructed during this thesis) The chloramphenicol resistance cassette was replaced by the neomycin phosphate transferase (*npt*), which is the same as in pRL278, pBWUV1 and pBWUV2. *npt* was amplified by PCR with pRL6 (see below) as template DNA (from a preparation according to Holmes and Quigley, see section 3.8.) and Kmcassette2 and Kmcassette3 as primers. Kmcassette2 contained a *Pvu*I site, Kmcassette3 contained a *Sca*I site, which are underlined below (see 3.4.3.). PCR was performed. Then the product was extracted with chloroform and precipitated with ethanol. The *npt* PCR product and pBWUV3 were restricted with *Pvu*I and *Sca*I, purified on a 0.7% gel, cut out and extracted.

pDU1 (Reaston et al, 1982) is a plasmid from the filamentous cyanobacterium *Nostoc* PCC7524. It can replicate in many filamentous cyanobacteria like PCC 7120 but not in unicellular strains like PCC6803.

pRL1 (Wolk et al, 1984): pBR322 (Bolivar et al, 1977) and pDU1 have been fused. Both have been linearized with *Eco*RV and then ligated. pBR322 has only one *Eco*RV site, whereas pDU1 contains 3 such sites. The latter plasmid was only partially digested, so it became linearized too. The fusion plasmid was called pVW1, which was specially treated with *Ava*II and *Sau* 96I and then the fragments were ligated again

together with pBR328. The new plasmid pRL1 lost all *Ava*I and *Ava*II sites and contained a resistance cassette for chloramphenicol derived from pBR328.

pRL6 The neomycin phosphate transferase from Tn5 of pColE1::Tn5 (Auerswald et al, 1981; Beck et al, 1982) was inserted into pRL1, introducing thereby an *Ava*II site (Wolk et al, 1984).

pRL7 the neomycin transferase from Tn5 (Auerswald et al, 1981; Beck et al, 1982) was inserted into pRL1 but in the other direction as in pRL6 (Wolk et al, 1988).

pRL25 is derived from pRL7, which has been partially digested with *Mst*I to remove the chloramphenicol acetyl transferase. The portion between *Nde*I and *Eco*RI had been replaced by the *Nde*I-*Eco*RI part of pRL1VB (Wolk et al, 1988).

pBWUV6 (Wurzinger, diploma thesis) *gtr* from PCC6803 was introduced into pRL25. Plasmid pGT12 (Zhang et al, 1989), which contains *gtr*, and pRL25 were isolated by Ish-Horowicz preparation and digested with *Bam*HI. The fragments were separated by a 0.7% agarose gel. The 2055bp fragment containing the *gtr* gene with its promoter (both derived from PCC6803) and the ~9800bp vector of pBWUV6 were cut out and ligated.

pBWUV4 (Wurzinger, diploma thesis) the downstream flanking sequence of *ptox* was amplified by PCR using primers Ptox downstream 5' and Ptox downstream 3'and PCC7120WT DNA as template. The PCR fragment was inserted into pRL278 between the *Eco*O109I and *Xho*I sites.

pPSUV1 (Wurzinger, diploma thesis) the upstream flanking sequence of *ptox* was amplified by PCR using primers Ptox upsream 5' and Ptox upstream 3' and PCC7120WT as template DNA. The PCR fragment was inserted into pRL278 between *Xho*I and *Bam*HI site.

pBWUV5 (Wurzinger, diploma thesis) both the upstream and the downstream flanking sequences were inserted into pRL278. They are connected at the *Xho*I site.

pRStUV1 (prepared during this thesis) The Gm^R cassette from pBBR1MCS-5 (Kovach et al., 1995) was amplified by PCR using the primers pbbr1-5 and pbbr1-3 and introduced into pBWUV5 at the *Xho*I site.

pRStUV2 like pRStUV1, however the Gm^R cassette has been introduced into the other direction

pUC19 contains *lacZα* including a multiple cloning site (MCS), an *ori* and an ampicillin resistance cassette (*amp*). Closely related pUC18 only differs at the orientation of the MCS (Messing, 1983; Norrander et al, 1983; Yanisch-Perron et al, 1985).

pGGUV1 (Graser, unpublished) a flanking sequence from *sll0813* was introduced into pUC19.

pGGUV2 (during this thesis): another flanking sequence of *sll0813* was introduced into pGGUV1.

pGGUV4 (during this thesis) a streptomycin resistance cassette was introduced between the two flanking sequences.

pGGUV5 (during this thesis) like pGGUV4, however the streptomycin resistance cassette has been inserted into the other direction.

3.3. Used resistance cassettes for molecular cloning

Kanamycin – Neomycin:

Tn5 (Auerswald et al, 1981; Beck et al, 1982) encodes a *neomycin phosphate transferase* (*npt*), which had been inserted into pRL6. *npt* from Tn5 is advantageous for PCC7120 compared to other *npt*s.

Streptomycin

pRL463(see above) contains the ω cassette, which confers resistance towards streptomycin.

Gentamicin

The gentamicin resistance cassette was derived from pBBR1MCS-5 (Kovach et al., 1995).

3.4. Primers

3.4.1. 7120coxB and 7120coxC

7120coxB:5′CGTCTAGAGCTGAACTTTGCGGCCCCTACCACGGCGC3′

7120coxC:5′CCTCTAGAGAAGGCCAGATATGCTCCGAACAAACC3′

7120coxB and 7120coxC were used for construction of pBWUV1. The underlined parts indicate *Xba*I sites in both primers.

3.4.2. Cm5 and Cm3

Cm5: 5′CCATCGATCGTTGATCGGCACGTAAGA3′

Cm3: 5′GGAGACCGGTTTTTATCAGGCTCTGGGAGG3′

Cm5 and Cm3 were used for construction of pBWUV2. The underlined parts indicate the *Pvu*I site in Cm5 and the *Age*I site in Cm3.

3.4.3. Kmcassette2 and Kmcassette3

Kmcassette2: 5′GCTTGCGATCGAACCTTTCATAGAAGGCG3′

Kmcassette3: 5′GCATAGTACTGGGCTATCTGGACAAGG3′

These primers were used for construction of pRStUV3. The underlined parts indicate a *Pvu*I site in Kmcassette2 and a *Sca*I site in Kmcassette3.

3.4.4. Ptox downstream 5' and Ptox downstream 3'

Ptox downstream 5': 5′ACTCGAGCCTCAAGAGTCCTCATAACCC3′

Ptox downstream 3': 5′GGAGGACCTGTGACGACCCAACAGTCTG3′

These primers were used for construction of pBWUV4 and pBWUV5. The underlined parts indicate an *Xho*I site in Ptox downstream 5' and an *Eco*O109I site in Ptox downstream3'.

3.4.5. Ptox upstream 5' and Ptox upstream 3'

Ptox upstream 5': 5′AGGATCCTCACATCCTGCTCATGTACC3′

Ptox upstream 3': 5′ACTCGAGCAACTCATTCCAAGACTCGC3′

These primers were used for construction of pPSUV1 and pBWUV5. The underlined parts indicate a *Bam*HI site in Ptox upstream 5' and an *Xho*I site in Ptox upstream 3'.

3.4.6. Pbbr 1-5' and Pbbr 1-3'

Pbbr 1-5': 5′GGTCTCGAGCCGGAAGCATAAAGTG 3′

Pbbr 1-3': 5′GGTTGTCGACAATCGGGATATGCAGG 3′

These primers were used for construction of pRStUV1 and pRStUV2. The underlined parts indicate a *Xho*I site in Pbbr 1-5' and a *Sal*I site in Pbbr 1-3'.

3.4.7. Graser 1 and Graser 2

Graser 1: 5′ GGCCACCGAAAGATTAGGAG 3′

Graser 2: 5′ AATAATCGGCACCACTGTCC 3′

These primers were used for construction of pGGUV1.

3.4.8. Graser 3 and Graser4

Graser 3: 5′ GAACTGCAGGGGAATTACCGAATCCG 3′

Graser 4: 5′ CCACCAGCCATAGACCATTG 3′

These primers were used for construction of pGGUV2. The underlined part indicates a *Pst*I site in Graser 3.

3.4.9. ARTOb-10 and ARTOb-11

ARTOb-10: 5′GGTCAGCCTTGGTAGCGTGGTC 3′

ARTOb-11: 5′AGCTCTATTGGCGAGTCCCAGG 3′

These primers were used for analysis of the *coxB2* locus of new mutant strains of PCC6803.

3.5. Cultivation of Cyanobacteria

Cyanobacteria can be cultivated on solid or in liquid medium.

3.5.1. Cultivation in liquid medium

Anabaena PCC7120 is cultivated in BG11 medium according to Rippka et al 1979, whereas *Synechocystis* PCC6803 is grown in BG11 medium supplemented with 3g/l sodium thiosulfate and 10mM TES buffer called BG11TS medium. 50ml liquid cultures are shaken in the light (10µmol $s^{-1}m^{-2}$) and 100rpm at 32°C. If necessary, appropriate amounts of antibiotics are added.

BG11 medium (Rippka et al, 1979):

K_2HPO_4 . 3 H_2O	40.00	mg/l	0.18	mM
$MgSO_4$. 7 H_2O	75.00	mg/l	0.3	mM
$CaCl_2$. 2 H_2O	47.50	mg/l	0.3	mM
$NaNO_3$	1500	mg/l	17.6	mM
$Na_2MgEDTA$	1.00	mg/l	2.8	µM
$FeNH_4$ citrate	6.00	mg/l	22.8	µM
Citric acid	6.00	mg/l	31	µM
Na_2CO_3	20.00	mg/l	0.19	mM
H_3BO_3	2.86	mg/l	46	µM
MoO_3	0.23	mg/l	1.6	µM
$MnCl_2$. 4 H_2O	1.81	mg/l	9.7	µM
$ZnSO_4$. 7 H_2O	0.22	mg/l	0.77	µM
$CuSO_4$. 5 H_2O	0.08	mg/l	0.32	µM
$Co(NO_3)_2$. 6 H_2O	0.05	mg/l	0.17	µM

3.5.2. Cultivation on solid media

For *Anabaena* PCC7120 BG11 is supplemented with 1% highly purified agar (HPA), whereas for *Synechocystis* PCC6803 1.5% bacto agar is added to BG11TS medium. HPA is made by washing “bacto agar” twice with water, once with ethanol and once with acetone. HPA or bacto agar gels are melted in the microwave. 30ml are poured into plastic petri dishes. If necessary, antibiotics are added. For aqueous

solutions of kanamycin, neomycin, chloramphenicol, streptomycin the stock solutions can be pipetted first, then the agar is added. For ethanolic stock solutions of erythromycin the antibiotic has to be added to the liquid agar in the petri dish. Solid cultures are incubated for at least 2 weeks at 32°C, constant light and 80% relative humidity.

3.5.3. Permanent storage

- 50ml of a liquid culture are centrifuged at room temperature.
- supernatant is removed.
- pellet is vortexed and resuspended in a mixture of 1ml BG11 and 300µl fresh DMSO inside a nunc tube.
- Pellet is shock frozen in liquid nitrogen and stored at -80°C.
- Fresh DMSO has been frozen to prevent oxidation and is thawed immediately before use.

3.6. Cultivation of *Escherichia coli*

E. coli cells are grown in LB medium (10g peptone, 5g yeast extract, 10g NaCl dissolved in 1l H_2O). For liquid cultures 3 to 6ml suspensions are shaken at 37°C, 200rpm over night (8 to 16 hours). For larger plasmid preparations larger cultures up to a volume of 1l are used. For solid media, plates with 30ml LB agar in petri dishes are used. LB agar means LB medium, which is supplemented with 1.5% (g/100ml) bacto agar. Bacteria are distributed over the agar and incubated over night at 37°C. For permanent storage 1ml from an over night culture is transferred into a nunc tube, shock frozen in liquid nitrogen and stored at -80°C.

3.7. Antibiotics

- **Kanamycin**
  stock solution: 5mg/ml, is used for *E. coli* (final concentration 50µg/ml) and PCC6803 (final concentration 20-50µg/ml). For a selection on a kanamycin resistance cassette in PCC 7120 the related neomycin is used.
- **Neomycin**
  stock solution: 3mg/ml is used for *Anabaena* PCC7120 (final concentration 30µg/ml).
- **Chloramphenicol**
  stock solution: 1mg/ml. Final concentrations used are 20µg/ml for PCC6803 and 25µg/ml for *E.coli*.
- **Streptomycin**
  stock solution: 5mg/ml. For *E. coli* 50µg/ml and for both PCC6803 and PCC7120 20µg/ml are used.

- **Erythromycin**
  stock solution: 50mg/ml in ethanol because the solubility in water is very low. Final concentrations used are 5µg/ml in PCC6803.
- **Gentamicin**
  stock solution: 1mg/ml. For *E. coli* 20µg/ml and for PCC7120 15µg/ml are used.
- **Other compounds that were added to the medium**
  Stock solutions of **glucose, fructose, sorbitol, 3-O-methylglucose** are prepared at a concentration of 1M, their final concentrations vary from 0.2mM to 200mM.
- **DCMU**
  stock solution: 20mM in ethanol. The final concentration is 10µM

3.8. Plasmid minipreparation according to Holmes and Quigley

- This preparation can be used for restriction enzyme digestions and polymerase chain reactions. This method does not ensure high purity, but very little DNA is lost and all proteins e.g. DNAses are denaturated by heat.
- 1.5 to 3ml of an *E. coli* over night culture are centrifuged in a 1.5ml Eppendorf tube and supernatant is removed (if 3ml are harvested this step is done twice). Pellet is resuspended in 350µl STET. Addition of 25µl of a 10mg/ml aqueous lysozyme solution is optional.

  STET: 8% w/v sucrose,
  10 mM Tris-HCl (pH 8.0)
  50 mM EDTA (pH8.0)

  Tris = tris(hydroxymethyl)aminomethane
  EDTA = Ethylenediaminetetraacetic acid
  After autoclaving 5(v/v)% Triton X-100 was added

- After incubation at RT for 5 min the tube is put into boiling water for 45s.
- The tube is put on ice immediately afterwards and centrifuged for 15min at 4°C, 14000rpm.
- The viscous pellet is removed by a tooth pick and 350µl of 2-propanol are added.
- The tubes are inverted, put on ice for a short time and centrifuged for 15min at 4°C, 14000rpm.
- The supernatant is removed and the pellet is washed with 100µl 70% (v/v) ethanol (-20°C) and centrifuged for 2min at 4°C, 14000 rpm. The supernatant is discarded.
- The remaining supernatant is removed in the vacuum desiccator at 25mbar.
- The dried pellet is dissolved in 50 to 100µl TE (Tris-EDTA) and stored at 4°C.

3.9. Plasmid minipreparation according to Ish-Horowicz

- 3ml of an over night culture of *E. coli* is harvested by centrifugation (twice) in a 1.5ml Eppendorf tube.
- The supernatant is resuspended in 100µl IH1 solution. 4mg/ml lysozyme may be added.

  IH1 solution: 50mM glucose
  25mM Tris-HCl (pH=8)
  10mM EDTA (pH=8)

  IH1 solution is stored at 4°C.
- After incubation at RT for 5 min 200µl of IH2 solution are added. The tubes are inverted several times and incubated on ice for 5 min.

  IH2 solution: 200mM NaOH
  1%(g/100ml) SDS

  IH2 solution is stored at RT.
- Then 150µl of IH3 solution are added. The tubes are inverted, put on ice for 5min and centrifuged for 15min at 4°C, 14000rpm.

  IH3 solution: 3M potassium acetate
  11.5% (v/v) acetic acid

  IH3 solution is stored at 4°C.
- The supernatant is transferred to a new tube. It is extracted twice with phenol, once with chloroform/isoamylalcohol (24:1) and once with chloroform. 400µl of the extraction medium is added, the tube is inverted, vortexed and centrifuged for 3min at 4°C, 14000rpm.
- The upper aqueous phase is transferred to a new tube. After the extraction with chloroform 1000µl ethanol are added. The tubes are put on ice for some min, afterwards centrifuged for 15min at 4°C, 14000rpm.
- The supernatant is removed and the pellet is washed with 100µl 70% (v/v) ethanol, centrifuged for 2min at 14000rpm, 4°C.
- The supernatant is removed, the pellet dried in the vacuum desiccator at 25mbar, dissolved in 20µl TE and stored at 4°C.

3.10. Plasmid maxipreparation according to Ish-Horowicz

- 1l of autoclaved LB medium in a Fernbach flask is inocculated with 1ml of a small over night culture and shaken over night at 37°C, 250rpm.
- On the next day it is divided into two autoclaved 500ml centrifuge tubes and centrifuged for 10min at 6000rpm at RT in the JA-10 rotor. The supernatant is discarded.

- The pellets are vortexed thoroughly and resuspended in 10ml IH1(see 3.9.) solution, then united in one tube.
- 5mg solid lysozyme is added. The sample is incubated 5min at RT.
- 40ml IH2 (see 3.9) solution is added. The sample is mixed carefully and incubated 5min on ice.
- 30ml ice cold (0°C) IH3 (see 3.9.) solution is added. The tube is inverted carefully and incubated 5min on ice.
- The sample is centrifuged for 20min at 10000rpm, 4°C (JA-10).
- The supernatant is transferred to a 150ml corex tube. If the supernatant is not completely clear it is centrifuged in the 150ml corex tube 15min at 4°C in the JA-14 rotor. Then the supernatant is transferred to another 150ml corex tube.
- 54ml isopropanol are added and incubated for 2min at RT. Then the samples are centrifuged for 15min at 7000rpm at RT (JA-14 rotor) and the supernatant is removed.
- The pellet is washed twice with 70% ethanol (–20°C), strongly vortexed and centrifuged 10min at 7000rpm at 4°C.
- Afterwards the pellet is dried 10min in the vacuum desiccator at 25mbar.
- The pellet is dissolved in 5ml TE while on ice.
- 2.5ml 7.5M ammonium acetate in TE is added and incubated on ice for 20min, then centrifuged for 20min, 7000rpm, 4°C.
- The supernatant is transferred into a 25ml corex tube.
- 15ml 95% ethanol is added. The tube is inverted and incubated at –20°C for 20min (if necessary the procedure can be stopped at that moment and the tube is stored over night at –20°C).
- The samples are centrifuged for 20min at 7000rpm, 4°C in the swing-out rotor.
- The supernatant is discarded and the pellet is dried for 10min in the vacuum desiccator at 25mbar.
- The pellet is dissolved in 2,5ml 50mM NaCl in TE, 2.5µl of a 10mg/ml RNAse A solution are added for an end concentration of 10µg/ml. The DNA + RNAse A solution is incubated for 30 min at 37°C.
- The solution is aliquoted to 5 Eppendorf tubes (to 500µl).
- Then the solution is extracted with various reagents.
- Twice with phenol equilibrated with an aqueous TE solution: 500µl phenol are added. The tubes are inverted several times, vortexed and centrifuged for 5min at 14000rpm. The upper aqueous phase is transferred to a new Eppendorf tube. The interphase is cotransferred with the aqueous phase: Once with chloroform/isoamylalcohol 24:1, once with pure chloroform. At the last two steps the samples are centrifuged for 1min only and the interphase is not transferred to the next tube.
- 10% (of the total volume of the aqueous phase) 3M sodium acetate is added.

- From 95% ethanol the double volume of the aqueous phase (after the chloroform extraction) is added. The tubes are inverted and incubated on ice for 10min.
- The samples are centrifuged for 15min at 14000rpm, 4°C.
- The supernatant is removed and the pellet is washed with 70% ethanol.
- The samples are centrifuged once more for 1min at 14000rpm, 4°C.
- The supernatant is discarded then the pellet is again centrifuged for a few sec. The remaining supernatant is removed and the pellet is dried in the vacuum desiccator for 2min at 25mbar.
- The pellets are dissolved in 20µl TE and stored at 4°C.

The yield of this preparation is ~ 1-2µg/ml.

3.11. Total genomic DNA preparation from cyanobacteria

- A liquid culture (20 – 50ml) is centrifuged in a 50ml Greiner tube at 1000rpm for 5min (filamentous strains) or 10min (unicellular strain).
- The supernatant is removed and the pellet is vortexed, resuspended in 1ml TE and transferred into a 1.5 ml Eppendorf tube.
- The tube is filled with TE and centrifuged for 2 min for filamentous strains like PCC7120 or for 10 min for unicellular strains like PCC6803.
- The supernatant is removed. The pellet is vortexed and resuspended in 400µl TE. 20µl SDS (10% = 10g/100ml H_2O), 150µl glass beads that are sterilized by heat and 450µl phenol/chloroform (1:1) are added.
- The samples are vortexed for one min, then immediately put on ice for one min. This step is performed five times for filamentous strains and 10 times for unicellular strains. Then the tubes are centrifuged for 15 min at 14000rpm, 4°C.
- The supernatant is transferred into another tube and extracted four times with 400µl of different media.
  phenol
  phenol/chloroform (1:1)
  chloroform/isoamylalcohol (24:1)
  chloroform
- After the last extraction step 40µl 3M sodium acetate and 1000µl ethanol are added. The tube is inverted, put on ice for 1 min and centrifuged for 15 min at 4°C, 14000rpm.
- The supernatant is removed and the pellet is washed once with 100ml 70% ethanol and centrifuged for 2min at 4°C, 14000rpm.
- Then the pellet is dried in the vacuum desiccator at 25mbar for 2min, dissolved in 50µl TE and stored at 4°C.

3.12. Polymerase chain reaction (PCR)

As template DNA plasmids from *E. coli* or the genomic DNA from cyanobacteria were used. PCR is performed in a total volume of 50µl in a mini Eppendorf tube specifically designed for PCR.

5µl of 10x Thermo buffer
0.5µl of both primers (100µM)
0.5µl (10mM) dNTP mix
0.5µl of Taq Polymerase (5units/µl)
1-2µl of the DNA in TE solution
filled up with H_2O to a total volume of 50µl.

Ideally the Taq polymerase is added last. When a higher amount of DNA is desired, a higher volume (e.g. 10x of all reagents) is prepared and aliquoted to 50µl. If two different DNAs are treated with the same program and reagents (primer), a mastermix is made. When n assay materials are used, the (n+1) times amount of the reagents (except the DNA) is mixed and then aliquoted to portions of 48-49µl (volume without the DNA) and the different DNAs are added last.

- First the samples are heated for 5min at 94°C.
- Then the cycles start. In each cycle the samples are first heated to 94°C for 30s (denaturating phase).
- Then it is cooled down to allow the annealing of the primers (5°C below the primer with the lower binding temperature) for 30s (annealing phase).
- Afterwards it is heated to 72°C (elongation phase). At that temperature the DNA polymerase of *T. aquaticus* has its optimum. The time depends on the size of the desired PCR product. About one min is necessary for synthesizing 1000bp. If two different products are made, the elongation time depends on the longer product. 30 to 50 cycles are performed.
- After the last cycle the temperature is held at 72°C for 7min to ensure that all DNA synthesis is completed.
- Then the samples are stored at 4°C.

The reactions are performed in a PERKIN ELMER cycler (Gene Amp PCR system 2400). For further treatment of the product gel electrophoresis or chloroform extraction + ethanol precipitation is performed.

3.13. Ethanol precipitation

Sometimes a DNA has to be treated with at least 2 enzymes that cannot be used simultaneously (e.g. Taq polymerase + restriction enzyme or 2 restriction enzymes, where no suitable buffer for both exists).

- In such cases the volume is filled up with TE or H_2O up to 400µl and extracted with an equal volume of chloroform to remove the enzyme of the past reaction.
- The sample is centrifuged for 3min, at 14000rpm, 4°C. The upper aqueous phase is transferred to a new tube and 40µl 3M sodium acetate and 1000µl ethanol are added.
- The tube is inverted several times, put on ice for one min and centrifuged for 15min at 14000rpm, 4°C.
- The supernatant is removed and the pellet is washed with 100µl 70% ethanol and centrifuged for 2min at 14000rpm, 4°C.
- The supernatant is removed and the pellet is dried in the vacuum desiccator at 25mbar.
- After that the DNA is dissolved in H_2O or TE and the next enzyme can be added.

3.14. Restriction enzyme digestion

Products or plasmids are cut with restriction enzymes from diverse bacteria, recognising a special DNA sequence. 2 units (u) of enzyme per µg DNA and 10% of the total volume from the appropriate 10x buffer are added. The reaction is performed in a volume of 10-50µl and the samples are incubated for 2 hours in a water bath at 37°C. The enzymes and buffers are from "New England Biolabs" (NEB). After digestion the cut fragments are purified with gel electrophoresis.

3.15. Gel electrophoresis

- 0.5-2.0% agarose in 1x TAE (Tris Acetate EDTA) buffer is boiled in the microwave and poured into a special apparatus, where the agarose forms a gel (becomes solid). 30ml are prepared for a minigel and 350ml for a big gel. The agarose concentration depends on the size of the DNA fragment. A higher concentration is applied for a more precise separation of shorter fragment lengths.
- While the gel is still liquid the slots are made with a comb. Afterwards the solid gel is covered with 1x TAE and the samples are pipetted into the slots.
- The samples are mixed with loading dye (10-50% of the total volume). A voltage of 3.4-5.5V/cm (80-120V) is applied.
- When the blue line of the loading dye reaches a sufficient distance from the loading slot, the gel is stained for 30min in a bath containing ethidium bromide (EtBr) and washed for 10min in H_2O.
- Then the gel is analysed with UV(302nm) and a picture is made. In the case of a preparative gel the fragments with the appropriate size are cut out with a razor blade.

3.16. DNA extraction from gel slices

- The weight of the gel slice is determined. 500µl gel solubilizer S (from Invisorb® Spin DNA Extraction Kit) is added for a weight up to 150mg. Between 150 and 300mg the volume of the solubilizer is increased proportionally (500-1000µl). Over 300mg the slice has to be divided and treated as two samples.
- The gel in the solubilizer is incubated in a water bath at 50°C for 10-15 min. In intervals of 5 min the tube is vortexed.
- 250-500µl (half of the volume of the solubilizer) of binding enhancer (from Invisorb® Spin DNA Extraction Kit) is added.
- The mixture is pipetted onto a spin filter tube (from Invisorb® Spin DNA Extraction Kit) that has been stuck on a 2 ml tube and centrifuged for 1min at 14000rpm. If the total volume is over 800µl (maximal volume that can be collected in the tube under the filter) the centrifugation has to be repeated until the whole mixture is filtrated.
- The filter is washed twice with 500µl washing buffer (from Invisorb® Spin DNA Extraction Kit). The tube is centrifuged for 1 min at 14000rpm and the filtrate is removed.
- After the second washing step the tubes are centrifuged 4 min at 14000rpm to remove all washing buffer.
- The spin filter is put onto a new 1.5ml tube. 20µl elution buffer (from Invisorb® Spin DNA Extraction Kit) are pipetted onto the filter. The tube is incubated at RT for 5min.
- The tube is centrifuged at 14000rpm for 1 min. The filter is removed.
- The extracted DNA is stored at 4°C. All centrifugations are performed in the Eppendorf centrifuge at RT.

3.17. Quantification of DNA on agarose gels

To determine the concentration of a DNA solution a defined volume is put onto a gel. A DNA standard length size with well defined DNA has to be copippetted to the gel. After separation of the marker's bands has taken place, the gel can be analysed by UV. The DNA concentration of our sample is estimated by comparing its intensity to that of the fragments of the marker. DNA ladders are specifically designed for qualitative but not for quantitative analysis. Usually one fragment has a higher (2-3 fold) mass. If lambda phage is restricted with *Hin*dIII it will be cleaved into the following fragments: 23130/9416/6557/4361/2322/2027/564/125bp. Each of them will be present in equimolar amounts. Therefore a broad range of DNA mass can be determined. The fragments 23130bp and 4361bp may unite to a 27,5kbp fragment due to 12bp single strand overhangs (cohesive ends), where lambda usually circularizes within the cell. If lamda/HindIII DNA is incubated for 3min at 70°C the 23130bp and 4361bp fragments will be separated.

3.18. Ligation

Restricted vector and insert that have been purified by gel electrophoresis are ligated. The vector (0.1-1.0µg) and the insert (about 2-4 fold the molar amount of the vector) are mixed with 1µl T4 ligase (400units/µl, from NEB), 1µl 10x ligase buffer (from NEB) and filled up with H_2O to a total volume of 10µl (yielding 1x T4 ligase buffer) and incubated over night at 4°C. Then electroporation is performed.

1X T4 ligase buffer: 50 mM Tris-HCl
10 mM $MgCl_2$
1 mM ATP
10 mM Dithiothreitol
pH 7.5 @ 25°C

3.19. Preparation of competent cells for electroporation

Escherichia coli cells are specially treated to become competent for electroporation.

- 2ml of SOB medium (+ antibiotics if necessary) are inoculated with the desired *E. coli* strain and incubated over night at 37°C, 200rpm.

  SOB-Medium: 20 g Bacto-trypton
  5 g Yeast extract
  0,5 g NaCl

  The above mentioned components are dissolved in 900ml H_2O. By titration with KOH the pH is adjusted to 7.5. The solution is filled up with H_2O to a total volume of 1l and autoclaved. Afterwards 20ml of a separately autoclaved 1M $MgSO_4$ solution is added.

- On the next day 1ml of the o/n culture is transferred into a Fernbach flask, containing 200ml SOB (+ antibiotics if necessary) that has been preincubated for 30 min at 37°C. The flask is shaken at 275rpm.
- At regular intervals the optical density (OD) at 550nm is measured. When an OD_{550} between 0.7 and 0.8 is reached, cells are harvested. They are transferred into an ice cooled 250ml centrifuge tube, incubated on ice for 10min and centrifuged for 5min at 7000rpm and 4°C.
- The supernatant is removed, the pellet vortexed, resuspended in 200ml ice cold glycerol.
- After the second washing step the supernatant is removed, the pellet vortexed and divided into aliquots of 80µl into 1.5ml Eppendorf tubes, which are shock frozen in liquid nitrogen and stored at -80°C. If electroporation is performed immediately, freezing is not necessary.

3.20. Electroporation

- For each electroporation an aliquot is thawed on ice.
- 1-2µl of the DNA to be transformed are added to the thawed competent cells and kept on ice for 10-15min.
- Then 60µl of the DNA cell mixture are transferred into a special metal cuvette designed for electroporation. The cuvette is put into a cuvette holder and electroporation is performed at 2.5kV, 200Ω, 25µF. The time constant should be 4.6-4.8ms. Immediately after the electroporation 1ml SOC medium is added and the whole suspension is transferred to a sterile tube and shaken at 200rpm for 1h, at 37°C.

  SOC-Medium:
  20 g Bacto-Tryptone
  5 g Yeast Extract
  0,5g NaCl

  These components are dissolved in 900ml H_2O. By titrating with KOH the pH is adjusted to 7.5. The solution is filled up with H_2O to 1l and autoclaved. Afterwards 10ml of a Mg solution (1M $MgCl_2$ and 1M $MgSO_4$) and 20ml of a 1M glucose solution are added (Mg and glucose solutions have both been autoclaved separately).
- Then the suspension is transferred to a 1.5ml Eppendorf tube and centrifuged for 1min at 14000rpm. The supernatant is removed, except for 100µl.
- The pellet is resuspended in the remaining supernatant and plated on 30ml LB medium, supplemented with 1.5% (g/100ml) bacto agar and the appropriate antibiotic. At this step a lower concentration of the antibiotics is often used than the usual standard (e.g. only 20µg/ml for Km and Sm).
- The plates are incubated at 37°C over night.
- For a liquid culture of the transformant a colony is picked and used for inoculation of a 3-6ml LB culture with the appropriate antibiotic, which is shaken over night at 37°C.
- For isolation of the newly constructed plasmid a plasmid preparation according to Ish-Horowicz (see 3.9.) or Holmes and Quigley (see 3.8.) is performed. To prove that the colony contains the correct plasmid a restriction enzyme digestion is performed and analysed by gel electrophoresis.

3.21. Transformation

When PCC6803 is genetically manipulated, transformation is used because this strain is naturally competent for transformation.

- 1.5ml of a PCC6803 culture is centrifuged for 10min at 14000rpm, at RT.
- The supernatant is removed. The pellet is vortexed and resuspended in 1ml BG11TS medium.

- OD_{730} is measured. An OD_{730} of 2.5 is desired. The density is adjusted either by dilution or by centrifugation and resuspension in a smaller volume.
- 150µl of the suspension with OD_{730} of 2.5 is pipetted into a completely transparent 15ml Falcon tube.
- 1-2µl of the DNA is pipetted at the top of the tube and centrifuged at 2000rpm for a few seconds.
- The cells and the DNA in the tubes are incubated over night in the light at 32°C. On the next day the cells are plated onto a sterile nitrocellulose filter placed on BG11TS agar in a petri dish.
- Two days later the filters are transferred to a new BG11TS plate, which includes the antibiotic.

3.22. Conjugation

As PCC7120 is not naturally transformable, conjugative gene transfer has to be performed.

- *E. coli* HB101(RP4+pRL528) and the *E. coli* strain with the appropriate plasmid to be transferred are grown over night in LB with the appropriate antibiotics (3ml).
- On the next day 750µl of both cultures are centrifuged for 1min at 14000rpm.
- The supernatant is removed and the pellet is resuspended in 1ml LB and centrifuged for 1min at 14000rpm.
- The supernatant is removed except for a small amount that covers the pellet. The pellet is resuspended in the remaining supernatant.
- The two resuspended pellets are mixed and incubated for 1 – 1.5h hours at RT.
- 1.5ml of a PCC7120 culture is centrifuged for 2min at 14000rpm, RT. The supernatant is removed and the pellet is resuspended in 1.5ml BG11 and centrifuged for 2min at 14000rpm at RT.
- The supernatant is removed and the pellet is resupended in 50µl BG11. From this PCC7120 suspension a 1:10 and a 1:100 dilution are prepared by adding BG11.
- From the original suspension as well as from the 1:10 and 1:100 dilutions 20µl are streaked on the nitrocellulose filter on the BG11 plate. They are streaked as lines next to each other on the filter.
- After the 1-1.5h incubation 1-2µl of the conjugational *E. coli* mixture are pipetted on each line of the cyanobacterial dilutions. As an additional test 1-2µl of the *E. coli* strain without RP4 are pipetted onto each dilution line.
- The plate is incubated for 2 days in permanent light at 32°C and 80% relative humidity.
- Then the filter is transferred to a new plate with BG11HPA containing the antibiotic selecting for conjugation.

- The exconjugants that have grown on the filter are transferred to a new plate without a filter containing the same antibiotic.
- Colonies from this plate are then used to inoculate a 50ml BG11 culture with antibiotic.

3.23. Selection for double recombination (Cai and Wolk, 1990)

- 100µl of a liquid culture of exconjugants (with integrated plasmid) are streaked on a plate containing the antibiotic and 5% (g/100ml) sucrose. If the cells express *sacB* (levansucrase), which is encoded by the plasmid's vector, the sucrose will be metabolized to polyfructans that kill the cells. Therefore the loss of the vector portion of the knock out plasmid by a second homologous recombination is necessary for survival of the cells in sucrose.
- Colonies from the antibiotic/sucrose plate are restreaked on a new antibiotic/sucrose plate. Colonies from these plates are used for inoculation of a 50ml liquid BG11 antibiotic/5% sucrose culture.
- To prove that the culture was homozygously mutated, the DNA is isolated. PCR is performed with primers amplifying the corresponding gene or the introduced resistance cassette and products are analysed by gel electrophoresis.
- To exclude the presence of any WT allele, 1ml of a liquid culture with antibiotic is centrifuged 2 min at 14000rpm at RT. The supernatant is removed and the pellet is resuspended in 1ml BG11.
- A new 50ml culture in pure BG11 without antibiotic is inoculated. When this culture has reached the stationary phase a further culture is inoculated.
- After a few cycles the DNA is isolated again and PCR is performed with primers flanking the locus of interest. If the strain is already homozygous the WT allele will not reappear.

3.24. Measurement of the optical density (OD)

For many experiments it is necessary to determine the concentration of cells by a spectral photometer. OD at 730nm (OD_{730}) is a measure of the cell density of cyanobacterial suspensions. 1ml of the suspension is pipetted into a quartz cuvette. At the same time 1ml of the growth medium (BG11 or BG11TS) is pipetted into another cuvette and the difference of absorption (= OD) at a special wavelength (for cyanobacteria at 730nm) is calculated (double beam photometry). If the value is over 1.0 the suspension is often diluted: 100µl suspension + 900µl medium, because the photometer measures most exactly an OD between 0.1 and 1.0. For experiments with *E. coli* a suspension is measured at OD_{550} with LB medium as the reference.

$$OD = -\lg (I/I_0)$$

OD…….absorption (optical density)

I……..intensity of light after leaving the sample

I_0……intensity of light after leaving the reference

3.24.1. Measurement of chlorophyll content of cyanobacterial suspensions

1ml is taken and centrifuged in a 1.5ml tube for 2min at 14000rpm at RT. The supernatant is removed. The pellet is vortexed for 1min and resuspended in 1ml methanol and again centrifuged at 14000rpm for 2min at RT. The OD_{665} of the supernatant is measured.

3.25. Growth measurement under photoheterotrophic and mixotrophic conditions

- Liquid cultures of 50ml in a 100ml Erlenmeyer flask are inoculated at an OD_{730} ~ 0.1.
- Appropriate amounts of sugars are added. For photoheterotrophic but not for mixotrophic conditions 10µM DCMU is added. If necessary antibiotics are added and the cultures are shaken in permanent light at 100rpm at 30°C.
- At regular intervals (2-3 days) 1ml of the culture is removed, pipetted into a quartz cuvette and OD_{730} is measured.
- The content of the cuvette is transferred into a 1.5ml tube and the concentration of chlorophyll is measured (see section 3.24.1.).

3.26. Growth measurement under chemoheterotrophic conditions

Cultures are inoculated at an OD_{730} ~ 0.1 and shaken in a gyrotory shaker in the dark at 70rpm at 30°C. Every 2-3 days 1ml is removed and measured at OD_{730}. Then the chlorophyll concentration is determined.

3.27. Isolation of membranes from *Anabaena* sp. PCC7120

- A big bottle (diameter = 23cm) with 10l BG11 (if necessary with antibiotics) is inoculated with a dense 50ml culture. The 10 liter culture is sparged with a CO_2/air mixture and illuminated with two lamps from the side (distance = 14cm) and one from above.
- When the culture has reached stationary phase the suspension is centrifuged in 500ml centrifugation bottles for 10min at 8000rpm at RT. 6 tubes are used at the same time (number of positions in centrifuge).
- The supernatant is removed and the remaining suspension is poured onto the pellet.
- The centrifugation is continued until the whole culture has been harvested. The pellets are resuspended in HEN. 3 pellets are united in one bottle, which is filled up with HEN to a total volume of 500ml. The resulting 2 bottles are then centrifuged for 10min at 8000rpm at RT.

HEN (HEPES, EDTA, NaCl): 20mM HEPES
10mM EDTA
5mM NaCl
pH was adjusted with KOH to 7.4
HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

- The supernatant is removed. The 2 pellets are resuspended in HEN, united in one bottle, filled up to a total volume of 500ml and centrifuged 10min at 8000rpm at RT.
- The supernatant is removed. The pellet is resuspended in 300ml HEN and 75g sucrose are added and dissolved.
- 1ml of the cell suspension in HEN + sucrose is pipetted into 2 hematocrit tubes. The tubes are centrifuged for 8min at 7000rpm at RT. The packed wet volume of cells (in µl cells /ml total volume) is read on the tubes and the average of the two tubes is determined.
- The cell suspension in HEN + sucrose is centrifuged, the pellet is resuspended in the calculated volume to yield a density of 100µl packed cells/ml. The cell suspension is then put on ice.
- 0.0075% (w/v) DNAseI and 1mM PMSF (phenylmethanesulfonylfluoride) (stock solution: 100mM) are added.
- If a 50ml culture instead of a 10l culture is harvested the 50ml are transferred to a 50ml Greiner tube and centrifuged 10 min at RT at 2000rpm. The pellet is washed twice with 50ml HEN and afterwards resuspended in 50ml HEN. PMSF and DNAseI are added as above.
- The culture is treated by the French press 2-3 times. Type of the French press: French ® pressure cell press, SLM AMINCO ®, SLM Instrumentals INC. For filling the chamber within the French press the Ratio selector is switched on “Down”, for pressing the cells out of the French press the Ratio selector is switched on “High”. A pressure of 680psi is chosen. First the French press is washed with HEN and afterwards the cell suspension is pressed. If the volume of the cell suspension is larger than 40ml (maximal volume within the French press) the procedure has to be repeated until the total volume has been pressed through. Afterwards the chamber is washed again with HEN to press the last cells out, which are also collected.
- The pressed cell suspension is centrifuged for 10min at 4000rpm at RT.
- The supernatant is filled into ultracentrifugation tubes to portions of 25ml and centrifuged for 2h at 60000rpm, 4°C.
- The supernatant is removed. The pellets are resuspended in 20mM HEPES (pH=7.4, adjusted with KOH) buffer, united to one tube and filled up with HEPES buffer to 25ml.

- Again the sample is centrifuged for 2h at 60000rpm, 4°C. The supernatant is removed and the pellet is resuspended in 1-5ml HEPES buffer (the smallest volume possible) and stored at 4°C.
- The protein concentration of this suspension is determined.

3.28. Measurement of the protein concentration of the isolated membranes

Two aqueous BSA stock solutions are prepared: 1mg/ml and 100µg/ml.

- A series of different BSA solutions (0, 0.5, 1, 2, 5, 10, 20, 50µg) in a total volume of 50µl is prepared.
- 950µl of Bradford solution are added, vortexed and incubated for 40min at RT.

Bradford reagent solution:
20mg Coomassie Brilliant Blue G are dissolved in 10ml 95% ethanol. 20ml of 85% H_3PO_4 are added. The solution is filled up with H_2O to a total volume of 200ml. The Bradford solution is stored at 4°C and protected against light.

- The OD_{595} is measured and a standard curve is calibrated according to the values of the different BSA concentrations. Similarly different dilutions of the membrane suspensions are prepared, mixed with Bradford solution and OD_{595} is measured. The measured OD_{595} values are listed in table 1 and the standard curve is illustrated in fig. 1.

µg/ml	OD_{550} (difference to pure Bradford solution)
0	0.00000 (determined)
2	0.15654
5	0.26439
10	0.42248
15	0.58285
20	0.67549
25	0.82509
30	0.97625
40	1.04989
50	1.18452

Table 1
Measurement of OD_{550} from different BSA concentrations.

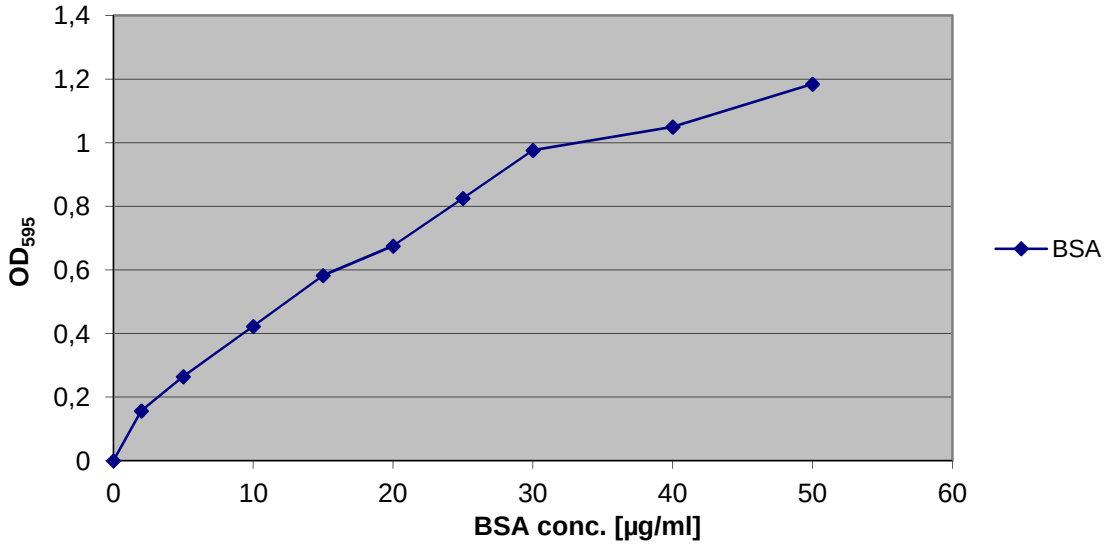


Fig. 1
Calibration of OD_{595} according to the used concentration of BSA

3.29. Measurement of the cytochrome *c* oxidase activity of isolated membranes

- 30mg of horse heart cytochrome *c* is dissolved in 1ml HEPES buffer (20mM, pH = 7.4).
- 5mg of sodium ascorbate is added.
- Then the cytochrome *c* is purified by Econopac columns.
- 1.5ml HEPES buffer (20mM) and 15µl of the eluted reduced cytochrome *c* solution are pipetted into a 2ml quartz cuvette with a magnetic stirrer.
- Small amounts of sodium ascorbate are added until the OD_{550} reaches its maximum.
- When no further increase occurs, solid $K_3Fe(CN)_6$ (potassium hexacyanoferrate (III)) is added until the OD_{550} reaches its minimum. Together with the maximum and the minimum the reduction status of the cytochrome *c* solution is calculated.

$$\% \text{ of reduced cytochrome } c = \frac{(OD - OD_{ox}) \times 100}{OD_{red} - OD_{ox}}$$

OD……..initial absorption of cytochrome *c* solution
OD_{red}…...maximal absorption after sodium ascorbate addition
OD_{ox}…...minimal absorption after potassiumhexacyanoferrate (III) addition

The optimal calculated percentage of the pre-reduced cytochrome *c* preparation for further use is greater than 90% but smaller than 100%. If a 100% reduction is calculated the removal of all sodium ascorbate molecules in the solution cannot be completely guaranteed.

- 1320µl of 20mM HEPES are pipetted into a 2ml quartz cuvette with a magnetic stirrer and measured in the spectral photometer with a dual wavelength spectroscopy technique. The difference of OD_{550} and OD_{540} is calculated. At 541.75nm there is the isosbestic point, where the reduced and the oxidized form of cytochrome *c* absorb at the same rate (extinction coefficient $\varepsilon = 9.9 mM^{-1}cm^{-1}$), whereas at 550.25nm the reduced ($\varepsilon = 27.7 mM^{-1}cm^{-1}$) but not the oxidized molecule ($\varepsilon = 9.0 mM^{-1}cm^{-1}$) has an absorbance maximum (Margoliash and Frohwirt, 1959). The difference between the actual isosbestic point or the absorption maximum and the wavelengths used for measurement ($\varepsilon_{550}red$ = 27.6, $\varepsilon_{550}ox$ = 9.1, $\varepsilon_{540}red$ = 8.8, $\varepsilon_{540}ox$ = 10.2) is negligible. For calculating the changes of the relation between the concentrations of oxidized and reduced cytochrome *c* molecules the OD_{550}-OD_{540} difference is measured at two different times.

  $\varepsilon = (\varepsilon_{550} - \varepsilon_{540})_{red} - (\varepsilon_{550} - \varepsilon_{540})_{ox} = (27.6 - 8.8) - (9.1 - 10.2) mM^{-1}cm^{-1} =$
  $= 18.8 - (-1.1) mM^{-1}cm^{-1} = 19.9 mM^{-1}cm^{-1}$

  $OD = \varepsilon \times c \times d \quad c[mM] = OD/\varepsilon \times d = 19.9^{-1} \times OD \quad (d = 1cm)$

  conc(oxidized cyt *c*) [mM] = conc(cyt *c* red)$_1$ – conc(cyt *c* red)$_2$ =
  $= [(OD_{550} - OD_{540})_1 - (OD_{550} - OD_{540})_2] \times 19.9$
  1 and 2 …… at time 1 and 2

  Program Cary WinUV is used with the following setup:
  Kinetics, user collect: Read(550)-Read(540), single collect, 30min per cycle are measured. The temperature is blocked at 38°C.

- When HEPES is added no difference between OD_{550} and OD_{540} is measured.
- 20µl of the cytochrome *c* solution derived from the Econopac column are added. The OD_{550}-OD_{540} increases and then stays constant at a higher level.
- Then 50-200µl of isolated membranes in HEPES buffer are added. At that moment the OD_{550}-OD_{540} decreases abruptly. If the membranes have no cytochrome *c* oxidase activity the absorption stays constant after the abrupt decrease. If there is any cytochrome *c* oxidase activity, the absorption continuously decreases further.
- To ensure that this decrease is due to a cytochrome *c* oxidase activity, 2µl of 1M KCN is added. Then the decrease stops.

3.30. Measurement of sugar uptake rates into cells

- Cells are cultivated in permanent light, at 32°C.
- When the culture has reached an OD_{730}=1.0 the cells are harvested and concentrated up to OD=2.0. The cells are centrifuged for 10min (for unicellular strains) and for 5min (for filamentous strains) at 2000rpm at RT.
- The supernatant is removed and the cells are washed once with BG11 or BG11TS (depending on the strain). Then the cells are centrifuged for 5-10min again at 2000rpm at RT.
- The supernatant is removed and the pellet is resuspended in the appropriate volume of BG11 or BG11TS for a desired end concentration of OD_{730}=2.0
- The cells are pipetted into the cell chamber and stirred with a magnetic stirrer for 5min. The cells are illuminated or not, according to the type of experiment (uptake in the light or in the dark).
- After an incubation time of 5min stable and radioactive ($U^{14}C$) sugar of the same molecule are added. The sugar concentration within the cell is only influenced by the stable molecules because the radio labelled sugar molecules are added in very small amounts.
- In regular intervals 1ml is taken and pipetted onto a nitrocellulose filter (pore size 0.45µm), which is connected to an oil vaccum pump apparatus. The supernatant is sucked off, while the cells are retained on the filter. The filter is washed immediately afterwards with 5ml medium (BG11 or BG11TS) and put into a scintillation vial, where it is mixed with 10ml scintillation cocktail. Therefore radioactivtiy from the supernatant is removed and only the incorporated radiolabelled molecules remain on the filter.

  scintillation cocktail: 60g Naphthalin
  4g PPO (2,5-Diphenyl-1,3,4- oxadiazol)
  dissolved in 1 l Dioxan.

- 0.5ml of the cell culture within the cell chamber is pipetted into a scintillation vial without any filter and mixed with 10ml scintillation cocktail.
- The samples are analysed over night in the scintillation counter. The counts per minute (cpm) are measured.

  % of incorporated molecules at a certain point of time:
  100x cpm (at that time) / 2x cpm of total suspension

  The time when the radiolabelled (and stable) sugar is added is set as 0. The % of uptake at different points of time is displayed in a diagram.

4. Experiments and Results

4.1. PCC7120 WT but not PCC7120*cox1*$^-$ has the capacity for chemoheterotrophic growth dependent on fructose

Anabaena sp. PCC7120 had been thought over decades to be obligately photolithoautotrophic (Rippka et al, 1979). Ungerer et al (2008) introduced the *frtRABC* genes from ATCC29413 into PCC7120, which conferred the competence for chemoheterotrophy dependent on fructose to the new transgenic strain. This clearly demonstrates that PCC7120 still retains some fructose metabolism within the cell. It results in the capacity for heterotrophy inhibited only by the failure to incorporate fructose from the environment. Considering that the two *Anabaena* strains PCC7120 and ATCC29413 are closely related the expression of *frtRABC* in PCC7120 was not unusual. We tried to transfer the glucose transporter gene *gtr* from *Synechocystis* PCC6803 into PCC7120 by conjugation to investigate whether the promoter of *gtr* was recognized in PCC7120 despite the fact that unicellular PCC6803 is not closely related to PCC7120. Another question was, whether PCC7120*gtr*$^+$ could use glucose for heterotrophic growth as Gtr is mainly a glucose transporter although it can also mediate fructose uptake in PCC6803.

4.1.1. Introduction of pBWUV6 into PCC7120 by conjugation

pBWUV6 (see section 3.2.) was introduced into PCC7120 by conjugation. It can replicate autonomously because it has the pDU1 origin.

Conjugational experiment:

E. coli HB101(RP4+pRL528) + Top10(pBWUV6) + PCC7120WT

Negative control experiment:

E. coli Top10(pBWUV6) + PCC7120WT

(strain with RP4 and pRL528 was missing)

The *E. coli*/PCC7120 mixture stayed 3 days without antibiotics. Then it was selected on 30µg/ml Nm and some colonies grew. No colonies were visible with the negative control. The colonies were streaked on a new plate and a liquid culture was inoculated with colonies growing on that plate. When the culture had become dense it was analysed for the presence of *E. coli* cells. 1ml of the culture was centrifuged, resuspended in remaining 100µl supernatant, streaked on an LB agar plate and incubated over night at 37°C. No colonies formed. Therefore all *E. coli* cells had already been removed.

To prove the presence of the plasmid in the cells the DNA from the transgenic culture as well as from a WT culture were isolated and PCR was performed with primers amplifiying *gtr*. Two different primers were used for the 5' end and were combined with the same 3' end primer. An annealing temperature of 45°C and an elongation time of 1min 20s were chosen. PCC7120WT and PCC7120*gtr*$^+$ were tested with both primer combinations. Transgenic PCC7120*gtr*$^+$ yielded a 1124bp fragment

with the old 5' primer and an 881bp fragment with the new 5' primer, which confirmed the expections. PCR with the WT DNA yielded none of the two fragments (see fig. 2).

gtr-5old: 5′ATGAATCCCTCCTCTTCTCC3′
gtr-5new: 5′GGGCGGATTAAAACGATG3′
gtr-3: 5′GTCACCAAAGCAATTATCCCAGC3′

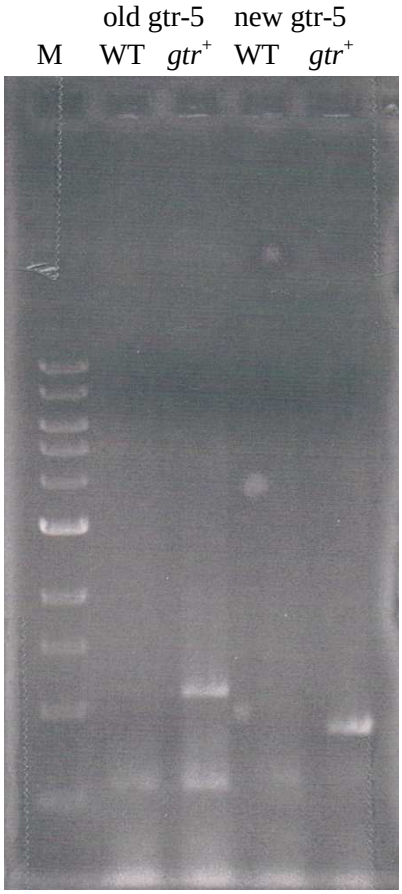


Fig. 2
Detection of *gtr* in new transgenic strain PCC7120*gtr*$^+$ but not in the WT by 2 primer combinations. M…1kb ladder (from NEB)

4.1.2. Comparison of PCC7120*gtr*$^+$ with PCC7120WT according to their trophies

Now it was investigated whether the new strain had won any capacity of heterotrophic or mixotrophic growth. As a control the same experiments were also performed with the WT.

4.1.2.1. PCC7120*gtr*$^{+}$ and the WT are capable of mixotrophic growth on fructose

First both strains were grown under mixotrophic and photolithoautotrophic conditions. They were cultivated in the light without DCMU. As a consequence both photosystems were active allowing non cyclic electron transport. Different amounts of fructose (10, 20, 50, 100 and 200mM) were added to look whether the growth rate was enhanced by fructose compared to strictly photolithoautotrophic growth in pure BG11. We can clearly recognize that 100mM fructose can double the growth rate of PCC7120*gtr*$^{+}$ but 200mM fructose has a toxic effect on the strain (see fig. 3). Very unexpectedly the WT has also the potential for mixotrophic growth (see fig. 4). The phenotypes of the two strains did not differ very much except for the fact that the WT tolerated 200mM fructose in the first two weeks. Afterwards the culture lysed and adopted a blue colour.

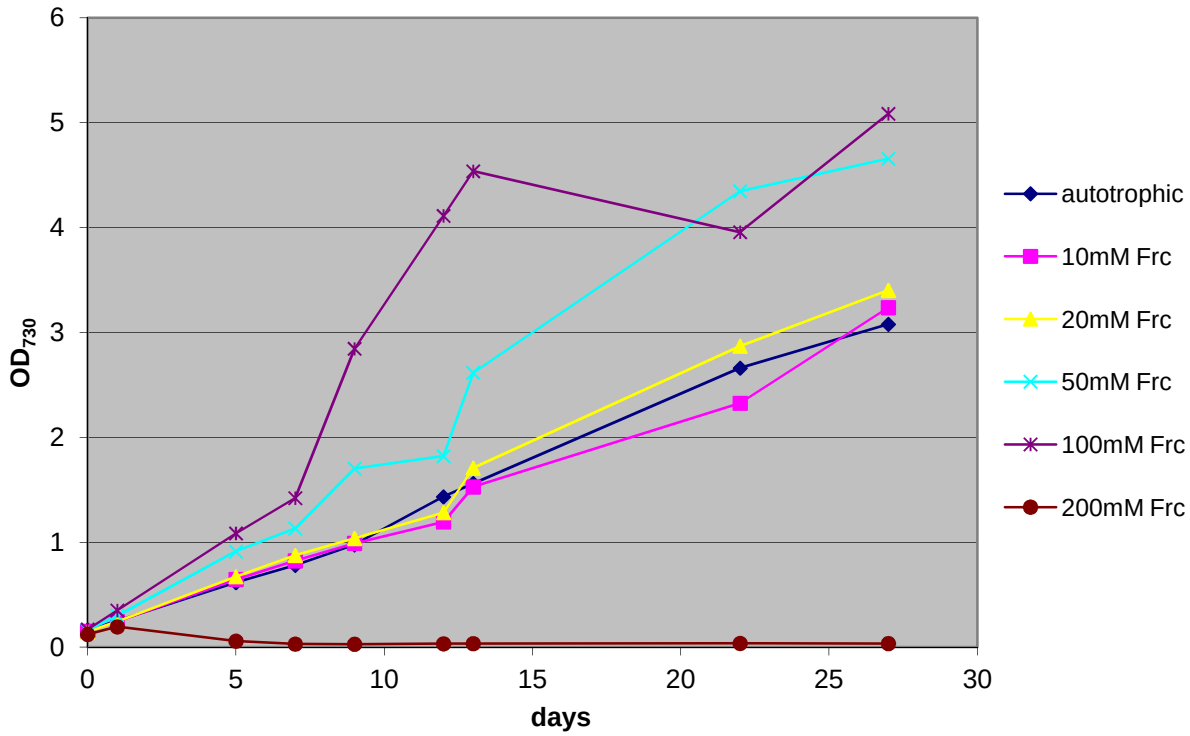


Fig.3
Mixotrophic growth (except for the curve marked "autotrophic") of PCC7120*gtr*$^{+}$ dependent on the fructose concentration

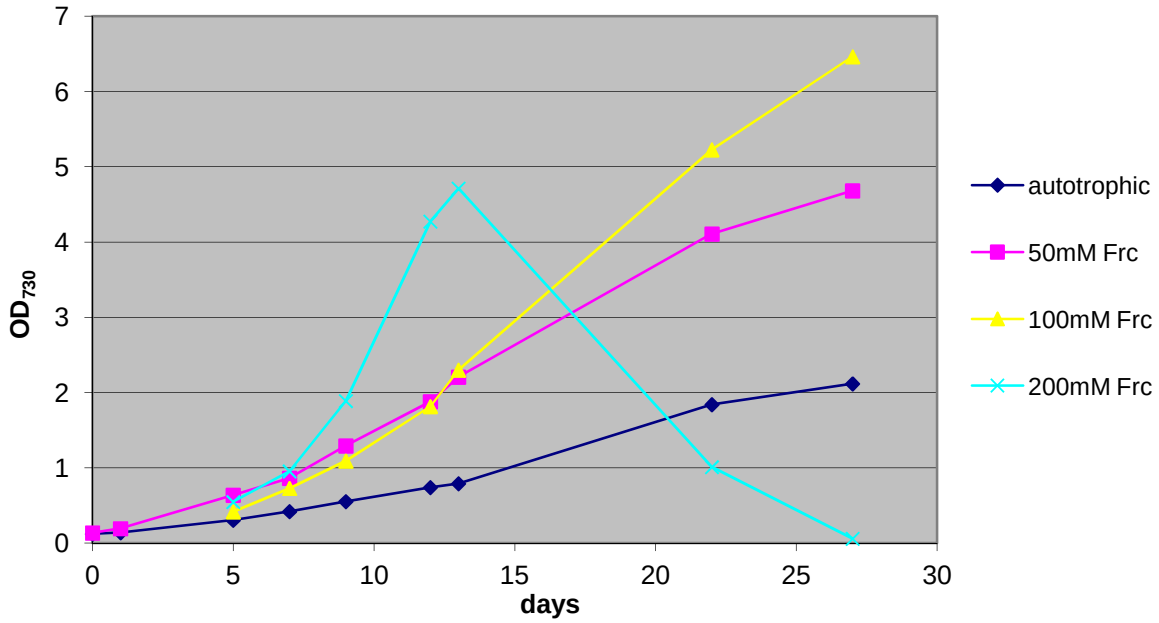


Fig. 4
Mixotrophic growth (except for the curve marked "autotrophic") of PCC7120WT. For two weeks 200mM fructose was beneficial for the WT contrary to PCC7120*gtr*$^{+}$.

As any mixotrophic and heterotrophic potential has been completely excluded in the past the experiment was repeated but this time the chlorophyll content was determined additionally. The results were reproduced (see fig. 5) and the chlorophyll content increased as well (see fig. 6).

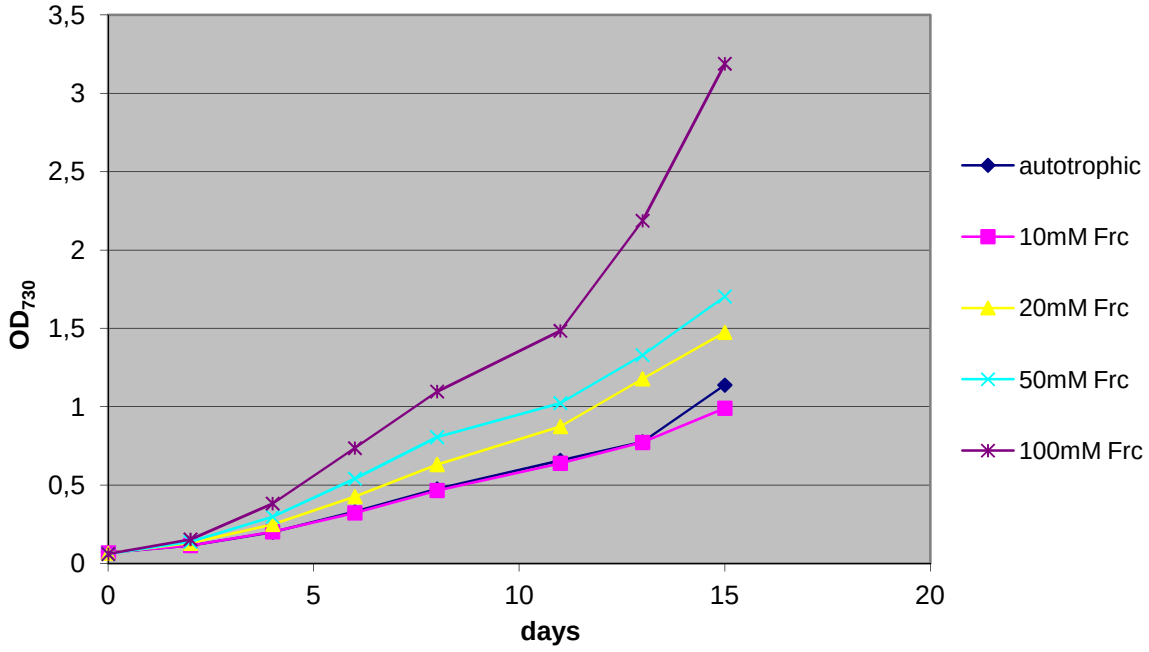


Fig. 5
OD_{730} during mixotrophic growth (except for the curve marked "autotrophic") of PCC7120WT dependent on the fructose concentration

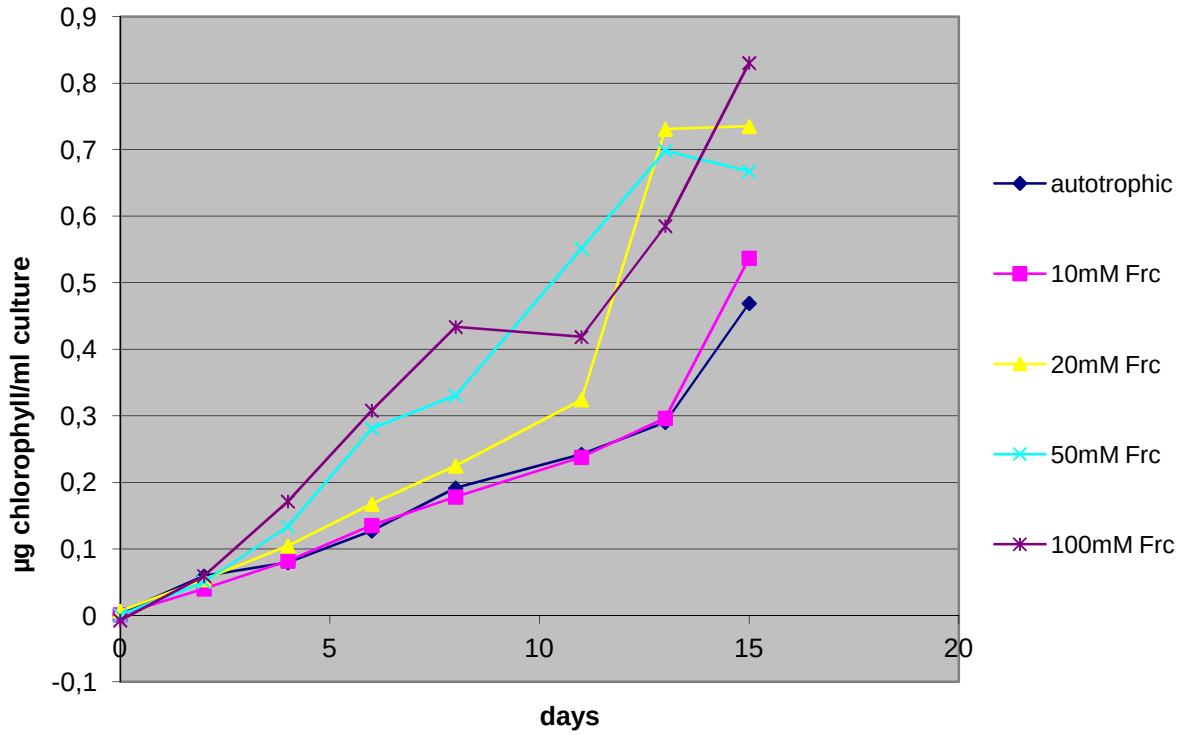


Fig. 6
Chlorophyll content during mixotrophic (and autotrophic) growth of PCC7120WT dependent on the fructose concentration

4.1.2.2. Glucose is toxic for PCC7120*gtr*$^+$ but not for PCC7120WT

After investigation of the fructose's role in mixotrophic growth the effect of glucose was investigated. When PCC7120*gtr*$^+$ was grown in 10mM glucose and 10µM DCMU (photoheterotrophic conditions) the cells died within a few days (data not shown). To exclude a harmful effect of glucose + DCMU the PCC7120*gtr*$^+$ strain was grown mixotrophically with 5mM or 10mM glucose without DCMU. Additionally the cells were grown in a 10mM glucose/ 50mM fructose mixture. Fructose and glucose are both imported by Gtr in PCC6803. In this strain additional glucose as a competitive substrate can relieve the toxicity of fructose by preventing fructose entry into the cell (Flores and Schmetterer, 1986). For PCC7120*gtr*$^+$ the opposite situation is assumed and fructose may protect from the deleterious effect of glucose. These predictions turned out not to be true. Glucose was toxic even at low concentrations of 5mM as well as at 10mM and addition of 50mM fructose did not prevent the effect of glucose (see fig. 7).

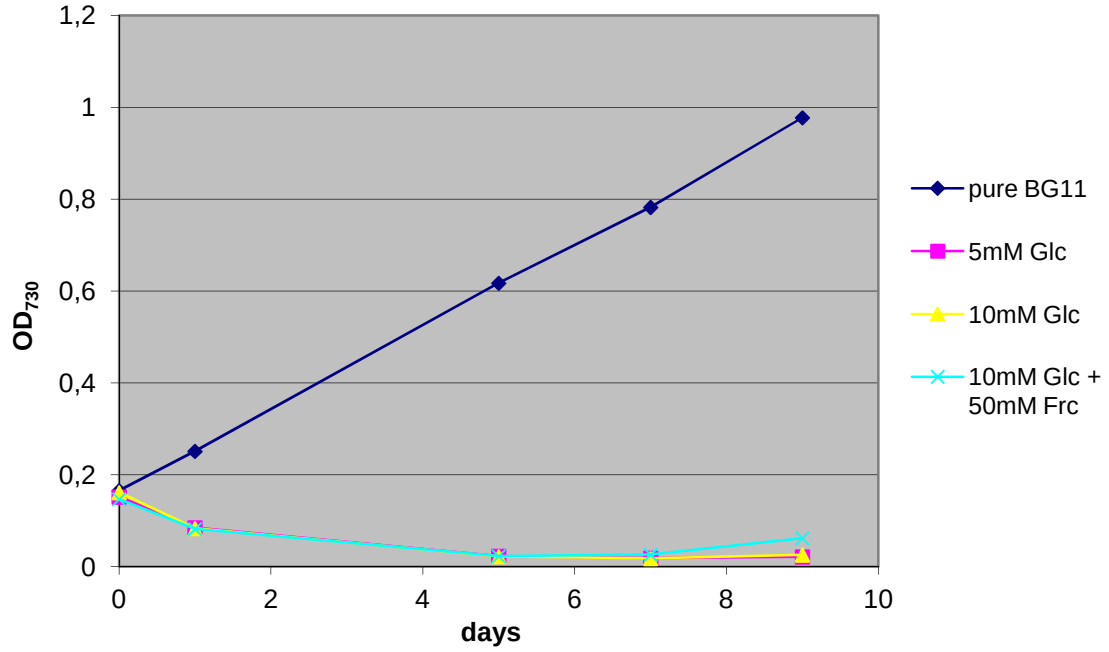


Fig. 7
PCC7120*gtr*$^{+}$ was grown mixotrophically at different glucose concentrations and compared with photolithoautotrophic conditions.

The effect of glucose on PCC7120WT was also tested. The strain was grown in the light with 10mM, 50mM and 200mM glucose (mixotrophy). None of these concentrations was toxic for the WT strain. On the contrary 200mM glucose even showed a small stimulating effect on growth, however, 200mM fructose, which was tested in the same experiment, was much more beneficial (see fig 8).

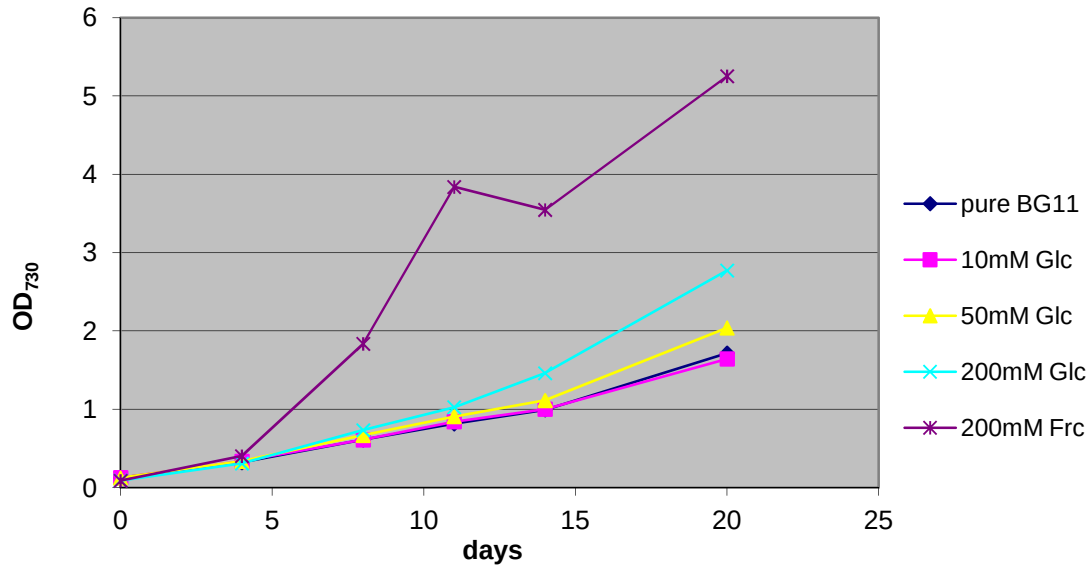


Fig. 8
Effect of glucose (different concentrations) and fructose on PCC7120WT in mixotrophic conditions.

4.1.2.3. PCC7120*gtr*$^+$ and the WT are capable of photoheterotrophic growth

In the next experiment the cells were grown in the light with DCMU, so that photolithoautotrophy was inhibited but photoheterotrophy was still possible. Both PCC7120WT and PCC7120*gtr*$^+$ had the capacity for photoheterotrophic growth. A control with DCMU but without any fructose, where no growth occurred, clearly demonstrated that cell reproduction was driven by fructose (see figs. 9 and 10).

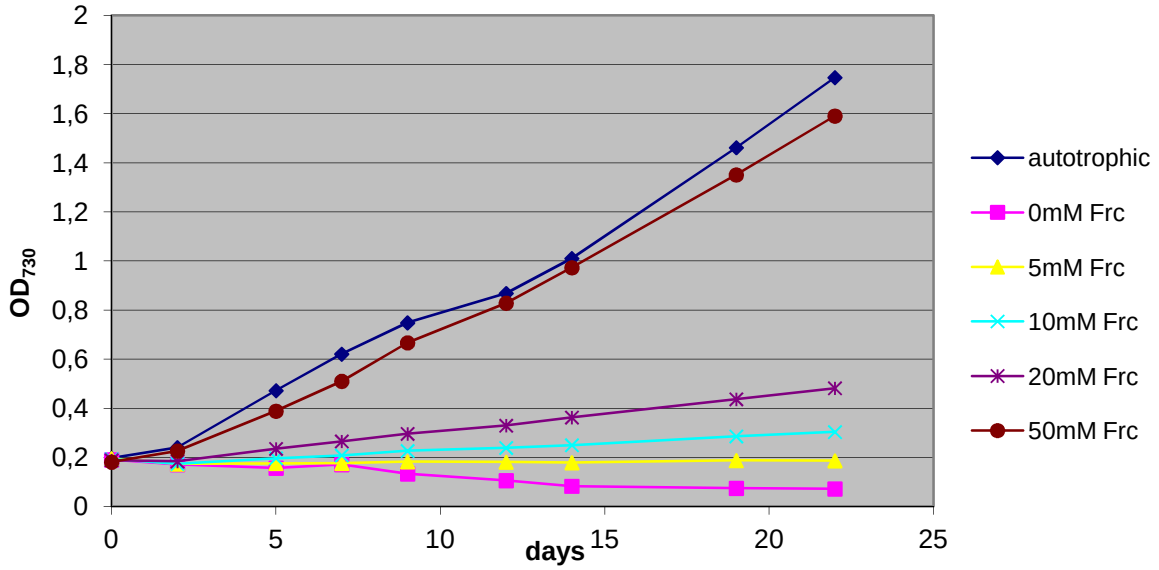


Fig. 9
Photoheterotrophic growth of PCC7120*gtr*$^+$. Cultures were grown at different fructose concentrations.10µM DCMU was added except for the curve marked "autotrophic".

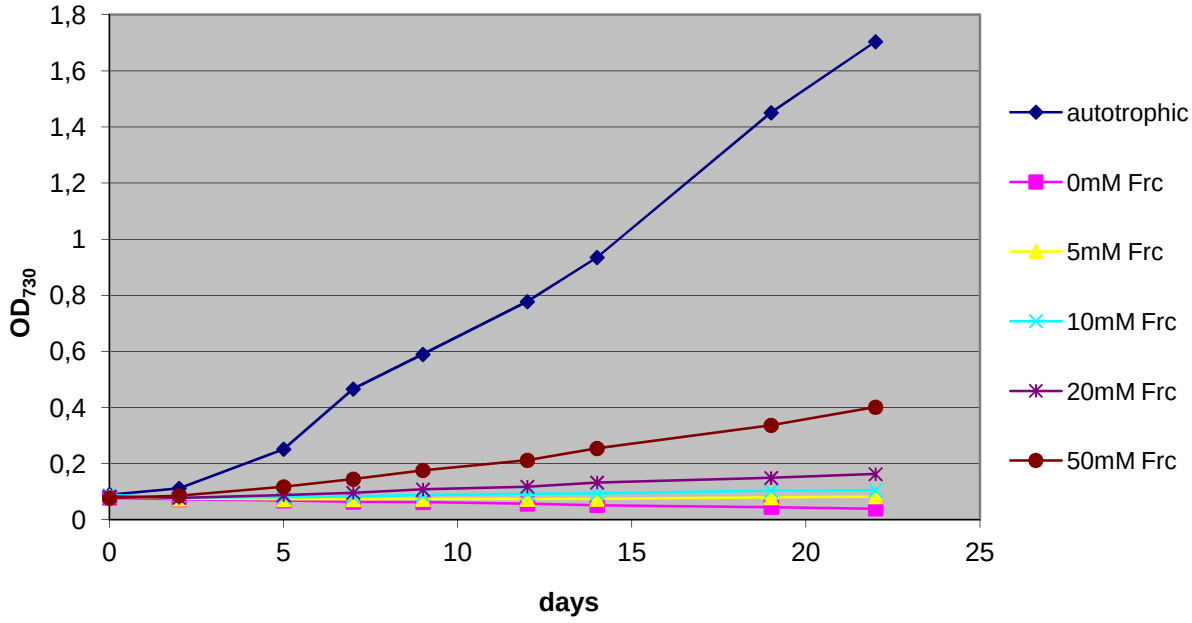
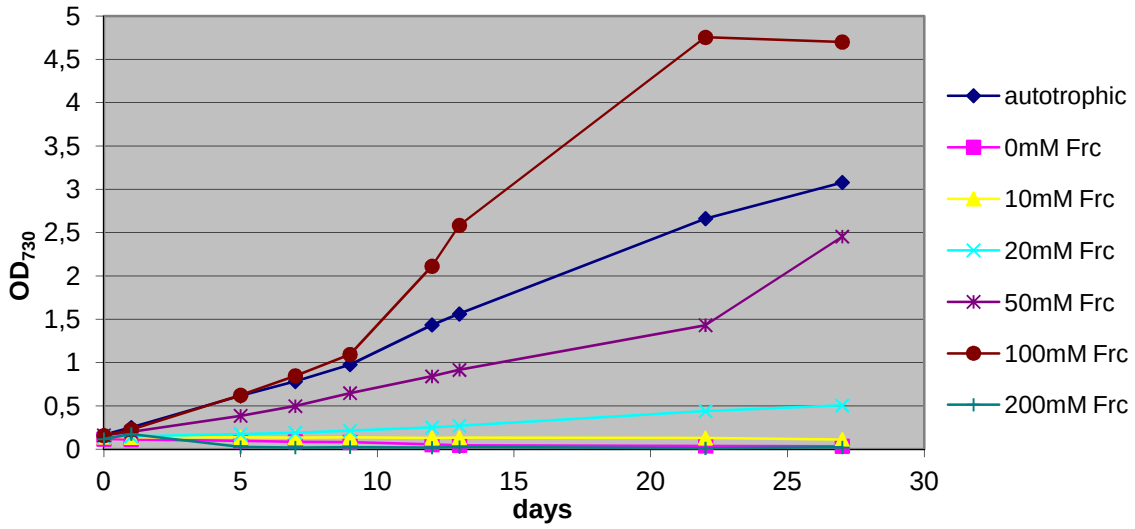


Fig. 10
Photoheterotrophic growth of PCC7120WT. 10µM DCMU was added except for the curve marked "autotrophic".

Both strains were capable of photoheterotrophic growth, however, a difference could be detected. The WT could obviously use the fructose as carbon and electron source when PSII was blocked by DCMU, whereas with DCMU without sugar no reproduction took place. Nevertheless the growth rate of PCC7120WT with 50mM fructose and DCMU was clearly below the rate of photolithoautotrophic growth (see fig. 10) while PCC7120*gtr*$^{+}$ grew at a rate that was only slightly below the photolithoautotrophic rate (see fig. 9). The presence of Gtr evidently facilitates the import of fructose but fructose uptake also occurs in the wild type. Since the capacity for photoheterotrophic growth of PCC7120 strains was quite unexpected, these experiments were repeated, using also higher fructose concentrations (see fig. 11).

A



B

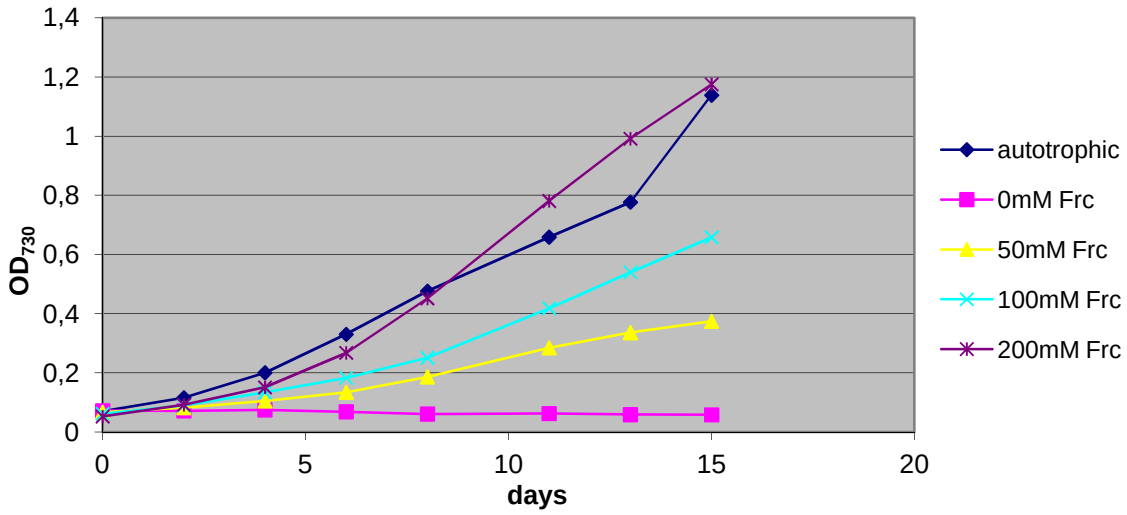


Fig. 11

Photoheterotrophic growth of PCC7120*gtr*$^{+}$ (A) and WT (B). Cultures were grown in 10µM DCMU + different fructose concentrations except for the curves marked “autotrophic”, where the cells were grown in pure BG11. The PCC7120WT but not PCC7120*gtr*$^{+}$ can tolerate 200mM fructose.

Again the WT tolerated 200mM fructose and growth was even most enhanced at that concentration, contrary to strain PCC7120*gtr*$^{+}$, which died at 200mM (see fig. 11). Therefore we can conclude that the differential behavior does not depend on DCMU. Besides the WT exhibited photoheterotrophic growth rates, which were either similar to (200mM) or below (50mM and 100mM) the photolithoautotrophic growth rate, whereas the PCC7120*gtr*$^{+}$ strain grew much faster in DCMU + 100mM fructose than in pure BG11 (see figs. 11B and 11A). To exclude an increase of OD_{730} by chemoheterotrophic contaminants the WT cultures were measured additionally for their chlorophyll content. The positive effect of fructose up to concentrations of 200mM was confirmed within the experimental error even at 200mM (see fig. 12).

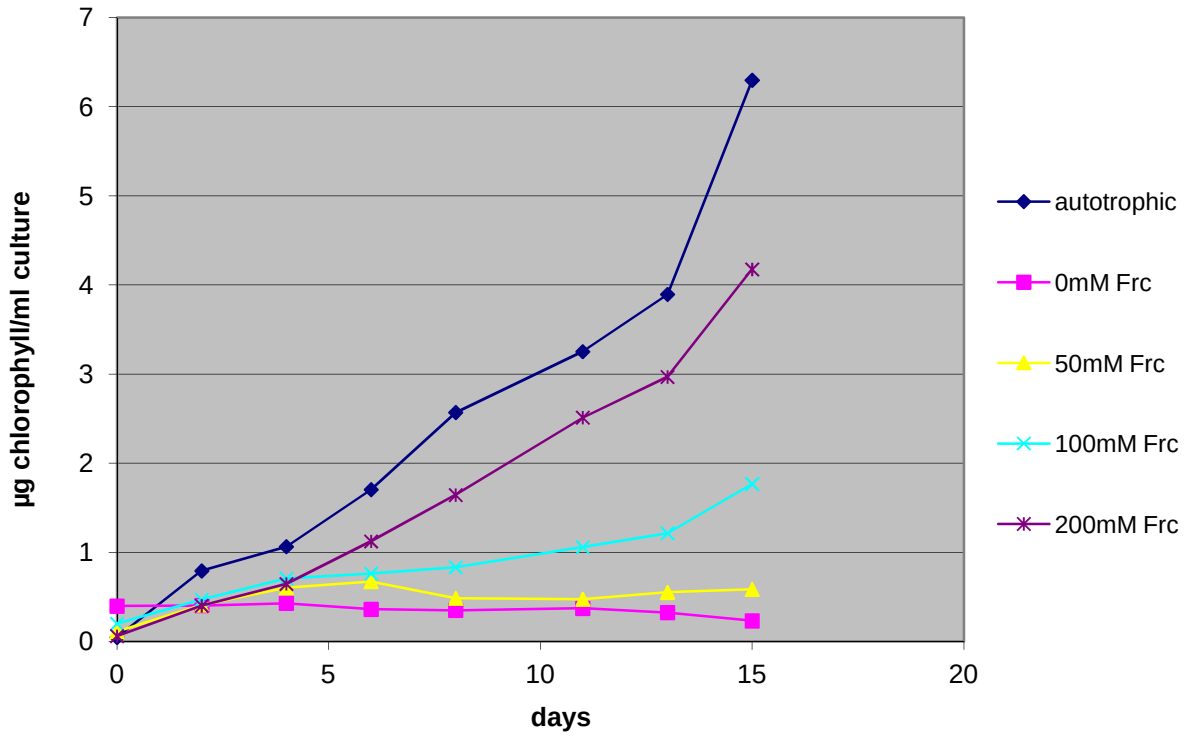


Fig. 12
Chlorophyll content of PCC7120WT, grown photoheterotrophically at different fructose concentrations compared to photolithoautotrophic conditions. 10µM DCMU were added except for the curve marked "autotrophic".

4.1.2.3.1. Sorbitol is not a substrate for photoheterotrophic growth: neither for PCC7120WT nor for PCC7120*gtr*$^{+}$

Other organic compounds than fructose were tried as possible substrates for photohetrotrophic growth. PCC7120*gtr*$^{+}$ and PCC7120WT were cultivated in the light in BG11 supplemented with 10µM DCMU and diverse concentrations of sorbitol (50, 100 and 200mM) and compared to cultures in BG11 with DCMU without any sorbitol or photolithoautotrophic cultures in pure BG11. Neither PCC7120WT (see fig. 13) nor

PCC7120*gtr*$^{+}$ (see fig. 14) were affected by sorbitol at any concentration and their OD_{730} did not differ from that in DCMU without any sorbitol. Besides PCC7120WT was incubated in photoheterotrophic conditions in the presence of DCMU and 200mM glucose and no growth occurred (data not shown).

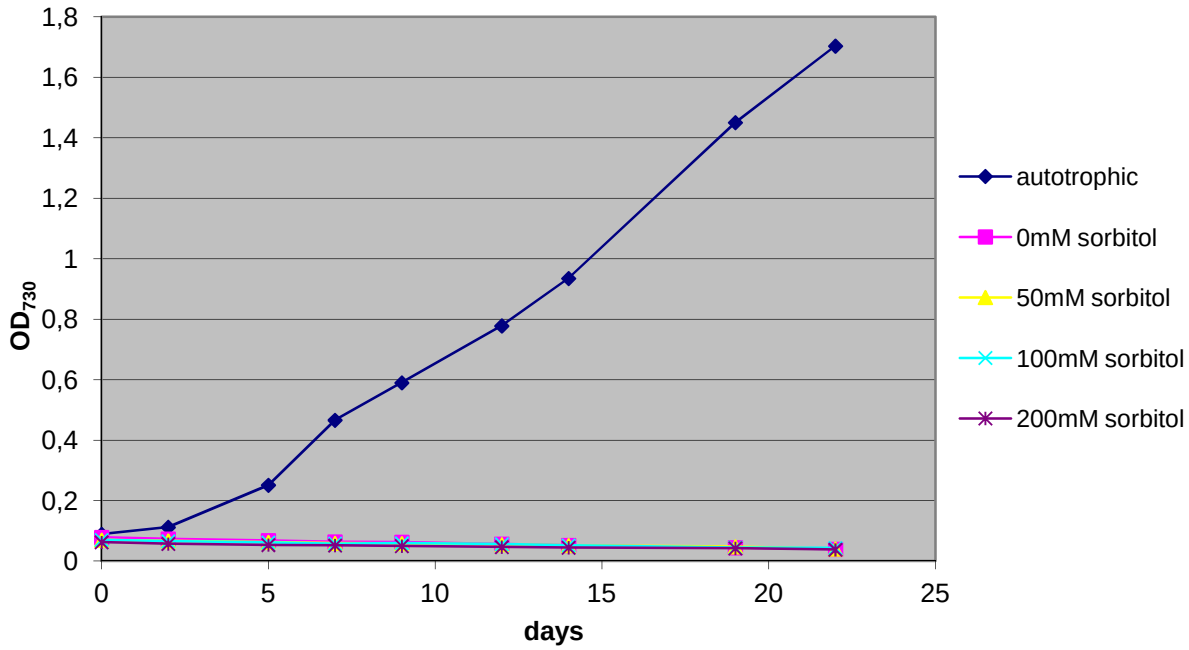


Fig. 13
Incubation of PCC7120WT under photoheterotrophic conditions dependent on sorbitol: Except for the curve marked “autotrophic” all cultures contained 10µM DCMU.

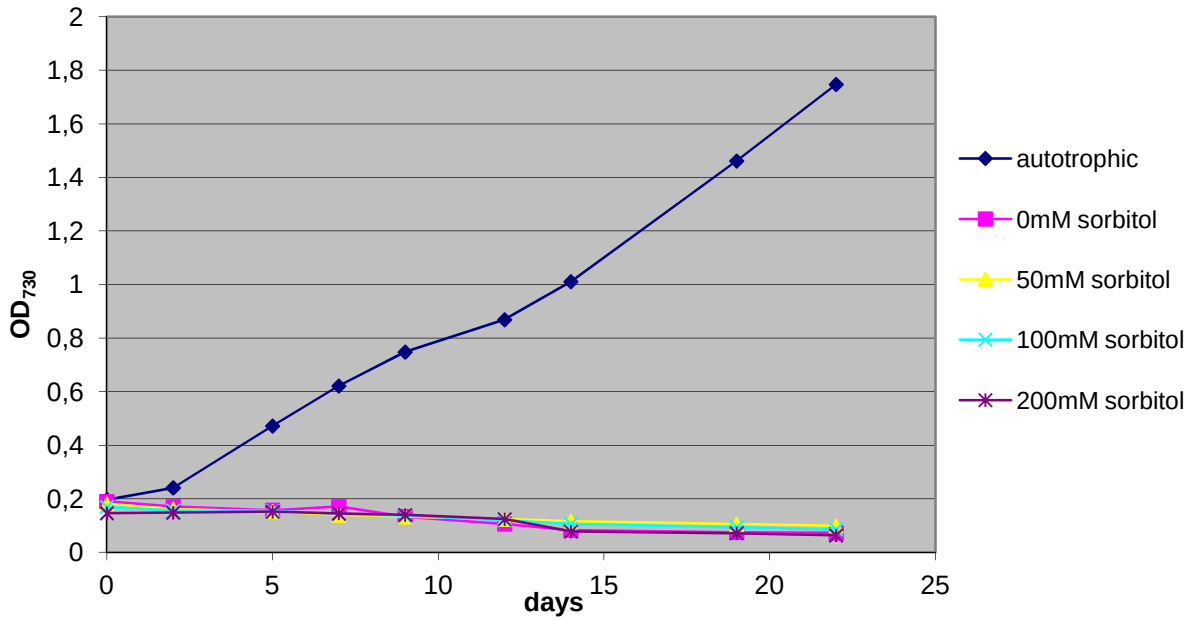


Fig. 14
Incubation of PCC7120*gtr*$^{+}$ under photoheterotrophic conditions dependent on sorbitol: Except for the curve marked “autotrophic” all cultures contained 10µM DCMU.

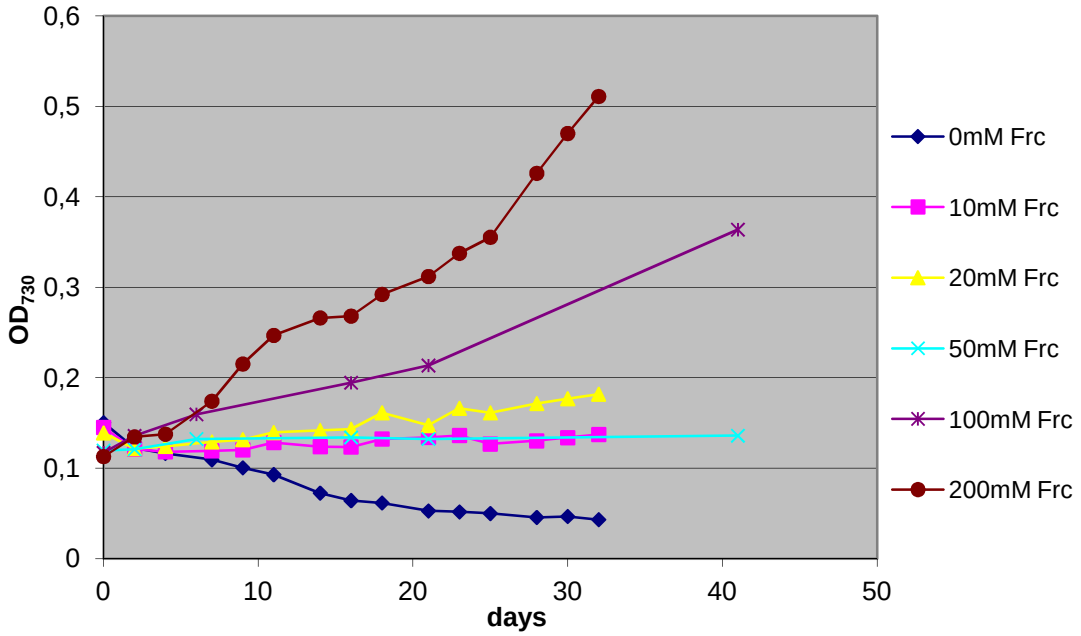
4.1.2.4. PCC7120WT but not PCC7120*gtr*$^+$ is capable of chemoheterotrophic growth

When PCC7120WT and PCC7120*gtr*$^+$ were grown in the dark on fructose most unexpected facts were observed. PCC7120WT grew slowly but continuously in the dark (see fig. 15 A and B), whereas strain PCC7120*gtr*$^+$ failed at dark growth with any fructose concentration (see figs. 16 and 17). Consistent with mixotrophic and photoheterotrophic growth the cells grew fastest at external concentrations of 200mM fructose. 100mM fructose supported the growth to a lesser extent. At 10mM, 20mM and 50mM the OD_{730} stagnated (their values differed within the normal variance), whereas the density declined when no fructose was added (see fig. 15A). Measurement of the chlorophyll content of chemoheterotrophic cultures confirmed these results (see fig. 15B). Even after incubation in the dark for nearly 4 months PCC7120WT cells grown in 50mM fructose kept the capacity to inoculate a photolithoautotrophic culture (data not shown). PCC7120*gtr*$^+$ grew within the first days, especially at 50mM. Later the cells died at all concentrations (see fig. 16). Not even a cultivation at lower concentrations could induce a proliferation, however. The cell density was constant in 5mM, whereas the density decreased when cultivated without fructose (see fig. 17). Since glucose increases the uptake of fructose when added simultaneously (see section 4.1.4.1.1.) PCC7120WT was also incubated in the dark in a mixture of 10mM fructose and 2mM glucose but no growth occurred (data not shown).

Although chemoheterotrophic growth of PCC7120 was proved without any doubt (growth took place dependent on the fructose concentration) it remained unclear whether it was real chemoheterotrophic growth or light activated dark growth because the cultures were unavoidably illuminated when measured. 4 cultures of PCC7120WT were inoculated at the same time in BG11-100mM fructose at an OD_{730} = 0.09 and grown in the dark. Every week one culture was measured, while the others remained untouched and were protected from light. Cultures that had already been opened once were measured again and compared to the culture that had just been opened for the first time. So it could be analysed whether the short illumination had any effect on the growth. Both the OD_{730} and the chlorophyll content increased continuously each week regardless of whether the culture had already been opened or not (see fig. 18A and B). As a consequence the growth can be considered a true chemoheterotrophic growth.

Glucose was also tested as a substrate for chemoheterotrophic growth of PCC7120WT and PCC7120*gtr*$^+$. The former strain was incubated in 200mM glucose the latter in 50mM glucose growth was not detected at any of the two strains (data not shown). For PCC7120*gtr*$^+$ it is not clear whether glucose could not be used as a substrate for chemoheterotrophic growth or it exhibited a toxic effect towards this strain in the dark as well, since glucose has been demonstrated to be toxic in the light (see section 4.1.2.2.).

A



B

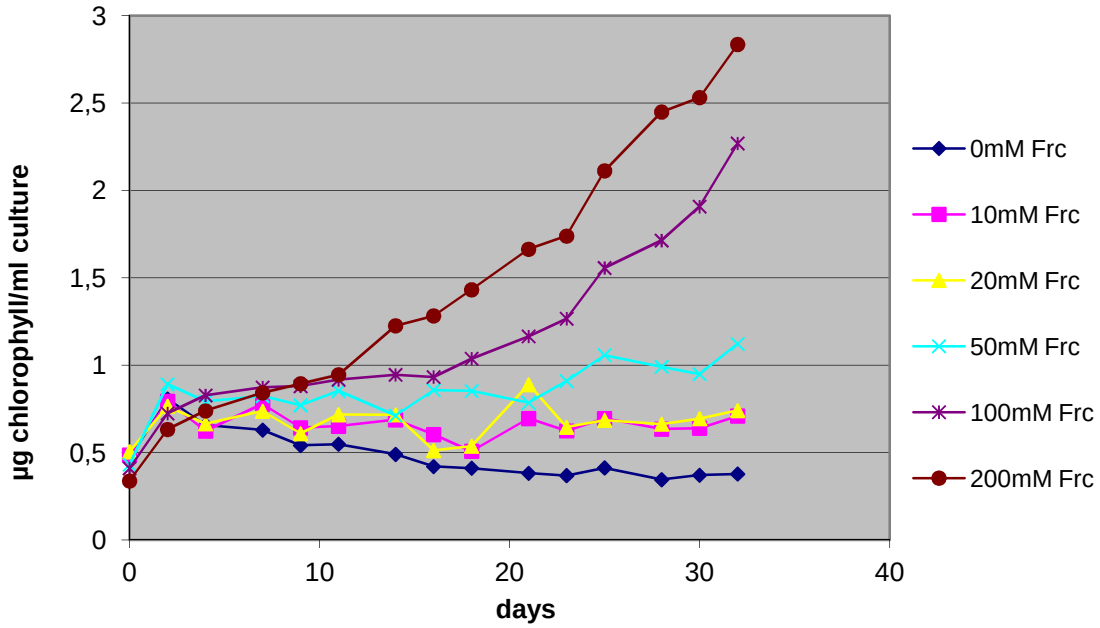


Fig. 15
Chemoheterotrophic growth of PCC7120 WT at different fructose concentrations.
A measurement of OD_{730}
B determination of chlorophyll after methanol extraction

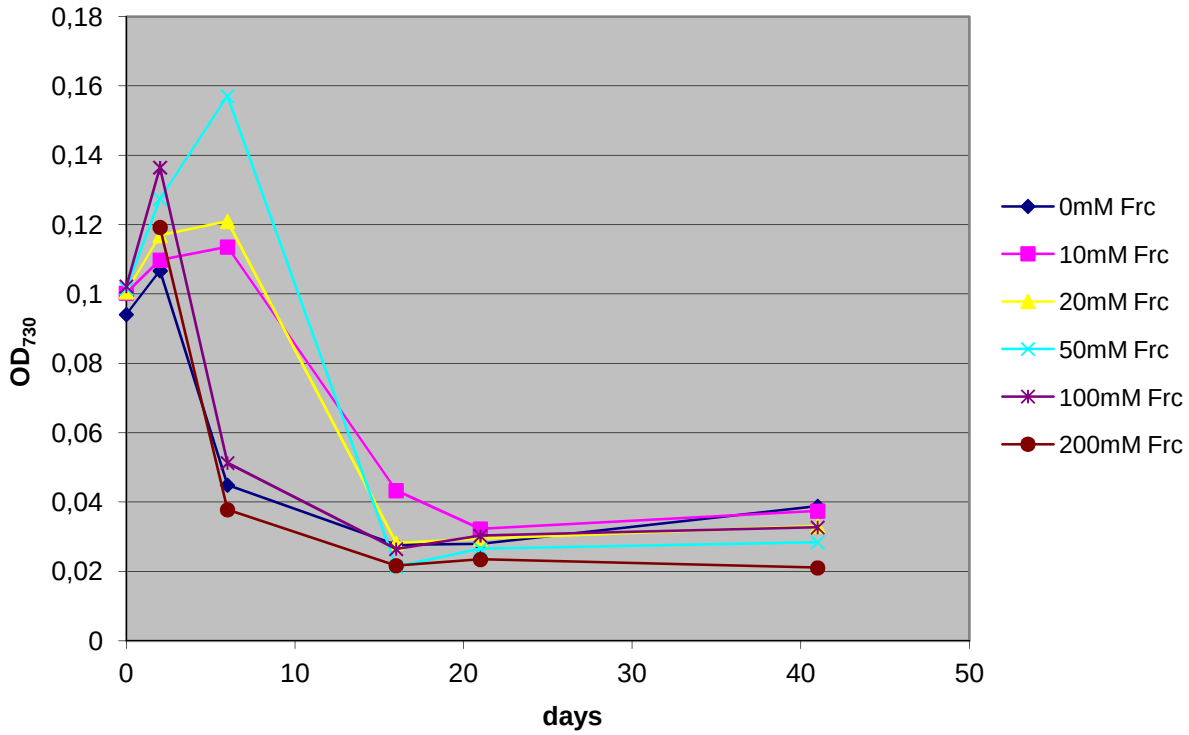


Fig. 16
PCC7120*gtr*$^{+}$ failed to grow in the dark

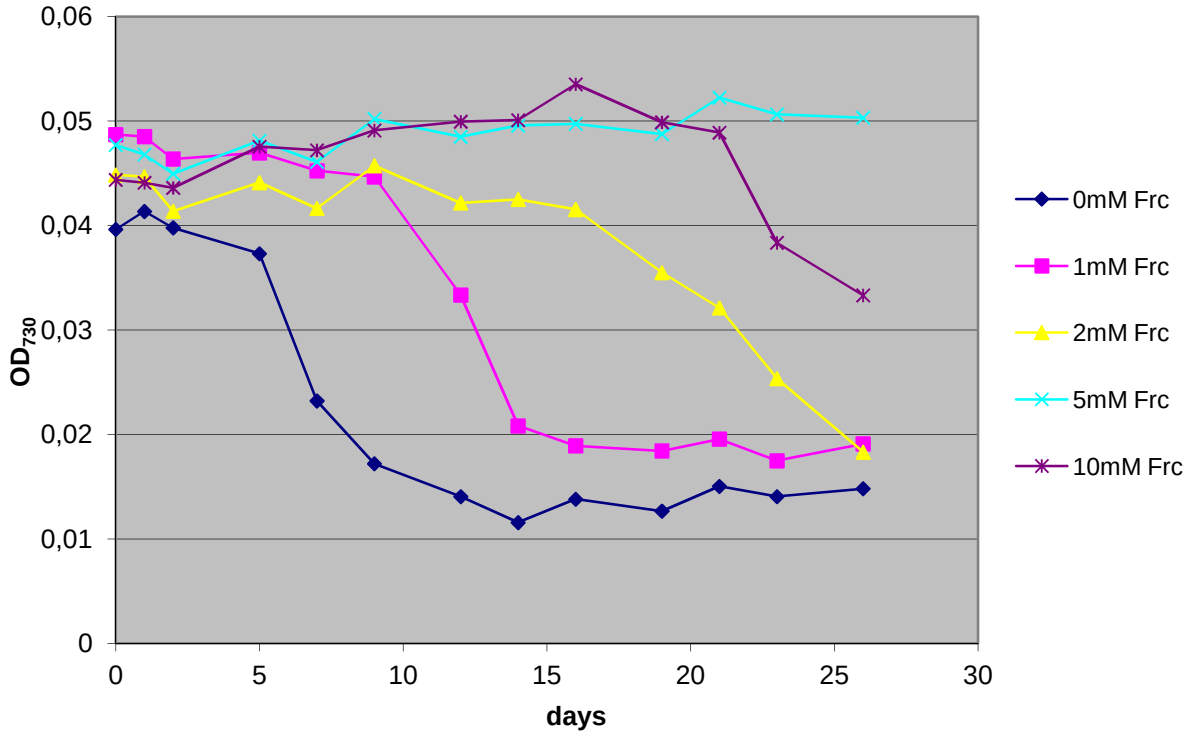
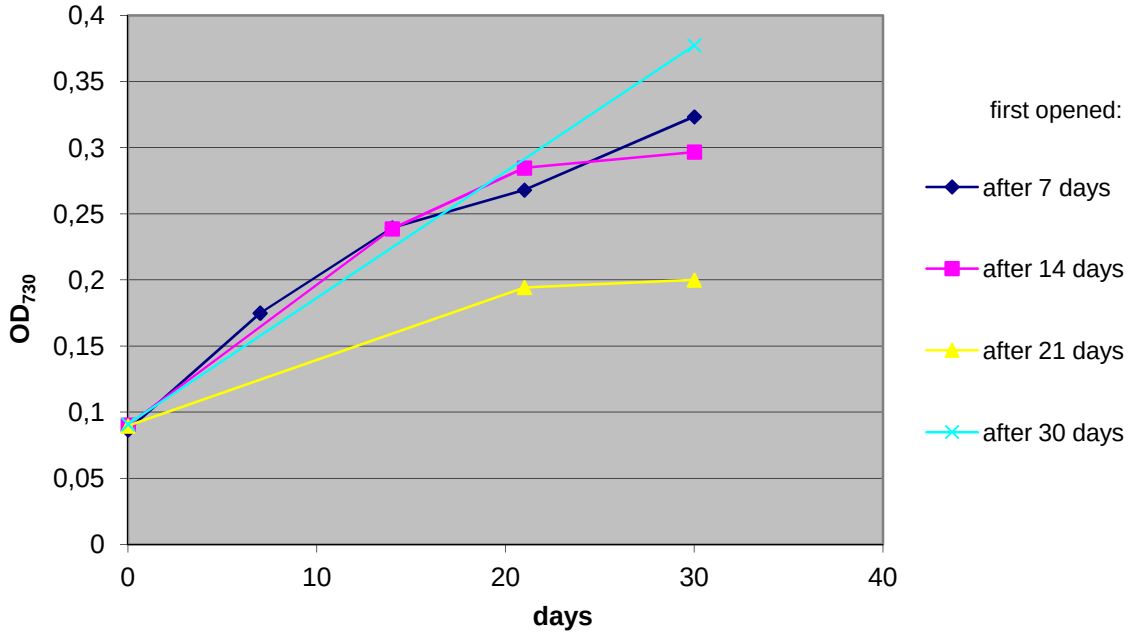


Fig. 17
PCC7120*gtr*$^{+}$ also failed to grow in the dark at low fructose concentrations.

A



B

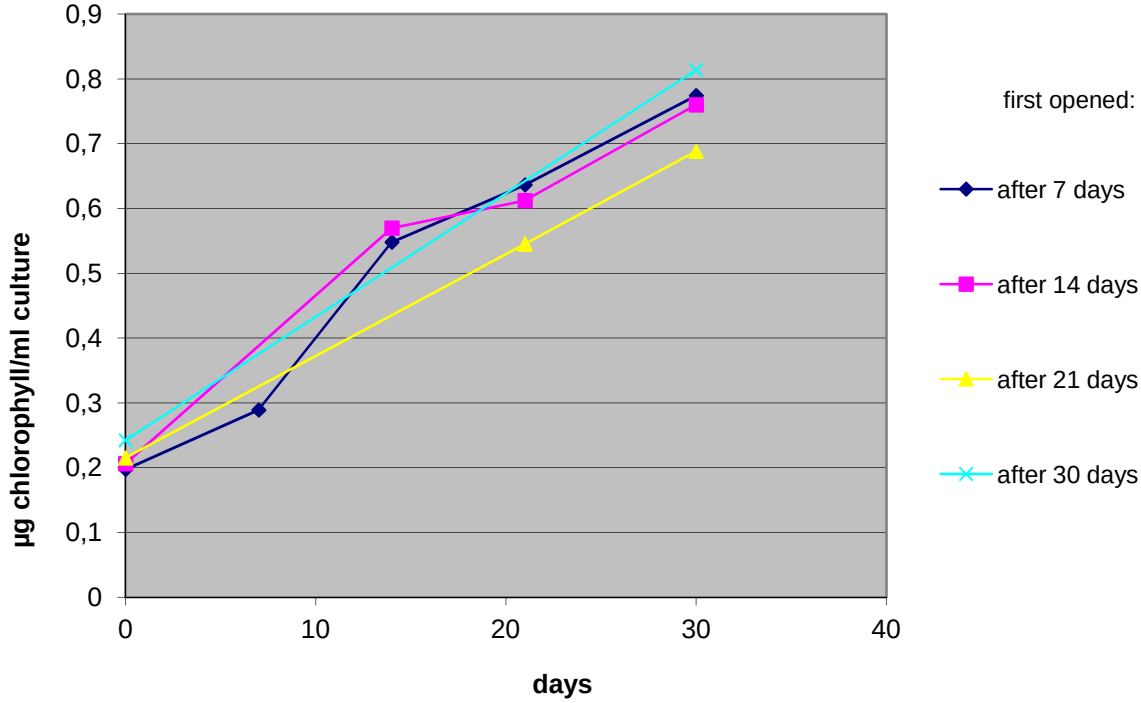


Fig. 18
Growth of PCC7120 WT in complete darkness
A OD_{730} measurement
B determination of the chlorophyll concentration after methanol extraction

4.1.2.4.1. Sorbitol is not a chemoheterotrophic substrate for PCC7120*gtr*$^{+}$ either

It was investigated, whether PCC7120*gtr*$^{+}$ could use sorbitol as a substrate for chemoheterotrophic growth. PCC7120*gtr*$^{+}$ was incubated in BG11 supplemented with 50mM, 100mM and 200mM sorbitol in the dark. These cultures were compared to PCC7120*gtr*$^{+}$ incubated in the dark in pure BG11. No chemoheterotrophic growth dependent on sorbitol was detected, however, a delayed cell dying was observed. While the OD_{730} of PCC7120*gtr*$^{+}$ rapidly declined in pure BG11 in the dark, the addition of sorbitol could delay the decline of the OD_{730}. This effect was the stronger the more sorbitol was added (see fig. 19).

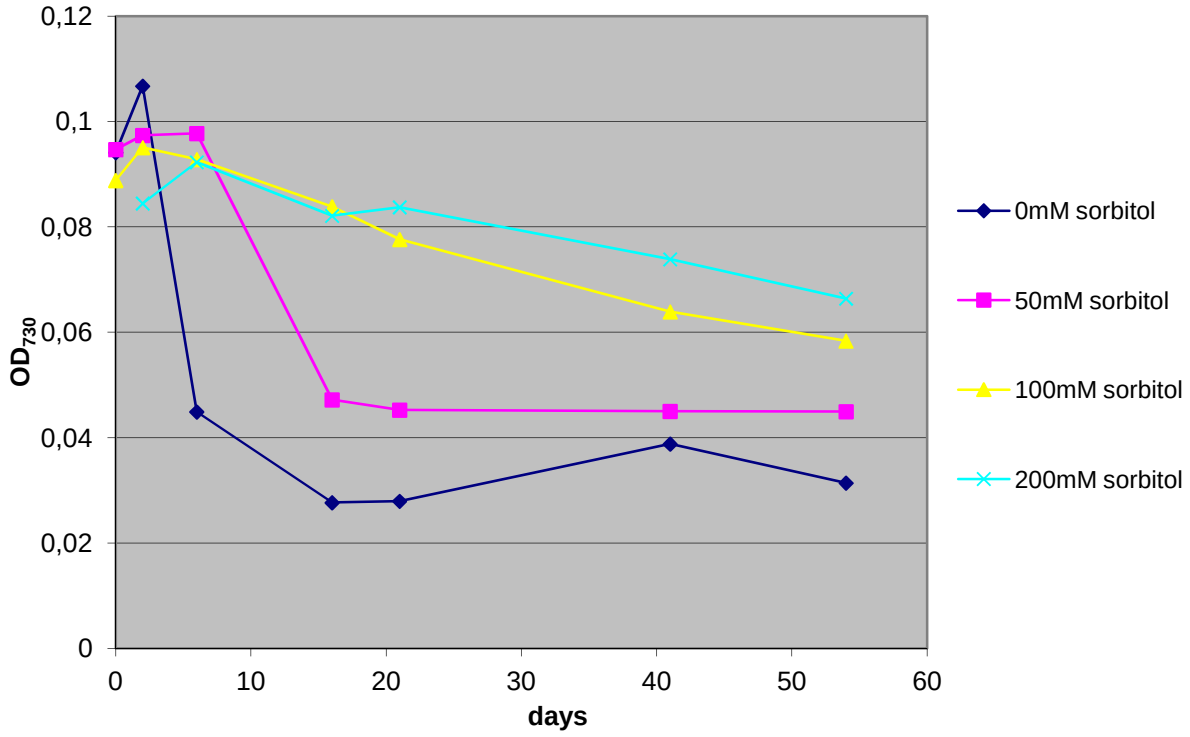


Fig 19
PCC7120*gtr*$^{+}$ grown in the dark on different concentrations of sorbitol

4.1.3. Construction of PCC7120*cox1*$^{-}$

4.1.3.1. Construction of the plasmid

Since knock-out mutants of *cox2* and *cox3* exist already in *Anabaena* sp. PCC7120 (Valladares et al, 2003), a knock-out mutant of *cox1* was constructed. PCR was performed with isolated genomic DNA from PCC7120 and primers 7120coxB and 7120coxC (see section 3.4.1.). Both primers introduced an *Xba*I site at the ends. The PCR product contained the complete *coxA1* ORF and parts of the adjacent *coxB1* and *coxC1*genes. pRL278 (see section 3.2.; Black et al, 1993) and the PCR product were digested with *Xba*I. pRL278 has 3 *Xba*I sites (see fig. 20). The largest fragment with 2448bp (containing neomycin phosphate transferase and the *sacB* gene) was ligated with the PCR product. The new plasmid was named pBWUV1 (see fig. 20).

In a second step a chloramphenicol resistance cassette (or chloramphenicol acetyl transferase = *cat*) replaced part of the *coxA1* ORF. The chloramphenicol resistance (Cm^R) cassette from pBR328B was amplified by PCR using the primers Cm5 and Cm3 (see section 3.4.2.).

Cm5 contains a *Pvu*I site. *Pvu*I cuts once in the entire plasmid pBR328, but not within the Cm^R cassette. Cm3 contains an *Age*I site. *Age*I cuts 1x in the plasmid within the region of the amplified PCR product but downstream of the Cm^R ORF. So for each enzyme one recognition site was introduced into the PCR product. The product was purified by gel electrophoresis and cut out from the gel as a fragment of 1076bp. The Cm^R cassette PCR product was restricted by *Pvu*I and *Age*I into four fragments: the short 7bp fragments on both ends and the 927bp and 135bp in between. pBWUV1 was digested by *Asi*SI and *Sgr*AI into two fragments: the larger 7684bp vector backbone and a small fragment of 215bp, which is in the middle of the *coxA1* ORF. Both digestions were purified on agarose gel. The 7684bp vector backbone of pBWUV1 and the 927bp fragment including Cm^R were cut out from the gel. The DNA was extracted and the two molecules were ligated. *Pvu*I and *Asi*SI sites have complementary sticky ends. So do *Age*I and *Sgr*AI. The new plasmid, which was named pBWUV2 (see fig. 20), was used to create a deletion-insertion mutation at the *cox1* locus leaving the genes *coxB1* and *coxC1* intact.

pBWUV2 was restricted with *Bam*HI and *Eag*I and the vector (without *npt* but with *sacB*) was treated with mung bean nuclease to remove ss overhangs (*Bam*HI and *Eag*I ends are not compatible). Then the remaining vector was ligated. The new plasmid was named pBWUV3 (see fig. 20). A conjugation was performed several times but never succeded, neither with pBWUV2 nor with pBWUV3.

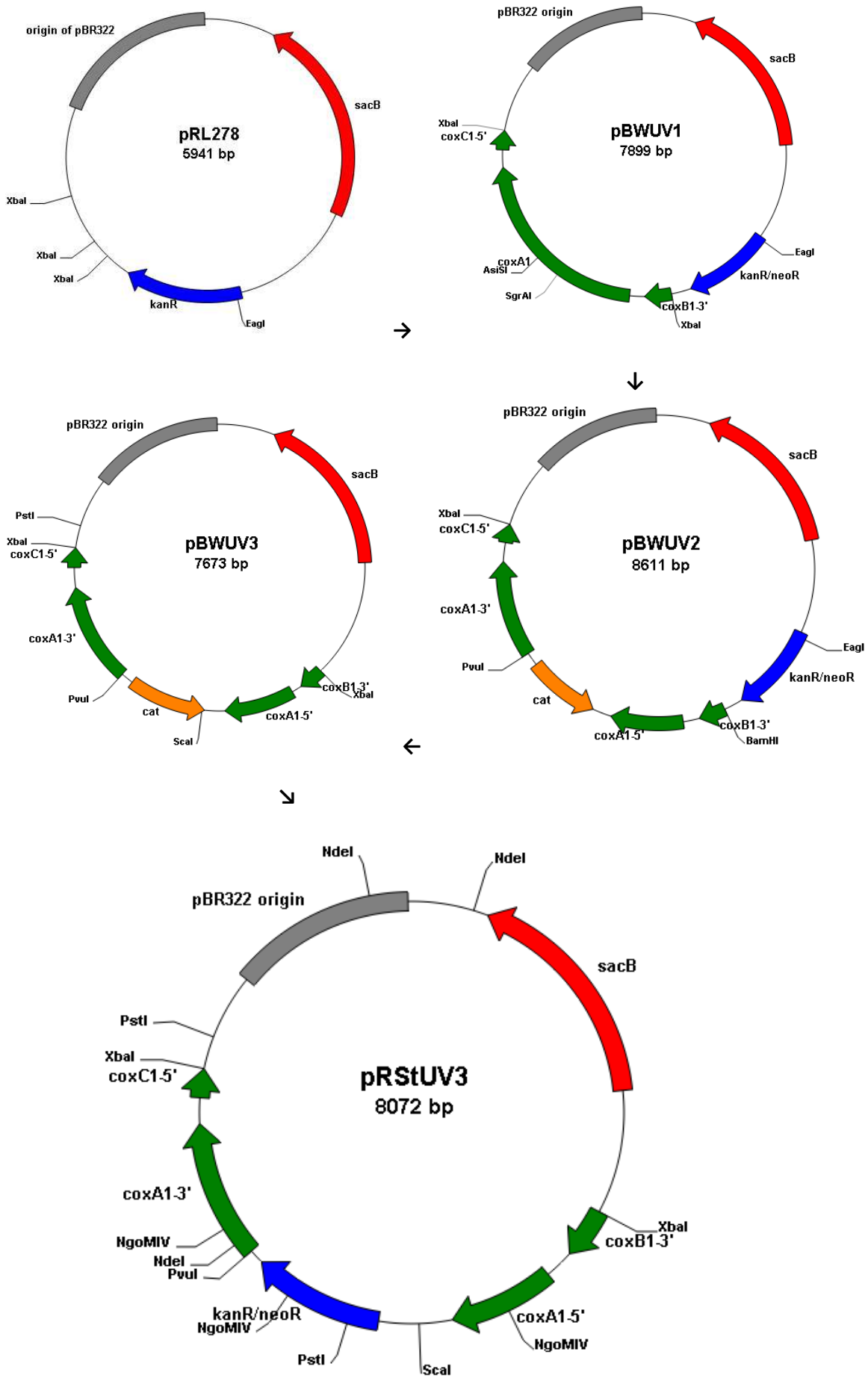


Fig. 20 Construction of pRStUV3

We decided to replace the chloramphenicol resistance cassette in pBWUV3 by a neomycin resistance cassette. The neomycin phosphate transferase (*npt*) cassette was obtained from pRL6 (see section 3.2.) by PCR amplification with pRL6 as a template. PCR was performed with primers Kmcassette2 and Kmcassette3 (see section 3.4.3.). 54°C were used as annealing temperature and 1min 10s as elongation time.

pBWUV3 and *npt* PCR product were both restricted with *Pvu*I and *Sca*I and purified by an agarose gel (see fig. 21). After DNA extraction from agarose gel 370ng of pBWUV3 vector (6934bp) and 105ng of *npt* (1137bp) were ligated, which means a 1.7 fold molarity of *npt*. The ligation mix was electroporated into *E. coli* Top10 and two colonies grew on an LB agar plate supplemented with 20µg/ml Km. After inoculation of small over night cultures (4ml LB with 50µg/ml Km) and plasmid minipreparations according to Holmes and Quigley (see section 3.8.) a restriction digestion with *Eco*RI was performed, which yielded the expected fragments 6785bp and 1287bp (see fig. 22). Ancestor pasmid pBWUV3 was also restricted with *Eco*RI and analysed on the same gel. From the expected restriction pattern 4951/1435/1287bp only the largest fragment could be detected (see fig. 22).

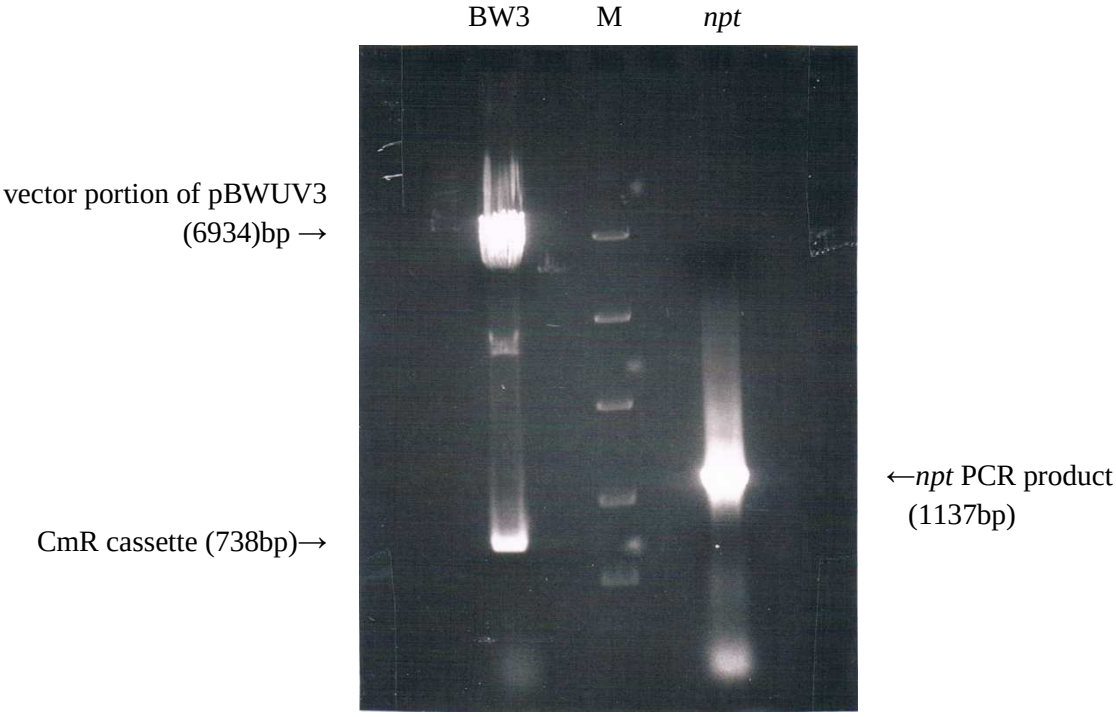


Fig. 21
pBWUV3 (BW3) and the PCR product of *npt* were digested with *Sca*I and *Pvu*I. The 6934 fragment of pBWUV3 and the restricted *npt* were cut out from the gel and the DNA was extracted.
M…. marker SM1123 (from Fermentas) contains the fragments 10, 4, 2, 1 and 0.5 kbp.

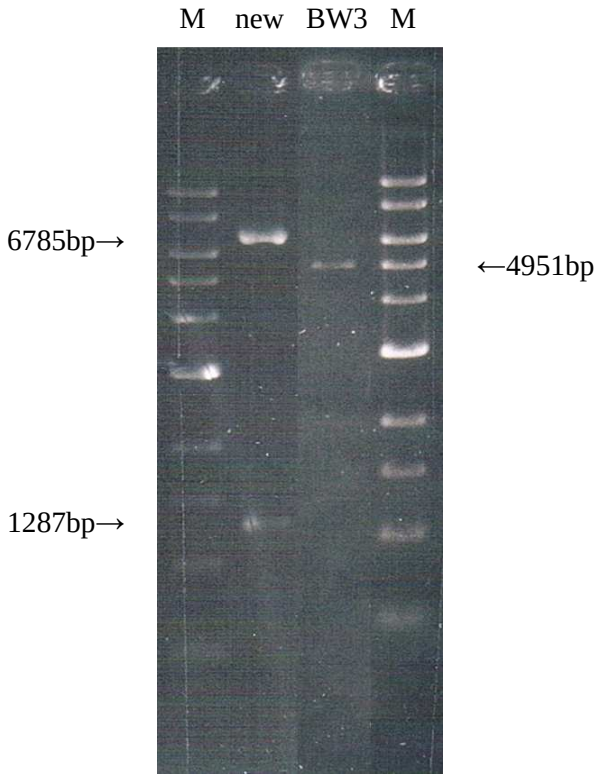


Fig. 22
Restriction digestions of the new plasmid (later called pRStUV3) and old pBWUV3 (BW3) with *Eco*RI , M…1kb ladder (from NEB): contains the fragments 10, 8, 6, 5, 4, 3 (thick band), 2, 1.5, 1 and 0.5 kbp.

Further restriction digestions were carried out with the newly constructed plasmid:

1) *Ngo*MIV 6162/1443/467bp
2) *Nde*I 4794/2648/630bp
3) *Pst*I 5971/2102bp

At the same time the ancestor plasmid pBWUV3 was also restricted with *Pst*I.

1) *Pst*I 7673bp

All enzymes yielded the expected pattern (see fig. 23). The digestion with *Pst*I clearly revealed the difference between the ancestor pBWUV3 and its derivative, which was named pRStUV3 (see fig. 20). A liquid culture of Top10 (pRStUV3) was frozen and stored at -80°C as No 1077.

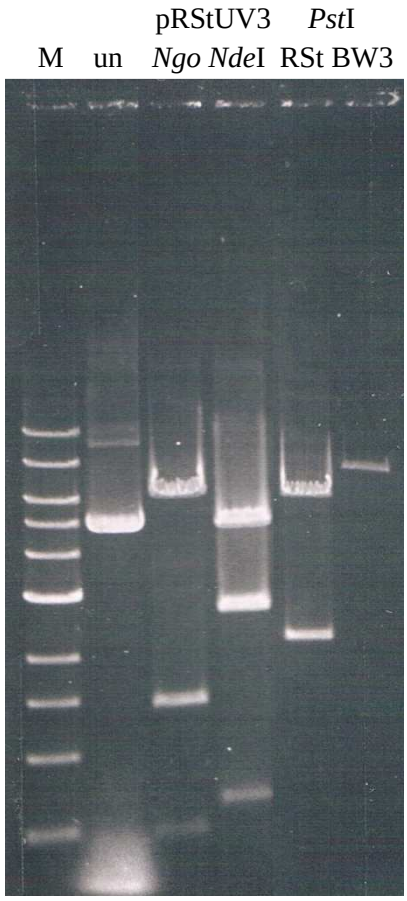


Fig. 23
Control digest of pRStUV3
M…1kb ladder (NEB), un...undigested pRStUV3, *Ngo*…*Ngo*IV, BW3…pBWUV3, RSt…pRStUV3
*Ngo*IV and *Nde*I were used for restriction of only pRStUV3, *Pst*I for both plasmids.

4.1.3.2. Conjugation of pRStUV3 into *Anabaena* sp. PCC7120

Conjugational approach:
HB101(RP4+pRL528) + Top10(pRStUV3) + PCC7120WT
Control approach:
Top10(pRStUV3) (twice the amount) + PCC7120WT (no RP4)

After incubation on a BG11HPA-30µg/ml neomycin plate some colonies had grown in the 1:100 dilution on the side, where RP4 had been present. On the control side, where no RP4 had been present, no colonies had formed. The colonies were streaked on a new plate with BG11HPA+30µg/ml Nm and inoculated a 50ml liquid culture, which was proved to be free of *E. coli* cells (see section 4.1.1.).

4.1.3.3. *Anabaena* sp. PCC7120*cox1*$^-$ was homozygously mutated in the *cox1* locus.

After the integration of pRStUV3 the culture was plated on BG11-5% sucrose with 30µg/ml Nm to select for the second recombination. Colonies were transferred to another BG11HPA plate with 30µg/ml Nm and 5% sucrose. Cell mass that had grown on the new plate was used for inoculation of a 50ml liquid culture. The DNA was isolated from a PCC7120*cox1*$^-$ culture with a high cell density. A PCR was performed with the same primers that had been used for amplifying the *cox1* locus of the WT (see section 3.4.1.). 2 approaches of strain PCC7120*cox1*$^-$ (5µl and 10µl of the template DNA) were compared with PCC7120WT DNA and knock out plasmid pRStUV3 as template. PCR with DNA from the new strain PCC7120*cox1*$^-$ (both with 5µl and 10µl) showed the same pattern as the PCR with pRStUV3: a fragment of 3559bp for the *cox1* locus interrupted by *npt* but no WT allele (2438bp) was detected (see fig. 24). Cultivation in the absence of neomycin did not change this pattern (see fig. 25), not even if cultivated a second time (inocculated by the first antibiotic less culture) without antibiotic (data not shown). This means that the WT allele was completely absent.

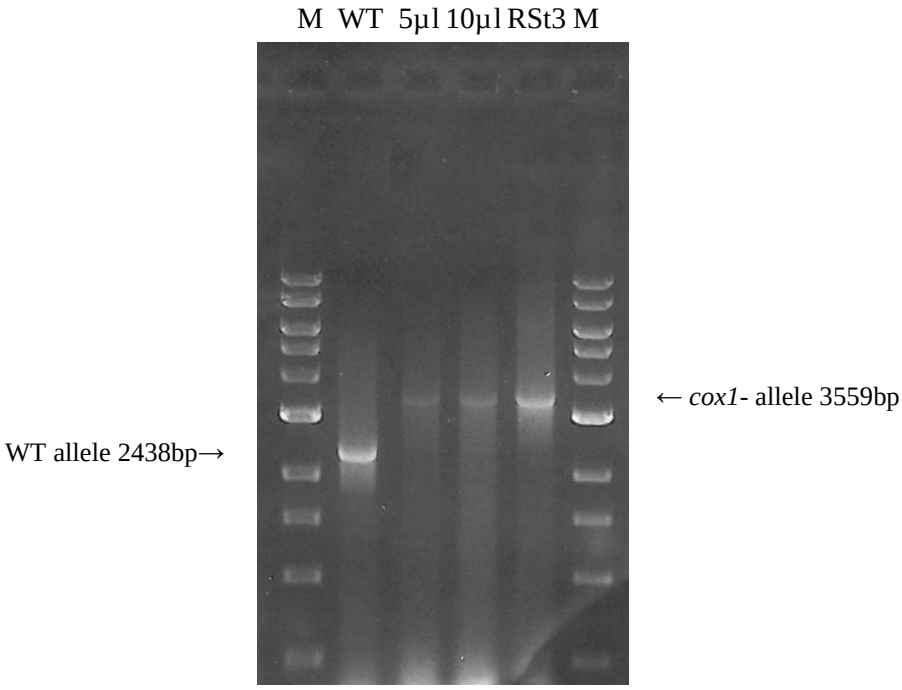


Fig. 24
PCR performed with DNA from PCC7120WT, PCC7120*cox1*$^-$ and knock out plasmid pRStUV3.
M….1kb ladder (NEB), 5µl and 10µl… from PCC7120*cox1*$^-$ DNA, RSt3...pRStUV3.

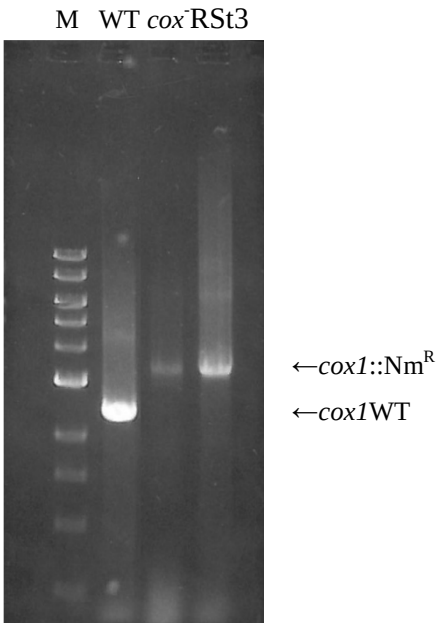


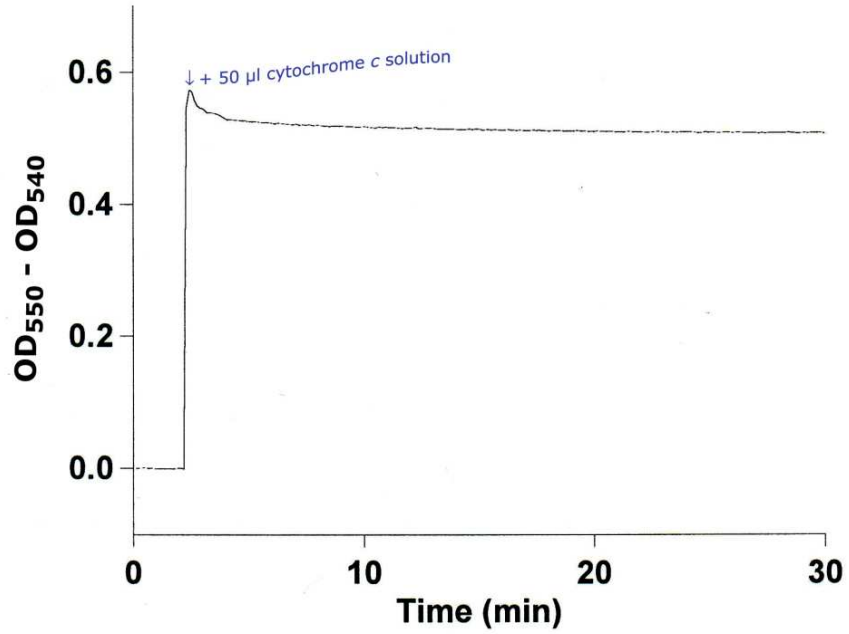
Fig. 25
PCR with isolated DNA from *cox1*$^-$ strain after growth in BG11 without Nm or sucrose
M….1kb ladder (NEB), *cox*$^-$: DNA preparation of liquid culture of PCC7120*cox1*$^-$.
RSt3: Plasmid preparation of pRStUV3

4.1.3.4. Cytochrome *c* oxidation reactions

4.1.3.4.1. Effect of KCN on cytochrome *c* alone

First the OD_{550}-OD_{540} difference of a cytochrome *c* solution in 20mM HEPES buffer was observed over 30 min without adding any further substances (membranes, KCN) to look whether the redox status of cytochrome *c* is changed by other components (e.g. HEPES) over a longer period. Within the first few minutes the values decreased indicating an oxidation of cytochrome *c* by air at a rate of 0.21nmol min^{-1} then flattened to a negligible rate of 0.023nmol min^{-1} (see fig. 26A). After this experiment the effect of KCN on cytochrome *c* absorbancy without any membranes was tested. Before KCN addition cytochrome *c* was oxidized at a rate of 0.11nmol/min, afterwards this trend was reversed to -0.21nmol min^{-1} (see fig. 26B). Obviously KCN alone had an influence on cytochrome *c*. KCN solutions, which are highly alkaline in aqueous solutions, change dramatically the pH value in the cuvette. As a consequence the absorption spectrum is changed.

A



B

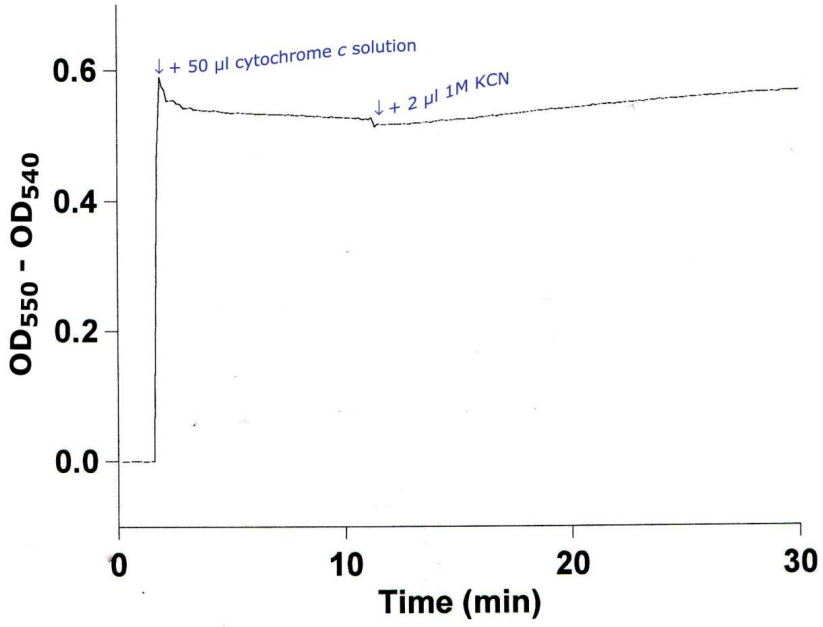


Fig. 26

A Measurement of cytochrome c alone.

B KCN reacts with pure cytochrome *c*. 50µl of cytochrome *c* solution and 2µl of 1M KCN were used.

4.1.3.4.2. Cytochrome *c* oxidase experiments with membranes from 50ml cultures

First the experiment was performed with 50ml cultures of both strains (WT and *cox1*$^{-}$). When the cultures had reached the stationary phase the membranes were

isolated (see section 3.27.). Then the protein concentration of the membrane suspension was determined (see section 3.28.). The calculated average values were 2.1µg/µl for *cox1*$^-$ and 2.5µg/µl for the WT see table 2 and 3).
The following protein concentrations were calculated for the suspension of the isolated membranes of PCC7120*cox1*$^-$:

volume used	measured OD_{595}		concentration in the cuvette		calculated to used volume		initial concentration
0.5µl	0.09075	→	1.1µg/ml	→	1.1µg in 0.5µl	→	2.2 µg/µl
1µl	0.11803	→	1.5µg/ml	→	1.5µg in 1µl	→	1.5 µg/µl
2µl	0.25039	→	4.7µg/ml	→	4.7µg in 2µl	→	2.35 µg/µl
5µl	0.42922	→	10.0µg/ml	→	10.0µg in 5µl	→	2.0 µg/µl
10µl	0.68262	→	20.0µg/ml	→	20.0µg in 10µl	→	2.0 µg/µl

Table 2 Protein concentration of suspensions of isolated membranes from 50ml culture of PCC7120*cox1*$^-$

From the WT of PCC7120 the protein content of the isolated membranes were measured as follows:

volume used	measured OD_{595}		concentration in the cuvette		calculated to used volume		initial concentration
0.5µl	0.13551	→	1.7µg/ml	→	1.7µg in 0.5µl	→	3.4 µg/µl
1µl	0.22596	→	3.6µg/ml	→	3.6µg in 1µl	→	3.6 µg/µl
2µl	0.23062	→	5.0µg/ml	→	5.0µg in 2µl	→	2.5 µg/µl
5µl	0.45999	→	11.1µg/ml	→	11.0µg in 5µl	→	2.22µg/µl
10µl	0.64057	→	17.8µg/ml	→	17.8µg in 10µl	→	1.78µg/µl

Table 3 Protein concentration of suspensions of isolated membranes from 50ml culture of PCC7120WT.

4.1.3.4.2.1. PCC7120*cox1*$^-$ does not exhibit any cytochrome *c* oxidase activity when grown in a 50ml culture

First the redox status of the cytochrome *c* solution was determined (see section 3.29.). OD_{550} = 0.35682 was measured for the untreated cytochrome *c* solution, while after the addition of sodium ascorbate or potassium hexa-cyanoferrate (III) OD_{550} = 0.47411 and OD_{550} = 0.12760 were obtained, respectively. The proportion of the reduced form can be calculated as 66.15% of total cytochrome *c*. 50µl of the cytochrome *c* solution and 200µl of the isolated membranes from the WT were added. At the moment of membrane addition the OD_{550}-OD_{540} difference dramatically

changed independently whether any Cox is present or not (see fig. 27). From that point any further decrease of measured OD_{550}-OD_{540} is caused by oxidation of cytochrome *c* by the membranes. An oxidase activity of 14nmol min^{-1} x [mg protein]$^{-1}$ could be observed that was stopped when 2µl 1M KCN were added after about 8min (see fig. 27). After KCN addition OD_{550}-OD_{540} began to increase like in section 4.1.3.4.1.

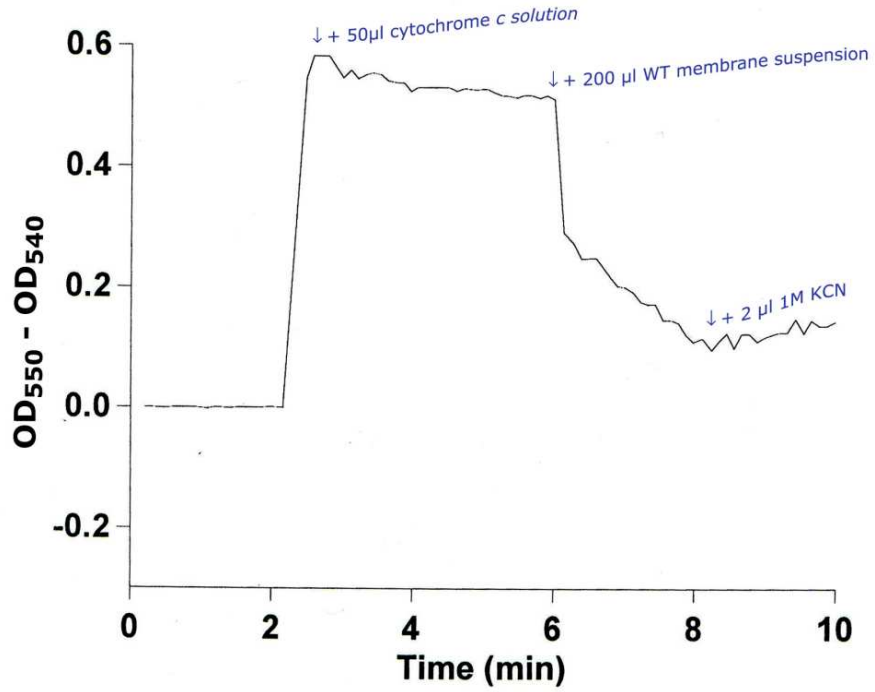


Fig. 27
Cytochrome *c* was oxidized by membranes of PCC7120WT. The reaction was inhibited by KCN. 50µl cytochrome *c* solution, 200µl of isolated membranes and 2µl of 1M KCN were used.

The experiment was repeated, however, this time the duration of the measurement was elongated to 30min and no KCN was added to analyse the change of the reduction state over a longer period. The proportion of reduced cytochrome *c* continuously decreased although the curve became flatter within 30 min (see fig 28). The initial rate of oxidation was 6.86nmol min^{-1} x [mg protein]$^{-1}$. Once more an experiment was performed with membranes derived from PCC7120WT but this time 2µl KCN were added after about 10 min. The measurement continued again for 30min in order to compare this experiment with the previous one. Until the moment of KCN addition, the experiment was similar to the preceeding one. Cytochrome *c* was oxidized, starting with a rate of 6.23nmol min^{-1} x [mg protein]$^{-1}$, which later decreased (see fig. 29). After KCN addition the OD_{550}-OD_{540} value began to increase (see fig. 29 and section 4.1.3.4.1.). These results clearly demonstrate that the decrease of the measured OD_{550}-OD_{540} difference is caused by a cytochrome *c* oxidase localized in the membranes that can be inhibited by KCN, as otherwise the oxidation continues.

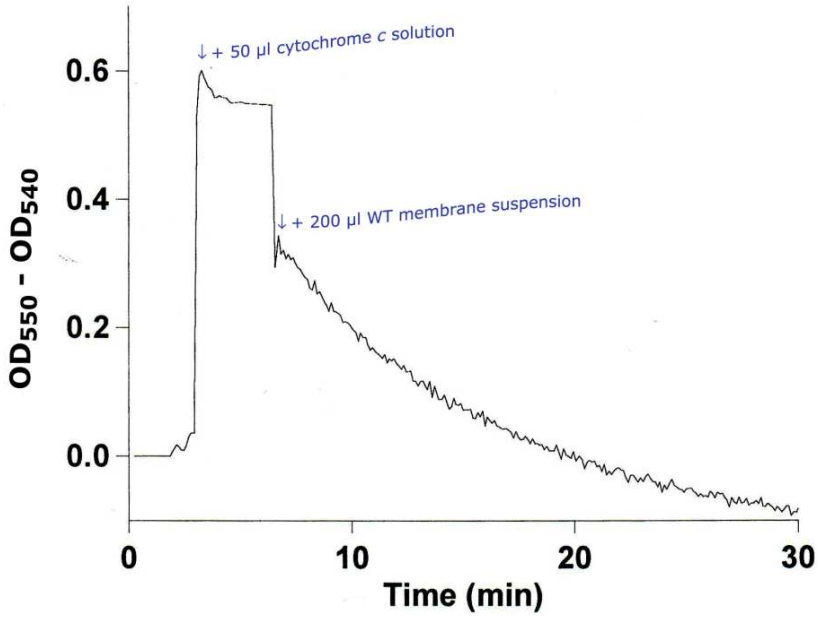


Fig. 28
Cytochrome *c* was oxidized by membranes of PCC7120WT. No KCN was added and oxidation continued within the first 30min. 50µl cytochrome *c* solution and 200µl from the isolated WT membranes were used.

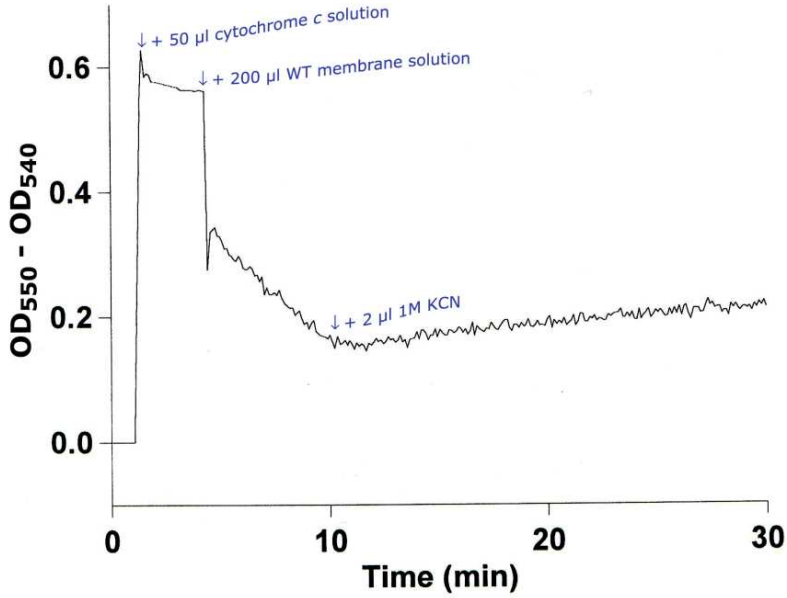


Fig. 29
Cytochrome *c* is oxidized by PCC7120 WT membranes, which is inhibited by KCN.
50µl of cytochrome *c* solution, 200µl of *cox1*$^-$ membranes and 2µl of 1M KCN were used.

The same experiments were performed with the membranes of the *cox1*$^-$ strain. Again 50µl of the cytochrome *c* solution and 200µl of *cox1*$^-$ membranes were used, because the protein concentrations of the isolated membranes from the different strains were similar (see table 2 and 3). The cytochrome *c* addition was performed in two steps. Hardly any cytochrome *c* oxidase activity could be detected (see fig. 30). No KCN was used to analyse whether the values continue to stay constant for half an

hour. Now the reaction towards KCN was investigated (see fig. 31). The addition of KCN led to a small increase of the measured OD_{550}-OD_{540} very similar to the value obtained without membranes (see fig. 26B).

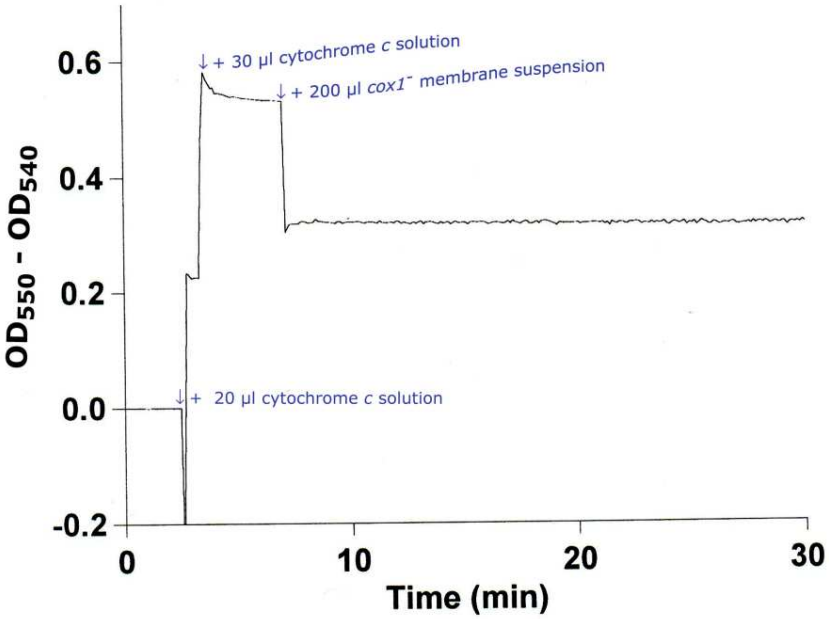


Fig. 30
No cytochrome *c* oxidation by membranes of *cox1*$^-$: 50µl (20µl + 30µl) of cytochrome*c* solution and 200µl of *cox1*$^-$ membranes were used.

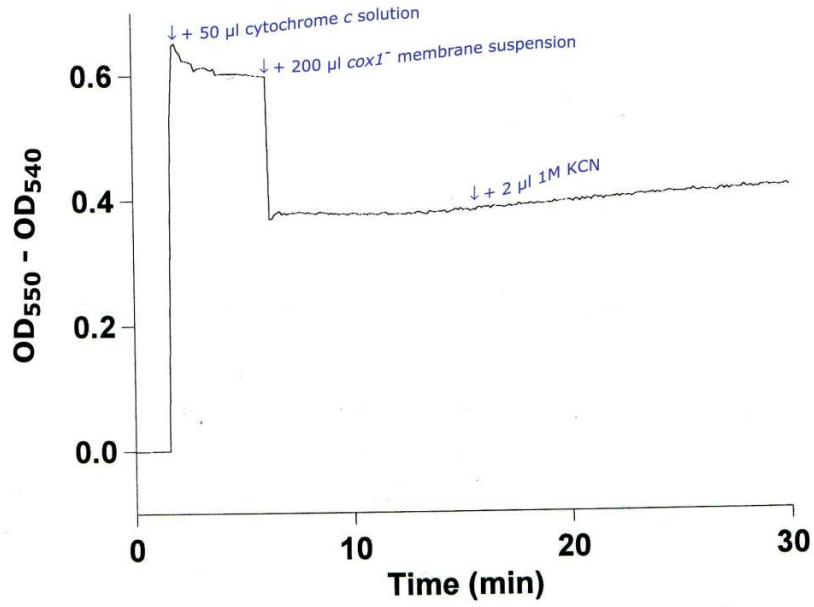


Fig. 31
No oxidation of cytochrome *c* by membranes of PCC7120*cox1*$^-$: 50µl cytochrome *c* solution, 200µl of *cox1*$^-$ membranes and 2µl 1M KCN were used.

4.1.3.4.3. Experiments with membranes from 10l cultures

From PCC7120WT and *cox1*$^-$ 10l cultures were grown. The *cox1*$^-$ culture was harvested at an OD_{730} of 0.730, the WT culture at an OD_{730} of 0.422. The membranes were isolated (as described in section 3.27.) and the protein concentrations of the membrane suspensions were determined (see table 4).

	used volume of suspension	measured OD_{550}	concentration in the cuvette	according to initial volume	initial concentration
	1µl	0.52423	13µg/ml	13µg/1µl	13 µg/µl
A	2µl	0.81454	25µg/ml	25µg/2µl	12.5µg/µl
	1µl	0.28879	6µg/ml	6µg/1µl	6µg/µl
B	2µl	0.50169	12µg/ml	12µg/2µl	6µg/µl

Table 4
Measurement of the protein concentration of isolated membranes of PCC7120*cox1*$^-$ (A) and WT (B).

First the redox status of the cytochrome *c* solution was measured (see section 3.29.).

basic OD_{550}: 0.34759
OD_{550} after sodium ascorbate addition: 0.36520
OD_{550} after potassium hexacyanoferrate addtion: 0.10381

This means that 93.26% of the cytochrome *c* were reduced. 20µl of cytochrome *c* and 10µl of isolated membranes were used for a total volume of 1350µl. At last 2µl 1M KCN were added. A peak down was observed on the graphic, where the magnetic rotator might have been touched (see fig. 32). While adding something into the cuvette the spectral photometer had to be opened. During this short period the measurement was interrupted. When the measurement was continued a line was connected between the values before and after opening. The quicker the experiment was performed the steeper the connection was. A real oxidation was observed. The OD_{550}-OD_{540} difference continously decreased from an initial value of 210.58nmol min^{-1} x [mg protein]$^{-1}$. After KCN addition the measured OD_{550}-OD_{540} increased again (see section 4.1.3.4.1.). The experiment was repeated with 20µl of isolated membranes, which leads to a faster oxidation and the effect of KCN is more significant (see fig. 33).

The decrease from the recovery after the down peak to the moment of KCN addition corresponds to the cytochrome *c* oxidase activity of the isolated membranes because of its intact Cox. Directly after membrane addition an oxidation rate of 233.5nmol min^{-1} x [mg protein]$^{-1}$ was calculated, which later flattened. After the addition of KCN the OD_{550}-OD_{540} increased again (see section 4.1.3.4.1.).

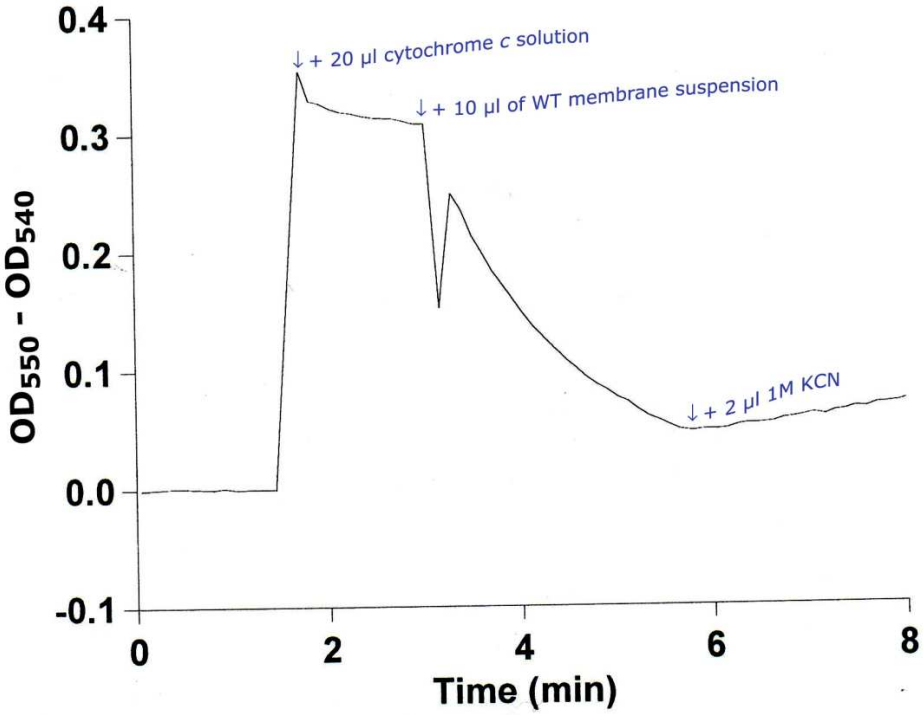


Fig. 32
Measurement of the cytochrome *c* oxidase activity with the membranes of the WT. 20µl of the cytochrome *c* solution, 10µl of the membrane suspension and 2µlof 1M KCN were added.

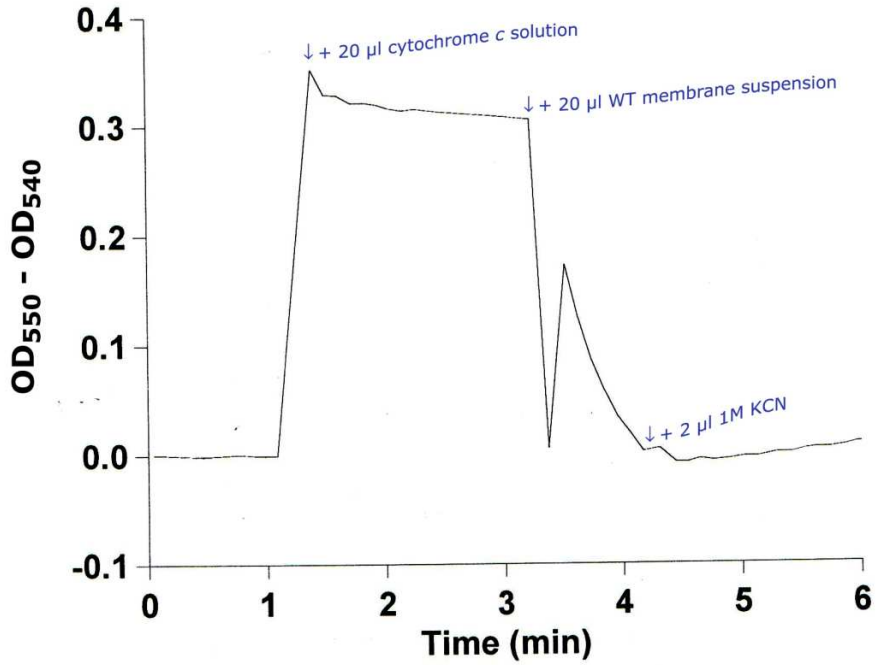


Fig. 33
Horse heart cytochrome *c* oxidation by isolated membranes from the WT: 20µl were added from cytochrome *c* solution as well as from the membranes. Again a small peak occurred after cytochrome *c* and a high peak after the membrane addition.

The same experiment was performed with the membranes of the mutated strain. The redox status of the cytochrome *c* solution was determined again. The initial OD_{550} was 0.28959, the highest value after sodium ascorbate addition was 0.35291 and the lowest value after potassium ferricyanate (III) addition was 0.08982. 75.9% of the total cytochrome *c* was reduced. 20µl were used of both the membrane suspension and the cytochrome *c* solution.

Unexpectedly an oxidation activity of 3.50nmol min^{-1} x [mg protein]$^{-1}$ was observed (see fig. 34) although this strain had been proven by PCR to be homozygously mutated in the *cox1* locus. After the addition of KCN the measured OD_{550}-OD_{540} increased again (see section 4.1.3.4.1.). The experiment was repeated and this time an oxidation of even 5.94nmol min^{-1} x [mg protein]$^{-1}$ was calculated (data not shown). Nevertheless the oxidation activity was very small compared to experiments with membranes of the WT.

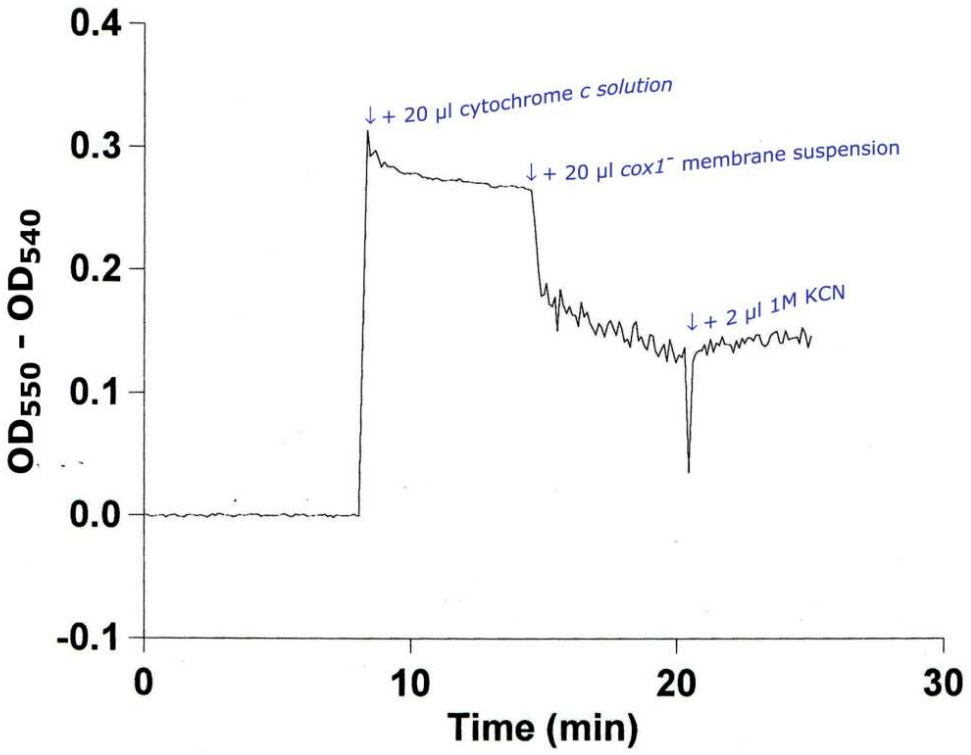
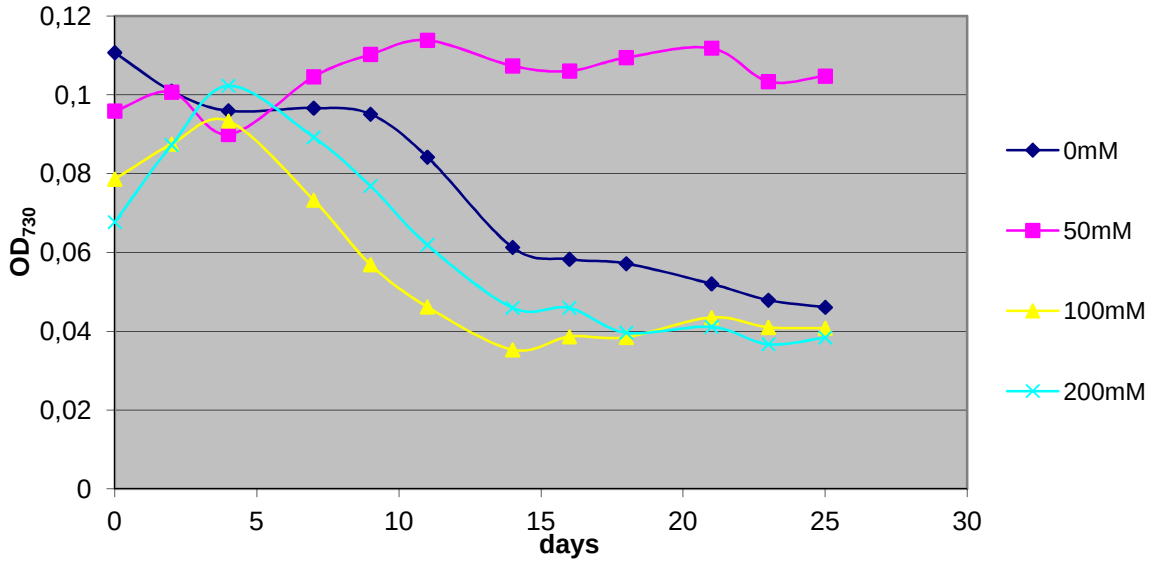


Fig. 34
Cytochrome *c* oxidation by membranes of *cox1*$^{-}$. 20µl cytochrome *c* solution, 20µl membrane suspensions and 2µl of 1M KCN were used.

4.1.3.5. If *cox1* is deleted, PCC7120 loses its capacity for chemoheterotrophic growth

PCC7120*cox1*$^{-}$ was also tested for chemoheterotrophic growth. 0, 50, 100 and 200mM fructose were used. Only at 50mM both the OD_{730} and the chlorophyll content kept constant, whereas without sugar or at higher fructose concentrations a reduction of both OD_{730} and chlorophyll content could be seen (see fig. 35).

A



B

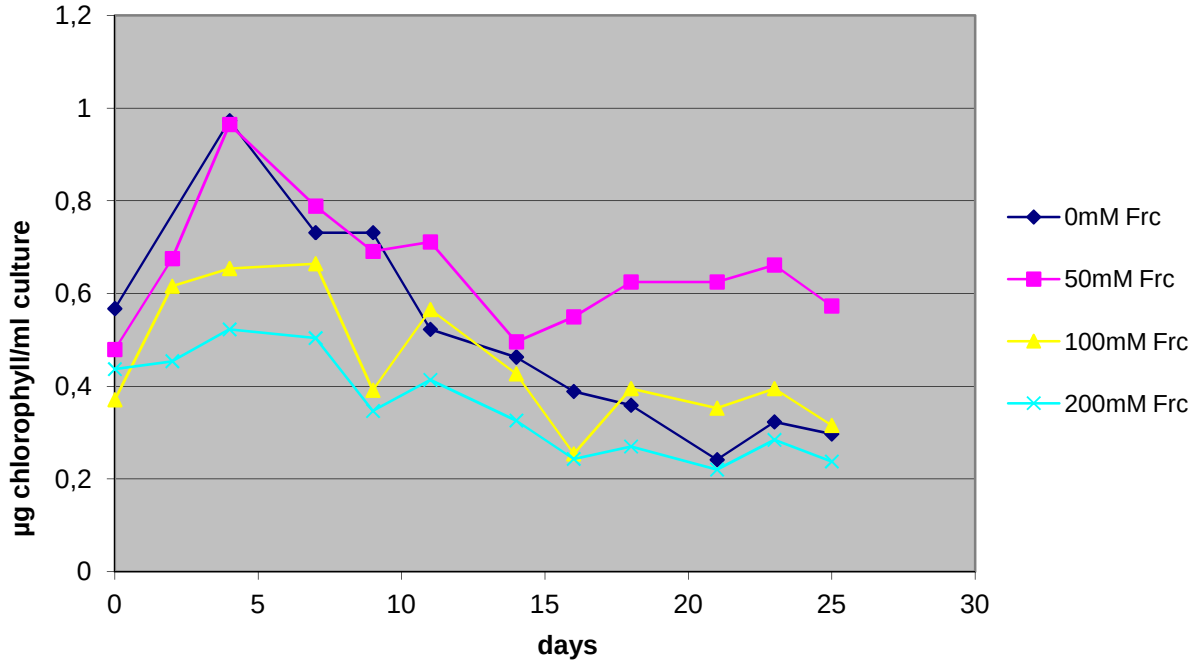


Fig. 35
Cultivation of PCC7120*cox1*$^-$ in the dark under different concentrations of fructose.
A measured OD_{730}
B chlorophyll concentration after methanol extraction

Without any fructose or at concentrations of 100mM and 200mM the cells died after 2 weeks, whereas at 50mM the culture could sustain its density within the first 2 weeks. Nearly no increase of chlorophyll was observed at 50mM fructose for the *cox1*$^-$ strain.

4.1.4. Uptake measurements of sugars

4.1.4.1. Uptake of sugars by the wild type

If the strain PCC7120 has the ability for heterotrophic growth dependent on fructose this sugar has to be taken up into the cell followed by its metabolism. Therefore experiments with radiolabeled fructose were performed. First an external concentration of 0.2mM was chosen. PCC7120 WT was harvested at an OD_{730} = 2.5167 and diluted to 2.0. After addition of 0.2mM fructose the cells were incubated in the light for 8 hours and every hour a sample of 1ml was taken.

The uptake of glucose by PCC7120 WT from 0.2mM solution was analysed the next day. The same liquid culture was used for the glucose and for the fructose experiments. The culture was harvested at OD_{730} = 2.7846 and diluted to OD_{730} = 2.0. The incorporated radioactivity was measured by the szintillation counter and the percentage of the total radioactivity incorporated into the cells was calculated (see fig. 36).

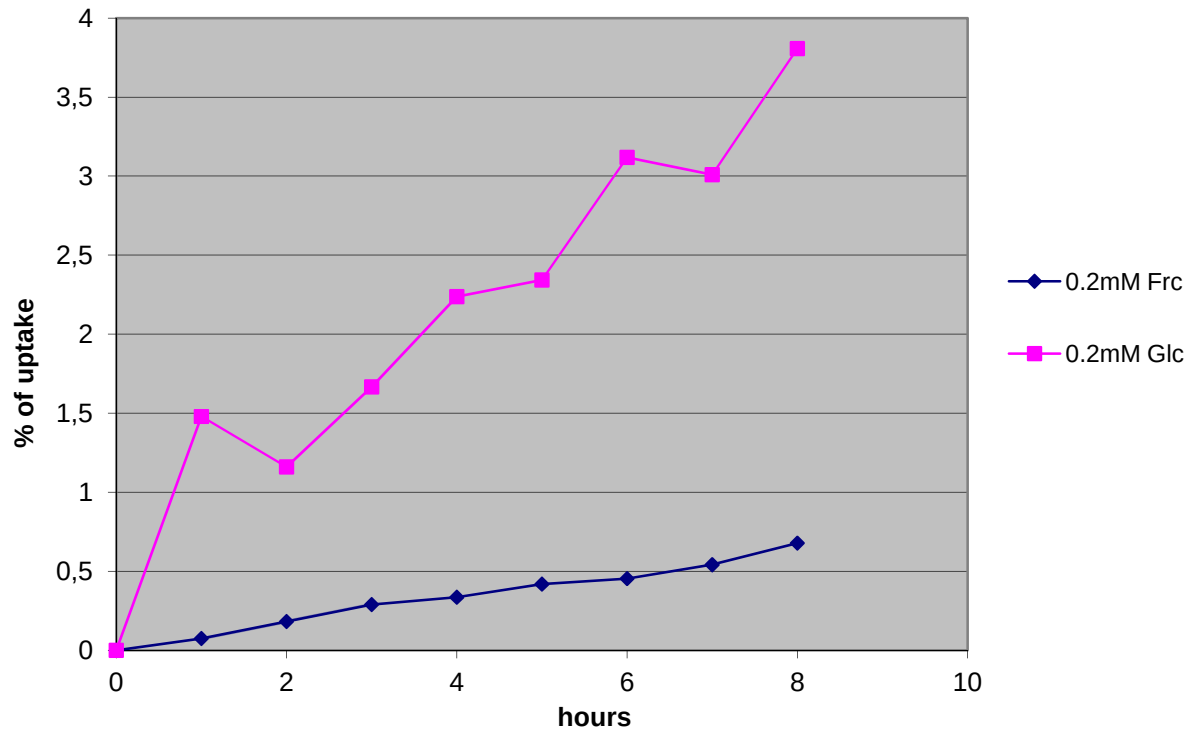


Fig. 36
Fructose and glucose uptake by PCC7120WT

Interestingly the uptake rate of glucose was much higher, although glucose cannot be used as a substrate for heterotrophic growth. However a fructose concentration of 0.2mM has not been observed to induce any mixotrophic growth. Therefore fructose uptake was analysed at a concentration of 2mM, 10mM, 50mM, 100mM and 200mM. The percentage of uptake was highest in 2mM and declined with an increasing concentration. The uptake rate was very similar at concentrations of

0.2mM, 50mM and 100mM (see fig. 37), while the cells took up only half the percentage at a concentration of 200mM Frc.

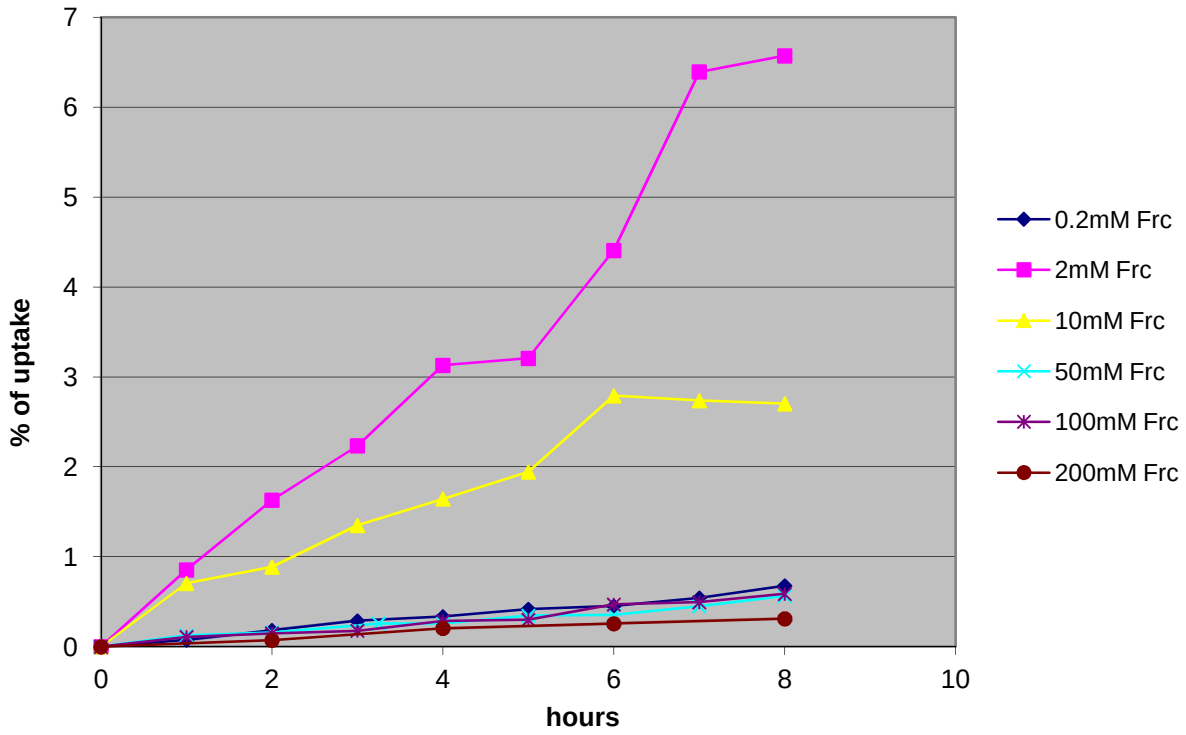


Fig. 37
Fructose uptake by PCC7120WT compared at different external concentrations

According to the percentage of uptake the amount of incorporated fructose molecules was calculated at 4h, 6h and 8h after fructose addition. Although the percentage of uptake decreases with a higher external fructose except for 0.2mM, the absolute amount of incorporated fructose increases (see table 5).

Concentration	4h	6h	8h
0.2mM	0.68	0.90	1.36
2mM	62.60	88.20	130.00
10mM	164.00	279.00	270.00
50mM	130.00	185.00	280.00
100mM	290.00	470.00	590.00
200mM	420.00	520.00	620.00

Table 5
Incorporated amounts of nmol Frc x [ml suspension of OD_{730} = 2.0]$^{-1}$

4.1.4.1.1. Effect of glucose on fructose uptake

Now the effect of glucose addition to fructose uptake was investigated. When there were external conditions of 2mM glucose and 0.2mM fructose the latter was incorporated at a much higher rate (about 18% after 8h). When the external

concentration of both glucose and fructose was 0.2mM an even higher fructose uptake of about 25% after 7h was observed (see fig. 38).

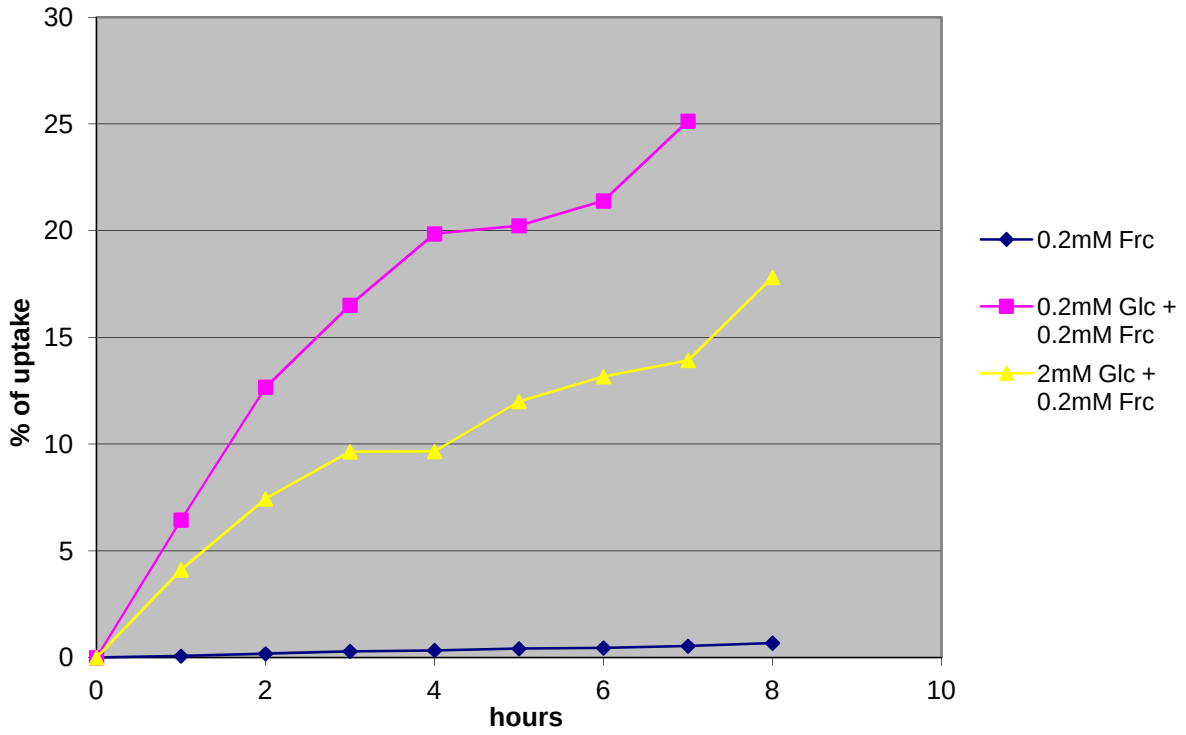


Fig. 38
Frc uptake dependent on the concentration of added Glc: Frc uptake at 0.2mM and 2mM Glc was compared to the Frc uptake in the absence of Glc. The Frc concentration was 0.2mM in every case.

4.1.4.2. Sugar uptake rates of PCC7120*gtr*$^+$

gtr was derived from PCC6803 (Schmetterer, 1990), where it is responsible for the strain's ability to take up glucose, 3OMG and fructose. *gtr* confers the capacity for heterotrophy with glucose as well as sensitivity towards fructose and both features were abolished in a PCC6803*gtr*$^-$ strain (Flores and Schmetterer, 1986, Joset et al, 1988; Zhang et al, 1989). However, when PCC6803WT was incubated in 10mM glucose in the light for 24-48h the strain took up nearly no 3OMG. When incubated in the light in the presence of both 10mM glucose and 10µM DCMU the glucose uptake was also reduced but not as much as with glucose alone (see fig. 39). It was analysed whether the introduction of *gtr* changes the phenotype of PCC7120 concerning the uptake of sugars. The uptake rate of glucose (external concentration = 0.2mM) by PCC7120*gtr*$^+$ was significantly higher than the rate of the WT (see fig. 40). The same experiment was performed with 3-O-methylglucose (3OMG). Again the uptake by PCC7120*gtr*$^+$ was much higher (see fig. 41), however, a difference to the preceeding experiment was observed within the following 4h. Within the first hour an uptake occurred that was comparable to the glucose import. Then the curve flattened and even decreased (see figs. 41 and 42). 3OMG cannot be metabolized. Therefore the concentration remains constant within the cell. At last the effect of cultivation in

fructose on the glucose uptake by PCC7120*gtr*$^{+}$ was tested (cultivation in glucose was not possible due to its toxicity). The strain PCC7120*gtr*$^{+}$ took up glucose at a much higher rate when grown mixotrophically in BG11 supplemented with 10mM fructose than after cultivation in pure BG11 (see fig. 43). In both cases no fructose had been added during the experiment itself and any fructose had been removed from the medium by washing before.

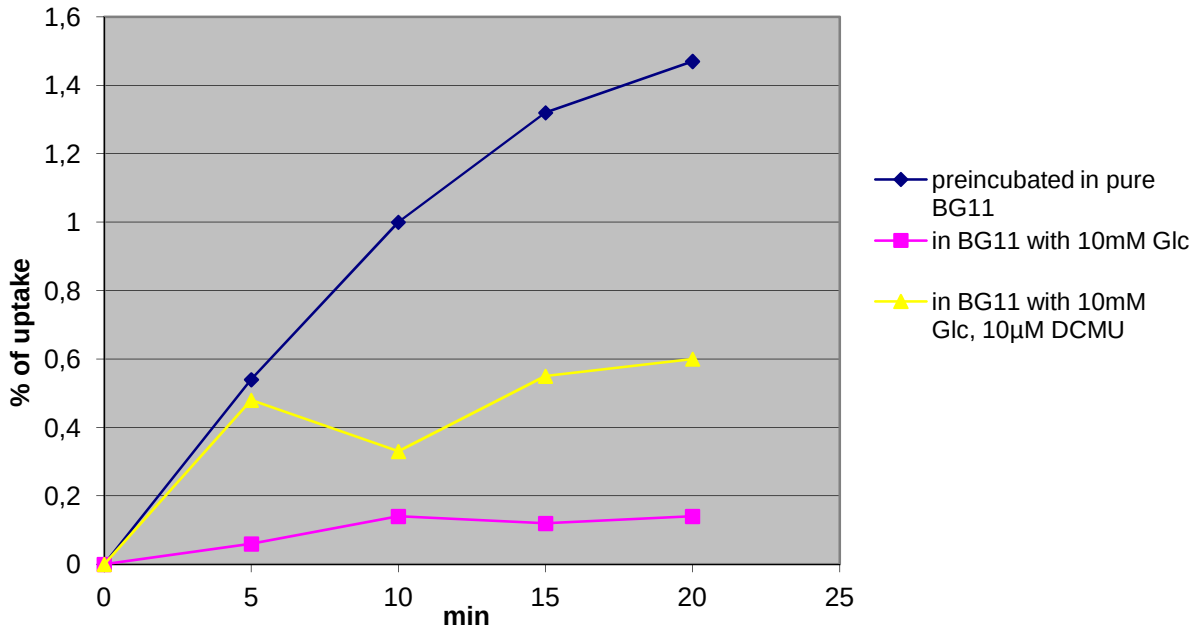


Fig. 39
3OMG uptake by PCC6803WT is influenced by preincubation in medium containing 10mM glucose (external concentration of 3OMG: 0.25mM)

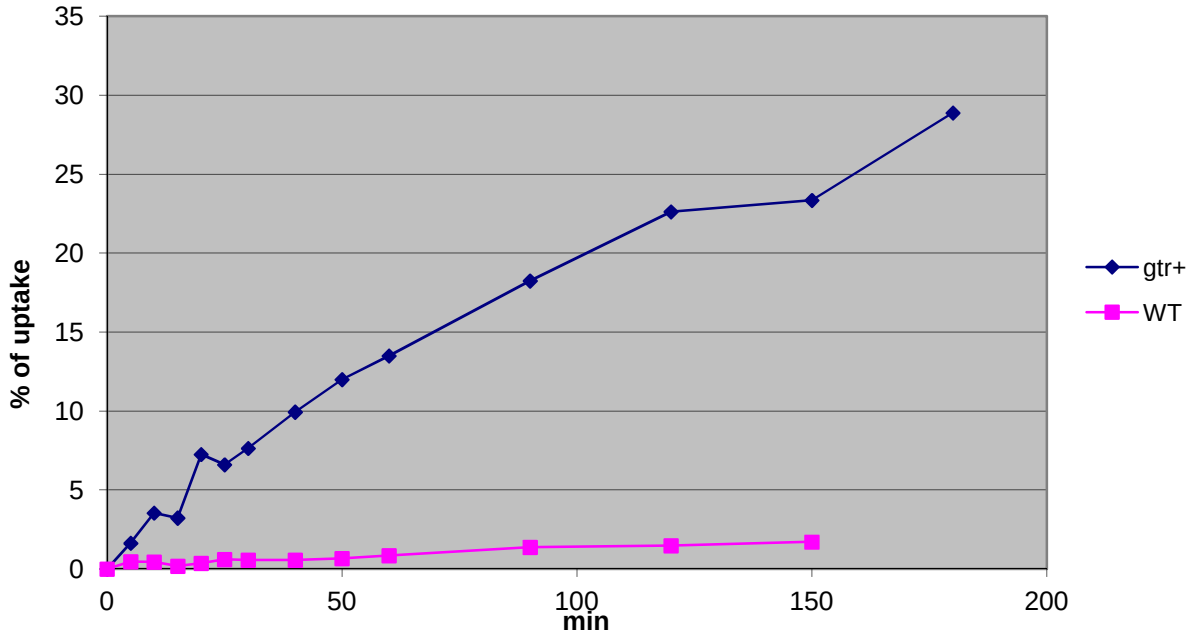


Fig. 40
Glucose uptake by PCC7120WT and *gtr*$^{+}$ at an external concentration of 0.2mM

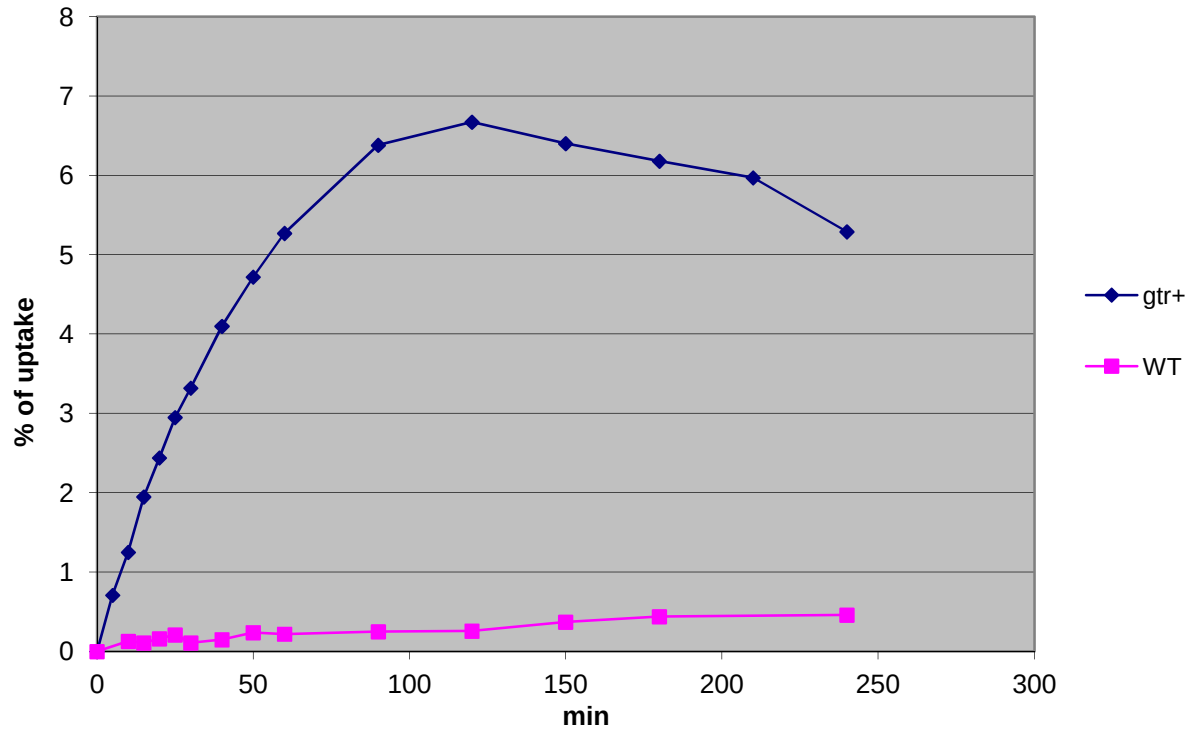


Fig. 41
3OMG uptake by PCC7120WT and *gtr*$^{+}$ at an external concentration of 0.2mM

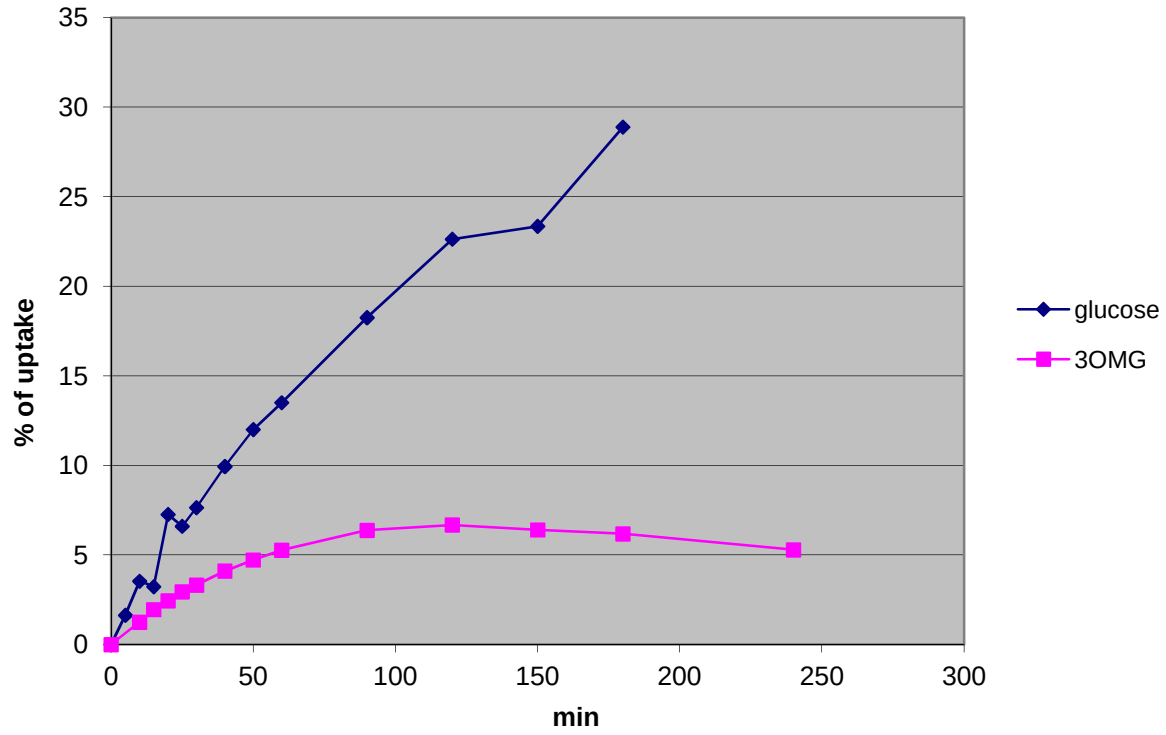


Fig. 42
Comparison of the uptake of glucose and 3OMG by PCC7120*gtr*$^{+}$: In both cases the external concentration was 0.2mM.

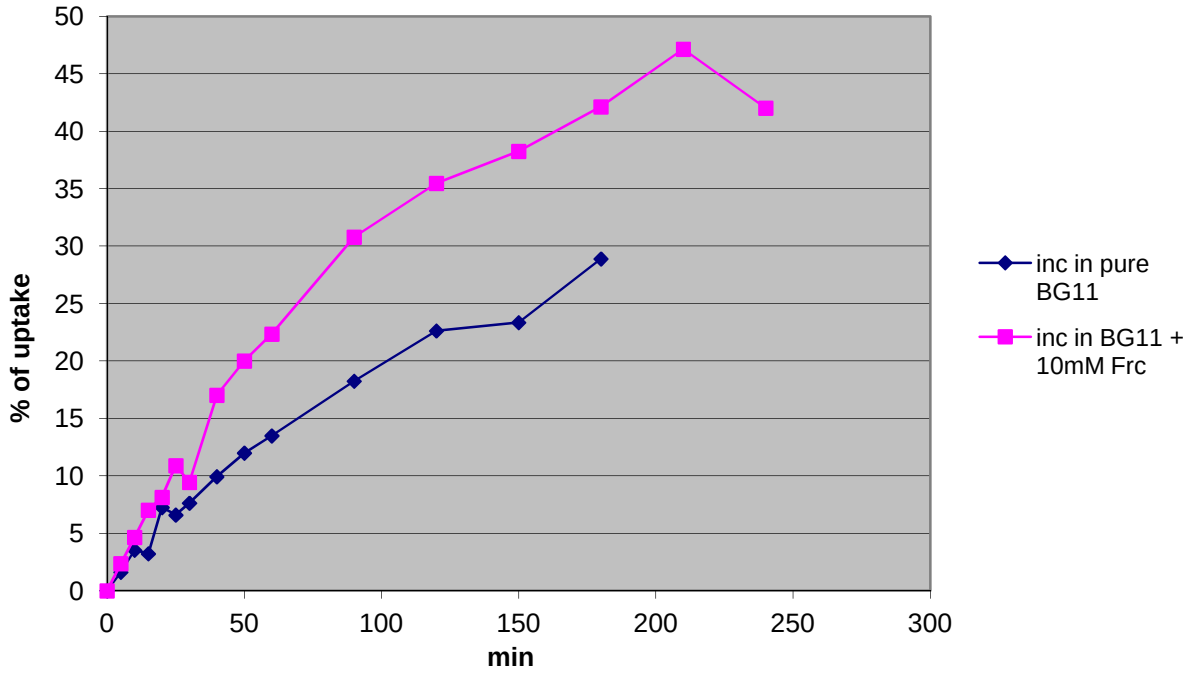


Fig. 43
PCC7120*gtr*$^+$ takes up glucose at a much higher rate when the culture has been incubated in 10mM fructose compared to a culture that had been cultivated in pure BG11.

Concerning the uptake of fructose by PCC7120*gtr*$^+$ experiments were performed with external concentration of 0.2mM and 50mM fructose. In contrast to the wild type (see fig. 37) fructose was incorporated at a higher percentage, when the external concentration was 50mM (see fig. 44). Under the latter conditions strain PCC7120*gtr*$^+$ takes up fructose at a 2-4 fold rate (see fig. 46), whereas under the former conditions PCC7120*gtr*$^+$ cells take up fructose only at half the rate (see fig. 45).

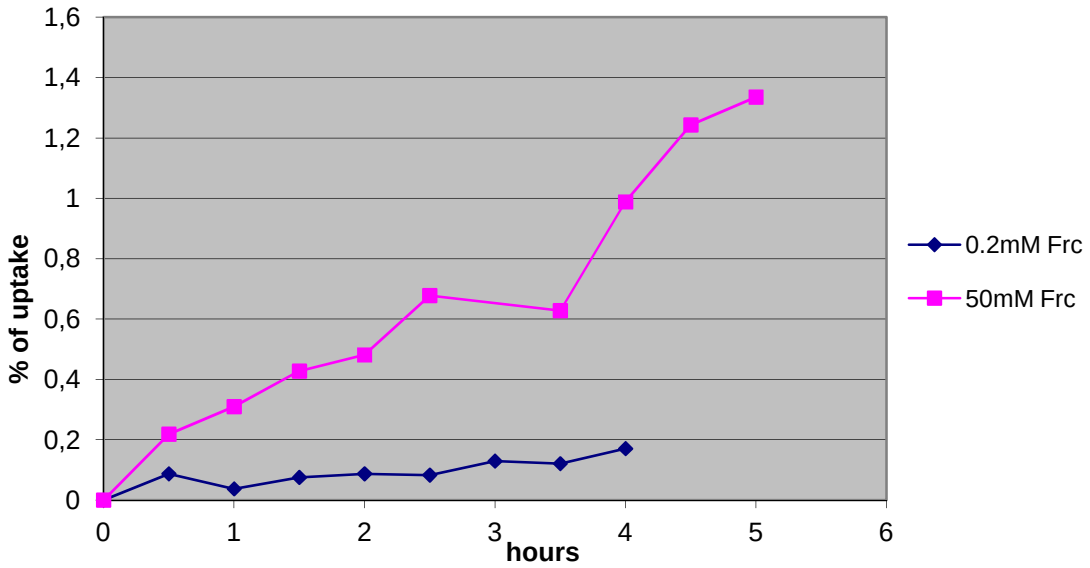


Fig. 44
Uptake rates of fructose by PCC7120*gtr*$^+$ at different external concentrations

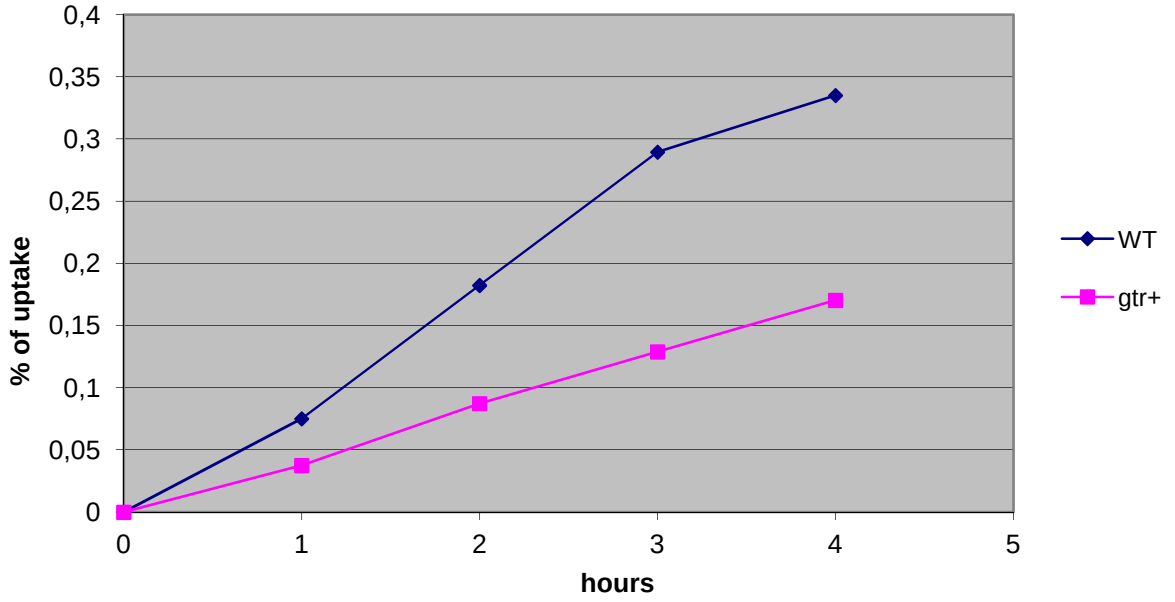


Fig. 45
Fructose uptake by PCC7120WT and *gtr*$^{+}$: The external concentration was 0.2mM in both cases.

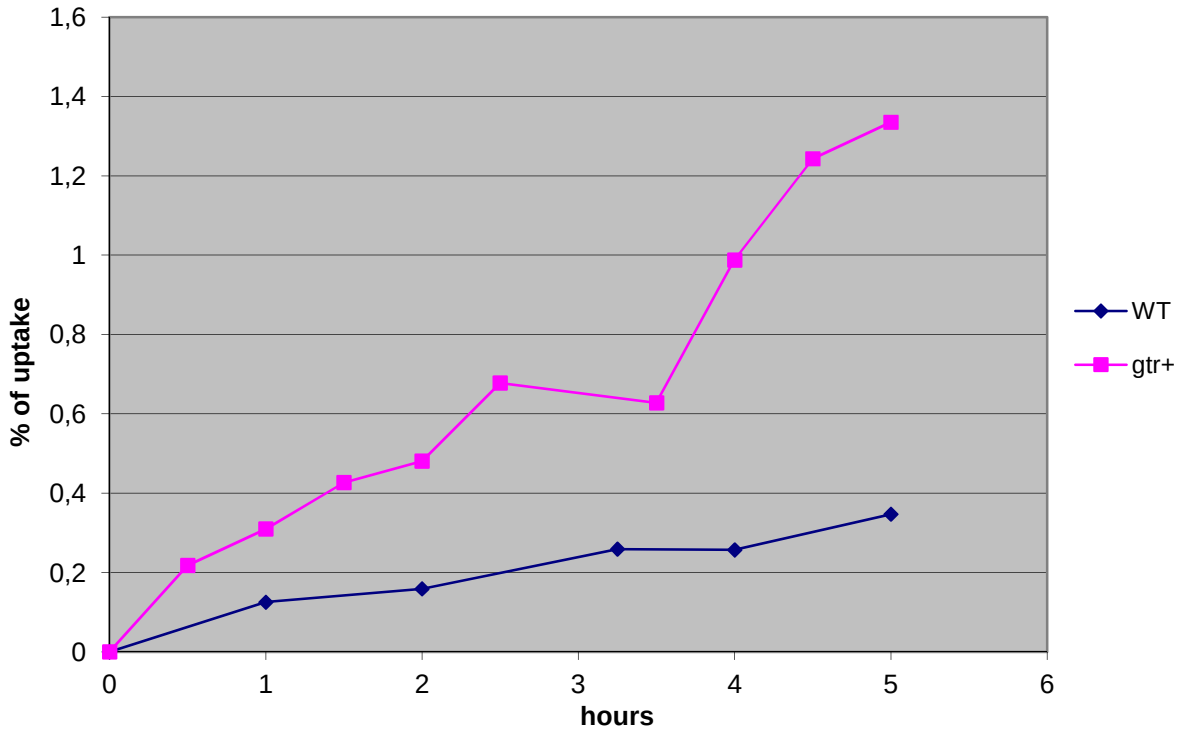


Fig. 46
Fructose uptake by PCC7120WT and *gtr*$^{+}$: The external concentration was 50mM in both cases.

4.2. Creation of a *ptox* knock out mutant

The ORF of the *ptox* gene (*all2096* according to Cyanobase, 2011) was disrupted by a gentamicin cassette. Two flanking sequences from the chromosome are adjacent to the gentamicin resistance cassette with plasmid pRL278 as the vector.

4.2.1. Construction of pBWUV4 (constructed by Wurzinger, diploma thesis)

First the downstream flanking sequence was amplified by PCR with DNA isolated from the WT of PCC7120 as template and the primers Ptox downstream 5' and Ptox downstream 3'(see section 3.4.4.).

pRL278, which had been isolated from *E. coli* Top10(pRL278) and the *ptox* downstream fragment were both cut with *Xho*I and *Eco*O109I. Both primers include restriction sites, which are not located at this locus on the chromosome. Primer Ptox downstream 5' contains a *Xho*I site, Ptox downstream 3' primer contains an *Eco*O109I site (underlined in section 3.4.4.). The digested DNA passed an agarose gel, DNA was extracted and the downstream flanking sequence was inserted into pRL278 vector (see fig. 47). Competent cells were electroporated with the ligation product and selected on an LB agar plate containing 50µg/ml kanamycin. Many colonies had grown over night. 24 minipreps were performed followed by restriction analysis. All 24 minipreps were digested with *Eco*O109I and analysed by agarose gel. Minipreparation No. 18 showed the expected pattern of 5649/1327bp. As a further control minipreparation No. 18 was digested with:

1) *Xho*I: 5649/1327
2) *Pvu*I: 6976
3) *Xho*I/*Eco*RI: 4813/2163
4) *Bsp*EI: 4987/1989
5) *Bsp*HI: 5189/1242/371/174

The numbers in the list indicate the calculated lengths of the fragments in bp. All digestions gave the expected pattern (data not shown). The new plasmid was named pBWUV4 (see fig. 47).

4.2.2. Construction of pPSUV1 (constructed by Wurzinger, diploma thesis)

The *ptox* upstream flanking sequence was amplified by PCR using PCC7120 WT as template and the primers Ptox upstream 5' and Ptox upstream 3' (see section 3.4.5.). Primer Ptox upstream 5' contains a *Bam*HI site, while primer Ptox upstream 3' contains a *Xho*I site (underlined in section 3.4.5.), which are both not present at these loci in the natural genome but will be introduced after PCR. pRL278 and the PCR fragment were digested with *Xho*I and *Bam*HI and the fragments were purified by an agarose gel. The pRL278 vector and the PCR product were cut out from the gel and extracted from the agarose. They were ligated (see fig. 47) and electroporation was performed with *E. coli* Top10 cells. Electroporated cells were selected on LB agar supplemented with 50µg/ml kanamycin. 55 colonies had grown over night at 37°C.

From one colony an over night culture was inoculated and an alkaline lysis minipreparation was made. Several restriction enzyme digestions were performed. The desired plasmid was predicted to show the following fragment lengths in bp.

1) *Bam*HI: 7070
2) *Xho*I: 7070
3) *Acc*I: 2202/1882/1639/650/353/335/9
4) *Pvu*I: 6009/682
5) *Bsp*I: 3137/2759/1174

The predicted patterns were confirmed (data not shown). The new plasmid was named pPSUV1 (see fig. 47).

4.2.3. Construction of pBWUV5 (constructed by Wurzinger, diploma thesis)

pPSUV1 and pBWUV4 were restricted with *Eco*RI and *Xho*I and purified by an agarose gel. The 4813bp fragment from pBWUV4 and the 3291bp fragment from pPSUV1 were cut out from the gel, extracted from the agarose and ligated (see fig. 47). After electroporation into *E. coli* Top10 the cells were selected on LB agar with 50µg/ml kanamycin. 3 colonies had grown over night at 37°C. From all 3 colonies minicultures were inoculated and minipreps were performed. The desired plasmid has three *Pvu*I fragments of 6281/1061/762bp. Only one minipreparation gave the expected result. Other restriction assays were carried out as additional controls.

1) *Xho*I/*Eco*RI: 4815/3291bp
2) *Bam*HI: 8104bp
3) *Pvu*II: 5298/1349/1313/144bp
4) *Hin*dIII: 5992/2112bp

On the control gel all expected patterns were detected (data not shown). The new plasmid was named pBWUV5 (see fig 47).

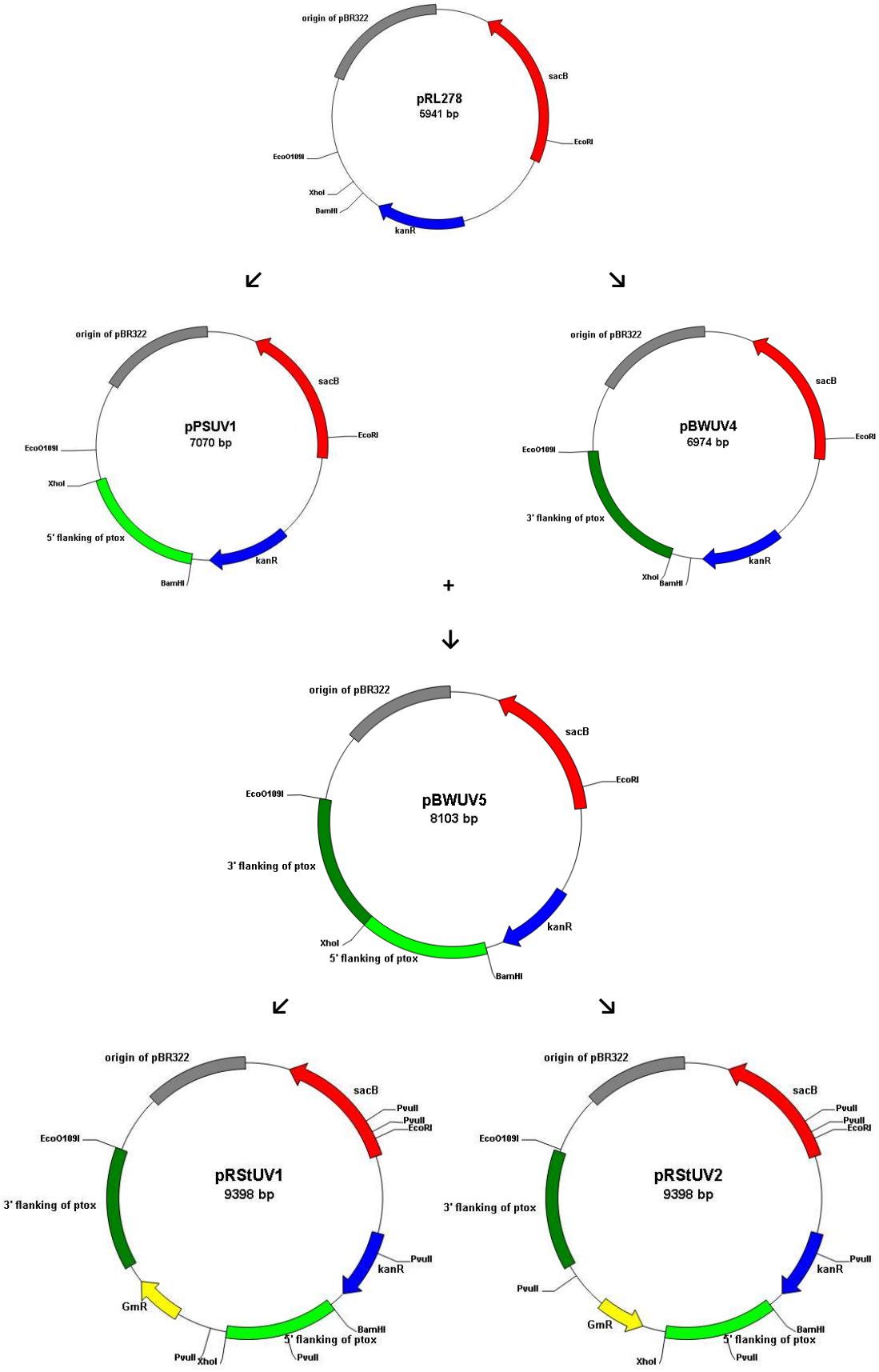


Fig. 47
Scheme of the construction of pRStUV1 and pRStUV2

4.2.4. Construction of pRStUV1 and pRStUV2

In pBWUV5 the upstream and downstream flanking sequences of *ptox* are directly linked at the *Xho*I site. A gentamicin resistance cassette was obtained from plasmid pBBR1MCS-5 (Kovach et al., 1995). JM109(pBBR1MCS-5) was streaked from a frozen stock culture to an LB plate containing 5µg/ml gentamicin. One colony was used for inoculation of an over night miniculture. An alkaline lysis minipreparation was performed and the gentamicin resistance cassette was amplified by PCR using pBBR1MCS-5 as template and the primers Pbbr 1-5' and Pbbr 1-3' (see section 3.4.6).

The 5' primer contains a recognition site for *Xho*I, the 3' primer for *Sal*I. The total amount of the PCR product was estimated to be about 500ng by comparison with a DNA standard marker. The cohesive ends after *Xho*I and *Sal*I digestion are complementary to each other. The PCR product was digested by both *Xho*I and *Sal*I, purified by gel electrophoresis and extracted from the agarose. A plasmid midipreparation of pBWUV5 was performed. Both the eluated PCR product and the midiprep were quantified by a 1% agarose gel (see fig 48).

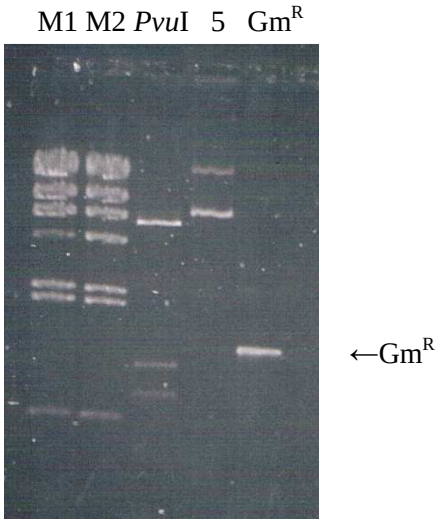


Fig. 48
M1 … λ*Hin*dIII, directly used, M2… λ*Hin*dIII, heated at 70°C before use, *Pvu*I…pBWUV5 cut with *Pvu*I, 5…pBWUV5 uncut, Gm^R…extracted PCR product of Gm^R cut with *Xho*I/*Sal*I

The concentration of the midipreparation was estimated at 2.6 ng/µl, the concentration of the PCR product was 18 ng/µl (see fig. 48). As the concentration of the midiprep was very low the complete yield of about 150ng was used for ligation. An equimolar amount of the PCR product (1308bp) was used. 1.4µl (at a concentration of 18 ng/µl) of the PCR product were cut with *Xba*I and *Sal*I and ligated to pBWUV5 restricted with *Xho*I. The ligation product (see fig. 47) was electroporated into competent *E. coli* JM109 with selection on LB agar with 5µg/ml gentamicin. Of 24

colonies inoculated in liquid cultures, only 3 cultures had grown over night (No. 9, 10, 13). Plasmids were isolated by plasmid minipreparation. The preparations were digested with the following enzymes:

a) *Pvu*I 6280/2057/1061bp
b) *Xho*I/*Eco*O109I/*Bam*HI 5511/2622/1265bp

All three samples gave the expected patterns (see fig. 49). The direction of the insertion cannot be revealed because none of these enzymes cuts within the cassette.

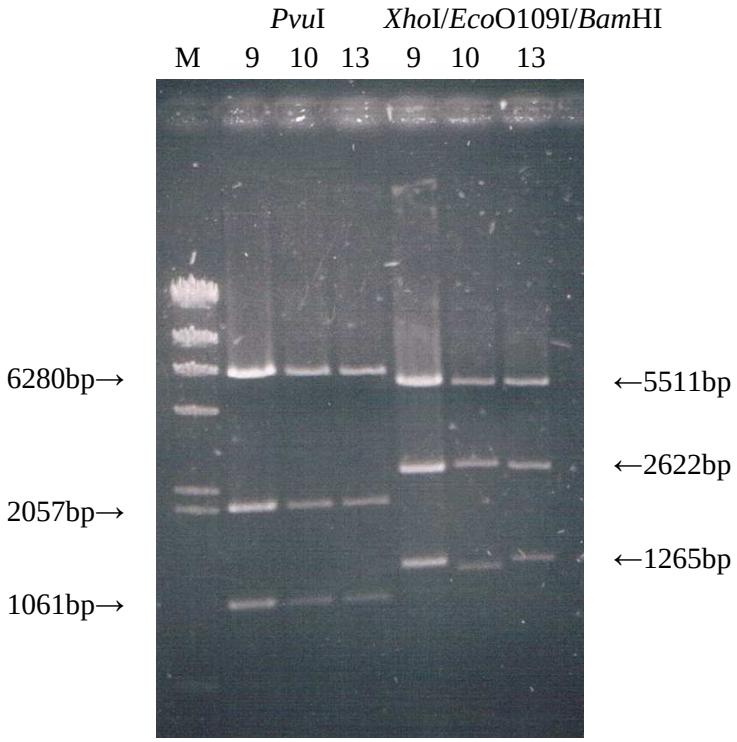


Fig. 49
All 3 minipreparations were digested with a) *Pvu*I and b) *Xho*I/*Eco*O109I/BamHI. Each of them was digested to the expected pattern. M…λ digested with *Hin*dIII.

*Pvu*II cuts within the 1308bp gentamicin resistance cassette dividing the cassette into fragments of 1185/110bp and 3 times within the vector. The very unsymmetrical division of the cassette allows the detection of its orientation within the new plasmid. Plasmid preparations No 9 and 13 led to the pattern 4719/1873/1349/1313/144bp, No 10 to 5794/1349/1313/798/144bp. The two fragments of 1349 and 1313bp appear as one fragment on the gel because of their similar length and the 144bp fragment is too small to be detected (see fig. 50). According to the restriction pattern it was concluded that both variants had been

successfully constructed. The plasmid isolated from colony No 9 (gentamicin cassette antiparallel to *ptox* ORF, see fig. 47) was named pRStUV2, whereas the plasmid from No. 10 (gentamicin cassette parallel to *ptox* ORF, see fig. 47) was called pRStUV1. Both plasmids were electroporated separately into *E. coli* strain HB101 (pRL528). The strains containing the new plasmids were shock frozen in liquid nitrogen and stored at -80°C.

JM109 (pRStUV1) = culture No. 10	→	No. 1014
JM109 (pRStUV2) = culture No. 9	→	No. 1015
HB101 (pRL528 + pRStUV1)	→	No. 1016
HB101 (pRL528 + pRStUV2)	→	No. 1017

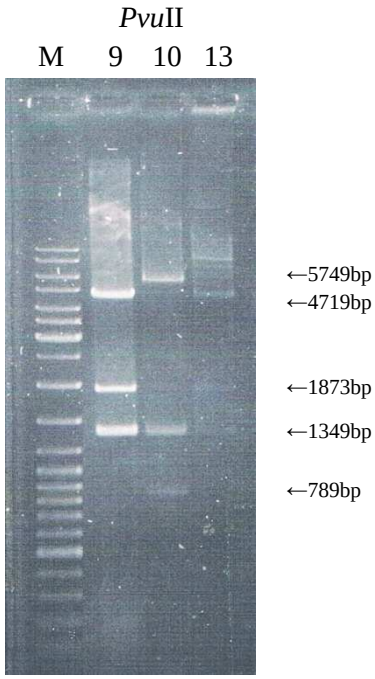


Fig. 50
The minipreparations were digested with *Xho*I/*Nsi*I (left) and *Pvu*II (right). *Pvu*II digestion led to the expected patterns. M…SM0332 (from Fermentas).

4.2.5. Conjugation of pRStUV1 and pRStUV2 into PCC7120WT

pRStUV1 and pRStUV2 were both conjugated into the WT of PCC7120.

Conjugation assays:

HB101(RP4) + HB101(pRL528 + pRStUV1) + PCC7120WT

HB101(RP4) + HB101(pRL528 + pRStUV2) + PCC7120WT

Control assays:

2x HB101(pRL528 + pRStUV1)

2x HB101(pRL528 + pRStUV2)

Conjugation was performed on a nitrocellulose filter (see section 3.22.). At first the filters were incubated on pure BG11HPA for 3 days. After that they were transferred to BG11HPA containing 15µg/ml gentamicin. Single colonies grew within the 1:100 cyanobacterial dilution on both the positive and the negative side of the pRStUV1 conjugation and no difference could be observed between the conjugational and the control side. Colonies from the positive side were restreaked onto a new BG11HPA plate containing 15µg/ml gentamicin. Colonies of this new plate were used to inoculate a liquid culture. When it had grown for about a month, an aliquot was streaked on a BG11HPA plate containing both 15µg/ml gentamicin and 5%(w/v) sucrose, which selected for the second recombination. A colony from the sucrose containing plate was used to inoculate a new liquid culture in BG11 with gentamicin and sucrose. DNA was isolated from the sucrose + gentamicin liquid culture and a PCR was performed to analyse its genotype.

The primers Ptox upstream 5' and Ptox downstream 3' (see sections 3.4.5. and 3.4.4.) were used for amplifying the region between the two flanking sequences. The PCR was performed using the following conditions: 1µl isolated DNA (from PCC7120 WT, the *ptox*$^-$ culture, plasmid preparation of pRStUV1). The elongation phase was 15min, the annealing temperature was 49°C. Such a long elongation phase was used to ensure the completion of all started products. DNA from *ptox*$^-$ as template yielded the 3896bp fragment, which is characteristic of the mutated allele and was also visible with knock out plasmid pRStUV1 as template DNA. No WT allele (3008bp) could be detected with the DNA of strain *ptox*$^-$. Another fragment appeared in all samples. As PCC7120WT and knock out plasmid pRStUV1 do not share any homologous sequences except the two flanking sequences it can be concluded that these PCR products derive from one of the flanking sequences. The length of the fragment was estimated to be about 1300bp. Since the size of the flanking sequences is 1282bp for the upstream one and 1344bp for the downstream one an annealing of the primers is possible at least within the downstream flanking sequence (see fig. 51A). For a final proof the culture was grown without antibiotics and the DNA was isolated and PCR

was performed with the new DNA. Strain PCC7120*ptox*$^-$ can be concluded to be homozygously mutated because the WT allele did not reappear (see fig. 51B).

The conjugation with plasmid pRStUV2 (see above) into PCC7120 was also attempted. Colonies from the positive side of the conjugational plate were restreaked, but inoculation of liquid cultures failed even before the selection on sucrose. Single colonies had grown on the negative side of both conjugational experiments. They were treated as described in section 3.22. and finally liquid cultures in gentamicin + sucrose were established. DNA isolation followed by PCR revealed their WT genotype on the corresponding locus (data not shown).

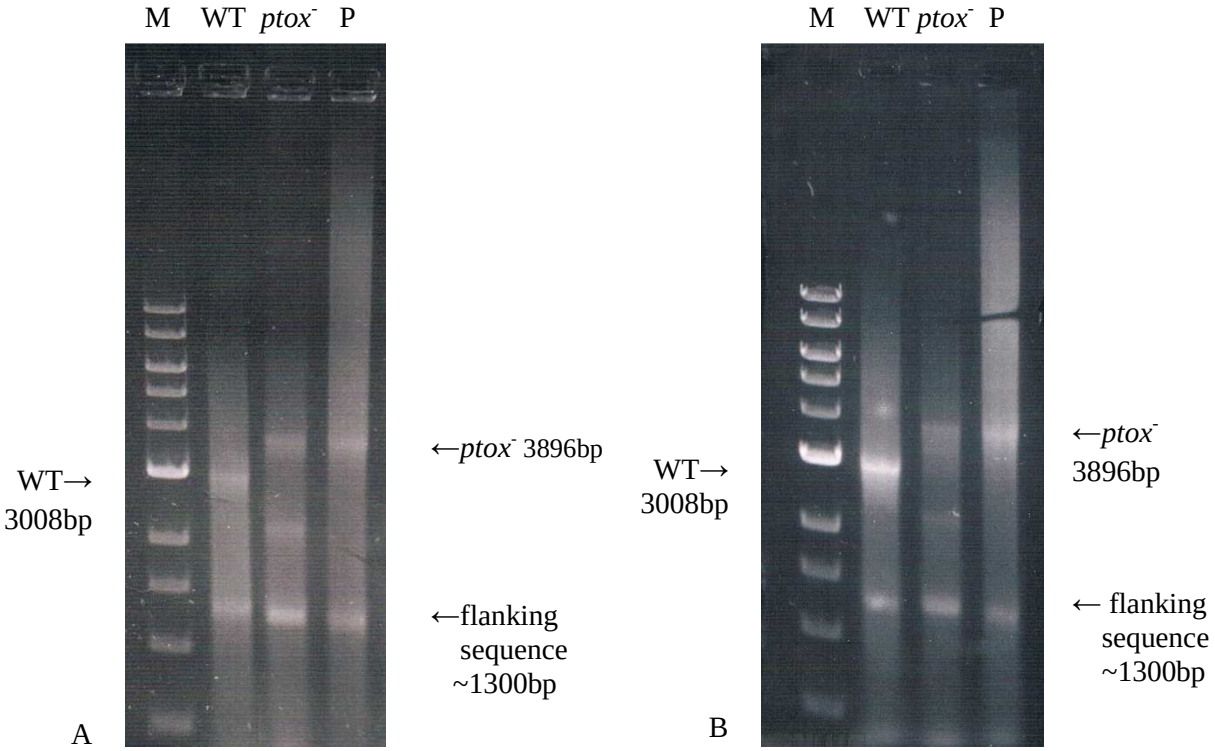


Fig. 51
PCR with PCC7120*ptox*$^-$ to prove homozygosity
A PCR after growth in medium with gentamicin and sucrose
B PCR after growth in medium without any antibiotics
M….1kb marker (NEB), *ptox*$^-$… PCC7120*ptox*$^-$, P….pRStUV1

4.3. Creation of a knock out plasmid for *Synechocystis* sp. PCC6803 gene *sll0813*

4.3.1. Construction of pGGUV1 (G. Graser, unpublished)

From strain DH5α(pUC19) a 1l LB over night culture was inoculated and a caesium chloride plasmid preparation (method not shown) was carried out. Genomic DNA from PCC6803 was isolated according to section 3.11. PCR was performed with PCC6803 DNA as template and primers Graser 1 and 2 (see section 3.4.7), which amplify the 5' flanking sequence region of *sll0813*, the gene which encodes the putative subunit B of the ARTO. It is located far away from the subunits A and C, which are adjacent on the chromosome. The importance of subunit B for the entire enzyme and the possibility of its replacement by subunit B of Cox (see aim of the thesis) were to be analysed.

The PCR product (1084bp) was purified by agarose gel electrophoresis and extracted from the gel. Both the PCR product and pUC19 (see fig. 52) were cut with *Hin*dIII and *Pst*I (both from New England Biolabs). *Hin*dIII requires NEB2, whereas for *Pst*I NEB3 is the appropriate buffer. The main difference is the double amount of NaCl in NEB3 compared to NEB2. Therefore the DNAs were digested with *Hin*dIII and NEB2 first, then the amount of NaCl was doubled (by supplementing 0.5µl of a 1M NaCl stock solution) and *Pst*I was added. Both restrictions were performed for 2h at 37°C. After this step the restricted DNAs were purified by gel electrophoresis with 0.7% agarose.

The desired fragments were cut out (919bp for the restricted PCR product and 2647bp for the vector portion of pUC19) and the DNA was extracted from the gel. 125ng of the linearized pUC19 were ligated to 200ng of the restricted PCR product, which means a 4.6 fold molarity for the PCR product. Electroporation was performed into *E.coli* JM109 and the cells were streaked on an LB agar plate supplemented with 50µg/ml ampicillin.

Many colonies grew on the plate. From 24 colonies a Holmes and Quigley plasmid minipreparation (see section 3.8.) was performed and the concentration was determined by gel electrophoresis of an aliquot.

The following restrictions were carried out:

1) *Nco*I
2) *Nco*I/*Eco*O109I
3) *Bgl*I
4) *Bse*YI

Analysis was performed by gel electrophoresis

Ad 1) *Nco*I only cuts once in the flanking sequences but not in the pUC19 vector portion. The restriction pattern was one fragment of 3593bp.

Ad 2) *Nco*I cuts once in the insert, *Eco*O109I once in the vector and once in the insert, however the site within the insert was blocked by overlapping methylation. As expected two fragments of 3111bp and 481bp were visible.

Ad 3) 2171/1118/304

Ad 4) 2608/733/252

The results (data not shown) demonstrated that the desired plasmid had been constructed. It was named pGGUV1 (see fig. 52). 1ml of a JM109 (pGGUV1) liquid over night culture was mixed with glycerol and stored at -80°C after shock freezing under the number 1018 in the list.

4.3.2. Construction of pGGUV2

JM109(pGGUV1) was streaked on an LB agar plate, supplemented with 50µg/ml ampicillin. From an over night grown colony a liquid miniculture was inoculated and a plasmid minipreparation according to Ish-Horowicz (see section 3.9.) was performed. The 3' flanking sequence of the *sll0813* gene was amplified by PCR with total DNA from PCC6803 as template and the primers Graser 3 and Graser 4 (see section 3.4.8). The annealing temperature was 47°C and the elongation time was 36s.

The 5' end of primer Graser 3 contains a *Pst*I site (underlined in section 3.4.8). Inside the amplified sequence there is a natural *Eco*RI site. pGGUV1 and the PCR product were digested with *Eco*RI and *Pst*I and the restricted DNAs were purified by two different agarose gels according to the size of the DNA (2% for the PCR product, 0.8% for pGGUV1). From pGGUV1 a fragment of 3554bp (see fig. 53B) and the 416bp PCR product were cut out (see fig. 53A), extracted from the gel and quantified by an agarose gel. 66ng of pGGUV1 vector and 96ng of the PCR product were ligated to each other, which means a 12 fold molarity of the insert.

A control assay was also performed, where the insert had been omitted to observe the frequency of self recirculation. Ligation and control mix were incubated over night at 16°C. Afterwards the ligase was inactivated by heating at 65°C for 10min. Both mixtures were electroporated into competent Top10 cells and streaked on a plate containing LB agar with 50µg/ml ampicillin. 7 colonies grew on the plate with insert, whereas on the control plate not a single colony appeared. From all colonies mini liquid cultures were inoculated. One culture did not grow over night. From the other six cultures plasmid preparations were performed, followed by restriction analysis. Enzymes *Nco*I and *Eco*O109I were used for single restrictions.

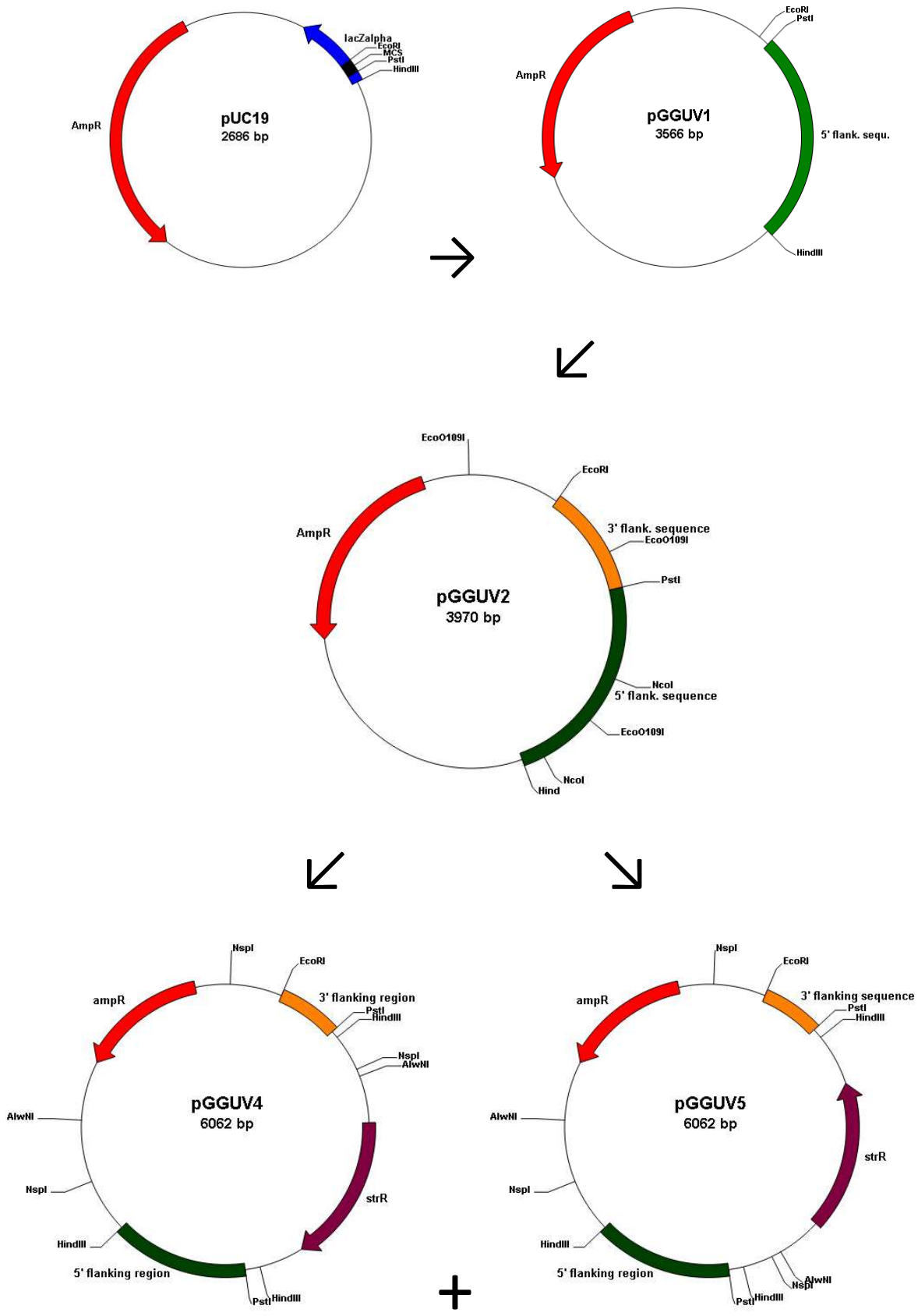


Fig. 52 Construction of pGGUV4 and pGGUV5

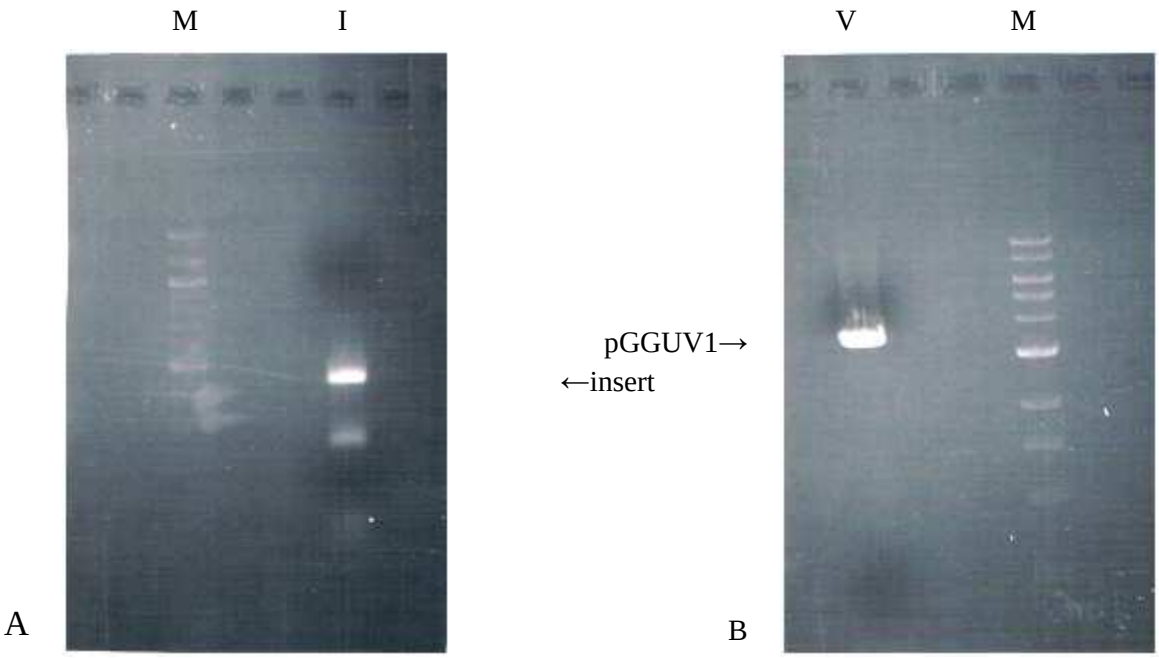


Fig. 53
A: restricted PCR product
B: pGGUV1 cut with *Eco*RI and *Pst*I.
M….1kb ladder (NEB), V…pGGUV1 (3554bp), I….PCR product (416bp)

*Nco*I cuts once in the 5' flanking insert and therefore in pGGUV1 and once in the 3' flanking insert yielding fragments of 3566/404bp. *Eco*O109I cuts three times, once in the pRL278 vector, once in the 5' flanking insert and once in the 3' flanking insert. Therefore three fragments were expected: 2532/738/700bp.

The digestions were performed with preparations 4 and 5, which had the highest DNA concentrations. Afterwards RNAse was added. *Nco*I restriction yielded the expected pattern of 3566/404bp (see fig. 54A on the right). However, only two fragments 3270/700bp were visible with the *Eco*O109I digestions (see fig. 54A on the left). Analysis by the NEB cutter program (http://tools.neb.com/NEBcutter2/) revealed that one site is overlapped by a *dcm* methylation site and thereby blocked. The 2532bp and 738bp fragments stayed connected as a 3270bp fragment. A double digestion of *Eco*O109I and *Eco*NI led to three fragments: 2783/700/487bp (see fig. 54A in the middle). *Eco*NI cuts once within the 738bp fragment dividing it into 487bp and 251bp but nowhere else in the desired plasmid. This restriction was performed to prove whether the small *Eco*O109I fragment in fig. 54A on the left corresponds to 700bp or to 738bp, as the two fragments could not be distinguished on the gel. The other part of the DNA was assumed to be still connected to the large fragment. The photo of the gel clearly demonstrates that the 700bp fragment was separated, while the 738bp fragment remained attached to the large one. The 487bp portion was cut off by *Eco*NI, but the remaining 251bp size DNA was still connected to the 2532bp DNA forming a 2783bp fragment, because the *Eco*O109I site is blocked by overlapping *dcm* methylation. As a further control, minipreps from pGGUV1 were digested with *Eco*O109I as well as

with *Nco*I. Both (single) digestions led to a linearization of the 3593bp fragment (see fig. 54B). pGGUV1 contains one *Nco*I site and two *Eco*O109I sites, however, one is blocked by *dcm* methylation.

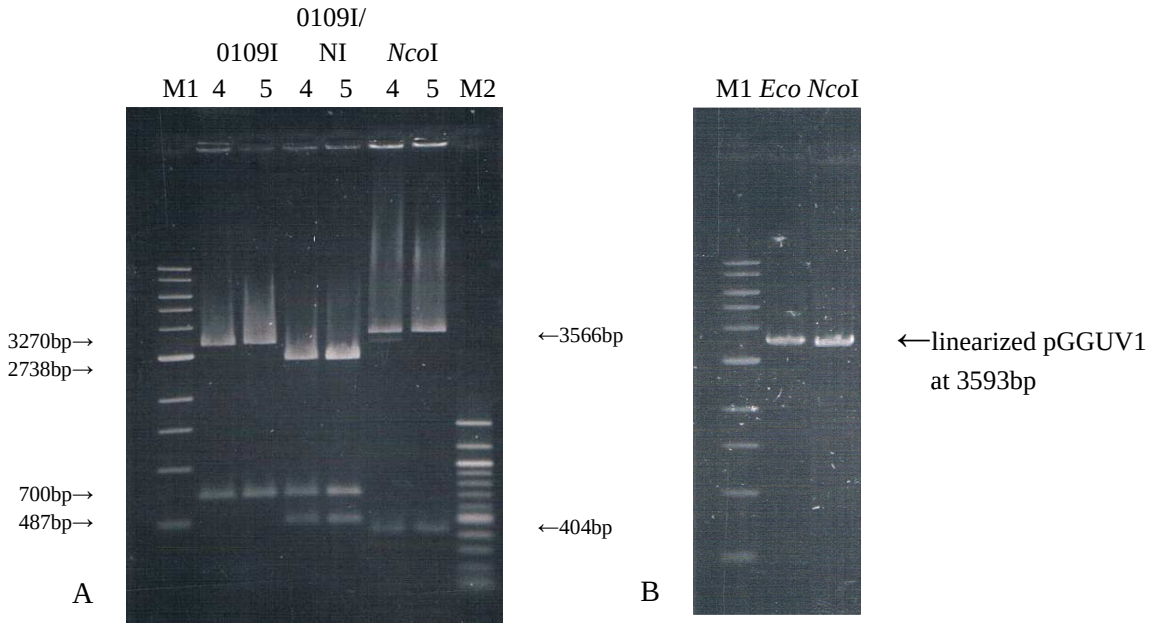


Fig. 54
A shows restrictions of Holmes Quigley preparations 4 and 5
B pGGUV1 was restricted with *Eco*O109I and *Nco*I.
M1…1kb ladder, M2...100bp ladder (both from NEB)

Since the construction of the desired plasmid had been proven, 1ml of an over night culture containing the plasmid was shock frozen in liquid nitrogen and stored at -80°C. It was named pGGUV2 (see fig. 52) and numbered 1020 within the list.

4.3.3. Construction of pGGUV4 and pGGUV5

The two flanking sequences are directly connected by a *Pst*I site on pGGUV2. For a complete knock out plasmid a resistance cassette had to be introduced into the *Pst*I site between the two flanking sequences.

pRL463 contains the ω cassette encoding a resistance against streptomycin. This cassette, derived from pHP 45Ω (Prentki and Krisch, 1984), has been inserted into the L.HEH inverted polylinker region of pRL138 (Elhai and Wolk, 1988) resulting in pRL463. A plasmid minipreparation of pRL463 was performed according to Ish-Horowicz (see section 3.9.). Three single digestions were carried out with the plasmid: *Pst*I, *Bam*HI and *Dra*I. The fragments were separated by an agarose gel (see fig. 53).

*Pst*I: 2662/2092

*Bam*HI: 2698/2056

*Dra*I: 1942/1203/898/692/19

The smallest fragment of 19bp could not be detected. This pattern clearly demonstrates the presence of a fragment of about 2000bp corresponding to the streptomycin resistance cassette, which is present in all digestions. As the cassette has been introduced into a reverse polylinker, it can be excised with all remaining enzymes of this linker. Luckily none of the three enzymes cuts within the cassette but all left an intact ORF (see fig. 55).

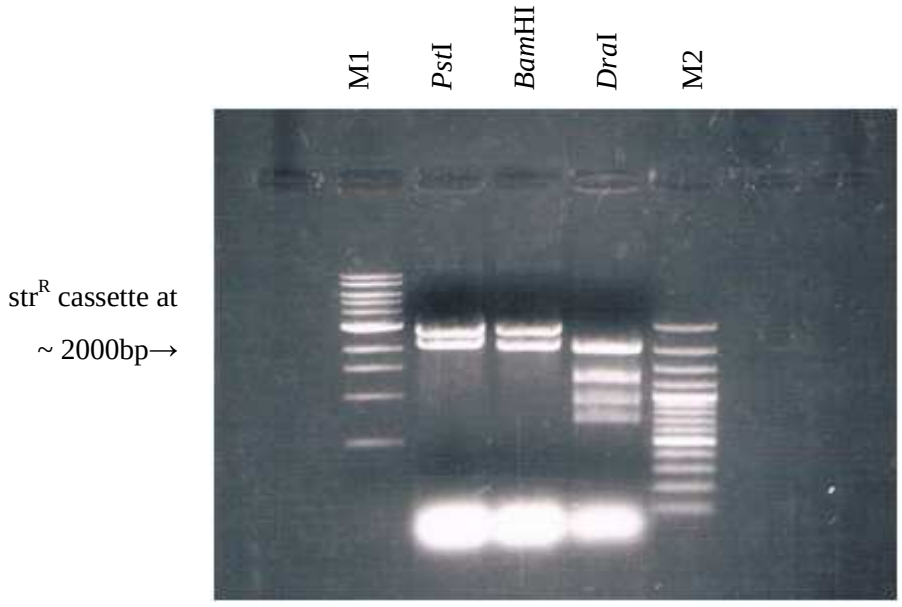


Fig. 55
pRL463 was restricted with a) *Pst*I, b) *Bam*HI, c) *Dra*I; digestions were analysed by an agarose gel M1…1kb DNA ladder, M2….100bp ladder (NEB). A fragment of about 2000bp is present in all 3 digestions (the ω strR resistance cassette)

pRL463 and pGGUV2 were both digested with *Pst*I. pGGUV2 was linearized at the site between the two flanking sequences, while the streptomycin resistance cassette was separated from the pRL463 vector portion. The digested plasmids were purified by electrophoresis (see fig. 56), the linearized pGGUV2 and the streptomycin resistance cassette were cut out from the gel, extracted and quantified by an agarose gel (data no shown). About 200ng from the pGGUV2 vector and 100ng from the streptomycin resistance cassette were ligated. This time equimolar amounts were mixed (3970bp pGGUV2: 2092bp ω cassette = 1.9:1) because it could be selected directly for the insertion of the resistance cassette. The ligation mix was incubated for 4h at room temperature. After that half of the ligation mix was inactivated by heating for 10min at 70°C, while the other part continued to ligate over night at 4°C.

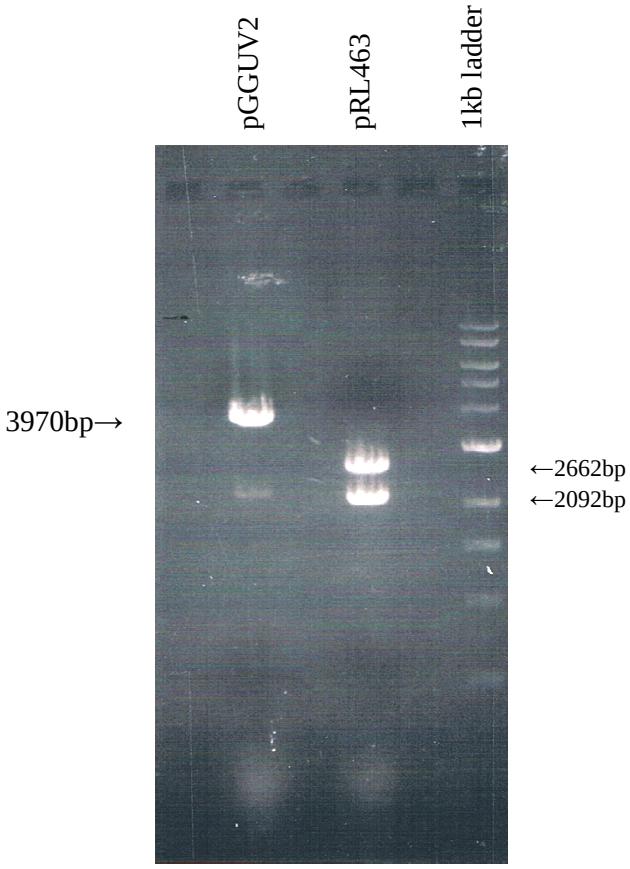


Fig. 56
pGGUV2 and pRL463 were cut with *Pst*I. Linearized pGGUV2 (3970bp) and ω cassette of pRL463 (2092bp) were cut out and ligated.

Electroporation of 2µl of the inactivated ligation mix into competent DH5α was carried out and cells were selected on an LB agar 20µg/ml streptomycin plate. Many colonies had grown over night. 24 small liquid cultures were inoculated, however, only two of them grew over night. The DNA was isolated by minipreparation after Holmes and Quigley (see section 3.8.) and a restriction analysis was performed with *Alw*NI, *Bsa*I and *Nsp*I (three single digestions). These digestions do not only prove the successful insertion, but also indicate the orientation of the cassette within the vector. As the insert has the same ends on both sides an insertion is possible in both directions. One preparation contained a clone, whose digestion pattern (see fig. 57A) clearly corresponded to the desired plasmid, where the streptomycin resistance cassette had been inserted in the same orientation as *sll0813*. This plasmid was named pGGUV5 (see fig. 52).

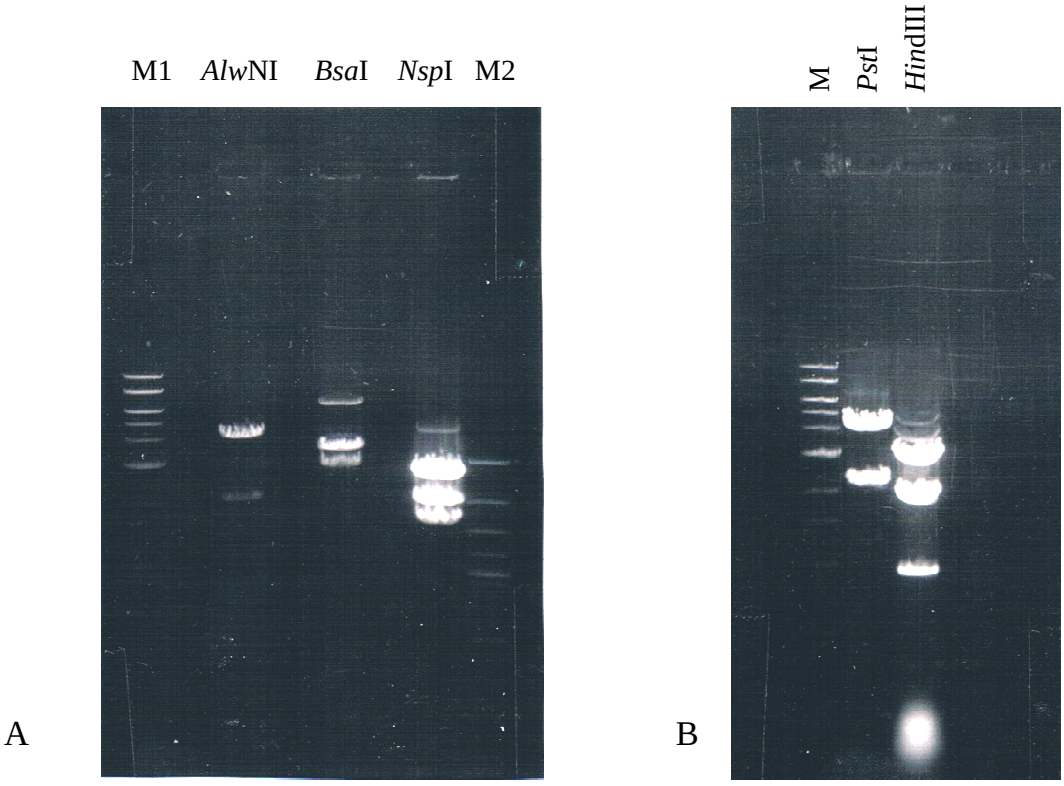


Fig. 57
Control digestions of presumptive pGGUV5
A Control digestions with a) *Alw*NI, b) *Bsa*I, c) *Nsp*I.
M1…1kb ladder, M2…100bp ladder (both from NEB).
B Control digestions with a) *Pst*I, b) *Hin*dIII.
M…1kb ladder (from NEB)

*Alw*NI: 4047/2015
*Bsa*I: 3323/2739
*Nsp*I: 2560/1917/1585

For further proof this minipreparation was digested with *Pst*I as well as with *Hin*dIII: Digestion to the predicted fragments occurred (see fig. 57B).

*Pst*I: 3970/2092
*Hin*dIII: 3090/2014/958

When digested with *Hin*dIII a large amount of RNA was visible because no RNAse had been added to this sample (see fig. 57B).

Electroporation was repeated to obtain a plasmid, where the ω cassette is inserted in the other orientation than *sll0813*. 2µl of the over night ligase reaction were electroporated into DH5α cells followed by selection on LB agar supplemented with 20µg/ml streptomycin. After growing liquid cultures and plasmid preparation, restriction digestions were performed with *Alw*NI, *Nsp*I (see fig. 58A) and *Hin*dIII (see fig. 58B). At least one preparation contained the right clone, which was named pGGUV4 (see fig. 52).

*Alw*NI: 3458/2604
*Nsp*I: 3063/1917/1082
*Hin*dIII: 3090/2014/958

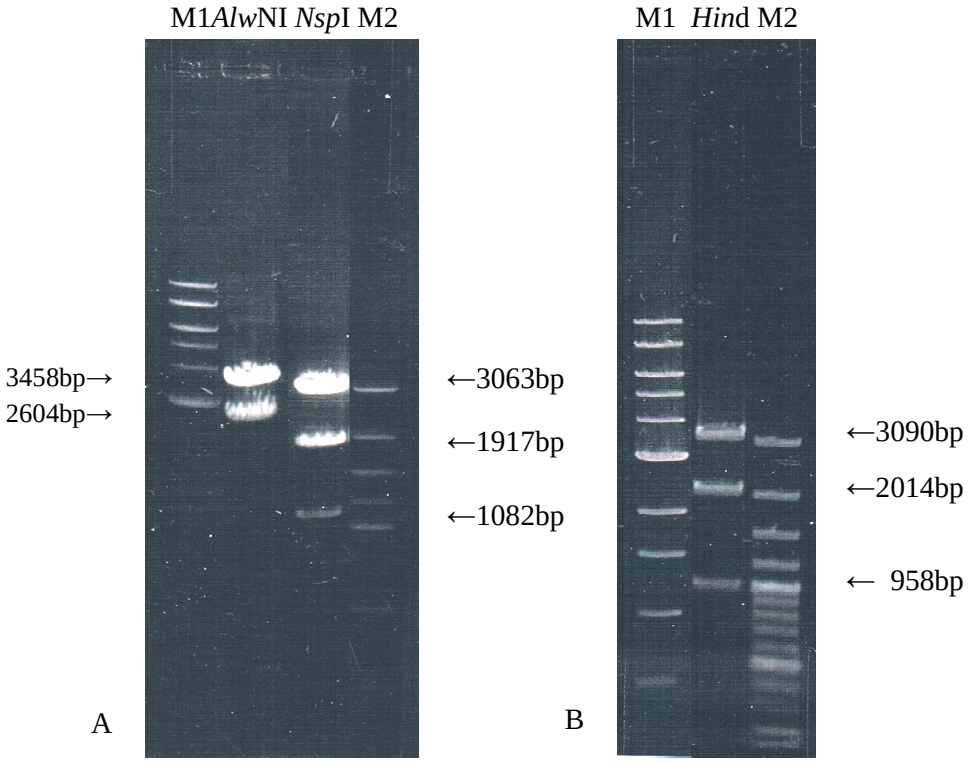


Fig. 58
Control digestions of the presumptive pGGUV4
A: with a) *Alw*NI and b) *Nsp*I
B: with *Hin*dIII
M1…1kb ladder, M2…100bp ladder (both from NEB)

Since both desired *sll0813* knock out plasmids had been constructed, parallel control digestions were carried out. pGGUV4 and pGGUV5 were restricted with *Alw*NI, *Nsp*I, *Pst*I and *Hin*dIII and their digestions were compared on the same agarose gel. The treatment with *Alw*NI and *Nsp*I revealed the different orientation of the ω cassette within the two plasmids (see fig. 59 on the left side and in the middle), whereas a *Pst*I digestion yielded the same pattern for both plasmids (see fig. 59 on the right side) because it just reverted the last cloning step and caused separation into the initial compounds (vector and insert).

*Alw*NI: 3458/2604 (pGGUV4) / 4047/2015 (pGGUV5)
*Nsp*I: 3063/1917/1082 (pGGUV4) / 2560/1917/1585 (pGGUV5)
*Pst*I: 3970/2092 (pGGUV4 and pGGUV5)

After this final confirmation the *E. coli* strains DH5α(pGGUV4) and DH5α(pGGUV5) were shock frozen and stored at -80°C, where they got the following numbers: DH5α(pGGUV5) → 1021 and DH5α(pGGUV4) → 1022.

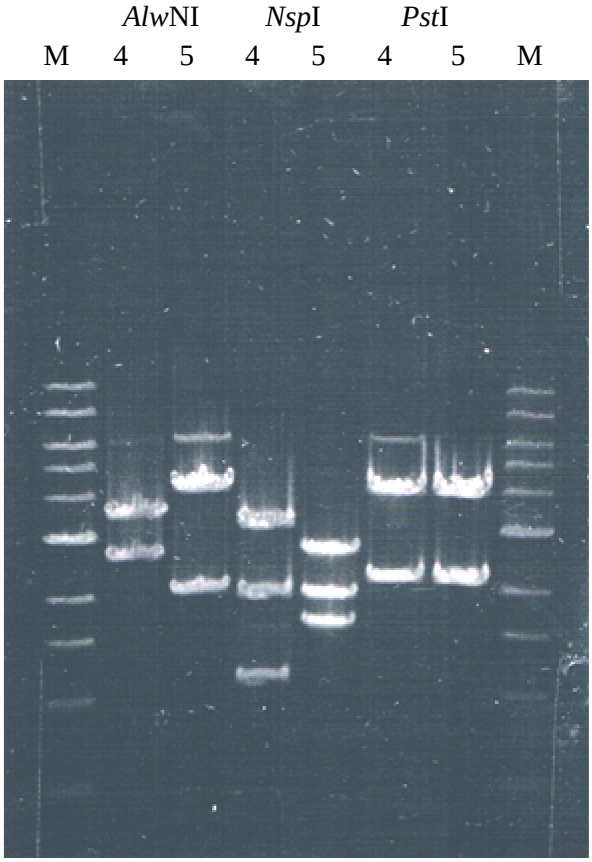


Fig. 59
pGGUV4 and pGGUV5 were both digested with *Alw*NI, *Nsp*I and *Pst*I.
*Alw*NI and *Nsp*I digestions resulted in different pattern, whereas *Pst*I digestions led to the same pattern (just as expected). 4 and 5 are abbreviations for pGGUV4 and pGGUV5. M…1kb ladder (from NEB)

4.3.4. Transformation of pGGUV4 and pGGUV5 into PCC6803BC2 and PCC6803BC3

In both strains BC2 and BC3 the Qox and the Cox had been inactivated leaving ARTO as the only remaining RTO. *cydA* and *cydB* (encoding Qox) were inactivated by an erythromycin resistance cassette. In BC3 the whole *cox1* locus (*coxB1*, *coxA1* and *coxC1*) was inactivated by a neomycin phosphate transferase (*npt*). In BC2 only *coxA1* and *coxC1* were inactivated by an *npt* cassette, but *coxB1* remained intact. Transformation of plasmids pGGUV4 and pGGUV5 into strains of PCC6803 leads to a knock-out of the *coxB2* gene (*sll0813*) that is located far away from the *coxA2* and *coxC2* genes.

Strains BC2 and BC3 were streaked on BG11TS agar plates supplemented with 20µg/ml kanamycin and 5µg/ml erythromycin. The cell mass that had grown on the plates was used for inoculation of a 50ml liquid culture supplemented with the same amounts of antibiotics.

The following transformations were performed:

PCC6803BC2 + pGGUV4 → PCC6803BC2*coxB2*$^{-}$::Sm^{R}direct

PCC6803BC2 + pGGUV5 → PCC6803BC2*coxB2*$^{-}$::Sm^{R}inverse

PCC6803BC2 no plasmid added

PCC6803BC3 + pGGUV4 → PCC6803BC3*coxB2*$^{-}$::Sm^{R}direct

PCC6803BC3 + pGGUV5 → PCC6803BC3*coxB2*$^{-}$::Sm^{R}inverse

PCC6803BC3 no plasmid added

After an over night incubation in the light at 32°C, the cells were plated onto filters placed on BG11TS agar with 20µg/ml kanamycin and 5µg/ml erythromycin and incubated for 48h in the light. Then the filter was transferred to a plate containing 20µg/ml kanamycin, 10µg/ml streptomycin and 5µg/ml erythromycin. After two weeks some colonies had grown on all transformation plates, whereas none were visible on the negative controls. From liquid cultures of transformants the DNA was isolated and the *coxB2* locus was analysed by PCR with primers ARTOb-10 and ARTOb-11 (see section 3.4.9) and compared to the DNA of the WT and the knock out plasmids.

The PCR yielded fragments of 761bp for the WT and of 2276bp for the mutated allele. The WT allele (761bp) was not visible in any of the mutant strains. As a control the PCR was also performed with plasmid pGGUV4 and pGGUV5 as the substrate (see fig. 60).

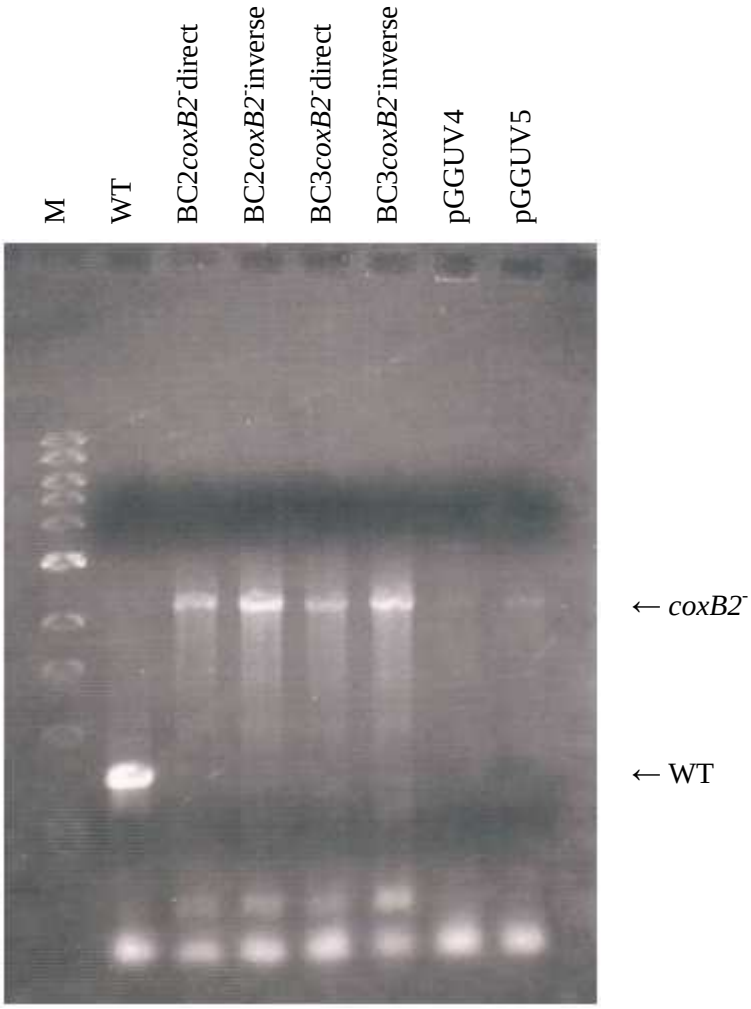


Fig. 60
PCR products with primers ARTOb-10 and ARTOb-11 with different DNAs: The WT allele was not detected in any of the mutant strains. M…1kb ladder (NEB).

4.3.5. No uptake of glucose or 3OMG by BC2 and BC3 was observed

Unfortunately the construction of strains PCC6803BC2*coxB2*⁻dir, PCC6803BC2*coxB2*⁻inv, PCC6803B3*coxB2*⁻dir and PCC6803BC3*coxB2*⁻inv did not lead to results that allowed an answer to a possible function of the CoxB1 subunit in the ARTO protein. Very small amounts of glucose or 3OMG were taken up by BC2 and BC3 cells in the dark. The results of Dietmar Pils could not be reproduced. Possibly my experiments concerning *coxB2* of PCC6803 failed, because complete exclusion of external light was not achieved. The uptake rates of glucose and 3OMG did not differ significantly between the BC2, BC3 and the ABC mutant. As a consequence the deletion of *coxB2* did not diminish the incorporations (data not shown).

5. Discussion

5.1. PCC7120WT is capable of heterotrophic growth (both chemo- and photoheterotrophic growth)

In the past PCC7120WT was considered to be strictly photolithoautotrophic (Rippka et al, 1979), however, the results described in this thesis, clearly reveal the strain's capacity for mixotrophy and photoheterotrophy as well as for chemoheterotrophy. Obviously the fructose metabolism is intact and the only barrier is the cytoplasmic membrane, which is passed at a very low rate by fructose molecules and extremely high concentrations are needed for a visible effect. It is very likely that fructose concentrations of 100mM and 200mM, which have been used in this thesis, have not been tested so far, because these conditions are very rare in nature.

The experiments indicate that PCC7120WT exhibits a significant heterotrophic growth only at concentrations of at least 50mM fructose (see section 4.1.2.) and the rate is increased with rising fructose concentrations of the medium (see figs. 4-6). This observation clearly corresponds to the uptake results, where the absolute amount of incorporated molecules per cell increases with the external concentration (see table 5), although the percentage of uptake (based on the total number of surrounding fructose molecules in the medium) diminishes with rising concentrations with the exception of 0.2mM fructose (see fig. 37). It can be calculated that 1.77µmol fructose per day and ml suspension of OD_{730} = 2.0 (incorporated amount at 100mM) have to enter the cells to allow a mixotrophic growth. The absolute uptake of fructose does not differ very much between 100mM and 200mM (see table 5). Nevertheless 200mM is the best of all tested concentrations for the wildtype under mixotrophic (at least within the first two weeks, see fig. 4) as well as under photoheterotrophic (see fig. 11B and 12) and chemoheterotrophic conditions (see fig. 15A and B). In contrast to the experiments showing mixotrophy (see fig. 4) the cells did not lyse at 200mM fructose when cultivated under chemoheterotrophic conditions (see figs. 15A and B). This can be explained by the fact that chemoheterotrophic growth was so slow that an OD_{730} of 4.0 was not surpassed within a month (duration of this experiment).

Based on the proved heterotrophy dependent on fructose two facts about the substrate molecule can be concluded. There has to be a fructose uptake mechanism into the cell as well as a corresponding metabolism. In the total genomic sequence (Cyanobase, 2011) several ORFs have been annotated as proteins involved in either sugar metabolism or uptake. *alr0517* codes for a putative fructokinase, however, its most closely related gene (98.5%) in ATCC29413 codes for a phosphofructokinase B (PfkB). *all5002* encodes a sugar kinase, which has many homologues in other organisms (Cyanobase, 2011 and NCBI Blast, 2011), predominantly ROK (repressor, open reading frames, kinase) family proteins in cyanobacteria. Taken into

consideration that glucose cannot be used for heterotrophic growth the sugar kinase might be the first enzyme in the unknown fructose metabolism. The experiments of this thesis indicating that simultaneously added glucose dramatically increases the uptake rate of fructose (see section 4.1.4.1.1.) can be linked to observations that there exists a sucrose metabolism in PCC7120 (Cumino et al., 2007; Curatti et al., 2002) and its closely related strain PCC7119 (Curatti et al., 2002). Sucrose has also been shown previously to support mixotrophic growth in PCC7120 (E. Flores, personal communication). Fructose seems to be important in heterocyst development as an overexpression of *susA*, which cleaves sucrose, impairs the development of heterocysts (Curatti et al., 2002). Both the uptake and the metabolism of sucrose and fructose may be linked. *all0261* codes for a sugar transport system permease protein, *all1823* for a sugar ABC transporter, ATP binding protein, *all1916* for a sugar transport system, sugar binding protein. In *Anabaena variabilis* ATCC29413 Ungerer et al. (2008) identified putative ABC transporter like genes: *ava2071*, *ava2072* and *ava2073*. These clustered genes are believed to be under the control of the upstream located *ava2070*, a transcriptional regulator of the LacI family (see fig. 61). *ava2172*, which encodes an ABC transporter-like protein, shows only 38.4% identity to PCC7120 gene *alr5362*, a sugar ABC transporter ATP binding protein. A gene set homologous to the fructose carrier genes in ATCC29413 has not been discovered in PCC7120 so far.

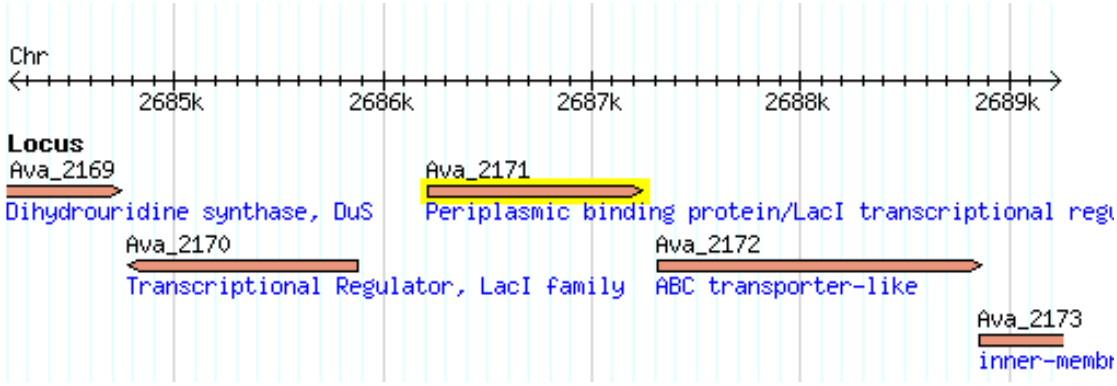


Fig. 61
Putative fructose ABC transporter genes *ava*2171, *ava*2172 and *ava*2173 and their regulator gene *ava 2170* in ATCC29413 (Cyanobase, 2011)

5.2. PCC7120 is capable of real chemoheterotrophic dark growth

Contrary to PCC6803, which cannot grow in complete darkness on glucose, but needs a daily illumination of 5min, called light-activated heterotrophic growth (LAHG) (Anderson and McIntosh, 1991), PCC7120 continually grows over a period of one month in complete darkness (see fig. 18A and B) like its closely related strain ATCC29413 (Haury and Spiller, 1981). Moreover, these two filamentous organisms have in common fructose as their carbon source for heterotrophic growth.

5.3. Introduction of *gtr* from PCC6803 changed the phenotype of PCC7120

gtr was derived from PCC6803, which is a unicellular organism. It had been identified by Schmetterer (1990). Since there are some plasmids like pDU1 (Reaston et al., 1982) that can replicate in filamentous strains like PCC7120, but not in unicellular PCC6803, it was not clear, whether *gtr*, which had been introduced with its original promoter from PCC6803 would be recognized in PCC7120 and expression could occur. However, the uptake of glucose (see fig. 40) and 3OMG (see fig. 41) by PCC7120*gtr*$^+$ was much higher than in PCC7120WT and the manifestation of the *gtr* gene within the new strain was demonstrated by PCR (see fig. 2). If there were no expression, no difference of glucose uptake between these two strains would have been detected.

At an external concentration of 0.2mM PCC7120*gtr*$^+$ took up about 15 times more glucose or 3OMG than PCC7120WT. In the latter case the uptake stopped after about 2h (see fig. 41), because 3OMG is not further metabolized and therefore accumulates within the cell. For fructose the uptake difference was not so significant. At an external concentration of 0.2mM PCC7120*gtr*$^+$ took up even less than the WT (see fig. 45), while at a concentration of 50mM PCC7120*gtr*$^+$ the uptake rate was 2-4 fold of that of the WT (see fig. 46).

As Gtr imports glucose into PCC6803 (the strain's only heterotrophic substrate) it is not surprising that its presence in PCC7120 affects mostly the glucose uptake. PCC7120*gtr*$^+$ exhibits a great sensitivity towards glucose (see section 4.1.2.2.). Concentrations of 5mM are able to kill PCC7120*gtr*$^+$ cells within 1 day (see fig. 7), whereas the WT does not seem to be impaired by glucose even at 200mM (see fig. 8). The behavior towards fructose also changes, when *gtr* is introduced into PCC7120. 200mM fructose has a toxic effect for PCC7120*gtr*$^+$ (see figs. 3 and 11A), but seems to be the optimal concentration for heterotrophic growth of the WT (see figs. 4, 11B and 12). Under mixotrophic conditions little difference concerning the growth rates was detected between the two strains except for an external concentration of 200mM fructose (see figs. 3-5). The growth stimulating effect of fructose was nearly the same in both strains (very little effect at 10mM Frc, significant enhancement at 50mM and 100mM). Regarding the photoheterotrophic experiments PCC7120*gtr*$^+$ grew better at 10-50mM fructose than the WT (see figs. 9 and 10). Under photoheterotrophic conditions at 50mM fructose PCC7120*gtr*$^+$ exhibited a growth rate nearly equal to the photoautotrophic growth (see fig. 9), whereas the growth rate of PCC7120WT was enhanced at 50mM under photoheterotrophy, but the level of photoautotrophic growth was not reached (see fig. 10). Higher concentrations (200mM) had to be added to PCC7120WT to achieve a growth rate in the range of photolithoautotrophy (see fig. 11B).

In the light PCC7120*gtr*$^+$ behaves just the opposite of PCC6803, the organism from which *gtr* was derived. For PCC6803 fructose is toxic (Flores and Schmetterer, 1986) and glucose is a heterotrophic substrate (Astier et al., 1984), whereas it is exactly the contrary for PCC7120*gtr*$^+$. In both cases the heterotrophic substrate is also toxic, when added at high concentrations. There are several possible explanations for the toxicity of glucose in PCC7120*gtr*$^+$. When glucose is taken up through the Gtr protein protons are cotransported. If this occurs at a very high rate the proton gradient may dissipate. It cannot be excluded that glucose is metabolized differently according to the way of its entry into the cell. The same can be true for fructose, where an outer concentration of 200mM is only toxic for PCC7120*gtr*$^+$ but not for PCC7120WT. A further aspect is the possible insufficient regulation of glucose metabolism in PCC7120. In PCC6803 a putative histidine kinase Hik31 has been identified to be important for glucose tolerance. When the gene encoding it was knocked out the cells were impaired in their growth after the addition of D-glucose but not 3OMG, which is not further metabolized (Kahlon et al., 2006). Besides PCC6803 lowers the glucose import rate when grown in the presence of glucose for 2 days (see fig. 39). When *gtr* is transferred to a new organism, where important regulators may be missing, problems may occur.

PCC7120*gtr*$^+$ has never grown successfully in the dark under chemoheterotrophic conditions regardless of the concentration of fructose used (see figs. 16 and 17). If PCC7120*gtr*$^+$ is grown in pure BG11 in the dark the cells do not survive, whereas low concentrations of fructose (5mM and 10mM) can delay the dying process (see fig. 17). However, an increase of the optical density did not take place. An addition of high sorbitol concentrations had also the effect of a slower cell dying (see fig. 19), which excludes a negative osmotic effect of high fructose concentrations. Given the possibility that fructose uptake through Gtr dissipates the proton gradient in PCC7120*gtr*$^+$, this might explain the strain's inability to grow in the dark, whereas in the light enough ATP is produced using the cyclic photosynthetic electron transport so that the cells are not significantly impaired by the uptake mechanism.

5.4. PCC7120*cox1*$^-$ loses cytochrome *c* oxidase activity

PCC7120 lost most of its cytochrome *c* oxidizing activity when the *cox1* region was replaced by a neomycin resistance cassette (see figs. 30, 31 and 34). The quantitative values could be reproduced within a factor of 2, which is small compared to the effects measured. When grown in a 50ml culture hardly any activity was measured (see figs 30 and 31), whereas membranes derived from a 10l culture of PCC7120*cox1*$^-$ exhibited a relatively low but still significant rate of cytochrome *c* oxidase activity (see fig. 34). This might be due to the fact that membranes isolated from 10l cultures generally tended to oxidize horse heart cytochrome *c* to a greater extent than membranes from 50ml cultures (see sections 4.1.3.4.2.1. and 4.1.3.4.3.). For 50ml cultures membranes from the wild type oxidize cytochrome *c* at a rate of

more than 100 fold higher compared with membranes from PCC7120*cox1*$^-$ (see section 4.1.3.4.2.1.). When membranes were obtained from a 10l culture of the wild type the cytochrome *c* oxidation rate was about 50 fold higher than that of the mutant strain (see section 4.1.3.4.3.). These two factors are within the same range. While the very small rates of 0.08nmol min^{-1} [mg protein]$^{-1}$ (see section 4.1.3.4.2.1.) are neglegible, rates of 4.72nmol min^{-1} [mg protein^{-1}] (see section 4.1.3.4.3.) are significant. The remaining oxidase activity of PCC7120*cox1*$^-$ cannot be explained by the presence of wild type copies of the *cox1* genes, as the PCR clearly demonstrated the homozygosity of the strain (see figs. 24 and 25).

Valladares et al. showed that *cox2* and *cox3* are exclusively expressed within the heterocysts (2003). The two loci code both for ARTOs but their type differs. *cox2* encodes a type II ARTO, *cox3* a type I ARTO. While type I ARTOs do not contain a Cu^{2+} binding motive essential for the oxidation of cytochrome *c*, ARTOs of type II possess a Cox like Cu^{2+} binding motive in their subunit II (see section 2.5.2.5.) thereby possibly allowing the oxidation cytochrome *c*. Under the assumption that heterocyst formation is never completely inhibited, even a BG11 grown culture develops heterocysts, where *cox2* and *cox3* are expressed. In contrast to Cox3 Cox2 might also contribute to the total cytochrome *c* oxidase activity. For the wild type the Cox2 mediated portion of the oxidizing activity may be very small, but it might explain the remaining activity after *cox1* deletion.

Interestingly membranes from 10l cultures exhibit a larger cytochrome *c* oxidase activity than membranes from 50ml cultures (see sections 4.1.3.4.3. and 4.1.3.4.2.1.), for PCC7120WT by a factor of 24, for PCC7120*cox1*$^-$ by a factor of 60. It can only be speculated, why the cells develop a higher cytochrome *c* oxidase activity, when cultivated in a larger vessel. The measured OD_{730} of 10l cultures never reached 1.0 (see section 4.1.3.4.3.), whereas 50ml cultures grew up to a density of OD_{730} = 5.0 (data not shown). Perhaps the stronger illumination of the 10l bottle (less self shadow at less dense cultures) enhances the activity of Cox1 (and maybe of Cox2) within the membranes.

5.5. Strain PCC7120*cox1*$^-$ fails to grow chemoheterotrophically

PCC7120*cox1*$^-$ fails at chemoheterotrophic growth (see section 4.1.3.5.). In diverse strains like PCC6803 (Pils et al., 1997) and ATCC29413 (Schmetterer et al., 2001), which are only distantly related among the cyanobacteria, it has been demonstrated that chemoheterotrophic growth is strictly dependent on the presence of the genuine Cox. Therefore the same situation was possible for PCC7120, which is closely related to *Anabaena* ATCC29413. Both strains have one genuine Cox, more than one ARTO, which belong to the subgroups 1 and 2, one Ptox and at least one Qox. Some of the ARTOs are involved in the development of heterocysts (Valladares et al., 2003). At the beginning of the thesis the question was not raised whether Cox is

essential for chemoheterotrophic growth, as the strain was believed to be strictly photolithoautotrophic, but during the work this strain was discovered to exhibit both photoorganoheterotrophic and chemoheterotrophic growth. These facts raised new questions concerning the role of Cox in chemoheterotrophy. For PCC7120WT concentrations of at least 50mM fructose have been observed to support growth in the dark. For that reason 50mM, 100mM and 200mM have been tested for the *cox1*$^{-}$ mutant (see figs. 35A and B). Interestingly 50mM fructose could keep constant the optical density over a long time, though no increase could be detected, whereas at 100mM and 200mM fructose the density decreased immediately, just as in the control experiment without fructose. This might have osmotic reasons. A medium osmolarity may save the cells. Too high concentrations could influence the pH inside a cell. Since Cox has a function in generating proton motive force it can regulate the pH. Absence of this enzyme might lead to an increased sensitivity towards deviation from normal pH.

5.6. A PCC7120*ptox*$^{-}$ strain could only be created, if Gm^{R} was inserted in parallel to the *ptox* ORF

Very little is known about the functions of RTOs of the Ptox type in cyanobacteria. Some evidence suggests their role in carotenoid biosynthesis (Carol et al., 1999; Wu et al., 1999). Initially we planned to knock out the *ptox* (*all2096*) gene by inserting a gentamicin resistance cassette in both directions into the ORF and both knock-out plasmids were successfully constructed. However, the creation of a mutant strain failed, where the resistance cassette is inserted with the reading frame antiparallel to that of the *ptox* gene (see section 4.2.5.). Markus Mikulic, who tried to inactivate *ptox* by insertion of a streptomycin resistance cassette, did not succeed in the antiparallel direction either. Analysis of the surrounding genes of the *ptox* locus according to Cyanobase (2011) showed a cell death suppressor gene (*all2097*), which is the neighbour on the 5' side of the *ptox* gene (see fig. 62). *all2097* shows some homology (30-40%, according to Cyanobase, 2011) to Lls1 (lethal-leaf spot 1) like cell death suppressor proteins in other cyanobacteria. Lls1 related non-heme oxygenases of cyanobacterial origin are also widespread in plants (Gray et al., 2004). In *Arabidopsis thaliana* Lls1 related Pao (pheophorbide *a* oxygenase) is responsible for the degradation of chlorophylls (Pruzinska et al, 2005). The cell death suppressor protein of PCC7120 is transcribed in the same direction as *ptox*. As a consequence a resistance cassette inserted in the opposite direction might inhibit the transcription of the cell death suppressor gene. In contrast to the ORF of the streptomycin resistance cassette the length of the transcriptional unit of the Gm^{R} gene is not elucidated. Given the possibility that important terminator sequences have not been cloned in pRStUV1 and pRStUV2 one cannot exclude that the mRNA of the resistance cassette might act as an antisense RNA towards the mRNA of the cell death suppressor protein.

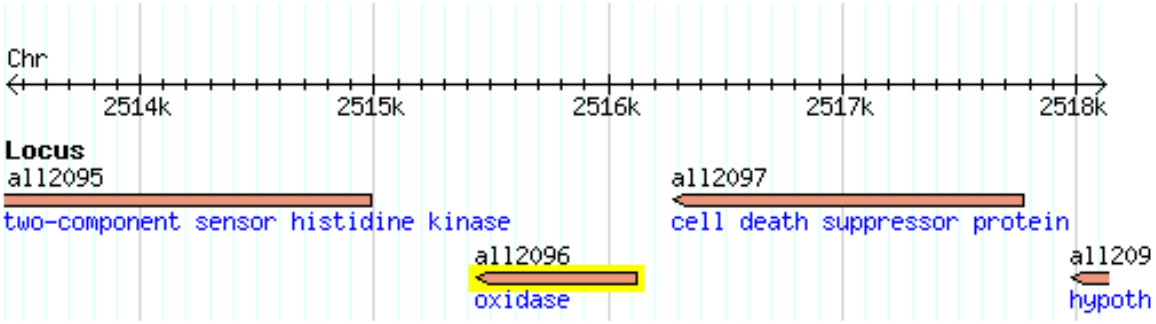


Fig. 62
Surounding genes of *ptox* (Cyanobase, 2011)

6. Zusammenfassung

Cyanobakterien sind Prokaryoten, welche oxygene Photosynthese betreiben. Dennoch können alle Cyanobakterien im Dunkeln atmen. Im Gegensatz zu photosynthetischen Eukaryoten (Algen, höhere Pflanzen), wo Photosynthese und Atmung in Chloroplasten und Mitochondrien räumlich voneinander getrennt stattfinden, laufen diese beiden Prozesse in Cyanobakterien in ein und demselben Kompartiment ab und einige Komponenten werden von beiden Elektronentransportketten verwendet. Die respiratorischen terminalen Oxidasen (RTOs) sind Schlüsselenzyme, da sie nicht direkt an der Photosynthese beteiligt sind, sondern die Elektronen zum terminalen Akzeptor O_2 leiten. Respiratorische Oxidasen in Cyanobakterien gehören verschiedenen Klassen an.

Wichtige Klassen sind die Häm Kupfer Oxidasen, welche homolog zur Cytochrom *c* Oxidase in Mitochondrien ist, die Chinon Oxidase, welche homolog zu Cytochrom *bd* in *E. coli* ist, sowie die Klasse jener RTOs, die homolog zu Plastid terminalen Oxidasen (Ptox) in Chloroplasten ist. Fast alle Cyanobakterien besitzen zumindest eine Cytochrom *c* oxidase vom Cytochrom *aa3* type.

Drei verschiedene Unterklassen von Cytochrom *aa3* Typ RTOs sind identifiziert worden:

a) echte Cytochrom *c* Oxidasen, welche die charakteristischen Motive enthalten;
b) eine 2. Unterklasse, welche alternative respiratorische terminale Oxidase (ARTO) genannt wird;
c) eine Oxidase vom cbb_3 Typ (zuerst in Purpurbakterien charakterisiert).

Anabaena PCC7120 gehört zu den unverzweigten filamentösen Cyanobakterien, welche zur Zelldifferenzierung befähigt sind. Bei einem Mangel an gebundenem Stickstoff (Nitrat, Nitrit, Ammoniak) können einige vegetative Zellen zu Heterocysten differenzieren (in nicht zufälliger Verteilung innerhalb eines Filaments). PCC7120 hat 5 verschiedene respiratorische terminale Oxidasen: 1 echte Cytochrom *c* Oxidase (Cox), 2 ARTOs, eine Chinon Oxidase (Qox) und 1 Plastid terminale Oxidase (Ptox). Da die Expression der beiden ARTOs auf die Heterocysten beschränkt zu sein scheint, verbleiben in den vegetativen Zellen 1 Cox, 1 Qox und 1 Ptox. Wenn der *cox* Locus durch eine Antibiotikumkassette ausgeschaltet wird, verliert der neue Mutantenstamm jegliche Cytochrom *c* oxidase Aktivität, wie Versuche mit isolierten Membranen aus dem Mutantenstamm und aus Pferdeherz isoliertem Cytochrom c_{550} bewiesen. Außerdem scheint Cox essentiell für das chemoheterotrophe Wachstum von PCC7120 im Dunkeln zu sein. Trotz bisheriger Untersuchungen, welche diesen Stamm als streng photolithoautotroph klassifizierten, zeigten neue Versuche, dass PCC7120 heterotroph wachsen kann, vorrausgesetzt hohe Mengen an Fruktose wurden dem Medium hinzugesetzt. Offenbar hat PCC7120 eine gewisse Kapazität für heterotrophes Wachstum und Fruktosemetabolismus im Inneren der Zelle. Sehr hohe Konzentrationen im Nährmedium reichen aus, dass Fructose über die Zellwand und die Membranen ins Innere gelangt. Experimente haben gezeigt, dass umso mehr Fruktose eindringt, je höher die Konzentration im äußeren Medium ist. Das Einschleusen des Glukose carrier Gens *gtr* aus PCC6803 führt zu einem transgenen Stamm, der im Gegensatz zum Wildtyp bereits bei geringeren Fruktosekonzentrationen photoorganoheterotroph wachsen kann. Glukose ist jedoch toxisch für PCC7120gtr^+, während der Wildtyp dieser gegenüber tolerant ist. Der Mutantenstamm, in welchem *cox* ausgeschaltet worden ist, kann nicht mehr chemoheterotroph wachsen. Ähnliche Ergebnisse gibt es auch von *Synechocystis* PCC6803 und *Anabaena variabilis* ATCC29413, in welchen Cox ebenfalls notwendig für das chemoheterotrophe Wachstum ist.

7. Abbreviations

ADP	adenosinediphosphate
AMP	adenosinemonophosphate
ATP	adenosinetriphosphate
ARTO	alternate respiratory terminal oxidase
ATCC	American Type Culture Collection
bp	base pairs
BSA	bovine serum albumin
CM	cell membrane, cytoplasmic membrane
Cm	chloramphenicol
Cox	cytochrome *c* oxidase
Cpm	counts per minute
Cyt	cytochrome
dcm	DNA cytosine methyltransferase
DCMU	3-(3,4-dichlorphenyl)-1,1-dimethylurea (diuron)
DMBIB	2,5-dibrom-3-methyl-6-isopropyl-p-benzoquinone (dibromothymoquinone)
DMSO	dimethyl-sulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxy-nucleosidetriphosphate
E. coli	*Escherichia coli*
EDTA	ethylene-diamino-tetraacetic acid
EtBr	ethidium bromide
Frc	fructose
Glc	glucose
GlcP	glucose-fructose permease
Gm	gentamicin
Gtr	glucose transporter
HEN	HEPES EDTA NaCl
HEPES	n-2-hydroxyethylpiperazine-N'-2-ethanesulfone acid
HQNO	2-heptyl-4-hydroxyquinoline-N-oxide
ICM	intracytoplasmic membrane
kbp	kilobasepairs
KCN	potassium cyanide
Km	kanamycine
LAHG	light-activated heterotrophic growth
LB	lysogenic broth, Luria-Bertani
LHC	light harvesting complex
M	molar
MCS	multiple cloning site
mM	millimolar

µM	micromolar
Mbp	megabasepairs
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced form of NAD^+
$NADP^+$	nicotineamide adenine dinucleotide phosphate
NADPH	reduced form of $NADP^+$
$NAD(P)^+$	NAD^+ or $NADP^+$
NAD(P)H	NADH or NADPH
NDH	NAD(P)H dehydrogenase
NEB	New England Biolabs
Nm	neomycin
nm	nanometer
npt	neomycin phosphate transferase
OD	optical density (extinction)
ORF	open reading frame
3-OMG	3-O-methylglucose
PCC	Pasteur Culture Collection
PCP	pentachlorophenol
PCR	polymerase chain reaction
pmf	proton motive force
PMSF	phenyl-methyl-sulfonylfluoride
PSI	photosystem I
PSII	photosystem II
Ptox	plastid terminal oxidase
Qox	quinol oxidase
RC	reaction center
RNA	ribonucleic acid
RT	room temperature
RTO	respiratory terminal oxidase
SIS	spectral index of the sample
Sm	streptomycin
SOB	super optimal broth
SOC	super optimal broth with catabolite repression
TAE	Tris acetate EDTA
TE	Tris EDTA
Tris	tris(hydroxymethyl) aminomethane
tSIE	transformed spectral index of the external standard
v/v	volume per volume (concentration)
w/v %	g/100ml (weight per volume)
WT	wild type

8. References

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