Functional analysis of the leader peptidases in cyanobacterium *Synechocystis* sp. PCC 6803.

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Abbreviations

General abbreviations, chemicals and enzymes

A	Absorbance
aa	amino acid
APS	Ammoniumperoxidosulfate
ATP	Adenosinetriphosphate
BG	Blue-green
BisTris	Bis-(2-hydroxyethyl) amino-tris (hydroxymethyl)-methane
BLAST	Basic Local Alignment Search Too
BN	Blue-native
BSA	Bovine-serume albumine
CHAPS	3-((3-Cholamidopropyl)- dimethylammonio)-1-propane-sulfonate
Chl	Chlorophyll
Cm	Chloramphenicol
DCBQ	2,6-dichloro-p-benzoquinone
DCPIP	Dichlorophenyl indophenol
DMSO	Dimethyl sulfoxide
DTT	Dithiotreitol
EDTA	Ethylenediamintetraacetate
ECL	Enhanced chemoluminescence
E. coli	*Escherichia coli*
Gm	Gentamycin
HEPES	2-[4-(2-hydroxyethyl)1-1-piperazinyl)-ethansulfonic acid
IPTG	isopropyl β-D-thiogalactoside
Km	Kanamycin
LB	Luria-Bertani-medium
Luminol	3-aminophtalhydrazide
MALDI	Matrix assisted laser desorption and ionisation
MES	2-N-Morpholinoethanesulfonic acid
MS	Mass spectroscopy
MV	Methylviologen, (1,1'-Dimethyl-4,4'- bipyridinium-dichloride)
NADP	Nicotine-adenine dinucleotide
NBT	Nitro blue tetrazolium chloride
NCBI	National center for biotechnology information
OD	Optical density
ORF	Open reading frame
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate bufferen saline
PCC	Pasteur culture collection (Paris, France)
PCR	Polymerase chain reaction
PMSF	Polymethyl sulfonic acid
Pre-	Precursor
PVDF	Polyvinyliden difluoride
S	Substrate
SDS	Sodium dodecylsulfate

SOD	Superoxide-dismutase
Synechocystis 6803	*Synechocystis* sp. PCC 6803
TCA	Trichloroacetic acid
TE	Tris-EDTA
TEMED	N, N, N', N'-Tetramethylethylendiamin
ToF	Time-of-flight
TMBZ	3,3',5,5'-tetramethylbenzidine
TMH	transmembrane helix
TMHMM	Transmembrane helices prediction based on hidden Markov model
Tricine	N-tris-(hydroxymethyl)-methylglycine
Tris	2-amino-2(hydroxymethyl) 1,3-propandione
Triton X-100	Octylphenoxy poly-(8-10)-ethyleneglycol
Tween 20	Polyoxyethylene sorbitan monolaureate
U	Unite
URL	Universal resource locator
WT	Wild type
ΔpH	proton gradient

Amino acids

A, Ala	Alanine	M, Met	Methionine
C, Cys	Cysteine	N, Asn	Asparagine
D, Asp	Aspartic acid	P, Pro	Proline
E, Glu	Glutamic acid	Q, Gln	Glutamine
F, Phe	Phenylalanine	R, Arg	Arginine
G, Gly	Glycine	S, Ser	Serine
H, His	Histidine	T, Thr	Threonine
I, Ile	Isoleucine	V, Val	Valine
K, Lys	Lysine	W, Trp	Triptophane
L, Leu	Leucine	Y, Tyr	Tyrosine

Nucleic acids

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
dNTP	Desoxynucleoside-5'-triphosphate

Units

bp	base pair
°C	grades Celsius
E	Einstein (mol of photons)
g	gram
g	gravity
h	hour
K	Kelvin
kb	kilo base pair
(k)Da	(kilo) Dalton
l	litre
m	meter

M	molar
mA	milliamper
mg	milligram
µg	microgram
µl	microlitre
mM	millimolar
µM	micromolar
nm	nanometer
rpm	rotations per minute
sec	second
v/v	volume per volume
w/v	weight per volume

Proteins

ATP-Se	ATP-synthase complex
C-	Carboxyl terminus of the protein
N–	Amino terminus of the protein
33 kDa	PsbO protein or manganese stabilising protein of photosystem II
CF_0II	Chloroplast F_0 ATP synthase subunit II
cyt $b_6 f$	Cytochrome $b_6 f$ complex
Cyt *f*	Cytochrome f
Ffh	Fifty-four homolog proteine
FtsY	Filamentous temperature sensitive mutant Y
Lep	Leader peptidase
OEC	Oxygen evolving complex
PC	Plastocyanine
PC/	Phycobiliprotein
PSI	Photosystem I
PSI-3	PsaF protein
PSII	Photosystem II
Rieske	Iron-sulphur protein of cytochrome b6f complex
Rubisco	Ribulose-1,5'-bisphosphate-carboxylase/oxygenase
SP	Signal peptidase
SPP	Stromal processing peptidase
SRP	Signal recognition particle receptor protein
SRP54	Signal recognition particle 54 kDa protein
Tic	Translocase of the inner chloroplast envelope membrane
TPP	Thylakoid processing peptidase
Ycf	Conserved chloroplast open reading frames

1. Introduction

1.1. Specific features of cyanobacteria important for this study

Cyanobacteria are aquatic and photosynthetic Gram-negative bacteria important to the food chain and the renewal of the oxygenic atmosphere of the planet. *Synechocystis* sp. PCC6803 (hereafter referred as *Synechocystis* 6803) is an unicellular freshwater inhabitant, which belongs to the phylum *Cyanophyta* and cannot fix nitrogen. The cyanobacteria of this phylum contain only chlorophyll *a* and various phycobiliproteins, which are assembled in the phycobilisomes on the thylakoid membranes. This is different from prochlorophytes, genera of cyanobacteria, which lack phycobilins and have both, chlorophyll *a* and chlorophyll *b*.

Like all Gram-negative bacteria, the cells of cyanobacteria are surrounded by two membranes, an inner membrane and an outer membrane with a cell wall, made of the peptidoglycan murein. Therefore, cyanobacteria possess a functional periplasm between the inner membrane and outer membrane. The thylakoids of cyanobacteria do not form grana, though the thylakoid membranes form the internal membrane structure, which resembles the layers (Fig.1). The model of the internal membrane structure which was proposed based on the electron microphotographs of the thin sections is represented at the internet page http://lsweb.la.asu.edu/Synechocystis/.

According to the endosymbiotic theory, cyanobacteria are considered as ancestor of chloroplasts (Schwartz et al., 1978). Taking this aspect into account, the cyanobacteria are suitable model object to study the oxygenic photosynthesis, the regulation of photosynthesis and cell development. The genome of *Synechocystis* 6803, a well known model object, was completely sequenced (Kaneko et al., 1996). The genomic DNA is 3.57 Mbp large; the genome encodes 3168 proteins. This bacterium is able to grow phototrophically and heterotrophically in the absence of photosynthesis. It is easily transformable (Shestakov and Reaston, 1987), and easily amenable for targeted gene modifications (Vermaas et al., 1996) and shares a large number of genes in common with plants (Martin et al., 2002). The intensive work on photosynthetic organisms including this cyanobacterium has clarified the function of many photosynthetic proteins (Pakrasi, 1995). The analysis of the role of the proteins related to the regulation of photosynthesis became recently one of the central research areas, in which different cyanobacteria are intensively used.

The cytoplasmic membrane separates the cytoplasm from periplasm and contains in cyanobacteria mostly the proteins of the respiratory electron transport chain. The thylakoid membrane system in cyanobacteria, which separates the cytoplasm from the thylakoid lumen, contains protein complexes of both, the photosynthetic and the respiratory electron transport chain. The photosynthetic electron transport chain of cyanobacteria is largely similar to that of plants, though there are differences in the composition of the protein complexes.

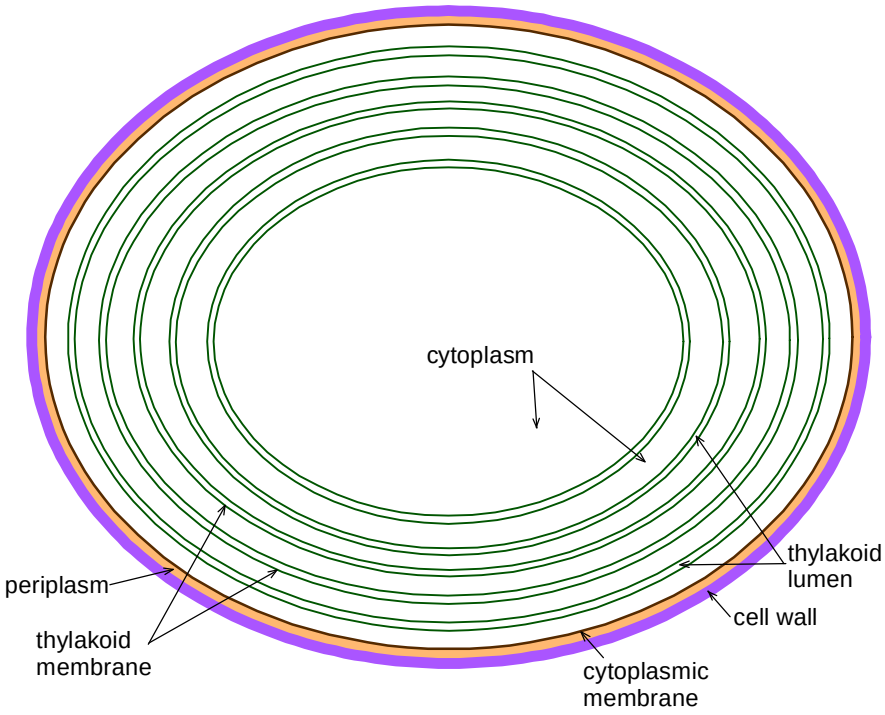

Figure 1. Schematic representation of the intracellular structure of cyanobacteria (based on Vermaas, 2001). Thylakoid membranes (indicated in green) occur in pairs and separate the cytoplasm from the lumen; the cytoplasmic membrane (brown) separates the cytoplasm from the periplasm; and the outer membrane (brown) forms the cell wall.

In cyanobacteria, several redox-active components of the thylakoid membranes are utilized by both, photosynthesis and respiration. These components are the plastoquinone (PQ) pool, the cytochrome b_6 *f* complex and the soluble electron carriers in the lumen.

The photosynthetic electron transport chain includes protein complexes of PSII, PSI, cyt b_6 *f* and ATP-synthase. The light harvesting antenna (LHC, light-harvesting complex), found in thylakoid of plants, is absent from the *Synechocystis* thylakoids. Instead, in *Synechocystis*, the phycobilisome is the major light-harvesting, multiprotein complex attached to the surface of photosynthetic membrane (Grossman et al, 1993). Photosystem II uses light energy for water

splitting and PQ pool reduction. Upon the water splitting, the protons are released into the thylakoid lumen. The electrons are transferred from the PQ pool to the cyt b_6 *f* complex. The proteins of photosystem II are encoded by *psb* genes which occur in cyanobacteria and also in higher plants and algae (Barber et al., 1997). The exceptions are several proteins like: the PsbT protein, which is not homologous in plant and cyanobacteria; the psbW protein, which has been found in plants but not in cyanobacteria, and the PsbU and PsbV proteins, which are present only in the cyanobacterial oxygen evolving complex (Thornton et al., 2004). About luminal proteins of PSII of *Synechocystis* 6803, like PsbO, PsbU and PsbV it is known that they are synthesized as precursors (Philbrick and Zilinskas, 1988; Shen et al., 1997; Shen et al., 1995).

The cyanobacterial cytochrome b_6 *f* complex is essential for the electron transport of the cell, thus it is indispensable for cyanobacteria, unlike, e.g., the cyt b_6 *f* complex of *Chlamydomonas reinhardtii* (Vermaas, 2001; Berthold et al., 1995). The *c*-type cytochromes of cyanobacteria (cytochrome *f*, cytochrome c_{550}, and cytochrome c_{553}) are localized on the lumenal side of the membrane and are synthesized as a precursor protein, whose N-terminal signal sequence is recognized by the Sec system of protein translocation and is cleaved by the signal peptidase (Tichy and Vermaas, 1999**;** Thöny-Meyer et al., 1995). From the cyt b_6 *f* complex the electrons are transferred to a soluble electron carriers, cyt c_{553} or plastocyanine (PC), located on the luminal side of the thylakoid membrane and synthesized as precursor protein (Varley et al., 1995). These proteins are responsible for further electron transport to PSI.

The core of the PSI complex is formed by the PsaA and PsaB subunits. In addition, the cyanobacterial PSI complex contains three peripheral proteins (PsaC, PsaD, and PsaE) and six integral membrane proteins (PsaF, PsaI, PsaJ, PsaK, PsaL, and PsaM) (Chitnis, 1996). PSI complex is monomeric in higher plants and green algae, unlike cyanobacteria, where the PSI is trimeric (Scheller et al., 2001) and contains most of the chlorophyll of the cell (Rögner et al., 1990). In some cyanobacteria, the ratio of PSI to PSII is higher than in plants. In *Synechocystis* 6803 this ratio is about 5 (Shen et al., 1993), whereas in plants an equal ratio is usual. Such high ratio is proposed to be necessary for cyclic electron flow from PSI/ferredoxin to cyt b_6 *f* and PQ and back to PSI. This is used to generate a proton gradient across the thylakoid membrane, and thus for ATP synthesis, but not for NADP reduction. On the other hand, the high number of PSI may provide the oxidized state of PQ pool in the light, which is important to minimize photodamage (Andersson and Barber, 1996).

Although in cyanobacteria, both the respiratory and photosynthetic electron transport chains use the same electron transport intermediates (Scherer, 1990), only the respiratory electron transport chain involves the activity of succinate dehydrogenase, NAD(P)H dehydrogenases (NDH-1 and NDH-2) and different terminal oxydases, whose activity was detected in both cytoplasmic and thylakoid membranes.

An interesting question is how photosynthesis and respiration are regulated in a cyanobacterium. If light is abundant, the photosynthetic electron transport chain has a much higher capacity of electron flow than has the respiratory chain, but at very low light intensity or in darkness respiratory rates are higher than those of photosynthesis (Vermaas, 2001**).** The analysis of the role of the proteins related to the regulation of photosynthesis became recently one of the central research areas where different cyanobacteria are also intensively used. Among these proteins are important factors of regulation of the post-translational membrane insertion and translocation of thylakoid proteins (Robinson et al., 1998; Wollman et al., 1999). An intriguing question by the study of these processes in cyanobacteria is the determination of the membrane where the photosynthetic complexes are forming, as there are two potential targets for protein export – thylakoid membrane and plasma membrane. For cyanobacteria it was recently proposed that initial steps of biogenesis of photosystems occur in the plasma membrane (Zak et al., 2001).

1.2. Translocation of proteins and biogenesis of thylakoid membrane.

Protein translocation in and across the membranes takes place in all living organisms including bacteria. Typically, about half of the cellular proteins need to be transported across or into membranes (Schatz and Dobberstein, 1996).

Firstly, it was proposed that proteins contain information within their amino acid sequences for protein targeting to the membrane (Blöbel and Sabatini, 1971). Unaware of this hypothesis, it was discovered that the light chain of kappa-immunoglobulin from myeloma cells was synthesized in a higher molecular weight form and was converted to its mature form when microsomes were added to the translation system (Milstein et al., 1972). Subsequently the signal peptides were later found to be cleaved from the exported proteins by specific signal peptidases in the processing step.

In both, prokaryotic and eukaryotic cells, proteins destined for secretion are initially made with an N-terminal signal peptide that serves to route the attached polypeptide into the

secretory pathway. The structure of the signal peptide determines the moment of the protein export, i.e., either during or after the translation, and the type of energy used for the translocation. The proteins can be translocated in either folded or unfolded state. For proper protein conformation, the function of chaperones can be required.

The translocation is an energy-dependent process. It can be carried out with the help of protein factors associated with the transmembrane channel, which use the energy of nucleosidetriphosphate hydrolysis. Another moving force used for protein translocation in thylakoid membrane and bacterial membranes is the proton gradient (Dalbey and Robinson 1999).

The systems of protein translocation in different membrane systems can be basically divided into two major groups: the export system and the import system (Schatz and Dobberstein, 1996). The export system transports proteins from the cytosol to an extracytosolic compartment. The export systems of eukaryotes have many common features with the export systems of bacteria. Since the export systems are phylogenetically related, the investigation of the bacterial and chloroplast protein transport systems complete the general knowledge in this research area. Though many components of the translocation machineries are known (Table 1), the mechanisms of the protein translocation are not yet sufficiently clarified. For better understanding of the role of the protein transport for photosynthetic organisms, new approaches are necessary. One of such is to study the translocation mechanisms in organisms whose genome has been completely sequenced. For the study of the thylakoid membrane biogenesis, the uni-cellular cyanobacterium *Synechocystis* 6803 is very suitable since not only the nucleotide sequence of this organism is completely sequenced, but also a large amount of data conserning physiology and biochemistry of photosynthesis is available. Cyanobacteria like other bacteria have the systems of protein export, and are of particular interest for the study of protein export in the thylakoid membrane. For proteins synthesized in cyanobacteria there are two potential targets for export – the thylakoid membrane and plasma membrane. Potentially, in both membranes the same component of the translocation machineries can be located (Howe et al., 1996).

Through the study of the protein translocation system in chloroplasts *in vitro* several pathways of protein integration in the thylakoids were discovered. The selection of the pathway of protein integration depends most likely on the protein nature (integral or peripheral), and the nature of the signal (i.e., the presence of the signal peptide in the protein). Four pathways of the protein translocation in or across the thylakoid membrane were found

(Fig. 2). These are: Sec, ΔpH, SRP and spontaneous mechanism, which will be discussed below.

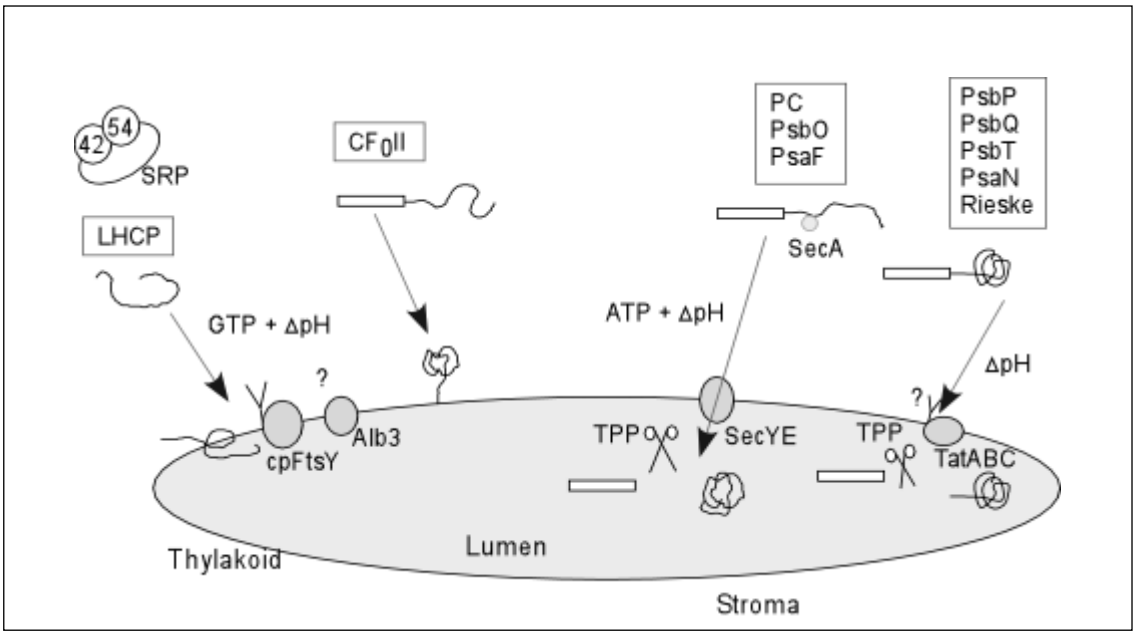

Figure 2. Protein translocation pathways in thylakoid membrane of chloroplasts
SRP - signal recognition particle.
TPP – thylakoid processing peptidase
Further abbreviations are given in text.

The similarity search of the known translocation protein factors from *E. coli* and plants revealed the presence of the homologous proteins in the genome of *Synechocystis* 6803. The data is summerized in the Table 1 (based on Robinson and Dalbey, 1999).

Table 1.

Proteins which are important for protein translocation in Gram-negative bacteria and chloroplasts.

Function	*E.coli*	*Synechocystis 6803*	Chloroplast
Recognition	SecB Ffh	- Ffh	- chlSRP54 chlSRP43
Translocation	SecA SecY SecE SecG SecD SecF YaiC	SecA SecY SecE SecG SecD SecF	SecA SecY SecE

	TatE (YbeC) TatA (YigT) TatB TatC FtsY	TatA TatB TatC FtsY	TatA (Tha4) TatB (Hcf106) TatC FtsY
N-terminal processing	SP type I - LepB SP type II – LspA -	SP type I - LepB1 - LepB2 SP type II – LspA -	TPP1 TPP2 - SPP

Abbreviations: Ffh – fifty-four homolog, SRP – signal recognition particle, Hcf – high chlorophyll fluorescence, Tha – thylakoid assembly, SP – signal peptidase, TPP – thylakoid processing peptidase, chl - chloroplast.

The Sec-pathway of the protein transport was intensively studied in the plant thylakoids and in the Gram-negative bacterium *E. coli*. The common principle of this pathway is that the substrate proteins are translocated in unfolded state. The number of the proteins involved in the pathway is relatively high in the *E. coli* cells (Table 1). Moreover, this pathway is important for the secretion of the proteins out of the cell. The thylakoid Sec-pathway involves the function of SecA, SecY and SecE proteins as it is known up to date.

In the genome of *Synechocystis* 6803, the genes encoding for the putative components of Sec-dependent translocation were also identified (table 1). These are the genes typical for plant *secA, secY, secE,* but also genes *secG, secD* and *secF*. Two facts suggest that the Sec-dependent translocation pathway operates in *Synechocystis* 6803: the presence of proteins translocated in chloroplast by Sec-dependent way (PsbO, PsaF and plastocyanin) and the structural similarities of the signal peptides of these proteins with those from plants (Howe et al., 1996).

The chloroplast signal recognition particle (SRP) pathway is responsible for targeting of integral thylakoid proteins, the LHCPs (Li et al. 1995). The membrane insertion of these proteins does not depend on the signal sequence (Lamppa, 1988). The SRP54 protein from chloroplast is homologous to the bacterial SRP pathways component ffh (SRP54 - fifty-four homolog) and forms a soluble complex with LHCP substrates in the stroma (Keegstra and Cline, 1999). A second soluble factor, FtsY, is also involved in the insertion mechanism (Kogata et al., 1999), which requires GTP hydrolysis (Hoffman and Franklin, 1994).

There are data suggesting that SRP could participate in the process of the membrane insertion of the chloroplast encoding protein D1. It remains however unclear whether the SRP43 subunit participates in this process too (Nilsson et al., 1999). In the *Synechocystis* 6803 genome, two genes were identified which encode the proteins Ffh and FtsY (Table 1), but it is unknown

whether the SRP-dependent mechanism is important for thylakoid membrane biogenesis of *Synechocystis* 6803.

The ΔpH-dependent pathway of protein translocation in chloroplasts is homologous to the TAT (twin arginine translocase) pathway of bacteria. The ΔpH-dependent pathway uses hydrophobic signal peptides of the transported proteins, similar to that of the Sec-dependent pathway. However, the ΔpH-dependent mechanism operates without a stromal factor or nucleoside triphosphates. The protein transport is carried out on the expense of the ΔpH-gradient across the thylakoid membrane (Fig. 2., Henry et al., 1994; Mould et al., 1991; Klösgen at al., 1992). ΔpH-dependent and Tat dependent systems translocate folded proteins whereas the Sec-dependent system transfers the unfolded proteins (Santini et al., 1998).

The plant proteins PsaN, PsbP, PsbQ, PsbT, which use the ΔpH-dependent translocation pathway, are absent in *Synechocystis sp.* PCC6803 (Nakamura et al., 1998). The Rieske protein, which is one of the subunits of cytochrome b_6 *f* complex, is present in both cyanobacteria and plants. In plant chloroplasts, the Rieske protein is transported to thylakoid lumen via ΔpH-dependent translocation pathway. The leader peptides of cyanobacterial and plant Rieske proteins serve to anchor the protein in thylakoid membrane and show a high degree of homology. This can be an argument in favor of existence of ΔpH-dependent translocation in cyanobacteria (Madueno et al., 1993).

Some thylakoid proteins like CF_0-II (ATP-synthase subunit), PsbW and PsbX (subunits of PSII) are synthesized as precursors in cytosol and contain a bipartite signal peptide, typical for proteins of the thylakoid lumen. The membrane insertion of proteins CF_0-II, PsbW and PsbX depends neither on protein factors of the stroma, nor on nucleoside triphosphates, nor on ΔpH in thylakoids, and is also not affected by protease-treatments of thylakoids (Fig. 2; Michl et al., 1994; Lorkovic et al., 1995; Kim et al., 1998). Therefore, it has been proposed that these proteins insert spontaneously into the thylakoid membrane. The genes encoding CF_0-II and PsbX were found in the genomes of cyanobacteria and plastid genomes of some eukaryotic algae, but these proteins are synthesized without any signal peptides. The signal peptides appeared after the transfer of the respective gene into the nucleus. Probably, the signal peptides provide in this case the insertion mechanism concerned with the more complex pathway of protein delivery from the cytosol to the thylakoid membrane.

1.3. Role of the signal peptidases for the protein transport processes.

1.3.1. Types of signal peptidases in bacteria

The translocation of the proteins in bacteria requires a cleavage of the N-terminal signal peptide. This function is performed by signal peptidases, which help the proteins to reach their final destination. There are different classes of the signal peptidases involved in the cleavage of the signal peptides in bacteria. The signal peptidases that employ a catalytic serine/lysine dyad and are inhibited by penem belong to the type I of the signal peptidase (also mentioned as leader peptidase). Signal peptidases of this type can process nonlipoprotein substrates that are exported by the Sec-pathway or the TAT-pathway. The specific feature of the signal peptidase of type II is the ability to cleave lipoprotein signal peptides. These enzymes can be inhibited by globomycin and also pepstatin suggesting that they are aspartic peptidases (Rawlings and Barrett., 1995). The signal peptidases of class III are responsible for the cleavage of the prepilins of type IV– outer membrane proteins excreted by Gram-negative bacteria (Nunn and Lory, 1991).

1.3.2. Specific features and role of different signal peptides

Signal peptides are important for the correct targeting of the proteins. It is believed that the secretory signal peptides of eukaryotic and prokaryotic proteins are formed by three distinct regions (von Heijne, 1989, Gierasch, 1989): i) a positively charged N-terminus (n-region), ii) a central hydrophobic region (h-region) and iii) a polar C-domain (c-region). These features determine the recognition of the signals by the respective translocation machinery. The hydrophobic amino acids of the signal peptides are important for initiation of the protein insertion into the membrane. In the positions -3 and -1 to the cleavage site, uncharged amino acids with small side groups are located (von Heijne et al., 1989). The analysis of the amino acids important for the cleavage has revealed a preference for alanine or an Ala-X-Ala motif (von Heijne, 1983). The amino acid composition of the signal peptides is variable among different proteins, though they can show some similarities depending on the translocation pathway. In Fig. 3, the signal peptides of the plant proteins of thylakoid lumen are shown. These proteins use the Sec-dependent or ΔpH-dependent pathways of protein translocation. The specific feature of the proteins translocated by the ΔpH-dependent mechanism is the presence of two arginine residues in front of the hydrophobic core of the signal peptide. The

comparison of the signal peptides for different pathways has shown that the hydrophobic domain, characteristic for SRP-specific translocation, is longer than that of Sec-dependent pathway both in eukaryotic and in bacterial systems (Zheng and Gierasch, 1996; Ng et al., 1996, Ulbrandt et al., 1997; Valent et al., 1998).

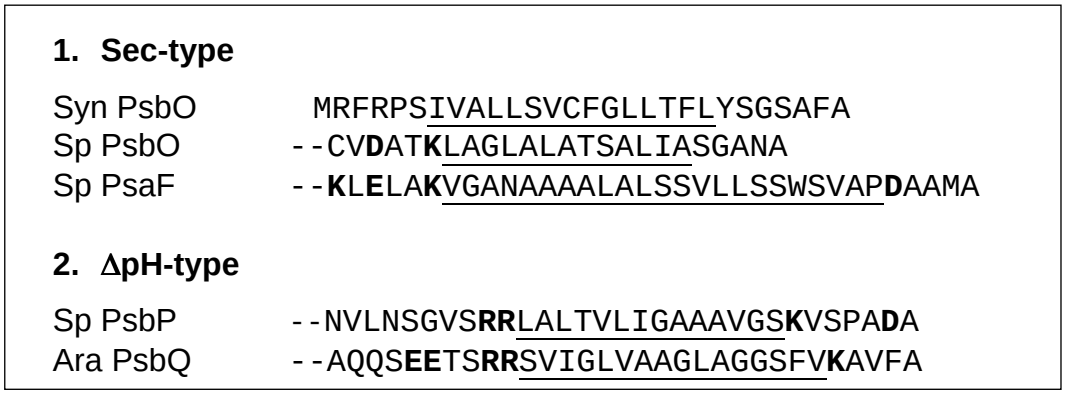

Figure 3. Signal peptides of proteins of the thylakoid lumen.
As example, the peptides of *Synechocystis* (Syn), *Spinacia oleracea* (Sp) and *Arabidopsis thaliana* (Ara) are shown. The hydrophobic regions are underlined; the charged amino acids are in bold.

The lipoprotein signal peptides contain a cystein residue at the C-terminus, modified by the prolipoprotein diacylglyceryltransferase. This modification is essential for the processing of the protein by the lipoprotein signal peptidase. In the cells of *E. coli* an additional modification of lipoproteins occurs: the aminoacylating of the diacylglycerylcysteine (Tokunaga et al., 1982). In the bacteria the signal peptidases of the type II play an important role for the protein secretion. Especially in Gram-positive bacteria these enzyme are very important as they are essential for development of competence and for sporulation (Sutcliffe and Russell, 1995).

The type IV prepilin signal peptides are characterized by a short basic region without any hydrophobic domain. The processing site is located at the amino-terminal side of the hydrophobic region within the mature protein.

The chloroplast proteins, which are transferred into or across the thylakoid membrane, have more complex signal peptides than cyanobacteria. These proteins have to be delivered from the cytosol into the stroma of chloroplasts, where the first signal peptide is cleaved by the stroma processing peptidase SPP (Dalbey and Robinson., 1999). In the thylakoid membrane,

the second part of the signal sequence is cleaved by thylakoidal processing peptidase (TPP), which belongs to the type I of signal peptidases.

Generally, the signal peptide serves for the effective protein translocation in the membrane. Some proteins carry the signal peptides that do not have any additional function and are processed after the translocation. Other proteins carry the signal peptides which serve to anchor the protein in the membrane. The signal peptide of Rieske protein is not deleted after the translocation and serves to anchor the protein to the thylakoid membrane. The transport of the proteins into the thylakoid lumen strongly requires leader peptidase function. For example, the proteins of the photosystem II complex (PSII) - PsbO, PsbP and PsbQ that are responsible for water splitting reaction and stabilization of the Mn-ion, or plastocyanin, which participates in electron transfer from the cytochrome b_6 *f* complex to photosystem II (PSII), undergo after the translocation the processing step. In all these cases the processing step is obviously needed to release the protein from the thylakoid membrane after the translocation thus converting it into the active state.

The protein translocation step can be accompanied by other processes like protein-cofactor interaction. An example is the biogenesis of the membrane protein cytochrome *f*, which comprises two key steps. The first one is the processing of apocytochrome *f*; the second is the transformation of the apocytochrome into the holocytochrome by covalent binding of c-heme with two cysteine residues of the holocytochrome. Both steps take place on the luminal side of the membrane, or after the translocation step (Howe and Merchant, 1994). The binding of heme-group by the apocytochrome can occur prior to the processing. This is testified by the ability of the cytochrome *f* precursor to bind the heme group in the cells of the mutant which is not capable to cytochrome processing (Wollman et al., 1999). Therefore, in this example, the processing is the final step of protein maturation.

1.3.3. Structural and functional similarities of leader peptidases from bacteria and thylakoid processing peptidase from higher plants.

The features of the signal peptidase type I from bacteria *Escherichia coli* are most well studied. It is an integral membrane protein with two transmembrane regions. The C-terminal part of the protein is located in periplasmic space, where it is catalytically active (Bilgin et al., 1990). The position of the signal peptidases relative to the membrane is shown on the Fig. 4. In contrast to the LepB from *E. coli*, the leader peptidases from cyanobacteria and plant

chloroplasts possess only one transmembrane region, though the active site is proposed to have the same orientation in the membrane.

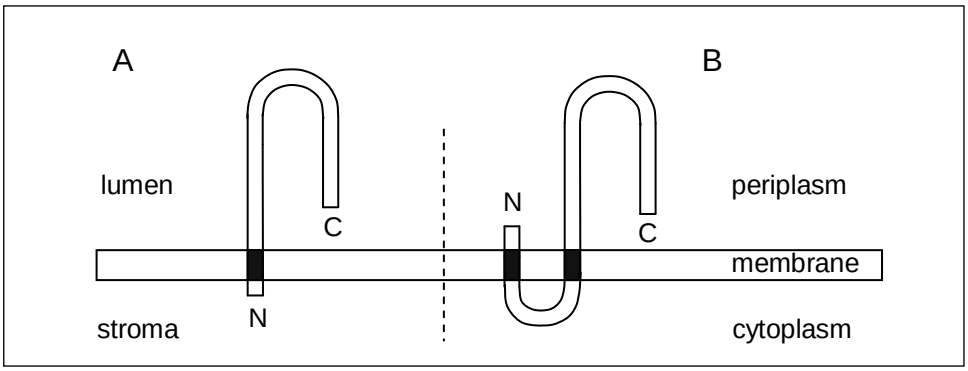

Figure 4. Illustration of topology of the signal peptidases in the membrane (adapted from Dalbey, 1997). Left, on part A, the presumable orientation of the thylakoid processing peptidase is shown. The orientation of the signal peptidase of *E. coli* is shown on part B. The transmembrane regions are filled with black colour.

The comparison of the different peptidases of type I revealed the presence of some conserved regions in the amino acid sequences (Paetzel et al., 2002). The site-directed mutagenesis of the *E. coli* signal peptidase in the conserved regions revealed that two amino acids are essential for catalytic activity: serine 90 and lysine 145 (Dalbey et al., 1997). Lysine residue is typical for the catalytic site of the mitochondrial and prokaryotic leader peptidases, whereas the homologous region of the leader peptidase from endoplasmic reticulum contains histidine residue instead (Paetzel and Dalbey, 1997). The substrate specificity of this peptidase is determined by the amino acids Ile144 and Ile86, and Ile144 is important for the cleavage at the correct site (Karla et al., 2005).

The thylakoid processing peptidase (TPP) has its catalytic site on the luminal side of thylakoid membrane (Kirwin et al., 1988). As well as the leader peptidase from *E. coli*, the TPP belongs to the type I of signal peptidases. TPP cuts off the transit peptide from the N-terminus of the precursor protein. The proteolytic mechanism of TPP is similar to that of leader peptidase of *E. coli*, as the catalytic active residues – serine and lysine are conserved in these proteins (Chaal et al., 1997). These catalytic residues are inhibited by the inhibitor penem which is known to inhibit activity of the signal peptidase from *E. coli* (Barbrook et al., 1996).

The signal peptidase is an essential enzyme for *E. coli*. The study of a conditional-lethal mutant has shown that, in the absence of signal peptidase, the protein substrates are not

released after the translocation, but remain bound to the membrane (Dalbey and Wickner, 1985). In contrast, *Bacillus subtilis* encodes five type I leader peptidases with overlapping substrate specificity and different importance for the cell (Tjalsma et al., 1998). This redundancy suggests differential roles for these enzymes in the cellular processes (Bonnemain et al., 2004).

In cyanobacteria, there are two independent membrane systems, which are targets for protein-carrying signal peptides: the cytoplasmic membrane and the thylakoid system. Both membranes carry leader peptidase activity, as was shown for example for *Phormidium laminosum* (Packer et al., 1995). In line with that, most cyanobacterial genomes analyzed so far contain at least two genes encoding proteins with homology to type I leader peptidases. According to Cyanobase, the genome of *Synechocystis* 6803 encodes leader peptidases of both type I and type II (table 1). In the cells of most cyanobacteria at least two genes encoding for leader peptidases are found, in *Synechocystis – lepB1* (sll0716) and *lepB2* (slr1377), which show homology to the unique leader peptidase of *E. coli* (Chaal et al., 1998). The signal peptidase of type II is encoded in *Synechocystis* by the *lspA* gene

1.4. Aims of this work

The translocation pathways in thylakoid membranes of plants are intensively studied. However, the information about the exact role of thylakoid processing peptidase in thylakoid membrane biogenesis is limited. For the study of photosynthesis related processes, a well-studied model object, *Synechocystis* sp PCC 6803 is very suitable as its genome was completely sequenced and it is easy amenable for targeted genetic modifications. In order to examine the specific function of the two putative leader peptidases (LepB1, LepB2) encoded in the genome of the cyanobacterium *Synechocystis* 6803, inactivation mutants were generated by insertions of kanamycin resistance cassettes into the respective open reading frames. The function of the leader peptidases was studied by characterization of the mutant phenotype with different physiological and biochemical approaches. In addition we analysed the complementation of LepB1 with homologous protein LepB from *Escherichia coli*.

2. Materials and Methods

2.1. Chemicals and enzymes

For this study chemicals of p.a. quality were used. The suppliers were:
Difco-Laboratories (Detroit, USA), Merck (Darmstadt), Serva (Heidelberg), Biomol (Hamburg), Applied Biosystems (Roche Diagnostics), Amersham Biosciences (Freiburg), Roth (Karlsruhe) and Sigma-Aldrich (Munich).
Enzymes were purchased from Sigma-Aldrich (Munich), Boeringer Ingelheim, MBI Fermentas (St.Leon) and New England Biolabs (Frankfurt am Main).

2.2. Bacterial strains and plasmids

Table 2

Bacterial strains and plasmids used in this work

A. Strains	**Genotype or phenotype**	**References**
Escherichia coli		
JM109	*recA1 endA1 gyrA96 thi hsdR17 (r_k- m_k+) relA supE44 λ- Δ(lac-proAB) [F' traD36 proAB+ lacqZ ΔM15]*	Yanisch-Perron et al., 1985
BL21(DE3)	*F- dcm hsdS gal(cIts857) ind1 Sam7 nin5 lacUV5-T7 gene 1*	Studier and Moffat, 1986
DH5α	*F- endA hdR17 supE44 thi1 λ- recA1 gyrA96 relA1 (argF-laczya) U169 φ80dlacz ΔM15*	Hanahan 1983
C600	*F- thi-1 thr-1 leuB6 lacY1 tonA21 supE44 λ-*	Sambrook et al., 1989
M15 (pREP4)	*F–*, NaIS, StrS, RifS, *thi– gal lac-* , Ara+, Mtl–, , RecA+, Uvr+, Lon+ [*lacl F-lacZ (am) pho (am) lon trp (am) tyrT[supC (ts)] rpsL (Str R)mal (am)*	Qiagen
***Synechocystis* sp. PCC 6803**	Wild type	Genetic dept. MSU, Russia

B. Plasmids	**Genotype or phenotype**	**References**
pACYC184	Tc^r Cm^r	Rose, 1988

pCRII	Ap^r Km^r	Invitrogen
pGEM-T Easy	Ap^r Lac^+	Promega
pUC-4K	Ap^r Km^r	Taylor and Rose, 1988
pSL762	Ap^r Gm^r	Schweizer, 1993
pVZ321	IncQ Km^r Cm^r	Zinchenko et al., 1999
pSLEP1	IncQ Cm^r *lepB1*	Zinchenko, pers communication
pELEPB	IncQ Cm^r *lepB* (from *E. coli*)	Zinchenko, pers communication
pET3a	Ap^r	(Rosenberg et al., 1987)
pET21		Novagen
R571	IncP Tp^r Tra^+	Meyer and Shapiro, 1980
pRD8	Ap^r	Dalbey, 1985

* Abbreviations: Inc, Incompartability group; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamycin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Tp^r, trimethoprim resistance; Tra^+, capability of the plasmid to conjugative transfer.

Table 3.

Mutant strains of *Synechocystis* 6803, used in this work

Strain	Genotype	Reference
LepB1::Km^R	Inactivation of *lepB1* (sll0716) Complete segregation	this work
LepB2::Km^R	Inactivation of *lepB2* (slr1377) Uncomplete segregation	this work.
TatA::Gm^R	Inactivation of *tatA* (slr1046) Uncomplete segregation	Zinchenko et al., isolated at year 1998
TatB::Km^R	Inactivation of *tatB* (ssl2823) Uncomplete segregation	Zinchenko et al., isolated at year 1998
TatC::Km^R	Inactivation of *tatC* (sll0194) Uncomplete segregation	this work
SecA::Km^R	Inactivation of *secA* (slr0616) Uncomplete segregation	Zinchenko et al., isolated at year 1997
SecY::Km^R	Inactivation of *secY* (slr1814) Uncomplete segregation	Zinchenko et al., isolated at year 1997
FtsY	Inactivation of *ftsY* (slr2102) Uncomplete segregation	Zinchenko et al., isolated at year 1997
Ffh	Inactivation of *ffh* (slr1531) Uncomplete segregation	Zinchenko et al., isolated at year 1998

slr1378::Km^R	Inactivation of the unknown gene downstream of slr1377, Complete segregation	Zhbanko et al., isolated at year 2002
pLepB1compl	LepB1::Km^R strain with sll0716 expressed from pVZ321	Zinchenko et al., isolated at year 2000
pLepBEcompl	LepB1::Km^R strain with *lepB* (E.coli) expressed from pVZ321	Zinchenko et al., isolated at year 2000

2.3. Oligonucleotides

Olygonucleotides for this work were synthesized by Metabion GmbH (Munich, Germany) or MWG Biotech GmbH (Ebersberg, Germany) and stored in aqueous solution at -20°C.

Table 4.

Oligonucleotides used in this work

Name	Sequence (5'-3')
sll0716F	5' CCTGCTGCTGCGTTTCTTTGT3'
sll0716R	5´ GGGGTGTCGGGTATTAGGTATTG 3´
lep1043rev	5' CTTTAGCTCTGGTCGCCGTGA 3'
SynLep1forw	5´ GCATATGCAAAATTCCCCCATCC 3´
SynLep1rev	5' GAAGCTTAGGTATTGATGATGGTACG 3'
SynLep1Bam	5'-CGGATCCTTAGGTATTGATGATGGTACG-3'
lepB2forw	5´ CCCACCAGGAAGAAGAAGAGG 3´
lepB2rev	5´ CTGTTGTTGCGGTTATCCCCTA 3'
slr1378forw	5´ CCCAGTGAAAGTGCCCGATG 3´
slr1378rev	5´ GTGGGCTGCTTTGGTTCCCC 3´
lepBforw	5' GCATATGGCGAATATGTTTGCCCTG 3'
lepBrev	5' AGTCGACGATGGCTATTAATGGATGCCG 3'
sll0194F	5' GCATATGTCAACCCAGCTTGATAAC 3'
sll0194R	5' GGATCCTATTTACCCAGTAAGCGCAC 3'

2.4. Molecular weight markers for gel electrophoresis

DNA-size marker: 1 kb ladder (Gibco BRL); fragment sizes 12216 bp, 11198 bp, 10180 bp, 9162 bp, 8144 bp, 7126 bp, 6108 bp, 5090 bp, 4072 bp, 3054 bp, 2036 bp, 1636 bp, 1018 bp, 517 bp, 506 bp, 396 bp.

To determine the size of the proteins, following molecular weight markers were used.

Table 5.

Molecular weight standards used for protein electrophoresis

Marker	Sizes of the fragments, kDa	used for
SDS-VII-L (Sigma)	66, 45, 36, 29, 24, 20.1, 14.2	SDS-PAGE
10 kDa protein ladder (Gibco BRL)	200, 120, 110, 100, 90, 80, 70, 60, 50, 40, 30, 20, 10	SDS-PAGE
SDS VII-B (Sigma)	118, 116, 84, 58, 48.5, 36.5, 26.6	Western Blots
HMW standard (Amersham Biosciences)	669, 440, 232, 140, 67	Blue native PAGE

2.5. Cultivation of *Escherichia coli* cells

E. coli strains were cultivated in LB medium. When necessary, the medium was supplemented with antibiotica solutions sterilized by filtration. For overexpression, different media were tested. For preparation of the competent *E. coli* cells for electroporation, S.O.B. medium was used. By the electroporation, SOC medium was used.

LB

1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl

S.O.B.

2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, after autoclaving KCl and MgCl2 were added to the end concentration of 2.5 mM and 10 mM respectively.

SOC

SOB medium with 20 mM glucose

All media were adjusted to pH 7.0 with NaOH.

2.6. Cultivation of *Synechocystis* sp. PCC6803 cells

Synechocystis sp. PCC 6803 cells were cultivated in BG11 medium (Rippka et al.,1979) at 30°C with stirring at 100 rpm. For photomixotrophic conditions, the growth medium was supplemented with 5 mM glucose. The mutant strain was kept under light intensity of 5 µE/m^2×sec. For photoautotropic conditions, the cultures were illuminated with 50 µE/m^2×sec. For photoheterotropic conditions the medium was supplemented with 5 mM glucose and 10 mM DCMU and the cultures were grown under light intensity of 50 µE/m^2×sec.

BG11-medium

			Trace elements	
$NaNO_3$	18	mM		
$K_2HPO_4×3H_2O$	0.23	mM		
Citric acid	0.031	mM	H_3BO_3	46 µM
Ammonium ferric citrate	0.02	mM	$MnCl_2×4H_2O$	9.1 µM
$Na_2MgEDTA$	2.8	µM	$ZnSO_4×7H_2O$	7.7 µM
Na_2CO_3	0.19	mM	$Na_2MoO_4×2H_2O$	1.6 µM
			$CuSO_4×5H_2O$	0.32 µM
$MgSO_4×7H_2O$	0.40	mM	$Co(NO_3)_2×6H_2O$	0.17 µM
$CaCl_2×2H_2O$	0.24	mM		

5× BG11 stock solution was prepared and autoclaved separately from the Mg and Ca salts and trace elements. 1000× concentrated solutions of $MgSO_4×7H_2O$, $CaCl_2×2H_2O$ and solution of trace elements were autoclaved separately too and were added to liquid or solid media just prior to use.

Solid media plates were supplemented with 1% agar (Difco). When necessary, sterile glucose in concentration of 5 mM or filter-sterilized $NaHCO_3$ in concentration of 10 mM were added prior to inoculation.

2.7. Transformation of *E. coli* cells

Transformation with *E. coli* cells was conducted according to Sambrook et al., (1989) using either the $CaCl_2$ method or the electroporation method with Bio-Rad Gene Pulser according to the instructions of the supplier (Biorad).

2.8. Transformation and conjugation of *Synechocystis* 6803 cells.

The cells of *Synechocystis* 6803 are naturally transformable (Shestakov and Reaston, 1987). The only request for successful transformation is the early logarithmic growth phase of competent cells. The cells from 1 ml of the culture with OD_{730} 0.3-0.5 (normally 3 days old culture) were centrifuged at 2,000 g for 5 minutes and the cell sediments were resuspended in 50 µl of fresh BG11 medium (1/20 of original volume). Then 0.1-0.3 µg DNA was added and the suspensions were incubated at 30°C for 18 h in the light of 20 $\mu E/m^2 \times sec$. Subsequently the transformation suspensions were plated on BG11 plates containing 10 µg/ml kanamycin. After 2 weeks the transformants became visible and were transferred to BG11 plates with higher concentration of antibiotic.

Since *Synechocystis* 6803 contains 12-15 chromosome copies per cell (Labarre et al., 1989), the higher selective pressure was needed for complete segregation of the mutant gene copies. Therefore, the antibiotic concentration in the medium was raised up to 100 µg/ml. After several rounds of restreaking, the segregation was analysed by PCR.

If the first attempts of segregation were unsuccessful, the antibiotic concentration was stepwise increased to 200 µg/ml. Segregation was monitored by PCR.

The plasmids pSLEP1 and pELEPB were introduced into *Synechocystis* 6803 *lepB1*::Km^R mutant via conjugation (Zinchenko et al., 1999). The donor *E. coli* culture and helper strain (with R751 plasmid) were grown over night. Then, 100 µl of each *E. coli* culture were mixed with 600 µl of *Synechocystis* 6803 culture grown until late exponential stage, centrifugated at 3000 rpm for 5 min and resuspended in 50 µl of saline. The conjugation mixture was dropped on the membrane filter with pore size of 0.45 µm and incubated on BG11 plates supplemented with 5% LB medium and 5 mM glucose. After 1 day of incubation under dim light, the membrane was transferred on the BG11 plates supplemented with antibiotic (for pVZ321M-

derivede plasmids, the antibiotic chloramphenicol (Cm) was used in concentration of 3-5 µg/ml. The transconjugants appeared normally after 2-3 weeks after incubation on selective medium.

2.9. Harvesting of *Synechocystis* 6803 cells

For physiological and biochemical analysis of the *Synechocystis* 6803cells the cultures were harvested as they reached the late logarithmic phase of growth. This growth stage corresponds to an optical density of the culture (OD_{730}) of 0.6-0.7. The cells were collected by the centrifugation of the cultures at room temperature for 10 minutes at 3000 g. The cell pellet was resuspended in fresh BG11 medium in 1/100 of original volume. After the determination of the chlorophyll concentration, the aliquots of cell suspension were prepared for an experiment. For later processing, the cell pellet could be stored in thylakoid buffer at -70°C.

2.10. Preparation of stock cultures

Both *E. coli* and *Synechocystis* 6803 cultures can be stored in stock cultures at low temperatures. For preparation of the stock cultures of *E. coli*, glycerole was added to the overnight culture to a final concentration of 10%. Then the vials were rapidly frozen in liquid nitrogen and stored at -80°C.

Cryopreservation refers to the indefinitely long storage of living organisms at ultra-low temperatures. After such preservation the cultures can be revived in the same state as before storage. The temperature of storage corresponds to the temperature of liquid nitrogen, approximately –130°C, at which frozen water no longer recrystallizes. To prepare stock cultures of *Synechocystis* 6803, the method of Brand (http://www-cyanosite.bio.purdue.edu/protocols/cryo.html) was used. The cultures of logarithmic phase of growth were pelleted and resuspended in fresh BG11 medium, which was diluted twice. To avoid the osmotic stress or the ice crystal damage during freezing and thawing processes, the cryoprotective compound DMSO was added to a final concentration of 8%. The tubes with cultures were gently shaken and protected from strong light, as the cells are highly light sensitive in the cryoprotective solution. The tubes were prechilled for 2 h at -70°C and then transferred for the storage in liquid nitrogen.

For re-thawing, the cryocultures were taken out and warmed rapidly to room temperature. The cell pellets were centrifuged at low speed (3000 rpm) for 5 minutes. The cells were resuspended in fresh BG11 medium and incubated for 1-2 days at 30°C in complete darkness. Subsequently, the cells were inoculated as usual in liquid BG11 medium or on the agar plates.

2.11. *Synechocystis* 6803 growth curves

To measure the growth features of *Synechocystis* 6803 cells the cultures were grown simultaneously until the cultures reached OD_{730} of 1.0. Then the cultures were diluted in fresh medium to the OD_{730} of 0.04. The growth was monitored twice a day by measuring of the optical density at 730 nm with a Schimadzu spectrophotometer (Schimadzu, Japan).

2.12. Molecular biology methods

2.12.1. Standard methods

Basic methods of molecular biology were conducted as described by Sambrook et al. (1989). Enzymatic modifications of DNA were performed according to the instructions of the suppliers. Plasmid DNA isolation was performed with the alkaline method from 1.5 – 3 ml over night culture (Birnboim and Doly, 1979). Recombinant plasmids based on pVZ321 were isolated by boiling method (Holmes and Quigly, 1981). DNA sequencing was carried out using ABI method and DNA sequencing Kit (ABI Prism dRhodamine).

2.12.2. Polymerase chain reaction

DNA fragments were amplified by PCR using specific primers and the *Taq* DNA polymerase from Promega (White et al., 1989). The reaction mixture of 20 to 50 µl contained 1 U/200 µl Taq-polymerase, 0.4 pmol of each primer, approximately 0.2-2.0 µg of genomic DNA in a buffer containing 3 mM $MgCl_2$ and 0.2 mM dNTP mixture. The reaction was carried out in a Biometra thermocycler. The reaction mixture was heated for 5 minutes at 95°C, then 30 cycles each with three steps were programmed 1) denaturation of DNA for 40 sec at 95°C, 2)

annealing for 45 sec at 56°C and 3) elongation for 1 min per 0.5 kb of DNA fragment at 72°C. The reaction was terminated after a final elongation step for 10 min at 72°C. DNA fragment of interest was isolated from the reaction mixture by extraction from the gel.

2.12.3. Isolation of genomic DNA from *Synechocystis* 6803 cells

For DNA isolation, the cultures were grown until the late logarithmic phase (i.e. OD_{730} 0.9-1.0). The cells from 3 ml of culture were pelleted by centrifugation for 2 minutes at 11600 g. Then the cells were washed with 1 ml Tris/EDTA buffer of pH 8.0 containing 50 mM Tris-HCl and 50 mM EDTA and resuspended in 270 µl of STET (8% (w/v) sucrose, 5% (v/v) Triton X-100, 50 mM EDTA and 50 mM Tris-HCl, pH 8.0). The cell suspensions were shaken for 5 minutes with 15 µl of chloroform. After this treatment, the suspensions were incubated for 30 minutes at 37°C upon addition of 30 µl of lysozyme (20 µg/ml). 100 µl 10% SDS was added for cell lysis and the suspensions were incubated for 60 minutes at 65°C. After addition of 100 µl 5 M NaCl, the solution was deproteinized three times by extraction with the same volume of chloroform. The chromosomal DNA was precipitated from the aqueous solution by addition of the same volume of isopropanol, centrifuged at 4°C and 13000 rpm for 20 min, washed three times with 70% ethanol and resolved in 30-40 µl of TE buffer.

2.12.4. Isolation of plasmid DNA from *Synechocystis* 6803 cells

For isolation of plamids by the alkaline method, the cells were grown in the presence of selective antibiotic for 2 weeks. Cells from 3 ml culture were pelleted and resuspended in 1 ml of Tris/EDTA buffer of pH 8.0 containing 50 mM Tris-HCl and 50 mM EDTA and centrifuged at 5000 rpm for 2 min. The cell suspensions were treated for 5 minutes with 200 µl of chloroform and the cells were collected from aqueous phase and resuspended in 280 µl of STET buffer (8% (w/v) sucrose, 5% (v/v) Triton X-100, 50 mM EDTA and 50 mM Tris-HCl, pH 8.0). Then the suspensions were incubated at 37°C for 20-40 min upon addition of 20 µl of lysozyme (20 µg/ml). For cell lysis, 600 µl of 0.2 M NaOH solution containing 1% SDS were added, and suspensions were incubated 10 min at room temperature. After addition of 450 µl of 3 M Na-acetate pH 5.2, the cell debris was precipitated by centrifugation for 15

minutes at 11600 g. Plasmid DNA was precipitated from the supernatant with same volume of isopropanol, centrifuged, washed three times with 70% ethanol and resolved in 50 µl of TE buffer.

2.12.5. Construction of recombinant plasmids

For cloning of genes of *Synechocystis* 6803 for subsequent inactivation, the DNA-fragments were amplified by PCR using appropriate primers (listed in table 5). The amplified fragments were cloned with the pGEM T-easy vector (Promega) after transformation of *E. coli* strain JM109. The resulting transformants were screened by PCR using M13 primers. Appropriate recombinant plasmids were isolated and sequenced.

For insertional inactivation of the gene, the recombinant plasmid was linearized at a unique endonuclease restriction site within the *Synechocystis* 6803 gene, and ligated with a kanamycin-resistance cassette, cutted from pUC4K using a corresponding cleavage site. After transformation of *E. coli* strain JM109 with the ligation mix, the clones containing the respective recombinant plasmid were selected on a medium with kanamycin. The plasmid DNA was dissolved in TE buffer and used for transformation of *Synechocystis* 6803 cells.

For introducing of the complementing genes into a *Synechocystis* 6803 mutant, the vector pVZ321M that allows expression of a cloned gene from regulatory elements of the aphII gene conferring the resistance to kanamycin was generated by group of Zinchenko V. It was constructed from the 9.2 kb Km^R Cm^R RSF1010-derived vector pVZ321 that replicates autonomously in cyanobacterial cells (Zinchenko et al., 1999; accession number AF100176). To generate pVZ321N, the *Nde*I site was introduced in front of the ATG start codon of the *aphII* gene of pVZ321 using the mutagenic primers 5'-CAGTAATACAAGGGGTCATATGAGCCATATTCAACGGG-3' and 5'-CCCGTTGAATATGGCTCATATGACCCCTTGTATTACTG-3', and the QuikChange site-directed mutagenesis kit (Stratagene).

Complementation studies were performed with the full-length *lepB1* gene of *Synechocystis* 6803 and full-length *lepB* gene of *Escherichia coli*. The *lepB1* coding region was amplified from genomic DNA of *Synechocystis* 6803 by PCR using the *Nde*I creating primer SynLep1forw to introduce an *Nde*I restriction site in front of the ATG start codon, and the *Hind*III creating primer SynLep1rev to generate a *Hind*III site downstream of the TAA stop codon (Table 4). Corresponding *Nde*I-*Hind*III fragment was cloned into the vector pVZ321N

opened with both *Nde*I and *Hind*III, yielding the plasmid pSLEP1. The *lepB* gene of *E. coli* was amplified by PCR using *Nde*I creating primer *lepB*forw to introduce an *Nde*I restriction site in front of the ATG start codon, and the *Sal*I creating primer *lepB*rev to generate a *Sal*I site downstream of the TAA stop codon (Table 4). As the template for this PCR, DNA of the pRD8 plasmid (Dalbey and Wickner, 1985) was used. Corresponding *Nde*I-*Sal*I fragment was cloned into the vector pVZ321N opened with both *Nde*I and *Sal*I, yielding the plasmid pELEPB. The plasmids pSLEP1 and pELEPB were introduced into Synechocystis sp. PCC 6803 *lepB1*::Km^R mutant via conjugation (Zinchenko et al., 1999).

For overexpression of LepB1 protein from *Synechocystis* 6803 in *E.coli*, the pET28 plasmid (Novagen, Madison) was used. The *lepB1* coding region was amplified from the genomic DNA of *Synechocystis* 6803 by PCR using the *Nde*I creating primer SynLep1Forw to introduce an NdeI restriction site in front of the ATG start codon, and the *Bam*HI creating primer SynLep1Bam to generate a *Bam*HI site downstream of the TAA stop codon (Table 4). After restriction enzyme digestion, the *Nde*I-*Bam*HI fragment was ligated in frame with the pET3a (pAR3040) plasmid (Rosenberg et al., 1987). Recombinant plasmids containing the correct insert were used for transformation of *E. coli* strain BL21(DE3) (Novagen). The resulting cell cultures were used for expression of the LepB1 protein.

The cells carrying the expression plasmids were grown at 37°C in LB medium containing 50 µg/ml ampicillin. The growth was monitored by measurement of the optical density at 600 nm (OD600). When the cultures had reached a density of 0.8, the expression of the protein was induced by the addition of the isopropyl-β-D-thiogalactopyranoside (IPTG) to the final concentration of 1 mM. The cultures were incubated for an additional time of 2 hours. The optical density of the cultures should not exceed 1.2 for effective protein expression. The cultures were collected by centrifugation at 6,000 x g for 15 minutes and were suspended in 20 mM Tris HCl, pH 7.5.

After that, lysozyme was added, and the cells were disrupted by sonication.

2.13. Biochemical methods

2.13.1. Determination of protein concentration

The protein concentration was determined using the Bio-Rad protein assay Kit according to the instructions of the supplier.

2.13.2. Protein precipitation

The proteins in solutions were precipitated with 1/10 volume of cold 100% trichloracetic acid TCA (w/v) for one hour on ice. The precipitated proteins were pelleted by centrifugation at 11,600 g for 15 minutes. The pellet was washed with acetone and dried in a vacuum dryer (Bachofer, Reutlingen, Germany). The proteins were resolved in a loading buffer (Laemmli, 1970).

2.13.3. Isolation of expressed protein from *E. coli*

Small scale isolation of expressed proteins

The plasmids carrying the recombinant genes for protein expression were transformed freshly into the *E. coli* strain BL21 (DE3). The colonies selected from LBG/amp plates were inoculated in 3 ml of fresh LBG medium supplemented with ampicillin to a final concentration of 100 µg/ml and grown overnight. The 1:100 diluted cultures were incubated at 37°C until they reached an OD_{600} of 1.0. Then, the cells from 1 ml of the culture were pelleted to prepare the (–) fraction in Laemmli buffer. For induction of protein expression 1 mM IPTG was added to 2 ml of culture and the cultures were incubated at 37°C for additional 3 h. The cells from these cultures were used to prepare the (+) fraction.

Large scale isolation of expressed protein in inclusion bodies

The overnight culture was diluted 1:100 in the large volume of fresh LBG medium containing 100 µg/ml ampicillin and incubated at 37°C until it reached an OD_{600} of 1.0. Expression of the protein was induced by the addition of IPTG to a final concentration of 1 mM. Then, the cultures were incubated at 37°C for 1-3 h. The cells were collected by centrifugation at 2,000 g for 10 min, washed in 200 ml of the 50 mM TrisHCl pH 8.0 and resuspended in 20 ml of the same buffer. The cells were twice disrupted using a French press at 1000 psi. After centrifugation of the cell lysate at 8000 rpm (7600 g) for 30 min, the pellet was resuspended in 50 mM TrisHCl pH 8.0, containing 20% saccharose. The suspension was layered on 20 to 40% sacharose gradient in 50 mM TrisHCl pH 8.0, 25 mM NaCl, 1.5 mM EDTA and inclusion bodies were centrifuged at 10000 rpm for 20 min. The pellet was washed 4 times with a solution containing 10 mM TrisHCl, pH 8.0, 0.1 mM EDTA, 50 mM NaCl, 0.5 mM PMSF. In the first washing step this solution was supplemented with 400 mM NaCl and 0.5%

NP-40. In the second step the solution was supplemented with 0.5% NP-40 and 1 mM DTT, in the third step with 1 mM DTT. The last washing step was carried out with a solution supplemented with 1 mM DTT and 1 M Urea. Finally the pellet was resolved in a solution containing 7 M Urea and 30 mM HEPES/KOH, pH 7.5. For the monitoring of protein isolation, aliquots equal to 1/1000 of original volume were prepared for SDS-PAGE.

Antibodies

Polyclonal antiserum was raised against expressed LepB1 protein. The protein was excised from the separation gel, and used for rabbit immunisation. This was performed by Dr. H. Schubert from the Instute für Versuchstierkunde of Friedrich-Schiller University in Jena.

2.13.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

For the separation of the polypeptides according to their molecular weight, SDS polyacrylamide electrophoresis with a Tris-glycine buffer system was used (Laemmly et al., 1970). The sample gel was made of 5% acrylamide, and the separation gel was made either from 12.5% acrylamide or from two acrylamide solutions, 10 and 17,5%, in case of gradient gels.

Samples were mixed with 4× sample buffer containing 0.25 M Tris-HCl, pH 6.8, 10% (w/v) glycerol, 8% SDS, 20% (v/v) β-mercaptoethanol, 0.016% (w/v) bromophenolblue and incubated in the 1× sample buffer at 90°C for 3 min. Prior to loading, the samples were cooled and centrifuged at 11,600 g for 3 minutes, and the supernatant was loaded on the gel. When samples contained membrane proteins, they were solubilized in the 1× sample buffer at 60°C for 15 minutes. The electrophoresis was conducted using a running buffer containing 25 mM Tris, pH 8.3, 125 mM glycine, 0.1% (w/v) SDS. For small gels (100×70×1 mm) the running conditions were 2 hours 30 mA/gel. The electrophoresis in standard gels (200×200×1 mm) was conducted at 160 V overnight.

2.13.5. Staining of polyacrylamid gels

The polyacrylamide (PAA) gels were stained after electrophoresis either with coomassie or with silver nitrate.

A coomassie staining solution containing 42.5% (v/v) ethanol, 5% (v/v) acetic acid, 0.4% (w/v) coomassie blue R-250, 0.04% (w/v) coomassie blue G-250 was used for incubation of the gel for one hour or over night. The gels were destained with a solution containing 20% (v/v) methanol and 7% (v/v) acetic acid.

To detect low amounts of the proteins in the gel, the gels were stained with silver nitrate according to Blum et al., 1987.

2.13.6. Staining of heme-containing proteins

For SDS electrophoresis of heme-binding proteins, the gels and the running buffer were supplemented with a reduced amount of SDS (0.01% (w/v) instead of 0.1% (w/v)). To protect the cytochromes from the loss of the heme group, the electrophoresis was conducted at 4°C and in the dark. The loading buffer contained 0.05 M Tris-HCl, pH 6.8, 2% (w/v) glycerol, 4% (v/v) β-mercaptoethanol, 0.004% (w/v) bromophenolblue and 0.1% SDS. The concentration of SDS in PAA gel was also reduced to 0.1% (Tichy and Vermaas, 1999). The weak peroxidase activity of the proteins containing a heme group allows the detection of these proteins in the gel with 3,3',5,5'-tetramethylbenzidine (TMBZ) and H_2O_2 (Thomas et al., 1976). A 6.3 mM TMBZ solution was freshly prepared in methanol. Immediately before use, 3 parts of the TMBZ solution were mixed with 7 parts of 0.25 M sodium acetate, pH 5.0. After electrophoresis, the gels were incubated in this mixture 1 to 2 hours with occasional mixing at room temperature in the dark. Then H_2O_2 was added to a final concentration of 30 mM, and the heme-containing protein bands became visible. The staining appeared within 3 min and increased in intensity over the next 30 min. To remove any precipitated TMBZ, the gels were placed in 30% isoropanol solution buffered wit 0.25 M sodium acetate pH 5.0. This solution was replaced to clear the gel background.

2.13.7. Western Blot Analysis

For immunological analysis, proteins were transferred on polyvinylidenfluorid (PVDF) membranes (Millipore Corporation, Bedford, MA) using the semi-dry blotting system from Bio-Rad. Prior to the transfer the gels were incubated for 30 minutes in blotting buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine and 10% (v/v) methanol). After the transfer of the

proteins onto the membrane, it was reversibly stained with Ponseau S solution (0.2% (w/v) Ponseau, acetic acid) to visualize the molecular weight standard VII-L. Prior to applying the first antibody, the membrane was blocked with PBST buffer (137 mM NaCl, 1.5 mM KH_2PO_4, 7.9 mM Na_2HPO_4, 2.7 mM KCl, 0.5% (v/v) Tween-20) containing 1.5% (w/v) skimmed milk. After one hour of blocking the first antibody was applied in the concentrations shown in the Table 6, and the gel was incubated for an additional hour.

After incubation with the secondary antibody (anti-rabbit horseradish peroxidase conjugate (Sigma) in the dilution 1:15000), the membrane was washed 4 times with the PBST buffer. To start the ECL reaction, the membrane was incubated with a solution containing 100 mM Tris-HCl, pH 8.5, 2.5 mM luminol, 0.4 mM p-coumaric acid and 0.01% (v/v) H_2O_2. The signal was documented using a X-ray film.

To estimate the protein size in the Western blot, the molecular weights of protein bands were compared with molecular weight standards: VII-L (Sigma), which was visualized by Ponseau staining, and prestained VII-B (Sigma).

The thylakoid membranes from spinach were used as positive control for Western blot analyses.

Table 6.

Antisera used for Western blot

Antiserum	Origen of the antigen	Dilution	Source of the antisera
PsbO	*Spinacia oleracea*	1:1000	Laboratory of Prof. Dr. R.B. Klösgen, University of Halle-Wittenberg
Anti-CF_0II	*Spinacia oleracea*	1:1000	Laboratory of Prof. Dr. R.B. Klösgen
Anti-PSI-3	*Spinacia oleracea*	1:1000	Laboratory of Prof. Dr. R.B. Klösgen
Anti-Rieske	*Spinacia oleracea*	1:1000	Laboratory of Prof. Dr. R.B. Klösgen,
Anti-PC	*Spinacia oleracea*	1:1000	Laboratory of Prof. Dr. R.B. Klösgen,
Anti-cyt *f*	*Spinacia oleracea*	1:1000	Laboratory of Prof. Dr. R. Malkin, University of California
PsaA/B	*Synechococcus elongatus*	1:1000	Laboratory of Y. Inoue, University, RIKEN, Japan
LepB1	*Synechocystis* 6803	1:1000	this work
slr0924	*Synechocystis* 6803	1:1000	Fulda S. et al., 2002
Anti -D1	*Spinacia oleracea*	1:300	Johanningmeier U., 1988
Anti-CP43	*Chlamydomonas reinhardtii*	1:300	Laboratory of Prof. Dr. U Johanningmeier, University of Halle-Wittenberg

2.13.8. Isolation of membranes from *Synechocystis* 6803

Cells from the late exponential phase were pelleted and were resuspended in thylakoid buffer (1/100 of the original culture volume) containing 50 mM Hepes-NaOH, pH 7.0, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 10% glycerol (v/v) and 0.5% DMSO. Cell suspensions (0.7 ml) were transferred to 2-ml microcentrifuge tubes, mixed with an equal volume of glass beads (0.1 mm diameter), and kept on ice. The cells were broken in a minibead beater (Thermo Savant, USA) two times at the highest speed for 30 sec with 2 min interruption for cooling on ice, and then centrifuged at 1,000 rpm for 1 min in a Heraeus centrifuge (Kendro). The glass beads were washed twice to increase the yield of thylakoid membranes. The homogenate was centrifuged at 4,000 rpm for 10 min at 4°C to remove cell debris. The supernatant was centrifuged at 14000 rpm at 4°C for 20 min to pellet thylakoid membranes. The pellet was resuspended in the original volume of thylakoid buffer.

For heme staining, the thylakoid membranes were isolated in the same manner as described above, with following buffer: 50 mM MES/NaOH, pH 6.0, 5 mM $MgCl_2$, 5 mM $CaCl_2$ and 10% glycerol. The thylakoid membranes were pelleted at 21,000 rpm in Super T21 centrifuge (Sigma) for 30 minutes.

2.13.9. Blue native PAGE

Blue native PAGE was carried out as described in (Berghöfer and Klösgen, 1999) with some modifications.

<u>The sample preparation</u>

The *Synechocystis* 6803 thylakoid membranes were prepared as described in 2.13.8. Thylakoid membranes corresponding to 30 µg chlorophyll were resuspended in 25 µl of lysis buffer (50 mM Bis-Tris pH 7.0, 1 M ε-aminocaproic acid, 5 mM EDTA, 0.5 mM $MgCl_2$, 1 mM PMSF, 1 mM DTT) and supplemented with 8 µl 5% (w/v) digitonin. Solubilisation was carried out on a rotating mixer at 4°C for one hour. Unsolubilized membranes were pelleted by centrifugation at 40,000×g for 1 h. The supernatant was supplemented with 1.5 µl 5% (w/v) Coomassie G-250 and incubated on ice for 10 min, and then loaded on the gel.

<u>Composition of the PAA gel for blue native electrophoresis.</u>

For blue-native separation of the protein complexes, the 5-13.5% gradient gel with a 4% stacking gel containing 0.03% (w/v) digitonin was prepared (Schägger and von Jagow, 1991; Schägger et al., 1994) (Table 7).

Table 7.

Solutions for blue native PA gel (200 x 200 x 1 mm)

Solution	Stacking gel	Resolving gel	
		5%	13.5%
H_2O	5.1 ml	5.42 ml	1.2 ml
0.5 M Bis-Tris pH 7.0	1 ml	1.3 ml	1.3 ml
30% acrylamide solution (30% acrylamide, 0.8% bisacrylamide)	1.3 ml	2.2 ml	5.85 ml
2M ε-aminocaproic acid	2.5 ml	3.25 ml	3.25 ml
87% glycerol	-	0.74 ml	1.3 ml
5% digitonin	60 µl	78 µl	78 µl
10% APS	90 µl	50 µl	40 µl
TEMED	9 µl	5 µl	4 µl
Volume	10 ml	13 ml	13 ml

Protein complexes were separated at 100 V and 4°C overnight with a cathode buffer containing 50 mM Tricine, 15 mM Bis-Tris, 0.0075% (w/v) coomassie brilliant blue G-250 and an anode buffer 50 mM Bis-Tris, pH 7.0. The electrophoresis was stopped after the blue front had reached the bottom of the gel.

The gels were scanned for documentation and were either frozen at -80°C or used immediately for separation in the second dimension. Prior to the second dimension the gel strips were equilibrated in a solution containing 1% SDS and 1% β-mercaptoethanol for 10 min at 40°C. The strips were sealed with 0.5% agarose melted in SDS-electrophoresis running buffer.

2.13.10. Determination of chlorophyll content

The chlorophyll concentration was determined after methanolic extraction according to Lichtenthaler, 1987.

Chlorophyll (µg/ml) = $16{,}72 \times OD_{665} - 9{,}16 \times OD_{652}$. The formula is generally used for calculation of the concentration of chlorophyll *a* and *b*, but can be applied also for cyanobacteria, which contain only chlorophyll a.

The 10 µl of a cell suspension or a membrane suspension were added to the 1 ml of methanol. The suspensions were vortexed and kept in the darkness on ice for at least 30 minutes. The cell debris was centrifuged at 11,600×g for 10 minutes, and the ODs of the clarified extract were measured at 665 and 652 nm.

2.13.11. Pigment analysis by HPLC

For qualitative analysis of pigments in *Synechocystis* 6803, reverse phase high performance liquid chromatography (HPLC) was used. The pigments were extracted with a mixture of acetone:methanol in the ratio 3:1 (both solutions were of HPLC-grade). After incubation on ice for 30 minutes, the cell debris was precipitated by centrifugation. To ensure the purity of the extracts, the probes were stored at -80°C for 24 h and centrifuged at 14000 rpm for 20 min. The pigment extracts were analysed in a gradient system with the HPLC pump 322 (Kontron, Munich, Germany) and Rheodine 7125 probe injector ventile (Rheodin Incorporated, USA). The volume of the probe was 20 µl. The pigment separation was carried out according to Doege (1999). To purge the solution from gas bubbles, the degas system DG 1210 was used. (UNIFLOWS, Co., Japan).The pigments were analysed with the diode array detector DAD440 (Kontron, Munich, Germany), which helps to identify the pigments by spectral features. The final identification of the pigments was done by comparison of the spectra with already known data. For management of the separation the programm DS 450-MT1-EMS (KONTRON Instruments, Munich, Germany) was used.

2.13.12. Determination of the cell densities

For the quick determination of the cell number, a rapid essay was developed. The cell density was measured as absorbance of the cell suspension at 730 nm. Then, the cell number was counted in a Thoma cell chamber (Bad Blankenburg, Germany). The dependence of the optical density at 730 nm from the cell number till OD_{730} of approximately 0.9 has linear character (Fig. 5). The cell number per ml was therefore calculated from OD_{730} with a formula 1 OD730 = 7×10^7 cells.

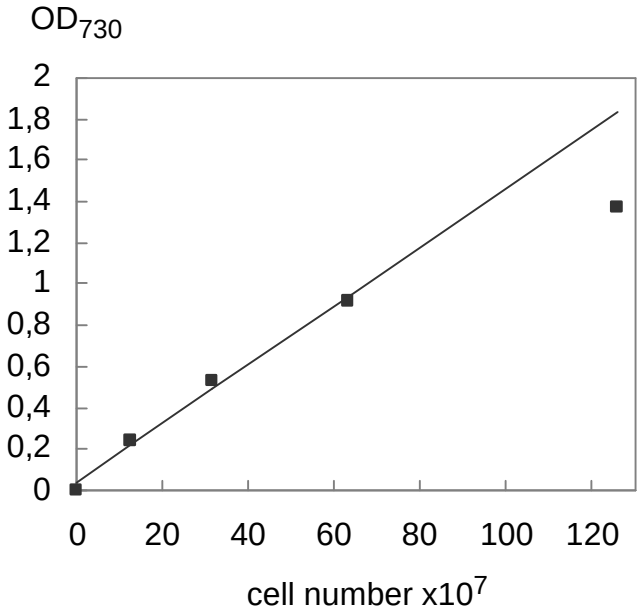

Figure 5. Graphic illustrating the relation between the cell number and optical density of *Synechocystis* 6803 cultures at 730 nm. The cell number was counted in the cell suspension, which was diluted, and the optical densities of the different dilutions were measured at 730 nm.

2.14. Proteomic methods

2.14.1. Two-dimensional gel electrophoresis

Two-dimensional (2D) gel electrophoresis is a powerful modern method, which was applied for proteomic analysis of the lepB1 mutant of *Synechocystis* 6803.

In 2D gel electrophoresis the following reagents were used:

for protein denaturation - 8-9 M urea, which can be partially substituted with thiourea to increase the solubility of the hydrophobic proteins;
detergents - zwitterionic detergent CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate) and non-ionic detergent Triton X-100.
reducing agents - 2-mercaptoethanol; dithiothreitol (DTT)
electrophoretic reagents - carrier ampholyte IPG-buffer (immobiline polyacrylamid gel) and as electrophoresis dye the bromphenol blue was used.

Sample preparation

Total proteins

The cells from 50 ml of culture were pelleted and resuspended in 300 µl chloroform/0.07% β-mercaptoethanol. The suspensions were frozen for 5 min in liquid nitrogen and were subsequently thawed by mixing. The proteins were precipitated by addition of 3 ml aceton/10% TCA/0.07% β-mercaptoethanol and incubation at -20°C for at least 1 hour. The proteins were pelleted by centrifugation at 4°C for 30 min. The pellets were washed with 1 ml of the mixture aceton/0.07% β-mercaptoethanol and were dried in vacuum for 30 min. The proteins were solubilized in 200 ml buffer containing 7 M urea, 2 M thiourea, 2% CHAPS and 2% β-mercaptoethanol. Protein concentration was determined using Bio-Rad Protein Assay Kit based on method of Bradford (BioRad, USA).

Membrane proteins

For the preparation of the samples containing membrane proteins, detergent solubilisation was used. Thylakoid membranes isolated as described in 2.13.9a were mixed with a solution, containing 9 M Urea, 2% triton X-100, 40 mM Tris (base). After allowing the solubilization of proteins for 1 hour at room temperature, the samples were centrifugated at 21000 rpm in ST-micro Rotor (Sigma). The supernatant was then used for application on the IPG-strip together with rehydration solution consisting of 8 M Urea, 0.5% triton X-100, 10 mM dithiothreitol, 0.5% carrier ampholyte (pH 3.0-10.0) and 0.001% bromphenol blue.

Isoelectric focussing

The first dimension was conducted using the immobilized pH gradient (IPG) strips (strip gels), pH 4-7/180 mm or pH 3-10/180 mm (Pharmacia). The strips were rehydrated with a buffer consisting of 8 M urea, 19 mM DTT (dithiothreitol), 2% (w/v) CHAPS, 0.001% (w/v)

bromphenol blue and 0.2% of the carrier ampholyte (Pharmalyte, corresponding to the pH range of the strip) at 20°C for 12 h. Additionally, 250 µg protein was added together with rehydration buffer. The focussing step was carried out in IPGphor (Amersham Biosciences, Sweden) at 20 °C using the program: step 1: 500 V/ 500 V/h for 1 h; step 2: 1000 V/ 1000 V/h for 1 h; step 3: 8000 V/ 30,500 V/h for 4 h.

Equilibration of the IPG-strips prior to 2nd dimension SDS-PAGE

Upon electrophoresis, the proteins on the strip were equilibrated for 15 minutes in 50 mM Tris-HCl (pH 6.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT followed by 10 minutes in 50 mM Tris-HCl (pH 6.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 2% iodine acetamide (IAA), 0.001% bromophenole blue.

Second dimension

In the second-dimension electrophoresis, the proteins were separated according to their molecular mass in 10.0 – 17.5% gradient gel at 20°C in chamber MULTI-Cell (BioRad, USA). Gels were run at 40 mA and stained with Coomassie Brilliant blue or silver nitrate (2.13.5). The stained gels were scanned with UMAC scanner with parameters settings 300 dpi and 16 bit greyscale and analysed using the ImageMaster 2D Elite software package (Amersham Biosciences).

2.14.2. Peptide mass fingerprinting (performed by Dr. Angelika Schierhorn)

Peptide mass fingerprinting (PMF) was carried out by Dr. Angelica Schierhorn at the Institute for Biochemistry of the Martin-Luther-University Halle-Wittenberg. The proteins from the spots in polyacrylamide gels were digested with trypsine and identified by MALDI-ToF MS.

The protein spots were washed three times in water, twice with 50 mM ammonium bicarbonate and finally with 50 mM ammonium bicarbonate in 50% acetonitrile. The gel pieces were dried under a gentle stream of nitrogen, reswollen in 20 µl 50mM ammonium bicarbonate (pH 8.0) and incubated with trypsin (Promega, Madison, WI, USA) overnight at 37°C.

MALDI mass spectra were recorded on a Bruker REFLEX II mass spectrometer (Bruker-Daltonik, Bremen, Germany) upgraded with a SCOUT ion source and pulsed ion extraction. Data were analysed with XMASS software supplied with the spectrometer. For analysis of the

tryptic digests a matrix thin layer preparation was made (Shevchenko et al.,1996). A saturated solution of α-cyano-4-hydroxycinnamic acid in acetone was mixed in a 4:1 (v/v) ratio with a 10 mg/ml solution of nitrocellulose (Transblot transfer medium, Bio-Rad, Hercules CA, USA) in acetone. 0.5 µl of the matrix was deposited onto the sample probe. 1 µl of the sample was injected into a small drop of 1% trifluoroacetic acid previously deposited onto the matrix surface in order to prevent dissolution of the matrix layer by basic pH of the digest solution. The sample was allowed to dry and the dried spot was rinsed three times with 10 µl 0.1% trifluoroacetic acid. Mass spectra were calibrated using trypsin autolysis products as internal standards.

The peptide mass fingerprint spectra were searched against the nonredundant protein data base (NCBInr) for exact matches using the MASCOT search program (Matrix Science Ltd.,London,UK) or MS-FIT (Protein Prospector, UCSF, San Francisco, CA, USA).

To confirm the results, peptide maps were desalted with ZipTip C18 (Millipore Corporation, Bedford, MA, USA), and MS/MS spectra were recorded on an ESI-Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a nanospray source.

Table 8.

Parameters used for identification of the proteins with MS-Fit

Database	NCBI
Species	*Synechocystis* 6803
Minimum peptide matches	4
Peptide masses	monoisotopic
Mass tolerance	+/-100 ppm
Digest	trypsin
Number of missed cleavages	1
Cysteins modified by	carbamidomethylation
N-terminus	hydrogen
Mass window	1 – 100 kDa
Possible modifications	peptide N-terminal Gln to pyroGlu oxidation of methionin Protein N-terminus acetylated

2.15. Physiological methods

2.15.1. Measurements of the absorption spectra

To measure the absorption feature of mutant strains, the cell cultures were diluted with fresh BG11 medium to an OD_{730} of 0.2. The spectra were recorded in UVIKON931 spectrophotometer (Kontron, Munich, Germany). The phycocyanin/chlorophyll (PC/Chl) ratios were calculated from the absorption spectra using the equations of Richaud et al. (2001). These equations are based on the index values of chlorophyll content (A_{680}-A_{750}) and of phycobiliprotein content (A_{620}-A_{750}).

2.15.2. Low temperature fluorescence emission spectra

The measurements of chlorophyll fluorescence emission spectra were carried out in the lab of Prof. Dr. Conrad Wilhelm at the Botanical Institute of the University of Leipzig. Fluorescence emission spectra were measured at 77K using a Hitachi fluorescence spectrophotometer (Tokio, Japan). Cell suspensions were diluted to a chlorophyll concentration of 1 µg/ml in 10 mM Hepes-NaOH, pH 7.0 containing 45% glycerol. After 10 min of incubation in the darkness, the samples were rapidly frozen in liquid nitrogen. The excitation and emission slitwidths were 10 and 3 nm respectively.

2.15.3. Measurements of the photosynthetic activity with Clark-electrode

Rates of the photosynthetic electron transfer reactions were measured using a Clark-type electrode (Hansatech, UK). The electrode chamber temperature was maintained at 30°C. Saturating red light (RG610 filter, Schott) was provided by a KL-1500 light source (Schott, Germany). Light intensity in the reaction chamber corresponded to about 1500 µE/m^{-2} sec^{-1}. The electron-transport activities of whole chain and PSII were determined in intact cells suspended in BG11 medium. The samples were adjusted to a chlorophyll concentration of 10 µg/ml for the suspensions of wild type cells and 5 µg/ml for the suspensions of lepB1::Km^R mutant cells. Whole chain electron transport rates were measured in the presence of 10 mM

sodium bicarbonate. PSII-mediated electron transport rates were measured in the presence of 0.5 mM 2,6-dichloro-p-benzoquinone (DCBQ) and 1 mM $K_3[Fe(CN)_6]$. The short-time measurements were conducted for at least 5 minutes.
Chlorophyll content of the cell suspensions and isolated membranes was determined accordingly the method of Lichtenthaler as described above.

Light treatment

The cells from the late-exponential growth phase (i.e. OD_{730} 0.9) were harvested by centrifugation and resuspended in fresh BG11 medium. The suspensions were subjected to heat-filtered white light with intensity of 1000 $\mu E/m^2$ s. Temperature was controlled so as not to exceed 32°C, and aliquots for various measurements were withdrawn after 30, 45, 60 and 120 min. The degree of inhibition of photosynthetic electron transport was monitored by the parameters of oxygen evolution (nmol O_2/A_{730} h L).

2.16. Electron-microscopy of the *Synechocystis* 6803 cells (performed by Dr. Gerd Hause)

The cells of *Synechocystis* 6803 were cryofixed for electron microscopy by high pressure freezing and followed by freeze substitution. The cells were embedded in Epoxy resin, which, upon polymerized, may be sectioned using an ultra microtome. For standard transmission electron microscopy, sections of approximately 60 nm were cut, labelled with uranyl acetate and Reynold's lead citrate and then viewed using a Zeiss EM900 transmission electron microscope.

2.17. Computer analysis of polypeptides

2.17.1. The search of *Synechocystis* 6803 proteins containing N-terminal signal peptides with the Signal-P3.0 program

The proteins of *Synechocystis* 6803 were analysed with the SignalP program version 3.0 to detect possible N-terminal signal peptides. This program is available online (http://www.cbs.dtu.dk/services/SignalP/).

The amino acid sequences of *Synechocystis* 6803 proteins were downloaded from Cyanobase and uploaded on the SignalP3.0 server. By uploading, the N-terminal sequences were automatically truncated to 70 aa.

The parameters were set for gram-negative bacteria. Both methods, neural networks and hidden Markov model described in (Nielsen and Krogh, 1998) were used to predict the presence and location of signal peptide cleavage sites.

2.17.2. Blast and ClustalW analysis

Nucleotide or amino acid sequences of *Synechocystis* 6803 were obtained from Cyanobase (URL: http://www.kazusa.or.jp/cyano/cyano.html).

The sequences of *Arabidopsis thaliana* proteins were obtained by *Arabidopsis* data base (URL: http://www.mips.biochem.mpg.de/proj/thal/db/index.html).

The sequence of *Escherichia coli* protein LepB was obtained on NCBI server.

For sequence alignment the following online tools were used: BLAST (URL: http://www.ncbi.nlm.nih.gov/BLAST/), ClustalW (URL: http://www2.ebi.ac.uk.clustalw/).

The analysis of different protein parameters like hydropathy plots (according to Kyte and Doolittle, 1982), molecular mass (Mw) and isoelectric point (pI) was performed using the ProtParam tools on Expasy (www.expasy.org).

The transmembrane helices (TMH) were predicted on TMHMM server 2.0 (Sonnhammer at al., 1998; Krogh et al., 2001).

3. Results

3.1. Analysis of the protein translocases and signal peptidases of *Synechocystis* 6803

By analysis of the Cyanobase database, 14 genes were identified which show homology in their encoded amino acid sequence to subunits of protein translocases and signal peptidases of bacteria and chloroplasts. These are SecA, SecD, SecG, and SecE, SecF and SecY homologues, Tat A, TatB, TatC homologues; SRP subunits and signal peptidases (Kaneko et al., 1996; Nakamura et al., 1998). In order to analyse the function of the corresponding proteins in *Synechocystis* 6803, a gene-disruption approach was applied, which was developed together with the group of Prof. Dr. V. Zinchenko (Moscow Universitiy). This approach is based on the interruption of the corresponding genes by insertion of an antibiotic resistance gene. The list of the interrupted genes (12 genes which show homology to protein translocases detected in chloroplasts and signal peptidases) and their gene products is shown in Table 9. With this approach it was impossible to eliminate all wild-type copies of the genes encoding translocases subunits. This indicates that the function of the most translocase subunits appears to be essential for survival of *Synechocystis* 6803 cells.

Table 9.

Overview of the putative translocases and signal peptidases in *Synechocystis* sp. PCC 6803

Group	Protein	Putative function	Gene	Homologous protein in *A. thaliana*	Identity to Syn	predicted localization
Sec	SecA	ATP-dependent translocase	sll0616	At4g01800	59%	cytoplasmic side of membrane
	SecE	Subunit of SecYEG translocase	ssl3335	At4g14870	19%	membrane protein
	SecG	Subunit of SecYEG translocase	ssr3307	not established		membrane protein
	SecY	Subunit of SecYEG translocase	slr1814	At2g18710	43%	integral membrane protein

Tat	TatA	Subunit of TAT-translocase	slr1046	At5g28750	52%	
	TatB	Subunit of TAT-translocase	ssl2823	At5g28750	42%	
				At5g52440	41%	
	TatC	Subunit of TAT-translocase	sll0194	At2g01110	61%	integral membrane protein
SRP	Ffh	SRP54 homolog	slr1531	At5g03940	54%	
	FtsY	SRP-receptor	slr2102	At2g45770	36%	
SP	LepB1	Signal peptidase I	sll0716	At2g30440	48%	membrane protein
				At1g06870	48%	
	LepB2	Signal peptidase I	slr1377	At1g06870	39%	membrane protein
				At2g30440	42%	
	LspA	Signal peptidase II	slr1366	not present in higher plants		integral membrane protein

Abbreviations:
Sec – Sec-pathway; **Tat** – TAT-pathway; **SRP** – SRP-pathway; **SP** – signal peptidases;
Syn – *Synechocystis* 6803

3.2. The strategy of the targeted gene inactivation

As an example, inactivation of the *Synechocystis* 6803 *tatC* gene (sll0194) will be described on *tatC* gene encoding subunit TatC of the TAT translocase. As insertion site, the *Pst*I restriction site was chosen. This site is located at 217 bp from the start codon of the *tatC* gene (Fig. 6A). The open reading frame sll0194 was amplified with the primers sll0194Forward and sll0194Reverse (Table 4) and cloned into the pGEMT-easy plasmid (Promega). The resulting plasmid was designated as p*tatC*.

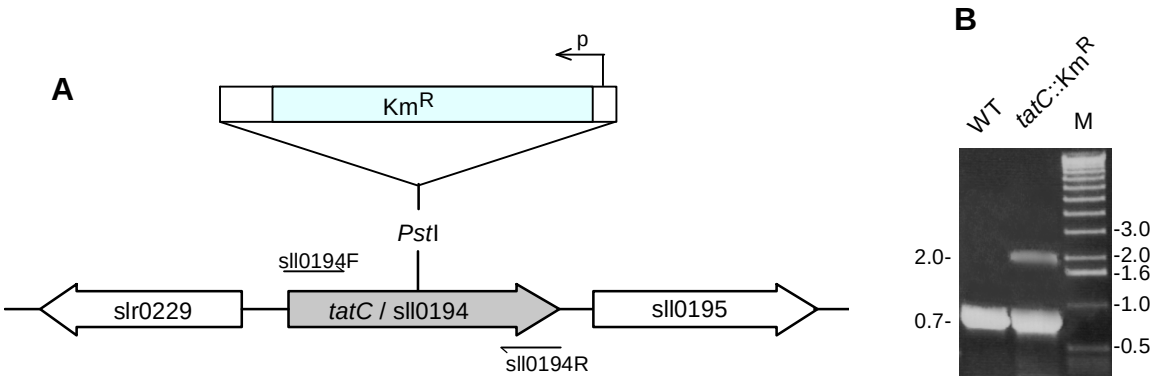

Figure 6. Insertional inactivation of the *tatC* gene of *Synechocystis* 6803. A. The *tatC* gene was amplified from *Synechocystis* 6803 genomic DNA with the primers sll0194FOR and sll0194REV. The 1.2 kb *Pst*I fragment containing the kanamycin cassette was excised from pUC4K and inserted into the *Pst*I restriction site of the *tatC* gene. The arrow "p" shows position of the kanamycine resistance gene promoter. **B. PCR analysis of the segregation of the *Synechocystis* 6803 *tatC*::Km^R mutant.** Chromosomal DNA from WT and the *tatC*::Km^R mutant was used to amplify the *tatC* gene with sll0194F and sll0194R primers. The sizes of the fragments amplified by PCR are given in kb, line M represents 1 kb DNA Ladder marker.

Subsequently, the kanamycin resistance cassette was excised from the plasmid pUC4K with restriction endonuclease *Pst*I. The *Pst*I fragment with a size of the 1240 bp containing the kanamycin resistance gene was ligated with the p*tat*C plasmid, which was linearized at the *Pst*I site. The *E. coli* cells were transformed with the ligation mixture and the resistant clones were selected on the medium containing kanamycin and ampicillin. The resulting plasmid DNA was isolated from was isolated from *E. coli* clones and examined by restriction or PCR analysis. Finally the cells of the *Synechocystis* 6803 were transformed with the appropriate recombinant plasmid. The kanamycin resistant transformants of *Synechocystis* 6803 were subjected to segregation analysis on BG11-plates in the presence of high concentrations of kanamycin at both photoautotrophic and photomixotrophic conditions. After several rounds of restreaking, segregation of the mutant and wild-type genes was monitored by PCR (Fig. 6B).
The disrupted gene containing the kanamycin insertion has a size of 2050 bp, and was detected in the mutant cell (Fig. 6B). It turned out that even after several rounds of restreaking the cells still contained the PCR fragment of 765 bp representing the wild type gene Though selective pressure was made for several weeks, it was not possible to eliminate the wild-type gene copy in the mutant cells. Therefore, we may conclude that the indispensability of the TatC subunit of the Tat-translocase for survival of *Synechocystis* 6803 cells prevents the complete segregation of the inactivation mutant.

3.3. Functional analysis of the two genes for type I signal peptidases of *Synechocystis* 6803.

3.3.1. Analysis of amino acid sequences of signal peptidases I.

According to Cyanobase, the genome of *Synechocystis* 6803 contains two genes encoding signal peptidases of the type I (Kaneko et al., 1996). The corresponding open reading frames are assigned as sll0716 for the gene encoding LepB1 protein, and slr1377 for the gene encoding the LepB2 protein (Nakamura et al., 1998). The analysis of amino acid sequences has revealed that the hydrophobic sequences at the N-terminus of LepB1 and LepB2 show characteristics of transmembrane domain described by Kyte and Doolittle (Fig. 7, Kyte and Doolittle, 1982).

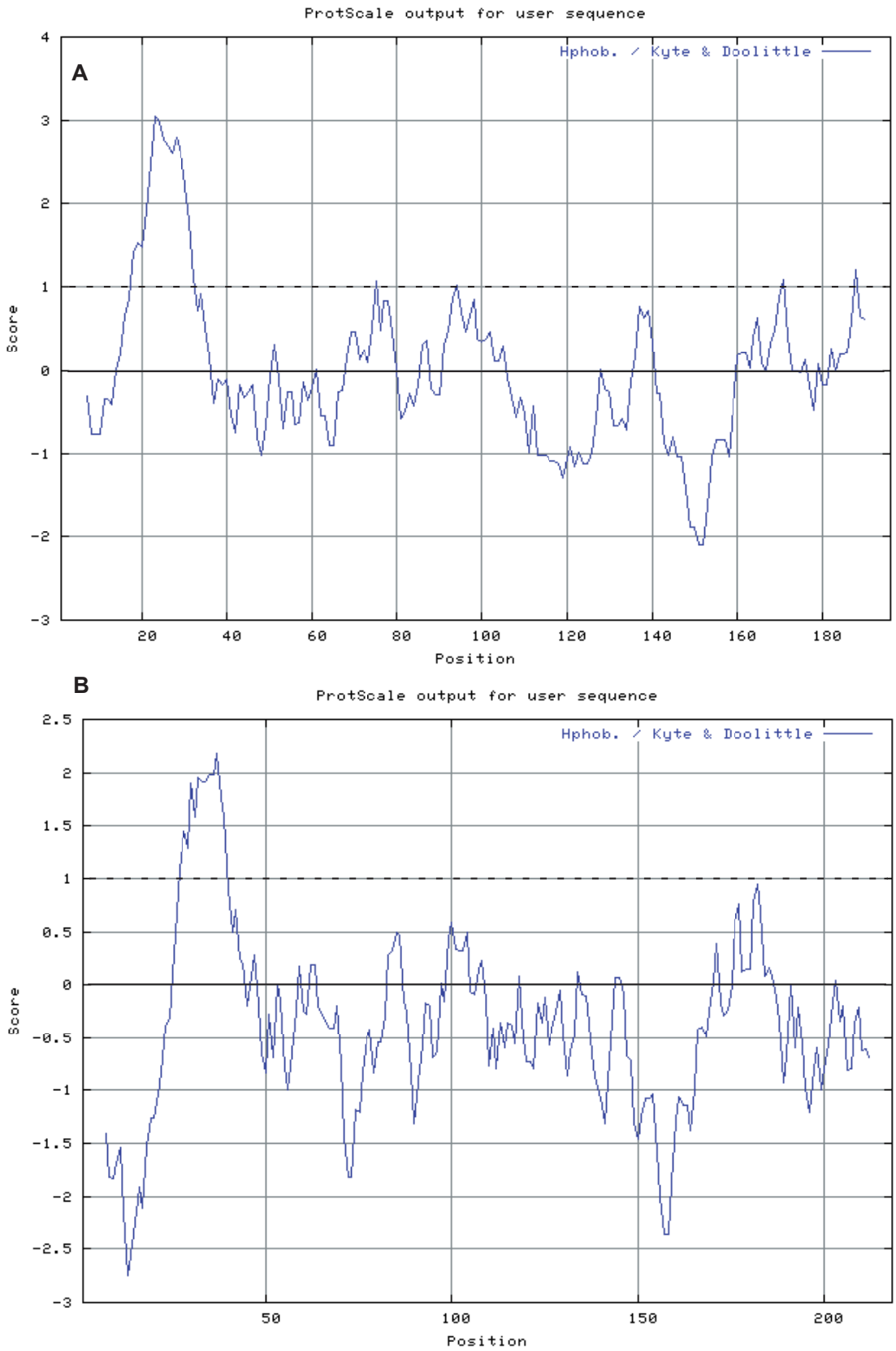

Figure 7. Hydropathy plots of LepB proteins of *Synechocystis* 6803 according to Kyte and Doolittle (1982). A) Analysis of LepB1, B) Analysis of LepB2. Amino acid positions are indicated at the bottom of the graph. For this analysis a scan window of 13 was used. Hydrophobic amino acids are shown above zero line. In this graphic presentation, a hydrophobicity index above 1.0 defines potential transmembrane domains.

The transmembrane helices near the N-terminus of the proteins are formed by residues 17-35 of LepB1 protein and residues 27-43 of LepB2 protein (Fig. 7). These data suggests the presence of only a single transmembrane domain in leader peptidases of *Synechocystis* 6803, unlike the leader peptidase LepB of *E. coli*, which spans the membrane twice (Table 10; Wolfe et al, 1983, Whitley et al., 1993).

Table 10.

Protein parameters calculated using the ProtParam tools on Expasy database

Protein	Cyanobase entry	Length (aa)	Mw (kDa)	pI	The number of TMH
LepB1	sll0716	196	22.230	5.67	1
LepB2	slr1377	218	24.733	5.40	1
LepB *E.coli*	-	324	37	6.8	2

TMH – transmembrane helix

The analysis of the amino acid sequences using the SignalP3.0 program showed the absence of a signal peptide in both, LepB1 protein and LepB2 protein. The data of the hydropathy analysis suggests that the C-terminal part of both proteins is soluble (amino acids 36-198 of LepB1 and 44-218 of LepB2 (Fig. 7). The active part of LepB peptidase of *E. coli* is located in the periplasm and is represented by the soluble large C-terminal part with the amino acids 76-324 (Bilgin et al., 1990; Dalbey, 1991). The amino acids which determine the substrate specificity in LepB of *E. coli* are located in the soluble part of the protein (Karla et al., 2005). The amino acids serine S90 and lysine K145, which are located in the soluble part of the LepB protein, are essential for catalytic activity (Dalbey at el., 1997). The analysis of the amino acid sequences of leader peptidases from different organisms has revealed that the catalytically active residues are located in the soluble part of the protein (Paetzel et al., 2002). Since the soluble C-terminal part of LepB1 protein includes the catalytically active residues, it might be located like LepB of *E. coli* in the periplasmic space. However, the cyanobacteria contain in addition to plasma membrane the thylakoid membrane system and the prediction of the topology of the leader peptidases LepB1, LepB2 and thylakoid processing peptidases (TPP) (Fig. 7; Chaal et al., 1998) suggest that cyanobacterial leader peptidases can potentially function in the luminal space of thylakoid membranes.

The similarity features of bacterial leader peptidases and thylakoid processing peptidases suggest that these enzymes may be functionally similar (Chaal et al., 1998). The multiple sequence alignment of the proteins has revealed a relative high level of homology between LepB1, LepB2 and thylakoidal processing peptidases excluding the N-terminal chloroplast targeting transit peptides of TPP (Fig. 8). On the protein level both cyanobacterial leader peptidases have 51% of identical amino acids and 17% of conserved substitutions. Interestingly, the TPP1 and TPP2 show higher homology among each other, namely 65% identity and 75% similarity. The homology level of LepB1 and LepB2 to the the TPP is significantly higher than to leader peptidase from *E. coli*, namely 39-49% in comparison to 26-27% respectively (Fig. 8). The LepB1 shows higher degree of identity to both TPP of plants (*Arabidorsis thaliana)* in comparison to LepB2 thus suggesting that LepB1 rather than LepB2 is related to the TPP ancestor protein**.**

LepB1 Syn	LepB2 Syn	LepB Ec	TPP1 Ath	TPP2 Ath	
100	51/68	26/36	49/67	49/67	LepB1 *Synechocystis* 6803 (LepB1 Syn)
	100	27/38	42/60	39/57	LepB2 *Synechocystis* 6803 (LepB2 Syn)
		100	37/51	37/52	LepB *Escherichia coli* (LepB Ec)
			100	65/75	TPP1 *Arabidopsis thaliana* (TPP1 Ath)
				100	TPP2 *Arabidopsis thaliana* (TPP2 Ath)

Figure 8. Homology of leader peptidases and thylakoidal processing peptidases. Analysis of identity and similarity. The identical and similar amino acids of the protein sequences of leader peptidases from *Synechocystis* 6803 (LepB1 Syn; sll0716 and LepB2 Syn; slr1377 , Cyanobase), from *Escherichia coli* (LepB Ec; Databank) and thylakoidal processing peptidases from *Arabidopsis thaliana* (TPP1 Ath, Databank; TPP2, Databank) were pairwise aligned.
The levels of identity/similarity are given in %

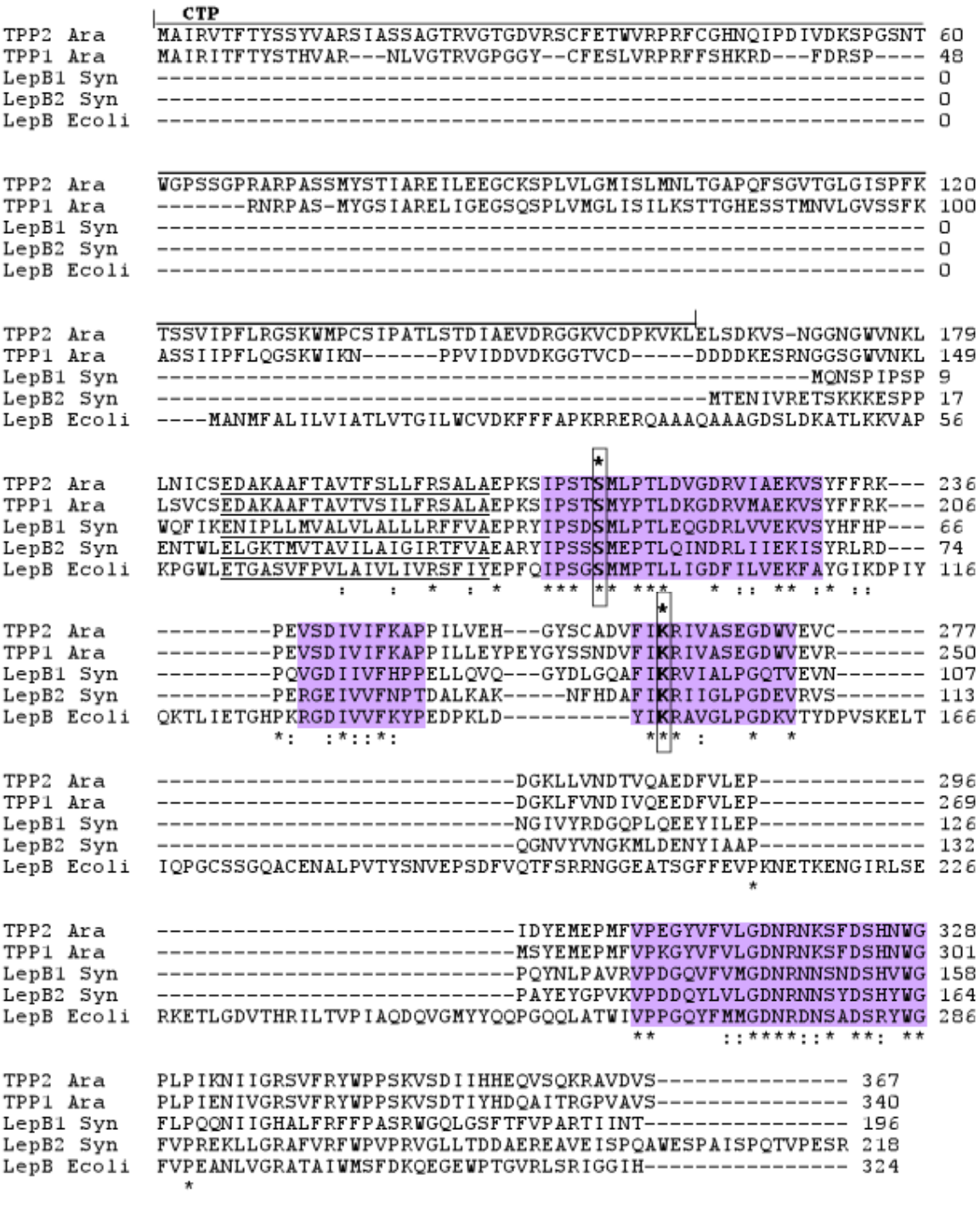

Figure 9. Comparison of the amino acid sequences of leader peptidases (LepB) from Gram-negative bacteria and the thylakoidal processing peptidases (TPP). Identical amino acids are labelled by star (*****), similar amino acids are labelled by dots (**:**). The residues essential for catalytic activity are shown in frames in bold letters and indicated by bold star. The conserve regions are shown in shaded boxes. The putative transmembrane domains are underlined. Abbreviations **Ara** – *Arabidopsis thaliana*, **Syn** – *Synechocystis* 6803, Ecoli – *Escherichia coli*. Accession numbers for the thylakoid processing peptidases are the following: TPP1 from *A. thaliana* - gi:22135950, TPP2 *A. thaliana* gi:21553622. The long N-terminal parts of the TPPs under the line represent the transit peptides for the import into chloroplast (CTP). For the further explanation see the text.

The comparison of the amino acid sequences of LepB1 and LepB2 proteins with those of homologous proteins from *E. coli* or the plant TPP showed the presence of four regions containing relative high number of identical amino acids and interchangeable substitutions (Fig. 9). These regions correspond with the conserved domains detected in bacterial signal peptidases of type I from different organisms (Paetzel at al., 2002). The catalytically important residues serine and lysine (showed in bold star on Fig. 9) and many amino acid residues in the first three boxes that form the substrate binding pockets S1 and S3, which bind the -1 and -3 residues of preprotein substrates, are identical in leader peptidases and thylakoidal processing peptidases. This suggests the existence of functional similarities of the signal peptidases LepB1, LepB2, LepB from *E. coli* and thylakoidal processing peptidases.

Interestingly, in the cDNA sequence of TPP1 there is region which shows strong identity to the coding region of both sll0716 and slr1377. The DNA section shown in Fig. 10 encodes the motif ***xVxGDNRNxSxDSHxWG*** in the last conserved domain. In contrast, the coding sequence of the *lepB* gene from *E. coli* does not show homology to coding sequences of TPPs and the cyanobacterial genes sll0716 and slr1377 in this region. The presence of homology on nucleotide level indicates that the plant genes encoding thylakoid processing peptidases have probably evolved from the common ancestor which is related to cyanobacterial genes.

```
   tpp2  TTCGTCCTAGGAGACAACCGCAACAAAAGCTTTGATTCTCATAACTGGGGT
         F  V  L  G  D  N  R  N  K  S  F  D  S  H  N  W  G

   tpp1  TTTGTCCTTGGTGATAACCGCAACAAAAGCTTTGACTCTCATAACTGGGGT
         F  V  L  G  D  N  R  N  K  S  F  D  S  H  N  W  G

   lepB2 TTAGTGTTAGGGGATAACCGCAACAACAGCTATGACTCCCACTATTGGGGC
         L  V  L  G  D  N  R  N  N  S  Y  D  S  H  Y  W  G

   lepB1 TTTGTCATGGGGGATAACCGCAACAACAGCAATGATTCCCATGTATGGGGA
         F  V  M  G  D  N  R  N  N  S  N  D  S  H  V  W  G

conserved
  motif  x  V  x  G  D  N  R  N  x  S  x  D  S  H  x  W  G
```

Figure 10. Conserved amino acid sequences derived from nucleotide sequences of cDNA of TPPs of *Arabidopsis thaliana* or leader peptidases of *Synechocystis* 6803. The conserved amino acid motif which is shown below is located in the forth conserced box on Fig. 9.

3.3.2. Inactivation of the genes encoding LepB1 and LepB2 proteins.

In order to examine the function of these two peptidases, both genes were independently inactivated by inserting a kanamycin resistance cassette into either of the two open reading frames via homologous recombination (Fig. 11A). After transformation, the cells were propagated under dim light conditions on agar plates supplemented with glucose and kanamycin. In order to promote complete genetic segregation of the mutated gene, the concentration of kanamycin in the medium was stepwise increased. After several rounds of restreaking, the insertionally inactivated *lepB1* gene had fully segregated giving rise to the homozygous mutant *lepB1::*Km^R. PCR analysis showed that the wild-type *lepB1* gene, which is represented by a fragment of 0.5 kb, is completely replaced by the mutant allele for which a PCR fragment of 1.8 kb is indicative (Fig. 11B)

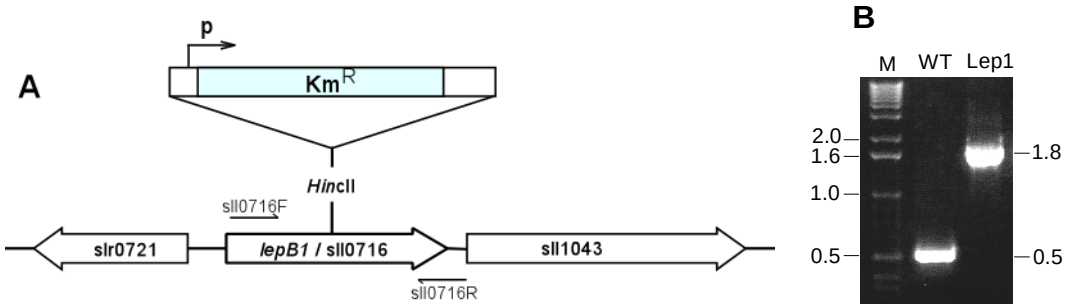

Figure 11. Insertional inactivation of the *lepB1* gene of *Synechocystis* 6803.
A. The chromosome region with open reading frames around sll0716/ *lepB1* gene. The unique restriction site *Hinc*II was used for insertion of the kanamycin resistance (Km^R) cassette from pUC4K. The positions of the primers, sll0716Forward and sll0716Reverse, are shown by small arrows. Arrow with "p" shows the position of Km^R gene promoter.
B. PCR verification of the complete genetic segregation of *lepB1*::Km^R mutant. The sizes of the fragments amplified by PCR are given in kb, line M represents 1 kb DNA Ladder marker.

In contrast, it was not possible with the same approach to completely eliminate the wild-type copy of *lepB2*. While the insertional inactivation of the gene by homologous recombination was successful also in this case, the cells remained heterozygous even if the concentration of kanamycin in the growth media was raised to 200 µg ml^{-1} to select for higher copy numbers of the mutant allele (Fig. 12B). This suggests an essential role of LepB2 for viability of *Synechocystis* 6803. However, since the open reading frame of *lepB2* overlaps by 49 nucleotides with that of the adjacent gene slr1378 (Fig. 12A), which encodes a protein of

unknown function (Kaneko et al., 1996, Nakamura et al., 1998), it could not be ruled out at this point that the insertional inactivation of *lepB2* had a polar effect on the transcription of slr1378 which itself might be essential for growth. In order to examine this possibility, slr1378 was inactivated in the same manner as described above by insertion of a kanamycin resistance cassette. PCR analysis of cultures selected on kanamycin containing media showed that the homozygous mutant slr1378::Km^R could easily be obtained (Fig. 12C). These cells did not show any phenotype different from that of wild-type cells (data not shown) which clearly demonstrates that it must be the function of *lepB2* and not any polar effect on the expression of slr1378 what prevents the elimination of the wild-type copy of slr1377. Thus, since one of the two leader peptidases of *Synechocystis* 6803 cannot be completely inactivated without loss of cell viability, it must be concluded that the two homologous proteins are not redundant in function.

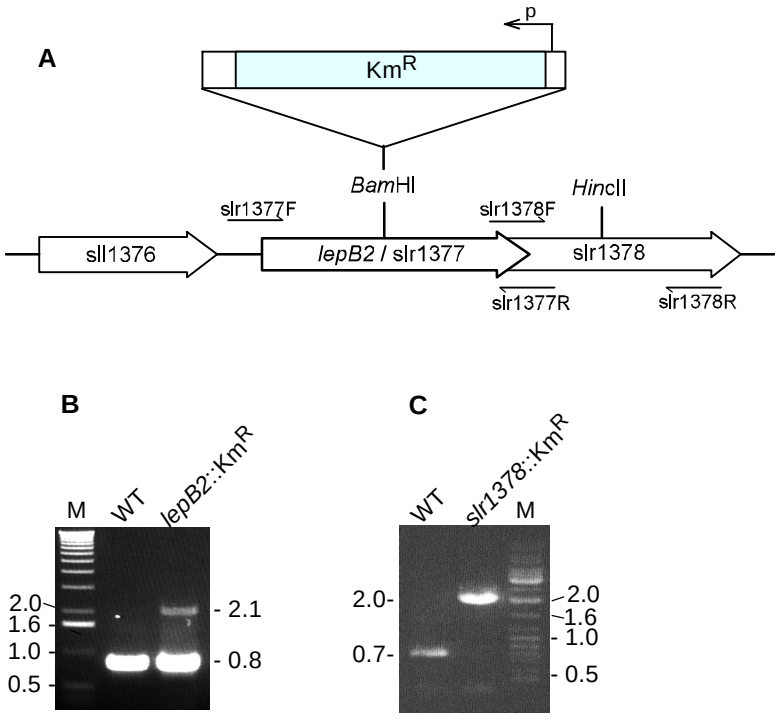

Figure 12. Insertional inactivation of the *lepB2* gene of *Synechocystis* 6803. Panel A. The chromosome region of the open reading frames around slr1377/ *lepB2* gene. The positions of forward and reverse primers (slr1377F and slr1377R; slr1378F and slr1378R, respectively) are shown by small arrows. The 1.2 kb *Bam*HI fragment containing the kanamycin cassette was excised from the pUC4K and inserted into the *Bam*HI restriction site of the *lepB2* gene. Similarly, the gene slr1378, whose 5'-region overlaps with 3'- region of slr1377, was inactivated. **Panels B and C.** PCR analysis of the segregation of *Synechocystis* 6803 mutants. Chromosomal DNA from WT and respective mutant strains was used to amplify the lepB2 gene with slr1377F/slr1377R primers (**B**) or the slr1378 gene with slr1378F/slr1378R primers **(C).**

3.3.3. Analysis of LepB1 antigen and production of antiserum.

For preparation of an antiserum against the LepB1 protein from *Synechocystis* 6803, the full-length nucleotide sequence of sll0716 was amplified by PCR with the primers SynLepB1forw and SynLepB1Bam generating *Nde*I and *Bam*HI restriction sites in front of start codon and after the stop codon, respectively (Table 4). After restriction enzyme digestion, the *Nde*I-*Bam*HI fragment was ligated in frame with the pET28 (pAR3040) plasmid (Rosenberg et al., 1987). Recombinant plasmids containing the correct insert were used for transformation of *E. coli* strain BL21(DE3) (Novagen). The resulting cell cultures were used for expression of the LepB1 protein.

Protein expression was carried out as described in Section 2.13.3. The cells of *E. coli* BL21 were transformed freshly with recombinant plasmid p*lep1* and after 3 h of growing the protein expression was induced with 1mM IPTG. The analysis of – and + fractions has shown the presence of the expressed protein with a molecular weight of 22 kDa in the + fraction (Fig. 13). The LepB1 was then expressed in a large scale. For the immunization of a rabbit, the proteins from overexpression were separated on a SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Schleicher and Schuell). The proteins were stained with Ponceau S and the band of interest was excised and handed over to Dr. H. Schubert from the Institute of Versuchstierkunde of the Friedrich-Schiller-University of Jena for immunization.

The antiserum was analyzed with Western blot of over expressed LepB1 and the mobility of the band recognized by the obtained LepB1 antiserum matches well the predicted molecular weight 22 kDa (data not shown). During the large scale preparation of the over expressed LepB1 and separation of the different fractions of the disrupted *E. coli* cells, the analysis of these fractions has revealed that LepB1 was located either in inclusion bodies or in membrane interphase. It could be resolved from the membrane proteins precipitated with TCA only after addition of thiourea in solubilisation buffer (Fig. 13B). This observation supports the idea that LepB1 is an integral membrane protein.

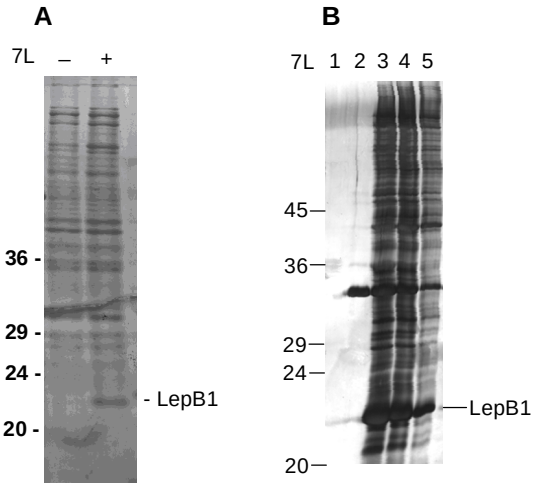

Figure 13. Overexpression of a full-length LepB2 protein.
A. For overexpression, the vector pAR3040 containing the full-length *lepB1* gene was transformed in the BL21 (DE3) strain of *E. coli*. Expression of LepB1 was induced by addition of 1 mM IPTG and was monitored after 3 h of incubation at 37°C on SDS PAGE.
B. The solubilisation of the precipitated with 10% TCA over expressed protein LepB1 from interphase fraction was monitored on SDS-PAGE. The supernatant was loaded after resuspension with buffer Lane 1: 20 mM TrisHCl buffer, pH7.5, 1/50 volume loaded; lane 2: supernatant after the pellet resuspended in 7M Urea prepared in same buffer, 1/50 volume loaded; lane 3, the pellet was resuspended in 7M urea/2M thiourea, 1/35 volume loaded; lane 4: the pellet was washed with 7M urea/2M thiourea, 1/10 volume loaded; lane 5: the pellet was resuspended in Laemmly buffer and 1/5 volume was loaded on the gel.
The position of the LepB1 protein with the molecular weight of about 22 kDa is shown by arrow. The positions of the molecular weight standards are indicated in kDa.

A corresponding band in the samples of thylakoid membrane of the wild type was not detected in the sample of the mutant, thus demonstrating the complete inactivation of *lepB1* gene (Fig. 14).

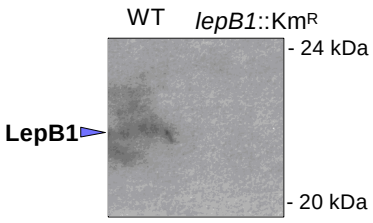

Figure 14. The leader peptidase LepB1 is absent in the *lepB1*::Km^R mutant cells. Thylakoid membranes were isolated from the cells of the wild type and *lepB1*::Km^R mutant and the proteins were separated on SDS-polyacrylamide gel. After the transfer of the proteins to nylon membrane, the LepB1 protein was detected by Western-analysis using an antiserum raised against LepB1 protein from *Synechocystis* 6803.

3.4. Phenotypic features of *lepB1::Km*^{R} mutant

3.4.1. Homozygous *lepB1::Km*^{R} cells are sensitive to high light intensities

Since it was impossible to fully inactivate the wild-type allele of *lepB2*, all further work was focused on the analysis of the homozygous *lepB1::*Km^R mutant. Remarkably, the complete segregation of this mutant could be achieved only in dim light (5 µE m^{-2} s^{-1}) in the presence of glucose. Similar features were observed for mutants, which are defect in photosynthesis, like for example a PSI-less mutant (Shen et al., 1993). Subsequent tests have confirmed that the *lepB1::*Km^R mutant was not capable to photoautotrophic growth indicating that it is not able to perform the photosynthesis at significant rates. The cells of the *lepB1::*Km^R mutant appeared to have a more bluish colour than the wild type cells, because the chlorophyll content in the mutant cells reached only 50% of that of the wild type. The cells of the *lepB1::*Km^R mutant grown in dim light in the presence of glucose showed a strong light sensitivity. After transfer of the cultures to the light stronger than 40 µE m^{-2} s^{-1} significant bleaching of the mutant cells was observed. This was presumably caused by degradation of the photosynthetic pigments (Fig 17). The light sensivity was also observed by the analysis of the photosynthetic properties of the *lepB1::*Km^R mutant.

3.4.2. The alterations in thylakoid membrane structure revealed in *lepB1*::Km^R mutant by electron microscopy of the *Synechocystis* 6803 cells

The cyanobacterium *Synechocystis* 6803 contains the intracellular system of thylakoid membranes, which form several layers parallel to the cell-membrane (Fig. 1). The phenotype of bacterial mutants can be analysed by the quantitative analysis of physiological or biochemical parameters. Also, it was interesting to observe the membrane structure of both wild-type and *lepB1*::Km^R mutant cells. Therefore the cells that were grown up at photomixotropic conditions were visualized by electron-transmission photography (Fig. 15).
On the Fig. 15, the overall reduction of thylakoid membranes of the *Synechocystis* 6803 *lepB1*::Km^R mutant cells could be observed. The mutant cells with different stages of the cell cycles showed different expression of the damage of normal thylakoid membrane structure, shown in Fig. 15. Remarkably, there were strong differences between single cells in the same samples: some mutant cells were similar to some extent to those of the wild type; some were almost empty of inner membrane structure. A more detailed observation of the mutant cells,

which did not have the drastic reduction of the membranes, has revealed that the space between thylakoid layers increased significantly in comparison to that of the wild type. The almost empty cells of the mutant showed very strong reduction of thylakoid membranes, and they contained instead granular electron-dense inclusions. In addition, some mutant cells displayed the intense light granular background. These features of the mutant cell structure are probably connected to the observed physiological features of the mutant.

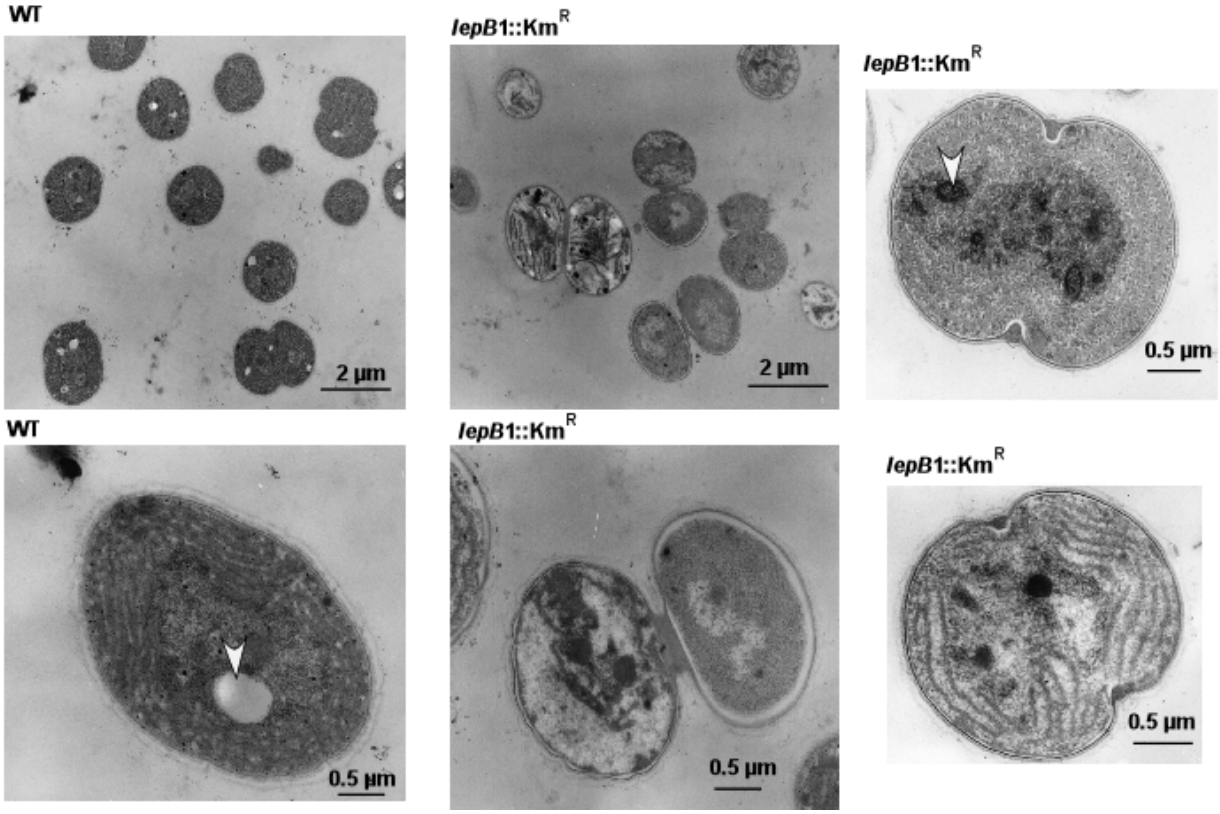

Figure 15. Inactivation of the leader peptidase LepB1 leads to a reduction of thylakoid membranes. The figure shows the electron microphotographs of wild type cells (WT) and lepB1::Km^R mutant grown in photomixotrophic conditions. Bars represent size markers. Different panel show most typical cells of the mutant. The arrowheads indicate the light inclusions, which represent starch granula, or dark inclusions, which are presumably carboxysomes.

Another specific feature in the *lepB1*::Km^R mutant cells is the absence of the transparent intracellular inclusions, presumably starch granules, which were detected almost in all wild-type cells (Fig. 15). This feature and the reduction of the thylakoid membranes might indicate that the mutant cells do not use the photosynthesis for the cell growth. In contrast, some of the mutant cells showed electron-dense crystalline inclusions similar to the wild-type cells, which

can be carboxysomes. The carboxysomes are inclusion bodies that contain the enzymes involved in carbon dioxide fixation including the ribulose bisphosphate carboxylase (RUBISCO). On the basis of the similarities observed in wild-type and mutant cell photographies, the significant alteration in carbon dioxide fixation cannot be expected.
The observed heterogeneity of the mutant cells might represent different situations. A possible explanation is that the culture of the *lepB1*::Km^R mutant is not monogenic. However, the function of LepB1 is absent since the *lepB1*::Km^R mutant had fully segregated and no LepB1 protein could be detected by the Western blot (as stated below). To find the explanation for the heterogeneity, the systematic analysis of the cell morphology in different growing conditions is necessary.

3.5. The complementation of the leader peptidase function in the *lepB1*::Km^R mutant.

After the complete inactivation of *lepB1* gene was achieved, the first observations have shown that the mutant was photosynthetically inactive. To confirm that the resulting phenotype was a direct consequence of the *lepB1* gene disruption, a complementing strain with wild type gene expressing on the plasmid pVZ321N was created in group of V. Zinchenko. The original vector pVZ321 (accession number AF100176) is based on RCF1010, a broad host range plasmid, which is capable of replicating autonomously also in cyanobacterial cells (Scholz et al., 1989; Marraccini et al., 1993). The vector pVZ321M differs from plasmid pVZ321 (Zinchenko et al., 1999) by the *Nde*I site, which was introduced in the start codon of the kanamycin resistance gene *aphII*. This modification facilitates the cloning of a gene of interest under the control of the Km^R gene promoter in *Synechocystis* 6803 cells (Fig. 16A). The resulting plasmid carrying the wild-type *lepB1* gene instead of the inactivated *aphII* gene was designated as pSLEP1 (Fig. 16). This plasmid was transferred into *lepB1*::Km^R mutant cells via triparental mating. The complementing cells were therefore resistant to both chlorampenicol (Cm^R gene on the plasmid) and kanamycin (Km^R gene on the chromosome).
The resulting strain carrying the plasmids pSLEP1 was designated as pLep1. The genetic background of the pLep1 strain was analyzed by PCR with specific primers. Although the reverse recombination events are unlikely, they cannot completely be excluded. Therefore primers were designed to detect the possible reverse recombination events between the chromosomal and plasmid DNA. The fragment, amplified with sll0716F and lep1043rev from WT genomic DNA, has an estimated size of 0.8 kb. A fragment of the same size could not be

detected in pLep1 cells, which indicate the absence of recombination between the pSLEP1 plasmid and the chromosome. The size of the PCR fragment obtained from the genomic DNA of the complementing strain pLep1 is about 2.1 kb and corresponds with that obtained from the mutant DNA (Fig. 16B). This result indicates that the plasmid pSLEP1 remained stable replicating in *Synechocystis* 6803 cells and the lepB1 gene is transcribed from the plasmid in the cells of the pLep1 strain.

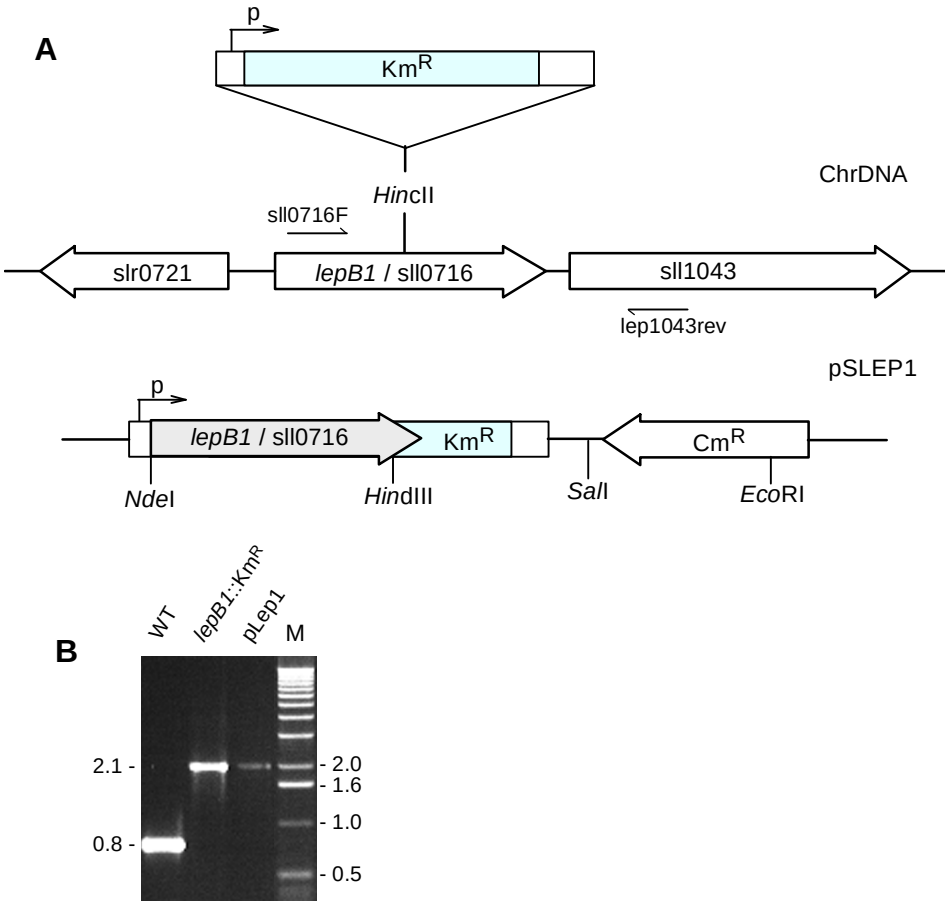

Figure 16. Complementation of the *lepB1*::Km^R mutant strain with wild type *lepB1* gene. Panel A: Construction of the complementing strain pLep1. The plasmid pSLEP1, which carries the LepB1 gene and Cm^R, was transferred in the cells of the mutant yielding the pLep1 strain. The schematic map shows positions of primers sll0716F and lep1043 rev which were chosen to analyse chromosome DNA (ChrDNA) for possible recombination with plasmid DNA. The promoter of kanamycin resistance gene is shown by "p".
Panel B. PCR analysis of the DNA isolated from wild type, *lepB1*::Km^R mutant and complementing strain pLep1. Total DNA was isolated from wild type, *lepB1*::Km^R mutant and complementing strain pLep1. M indicates the position of 1 kb ladder marker. PCR was carried out with the primer pair sll0716F and lep1043rev which are able to anneal only chromosomal region containing the *lepB1* gene resulting in fragments of either 0.8 kb or 2.1 kb.

The resulting strain pLep1 resembled the wild-type phenotype, i.e it was capable to grow photoautotrophically similar to wild type and did not show the light sensitivity observed in the *lepB1*::Km^R mutant cells. The concentration of chlorophyll and the photosynthetic properties of the pLep1 strain were practically the same as of the wild type cells (in detail described in chapters 3.7; 3.8).

3.6. Characterization of the *lepB1::Km*^{R} mutant strain of *Synechocystis* 6803

3.6.1. The *lepB1*::Km^R mutant strain is incapable of photoautotrophic growth

The *lepB1*::Km^R mutant differed significantly from the wild type already with respect to its growth behavior. Under photoautotrophic conditions, the mutant did not show any measurable growth (Table 11) indicating that it is not able to perform photosynthesis at significant rates. Furthermore, even if the cultures were supplemented with fermentable carbon sources like glucose (photomixotrophic conditions), the mutant cells could be propagated only if they were exposed to dim light (5 µE m^{-2} s^{-1}) which is not sufficient for efficient photosynthesis (Anderson and McIntosh, 1991). Under these conditions, the growth rates of wild-type and mutant cultures were essentially identical (Table 11) thus demonstrating that the mutant is not affected in energy metabolism in general. However, if incubated in the presence of glucose but at light intensities allowing efficient photosynthesis (40 µE m^{-2} s^{-1}), growth of *lepB1::*Km^R was again completely abolished (Table 11). This indicates that the mutant is not only incapable of performing photosynthesis but that light, even at relatively moderate intensities, is harmful for the cells.

Table 11.
Growth rates of wild type and homozygous *lepB1*::Km^R mutant

	wild type	***lepB1*::Km^R**
	doubling time [h]	
photoautotrophic growth (- glucose/ - DCMU; 40 µE m^{-2} s^{-1})	12.3 ± 1.3	No growth
photomixotrophic growth (+ glucose/ - DCMU; 5 µE m^{-2} s^{-1})	20.9 ± 2.2	20.9 ± 1.0
photomixotrophic growth (+ glucose/ - DCMU; 40 µE m^{-2} s^{-1})	8.0 ± 0.9	No growth
photoheterotrophic growth (+ glucose/ + DCMU; 40 µE m^{-2} s^{-1})	16.2 ± 1.8	26.6 ± 1.2

Remarkably, supplementation of the medium with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) restored the property of the mutant cells to grow at such conditions, though at somewhat reduced rates as compared to wild-type cultures (Table 11). DCMU leads to inhibition of photosystem II electron transfer to plastoquinone, therefore the photohetrotrophic growth upon addition of this inhibitor is non-photosynthetic (Herranen et al., 2004). Moreover, the observed growth behavior of the *lepB1*::Km^R mutant cultures suggests that the electron flow from photosystem II caused photodestruction in the mutant cells.

3.6.2. The mutant cells show the altered pigment composition and PSI/PSII ratio

Analysis of the absorption spectra

The photosynthetic properties of cyanobacterial cells can be analysed using the absorption spectra of the whole cells. The comparison of the absorption features of the wild type and *lepB1*::Km^R mutant has revealed that the light-sensitivity of the mutant was reflected also by the variability of its pigment content in response to different light intensities**.** If propagated in dim light (5 µE m^{-2} s^{-1}), all photosynthetic pigments were present in the mutant, though in a composition that was somewhat different from that of wild-type cells (Table 12). In particular, the peaks characteristic for chlorophyll at 442 and 681 nm were decreased in the *lepB1*::Km^R mutant cells demonstrating that the chlorophyll content was reduced (Fig. 17A). In addition, the absorption of the phycobiliproteins at 630 nm has increased in the mutant cells. These alterations lead to an increase of the phycobiliprotein/chlorophyll ratio from 0.85 in wild type to 1.16 in mutant cells (Table 12) and, consequently, to a bluish phenotype. The higher phycobiliprotein to chlorophyll (PC/Chl) ratio is an indicator for a higher PSII/PSI ratio or a decrease of the chlorophyll content per photosystem (Li and Sherman, 2000). Indeed, the 50% reduction of chlorophyll content per cell was confirmed for the cells of the mutant by a methanolic exctraction of chlorophyll (Table 12).

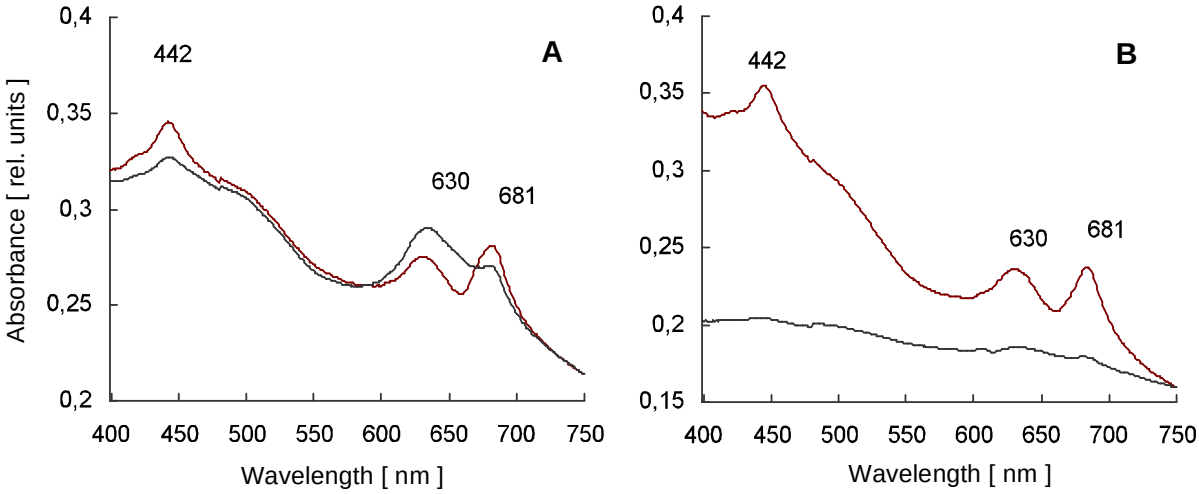

Figure. 17. Absorption spectra of the wild-type and *lepB1*::Km^R mutant cells.
Wild-type (dotted line) and mutant cells (solid line) were grown up in photomixotrophic conditions in dim light until the cultures densities reached OD_{730} 0.6. The spectra are shown in panel A. Then the cultures were transferred to the standard light (40 mE/m2 sec) and after 24 h of incubation the spectra were measured again (panel B).

After transfer of the cultures to higher light intensities (40 µE m^{-2} s^{-1} or more), significant bleaching of the mutant cells was observed. This was presumably caused by degradation of the photosynthetic pigments. This fact was confirmed by spectral analyses that demonstrated the complete loss of the indicative absorption peaks for chlorophyll and phycobiliproteins in the mutant samples (Fig. 17B).

Table 12.

Pigment characteristics of the wild type and *lepB1*::Km^R mutant strains

	pigment characteristics	
	wild type	***lepB1*::Km^R**
chlorophyll content[a] (µg ml^{-1} A_{730}^{-1})	3.8 ± 0.2	1.7 ± 0.1
phycobiliprotein/chlorophyll ratio[b]	0.85	1.16

[a] chlorophyll was extracted from cells grown in photomixotrophic conditions at 5 µE m^{-2} s^{-1}
[b] determined from intact cells according to the equation of Richaud *et al*. 2001.

Analysis of the chlorophyll fluorescence emission spectra

Since the chlorophyll concentration in the *lepB1*::Km^R mutant cells was significantly reduced in comparison to the wild type, the chlorophyll distribution between the two photosystems in

the *Synechocystis* 6803 cells had to be analysed. This was made by the measurement of the low temperature (77K) fluorescence emission spectra of chlorophyll. The wave length 435 nm was chosen for excitation (light absorbed primary by chlorophyll *a* in PSII and PSI core complexes). On the Fig. 18, two characteristics peaks originating from PSII fluorescence appeared at 685 nm and 695 nm and another fluorescence peak originating from PSI appeared at 725 nm of the spectra of *Synechocystis* 6803 cells.

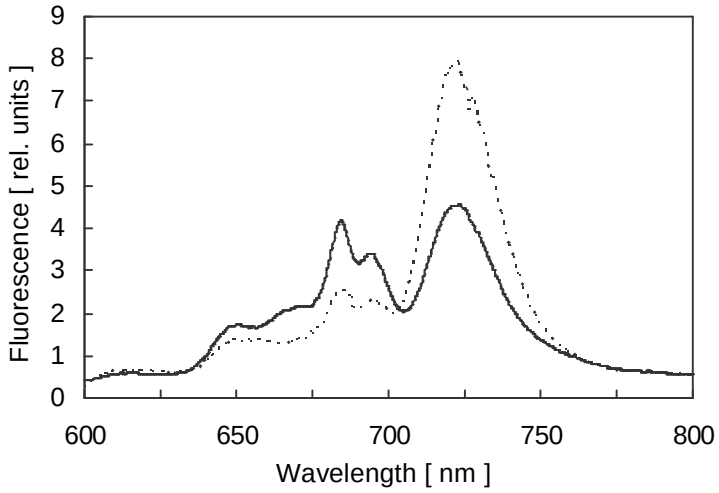

Figure 18. Low-temperature (77K) fluorescence emission spectra of wild-type and lepB1::Km^R mutant cells. The 77K fluorescence emission spectra of the wild type (dotted line) and *lepB1*::Km^R mutant (solid line) were recorded after excitation of chlorophyll at 435 nm. The spectra were normalized for the same cell number.
Wild-type (dotted line) and mutant cells (solid line) were grown up in photomixotrophic conditions in dim light until the cultures reached OD_{730} 0.6.

In Fig. 18 the following alterations in the intensities of the fluorescence peaks of wild type and *lepB1*::Km^R mutant could be observed: in comparison to the wild type, the mutant showed a significant decrease in fluorescence emission from PSI in relation to fluorescence emission from PSII. The decreased ratio of PSI to PSII and the 50% reduction of the chlorophyll content in the *lepB1*::Km^R mutant cells might reflect a decrease in the PSI content, as most chlorophyll molecules in the *Synechocystis* 6803 cells are associated with reaction centers of PSI. (Pakrasi, 1995; Rögner et al., 1990).

The cells of the *lepB1*::Km^R mutant showed altered ratios of the photosystems and a reduction of chlorophyll content when grown in photomixotrophic conditions. These changes may be due to a direct or indirect effect of leader peptidase inactivation. On the one hand, LepB1 could be involved directly in biogenesis of photosynthetic complexes. On the other hand, the

decrease of the chlorophyll content could be a response to light stress, caused by a deregulation of the electron transport already under weak light conditions. The harmful effect of light on the mutant cells can be demonstrated by the pigment analysis (chlorophyll and carotenoids) using high performance liquid chromatography (HPLC).

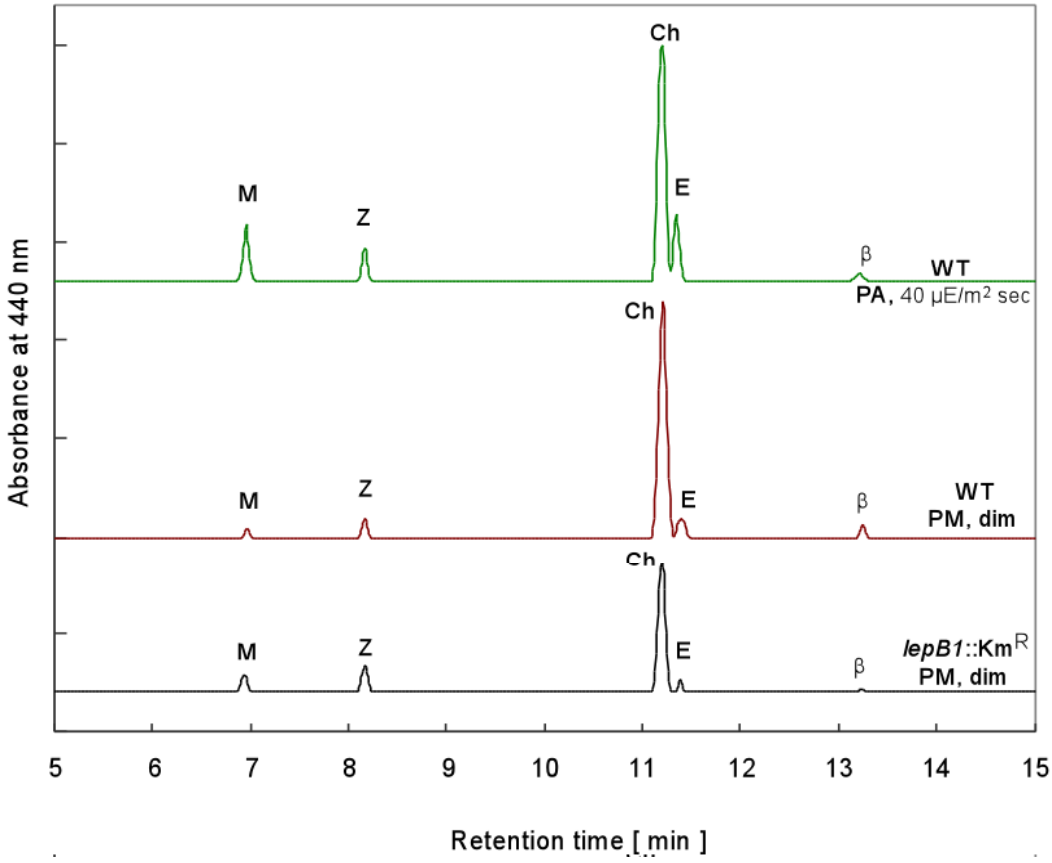

Figure 19. Pigment analysis of *Synechocystis* 6803 cells with HPLC.
The pigments were extracted from the same cell amount with mixture of acetone and methanol in the ratio 3:1 and analysed with HPLC (Kontron, Milan, Italy) after Gilmore and Yamamoto (1991). The pigments were identified by analysing of absoroption spectra.
Abbreviations:
M – myxoxanthophyll, Z – zeaxhantine, Ch- Chlorophyll a, E- echinenone, β – β-carotene.
WT, PA – wild type cells grown in photoautotrophic conditions
PM, dim – cells were grown in the photomixotrophic conditions under dim light (5 µE m^{-2} sec^{-1}).

The analysis of the pigment composition of *Synechocystis* 6803 cells has shown that the cells of the mutant grown in the presence of glucose at the dim light intensity 5 mE m^{-2} sec^{-1} (PM, dim) have not only a decrease of chlorophyll content. The detailes comparison of the HPLC data of the pigments of the wild-type and mutant cells has revealed in the mutant a decrease of β-carotene content and an increase of myxoxanthophyll content in relation to other pigments

(Fig. 19). The carotenoid myxoxanthophyll is known to be a stress pigment that comprises a quarter of the total carotenoids when the cells of wild type are grown under high light conditions (Mohamed and Vermaas, 2004). The function of this pigment is to protect the electron transport chain from photoinhibition (Schafer et al., 2005). Indeed, wild-type cells grown under higher light intensity (PA, 40 µE m^{-2} sec^{-1}) showed a higher myxoxanthophyll/chlorophyll ratio than the wild-type cells grown in the photomixotrophic conditions under dim light (PM, dim). An increase of the myxoxanthophyll suggests that the cells of the mutant suffer from stress already in weak light.

The biochemical properties of thylakoid membranes from the wild-type and *lepB1*::Km^R mutant cells were also studied in respect of their pigment composition. The membranes were resuspended in a buffer and aliquots were taken to determine concentrations of proteins and chlorophyll in the samples. When compared on the same protein level, the membranes of the wild type have a 2 times higher chlorophyll concentration than the mutant cells. Therefore the ratio of protein to chlorophyll in the membranes of the wild type was 7.7, in the membranes of mutant 13.2. This feature of the mutant cells is additional evidence that a decrease of the chlorophyll concentration may cause the differences in the protein composition of the photosynthetic membrane.

3.6.3. The photosynthetic electron transport in *lepB1*::Km^R is inhibited by strong light

In order to examine the photosynthetic properties of the mutant in more detail, photosynthetic oxygen evolution was determined with the Clark type oxygen electrode for wild-type and mutant cells that were grown photomixotrophically at low light intensities (5 µE m^{-2} s^{-1}). Unexpectedly, the mutant was able to perform photosynthetic electron transport rather efficiently in short term experiments (the measurements for at least 5 minutes). Comparing identical cell numbers, the photosynthetic oxygen production of the mutant reached approximately 80% of the wild-type level, irrespective of whether the entire photosynthetic electron transport chain or solely the PSII-mediated electron transport was measured for 5 minutes (Table 13). Thus, despite the lack of competence to grow photoautotrophically the mutant is competent for photosynthetic electron transport which indicates that the photosynthetic complexes of the thylakoid membrane are able to operate.

Table 13. Rates of electron transfer reactions in *Synechocystis* 6803 cells.

	oxygen evolution* [µmol O_2 A_{730}^{-1} L^{-1} h^{-1}]	
	wild type	***lepB1*::Km^R**
whole chain electron transport	995 ± 53	783 ± 51
PSII-mediated electron transport	1956 ± 173	1543 ± 187

* determined in four independent short term experiments (5 min) with cells grown in photomixotrophic conditions at 5 µE m^{-2} s^{-1}

However, photosynthetic electron transport is inhibited in the mutant cells by high light intensities, as shown by two complementary approaches. In the first approach, the measurement of the entire photosynthetic electron transport chain was repeated with diluted culture samples which allowed to determine photosynthetic oxygen evolution with the Clark electrode for extended periods of time. The idea behind this experiment was to potentially identify effects on electron transport which become visible only after long-term exposure of the cells to high light intensities (e.g., 100 or 500 µE m^{-2} s^{-1} in the example shown in Fig. 20).

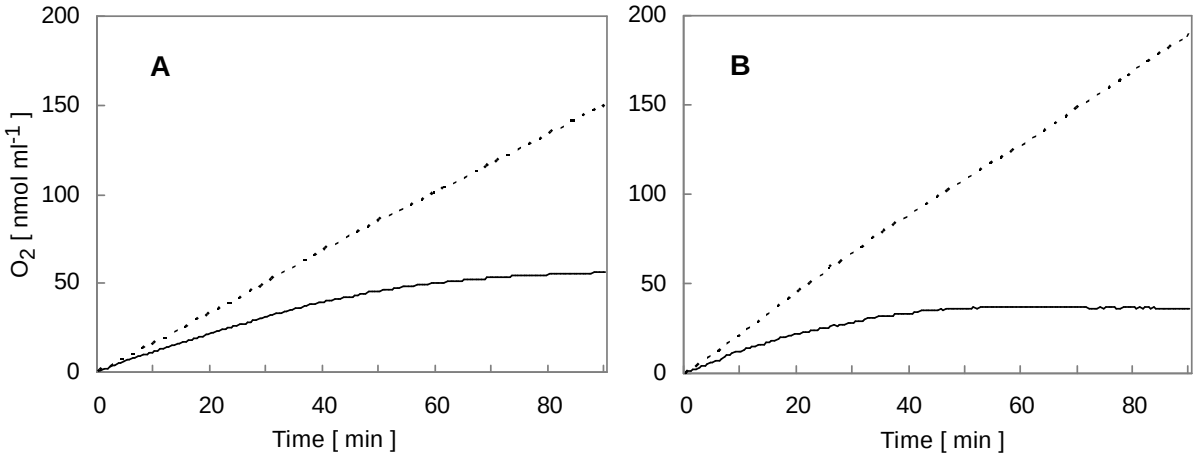

Figure 20. Photoinhibition of the oxygen evolution in the lepB1::Km^R mutant. Photosynthetic oxygen evolution of wild type (dotted line) and lepB1::Km^R cultures (solid line) which were exposed to continuous light (panel A – 100 µE m^{-2} s^{-1}; Panel B - 500 µE m^{-2} s^{-1}) was determined for identical amounts of cells using a Clark electrode.

Indeed, with this experimental design stronger differences between mutant and wild-type cells became apparent. While oxygen production in wild-type cultures remained essentially unaltered during the entire incubation time, the mutant cells showed a clear drop in oxygen evolution after approximately 20 minutes (Fig. 20A). When incubated for more than 60 minutes, photosynthetic oxygen production was even terminated in the mutant culture. Essentially the same results were obtained when the experiment was performed with more

moderate light intensities (100 µE m^{-2} s^{-1}), except that the effects were slightly retarded (Fig. 20).

The cells of *Synechocystis* 6803 are capable to accumulate carbohydrates in form of glycogen if they are incubated in medium grown in the presence of glucose (Yoo et al., 2002). To prevent the consumption of glycogen reserves, the glucose was added during the measurement, but it did not support the stable photosynthetic activity of the mutant, which become completely inhibited after 60 minutes of incubation in the light (Fig. 21). The observed inactivation of the photosynthetic electron transport machinery of the mutant cells occurs during extended exposure to light irresepective if the additional energy source is added to the medium. These data suggests that the photosynthetic electron transport chain of the mutant can not be used for cell metabolism.

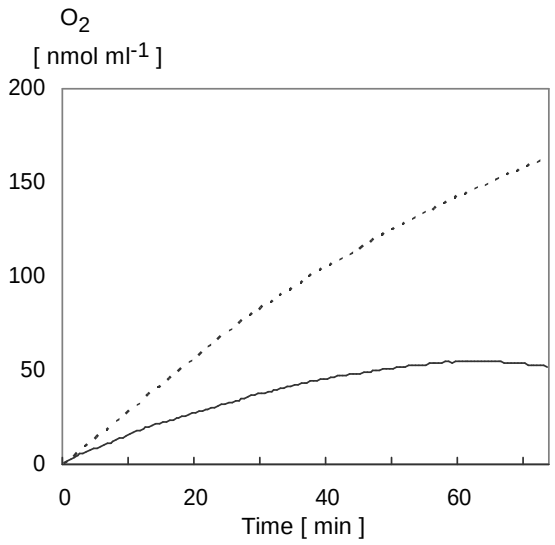

Figure 21. Oxygen evolution of *Synechocystis* 6803 cells in the presence of glucose. Wild-type (dotted line) and *lepB1*::Km^R cultures (solid line) were exposed to continuous light (1000 µE m^{-2} s^{-1}). The O_2 evolution activity was determined for identical amounts of cells upon addition of 10 mM glucose using a Clark electrode.

This was confirmed by an independent approach in which the oxygen evolution was determined for cultures that were exposed to light stress (30-120 minutes at 1000 µE m^{-2} s^{-1}) prior to the actual measurement. While the wild type was only mildly affected by this treatment, preserving more than 90% of the initial photosynthetic activity even after 120 minutes of light stress, the activity of the mutant declined gradually with increasing incubation time (Fig. 22). After 120 minutes, the residual photosynthetic activity was reduced to approximately 15% of that obtained with untreated samples. From these data it must be

concluded that the mutant cells are able to assemble a functional photosynthetic electron transport chain when grown in dim light but that they are apparently not able to preserve its activity when exposed to high light intensities.

From these data it seems likely, that the functioning of the photosynthetic electron transport chain is harmful for the mutant cells. Inhibition of the photosynthetic activity by the light occurs even in moderate light intensities, thus reflecting that the cells of the mutant cannot use the energy of photosynthesis for the growth.

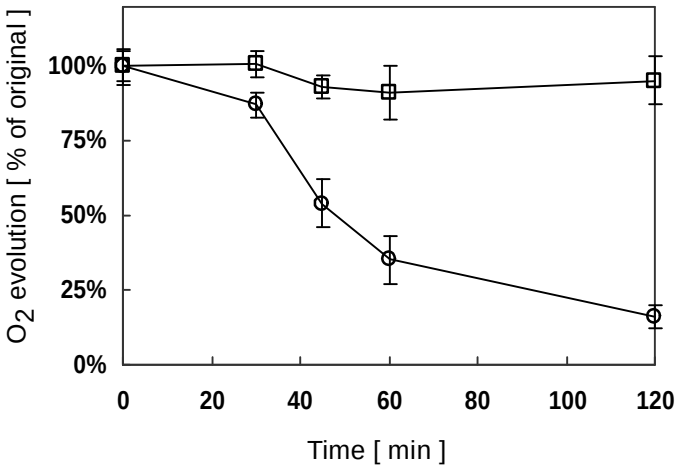

Figure 22. Photosynthetic oxygen evolution of *lepB1*::Km^R mutant cells is inhibited by light stress. Panel A- whole chain electrone transport, Panel B – photosystem II activity. Cultures of *Synechocystis* 6803 wild type (squares) and *lepB1*::Km^R mutant (circles) propagated under photomixotrophic conditions in dim light (5 µE m^{-2} s^{-1}) were exposed to light stress (1000 µE m^{-2} s^{-1}). Samples containing identical amounts of cells were taken at the time points indicated and photosynthetic oxygen evolution was immediately determined using a Clark electrode. For each time point, the average of at least five independent measurements is given. The error bars show the standard error.

3.6.4. The assembly of the core proteins of photosystems is not significantly affected in the mutant.

3.6.4.1. Analysis of thylakoid membrane proteins using SDS-PAGE

As a first step to analyse *Synechocystis* 6803 proteins, the thylakoid membranes were isolated and thylakoid proteins were separated on SDS-PAGE. After electrophoreses, the proteins were stained by Coomassie, and the resulting protein patterns of wild type and mutant were compared (Fig. 23). The analysis of the proteins from both wild type and *lepB1*::Km^R mutant did not reveal any significant differences, which suggests that *lepB1*::Km^R mutant cells are

capable of, for example, forming photosynthetic complexes in the thylakoid membrane when grown in dim light in the presence of glucose (Fig. 23A). However, after incubation of the cultures in photoautotrophic conditions, the strong protein degradation in the cells of the mutant *lepB1*::Km^R could be observed (Fig. 23B). This feature of the mutant suggests that the proteins, which are synthesized under non-photosynthetic conditions, are not stable in the light, thus confirming the incapability of photoautotrophic growth.

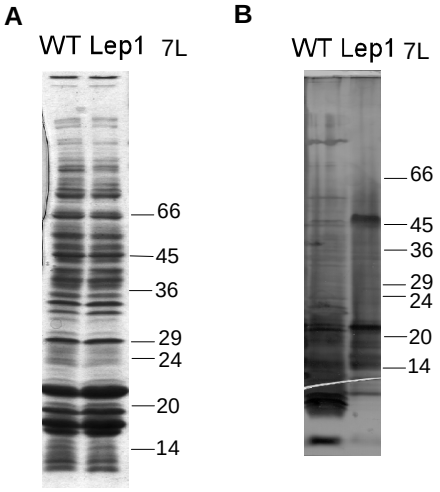

Figure 23. Protein analysis of total membrane preparation from the wild type *Synechocystis* 6803 and the *lepB1*::Km^R mutant. Coomassie-stained gel of total protein from wild type membranes (WT) and the membranes of *lepB1*::Km^R mutant (lepB1) were prepared either from cells grown in photomixotrophic conditions (panel A) or from the cells that were placed for 3 days in photoautotrophic conditions (light intensity 40 µmol/m^2 sec) (panel B). in total, 100 µg protein were loaded on the gel. Mobilities of molecular weight markers are indicated in kDa.

3.6.4.2. Analysis of membrane protein complexes by blue-native PAGE.

For analysis of the protein composition of the thylakoid membrane, blue-native PAGE was chosen (Schägger et al., 1994, Schägger and Jagow, 1991). This method allows separation of the native protein complexes of the thylakoid membrane (Kügler et al., 1997; Berghöfer and Klösgen, 1999). The detergent digitonine was used for solubilization of the thylakoid membranes of *Synechocystis* 6803. The solubilized complexes were charged with Coomassie-dye prior to native electrophoresis. In blue-native PAGE the complexes of the thylakoid membrane are visible either as blue-stained (like ATP-synthase or cytochrome b_6f complex) or blue-green stained bands (like PSII or PSI complexes which contain chlorophyll molecules).

During the adaptation of the solubilization step for *Synechocystis* 6803 thylakoids it became notable that it was difficult to resuspend the membranes in the same volume of solubilization buffer as that used for samples of spinach thylakoids (data not shown). For optimisation of this step, the thylakoid membranes of *Synechocystis* 6803 were resuspended in a higher volume of lysis buffer (Fig. 24A). Finally, a buffer volume of 25 µl instead of 15 µl (as used for spinach thylakoids) was chosen to resuspend the membranes containing 30 µg of chlorophyll *a*.

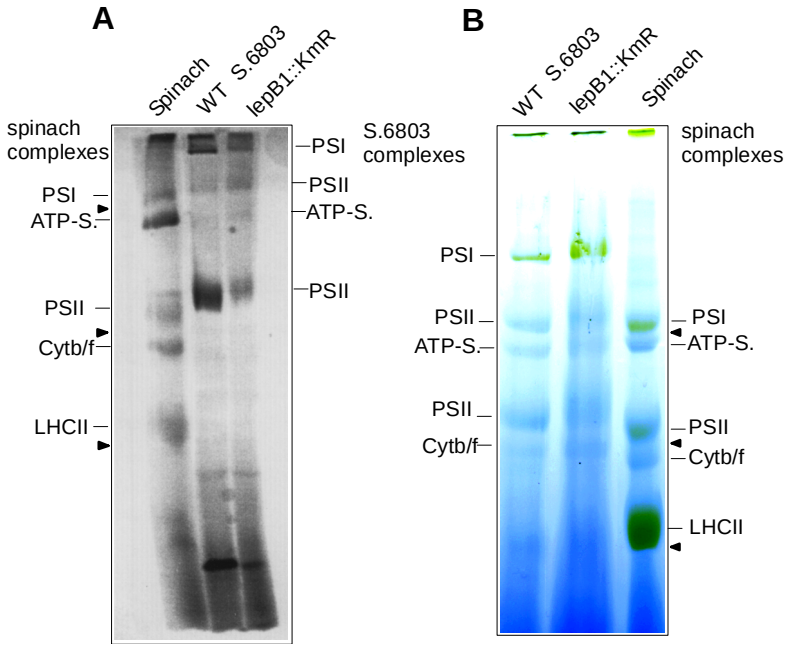

Figure 24. Blue-native PAGE of the thylakoid membranes of spinach and *Synechocystis* 6803 wild type and *lepB1*::Km^R mutant. The thylakoid membranes of *Synechocystis* 6803 wild type and *lepB1*::Km^R mutant were solubilized in different volumes of buffer with 1% digitonin. The protein complexes of *Synechocystis* 6803 (the positions are shown in center) and spinach (the positions are shown on the sides) were separated by blue-native PAGE. Thylakoid membranes of *Synechocystis* 6803 with chlorophyll concentration 30 µg were resuspended in 42 µl of solubilization buffer (Panel A) or in 30 µl of solubilization buffer (Panel B). The positions of the molecular weight standards are shown by arrowheads (from the top to the bottom: thyroglobulin (669 kDa), ferritin (440 kDa), and catalase (232 kDa)).

After the separation of the complexes in the first dimension, the differences in the mobilities of protein complexes from *Synechocystis* 6803 and spinach thylakoids could be observed (Fig. 24). Though the number of the complexes was identical, the order of the complexes was different in spinach and *Synechocystis* 6803 samples (Fig. 24; Berghöfer and Klösgen, 1999). For instance, the green complex in *Synechocystis* 6803 samples was absent in spinat, and the green-coloured band in spinach sample with molecular weight of 240 kDa, which corresponds

to LHCII complex did not appear in *Synechocystis* 6803. Notably, the bands of *Synechocystis* 6803 samples were less sharp in the first dimension of blue-native gels than the bands of spinach samples.

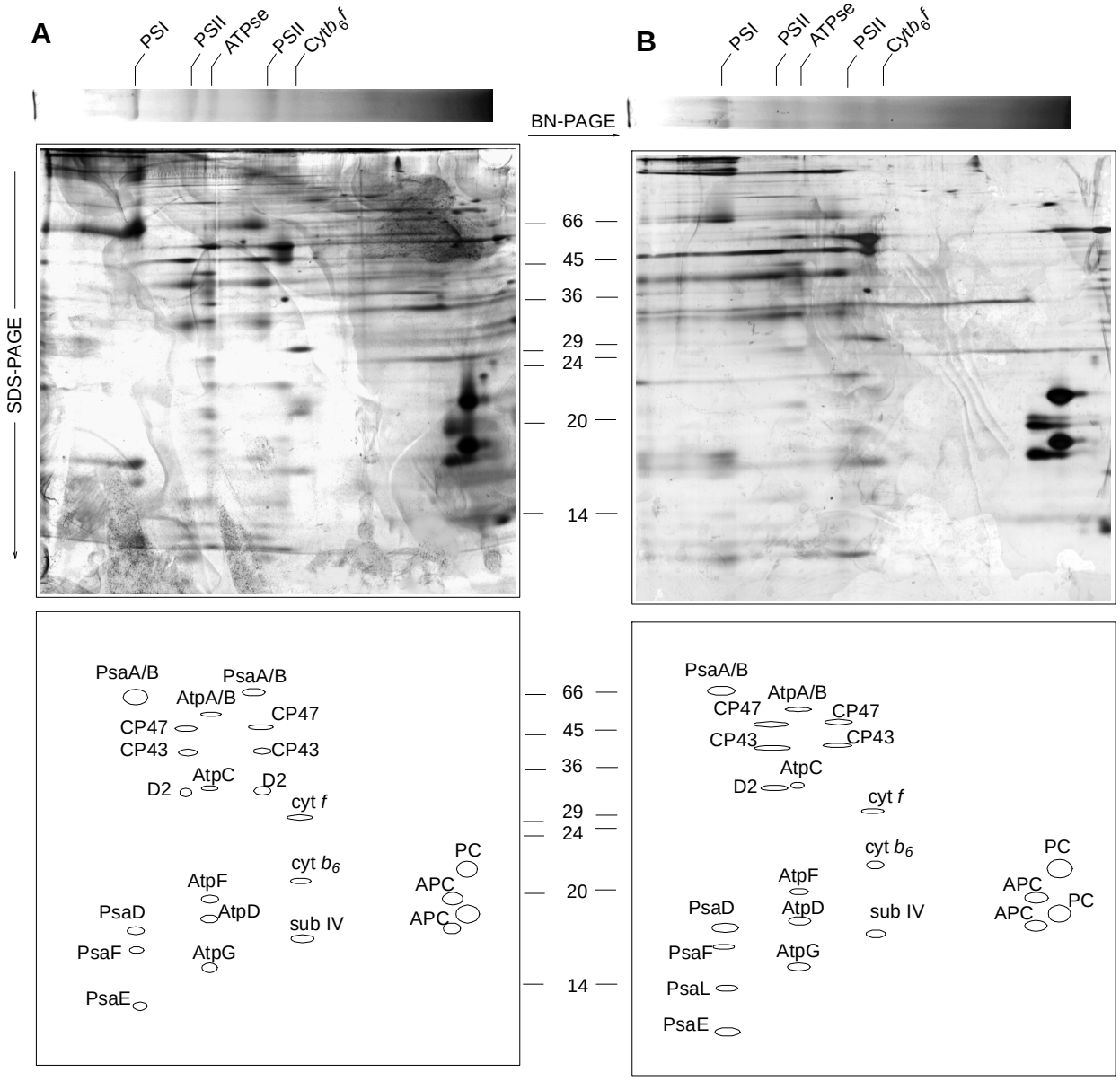

Figure 25. Blue-native PAGE of the protein complexes of *Synechocystis* 6803 wild type and *lepB1*::Km^R mutant. The thylakoid membranes of the wild type (panel A) and *lepB1*::Km^R mutant (panel B) were solubilized with digitonin and separated on the blue-native PAGE (BN-PAGE). Then the gel strips were denatured and placed on SDS-PAGE and the proteins were separated according to their molecular weight. The molecular masses of the standard proteins are given in the middle. The imprints under the gel pictures show the protein spots, for abbreviation of the protein names see text.

The separated complexes of the wild type and *lepB1*::Km^R mutant were compared after the first dimension. The concentration of the photosystetic complexes seemed to be lower in the pattern of the mutant when compared with the wild type, though the samples contained same quantities of chlorophyll (Fig. 25B).

After the first dimension the gel strips were excised from the BN PAA gel and the proteins were separated in the second dimension by SDS-PAGE according to their molecular weights. The protein complexes of *Synechocystis* 6803 were identified by analysis of the molecular weight of the proteins from the second dimension. The mobilities of the proteins on SDS-PAGE were compared with those observed previously (Kruip et al., 1997; Herranen et al., 2004; Huang et al., 2002; Peterman et al., 1998) and the proteins were assigned to the complexes of the first dimension (Table 14).

Table 14. Protein assignment in the *Synechocystis* 6803 complexes of BN-PAGE

Protein complex	Molecular weight of the proteins on SDS-PAGE	Corresponding protein
1. PSI	65 kDa 17 kDa 16 kDa 14 kDa 12 kDa	PsaA/PsaB PsaD PsaF PsaL PsaE
2. and 4. PSII	48 kDa 37 kDa 31 kDa	CP47 CP43 D2
3. ATP-synthase	59 kDa 31 kDa 19 kDa 18 kDa 15 kDa	AtpA/AtpB AtpC AtpF AtpD AtpG
5. Cytb_6f	29 kDa 22 kDa 17 kDa	cyt*f* cytb_6 subunit IV

The proteins complex of *Synechocystis* 6803 with highest molecular weight had green color. The subunits of photosystem I, PsaA and PsaB have predicted molecular weight of about 82 kDa, but on SDS electrophoresis they appear usually as band at approximately 65 kDa (Kruip et al., 1997; Sun et al., 1997). This band and the remaining protein bands below 17 kDa were referred to the proteins of photosystem I (Fig. 25, Table 14).

Another green complex, which has molecular weight of 670 kDa, represents photosystem II with the proteins CP47, CP43 and D2 (Fig. 25). D1 protein, which has an approximate size of 29 kDa, could not be detected in samples of *Synechocystis* 6803 wild type and *lepB1*::Km^R mutant after second dimension. The green complex with the molecular weight of about 450 kDa, which was also assigned to PSII, comigrated with the proteins of PSI complex (Fig. 25).
ATP-synthase complex migrates most likely next to the photosystem II complex as blue-coloured band with the molecular weight of 600 kDa. Though the subunits observed on the SDS-gel and listed in the Table 14 could match the subunits of ATP-synthese, it is not definitive that these proteins solely belong to ATP-synthase complex.
Cytochrome b_6f complex was represented by three proteins, which correspond to cytochrome *f*, cytochrome b_6, and subunit IV (Peterman et al., 1998; Herranen et al., 2004). This complex migrated at 420 kDa. The phycobilisome complex containing phycocyanin (PC) and allophycocyanin (APC) migrates right at the bottom of SDS gel as seen from the second dimension (Fig. 25).
The detailed examination of the composition of the separated thylakoid membrane complexes of the wild type and *lepB1*::Km^R mutant showed no significant differences. Taken together, the data suggest that the core complexes of thylakoid membrane have idenical composition in the *lepB1*::Km^R mutant and wild type if the cultures are grown in dim light with glucose, the assembly of the core complexes in the thylakoid membranes of *lepB1*::Km^R mutant is not really affected in the tested growth conditions. Unfortunately, the attempts to analyse thylakoid membranes from the cells exposed to the higher light have failed, since the optimal time for harvesting of the mutant cells without complete degradation of the pigments and proteins in thylakoid membranes was not reached (data not shown).

3.6.4.3. Analysis of cytochrome b_6f complex by specific staining

In order to clarify how the leader peptidase inactivation influences the photosynthetic properties of the mutant, an analysis of the cytochrome b_6f complex using heme-staining was performed. Since tetramethylbenzidine (TMBZ) covalently binds heme-groups in proteins, the concentration of heme-containing proteins in wild type and mutant cells could be estimated after staining of the PAA gel with TMBZ (Thomas et al., 1976) (Fig. 26). This analysis showed that the concentrations of protein bands stained by TMBZ were at least two times lower in the *lepB1*::Km^R mutant than in the wild type when the membrane samples containing the equal protein amount were loaded on the gel. This result indicates that there is significant

reduction of the heme-containing proteins in the thylakoid membranes of the *lepB1*::Km^R mutant.

The content of the proteins with heme-group was estimated and the bands were compared with those observed previously by Tichy and Vermaas (1999). According to molecular weight the respective proteins were assigned to cyt *f*, cyt b_6, cyt$_{550}$ and cyt $_{553}$. The decrease in concentration of the cyt *f*, cyt b_6 and cyt$_{553}$ proteins in the mutant cells compared to the wild type suggests a reduction in the content of cytb_6 *f* complex (Fig. 26A). The same samples were also analysed by staining of the proteins in the PAA gels with Coomassie (Fig 26B). It was confirmed that the concentration of the total protein in samples of wild-type and mutant was equal. Thus the decrease in the concentration of cytochromes could be observed only with the heme-specific reaction.

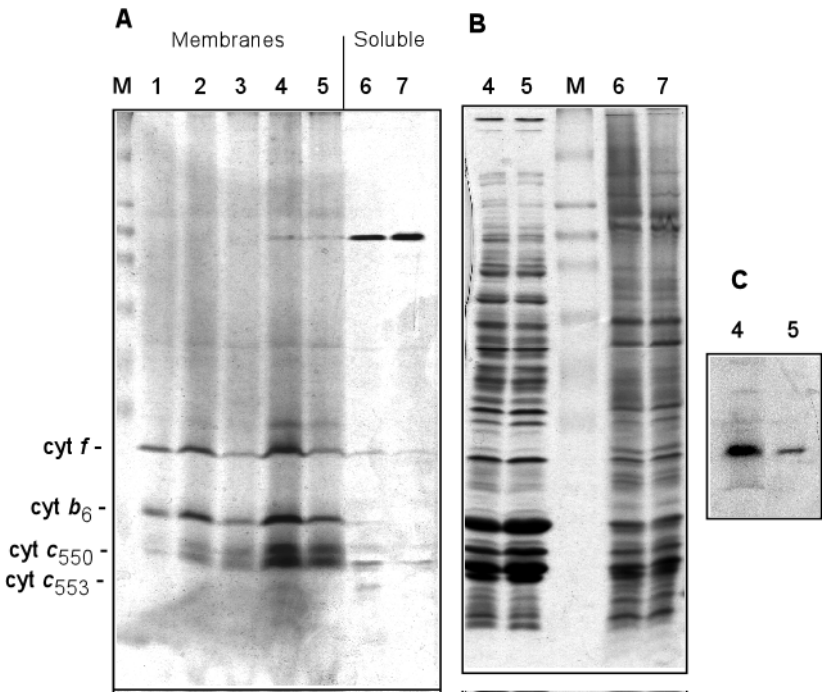

Figure 26. Heme staining of the total membrane proteins from wild type *Synechocystis* 6803 and the *lepB1*::Km^R mutant. Thylakoid membranes were isolated, and the samples were loaded on a polyacrylamid gel, which contained 25 µg (lane 1), 50 µg (lanes 2,3) and 100 µg of protein (lanes 4,5). The cytosolic samples containing soluble proteins (100 µg) were collected during the thylakoid membrane isolation. The proteins from wild-type (WT, lanes 1,2,4 and 6) and *lepB1*::Km^R mutant samples (M, lanes 3,5 and 7) were identified by heme-staining (panel A), or by coomassie-staining (panel B), or by cross-reaction with a cyt f antibody (panel C).

A similar result was obtained after immunoblotting analysis using an antiserum raised against the spinach cyt *f* protein. The accumulation of the cyt *f* protein was strongly decreased in the membrane samples of the mutant (Fig 26C). However, the obtained result showed no direct connection between the inactivation of LepB1 and decrease of accumulation of cytochrome b_6f complex. Since no precursor form of cyt *f* protein could be detected in the *lepB1*::Km^R mutant**,** the leader peptidase LepB1 is apparentely not involved in apocytochrome processing (Fig 26C).

3.6.4.4. Immunological analysis of thylakoid proteins

The gene *lepB1* encodes a putative peptidase, which might be required for removal of signal peptides from precursor proteins that need to cross the membrane to reach their final destination in the cell. Considering the phenotype of the *lepB1*::Km^R mutant described so far, LepB1 appears to be important for the processing of precursor proteins of the photosynthetic machinery in the thylakoid membranes. This function can be assumed to be essential for the biogenesis of the photosynthetic electron transport chain and thus to be particularly important under conditions in which the photosynthetic machinery is damaged like, for example, after photooxidation of the membrane complexes by light stress. Under these circumstances, the number of defective protein subunits that need to be replaced will be significantly higher.
To investigate the function of the LepB1 protein, the protein composition of thylakoid membranes of *Synechocystis* 6803 wild type and mutant was analysed in more detail using antisera raised against various proteins. Mainly, proteins synthesized with N-terminal signal peptide were examined. The obtained results can be divided in three groups.

1) no difference in the accumulation of the tested proteins in the samples of the wild type and the *lepB1*::Km^R mutant: Rieske protein and Slr0924 (Fig. 27)

2) a decrease in accumulation in the samples of the *lepB1*::Km^R mutant: PsaA/B and PsbO, (Fig. 28, Fig. 29)

3) no any crossreaction of the *Synechocystis* 6803 samples with following antisera:
Anti-CR43, Anti D1, Anti-CF0II , Anti-PSI-3 and Anti-Plastocyanin (data not shown)

The first group of analysed proteins showed no difference in the accumulation in wild type and *lepB1*::Km^R mutant cells. The protein Slr0924 shows similarity to the plant chloroplast protein Tic22 (Fulda et al., 2002). The synthesis of Slr0924 is induced by the high salt

concentrations (Fulda et al., 1999). This protein is mainly localized in the thylakoid lumen though it was also detected in the periplasmic space and is synthesized with an N-terminal signal sequence, which is similar to those of Sec-pathway (Fulda et al., 2002; Fulda et al., 2000). This protein Fig. 27A shows the Western blot analysis with antiserum raised against Slr0924 protein of *Synechocystis* 6803. It is apparent that no alterations in accumulation or processing of this protein in the *lepB1*::Km^R mutant cells could be detected. The specific reaction band corresponds to the protein with the size of 26 kDa. Since the absence of leader peptidase LepB1 did not lead to any alterations of Slr0924 protein, it seems that another leader peptidase LepB2 is responsible for processing of this protein.

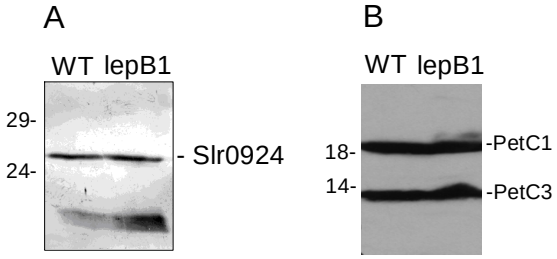

Figure 27. Western blot analysis of the protein encoded by slr0924 and of Rieske protein. Panel A. The thylakoid membrane samples of the wild type (WT) containing 100 µg of total protein (or 13 µg Chl a) and *lepB1*::Km^R mutant containing 170 µg of total protein (which corresponds the same chlorophyll concentration as in wild type sample 13 µg Chl a) were separated on SDS PAA gel. Panel A.The specific band which is recognized by slr0924 antibody (about 26 kDa) as well as mobilities of molecular weight standards are indicated.
Panel B. The same membrane samples were tested with antiserum raised against the Rieske protein from spinach: PetC1 – encoded by sll1316, PetC3 – by sll1182. The positions of the protein weight standards are indicated.

The Rieske protein is an important subunit of the cytochrome b_6f complex. In plant chloroplasts, this protein is transported into the thylakoid membrane via the delta-pH way. The signal peptide of the protein undergoes no cleavage and serves to anchor the protein in the thylakoid membrane. In *Synechocystis* 6803, 3 Rieske proteins were identified (Nakamura et al., 1998). All three proteins consist of the N-terminal transmembrane α-helix, which contain RR-motif and serve to anchor the protein in the membrane (Schneider et al., 2002). However, only two of them, namely PetC1 and PetC3 with approximate molecular weights of 19 kDa and 13 kDa, respectively, could be detected in thylakoid membranes of *Synechocystis* 6803 by Western blot analysis using specific antisera (Schneider et al., 2002). In order to analyse the translocation of Rieske protein in the *lepB1*::Km^R mutant strain, the Western blot analysis was carried out with an antiserum raised against Rieske protein of spinach (Fig 27B).

The antiserum crossreacted with two proteins with approximate molecular weights of 19 kDa and 13 kDa. These proteins were assigned to PetC1 and PetC3 based on the results obtained previously by Schneider et al. (2002). Thus, in the cells of the *lepB1*::Km^R mutant no alterations in accumulation of Rieske proteins could be detected.

Furthermore, several proteins were analysed by Western blot although they did not contain N-terminal signal peptides. The integral membrane proteins PsaA and PsaB insert in the thylakoid membrane co-translationally; these proteins form the core of photosystem I and were therefore chosen to analyse the corresponding complex in the cells of the *lepB1*::Km^R mutant. The antisera raised against PsaA/B heterodimer of *Synechococcus* recognized PsaA/B proteins in *Synechocystis* 6803, and was therefore used for immunoblotting of thylakoid membrane samples.

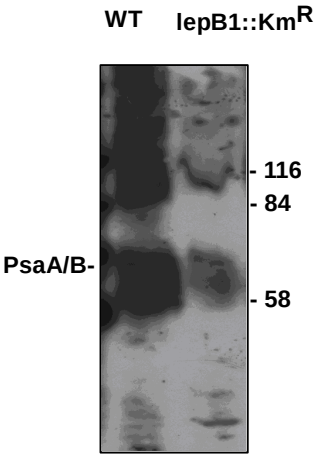

Figure 28. Western blot analysis of PsbA/B proteins in thylakoid membranes of *Synechocystis* 6803. The thylakoid membrane samples of the wild type (WT) and sll0716:: Km^R mutant containing100 µg of total protein were solibilized with Laemmli buffer and loaded on SDS-PAA gel. Mobilities of molecular weight standarts (in kDa) and the reacting band, which correspond to the PsaA/B dimer (with mobility of about 62 kDa) are indicated.

This analysis revealed that the PsaA/B content was significantly lower in the samples of the thylakoid membranes from *lepB1*::Km^R mutant if the same protein amount as in the membranes of wild type was loaded (Fig. 28). Such decrease in concentration of the core Photosystem I proteins implies the decrease of PSI content, which was also suggested in previous experiments. (3.2.)

As next step to examine whether LepB1 is involved in the processing of thylakoid proteins, Western blot analysis was performed for the subunit PsbO of the oxygen evolving system associated with photosystem II. In *Synechocystis* 6803, this protein is synthesized as a precursor polypeptide carrying a signal peptide for the translocation across the thylakoid membrane (Erickson and Rochaix, 1992). In wild-type cells, a single protein was recognized

by the antiserum which was raised against PsbO from spinach (Fig. 29). According to its mobility upon SDS-PAGE, its size was estimated to be approximately 29 kDa which is in line with the molecular mass predicted for mature PsbO (26.5 kDa). This protein is also present in the *lepB1*::Km^R mutant. However, in these samples a second polypeptide is detected by the antibodies which has a size of approximately 32 kDa. It probably represents the unprocessed precursor of PsbO that still carries the PsbO signal peptide with a predicted mass of 3 kDa. This result indicates that LepB1 plays an important, though not an essential role in the maturation of PsbO.

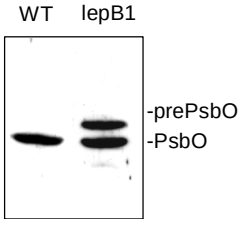

Figure 29. Western analysis of thylakoid proteins in wild-type and *lepB1*::Km^R mutant. Thylakoid proteins isolated from cultures propagated under photomixotrophic conditions in dim light were separated by electrophoresis on 10% - 17.5% SDS-polyacrylamide gradient gels, transferred to nylon membranes and analysed with antisera raised against PsbO. The position of the presumed precursor and mature proteins is indicated.100 µg of protein was loaded on the gel.

3.6.4.5. In the *lepB1*::Km^R mutant cells some proteins, which are synthesized with the signal peptides, accumulated in reduced amounts

In order to allow for the simultaneous analysis of a large number of proteins from *Synechocystis* 6803, two-dimensional electrophoresis was chosen, which combines isoelectric focussing and SDS-PAGE.

The solubilization of the native thylakoid membrane was carried out with mild nonionic detergent triton X-100, which is widely used for solubilization of the membrane proteins (Phadke et al., 2001). However, in the two-dimensional gel with an isoelectric focusing range of pH 3-10 and a protein load of 100 µg no more than 100 spots could be resolved (Fig. 30). The analysis of the protein patterns of the wild-type or *lepB1*::Km^R mutant thylakoid membrane proteins did not show significant differences. To prove how this approach can be applied for the separation of the membrane proteins, a Western blot analysis of the protein patterns was performed with the antiserum raised against PsbO protein of spinach (Fig. 30).

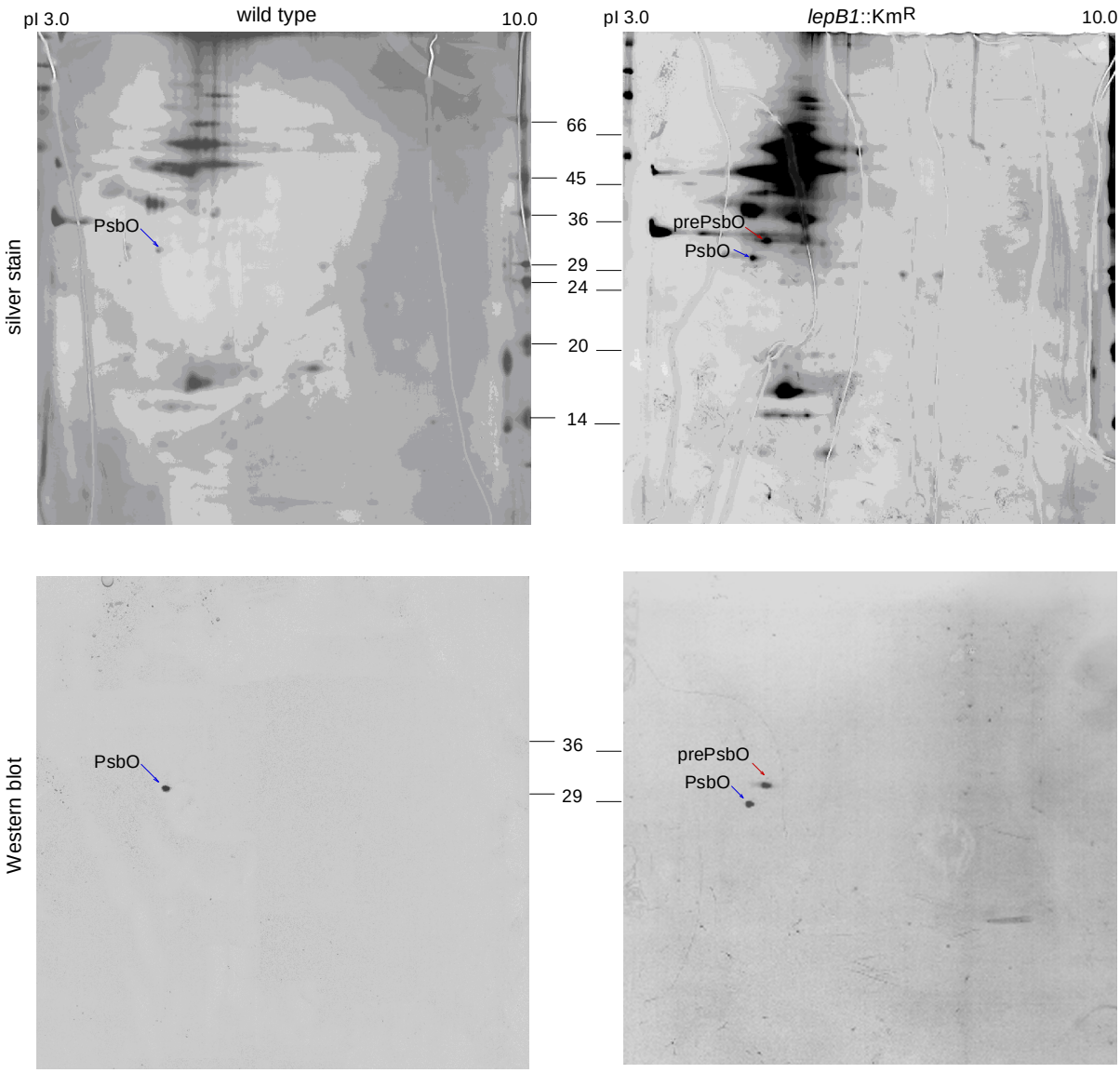

Figure 30. Two-dimensional analysis of thylakoid proteins from *Synechocystis* 6803 wild type and the *lepB1*::Km^R mutant. Thylakoid membranes from the cells grown under photomixotrophic conditions in dim light were solubilized in Triton X-100 containing buffer. The proteins were separated in two dimensions combining isolelectric focussing (pI 3.0 - 10.0) and SDS-polyacrylamide 10% - 17.5% gradient gel. In each case, 100 µg protein was loaded. In both panels, examples of gels obtained with proteins isolated from either wild type cells (left side) or *lepB1*::Km^R cells (right side) are shown after silver staining. Lower panels show the Western blot analysis with antiserum raised against PsbO protein from spinach. NB: the stronger intensity of the protein spots on the 2D gel with mutant sample is due to a little longer incubation time in developing solution by the silver staining; the vertical strips on the 2D gel with wild type sample are due to an interferention of the chlorophyll by the silver staining.

This analysis showed that an additional spot can be recognized by PsbO antibody in the protein pattern of *lepB1*::Km^R mutant. This protein spot has a mobility of 32 kDa which is approximately 3 kDa higher than that of the mature protein. The pI shift of about 0.2 to basic

pH could be also observed for this additional protein spot. These parameters were compared with predicted parameters for precursor and mature PsbO protein. The PsbO precursor protein has a predicted molecular weight of 29.9 kDa and pI of 4.82, which is higher than the predicted parameters of PsbO mature protein (26.7 kDa and pI of 4.7). The predicted difference of 3 kDa is in line with the difference observed in the pattern of *lepB1*::Km^R mutant sample. The increased pI of the additional protein spot fits well with higher pI predicted for the precursor protein (Fig. 30). Therefore, these observed differences of molecular weight and pI between both protein forms therefore allowed us to assign this additional spot detected in the *lepB1*::Km^R mutant sample as the precursor form of PsbO protein. Apparently this method of separation allows detecting some membrane-bound proteins, though the extremely low number of proteins which can be analysed required further optimisation of the separation method. Another disadvantage of solubilization of the native membranes is the influence of the pigments on the protein separation. For instance, the chlorophyll in the gel of the second dimension interferes during the subsequent silver staining. To increase the number of proteins which can be analysed, additional methods for sample preparation were tested. The precipitation of the proteins with trichloracetic acid (TCA) permits avoiding the interfering substances in isoelectric focussing (IEF). A sample of total proteins isolated from whole *Synechocystis* 6803 cells was chosen for this method. For solubilization of precipitated proteins, the zwitterionic detergent CHAPS was applied, which is apparentely more effective than Triton X-100 (Perdew et al., 1983).

2D gel electrophoresis of total protein was conducted first within pH range of 3.0 to 10.0 and the gel was stained with silver (Fig. 31). Since the most proteins were separated in the range of pI 4.0-7.0, the protein samples were subsequently applied on IPG strips in the pH range of 4.0 to 7.0. The silver straining of the gels allowed detection of about 800 proteins. The attempts to identify the proteins stained by silver nitrate using mass-spectrometry were unsuccessful. To analyse if this staining approach can be used further, one known protein was excised from the silver-stained 2D-gel and its identification was carried out using mass-spectrometry. The obtained results gave only 3 matching peptides after tripsin digestion, which is insufficient for reliable identification of the protein. According to this result the staining was subsequently always performed with coomassie (Fig. 33).

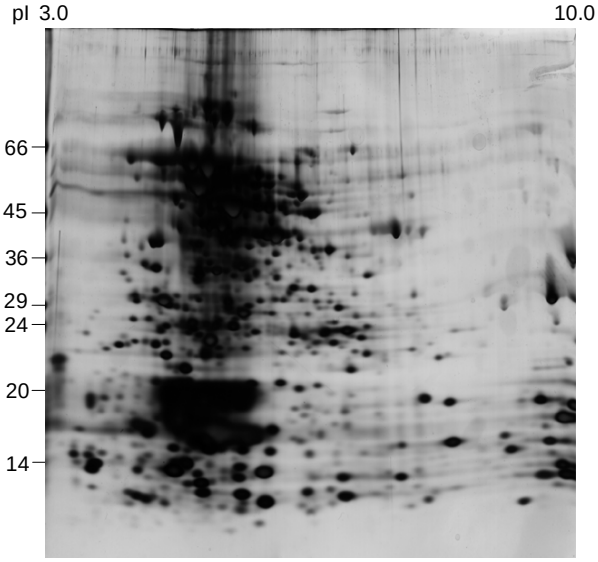

Figure 31. Two-dimensional analysis of the total proteins from *Synechocystis* 6803. Total proteins were isolated from wild-type cells grown under photomixotrophic conditions in dim light and separated in two dimensions combining isolelectric focussing (pI 3.0 - 10.0) and SDS-polyacrylamide 10% - 17.5% gradient gel.

The Western blot experiments with anti-PsbO antiserum were conducted with samples prepared for IEF (Fig. 32) or with the Western blot with proteins obtained after second dimension (Fig. 33). The Western blot analyses showed the presence in both wild type and *lepB1*::Km^R mutant only one form of the mature PsbO protein (Fig. 33), whereas the IEF sample of the mutant loaded on the SDS-PAGE still contaned the precursor of PsbO (Fig. 32). The absence of the precursor form of PsbO protein after the second dimension suggests that the precursor is lost during IEF. This loss is presumably caused by the treatment during precipitation of the proteins prior to solubilisation.

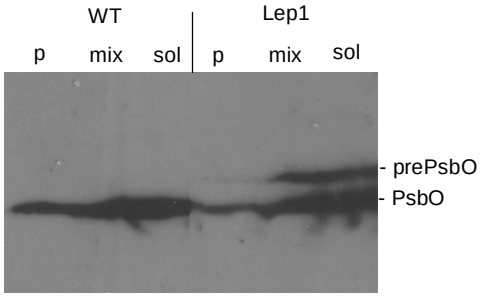

Figure 32. Western blot analysis of the samples for 2D analysis. Abbreviations: WT – wild type, Lep1 – *lepB1*::Km^R ; p – pellet, mix – solubilization mix; sol – solubilized protein.

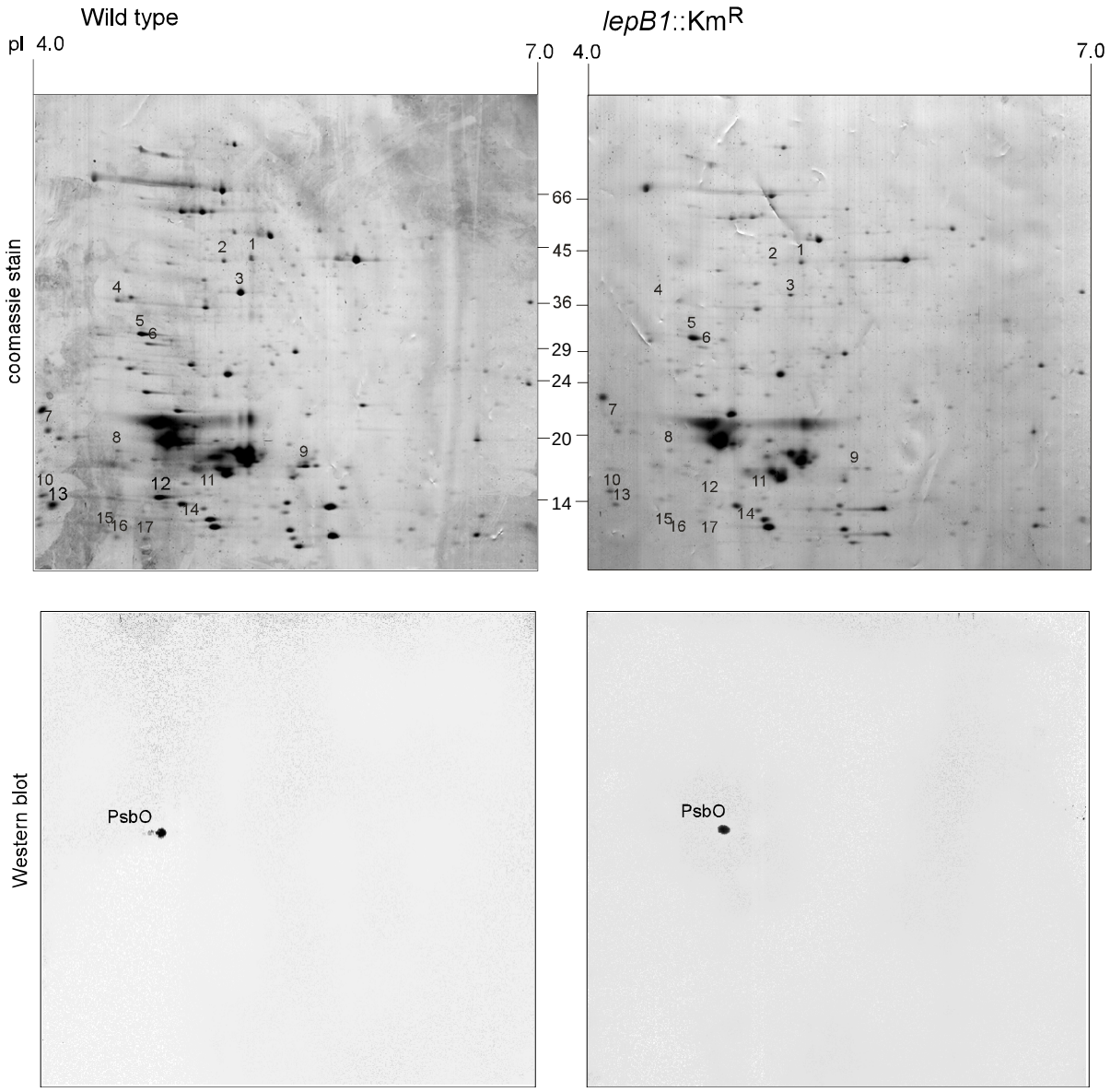

Figure 33. Two-dimensional analysis of the total proteins from *Synechocystis* 6803. Total proteins were isolated from wild-type and *lepB1*::Km^R cells grown under photomixotrophic conditions in dim light and separated in two dimensions combining isolelectric focussing (pI 4.0 - 7.0) and SDS-polyacrylamide 10% - 17.5% gradient gel. In each case, 200 µg protein was loaded. In both panels, examples of gels obtained with proteins isolated from either wild type cells (left panel) or *lepB1*::Km^R cells (right panel) are shown after staining with Coomassie Brilliant Blue. Numbers indicate protein spots that in five independent experiments always showed significant differences. These spots were recovered and analysed by MALDI-TOF-MS (see Table 15).

The 2D gel with a protein load of 200 µg and isoelectric focusing range of pH 4-7 results in a gel with about 200 spots visualized by coomassie staining (Fig. 33). After four independent experiments with the two-dimensional separation of the proteins from the wild type and

lepB1::Km^R mutant were performed, the resulting gels were computationally analysed with the Image 2D software (Amersham Biosciences). The comparison of the protein patterns of the mutant and the wild-type gels revealed that the concentration of about 17 proteins was altered in the *lepB1*::Km^R mutant cells (Fig. 33). That corresponds to 8% of the total number of proteins resolved in this experiment.

Table 15.
Proteins with altered accumulation in homozygous *lepB1*::Km^R cells.

Spot no.	**pI**	**Mol. mass** [kDa]	**ORF**	**Protein name**	**Localization**	**Signal peptide**
reduced amount in the *lepB1*::Km^R mutant (about 50 %)						
1	5.29	43.2	ni			
2	5.13	42.8	slr0394	phosphoglycerate kinase	cytoplasm	
3	5.23	36.8	slr0513	FutA2 (iron-binding transporter)	periplasm	31 aa
4	4.49	35.2	ni			
5	4.65	30.1	sll0427*	PsbO (33 kDa protein of PSII)	thylakoid lumen	28 aa
6	4.69	28.8	sll1784	unknown protein	periplasm	33 aa[1]
7	4.08	19.5	ni			
8	4.49	17.5	ni			
9	5.6	16.3	sll1578	CpcA (phycocyanin α chain)	phycobilisome	
11	4.04	14.3	ni			
12	4.74	14.0	sll1746	Rpl12, (ribosomal protein L12)	cytoplasm	
13	4.1	13.7	sll1194	PsbU (12 kDa protein of PSII)	thylakoid lumen	36 aa
14	4.93	12.6	ni			
15	4.41	12.0	ni			
16	4.48	11.7	ssl2501	unknown protein		
17	4.66	11.6	ni			
increased amount in the mutant (about 50%)						
10	5.04	14.3	ni			

ni – not identified proteins
* - confirmed by Western blot
[1] – according to SignalP (http://www.cbs.dtu.dk/services/SignalP-2.0)

As it can be seen from Table 15, almost all of the proteins with altered accumulation in the mutant cells, notably 16 out of 17, showed reduced concentrations**.** Eight of these proteins were subsequently identified by mass spectrometry from which four turned out to be synthesized with signal peptides (Table 15). Taking into consideration that only about 10% of

the proteins encoded by *Synechocystis* 6803 are predicted to carry signal peptides (Table 16, Table A), four out of eight proteins appears as a remarkably high value, although a serious statistical evaluation can not be made due to the limited size of the sample**.** Still, these results allow several interesting conclusions. First, the processing of neither of the precursor proteins identified is completely abolished in the mutant, in line with the results obtained by Western analysis for PsbO (Fig. 30). Instead, the accumulation of the respective terminal processing products is only reduced thus suggesting that an independent processing activity, presumably provided by LepB2, can replace the function of LepB1 to some extent. Second, PsbO, as components of the thylakoid system, was also detected in periplasm (Zak et al., 2001). FutA2, for example, is an iron-binding protein that is located in the plasma membrane of *Synechocystis* 6803 (Huang et al., 2002), but it was also detected in intrathylakoid space (lumen) (Tolle et al., 2002). This indicates that either LepB1 is involved in the processing of proteins from both membrane systems present in cyanobacteria or the thylakoid lumen and periplasmic space are connected (Zak et al., 2001). Therefore additional analyses of localization of LepB1 peptidase are necessary. Finally, among the polypeptides accumulating to a lesser extent in the mutant are also cytosolic proteins like phosphoglycerate kinase (Table 15) or ribosomal protein L12 which are not directly related to functions associated with the thylakoid system or the periplasmic space of the cells. So far, it is unknown why their accumulation is affected in the absence of LepB1 and it remains to be shown whether it is a direct consequence of the mutation or a secondary effect instead.

Not all proteins which are synthesized with a signal peptide and are present in lower concentration in the mutant cells have direct connection to photosynthetic process. The protein encoded by sll1784 is synthesized with a presequence but nothing is known about the function of this protein whether or not it has any relation to photosynthesis**.** Probably, this protein might be involved in response or in adaptation of the cells to strong light, since it was observed that the expression of this gene is activated after the transfer of the cells to strong light (Hihara et al., 2001). This protein was localized in the periplasmic space by two-dimensional separation of the membranes of *Synechocystis* (Huang, 2002). In this context, the study of the function of the protein encoded by sll1784 would be very interesting for a better understanding of the phenotype of the *lepB1*::Km^R mutant and of the biogenesis of the photosynthetic machinery.

3.6.5. Search of the full protein complement of *Synechocystis* 6803 for prediction of proteins with N-terminal signal peptides.

In order to obtain a general idea of how many proteins in *Synechocystis* 6803 are actually synthesized with a signal peptide and could be thus potential substrates for LepB1, a computational analysis was carried out with the machine learning techniques: the neural networks model (Nielsen et al., 1997) and the hidden Markov model (Nielsen and Krogh, 1998). For prediction of the presence of signal sequences the amino acid sequences can be analysed online with the SignalP program (http://www.cbs.dtu.dk/services/SignalP/).

It turned out that about 13% of the proteins (total 443) are predicted to be synthesized with N-terminal signal peptide, among those, most (65%) with unknown or hypothetical function (Table 16, Table A). Less than 4% (17 proteins) are photosynthetic proteins, subunits of the PSII, PSI, cyt $b_6 f$ and ATPase complexes. A rather big proportion, 12% of the potential substrates of the leader peptidases (54 proteins) play an important role in the transport of different cofactors and ions. Interestingly, to the potential substrates of leader peptidase belong the proteins, which are involed in processes of the cell development such as biosynthesis of cofactors, prosthetic groups, or components of the cell envelope (Table 16). The function of the leader peptidases might be of some importance for processing of the proteins involved in some cellular processes like for example cell division and chemotaxis or for some metabolic processes of *Synechocystis* 6803 cell.

In the analysis of the protein patterns of 2D gels, the concentrations of two photosynthetic proteins were altered. When compared with predicted data, the group of photosynthetic proteins is presented in higher number (4% against 11%, which corresponds to 2 of 17 proteins). Among proteins with decreased concentration in the mutant there were one transport protein FutA2 and one hypothetical protein, which are synthesized with a signal peptide. The proportions (in each case 6% of all proteins with altered concentration), which respresent one transport protein and hypothetical protein, appears to be lower in contrast to predicted proportions (12% and 34%, respectively).

About 14% of the potential subtrates contain the RR-motif in the signal peptide indicating that they may be transported via TAT-dependent protein translocation pathway in *Synechocystis* 6803 (Table A). The majority of the proteins listed in Table A belongs thus to the potential substrates of the Sec-pathway.

Table 16. Classification of the proteins, which might be potential substrates of leader peptidases in *Synechocystis* 6803.

Protein category	Subgroup	Number of the proteins
Biosynthesis of aromatic amino acids		1
Biosynthesis of cofactors, prosthetic groups, and carriers	*Cobalamin, heme, phycobilin and porphyrin*	1
	Carotenoid	3
	Nicotinate and nicotinamide	1
Cell envelope	*Membranes, lipoproteins, and porins*	8
	Murein sacculus and peptidoglycan	8
	Surface polysaccharides, lipopolysaccharides and antigens	1
	Surface structures	1
Cellular processes	*Cell division*	3
	Detoxification	4
	Transformation	1
	Chemotaxis	4
Central intermediary metabolism	*Polysaccharides and glycoproteins*	1
Energy metabolism	*Amino acids and amines*	1
	Sugars	2
	Glycolate pathway	1
Fatty acid, phospholipid and sterol metabolism		6
Photosynthesis and respiration	*ATP synthase*	1
	Respiratory terminal oxidases	1
	CO2 fixation	1
	Cytochrome b6/f complex	4
	NADH dehydrogenase	1
	Photosystem I	1
	Photosystem II	6
	Soluble electron carriers	2
Purines, pyrimidines, nucleosides, and nucleotides	*Purine ribonucleotide biosynthesis*	1
	Regulatory functions	8
Translation	*Degradation of proteins, peptides, and glycopeptides*	7
	Protein modification and translation factors	1
	Ribosomal proteins: synthesis and modification	1
Transport and binding proteins		54
Other categories	*Adaptations and atypical conditions*	2
	Drug and analog sensitivity	5
	Other	10
	WD repeat proteins	3
	Hydrogenase	1
Hypothetical		152
Unknown		134

3.7. Complementation of the *lepB1* mutant leads to reconstitution of the wild type phenotype

The cells of complementing strain pLep1 (see chapter 3.8) were able to grow like wild-type cells in photoautotrophic conditions (Fig. 37). The expression of the *lepB1* gene on the plasmid pSLEP1 in the cells of the *lepB1*::Km^R mutant could provide the same growth behavior as of wild type cells also in other conditions tested (Fig. 36). From these data it can be concluded, that the product of *lepB1* gene determines ability to photosynthetic growth.

Analysis of the absorption features of pLep1 complementing strain confirmed that the pigment content in the *lepB1*::Km^R mutant strain is identical to that of the wild type (Fig. 37). Moreover, the cells of the pLep1 strain did not show the light sensitivity, which was observed in the *lepB1*::Km^R mutant. Thus, the leader peptidase LepB1 function is important for proper pigment composition of *Synechocystis* 6803 cells and for resistance to strong light.

The photosynthetic activity of pLep1 cells grown in photomixotrophic conditions was identical to that of the wild type (Table 17). The slight decrease of photosynthetic activity of pLep1 cells grown in photoautotrophic conditions (about 75% of wild-type level, Table 17) might be caused by insufficient expression of the *lepB1* gene, when this gene is transcribed from the plasmid.

In the cells of the complementing strain containing the wild type gene *lepB1*, there was no accumulation of the precursor form of PsbO, irrespective of the growing conditions (Fig. 38). Taken together the data of complementation with the wild type *lepB1* gene suggest that the phenotype of *lepB1*::Km^R mutant derives from the *lepB1* gene inactivation.

3.8. Complementation with LepB from *E. coli*

The cloning of the homologous gene *lepB* from *E. coli* was performed similar to the cloning of *lepB1* gene from *Synechocystis* 6803 except that the insertion of the *lepB* gene was performed into the plasmid pVZ321M linearized with the NdeI and SalI restriction nucleases (Fig. 34). The resulting strain carrying the plasmids pELEPB was designated as pLepEc.

The genetic background of the pLepEc strain was analyzed using PCR. PCR analysis with primers specific to the *lepB* gene from *E. coli* showed that the fragment containing this gene was succesfully introduced in the cells of the *lepB1* mutant (Fig. 34).

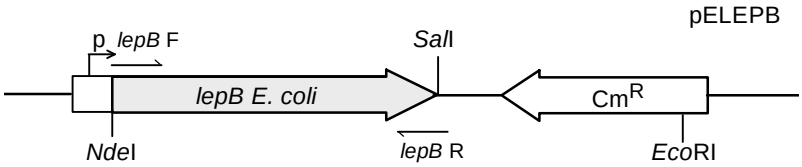

Figure 34. Complementation schemes of the pELEPB plasmid with *lepB* gene from *Escherichia coli.* Schematic map shows position of primers to lepB gene as *lepB* F (forward primer) and *lepB* R (reverse primer) that were used for PCR analysis of plasmid DNA with *lepB* gene from *E. coli*.. The plasmid pELEPB was transferred in the cells of the mutant yielding pLepEc strain.

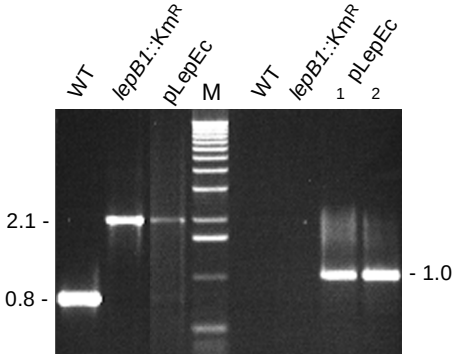

Figure 35. PCR analysis of the DNA isolated from wild type, *lepB1*::Km^R mutant and complementing strain of *Synechocystis* 6803. Total DNA was isolated from wild type, *lepB1*::Km^R mutant, complementing strain pLep1 and complementing pLepEc strains. M indicates the position of 1 kb ladder marker. PCR was carried out with primer pair sll0716F and lep1043rev which are able to anneal only chromosomal region containing *lepB1* gene resulting in the fragments either of 0.8 kb or 2.1 kb (see Fig. 16). For another PCR the primers for amplification of *lepB* gene of *E. coli* were used yielding the fragment of 1 kb.

The analysis of genomic DNA of complementing strain pLepEc was carried out with primers sll0716F and lep1043rev. The size of the PCR fragment obtained from the genomic DNA is about 2.1 kb and corresponds with that obtained with the mutant DNA (Fig. 35). This result indicates that the plasmid pELEPB remained stable replicating in *Synechocystis* 6803 cells and the *lepB* gene is transcribed from the plasmid.

The cells of complementing strain pLepEc were able to grow in photoautotrophic conditions, albeit the growth rates of this complementing strain was somewhat reduced when compared with that of pLep1 cells or wild-type cells, but not significantly (Fig. 36). In all other conditions except the photoautotrophic the growth rates of the complementing strain pLepEc were practically identical to that of the wild type (Fig. 36,A,C and D). Thus, the expression of

the homologous *E. coli* gene *lepB* in the cells of the *lepB1*::Km^R mutant can provide the same growth behavior as of wild type cells. These data demonstrate that the leader peptidase of *E. coli* can substitute the function of the inactivated gene *lepB1*.

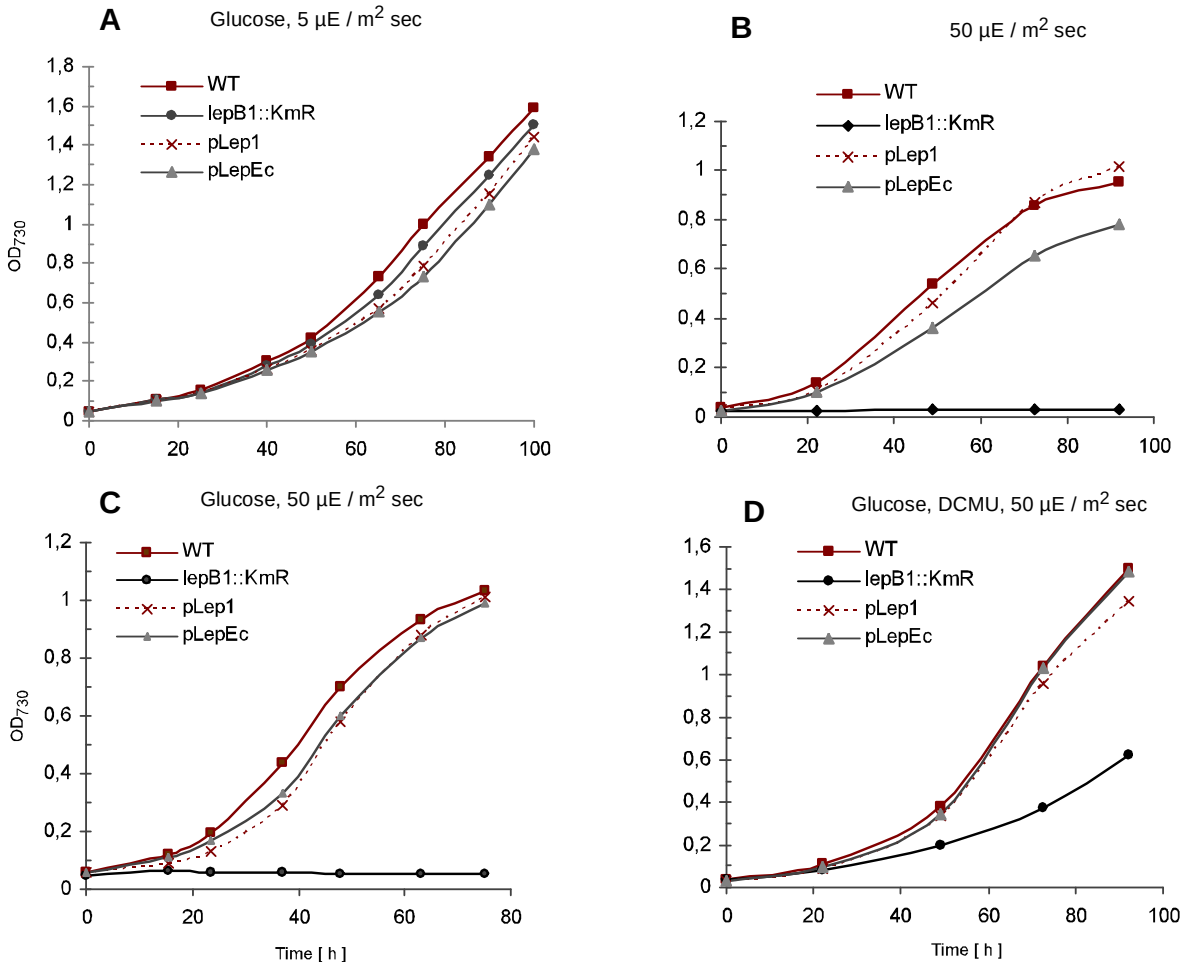

Figure 36. Analysis of the growth features of the wild type and different mutant strains under different growth conditions. The growth of wild type (WT), *lepB1*::Km^R mutant, complementing strains pLep1 and pLepEc was monitored in photomixotrophic conditions at dim light (panel A), in photoautotrophic conditions (50 µE m^{-2} s^{-1} light) (panel B), in photomixotrophic conditions (50 µE m^{-2} s^{-1} light) (panel C) and in photoheterotrophic conditions (panel D).

The absorption features of the complementing cells containing *lepB* gene from *E. coli* differed from that of the cells of the wild-type and pLep1 complementing strain, when the cells were grown in photomixotrophic conditions. Though the pLepEc cells were able to grow photoautotrophically, the concentration of chlorophyll in the cells of pLepEc was reduced. The cells of pLepEc grown in photomixotrophic conditions in low light showed only about 70% and the cells grown in photoautotrophic conditions only about 60% of the chlorophyll content of the wild type cells as measured by methanol extraction (Table 17). Since the

absorption features of pLepEc cells are not identical to that of the wild-type or of pLep1 complementing strain, the proper pigment composition of *Synechocystis* 6803 cells could not be reached if homologous *E. coli* gene *lepB* is expressing in the mutant cells

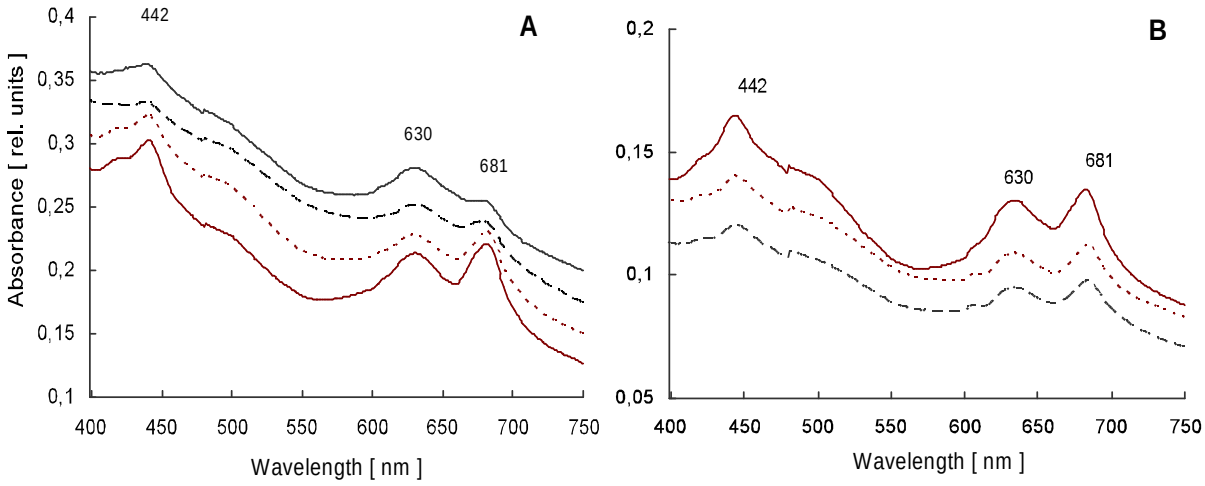

Figure 37. Absorption spectra of *Synechocystis* 6803 cells grown in the photomixotrophic conditions (A) or in photoautotrophic conditions (B). Absorption spectrum of *lepB1*::Km^R mutant is shown by black solid line, pLepEc cells absorption – by dashed line, and of pLep1 cells - by dotted red line. Wild type cells absorption spectrum is shown by red line. The absorption maxima for chlorophyll (at 442 and 681 nm) and for phicobiliproteins (at 630 nm) are indicated on the graph.

Table 17. Photosynthetic properties of complementing strains.

	wild type	pLepB1	pLepBEc
Photomixotrophic conditions, dim light			
chlorophyll content (µg ml^{-1} A_{730}^{-1})	3.8 ± 0.2	3.5 ± 0.15	2.7 ± 0.1
phycobiliprotein/chlorophyll ratio[a]	0.85	0.89	1.03
Photoautotrophic conditions, 40 µE/m2 sec			
chlorophyll content (µg ml^{-1} A_{730}^{-1})	3.3 ± 0.1	3.0 ± 0.12	1.9 ± 0.1
phycobiliprotein/chlorophyll ratio[a]	0.74	0.74	0.76
oxygen evolution[b] [µmol O_2 A_{730}^{-1} L^{-1} h^{-1}]			
whole chain electron transport, PM	995 ± 53	941 ± 48	850 ± 55
whole chain electron transport, PA	950 ± 66	705 ± 49	460 ± 32
PSII-mediated electron transport, PM	1956 ± 173	1947 ± 156	1768 ± 147

[a] determined from intact cells according to the equation of Richaud *et al*. 2001
[b] determined in short term experiments (5 min) with cells grown in photomixotrophic conditions at 5 µE m^{-2} s^{-1}
All parameters were calculated from four independent experiments.

The decrease of photosynthetic activity was observed in complementing strain pLepEc, if the cultures were grown in photoautotrophic conditions (Table 17). The strong reduction of the oxygen evolving activity of pLepEc cells can be explained either by specific features of *lepB* gene product, which is unable to substitute LepB1 function completely, or by differences in expression of *lepB1* gene in wild type and *lepB* gene from the plasmid pELEPB. Therefore the additional analyses including the study of the expression of the homologous genes from the plasmid in the *lepB1*::Km^R mutant cells are needed.

To determine the function of LepB from *E. coli* in the cells of the *lepB1*::Km^R mutant, an analysis of the PsbO processing in the thylakoid membranes of the complementing strains was performed.

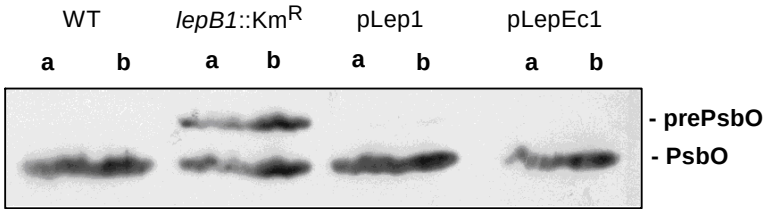

Figure 38. Western analysis of thylakoid proteins in wild-type, *lepB1*::Km^R mutant and complementing strain. Thylakoid proteins isolated from cultures propagated under photomixotrophic conditions in dim light were separated by electrophoresis on 10% - 17.5% SDS-polyacrylamide gradient gels, transferred to nylon membranes and analysed with antisera raised against PsbO. The position of the presumed precursor and mature proteins is indicated. (lanes a: 50 μg protein, lanes b: 100 μg protein).

The Western blot, which is shown in Fig. 38, demonstrates that there was no accumulation of the precursor form of PsbO in the cells of the complementing strain pLepEc. The absence of accumulation of PsbO precursor form was observed also under photoautotrophic growth conditions (Fig. 39). The obtained results imply that the function of the LepB1 peptidase can be substituted by the peptidase LepB from *E. coli*.

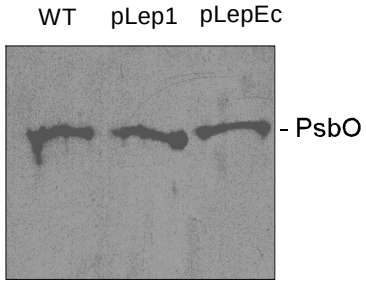

Figure 39. Western analysis of thylakoid proteins in wild type *Synechocystis* 6803 and complementing strain grown in photoautotrophic conditions. Thylakoid proteins isolated from the cells grown in photoautotrophic conditions were separated by electrophoresis on 10% - 17.5% SDS-polyacrylamide gradient gels, transferred to nylon membranes and analysed with antisera raised against PsbO. The position of the mature protein is indicated.

Taken together the data of complementation with *lepB* gene from *E. coli* demonstrate that LepB can partially substitute the function of LepB1 in *Synechocystis* 6803 cells. However it was not possible to distinguish at this step, why the phenotype of the pLepEc strain is not completely identical of that of the wild type or pLep1 complementing strain. To answer this question a number of experiments are needed, which can provide the possibility to analyse the expression of the genes encoding leader peptidases from the pVZ321M-derived plasmid.

4. Discussion

4.1. Two putative leader peptidases of *Synechocystis* 6803 are not redundant in their function

In this work, I have analyzed the function of the two genes encoding leader peptidases in *Synechocystis* sp. PCC 6803. This was done by inactivation of the genes by insertional knock-out. The complete inactivation was successful only for one of the two genes encoding leader peptidases, namely *lepB1*.

The segregation of the *lepB1*::Km^R mutant was achieved in dim light conditions in a medium containing glucose. The absence of segregation in photoautotrophic conditions can be explained by the photosynthetic deficiency of the mutant. Indeed, the growth features demonstrate that leader peptidase LepB1 is essential for growth in photoautotrophic conditions. The second leader peptidase, LepB2, is indispensable in all growth conditions, since it was not possible to eliminate the wild-type copies of *lepB2* gene completely. From these segregation experiments it can be concluded that LepB1 cannot perform the function of the peptidase LepB2. Similarly, LepB2 cannot functionally replace LepB1, as the cells with inactivated *lepB1* gene were unable to grow photoautotrophically. Thus, although both LepB1 and LepB2 belong to the same class of leader peptidases, they cannot functionally replace each other. The presence of two genes encoding leader peptidases of type I in genome of *Synechocystis* 6803 suggests that their functions are not identical.

The lack of complementation can have various reasons. It might be due to different expression rates of *lepB2* gene and that of the *lepB1* gene. This problem has to be clarified by analysis of the expression of the *lepB2* gene from the pVZ321M-derived vector in the *lepB1*::Km^R mutant. Alternatively, the lack of complementation could be due to a partial specialisation of the functions of two peptidases in *Synechocystis* 6803 cells. LepB2 seems to be responsible for the processing of essential proteins which carry out housekeeping functions. Therefore the attempts of segregation failed in all conditions tested. LepB1 might be responsible for processing of proteins essential for photosynthetic growth.
Indeed, one photosynthetic protein PsbO of the oxygen-evolving system is considered to be the putative substrate of LepB1 peptidase.

The complementation of the *lepB1*::Km^R mutant with the plasmid carrying the wild-type *lepB1* gene and the full restoration of the wild-type phenotype serves as evidence that the observed mutant phenotype is a direct consequence of the *lepB1* inactivation. The slight

difference of the photosynthetic activity of pLep1 complementing cells and WT cells, when grown in photoautotrophic conditions, might be due to differences in gene expression. The expression of the *lepB1* gene from the kanamycin resistance gene promoter Tn903 (in pLep1 strain) can be different to the expression from the chromosome (WT). To check this possibility, the *lepB2* gene has to be placed under the control of the kanamycin resistance gene promoter and introduced in the cells of the *lepB1*::Km^R mutant**,** and the phenotype of the complementing strain has to be studied. This would help to answer how the additional copy of *lepB2* gene can influence the expression of the LepB2 and if it can provide the capability to photoautotrophic growth and light resistance to the *lepB1* inactivation mutant.

4.2. The function of LepB1 is important for photoautotrophic growth and light tolerance of *Synechocystis* 6803 cells.

Although the homozygous *lepB1*::Km^R mutant strain cannot grow photoautotrophically, it is able to perform photosynthetic electron transport at significant rates (Table 13). The decrease of photosynthetic activity per cell is represented by the 50% reduction of chlorophyll content in the mutant strain in comparison to the wild type (Table 12). This photosynthetic activity, however, is not stable and takes place only for short periods of time (Fig. 20). After longer incubation in strong light the photosynthetic activity decreases gradually and finally disappears. This suggests that the mutant is highly sensitive to light, which is indicative for photooxidative stress.

In line with that, the *lepB1*::Km^R mutant was able to grow in the presence of carbohydrates only if either kept in dim light or if photosynthetic electron transport was inhibited by DCMU **(**Table 11**)** which blocks the Q_B binding site at photosystem II**.** The dim light conditions are not sufficient for efficient photosynthesis (Anderson and McIntosh, 1991), and DCMU blocks the linear electron transport chain. These data suggest that the *lepB1*::Km^R mutant cannot grow on the expense of photosynthesis and the electron flow from photosystem II caused photodestruction in the mutant cells. Interestingly, the same effect of DCMU on growth behaviour was observed in *Synechocystis* 6803 mutants with affected photosystem I. Similar phenotype, i.e. light sensitivity which is suppressed by inhibition of photosystem II, was described also for mutants which either lack photosystem I (Shen et al, 1993) or show an imbalance in the ratio of the two photosystems (Sonoike et al., 2003). The enhanced light

sensitivity of these strains could be decreased by addition of DCMU to the growth medium. The reduction of the size of the light-harvesting antenna complex by deletion of *apcE* gene coding for the anchor protein linking the phycobilisome to the thylakoid membrane, leaded to the suppression of the activity of photosystem II (Shen et al., 1993). A similar effect of DCMU, notably the restoration of the growth in high light conditions was observed also for the *pmg*A mutant. This strain is not capable to properly adapt the stoichiometry of two photosystems in response to different light conditions, which presumably leads to an imbalance in the photosynthetic electron flow (Hihara et al., 1998; Sonoike et al., 2003). The mutation of the *pmgA* gene leads to cell death after prolonged incubation in the strong light, but addition of DCMU restored cell growth under these conditions.

It was often observed that the abundance of carotenoids can be increased to protect cells facing photodamage. This pigment accumulates typically in high light conditions, therefore it was suggested that caroteinoid myxoxanthophyll is responsible for protection from the high light (Mohamed and Vermaas, 2004). The function of myxoxanthophyll, namely the protection of the electron transport chain from photoinhibition was confirmed also by other investigators (Schafer et al., 2005). Indeed, the cells of the *lepB1*::Km^R mutant have under weak light conditions an increased level of myxoxanthophyll in comparison to the wild-type cells (Fig. 19). Therefore the increase of this pigment in the *lepB1*::Km^R mutant cells suggests that they require the protection of the electron transport chain against photoinhibition.

In the *lepB1*::Km^R mutant cells a decrease of PSI content was revealed by 77K fluorescence emission spectra as well as by Western blot analysis using PsaA/B antiserum (Fig. 18; Fig. 28). However, the analysis of the thylakoid membrane complexes by the blue-native PAGE did not show any significant alterations in the accumulation of the photosynthetic proteins including PSI. Also, a direct connection of the *lepB1* inactivation and the PSI biogenesis was not detected. Still, the strong degradation of the photosynthetic pigments and proteins in the light suggests an important role of the LepB1 for the functioning of the photosynthetic apparatus in *Synechocystis* 6803. Therefore, the exact way of influence of the *lepB1* inactivation on the two photosystems as well as the exact cause for photoinhibition remains to be elucidated.

Similarly, the decrease of the subunits of cytochrome b_6f complex in the *lepB1*::Km^R mutant, revealed by heme-staining of thylakoid proteins (Fig. 26A), cannot be recognized as direct involvement of the LepB1 in biogenesis of these subunits. Since the Western analysis of

cytochrome *f* did not show any alterations in the processing of this protein in the *lepB1*::Km^R mutant (Fig. 25C), the apocytochrome *f* processing might be performed by LepB2.

4.3. The processing of PsbO is affected in the *lepB1::Km^R* mutant

The analysis of phenotypic features of *lepB1*::Km^R mutant has shown that LepB1 is required both for photoautotrophic growth and protection against light stress. This suggests that the enzyme might be preferentially involved in the maturation of components of the photosynthetic machinery. Indeed, subunit PsbO of the oxygen evolving system associated with photosystem II could be identified as the potential substrate of the LepB1 (Figs. 29, 30; 37). The PsbU subunit of the oxygen evolving complex system of PSII was identified in the 2D-analysis of proteins of the *lepB1*::Km^R mutant and wild type. Since the content of this protein was reduced in the mutant samples, PsbU can be also considered as another potential substrate of LepB1 (Fig. 33, Table 15). Substrate specificity might, however, also be mimicked by different localization of the two leader peptidases in the cyanobacterial membranes. In line with that, LepB2 was identified in cell fractions that were enriched with the inner plasma membrane of *Synechocystis* 6803, whereas LepB1 was not found in these samples (Huang et al., 2002) suggesting that it is located in the thylakoid membranes instead. However, the suggested classification in the subcellular topology of the two leader peptidases is probably not strict, because (i) among the presumed substrates of LepB1 is also a periplasmic protein, notably FutA was found (Table 15), (ii) processing of thylakoid proteins like PsbO or cytochrome *f* is not completely abolished in homozygous *lepB1::*Km^R cells **(**Fig. 29,30, Fig. 26) and (iii) PsbO protein was also detected in the periplasmic fraction (Huang et al., 2002). Interestingly, some authors have suggested the existence of a connection between the thylakoid lumen and periplasmic space (Zak et al., 2001; Tolle et al., 2004**)**. According to this idea, the proteins may be delivered from the plasma membrane to their final destination thylakoid membrane either by lateral movements of the membrane or the vesicle transport (Huang et al., 2002).

Another important fact concerns the proteins Slr0924 and FutA2, which were detected in the periplasm, but subsequently also in thylakoid lumen (Fulda, 2000, Fulda 2002, Katoh 2001, Tolle 2004). LepB1 is probably involved in the processing of FutA2 as determined from the 2D analysis (Fig. 33). But in the absence of LepB1, another leader peptidase, LepB2, is capable to process this proteins. In contrast to the FutA2 protein, the Slr0924 protein, which is

involved in salt response, is essential protein in *Synechocystis* 6803. It is obviously that LepB2 carries out the processing of Slr0924 preprotein (Fig. 27). Therefore, based on available data, the classification of the functions of both leader peptidases appears more likely, according to which LepB2 processes essential proteins while LepB1 is responsible for processing of non-essential proteins. On this regard, the analysis of the Slr1784 protein with a hypothetical function appears to be important in order to verify the classification of functions of two leader peptidases.

The genome of *Synechocystis* 6803 encodes 3667 proteins (Nakamura et al., 1998). According to the SignalP prediction, about 13% of *Synechocystis* 6803 proteins are synthesized as precursors with N-terminal signal peptides (Table 16, Table A). This value represents the approximate proportion of the proteins, which are potentially transported across the thylakoid membrane or the inner plasma membrane in this unicellular cyanobacterium. The proteins with N-terminal signal peptides belong to different groups, which are involved in different cellular processes (Table 16). The most numerous group includes the proteins with unknown and hypothetical function. The photosynthetic proteins PsbO and PsbU, and the transport protein FutA2, all of which accumulate in the mutant in reduced amounts (Table 15**)** were shown not to be indispensable for photoautotrophy of *Synechocystis* 6803 (Burnap and Sherman, 1991; Shen et al., 1993; Katoh et al., 2001) and, thus, cannot be initiative for the observed defects of *lepB1*::Km^R mutant. The function of other proteins affected, like for example of the gene product of sll1784, is so far not even known. Furthermore, only a very limited number of proteins could be examined so far. Taking into consideration the complexity of the processes involved in the biogenesis of the photosynthetic apparatus, it will therefore not be trivial to conclusively answer these questions. Further work will therefore be required to finally identify the primary cause for the lack of photoautotrophic growth of the *lepB1*::Km^R mutant.

4.4. LepB from *E. coli* can functionally replace the leader peptidase LepB1.

Alignment of the two putative leader peptidases from *Synechocystis* 6803 with homologous proteins revealed a high level of identity in four conserved domains of the protein sequence (Fig. 9). The catalytically active amino acids serine and lysine were identified in both peptidases LepB1 and LepB2 as well as in the thylakoidal processing peptidases. These facts support the idea that LepB1 and LepB2 indeed function as leader peptidases in *Synechocysis*

6803 cells. The signal peptidase of *E. coli*, LepB, consists of a polypeptide chain with the molecular mass of 37 kDa (Wolfe et al., 1982). This enzyme is an integral membrane protein in the inner plasma membrane, which spans the membrane twice, and the small N-terminus as well as the large carboxyl-terminal domain with the active site is located in the periplasmic space (Wolfe et al., 1983; Bilgin et al., 1990). In contrast, the signal peptidase of cyanobacteria like for example *Phormidium laminosum* (Packer et al., 1995) appears to have only one membrane-spanning region (Fig. 4).

The wild-type gene *lepB1* can complement the *lepB1*::Km^R mutant. However, the slight differences in the photosynthetic activity of the cells grown in photoautotrophic conditions suggest that the expression of the *lepB1* gene from the kanamycin resistance promoter Tn903 might be different than its expression from the chromosome. For better understanding of the mutant phenotype, the construction of a complementing strain expressing LepB2 from a plasmid and the analysis of the photosynthetic properties of such strain are necessary.

The complementation experiments showed that the leader peptidase from *E. coli* can partially substitute the function of the leader peptidase LepB1. If the leader peptidase LepB was expressed from a self-replicating vector, it restored photoautotrophic growth of mutant cells. From the alignment of the leader peptidases from *Synechocystis* 6803 and *E. coli* it appears likely that these enzymes have similar function. However, the comparison of the function of LepB1 and LepB peptidases in complementation experiments indicates that the function of both enzymes is not identical, as the photosynthetic performance of the strain pLepEc, grown in photoautotrophic conditions was reduced when compared to the wild-type cells or pLepB1 complementing cells. Therefore it is most likely, that the homologous leader peptidase from *E. coli* can substitute the function of endogenous protein, though only partially**.**

The identity of the most important residues suggests a similar mechanism for the leader peptidases which operate in plants, cyanobacteria and other bacteria. Therefore the LepB peptidase from *E. coli* was able to process quantitatively *in vivo* PsbO precursor (Fig. 38). This is in line with other experiments mainly done *in vitro*, which have shown that precursor proteins from different sources are substrates for heterologous leader peptidases (Halbig et al., 1999; Halpin et al., 1989; Kuwabara et al., 1989; Wexler et al., 1998).

It remains unclear, however, why the photosynthetic activity of the pLepEc complementing strain is low, though leader peptidase LepB from *E. coli* can perform processing of *Synechocystis* 6803 proteins also under photoautotrophic growth conditions (Fig. 39). Therefore the question arises, why the photosynthetic properties are affected in the complementing strain pLepEc. On the one hand, the efficiency of LepB peptidase from *E. coli*

for processing of the photosynthetic proteins in the strong light might be lower than that of LepB1. On the other hand, the rate of the expression of the *lepB* gene from *E. coli* in *Synechocystis* 6803 cells from the plasmid has to be compared with the expression rate of *lepB1* gene from the chromosome. The expression of *lepB1* gene might be regulated in response to different conditions of growth.

In *E. coli*, the signal peptidase is an essential enzyme (Date, 1983). In most cyanobacterial genomes analysed so far, two genes encode for putative leader peptidases of type I. Taking in account that cyanobacteria contain two types membranes, plasma and thylakoid membranes, the existence of two leader peptidases may originate from division of the functions between two peptidases. Similarly, *Bacillus subtilis* contains five leader peptidases of type I, which have overlapping substrate specificity (Tjalsma et al., 1998). Such a large number of signal peptidase-encoding genes may be correlated with ability of this bacterium to secrete large amounts of protein. The expression of these genes could be regulated for effective protein export. In addition, the signal peptidases from *B. subtilis* appear to have preference for different pre-proteins, indicating that they have different though overlapping substrate specificities (Bolhius et al., 1996). Similar facts concerning different functions of several leader peptidases were reported for *Listeria*, and the observed functional redundancy suggests that these enzymes can play different roles in the cellular processes (Bonnemain et al., 2004).

Taking in consideration the data reported so far, it can be concluded that the leader peptidase LepB1 is involved in maturation of photosynthetic proteins, but the exact role of this protein in *Synechocystis* 6803 cells remains to be further elucidated.

4.5. Outlook

The accumulation of the PsbO precursor protein in *lepB1*::Km^R mutant suggests that LepB1 is involved in processing of thylakoid proteins. However the mature PsbO protein is located in both thylakoid lumen and periplasmic space (Zak et al., 2001; Huang et al., 2002). Therefore it is important to find out the localization of the PsbO precursor in the cells of the *lepB1*::Km^R mutant and determine if LepB1 is functioning in thylakoid membrane or periplasmic membrane or in both. To determine the localization of LepB1 protein, the immunolabeling experiments should be carried out.

In addition, the function of Sll1784 has to be determined using targeted gene inactivation. This will help to ascertain whether the role of this protein might be important for photoautotrophic growth and the light tolerance.

The isolation of possibly entire photosynthetic complexes from the *lepB1*::Km^R mutant and wild type, comparison of their spectroscopic features and analysis of their protein composition by SDS-PAGE shall be carried out. The complexes can be isolated from the cells grown in different conditions using different approaches like for example some additional to BN-PAGE native gel electrophoresis systems, or gradient centrifugation.

Finally the processing of other thylakoid proteins in *lepB1*::Km^R mutant has to be studied further. For this purpose, the available antibodies against *Synechocystis* 6803 thylakoid membrane proteins shall be requested from other investigators and tested for the wild type and the *lepB1*::Km^R mutant. Alternatively the isolation of the thylakoid membrane complexes using different detergents may be applied.

5. Summary

The N-terminal processing step is important for successful transport of proteins, which are located in the thylakoid lumen or periplasmic space. In the thylakoid membranes several transport pathways exists, but the exact degree of involvement of the processing peptidases in the biogenesis of the thylakoid protein complexes and their regulation are not well studied.

In this work, the function of leader peptidases in the photosynthetic cyanobacterium *Synechocystis* 6803 has been studied. Apparently there is a division of function of the two leader peptidases, which are encoded in the genome of this cyanobacterium. The leader peptidase LepB2 is an essential protein of *Synechocystis* 6803, as it was not possible to achieve complete segregation of the mutant allele in all conditions tested. In the absence of LepB1, LepB2 is not capable to substitute the function of the leader peptidase LepB1 in photoautotrophic conditions, but is rather important for maintenance of housekeeping proteins.

Leader peptidase LepB1 is important for the optimal performance of photosynthesis and therefore for photoautotrophic growth. Analysis of the complementing strain pLep1 proved that the observed phenotypic features of the mutant derive from gene inactivation. The immunoblotting analysis has confirmed that LepB1 is a membrane protein, and according to the appearance of the mutant cells in electron microphotographs, LepB1 might be involved in the development of the thylakoid membrane system of *Synechocystis* 6803. However, the assembly of the photosynthetic complexes was not affected in the mutant cells upon the photomixotrophic growth under weak light conditions. The 50% chlorophyll reduction in the cells of the *lepB1*::Km^R mutant was accompanied by reduction of PSI content and thus by an alteration of the PSII/PSI ratio. The concentration of the cytochrome b_6f complex subunits was also decreased. It remains, however, unknown how these features of the mutant are connected with the LepB1 inactivation.

The inactivation of the leader peptidase LepB1 leads to a high light sensitivity of *Synechocystis* 6803 cells, which can be observed as a strong degradation of the photosynthetic complexes in the light. An excess of light caused cell death even in the presence of glucose in the growth medium, but can be prevented to some extent by addition of DCMU, which blocks the PSII electron transport. The photosynthetic activity becomes inhibited by light in the mutant cells. These observed features were indicative of photooxidative damage, which was probably caused by inefficient replacement of damaged components of the photosynthetic

machinery due to the lack of a leader peptidase removing the signal peptides from photosynthetic precursor proteins. Indeed, the proteomic and immunological studies revealed that LepB1 is involved in processing of photosynthetic proteins, i.e. the PsbO protein, the subunit of oxygen-evolving complex of PSII protein and apparently of another protein of this complex, PsbU. The results of this work strongly suggest that LepB1 is important for removal of the signal peptides after membrane transport of the components of the photosynthetic machinery, which in turn is a prerequisite for the biogenesis of a functional photosynthetic electron transport chain.

The computational analysis of proteins of *Synechocystis* 6803 has revealed that about 13% of all proteins are synthesized as precursor proteins and are thus potential substrates of leader peptidases. Thus the function of leader peptidases in *Synechocystis* 6803 might be important for different cellular processes. The proteins PsbO, PsbU and FutA2, which accumulated in reduced amount in the mutant cells, are not essential proteins of *Synechocystis* 6803. In line with this, the function of the forth potential substrate of LepB1, the Sll1784 protein shall be clarified to find out how the functions of two leader peptidases are divided in cyanobacteria.

The complementing experiments have shown that homologous LepB protein from *E. coli* can functionally substitute LepB1, which restores the photoautotropic growth and complete PsbO processing. However in photoautotrophic growth conditions the phenotype of the complementing strain is not identical to that of the wild type, suggesting that the functions of homologous proteins are not absolutely identical.

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Appendix

Sequences of genes and proteins

LepB1

> *Synechocystis* sp. strain PCC6803 (99199-98609) - sll0716
ATGCAAAATTCCCCCATCCCTTCCCCTTGGCAATTCATCAAGGAAAACATCCCCCTGCTCATGGTGGCCCTGGTG
CTGGCCCTGCTGCTGCGTTTCTTTGTGGCGGAACCCCGCTACATTCCCTCCGATTCCATGTTGCCCACCCTCGAA
CAAGGCGATCGCCTGGTGGTGGAGAAGGTGTCCTACCATTTCCATCCACCCCAGGTGGGGGACATTATTGTGTTC
CATCCGCCGGAGTTGCTGCAAGTACAAGGCTATGATCTAGGGCAGGCATTTATTAAACGAGTCATTGCGTTGCCG
GGGCAAACAGTGGAAGTTAACAACGGCATTGTTTACCGGGATGGGCAACCTTTACAGGAAGAATATATTTTGGAG
CCGCCCCAATATAATTTGCCCGCAGTGCGGGTGCCGGACGGTCAGGTGTTTGTCATGGGGGATAACCGCAACAAC
AGCAATGATTCCCATGTATGGGGATTTTTACCCCAGCAAAATATCATCGGCCATGCCCTATTTCGTTTTTTCCCC
GCCAGCCGTTGGGGCCAACTGGGTAGCTTCACCTTTGTCCCAGCCCGTACCATCATCAATACCTAA

> LepB1, sll0716 (98609 – 99199) translated protein sequence
MQNSPIPSPWQFIKENIPLLMVALVLALLLRFFVAEPRYIPSDSMLPTLEQGDRLVVEKVSYHFHPPQVGDIIVF
HPPELLQVQGYDLGQAFIKRVIALPGQTVEVNNGIVYRDGQPLQEEYILEPPQYNLPAVRVPDGQVFVMGDNRNN
SNDSHVWGFLPQQNIIGHALFRFFPASRWGQLGSFTFVPARTIINT
predicted transmembrane region is underlined

LepB2

> *Synechocystis* sp. strain PCC6803 (672291-672947) – slr1377
ATGACCGAAAATATTGTCCGCGAAACCAGCAAGAAAAAAGAAAGCCCCCCGGAAAATACCTGGTTAGAGTTGGGC
AAAACCATGGTTACCGCGGTTATCCTGGCGATCGGTATTCGGACTTTTGTGGCCGAAGCTCGCTACATTCCCTCT
TCCTCCATGGAGCCGACCCTGCAGATTAACGATCGCCTGATCATTGAAAAGATCAGTTACCGATTGCGGGATCCG
GAGCGGGGAGAAATTGTGGTCTTTAACCCCACTGACGCCCTAAAAGCAAAAAACTTCCACGATGCTTTTATTAAG
CGCATTATCGGTTTACCGGGGGATGAAGTTAGGGTTTCCCAAGGCAACGTTTATGTCAACGGCAAAATGCTGGAC
GAAAATTACATTGCGGCTCCTCCCGCCTATGAATATGGCCCAGTGAAAGTGCCCGATGATCAGTATTTAGTGTTA
GGGGATAACCGCAACAACAGCTATGACTCCCACTATTGGGGCTTTGTCCCCCGGGAAAAATTGCTCGGCCGTGCC
TTTGTCCGTTTTTGGCCCGTACCCCGGGTAGGCCTGTTGACTGATGATGCTGAGCGAGAAGCAGTAGAAATTAGC
CCCCAAGCATGGGAAAGTCCAGCCATTTCCCCCCAGACAGTGCCGGAGAGTCGTTAG

> LepB2, slr1377 (672291 – 672947) translated protein sequence
MTENIVRETSKKKESPPENTWLELGKTMVTAVILAIGIRTFVAEARYIPSSSMEPTLQINDRLIIEKISYRLRDP
ERGEIVVFNPTDALKAKNFHDAFIKRIIGLPGDEVRVSQGNVYVNGKMLDENYIAAPPAYEYGPVKVPDDQYLVL
GDNRNNSYDSHYWGFVPREKLLGRAFVRFWPVPRVGLLTDDAEREAVEISPQAWESPAISPQTVPESR
predicted transmembrane region is underlined

TatC

> *Synechocystis* sp. strain PCC6803 (2531666-2532430) - sll0194
ATGTCAACCCAGCTTGATAACATCACTTCCGCGGAAACTGCCCCCGATTATTTGGACGAAGTGCCCGATGACGTG
GAAATGTCTTTGTTTGATCACCTCGACGAACTCAGAACTCGTATTTTTCTGTCTTTGGGGGCGGTCTTGGTCGGG
GTAGTGGCCTGCTTTATTTTCGTTAAACCCTTGGTGCAATGGCTTCAAGTCCCGGCAGGCACAGTTAAATTTCTG
CAGCTCTCCCCAGGGGAATTCTTTTTCGTTTCCGTCAAAGTAGCAGGCTACAGCGGCATTCTGGTGATGAGCCCC
TTTATCCTCTACCAAATTATTCAATTCGTTCTACCCGGTCTAACCCGTCGGGAACGCCGATTACTGGGACCGGTG
GTGCTAGGCTCCAGTGTACTTTTTTTTGCTGGACTGGGCTTTGCTTACTATGCTCTCATCCCCGCCGCACTCAAG
TTTTTTGTCAGCTACGGAGCGGATGTGGTGGAGCAACTCTGGTCGATTGATAAATATTTTGAGTTTGTGCTGTTG
TTGATGTTTAGCACCGGGCTAGCTTTTCAAATTCCGATTATTCAAGTGGTTCTCGGCTTTTTGGGCATCGTTTCC
TCCGAACAAATGCTCAAGGGCTGGCGCTTTGTCATTTTGGGGGCGATGGTGCTGGGGGCAATTCTCACCCCTTCC
ACGGACCCCCTGACCCAGTCCCTTCTAGCTGGGGCAGTGCTGGGGCTTTACTTTGGCGGGATCGGTTGCGTGCGC
TTACTGGGTAAATAG

> TatC, sll0194 (2531666 – 2532430) translated protein sequence
MSTQLDNITSAETAPDYLDEVPDDVEMSLFDHLDELRTRIFLSLGAVLVGVVACFIFVKPLVQWLQVPAGTVKFL
QLSPGEFFFVSVKVAGYSGILVMSPFILYQIIQFVLPGLTRRERRLLGPVVLGSSVLFFAGLGFAYYALIPAALK
FFVSYGADVVEQLWSIDKYFEFVLLLMFSTGLAFQIPIIQVVLGFLGIVSSEQMLKGWRFVILGAMVLGAILTPS
TDPLTQSLLAGAVLGLYFGGIGCVRLLGK
(The predicted transmembrane regions are underlined)

Table A.
Proteins of Synechocystis sp. PCC 6803 synthesized with N-terminal signal peptides (predicted with SignalP 3.0).

ORF	Protein function	Gene	Signal peptide	SPL	aa
Biosynthesis of aromatic amino acids					
slr2081	prephenate dehydrogenase	tyrA	MKIGVVGLGLIGASLA-GD	16	279
Biosynthesis of cofactors, prosthetic groups, and carriers					
Cobalamin, heme, phycobilin and porphyrin					
sll1091	geranylgeranyl hydrogenase 1	chlP	MVLRVAVVGGGPAGSSA-AE	17	407
Carotenoid					
slr0940	zeta-carotene desaturase 2	crtQ-2	MRVAIVGAGLAGMATA-VE	16	489
slr1254	phytoene desaturase	crtP	MRVVIAGAGLAGLACA-KYLA-DA	16	472
slr1293	C-3',4' Desaturase Myxoxanthophyll biosynthesis	crtD	MVPSNAESQSVVVIGAGIGGLTTAALLA-QQG	28	507
Nicotinate and nicotinamide					
slr1434	pyridine nucleotide transhydrogenase ßsubunit	pntB	MSNSLQTVAYVAASILFIFSLGGLA-NQ	25	480
Cell envelope					
Membranes, lipoproteins, and porins					
sll0772	probable porin; major outer MP		MGLICGLAIASGLTVTIPSEA-QT	21	546
sll1271	probable porin; major outer MP		MNFTKQFTCLSSLGITVLITAIVGG-GNTEPIFAQS	25	572
sll1550	probable porin; major outer MP		MRTPMLRKLSLVWGLGALMAIAGGGGEALA-EP	30	544
slr0042	probable porin; major outer MP 4		MKQYRFTWLAGFATVTSLTTFPGSAGA-QM	27	576
slr1227	chloroplastic outer envelope MP homolog		MSNQNKSVILSPYYRLLLLTSGLVLGASPAQA-TQ	32	861
slr1272	probable porin; major outer MP		MINRSMKSCLLAALGGLLLGGMTPPAIA-DP	28	254
slr1841	probable porin; major outer MP		MLKLSWKSLLVSPAVIGAALVAGAASA-AP	27	630
slr1908	probable porin; major outer MP		MNKLTSHLLKLFPLALGSSLAIVPGAMA-QS	28	691
Murein sacculus and peptidoglycan					
sll0016	probable membrane-bound lytic transglycosylase A		MIKQFFPLLALLLVFGFHGSGLRA-QS	24	383
sll0657	phospho-N-acetylmuramoyl-pentapeptide-transferase		MANAKSSSLPSWKNPSGKTLLILLWALALALMALLSSWA-DM	39	365
slr0191	amidase enhancer, periplasmic protein		MFISPPRNLLKVAVQRVGGLVIALGLVAGGALPSFA-KD	36	535
slr0804	probable D-alanyl-D-alanine carboxypeptidase		MVRGSELYWKPMSKKCSPWLKVGVLATSAALLSTTPALA-NQ	39	314
slr0891	N-acetylmuramoyl-L-alanine amidase		MMAMESWLGTMIGVGAAVVFTMPAVA-QS	26	591
slr1744	N-acetylmuramoyl-L-alanine amidase		MSRLPGFALTFLSVLLTSLPAMA-GQ	23	649
slr1910	probable N-acetylmuramoyl-L-alanine amidase		MNYFSCSLPKSLGLICASLLSLPALAPPVWA-GS	31	338
slr1924	D-alanyl-D-alanine carboxypeptidase		MKKSLFMVGTLAFFSGLPAGGAWG-ES	24	400
Surface polysaccharides, lipopolysaccharides and antigens					
sll0380	probable glycosyltransfrase		MTGSHLLVNLSFLLAQPTGLSVYA-SN	24	365
Surface structures					

slr1962	probable extracellular solute-binding protein		MK**RR**QLLGQSAIAAGTAAGLVA-CGKATTSN	22	370
Cellular processes					
			Cell division		
slr0228	cell division protein FtsH 5	ftsH	MKFSWRTALLWSLPLLVVGFFFWQGSFGGADA-NL	32	627
slr1390	cell division protein FtsH	ftsH	MGLLVAGTLALPVSTLA-QE	17	642
slr1604	cell division protein FtsH, salt responce	ftsH	MSKNNKKWRNAGLYALLLIVVLALA-SA	25	616
			Detoxification		
sll1980	thiol_disulfide interchange protein	trxA	MTPAKIRNALLAVVAIALSAAVYLG-FQ	25	180
			Protein and peptide secretion		
slr0775	protein-export MP SecF	secF	MKLDLFKWEKPAWIVSSLLVLISIFAMAISWA-QF	32	315
slr1277	Pilus assembly protein (general secretion pathway protein D) 6 out membrane		MRSNSVKNFRFWLTTEIATCCLLALAPAQA-ET	30	785
ssr3307	preprotein translocase SecG subunit	secG	MTLITVLRIIWMASA-ALLTVLVLLHS	15	77
			Transformation		
sll1929	competence protein ComE	comE	MRFHPLALICLSFILGLLG-TGL	19	709
			Chemotaxis		
sll1694	pilin polypeptide PilA1 6 outer membrane	pilA	MASNFKFKLLSQLSKKRAEGG-FT	21	168
slr0163	a part of pilC, pilin biogenesis protein	pilC	MARLQNQIKSAMAYPVAVGFLAVVAFLGMTIFLIPVFA-GI	38	247
slr1120	type 4 prepilin-like proteins leader peptide processing enzyme type IV		MDPLIAPLAFLLAIALGCAVGSFLNVVA-YR	28	269
slr1929	type 4 pilin-like protein	pilA6	MVMKLPNPTFLLKLVFNPHPSTSSQGWTLIEIGVVTVIVGILAAMAFPSLA-GI	51	183
Central intermediary metabolism					
			Polysaccharides and glycoproteins		
slr0518	similar to alpha-L-arabinofuranosidase B		MKLLPLLPLFGLTVALAIPSMAILGGPRQIQA-QS	32	177
Energy metabolism					
			Amino acids and amines		
slr0229	3-hydroxyisobutyrate dehydrogenase		MNVSKIAVFGLGVMGSPMA-QN	19	290
			Sugars		
sll0244	UDP-glucose 4-epimerase		MATQQTILVTGGAGYIGSHGVLA-LQ	23	338
slr1617	similar to UDP-glucose 4-epimerase		MKIRWITPLLGTAAFNAVQ-NI	19	411
			Glycolate pathway		
sll1831	glycolate oxidase subunit, (Fe-S) protein		MFFPMVPMSAPPSFTALAQA-VN	20	460
Fatty acid, phospholipid and sterol metabolism					
sll0330	sepiapterine reductase		MNLLNKTALVTGSSRGIGRAIA-EEL	22	259
sll0418	2-methyl-6-phytylbenzoquinone methyltransferase 7		MPEYLLLPAGLISLSLAIA-AG	19	318
sll1522	CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase	pgsA	MNIPNWVTVSRLLALPLLFLWLPTPEA-ET	27	179
slr0089	gamma-tocopherol methyltransferase		MVYHVRPKHALFLAFYCYFSLLTMASATIASA-DL	32	317
slr1369	phosphatidate cytidylyltransferase	cdsA	MPTQRIISAVIGIALAFSLLILGGWYFSAA-IA	29	293
slr2124	3-oxoacyl-[acyl-carrier protein] reductase		MKPVVLITGIAGGIGQATA-EL	19	249
Photosynthesis and respiration					
			ATP synthase		
ssl2615	ATP synthase c chain of CF(0)	atpH	MDSTVAAASVIAAALAVGLGAIGPGIGQGNA-SG	31	81
			Respiratory terminal oxidases		
sll0813	cytochrome c oxidase subunit II	ctaC	MSRKNLILLAVYIVFTVGA-SLWLGQRA-YQWLPPAAA-QE	19	300
			CO_2 fixation		

slr1838	CO_2 concentrating mechanism protein CcmK homolog 3	ccmK3	MPQAVGVIQTLGFPSVLAA-ADAM	19	103
Cytochrome b6/f complex					
sll1182	Rieske iron sulfur protein	petC3	MVK**RR**KLISYTAFSTAIA-VITG	18	133
sll1317	apocytochrome f	petA	MRNPDTLGLWTKTMVAL**RR**FTVLAIATVSVFLIT DLGLPQAASA-YP	44	328
slr1185	Rieske iron sulfur protein	petC2	MDNTQAIAPPSYS**RR**QLLNFLAGTTVAVTASAGA YA-MG	36	178
smr0010	cytochrome b6f complex subunit 5	petG	MIEPLLLGIVLGLIPVTLA-GL	19	38
NADH dehydrogenase					
slr2009	NADH dehydrogenase subunit 4	ndhD 6	MNFPTAIAPNNLLIAIVALLVLALMGAFG-GY	29	495
Photosystem I					
sll0819	PSI reaction center subunit III	psaF	MKHLLALLLAFTLWFNFAPSASA-DD	23	165
Photosystem II					
sll0258	cytochrome c550	psbV	MKRFFLVAIASVLFFFNTMVGSANA-VE	25	160
sll0427	photosystem II manganese-stabilizing polypeptide	psbO	MRFRPSIVALLSVCFGLLTFLYSGSAFA-VD	28	274
sll1194	PS II 12 kDa extrinsic protein	psbU	MKFISRLLVACSLLIGLMGFLGADLAQALTPNPI LA-EL	36	131
sll1418	similar to PS II OEC 23 k protein	psbP 2	MLKKSLSTAVVLVTLLLSFTLTA-CG	23	188
slr1645	PS II 11 kD protein, Psb27	psbZ	MMSFLKNQLSRLLALILVVAIGLTA-CD	25	135
slr2034	PSII stability assembly factor HCF136 homolog, ycf48	ycf48	MPVKFPSLKFEQLKQLVLVAAIAVFCVSCSHVPD LA-FN	36	342
smr0009	photosystem II PsbN protein	psbN	MESATVLSITFAVILIAITGLAVYTSFGPPSA- ELGDP	32	43
Soluble electron carriers					
sll0199	plastocyanin	petE	MSKKFLTILAGLLLVVSSFFLSVSPAAA-AN	28	126
sll1796	cytochrome c553	petJ	MFKLFNQASRIFFGIALPCLIFLGGIFSLGNTAL A-AD	35	120
Purines, pyrimidines, nucleosides, and nucleotides					
Purine ribonucleotide biosynthesis					
slr1164	ribonucleotide reductase subunit α		MHPTLISAPISSSANDAHA-GT	19	767
Regulatory functions					
sll0474	two-component hybrid sensor and regulator		MVQEKPRFTRPLRYQLLAGCALILMITSAIA-VV	31	806
sll0798	Ni(II)-sensor and/or redox sensor, two-component sensor histidine kinase	nrsS, rppB, hik30	MNT**RR**LFARSRLQLAFWYALVMGGILTLLGLGVY RA-IVQA-NW	40	454
sll1871	two-component system sensory histidine kinase		MVSIIKSV**RR**SLLLKFVLSYFCLSSVVVA-SI	29	674
sll1872	transcriptional regulator		MMAKNSNKRGGDSHNGKKSPRKGKFQGGKWLLTG FALTAIA-LVSAGAGSLLA-MYS	41(52)	463
slr0527	transcription regulator ExsB homolog		MTKTAVVLLSGGLDSATVA-AI	20	232
slr0640	two-component sensor histidine kinase		MFQAT**RR**RLALWYTLVTAVLLLLFA-SGV	25	441
slr1860	carbon metabolisms regulatory protein IcfG	icfG	MKMKLIQPFIQSIRFRIVGLLLLCLIPPTLG-GI	31	634
slr2104	two-component hybrid sensor and regulator	hik22	MKSFFLNTFSPLRSTARFWVLVALLTSMELSA- SQ	32	950
Translation					
Degradation of proteins, peptides, and glycopeptides					
sll1427	protease,	hhoB	MAIHLKASHLGVAVLLLLFGGAIGA-AG	25	416
sll1679	periplasmic protease HhoA	hhoA	MKYPTWL**RR**IGGYLLAFAVGTAFGIANLPHAVAA -ADD	34	384
sll1703	protease IV	sppA 1	MKNFFQQMVASFFGTLAAIVVLLSLGATGLVLLF ILVSAEA-DP	41	610
slr0008	carboxyl-terminal processing protease	ctpA	MG**KR**T**RR**FWALAFSLLMGALIYLGNTPSALA-FT	31	427
slr0257	peripl carboxyl-terminal protease	ctpB	MSPHLLCLRPLVAALVFGLGLGTALRPALS-AP	30	462

slr0535	protease	spr	MI**KR**FFLTILFFAGLWFA-LNLY	18	613
slr1751	periplasmic carboxyl-terminal protease	ctp	MLKQ**KR**SLILGTTALLLTTVAVT-GV	23	423
Protein modification and translation factors					
sll0227	peptidyl-prolyl cis-trans isomerase B, periplasmic protein	ppiB	MRILPNISRATWFVGIFFVVNILLTACNQPSANSSA-EP	36	246
Ribosomal proteins: synthesis and modification					
sll1824	50S ribosomal protein L25	rpl25	MWPKIAFPLLTLIIIFTSITMA-LS	22	118
Transport and binding proteins					
sll0064	putative polar aa transport system periplasmic substrate-binding protein		MLLKSAFTWLALTFTTGLVAIAPVQA-ET	26	275
sll0108	ammonium/methylammonium permease	amt1	MSNSILSKLNVGERSPVNRTHSSRTEAEKSSLFRFV**RR**KINSPWLACVPLTALIVAIWNAAAIA-QD	64	507
sll0142	probable cation efflux system protein		MSSPNSVFSLSGLAI**RR**HIATLMLTLAIIVLGVFAVFS-LP	38	1075
sll0221	bacterioferritin comigratory protein		MTSKKFSWPKTIIALLLTLGLWLGLA-DLP	34	184
sll0224	amino-acid ABC transporter binding protein		MKKFACLALSVLLSGAASLPSWA-GE	23	298
sll0537	ammonium/methylammonium permease	amt3	MTSIDTLWLLLCAGLVFFMQA-GFMCLE-SGLTR	27	541
sll0540	phosphate-binding protein PstS homolog		MGQKNEAVILIGALAITGVV-VA	22	307
sll0679	periplasmic phosphate-binding protein of ABC transporter		MFDLSRLSRGIVPMALLLLGISACTPSQT-SQ	29	336
sll0680	phosphate-binding periplasmic protein precursor (PBP)		MTTFKQIKKLSKHLVPTASILALTVGLA-ACGGGGGGGDTA-QT	28	383
sll0682	phosphate transport system permease protein PstA homolog		MTAVNLQKKKSDLRAIFGYSMTAVSAACLLATVIPLFA-VL	38	287
sll0738	molybdate-binding periplasmic protein		MKLAWFWLLILVGLLTACGVSSFN-QL	24	270
sll0739	ATP-binding protein of molybdate ABC transporter		MATDLTPLWISLKTAGLATVVTFILGIAAAYG-ML	32	615
sll0771	glucose transport protein	glcP	MNPSSSPSQSTANVKFVLLISGVAAL-GG	26	468
sll0833	probable oligopeptides ABC transporter permease protein		MDWWKKL**RR**NSLARWGAILLLLFYVAVIG-AD	29	371
sll0834	low affinity sulfate transporter		MQITNKIHFRNLQGDLFGGVTAAVIALPMALAFGIASGAGA-TAGL	41	564
sll1017	ammonium/methylammonium permease	amt2	MKPKNFPLARYVLGAMLAFLFVGVAQA-QT	27	442
sll1104	substrate-binding periplasmic protein of a TRAP-type permease Na-dep.tr.	gtrC	MFNKVINFLPMGKFFCFFTYTTLSMRSLIVASCLLFLIPGLTVSATA-QT	47	296
sll1202	iron(III) dicitrate-binding protein of ABC transporter		MKKYKINYFSTLMIFMTSLL-TSCNT	21	347
sll1263	cation efflux system protein		MTARLARPYAVLSIGAALATMGLKLGAYA-IT	29	310
sll1270	substrate-binding protein of the ABC-type Bgt permease	bgtB	MKGMVKLGHWGKTWRYYLLLALGVLLAIA-IPLL	29	530
sll1404	biopolymer transport ExbB protein homolog	exbB	MAGGIVAVPLLGFSLLAVA-LI	19	210
sll1406	ferrichrome-iron receptor, outer membrane	fhuA	MNTKISLGLTICCLCSGLVAPLPILA-QI	26	828
sll1409	ferrichrome-iron receptor	fhuA	MWGAQKMIKILEQTSLAVLIGLTALHSGVALG-SV	32	863
sll1428	probable sodium-dependent transporter		MESNFLTTIFLPLALFLIMFGMGLG-LT	25	292
sll1450	nitrate/nitrite TS substrate-binding protein	nrtA	MSNFSRST**RR**KFMFTAGA-AAIGGVVLHG-CT	18	446
sll1482	ABC transporter permease protein		MKIPLAWHQLFHERMRLLAAIAGIAFA-DV	27	383
sll1598	Mn transporter MntC	mntC	MATSFASRGGLLASGLAIA-	19	330
sll1762	putative polar aa transport periplasmic substrate-binding protein		MLKQFSATFIGLLLATVGAQA-AI	21	299
slr0040	bicarbonate TS substrate-binding protein, outer membrane	cmpA	MGSFN**RR**KFLLTSAATATGALFLKGCAGNPPDPNA-ASTG	35	452

slr0401	periplasmic polyamine-binding protein of ABC transporter		MNLPCYS**RR**HFLQSAAAVGLATGLGGCWPGQGSS-KE	34	384
slr0447	ABC-type urea TS substrate-binding periplasmic protein	urtA	MTNPFG**RR**KFLLYGSATLGASLLLKA-CG	26	446
slr0513	Fe-TS substrate binding peripl protein	sfuA	MTTKIS**RR**TFFVGGTALTALVVANLPRRASA-QS	31	346
slr0529	glucosylglycerol substrate-binding protein	ggtB	MKFFKITTLIISLIVLTSCQGPGVNG-DE	26	421
slr0559	binding protein of ABC transporter for natural aa	natB	MNNLVGDFFRMRLFWPQS**RR**RLTAIALNLALA-GTTAG	32	454
slr0681	probable Na/Ca exchanger protein		MMTWLTIPFLILGLGILVAGA-EI	21	368
slr1200	urea TS permease protein		MSVLLEGIFNGLSTSSVLLIAALGLAIVFGLMGV INLA-HGEL	38	388
slr1229	sulfate permease		MGLYASFTIAVLTAFFGGRSGSISA-ATG	25	453
slr1247	phosphate-binding periplasmic protein (PBP)	pbp	MLSSLQKVATFSVVSVIVGFGGIGQAIA-GT	28	333
slr1249	phosphate TS permease protein PstA homolog		MKSSAPSSLLAPLSFFRNGFA-WVM	21	290
slr1295	iron TS substrate-bindingouter membrane protein	futA1	MVQKLS**RR**LFLSIGTAFTVVVGSQLLSS-CG	28	360
slr1316	ABC-type iron(III) dicitrate TS permease protein		MPFLQCIMRSSLYFRAKSPGYLALGLVLGATVLF A-CL	35	343
slr1319	iron(III) dicitrate transport system substrate-binding protein	fecB	MKSKLIIFTFCLVLFGCA-KQ	18	315
slr1336	H+/Ca2+ exchanger		MSTKSKIFLVLLVFCPLSFA-AHW	20	372
slr1452	sulfate TS substrate-binding protein	sbpA	MARSAFGWGFSVIAVLMVGSITA-CN	23	352
slr1454	sulfate TS permease protein	cysW	MLTINLPKTFKVKYLLIALALFYLILVLLLPAIA -VFYE	34	276
slr1490	ferrichrome-iron receptor		MQGVIMNQVQWSVLLMGIVSLLCAPRAWA-ET	29	853
slr1491	iron(III) dicitrate TS substrate-binding protein		MHRSG**RR**FRLFTLTILTIVFFSACVGSTS-QN	29	330
slr1509	membrane subunit of a Ktr-like ion TS	ntpJ	MTISRTICLGFIAAIAGGTLLLLMPLATST-GE	30	444
slr1723	permease protein of sugar ABC transporter		MKHWKTYLVLVLLAIAMLLPLLWLVATA-LK	28	270
slr1730	K-transporting ATPase C chain	kdpC	MIRNFVISLRSTALLWILTALI-YPAI	25	190
slr1740	oligopeptide binding protein of ABC transporter		MLLNLPATVKSRSCQKLIGGLLPLLLFA-CG	28	582
slr1897	sugar-binding periplasmic protein of ABC transporter		MVSWCRWRSP**RR**WFLFACLGLLLSGLISC-QS	29	433
slr2057	water channel protein	apqZ	MKKYIAEFIGTFWLVLGGCGSAVFA-AF	25	247
slr6042	probable cation efflux system protein, czcB homolog		MQNRIIVQGWAFPLLLGLLVMSPQATLAHA-GHG	30	545
Other categories					
			Adaptations and atypical conditions		
sll1283	similar to stage II sporulation protein D		MRKFVMEFTTKTGGSLPTNLRVLLPWLQLIGKSS FFGLIISLAWTLSAQA-ME	50	391
sll1677	similar to spore maturation protein B		MLNYIWFAIILLSVIAGTVTG-KI	21	203
			Drug and analog sensitivity		
sll0210	bacitracin resistance protein		MSPRQLNFLSAFSLSVAIA-VVYH	19	326
sll1154	putative antibiotic efflux protein		MSSFQTLRQLSPSTQKTFALLFASVLMFWLSLTA LLPTLPMYA-QD	43	418
sll1434	penicillin-binding protein		MSSTSTLKPSSTKVKAKAQPNFFQRVLCTTGTTF LGVGLVSTAVVA-GG	46	650
slr0319	beta-lactamase		MNHRFLLLVSLVVGSAEMLLTSPVSA-EL	26	304
slr0378	similar to 7-beta-(4-carbaxybutanamido)cephalosporanic acid acylase		MLVQIRGKL**RR**GVGLVLLFVLTVGLGIGNPVWA-NG	33	704
			Other		
sll0222	putative purple acid phosphatase		MGLSRLPVIGLFITLVTIAFLLVA-NF	24	326
sll0576	putative sugar-nucleotide		MIMKILITGGGGYIGSVLTPTLLA-AG	24	312

	epimerase/dehydratease				
sll0686	probable cytochrome c-type biogenesis protein		MLNRA**RR**SFSSMAPWAFCLILFLGALSLVVLTSQWA-SLTA	36	275
sll0915	putative zinc protease periplasmic protein	pqqE	MPLLVFRSLSRCLLVTALILVLHGPGLWSHPAIA-AD	34	524
sll1369	putative peptidase		MVRIFGATVLALLSWGGAG-EP	24	430
sll8034	2-nitropropane dioxygenase		MLTTQITQTYHLTTPIISAGMAFVATPKLA-AAV	30	344
slr1106	prohibitin		MSKQPSFDGWQSIVGGLIAALLVLLSFNSFV-VI	31	282
slr1761	FKBP-type peptidyl-prolyl cis-trans periplasmic isomerase		MRGRTHGIIRPTLLRPIFLMKFFTAMRDILISLTVVTFFSLVLISVAIFGKSSPSAIAA-PQ	59	201
slr1772	probable periplasmic hydrolase		MVGAIARWASLKIVMNPRQLTKTMNIQFLGNITTAILLAVVLQITLWPSAAQA-CT	53	377
slr5093	probable flavin-containing amine oxidase, periplasmic protein		MI**RR**RSFFKLSQLMFVSYLLSTSCGKNNTPVTANDAPSILIIGAGLAGLAAA-QS	52	458
			WD repeat proteins		
sll1491	WD-repeat periplasmic protein, salt responce		MNNYFPRLKQFSAPATFFLTVACLVYPGENAHA-NA	33	348
slr1409	periplasmic WD-repeat protein		MRIFPVFLLTFSLFLIKEEIVTA-EVK	23	326
slr1410	WD-repeat periplasmic protein		MKHKFLVSFLLGLTISFAGA-QVIA	24	334
			Hydrogenase		
sll1224	hydrogenase subunit of the bidirectional hydrogenase	hoxY	MAKIRFATVWLAGCSGCHMSFL-DM	22	182
Hypothetical					
sll0148	hypothetical protein		MTFD**RR**TFLRSGMALGLGSWAWA-WSGRN	23	375
sll0173	periplasmic virginiamycin B hydrolase	vgb	MSPFPAKSLLIALVLSWPASFDAALA-AP	26	352
sll0174	hypothetical protein		MNLFRKCLLILGLASPLYLGLGQTSAHA-EA	28	284
sll0180	membrane fusion protein		MVR**KR**SQFPVIGSMVALA-LLNT	18	501
sll0183	hypothetical protein	rfrB	MKIRPFLVALGLTTFAGAAHG-AN	21	259
sll0269	hypothetical protein		MTEPLFWLGLSLTLVSISLTAVLVVA-LP	26	137
sll0274	hypothetical protein	rfrC	MLMNFLVICQKFFTPNLFPWKAIARVQREKPQSLGRWQFVVRTGILVATFILALGSLASPSLA-LD	63	196
sll0301	hypothetical protein	rfrN	MSSFLVFSW**RR**WLQILILAIALVLLWAPSALA-QA	32	169
sll0314	hypothetical periplasmic protein		MLVFLTRFTPMNLIRNRWAQIFTQSILGVLIAGGTAWA-GD	38	314
sll0319	hypothetical periplasmic protein		MNWKFFPHPFLAMAVGAILAPFTPVSA-ST	27	297
sll0355	hypothetical protein		MQIESKTNTNIRSGLTLIAPFFLWGTAMVAMKGVLA-DT	36	330
sll0381	hypothetical protein		MPPRKSMRSPFKSGWSLVSLLFVLLSGCG-GE	30	294
sll0382	hypothetical protein		MKFNLALFCLAGGILTQTVSVLA-HG	23	176
sll0470	hypothetical protein		MSMPSMPKFSQAKLSKKLLALLSLSGVLVMGLPAIGFA-QG	38	186
sll0471	hypothetical protein		MITYC**RR**TVAAIFLAIFSWPIAPG-QT	24	684
sll0498	hypothetical protein		MKNRFVALIFALLFGSFGLHK-FYLG	21	150
sll0564	hypothetical protein		MPRQVWIFQRQFSLVPLKHSMTVSTATPAPTAMS-QV	34	327
sll0577	hypothetical protein	rfrO	MLKVFRQSFLVVLTVACLIWSAPAIA-AS	26	169
sll0638	hypothetical periplasmic protein		MGQSASGD**RR**GLSRSLLNKLMAVGMGLTLASFSMAPAFA-QT	39	449
sll0676	hypothetical protein		MAKNDLQSLLSASAMTTAEA-LS	20	180
sll0685	hypothetical protein		MKTTRSFLTSTLAIAFLGGALGVASVSLSSPQVSVA-QM	36	162
sll0691	hypothetical protein		MGASFRAEFPLWIHLAGIVVVPLALLTMMLGLALGTPFSVYG-LEL	42	200
sll0727	hypothetical protein		MPRSFLLLLLWMVIA-AGLRSWN	20	580
sll0736	hypothetical protein		MWTPWHYLGGKLWRLLVLLVALLVVLTTVFLGQNSSA-SP	37	408
sll0749	hypothetical protein		MEKIMSEQKSSSSLTGFALAALMVALVGTGFA-FWTG	32	199

sll0751	hypothetical protein	ycf22	MTAGRNQRLSPVLLQSSIGLLILTALILLA-FS	30	169
sll0788	hypothetical protein, salt responce		MGNQFWIYGLAGLLLSGSAAG-VT	21	196
sll0837	hypothetical protein		MEKFTVS**KR**HWLLLWGLQLGLAGGAAPVFS-QA	30	294
sll0839	hypothetical protein		MPRPCCLKLLGFSSVVKFGLSVTGFALILVTGLG LSSAQA-RN	40	197
sll0858	hypothetical protein		MVT**KR**SPTGIRQFVLFNHFSVNFLRNLLIMKNII IAGLLSGLACTVSTMPAMA-QN	53	197
sll0875	hypothetical protein		MDNSLLSEIWRQSLAFPWLSAVILNSFLLALA- AI	32	258
sll0886	TPR-family membrane protein 8 LAHG required	ogtA	MLSLLRKYFALLMPSLWGIVLVGLLLFFPSPVWA TE-SP	34	279
sll0931	hypothetical protein		MASTKKILQKTFIIASGLAFLGMMTVPMLTVLRG NA-NQ	36	156
sll1002	hypothetical protein	ycf22	MLRPRTIKEGSVGLFALLGLFIIGGIVLWLRGGA FG-NP	36	456
sll1004	hypothetical protein		MLIIAILCLGLLVAQGVAS-LI	19	324
sll1052	hypothetical protein		MAINTFTIISFIALGLIAGFA-SG	21	124
sll1071	hypothetical protein		MAVHLPSSATRFSKNFVWLLAASLSLMLWWGPAS PAQA-VN	38	275
sll1123	hypothetical protein		MLFLCNLLWLGIARSPTLA-ES	19	256
sll1158	hypothetical protein		MISQSIIPIPKNSMLRLKTVSSLLTPLILA-GMI	30	138
sll1160	hypothetical protein		MNIRPLVSSLSALAISVALLPLANFPAQA-QM	29	160
sll1191	hypothetical protein		MEPTAEGSFRWML**RR**MSQLAIAMVLALGLISLA- LL	33	103
sll1254	hypothetical protein		MLEIVAAIFIVLLGSGICSCAEA-AL	23	346
sll1307	hypothetical periplasmic protein		MLKFTFTPLLTTIALTGLALPALA-LE	24	175
sll1314	putative C4-dicarboxylase binding periplasmic protein		MKHS**RR**NFLALAGASSLLAIA-APK	21	369
sll1319	hypothetical protein		MRILSNVNLMGLLIVLLAAIFFCFHNVIV-RIL	29	329
sll1358	putative oxalate decarboxylase, periplasmic protein		MVNSVIGWL**RR**RFLLVGLSVLLITFLGIFTPTIA -QS	34	394
sll1378	hypothetical periplasmic protein		MALTMPRFFSAFLSPLSVVLLVAGMGFPAVQA- LE	32	300
sll1390	hypothetical protein		MEFHRQLPMTVAPHHPFNLSIFC**KR**LLSFLFLTL VLLGLSPAPSLA-TG	46	249
sll1424	hypothetical protein		MTSAYILVLSVLVLGGIIAALG-DH	22	491
sll1447	hypothetical protein		MRMLSVFGAIGGVMVLASGALMA-IG	23	126
sll1477	hypothetical protein		MKQWRWRMVLVAIAALVVISLA-TLF-QP	22	832
sll1483	similar to transforming growth factor induced periplasmic protein		MKTAARIVAFTALTGFALGMPTVAMA-EM	26	180
sll1488	hypothetical protein		MNSFWRTCRQKHWLSRNLSRPRVRKCRLWSLISL LALVMASSTLTIALA-SN	49	221
sll1507	salt induced periplasmic protein		MDLVVMNVRFPHFCLSLMFASLLAGAGTLPLAA- AE	33	181
sll1509	hypothetical protein	ycf20	MQRTRLNTIVEVRGQQLSQFFRNPW**RR**ISLSLLS FLFGFFVGTAVA-TT	46	109
sll1532	hypothetical protein		MFKPLFPLTLLLGCLSFLLTMLLHSPGQWG-AV	30	211
sll1581	GumB outer membrane protein		MNAMNPSQSFPSKLGGAVLVTTMACLGLWTQEAF A-QI	35	504
sll1618	hypothetical protein		MLPLILPALLSLSPAA-HP	16	200
sll1620	hypothetical protein		MGALLAVLLSGMVWFA-ALPGF	16	158
sll1638	hypothetical outer membrane protein		MSRLRSLLSLILVLVTTVLVSCSSPQV-EI	27	149
sll1667	similar to mitochondrial outer membrane 72K protein, periplasmic		MLILKNSMASPHPFPWPQRFTVMALTFAWGLSLP VLA-QT	37	266
sll1680	hypothetical protein		MK**RR**YLLEVGTASLGAFWLAQYVNS-TN	25	176
sll1696	hypothetical protein		MVFKLNLHARFVFQNLFSPCLAFASLIGLITLQR PALQ-AP	38	270
sll1736	hypothetical protein		MNNFSPKFVTVNQSGLGCGLTVLAVAMLLSA-IG	31	128
sll1738	hypothetical protein		MSDSRLNLVAIAIFLMMMSALV-GPI	22	231

sll1783	hypothetical protein		MRADFFLSDNRAALL**KR**GLTIILSFLVFTSIFLLPSPSLA-ED	40	147
sll1835	hypothetical periplasmic protein		MATHNLDRVAAPLISKLFPFFLVLAGMFSGTLAAQA-QG	36	265
sll1886	c-type cytochrome??? 9		MRIWPVVLTGLVIVFLSLGGGWAMA-QT	25	188
sll1940	hypothetical protein		MFRLFNSGKLDKVVLFWMGLTSALVTFYPTPSYG-QN	34	157
sll1973	hypothetical protein		MTTALLLCLCLLLITYLMGSIPTGYLA-GK	27	222
sll5033	hypothetical protein, on pSYSM		MKSILSFIKSVFILGILVGVLIINLAVSPAQA-VI	32	179
sll5034	hypothetical protein		MQTLPQRILSLMLIIGISLLLLGGNAAIATA-AP	31	207
sll7069	hypothetical protein		MKIIITAKGALLRNLATALVNGAITLTILLIAPLGLLA-VITNT	38	104
sll8025	hypothetical protein		MRLKIS**KR**SWLLSLHIATGGVWFGTALCSVALA-LS	33	171
slr0049	hypothetical protein		MAKVMIVGAGGVGSVVAHK-CA	19	398
slr0121	hypothetical protein		MITSMP**RR**PKS**RR**TQ**RR**NLRVISGTSTPPTGVTIA-EV	31	495
slr0142	hypothetical protein		MKLLSSGQLVAGVLFSGVAGFIDA-AS	24	244
slr0197	competence protein 6	comA	MGKLNRGWKPNPVVIFVALALLVAIA-VI	26	553
slr0238	hypothetical protein		MRLIKQIVAFLLLSLGIPLGLYCVV-EI	25	140
slr0243	hypothetical protein		MRKSLSGKQILQPWQSFSVNLIDFYRYRLLNWLTLGLICFSLNA-CQS	44	175
slr0250	hypothetical protein		MVRLLMVFPQRFSPSHLLSRHWGWLAVAMASLGSLSSLPVNV-NS	42	178
slr0280	hypothetical protein		MALGDLLKMVNWTRNWFGFSCLFVLTLAVMLGLKLFLPSPVVA-QS	43	610
slr0380	hypothetical protein		MSRFCPRFRLWGRLVPGVILGLLLFIGLAPWVQA-QI	34	459
slr0404	hypothetical protein		MLKMLKNLPLPKNITWRSVWMRGTSVVIIFVLAFTLVFTPTFE-AE	43	333
slr0431	hypothetical outer membrane protein		MRPKFFS**RR**PTMGISKLSKFSASVLLSGAILTTLPPSPLWA-NE	41	250
slr0453	hypothetical protein		MGSTLVGKCTSLGVFSMVTSPFSLSPFGQA-RS	30	821
slr0503	hypothetical protein YCF66	ycf66	MVNFGLNSASILGIFLAVA-GA	19	337
slr0516	hypothetical protein	rfrD	MNNKKLSLTLPLVGILALTA-CN	20	166
slr0565	hypothetical protein		MV**RR**RSVPWIHRYSRFIMGAIAVLGILITSYLA-YI	33	325
slr0575	hypothetical protein		MLPKISLAAVGLTVGGILTITGFVAYA-LD	27	184
slr0594	hypothetical protein		MFVNSSPIADGLSALVGLDSQPLQFYERGDVVPPKDQG-CW	38	407
slr0625	hypothetical protein		MLSLINVLFAFITIAILILAGRFLKQKIKLFQKLYLPESIIAGAIALLLGPGVFG-AI	55	487
slr0643	hypothetical protein		MWLLLLILAVITYFVVKSNA-APITR	20	493
slr0695	hypothetical outer membrane protein		MR**KR**LTRFLSLALVLGLLWFGTAACA-SQ	24-6	173
slr0712	hypothetical protein		MTAWQKIQKIAPAAIGACLFVLSIGAIN-SE	28	322
slr0734	hypothetical protein		MPNIAVIGAGVVGAAIA-YE	17	372
slr0765	hypothetical protein		MPLITKLKKY**RR**KYKRFFWLGCVVLALLLIIPHPSQA-QF	37	617
slr0769	hypothetical protein		MQKQVSPLARVALWLGGVSLTATSLVIPTAAQA-QS	33	204
slr0779	hypothetical protein		MSTLLGILAILSAAAAAGMRIA-LP	22	206
slr0784	hypothetical protein		MMKLSQWLGLACLAMAAYVAWEIRQLLLLLFLAIVLSTA-LN	39	340
slr0815	hypothetical protein		MFFCCPLFPFVIPGKTTM**RR**IDALAIALGFFLLGGVVYA-GL	39	121
slr0924	periplasmic(lumen) salt induced protein (Fulda et al., 2002)		MKSLLRIGATLGLIGTTAIGTWLGTTLQALA-LP	31	242
slr0967	hypothetical salt responce protein	rfrP	MMKTIKSPLIALGLIFASPLLWGGTAIA-EN	28	150

slr1025	hypothetical protein		MKNKLGIILVCNCLFSLFINRGVGA-EE	25	112
slr1160	hypothetical periplasmic protein		MLQRLVHILALTVCGLAVVSSLTPSVRG-QG	28	204
slr1170	hypothetical protein		MSPPLPLLLPSSQTAVS-QD	17	117
slr1173	hypothetical protein		MDKAIRKIFAYLRPGVILVACLLSLLFFVRPALA-FCG	34	463
slr1178	hypothetical protein		MLTSMKGLSGLLQFFIGFILGVTLFVGGISLAGYLVFNRFA-AS	41	155
slr1184	hypothetical protein		MASIVFRYLTVLVFSLCLMGFFSPPTLA-AG	28	164
slr1207	hypothetical protein		MPMAMPLVQSMKKPLPILLSLLGLGILVVGIFAYRSA-YG	37	514
slr1215	hypothetical protein		MVLNRLALACLWLALVIFAFGFAPPSNP-QT	28	222
slr1236	hypothetical salt responce protein		MTPLTFRNLGQSKAPSIARTLVTIGLGIGLAMGTVGCSPSYS-RS	42	177
slr1260	hypothetical protein		MVSWCQSLFLRWGKVLTLTVLSFGLCCACLTLWPVATPALV-RE	41	177
slr1262	hypothetical protein		MALQVVGYFLASCIGLSLGLLGGGGSVLA-LP	29	288
slr1266	hypothetical protein		MSKNFNLLLGTLVGVSLVFPQAIA-VQ	24	185
slr1270	hypothetical periplsamic protein	tolC	MKSIHPLKFWSSSTLLLLLSTSVGVFLPGFSGGQGAIAVA-QS	40	536
slr1273	hypothetical protein		MFLTALRSFLLFLAVTCLSLAIAMPAWA-LT	28	146
slr1306	hypothetical required for optimal photoautotrophy (Zhang et al2004		MGQPGLTMGFKTVFKKPLVMGGIGLSVLLGLWSTTHSA-EQ	38	485
slr1406	hypothetical periplasmic protein		MKVS**KR**FFQPCLVLGISLGLSLALVPDALA-LK	30	181
slr1415	hypothetical protein		MGLDPPLPMLFKS**RR**NLVILLAIAVVAVAALLWTGPGQA-DH	39	382
slr1428	hypothetical protein		MGILILPIAGVISFLLVAASWQINA-WH	25	624
slr1506	hypothetical outer membrane protein		MVTFPLNL**RR**WLQSVCLGALTAIA-VQ	24	577
slr1608	putative glucose dehydrogenase-B, periplasmic salt responce protein		MLIPFLFKLTLPLASGIALS-SC	16	412
slr1624	hypothetical protein		MLDRHWHNQNNCRPSYWSHVTTVLTICLLAIA-MG	32	458
slr1649	hypothetical protein		MSHSTDLSALARWMAADFSNQAQA-FE	24	196
slr1661	hypothetical protein		MPRAMLSCRYTLQLALCFCLPLGLVNQALA-TG	30	654
slr1704	hypothetical protein		MFNSFSFPKLHWQA**RR**STALLTTVLLMGGLVGSFTNPVEA-RP	40	274
slr1753	hypothetical P outer membrane protein, osmotic stress responce		MVEILLISPLIAVLNPSLRLFCLALLACSSSFSGNVLA-QN	38	1749
slr1796	hypothetical protein		MIMSVCLPWLARC**RR**FLIVSLAFAMLLLGIWGTLPFSLSDHGTAIA-AL	42	201
slr1820	hypothetical protein		MKLTWLNSS**RR**LISVIIYLTIFIRVLTLGLYAVA-DR	34	490
slr1927	hypothetical protein		MRKQRSKENWSPFSILINSRDLNFADWLE**RR**WVTPSYAGWLLLVLAVCLFGAATNSMA-GW	58	406
slr1940	hypothetical periplasmic protein		MPQFPMGFCIV**RR**INLRFASILALAIPLNLVASSLTA-AP	38	482
slr1944	hypothetical periplasmic protein		MIDF**KR**LLGIALTSSLLVLA-SP	20	538
slr1946	hypothetical protein		MNKKIKLWGWCLALFLLTLLLVSLPAIA-NN	28	261
slr1971	hypothetical protein		MGF**RR**YQSLFAWILTVVLTATVTVAIA-SPV	27	283
slr1972	hypothetical protein	ycf81	MLDAVIILLSIFTFAGVGFA-VV	20	359
slr2000	hypothetical protein		MAQKSSSFFPYLIAGLCALLGACSGNQA-LQ	28	321
slr2005	hypothetical periplasmic protein		MK**RR**KFIRTAGAGLLAVA-GV	18	261
slr2013	PSII assemebly regulation 11		MVPTLRFYICLMLIGGGAMVVAQV-DTV	24	435
slr2052	hypothetical protein		MIAPICRALRSRLPLAAMLMIAATATPALA-NS	30	298
slr2101	hypothetical protein		MKFISSFFALATVLACQPTVFA-FE	22	143
slr2105	hypothetical protein		MARLTALFRTYGKYAFLPAIA-FGL	21	595
slr6039	hypothetical protein on pSYSX		MGNQFWIYGLAGLLLSGSAAGVTA-VYR	24	196
slr6044	hypothetical protein on pSYSX		MKFSIPTNPVILLLIVFLLNACG-NQ	23	159
slr7102	hypothetical protein on pSYSA		MKKLLKSLKWPALFVGIILLLAACHRAPTRTA-KE	32	338

smr0015	hypothetical protein		MAE**KR**KK**RR**SSSSLFGKPLLGLVGLTLALYLLRG WG-II	36	67
ssl0461	hypothetical protein		MSC**RR**LSTSGVLSMSVQNLSK-QD	21	83
ssl1792	hypothetical protein		MAFFKSLFLTSIFCFVLTAIPSQA-KE	24	99
ssr1966	hypothetical protein		MATNMGSIDRLIRLVIASALLYLGLFFYSGTSLG -LG	34	64
ssr2340	hypothetical protein		MEIVLFLATILIIGLVLRWFFNVTA-AT	25	64
ssr2611	hypothetical protein		MTPKSGILLLLSCVSAIA-AV	18	65
ssr3409	hypothetical protein		MTVLLWTMLLAVFIPLLA-TLQ	18	83
ssr5121	hypothetical protein on pSYSM		MLKFIGIALLAALVGYAFGVMA-GI	22	71
unknown proteins					
sll0008	unknown protein		MVSIFHQLVKLFLVFIFVLTAFVGFSAQAIA-GE	31	169
sll0167	unknown protein		MFTKIRDLASPLPLMAAMAALGGMLFAPVVRS- QE	32	164
sll0172	unknown protein		MKINLIFMICAVLFAGTAAA-ET	20	152
sll0237	unknown protein		MLLSTRKQWLLTLISLLAIA-VN	20	343
sll0238	unknown protein		MRSPTSFGGYWEKLGLVVSALVIFSPLLA-LV	29	534
sll0252	unknown protein		MKKLTSFPFPLFKLFKSLKSSKSFSFQAIAGRWP MSRKWALMLIPLASIGTIPLLLA-WS	57	428
sll0293	unknown protein, osmotic stress responce		MPNIRPLTASLSLLTTAFVGLTPALYATIAEA- QD	32	170
sll0394	unknown protein		MRNQYFSLISPALWPAVVPTLASVYLGTLVISSP AIA-LD	37	208
sll0419	unknown protein		MYFLLVTLVILVFPLLSIA-LE	19	154
sll0426	unknown protein		MKYHYSPWKLVLLAGTAFVLGTPTTVTA-QS	28	120
sll0443	unknown protein		MKSWILFSLLALVLAGCGQR-GG	20	469
sll0448	unknown protein		MNRLRNFAIVFIVVIIRSISPVYA-QQ	24	401
sll0473	unknown protein		MKYSLKKLLGFGLILILTAIA-LI	21	329
sll0479	unknown protein		MKFRHRQDRDKLPELEITPMLNVMLVVLAFFVAV A-AN	35	150
sll0494	unknown protein		MVARAILLQLILLLFSNNLIHA-QE	22	301
sll0518	unknown protein		MTRAFLLGFVISPPLALE-VI	18	385
sll0539	unknown protein		MMELNFFLRNQNLCVYSPSTIFSKGKLAKSTVIK LLVSALAVVFMGSEATG-AE	51	397
sll0552	unknown protein		MNPRHRTTTNHKLKEQLIYNQVSKNPPWDKLTAL VPGGAWLQTPTAMA-FP	48	168
sll0588	unknown protein		MSLFPLLTALLGIMPANT-IE	18	153
sll0590	unknown protein		M**KR**IGHMLVTFTIFLLLVLTIPTVGVA-QN	27	564
sll0595	unknown protein		MVWANLGHCCRWLGALTVATTLLAPIPLKA-GS	30	143
sll0630	unknown protein		MGYCRFLPTCLIGVLLSSLMAIAPANA-GK	27	145
sll0645	unknown protein		MSNRPGKLLFAGLMGLALA-FPF	19	255
sll0647	unknown protein		MAANLTFLGSGSAFT-VGA	15	256
sll0702	unknown protein		MAIVINYRSPIAVLGACLCISAIAPVQA-IP	28	130
sll0761	unknown protein		MTMKTAQITRCLALSALTTGLFSFAPVQG-QS	29	119
sll0762	unknown protein		MVLKL**KR**GTWLLWTVALMLGLGVVA-WE	25	116
sll0775	unknown protein		MKIIFSPKNVLSISGLSLLSTCLAASPALA-GY	30	125
sll0779	unknown protein		MAISAQSFINVANESLVSTLAIAALGLSLLVILS LA-VI	36	884
sll0843	unknown protein		MKLAFFQKCLSFLGFIWLLSWEAPPVQA-FS	28	365
sll0914	unknown protein		MPAPKIIKIGQNTALILASIVLSLVIA-EI	27	376
sll0943	unknown protein		MLKILQKLLIGVLTLCPSMALA-GV	22	220
sll0985	unknown protein		MLPTNRKNYIFRHQRLNLSQLIALTLLTLLLSLG FAQA-QS	38	680
sll1040	unknown protein		MPMGCRFATV**KR**LILLGLLAFTLAVLIPPATA- QI	32	765
sll1068	unknown protein		MASTVNRLFLAILTIASFSLSGGYGYVPVPMAIA -AD	34	143
sll1086	unknown protein salt response		MLLFLLPGSGDRLAKFSTALLSFAFMGLGNLITP MAVA-AQ	38	155

sll1089	unknown periplasmic protein		MKYSLFVATAIATVVGGSTTGQVMA-QS	25	213
sll1135	unknown protein		MKQISVVIVGGGLCGLMAAVVLQ-LQ	26	334
sll1306	unknown periplasmic protein		MQ**RR**DLFKYGLATGAGAIASYALMGNKPLLA-QQS	31	335
sll1315	unknown protein		MKHIFRFKNYFFHSKNNQL**KR**LLLVAFLLTGLWLTPQQG-RV	39	352
sll1333	unknown protein		MTVIFRGWLSLLPSSLVIFSAIASVGSVGA-QE	30	235
sll1338	hypothetical outer membrane protein		MLNKSVQILSGVVLAAAALGFTTPAQA-EP	27	187
sll1344	unknown protein		MDRCKFSNVKTIAKISYQHKQNPMRTFTLALLAIGTLAPSASA-QT	43	253
sll1359	unknown protein		MKIGRITAYALVVIIFVLFFSLVTPVSS-KT	28	678
sll1426	unknown protein		MKSLLRGIALSVSVLMATGGYGLTTSAQV-AP	29	155
sll1511	unknown protein		MTLLSKLLALFPGLVITLALSCVIFWAMTRA-WW	31	205
sll1531	unknown protein		MLVPLLGLAIGFLLLTFSLLVYK-GV	23	608
sll1570	unknown protein		MVKSESGISMQTHLLPKKVASWITSLGLAGLSILALDPLASRA-DS	43	243
sll1665	unknown protein		METLSLVLAVIIGLVVGFA-GT	19	589
sll1761	unknown protein		MNRLSKMYVKSALLAPLTIVLGLLGAARVEA-EP	31	204
sll1763	unknown protein		MNSNQLSWTAFLVAIAGVTSASAQA-AT	25	134
sll1764	unknown protein		MAINNRSSWTAFVIALTAIGTVAEPGNALA-TN	30	144
sll1765	unknown protein		MIVSLAIVGYQLLPSQPLLA-EP	20	159
sll1784	unknown periplasmic protein		MKTLRLSPLLSKLCLILLIVLGTLTIAIPKALA-IS	33	267
sll1785	unknown periplasmic protein		MLLKVKLWGIGLVLTLTLGTILFLQNFSVA-A-ET	30	268
sll1837	unknown protein		MVNKGLWSAAVMVLGSTLVGSVFA-PT	30	145
sll1885	unknown protein		MRNNRMTFKKPAQMLGLLGAWLVLGAWSSNTDHHGISSPSALA-DP	43	845
sll1891	unknown protein		MVNHPLPLLLATALTFTVSA-PQT	20	252
sll1949	unknown protein		MK**KR**SLFSLLTVSLLMFSGGCGFLPGGGSG-EE	30	247
sll6010	unknown protein on pSYSX		MTLIAIMKYKILSSLALVTLGITAIAPMVNAGMA-KD	34	227
slr0019	unknown protein		MVFRFPSPVLVIILANCLMTVVGLILPQSA-RT	30	892
slr0060	unknown protein		MKPKVAIACQGGGSQTAFT-AG	19	369
slr0061	unknown protein		MLKKIFILVMLLAIAGAGLG-YY	20	221
slr0112	unknown protein		MVMRNALTIFALLLSSTGIFVSLA-RE	24	185
slr0114	unknown protein	ppcC	MMFRPLSRAIQVAMYYPIRIVAAAIILTALLFIPLGWS-SW	38	510
slr0262	unknown protein		MANEVVKAK**RR**SKFNFGDFVFSPLALFLAVLAGMGATA-FI	38	134
slr0271	unknown protein		MRINHKPLLIATALLTLVPNVVWS-SP	24	172
slr0306	unknown protein		MVKNQGAWLSRGLWSVLIWGAMATVCRA-ET	28	506
slr0333	unknown protein		MTLDKLGSAFLLGIALASLGVPALA-RG	25	106
slr0334	unknown protein		MKKFSRTVSALILSISGAIAWCLPSFA-AI	27	110
slr0341	unknown protein		MSNSSGSRLPQPKSFPILRPLLTLSAWALQSLVVPPVQA-ET	39	318
slr0353	unknown protein		MKIDYKLTLLLFCLCVPGVIA-SS	21	121
slr0386	unknown protein		MPPFSAIVLFSMTKSPQKKQWLANALLVLGSLGFAVVVA-EV	39	412
slr0442	unknown protein		MNTRFFLNFLTAP**KR**NAGFAMPMVIMGGLVITVAAA-AI	36	611
slr0489	unknown protein		MATFLALLPRSLTTFLYAVA-AL	20	151
slr0522	unknown protein		MKTSILSASTLLILGLTSLIPAMA-QS	24	294
slr0579	unknown protein		M**KR**NYLLTAIATLGILASLAPNANA-QF	25	117
slr0582	unknown protein		MAGLRQNLIMFIPSEDINAMVTTSTDQT**RR**SPTFLLVTLLSLLTAALVFFPVLAQA-GE	56	126
slr0587	unknown protein		MITVARKTTITLGLMAIAVSSVWPAQA-QM	27	110
slr0593	cAMP binding membrane protein	samp	MELGKISPIIINLLKTVENFALTAFLQGILGASSMALGALIA-VA	44	434

slr0602	unknown protein		MLSSLFSGGLTLTLALAPVGAFT-VE	23	129
slr0699	unknown protein		MFKNGYLLMGIFSATFFWLSATISA-ET	25	140
slr0708	unknown protein		MSQNFFANLPKTRLLARLNDGWLGKAVLALALVL LMAFPTWA-LL	42	402
slr0829	unknown protein		MLQVLLVMAVLVFSALS-LD	17	829
slr0841	12 periplasmic protein		MRLNFSRTLFFAAIMGGFALA-MG	21	291
slr0913	unknown protein		MNKLPKITNTNNEFFVSILALLLFIGIFFITASY S-DL	35	539
slr1079	unknown protein		MASFLALLPRSLTTFLYAVA-AL	20	164
slr1082	unknown protein		MASFLALLPRSLTTFLYAVA-AL	20	136
slr1150	unknown protein		MDMKFFILCRPLISFGLVSTISLALPATA-QG	29	170
slr1257	unknown protein		MLIL**RR**LILVVSLSFATIA-ID	19	327
slr1258	unknown protein		MSTIKALLPPKFPQLLTGLALLSLSLVVSTAIA- AK	33	251
slr1391	unknown protein		MSDPRTIYFQPRKPENSPKGGKFWGVLLWLSLLS MMPVQG-CA	40	156
slr1407	unknown protein		MKQVNFLAGFLSNSLITLVIIFVVTVAT-VT	28	289
slr1450	unknown protein		MFNFFVRLSAIGGMGVGAAVAIVSGA-CS	26	104
slr1485	putative phosphatidylinositol phosphate kinase (fulda 1999) periplasmic salt induced protein		MFKFAQIIFLAGSMVCLSSLGHQSLA-GN	26	345
slr1567	unknown protein		MKLFHWLLYFTTFSLPGIIPCPVLA-NE	25	765
slr1576	unknown protein		MGNRSRFGALTSLGLIVVAPLVLILSPNFSAMA- TE	33	106
slr1627	unknown protein		MLTMGWSNILSLFGAMLILAALPSLSVLT-VS	29	106
slr1667	(target gene of sycrp1) 13		MFTKFNQVLLASGLVLTSLVGFGSSAFA-EG	28	178
slr1681	unknown protein		MSVKLAVLSLLASTFTLPLLLPSSVQA-QT	27	320
slr1773	unknown protein		MLKNIFALTIFGGIFGAFPVLS-QT	22	258
slr1774	unknown protein		MGPTMKSLVRQTACLFSILILGLTLLPMDSALA- RG	33	289
slr1866	unknown protein		MQSWRHRFLSYVLLACLSCTSFTA-FI	24	333
slr1869	unknown protein		MNFKLEQALAMVFIIAPPLA-ST	20	249
slr1928	type 4 pilin-like protein	pilA5	MFEVLIALMISFLFLTGTLNA-MV	21	152
slr1931	type 4 pilin-like protein	pilA8	MKILWYLMTRNSSKGFTLLELLLASIMTFFVVSA -TG	34	305
slr1956	unknown protein		MVNHGYPLGRSLLSIAMAMLGALSLVFPLPLLT- QD	33	172
slr2004	unknown periplasmic protein		MHQISMKQTRFVQVLIVSIFLVFGMAIGLQQLSA -QP	34	474
slr2018	unknown protein		MKNKAIGNLRLTLFLFSRNPKSNPRGYMLFLVVS LLITLSGLLVA-YA	45	799
slr2048	PratA, processing accociater TRP periplasmic protein	pratA	MNLRYLWSGKQILIGGGLIPLLFGAAVSA-QS	29	383
slr2071	unknown protein		MFKFLANNYSQSLGLTACLGLFTYLAIA-VP	28	107
slr5085	unknown protein on pSYSM		MTLPAIMKYKILSSLALVTLGITALAPGAFA-NP	31	106
slr5111	unknown protein on pSYSM		MQINYYTAKPMNNPLLMLKDKTGSMKIKLLISLL ILTPITALSSVVFA-QS	48	150
slr5126	unknown protein		MKKLLLLLTTILLIPLNATPSNA-GA	23	156
slr5127	unknown protein on pSYSM		MRKISINRLLAIKVAGSFAAATICGFAGYA-IF	30	256
slr6006	unknown protein on pSYSX		MLTITHLLVGAASVGMAAQT-TNP	20	342
slr6008	unknown protein on pSYSX		MNWRALLLAVILGTVVFGGLHA-QT	22	343
slr6065	unknown protein on pSYSX		MLTITHLLVGAASVGMAAQT-TNP	20	342
slr6067	unknown protein on pSYSX		MNWRALLLAVILGTVVFGGLHA-QT	22	343
ssl2100	unknown protein		MKKNAGQQVTRSKQELVIRSFFGLFFLALA- IAILV	30	78
ssl2384	unknown protein		MFDRSNKDLISAGLVSALGAGIVTSFA-VN	27	57
ssl2502	unknown protein		MRFP**RR**ILGFCGGLLLCQNCLNLPSHT-AQ	27	78
ssl3383	unknown protein		MRILAPHPLGQTLVFTAGIMLAAGMGFGMALRSA PPRVVA-DP	40	90

ssl3769	unknown protein		MLVLSVLSLALASFSAIL-CF	18	74
ssl6018	unknown protein on pSYSX		MLKFIGIALLAALVGYAFGVMA-GI	22	71
ssl6077	unknown protein on pSYSX		MLKFIGIALLAALVGYAFGVMA-GI	22	71
ssr0536	unknown protein		MKNFPAMIGGSLIVGSFALFSGAMPGMA-QE	28	84
ssr0693	unknown protein		MKVLHLALMATFSVVYATVAVA-VP	22	95
ssr1049	unknown protein		MASLFKILRSLAIPAVIA-GL	18	73
ssr2153	unknown protein		MKLQHPKPHNSV**RR**ALRSWAGPLAIALVMIIGLETAA-TA	36	65
ssr2318	unknown protein		MKSY**RR**LKQLLFFTNIGDKSKQIRQKTGAGKTKIYLSMAVFLLCPLTLWG-TT	50	87
ssr2912	unknown protein		MPFPQRFFPTFSFTPTPMFSTSKTSVIGRIILLATLCNLAIASPIYA-AG	47	85
ssr3402	unknown protein		MKQIIPALITLSFSPMAIA-AL	19	96
ssr5074	unknown protein on pSYSM		MFKFIGIVLIGGLAGYALGVAA-GI	22	71

Abbreviations: SPL – length of the signal peptide, aa. Twin arginine motifs are shown in bold.

Publikation

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Curriculum vitae

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Education

1981-1991	Secondary school 858 in Moscow

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1992-1997	Study of Biology at the Lomonossov-University Moscow
1997	Diploma Thesis: „Cloning und molecular-genetic Analysis of Gene of Resistence to Amitrol by the Cyanobacterium *Synechocystis* sp. PCC6803“at the Department of Genetics of Lomonossov-University Moscow. Supervisor Dr. M. Babykin.
1998-April 2004:	Scientific employment at the Department of Genetics of Lomonossov-University Moscow
1.10.1998-31.01.2000	DAAD Fellowship at the Institute of Plant Physiology of MLU Halle-Wittenberg
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2002-2005	Stays in the working group Prof. Dr. R.B. Klösgen at the Institute of Plant Physiology MLU Halle as a visiting scientist.

Darmstadt, den October, 2005

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Ausserdem erkläre ich, dass ich mich früher nicht um den Doktorgrad beworben habe.

Maria Zhbanko