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Analysis of the role of PilA proteins in the cyanobacterium *Synechocystis* sp. PCC 6803

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Annotation

The PilA proteins are major components of pili fibres which are essential for motility in bacteria. This project was focused on the role of the PilA proteins in cyanobacteria, specifically in the alternative functions that are not directly related to motility. The major task of this project was also to develop a method for the purification of the PilA1 protein from *Synechocystis* under native conditions and to analyze purified PilA1 protein in detail.

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CONTENT

LIST OF ABBREVIATIONS

AMS	4-acetamido-4'maleimidyl-stilbene-2,2' disulfonate
BG11	blue green medium
cAMP	cyclic adenosine monophosphate
DNA	deoxyribonucleic acid
dNTPs	deoxynucleosides
DDM	dodecylmatoside
DM	decylmatoside
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
e.g.	for exemple
Ery	erythromycin
GT	glucose tolerant
HABA	hydroxy-azophenyl-benzoic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MES	2-(*N*-morpholino)ethanesulfonic acid
mRNA	messenger ribonucleic acid
OD_{730}, OD_{663}	optical density at 730 nm, 663 nm
OG	octylglucoside
PCC	Pasteur Culture Collection
PCR	polymerase chain reaction
PSI	photosystem I complex
PVDF	polyvinylidene fluoride membrane
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS	10 mM Tris-HCl pH 7.6 and 150 mM NaCl buffer
TCEP	tris(2-carboxyethyl)phosphine
TES	N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
Tfp	type IV pili
TTBS	0.05 % $tween_{20}$ solution in TBS

1. INTRODUCTION

1.1 Pili

Many bacteria display surface appendages called pili (Figure 1.1) that support inter-bacterial attachment, conjugative transfer of DNA, or adherence of pathogenic bacteria to host tissues. In addition to these functions, pili are thought to play a role in bacterial twitching or gliding motility. Pili are important in cell structure, but may also have specialised functions including biofilms production, the formation of spore-filled fruiting bodies in *Myxococcus xanthus* (Bonner, 2006), and Type II secretion machinery. Pili formation in pathogenic bacteria has traditionally received most attention.

Thousands of PilA polypeptides (pilins) are the major structural components of pili. Pili have been divided into distinct families based on their immunological characters or comparing the amino acid sequences of pilin structural genes. Pili are classified into families which include divergent sets of microorganisms (Strom and Lory, 1993).

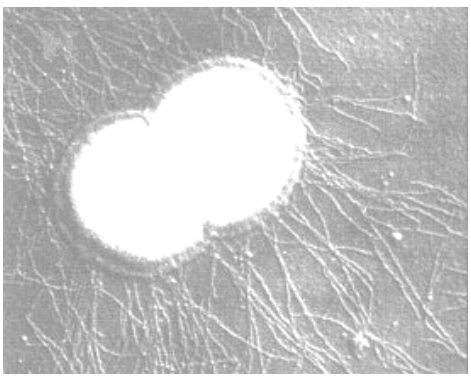

Figure 1.1. The pili of *Neisseria gonorrhoeae* allow the bacterium to adhere to tissues. Electron micrograph by David M. Phillips, Visuals Unlimited, http://www.visualsunlimited.com.

1.2 The Type IV pili

Type IV pili (Tfp) are expressed by a variety of Gram-negative ($Gram^-$) bacteria and related through the conservation of assembly and regulatory pili components. This group is typical for such pathogens as *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, and some enteropathogenic strains of *Escherichia coli* (Craig *et al.*, 2004) but occur also in non-pathogenic bacteria as *Myxococcus xanthus* (Wu and Kaiser, 1997). Proteins that share extensive homology with genes encoding Tfp have been recently discovered in cyanobacteria: *Synechocystis* sp. PCC 6803 (Bhaya *et al.*, 2000), *Thermosynechococcus elongatus* BP-1 (Iwai *et al.*, 2004), *Microcystis aeruginosa* PCC 7806 (Nakasugi and Neilan, 2005), and *Nostoc punctiforme* (Duggan *et al.*, 2007).

The Tfp apparatus is composed of a set of proteins usually named PilA-E, T, and Q. Small polypeptides as well as large motor proteins and enzymes, depicted in Figure 1.2, also belong to the Pil family.

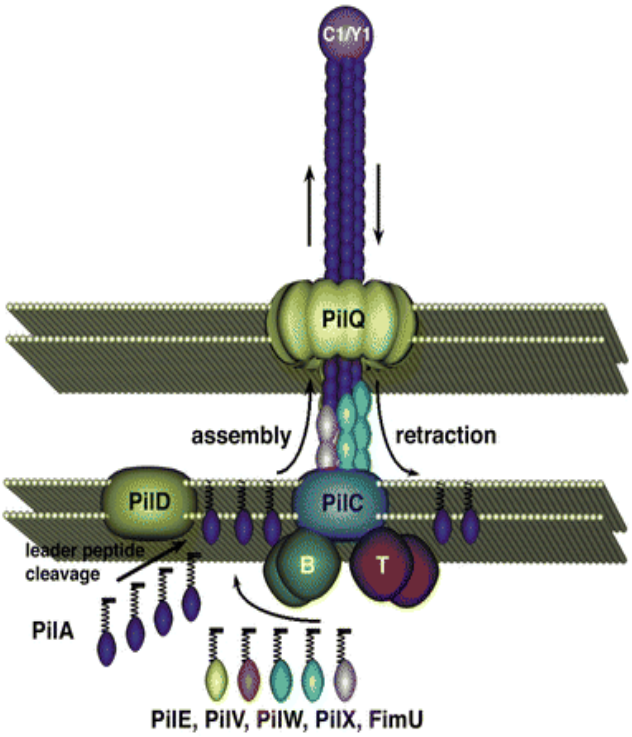

Figure 1.2. Structure of the Tfp apparatus. Prepilin PilA is targeted to the cytoplasmic membrane where it is maturated by the PilD peptidase. The PilD cleaves the PilA leader peptide and then methylates the N-terminus. Mature pilin remains in the membrane until processed into the growing filament by the PilB motor protein responsible for pilus extension. The PilB motor is structurally similar to the PilT motor, which is responsible for pilus retraction. The PilC has a capping and stabilization function in the filament. The filament passes through the outer membrane at a multimeric pore formed by the PilQ protein. The PilE, PilW, PilW, PilX, and FimU proteins can be incorporated into the filament in addition to PilA (Mattick, 2002).

Posttranslational modification of the most abundant pilin protein PilA forming the filament is typical for the Tfp group (Figure 1.2 and 1.3). This protein is initially synthesized as prepilin and undergoes two modifications where the N-terminal leader peptide is removed by proteolytic cleavage and the resulting N-terminal amino-acid (phenylalanine or methionine) residue is methylated prior to assembly of the pilins into filaments (Lory and Strom, 1997). All pilins share a conserved N-terminal region of about 60 amino-acids around the cleavage site. The N-terminus of the PilA proteins forms an α-helix that is capable of staggered coiling with several copies of itself (Craig *et al.*, 2004).

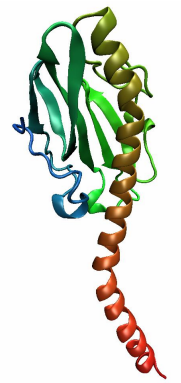

Figure 1.3. Structure of the PilA protein from *Neisseria gonorrhoeae*, a parasitic bacterium that requires functional pili for pathogenesis. The PilA protein is composed of a very long N-terminal α-helix and several β-sheets. A portion of the helix is cleaved by the PilD peptidase during maturation of the pilin. The pilus is assembled through non-covalent protein-protein interactions between the N-terminal helices of the pilin proteins. Helices are set in the center of the pilus fiber during staggered coiling with several PilA proteins, while β-sheets are organized on the exterior (source: http://en.wikipedia.org. pilin).

Twitching (gliding) motility (Figure 1.4) enables movement on solid surfaces in bacteria expressing the Tfp. Motility is mediated through reversible depolymerization and assembly of the Tfp filament (Strom and Lory, 1993). Twitching motility can also involve contact with another cell and social motility. The mechanism by which twitching motility is achieved has not been fully described but the ability of the Tfp to attach to surfaces is an important part of pili function (Nudleman and Kaiser, 2004).

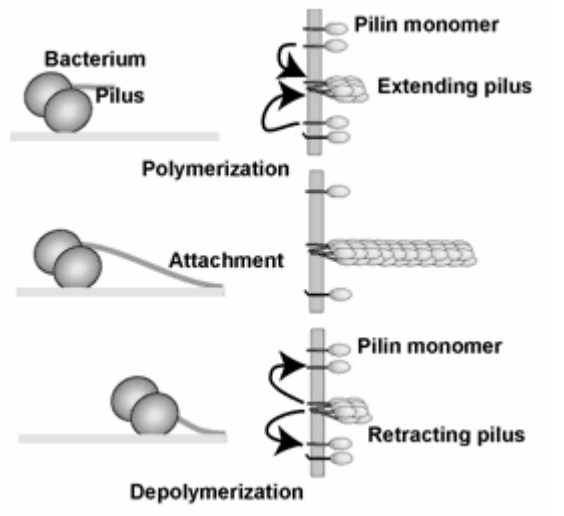

Figure 1.4. The proposed model for *Neisseria gonorrhoeae* twitching motility. *Neisseria* can move on surfaces through pilus extension, adhesion and retraction. The ability to switch between pilus polymerization and depolymerization is essential for cell motility. The mechanism which allows the bacterium to attach to surfaces is not known (Maier, 2005).

There is a relationship between Tfp biogenesis and the general secretory pathway also known as the Type II export machinery. Gram$^-$ bacteria have an outer membrane that protects them against the wide range of cytotoxins and hydrolytic enzymes that are exported by their secretory machinery. Most of secretory proteins (called also pseudopilins) are homologous to Tfp proteins and some (*e.g.* PilD) are shared by both Tfp and secretory systems. The general secretory pathway has not been completely described, but it is believed to be constructed from similar protein subunits as the Tfp and some models even presume the formation of a fibre composed of psedopilins - pseudopilus (Nunn, 1999). According to this theory, an exported protein present in the periplasm is pushed out by the pseudopilus piston through the secretin channel in the outer membrane (Nunn, 1999). It is not known whether secretory proteins can polymerize into the pseudopili or whether the pseudopili interacts directly with the exported proteins.

1.3 Tfp in cyanobacterium *Synechocystis* PCC 6803

The Tfp (or Tfp-like) proteins were shown to be essential for motility and extracellular DNA uptake in cyanobacteria (Bhaya *et al.*, 1999; 2000; Yoshihara *et al.,*

2001; Iwai *et al.* 2004), and they were also identified as a part of protein-secretion machinery (Sergeyenko and Los, 2000). Cyanobacterial Tfp proteins are best studied in *Synechocystis sp.* PCC 6803 (*Synechocystis*). This unicellular cyanobacterium is used as a model organism for studies related to photosynthesis (Williams**,** 1988; Komenda, 2004). It was also one of the first organisms for which a completely sequenced genome was determined (Kaneko *et al.*, 1996,). The *pil* genes are not named consistently in different bacteria. Also the *pil* gene products are not the same in different bacteria and therefore the description of the Tfp here is according to *Synechocystis* (http://bacteria.kazusa.or.jp/cyanobase/).

Synechocystis was isolated from a freshwater lake and deposited in the Pasteur Culture Collection (PCC) in 1968. It contains a single circular genome of about 3.6 Mbp, maintained in 6 to 10 copies per cell, and is capable of integrating exogenous DNA into its genome through active homologous recombination (Grigorieva and Shestakov, 1982). This competence, called spontaneous transformability, allows targeted gene replacement.

Synechocystis can be easily cultivated under autotrophic conditions. In addition to the original wildtype PCC strain, a glucose-tolerant (GT) strain exists which can be cultivated under autotrophic, mixotrophic, or heterotrophic growth conditions due to the presence of unidentified mutation(s).

Two types of Tfp can be observed on the surface of the motile wildtype *Synechocystis* cells: thick pili with a diameter of 5-8 nm and a length of 1-2 µm and more abundant thin pili with a diameter of 2-4 nm and shorter length of 0.5-1 µm. Thick pili have most of the unctional and morphological characteristics of Tfp and are probably encoded by the *pilA1* gene. This type of pilus appears to be twisted or knotted which may be an indication of flexibility. Is not known whether they are concentrated at particular pole of the cell or why they form links between cells. Thick pili could also serve as the apparatus used for DNA uptake and twitching motility. Thin pili are profuse and distributed about the entire surface of the cell, they are wavy or stretched straight probably depending on growth conditions and pH (Bhaya *et al.*, 2000). Thin pili can additionally form bundles of pili with a diameter up to 45 nm (Yoshihara *et al.*, 2001).

In comparison to other bacteria, the *Synechocystis* genome carries 11 *pilA* genes (Table 1.1) that is unusually large number. The conserved region of the PilA1, PilA2, and PilA4 proteins has the great similarity to the PilA proteins of *Pseudomonas aeruginosa*

and *Myxococcus xanthus* (Figure 1.5). It is not clear whether there is a distinct function for each of 11 PilA proteins. However, it was reported that the PilA1 protein plays an important role in the biogenesis and motility of both thick and thin pili (Bhaya *et al.*, 2000, Yoshihara *et al.*, 2001). The double *pilA1/A2* mutant exhibit few thin pili, and therefore remaining pili may be encoded by other *pilA* genes.

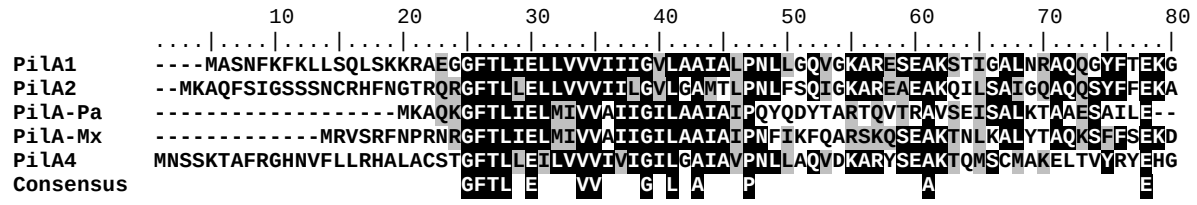

Figure 1.5. Comparison of the PilA1, PilA2, PilA4 protein sequences from *Synechocystis* and pilins from *Pseudomonas aeruginosa* and *Myxococcus xanthus*. Pa - *Pseudomonas aeruginosa*, Mx - *Myxococcus xanthus*. Residues conserved in 50% were highlighted in black (identical) or grey (similar). Only conserved part of the alignment is presented.

The *Synechocystis* PilA1 protein is probably processed by the PilD peptidase as mature PilA1 was detected in both the membrane and the growth medium into which it was secreted (Sergeyenko and Los, 2000; He and Vermaas, 1999). High levels of the *pilA1* mRNA accumulates in both the wild type cells and *pilA2* mutant (Bhaya *et al.*, 2000). Contradictory results were published for the *pilA2* mRNA (Bhaya *et al.*, 2000; Yoshihara *et al.*, 2001). The *pilA2* mRNA was not detected in either the wildtype or the *pilA1* mutants by Bhaya and coworkers (Bhaya *et al.*, 2000). However, Yoshihara and coworkers reported that the *pilA2* is highly expressed (Yoshihara *et al.*, 2001). Although a few authors imply that the *pilA2* gene has no function (He and Vermaas, 1999; Bhaya *et al.*, 2000), it has lower (52 %) transformation efficiency relative to the wildtype strain (Yoshihara *et al.*, 2001). PilA2 is thus a transformation competency protein which can be expressed in low level. Similarly to the PilA1 protein, PilA9, PilA10, and PilA11 are also required for cell motility. However mutants of these genes do retain thick and thin pili on the surface of the cell (Yoshihara and Ikeuchi, 2004). Other *pilA* genes have not been studied extensively. However, they do not appear to be essential for motility or phototaxis (Yoshihara *et al.*, 2001). The similarity of *pilA3* to other *pilA* genes is extremely weak. The PilA3 protein is much shorter than is typical for Tfp proteins and it is probably involved in the twin-arginine targeting secretory pathway (http://bacteria.kazusa.or.jp/cyanobase/). The remaining *pilA* genes are arranged

together in three gene clusters with the exception of *pilA3* and *pilA4*: *pilA1* and *pilA2*; *pilA5 - pilA8*; *pilA9 - pilA11* (Figure 1.6). The *pilA3* gene is located in a gene cluster encoding proteins involved in chemotaxis and the *pilA4* gene is located in a gene cluster encoding proteins involved in sulphate and chromate transport.

Figure 1.6. The arrangement of *pilA* genes into 5 gene clusters spread throughout the *Synechocystis* genome. *pilA1/A2* (*sll1694-5*), *pilA3* (*slr1046*), *pilA4* (*sll1456*) *pilA5/A6/A7/A8* (*srl1928-31*), *pilA9-11* (*slr2016-8*) (http://bacteria.kazusa.or.jp/cyanobase/).

A number of other genes was found to be required for cell motility, pilus biogenesis, and natural transformation competency of *Synechocystis* in addition to *pilA*. The organization of the Tfp apparatus in *Synechocystis* is probably similar to the typical structure (Figure 1.2). However, details on pilus structure in this cyanobacterium remain to be elucidated. Two copies of the *pilB* and *pilT* genes are present in the *Synechocystis* genome in contrast to other bacteria. The *pilB1* and *pilT1* genes encode motor proteins, containing Walker box motifs, that are responsible for the extension or retraction of pili. Disruption of the *pilB1* or *pilT1* genes resulted in a complete loss of motility. The *pilB1* mutant showed a non-piliated phenotype, in contrast to the *pilT1* mutation which resulted in a hyper-piliated phenotype. Interestingly, the *pilT2* mutant is also motile but negatively phototactic in conditions in which the wildtype cells are positively phototactic. A *Synechocystis* mutant of the *pilB2* gene has not yet been described (Bhaya *et al.*, 2000). Disruption of the *pilC*, *pilD*, *pilM*, *pilN*, and *pilO* genes in *Synechocystis* resulted in the total loss of motility and both types of pili on the cell surface (Bhaya *et al.*, 2000; Yoshihara *et al.*, 2001). The same effect was achieved through the deletion of the *pilQ* gene, which belongs to a large secretin family forming pores in the outer membrane and responsible for transport of the pilus across the outer membrane. Another gene, *pilD*, encodes a prepilin peptidase and pilin methylase. The *pilD* mutant lacked both thick and thin pili and displayed a non-motile phenotype. Interestingly, a number of *Synechocystis* non-motile *pil* mutants also lost natural transformation

competency, which suggests an important role of pilins in DNA uptake (Bhaya *et al.*, 2000; Yoshihara *et al.*, 2001).

The Tfp apparatus is complicated and tightly regulated by various families of genes, that are in consequence important for motility, including chemosensor-like elements - *che* genes (Bhaya *et al.*, 2001), sigma factors - *sigF* (Bhaya *et al.*, 1999), cAMP receptor - *sycrp1* (Yoshimura *et al.*, 2002), and serine/threonine protein kinases - *spkA* (Panichkin *et al.*, 2006).

Table 1.1. *pil* genes in the *Synechocystis* genome.

Genes involved in the pilus biogenesis and motility					**Other genes***	
Gene ID	Gene name	Loss of motility	Loss of transformability	Domed colonies	Gene ID	Gene name
sll1694	*pilA1*	+	+	+	*sll1695*	*pilA2*
slr2015	*pilA9*	+			*slr1046*	*pilA3*
slr2016	*pilA10*	+			*slr1456*	*pilA4*
slr2017	*pilA11*	+			*slr1928*	*pilA5*
slr0063	*pilB1*	+		+	*slr1929*	*pilA6*
slr0162-3	*pilC*	+	+		*slr1930*	*pilA7*
slr1120	*pilD*	+	+		*slr1931*	*pilA8*
slr1274	*pilM*	+	+	+	*slr0079*	*pilB2*
slr1275	*pilN*	+	+	+	*sll1533*	*pilT2*
slr1276	*pilO*	+	+	+		
slr1277	*pilQ*	+	+	+		
slr0161	*pilT1*	+				

* genes encoding for Pil proteins not essential for pilus biogenesis nor motility

The DNA uptake apparatus cooperates with the Tfp, but two unique genes for DNA uptake have been described recently in *Synechocystis*. The *comA* gene (*slr0197*) is essential for transformation competency but not for motility. The *comA* gene codes for a protein that was predicted to bind exogenous DNA to the cell surface. The *comA* mutant completely lost transformation competency and had decreased number of pili compared to the wildtype (Yoshihara *et al.*, 2001). The second competence-related gene was designated *comF* (*slr0388*). The *comF* mutant lost transformation competency, appeared to aggregate, and was not phototactic. Surprisingly, a 3.5-fold increase in *pilA1* mRNA was reported in this mutant implicating a direct cooperation of the DNA uptake apparatus with Tfp (Nakasugi *et al.*, 2006).

1.4 Tfp subunits are implicated in photosynthesis

Several authors have reported a phenotype related to photosynthesis as a consequence of *pil* mutations. The most striking results concern the functions of pilins in photosynthesis has been reported by He and Vermaas (1999). A *Synechocystis* mutant which was able to produce chlorophyll only at light was used in this study. This mutant lacked photosystem I (PSI) and the *chlL* gene, encoding for a component of light-independent protochlorophyllide reductase. Chlorophyll biosynthesis was thus fully light-dependent with the accumulation of protochlorophyllide under dark growth but no detectable levels of chlorophyll. Chlorophyll was synthesized at the expense of protochlorophyllide at a rate independent of the presence of photosystemI after returning the *PSI-less/chL*$^-$ mutant to continuous light (Wu and Vermmas, 1995).

Surprisingly, the PilA1 protein was identified by N-terminal sequencing in a previously unreported chlorophyll-protein complex that emerged in the *PSI-less/chL*$^-$ strain after two hours of illumination. In addition, the deletion of the *pilA1/pilA2* and *pilA4* genes in this *PSI-less/chL*$^-$ strain resulted in a 30% decrease in chlorophyll synthesis rate during the first six hours of illumination (He and Vermaas, 1999). This report suggests that absence of *pilA1/pilA2* and *pilA4* influences the chlorophyll metabolism at least during the early stages of chlorophyll biosynthesis (greening). None of the phenotypic effects were observed in the *pilA1/A2*$^-$ strain when fully green. Thus, the *pilA1* could be involved in delivering chlorophyll to nascent photosystems and antennae (He and Vermaas, 1999).

Another finding is related to the chlorophyll-binding IsiA protein that is induced in *Synechocystis* under iron deficiency, high salt, and oxidative conditions. IsiA probably functions as a dissipator of light energy and thus protects photosystem II from excessive excitation under iron-deficient conditions (Sandstrom *et al.*, 2001). Interestingly, the gene cluster containing *sll1693*, *pilA1*, *pilA2*, and *sll1696* was highly induced in the absence of *isiA* gene under no stress conditions (Singh *et al.*, 2005). *sll1696* has an unknown function and sll1693 strongly resembles a S-adenosylmethionine-dependent methyltransferase. An increased transcription of two additional *pil* genes - *pilA4* and *pilB2* was also identified in the absence of *isiA* gene (Singh *et al.*, 2005).

2. AIMS OF THE PROJECT

This project will analyze functions of the pilA proteins that are not directly or indirectly related to defects in motility of the cyanobacterium *Synechocystis*.

Aims of my diploma thesis were as follows:

1) To explore phenotypes of *pilA* mutants with an emphasis on the structure of the cell surface, metabolisms of chlorophyll and other photosynthetic pigments and possibly also effects on protein secretion.

2) To purify the PilA1 protein under native conditions and to characterize it in order to identify a putative protein complex or other structural details.

3. MATERIALS AND METHODS

3.1 Mutant strains

I used the glucose tolerant (GT) strain of *Synechocystis* as a control wild type strain and as a starting background for all the mutants constructed in this study. The *pilA4* deletion mutant possessing kanamycin resistance cassette was a generous gift of professor Wim Vermaas (He and Vermaas, 1999). The preparation of all other strains used during this project is described in chapters 3.4, 3.5 and in Table 3.1.

3.2 Culture and growth conditions

Synechocystis cells were grown under photoautotrophic or photomixotrophic conditions on agar plates or in BG11 medium (Rippka *et al.*, 1979) at 30°C and 40 µmol m^{-2} s^{-1} photons, unless otherwise indicated. The BG11 growth medium was supplemented by 10 mM TES (N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid in NaOH buffer, pH 8.2). 5 mM glucose was added to the medium for photomixotrophic growth.

3.3 Transformation and segregation of *Synechocystis*

Synechocystis cells growing on a plate were resuspended in 100 µl of BG11 and incubated with 100 ng of chromosomal (Table 3.1) or plasmid DNA for one hour. The suspension was streaked on a BG11 plate containing 5 mM glucose and cultivated overnight and then transferred onto a new plate with a starting concentration of 4 µg ml^{-1} for zeocin and 5 µg ml^{-1} etrythromycin and kanamycin.

Table 3.1. The preparations of mutant strains. The background strain was transformed by chromosomal DNA of another mutant in order to obtain a resulting strain.

Chromosomal DNA used for transformation	Used background	Resulting strain
pilA1/A2–	*pilA4–*	*pilA1/A2/A4–*
pilA1/A2–	*pilA1-StrepII*	*pilA1/A2–pilA1-StrepII*
pilA4–	*pilA1-StrepII*	*pilA4–pilA1-StrepII*
pilA1/A2–	*pilA4–pilA1-StrepII*	*pilA1/A2/A4–pilA1-StrepII*

The antibiotic resistance cassette is incorporated into chromosome by homologous recombination and the resistance cassette replaces the original gene sequence. Resistant colonies were transferred onto plates supplemented with increasing concentration

of relevant antibiotics every ten days, until complete segregation of all chromosomal copies was confirmed by PCR. Concentrations of 5, 10, 20, 50, and 100 µg ml^{-1} of etrythromycin or kanamycin, and 4, 7, 9, 12, and 20 µg ml^{-1} of zeocin were used.

3.4 Preparation of the *pilA1/A2* mutant

To delete the gene cluster encoding for the PilA1 and PilA2 proteins, a megaprimer mutagenesis based on PCR (Lee e*t al.*, 2004) was adapted resulting in a linear construct containing zeocin resistance cassette and ~ 400 bp flanking sequences to allow integration of the cassette into the genome by homologous recombination (Figure 3.1). This procedure consisted of three steps (Table 3.2, Figure 3.2). In the first step, DNA from the GT *Synechocystis* was used as a template in two successive PCR reactions. Fusion primers containing part of the zeocin cassette (Table 3.3) were combined with other primers to amplify two ~ 500 bp PCR products, so called megaprimers for the next step. The resulting megaprimers were purified using the GenElute PCR Clean-Up Kit (Sigma). In the second step, both megaprimers were extended using the plasmid pZeo (Invitrogen), which contains the zeocin resistance cassette, as a template. These new prolonged PCR fragments were purified again by the same way as previously. In the last step, the complete construct was finished in a single PCR reaction which contained both the extended megaprimers from the second step and flanking primers used for the first step. The PCR product from the final step was used directly for transformation of the GT strain and the resulting resistant colonies were segregated on increasing concentration of zeocin.

Table 3.2. Megaprimer PCR mutagenesis. The combination of primers and templates used for the deletion of the pilA1/A2 gene cluster.

	Step1	**Step2**	**Step3**
Template	GT chromosomal DNA	pZeo	2 extended megaprimers
Primers	1) PilA1-Z1 + PilA1-Z2 2) PilA1-Z3 + PilA1-Z4	1) Megaprimer 1 + Zeo 5 2) Megaprimer 2 + Zeo 3.1	PilA1-Z1 + PilA1-Z4
Extension step in PCR*	45 s	60 s	180 s
PCR product	2 Megaprimers	2 extended megaprimers	*pilA1/A2* zeocin resistance cassette

* Each 50 µl of PCR mixture contained: 25 µl of Jump Start REDTaq Ready PCR Reaction Mix (Sigma), 1 µl of 20 µM primers and 1 µl of DNA template. PTC-100 cycler (MJ Research) was used for amplification in three steps: denaturation (94°C, 45s), annealing (60°C, 45s), extension (72°C, time in table), cycles 29x.

Table 3.3. Sequences of primers used for the deletion of the *pilA1/A2* gene cluster by zeocin resistance cassette. The portion of each primer complementary to the zeocin cassette is highlighted in grey. The rest of the primer sequence is complementary to the *pilA1/A2* cluster.

Name	Sequence
PilA1-Z1 (p1)	GTTCAGCCATTGCAATGTTC
PilA1-Z2 (p2)	ACATTAATTGCGTTGCGCTCACTGCCAACCAGCAGTTCGATTAAG
PilA1-Z3 (p3)	CAACTTAATCGCCTTGCAGCACATCCAAGCAGATACTGAGTGCG
PilA1-Z4 (p4)	CCAATTAGACTGGCAAATGC
Zeo 5 (p5)	CGGGTCGCGCAGGGCGAAC
Zeo 3.1 (p6)	TGACCAGCGCCGTTCCGGTG

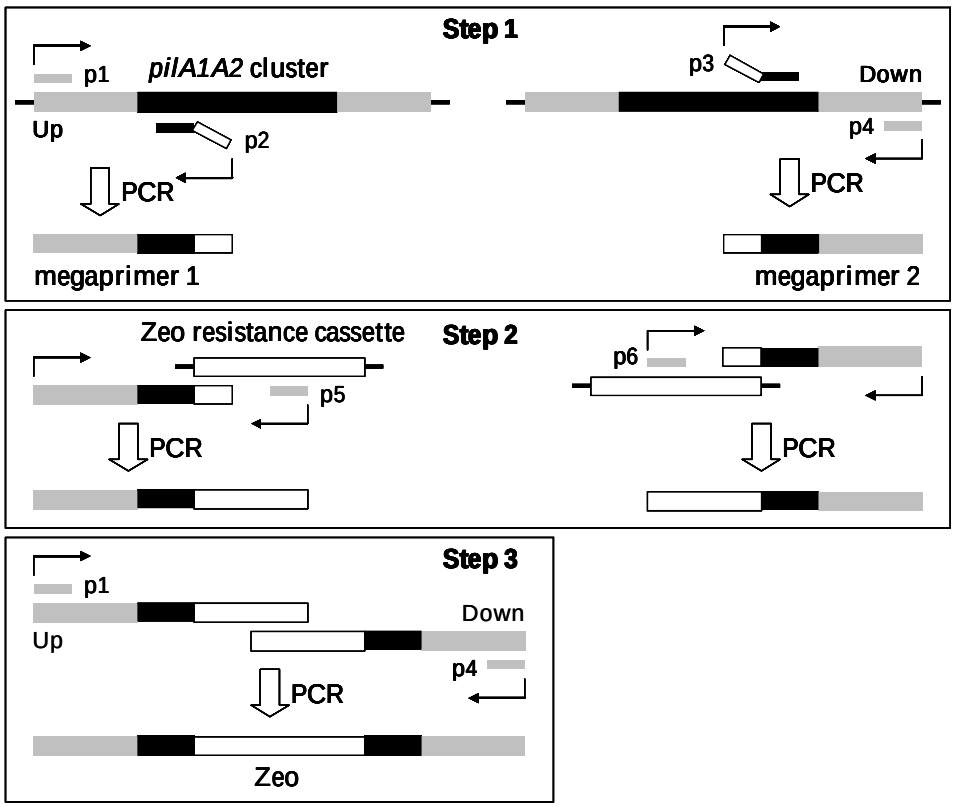

Figure 3.2. Three steps of the megaprimer mutagenesis. This method was used to prepare zeocin resistance cassette construct for disruption of the *pilA1/A2* genes. Letters p1-6 indicate the primers used in PCR reactions as listed in Table 3.2.

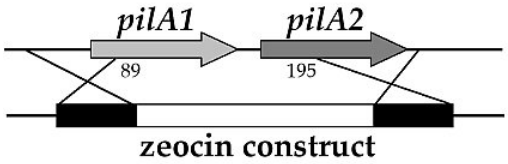

Figure 3.1. Deletion the *pilA1* and *pilA2* genes cluster by homologous recombination using a construct with zeocin resistance cassette.

3.5 Preparation of the *pilA1-StrepII* strain

In order to prepare a mutant containing the *pilA1* gene prolonged by the *StrepII* tag sequence on its C-terminus, I had to first prepare an appropriate *E. coli* plasmid for the *pilA1-StrepII* cloning containing a *Synechocystis* promoter, flanking sequences to allow homologous recombination and a gene for antibiotic resistance. Such a construct was made by modification of the pS2 plasmid (Lagarde *et al.*, 2000; Figure 3.4A), which already contained a strong constitutive *psbAII* promoter from *Synechocystis* and flanking sequences promoting incorporation into the *Synechocystis* genome.

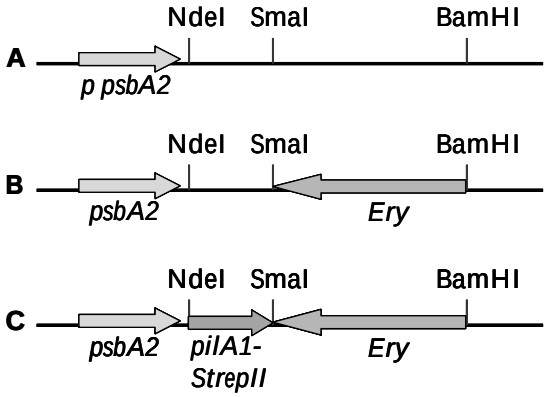

Figure 3.4. Preparation of the pS2-Ery-*pilA1-StrepII* plasmid. Part of the original plasmid pS2 (**A**) was replaced with an erythromycin cassette resulting in the pS2-Ery plasmid (**B**). The *pilA1-StrepII* gene was inserted into the pS2-Ery plasmid using the *NdeI* and *Cfr9I* restriction sites (**C**).

To insert a resistance cassette, part of the pS2 plasmid was digested out and replaced by the erythromycin resistance cassette (Figure 3.4B) obtained as a PCR product using the plasmid pERY (Thiel, 1993) as a template. The Ery cassette and the pS2 plasmid digested by appropriate restriction enzymes were mixed in a molar ratio of 8:1 and then ligated together for two hours at room temperature. Insertion of the Ery cassette was verified by PCR using Ery2 and W2 primers (Table 3.4).

Table 3.4. Sequences of primers used for insertion of the pilA1-StrepII and erythromycin resistance cassette into the pS2 plasmid. Restriction sites are highlighted in grey and the StrepII-tag sequence is underlined.

Name	Sequence
EryBgl-F	TATTCAAAGATCTCATCCGATTG
ErySma-R1	AACAAGTTAACCCGGGATGCAGTTTATG
Ery2	CTGATAAGTGAGCTATTCAC
W2	ATCCGCCGGCAGACGTTCTTCC
PilA1-NdeIf	GACAATCATATGGCTAGTAATTTTAAATTC
PilA1-Strep-Cfr	CATAATACCCGGGCTATTTCTCAAATTGCGGATGGGACCAAATTACTTCAGCACC
pilA1-1r	CTTCAAGCGTTTCCGTATCG
W1	TGTCATCTATAAGCTTCGTG

Two other primers (Table 3.4), containing *NdeI* and *Cfr9I* restriction sites and the StrepII tag encoding for WSHPQFEK amino acid sequence, were designed to prepare *pilA1-StrepII* gene construct for insertion into the pS2-Ery plasmid (Figure 3.4C). The product of this PCR and the pS2-Ery plasmid were digested using the by *NdeI* and *Cfr9I* endonucleases, mixed and ligated as described above. Insertion of the *pilA1-StrepII* into pS2-Ery plasmid was verified by PCR using PilA1-1r and W1 primers (Table 3.4). The resulting pS2-Ery-*pilA1-StrepII* plasmid was used to transform the *Synechocystis* GT cells. Obtained resistant colonies were segregated on increasing concentration of erythromycin as described earlier.

3.6 Inhibition of carotenoid biosynthesis

Fluridone is a chemical compound that blocks the synthesis of carotenoids, which causes photodamage of the photosynthetic apparatus in cyanobacteria even at low concentrations (< 1 µM) of this compound (Chamowitz *et al*., 1993). To compare resistance to fluridone in different strains, exponentially growing cultures were diluted at OD_{730} = 0.01 and then 15 µl of the culture was dropped onto autotrophic and glucose plate supplemented by 0, 0.01, and 0.2 µM fluridone. Plates were cultivated under 30 µmol m^{-2} s^{-1} photons for seven days.

3.7 Chlorophyll content

Chlorophyll content was measured in *Synechocystis* cells or in solubilized membranes. In order to measure the cell chlorophyll content, 1ml of suspension with a known optical density was centrifuged at 6000 rpm for 10 min and the resulting pellet was resuspended in 1 ml of 100% methanol. The sample was centrifuged again after one minute of shaking in methanol. Chlorophyll absorbance in the methanol supernatant was measured at 663 nm (OD_{663}). Chlorophyll content of cell suspension (µg ml^{-1}) was counted according to following equation (Porra *et al.*, 1989):

Chlorophyll content = OD_{663}/0,082 (milimolar extinction coefficient of chlorophyll)/OD_{730}

In order to measure the chlorophyll content in the solubilized membrane fraction, 5 µl of the membrane solution was added to 995 µl of 100% methanol, mixed and centrifuged at 10000 rpm for 5 min. Chlorophyll concentration was measured at 663 nm as described above.

3.8 SDS-PAGE and immunodetection

Protein samples for electrophoresis were denaturated by 30 min incubation with 1% SDS (and 1% dithiothreitol - DTT, if indicated) at room temperature. Precipitates in the sample were removed by centrifugation at 10 000 x g, 10 min, before loading on the gel. Proteins were separated on a gel using SDS-PAGE (BioRad-MiniProtean). The 1.5 mm thick gel included 4% acrylamide-bis solution for stacking and 12.5 % for resolving bands. Electrophoresis was run using a tris-glycine buffer at 15 mA for 30 min and 30 mA for 1 h. Proteins were stained by Commassie brilliant blue or by SYPRO stain following the manufacturer's instructions (Sigma, Germany).

Proteins were transferred from gel onto PVDF membrane (Amersham) using BioRad-Protean blotting system and carbonate buffer (3 mM Na_2CO_3, 10 mM $NaHCO_3$, 10% MeOH) at 0.85 A for 3 hours. The membrane was blocked in 0.2 % $tween_{20}$ solution in TBS buffer (10 mM Tris-HCl pH 7.6 and 150 mM NaCl) for one hour and then the membrane was washed 3 x 5 min in TTBS (0.05 % $tween_{20}$ solution in TBS buffer). To immunodetect the StrepII epitope, membranes were incubated for one hour at room temperature with anti-StrepII antibody conjugated with horse radish peroxidase (IBA) diluted 1:40 000 in TTBS. The incubation was followed by 3 x 10 min TTBS washing. Chemoluminescence reaction on the membrane was developed using SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce) and signal was visualised by exposure to an X-ray film.

3.9 Preparation of membrane and cytoplasmic cell fractions

400 ml of cells were grown until the culture reached an OD_{730}~0.6, and then the culture was centrifuged at 6000 x g for 10 min. The rest of the protocol was made at 4 °C. Cells were resuspended in 2 ml of thylakoid buffer containing a 1x protease inhibitor cocktail (Complete EDTA-free, Roche). The composition of buffer varied depending on the purpose of the isolation. Cells were broken using a BeadBeater and 0.1 mm glass beads by 5 cycles of 20 s each, with 2 min chilling interval between cycles. Unbroken cells and glass beads were centrifuged at 3000 x g for 5 min. The supernatant was centrifuged at 18 000 x g for 20 min to separate membrane and cytoplasmic proteins. Soluble proteins were transferred into a new tube and centrifuged at 50 000 x g for 1 h to remove membranes. Pelleted membranes were washed, resuspended in the same volume of thylakoid buffer and resolubilized using a detergent according to giving method (see chapter 3.11).

3.10 Localization of the PilA1-StrepII protein

Cytoplasmic and membrane proteins were prepared from the *Synechocystis* strain expressing the PilA1-StrepII protein as described in chapter 3.9. To discard peripheral proteins associated with the membrane, 100 µl of isolated membrane solution were resuspended and incubated for four hours in the same volume of 1M $CaCl_2$ or 0.1 M Na_2CO_3 solution in MES buffer pH 6.5 (modified according to Dobáková *et al.*, 2009). In order to remove salts after treatment, samples were dialyzed at room temperature for 18 hours against 150 ml of MES buffer (pH 6.5). Dialyzed samples were centrifuged at 18 000 x g for 20 min and supernatants were collected as peripheral protein fractions. Pellets, containing integral membrane proteins, were resuspended again in 100 µl of MES buffer (pH 6.5). All samples were then separated on SDS-PAGE, electroblotted and incubated with anti-StrepII antibody to detect the presence of the PilA1-StrepII protein.

3.11 Purification of the PilA1-StrepII protein

Strep-tag purification system is based on the highly selective binding of engineered streptavidin (Strep-tactin) to Strep-tag II fusion proteins. Gentle elution of purified recombinant protein is performed by addition of 2.5 mM desthiobiotin after application of crude extract on a Strep-tactin column and a short washing step. The resin was regenerated by washing with 1 mM HABA (hydroxy-azophenyl-benzoic acid). Purification of the PilA1-StrepII protein was tested on three different Strep-tactin resins listed in Table 3.4 (all purchased from IBA).

Table 3.4. Resins used for the PilA1-StrepII protein purification.

Resin	Support	Bead size	Recommendation
Sepharose	Sepharose 4FF, 4% agarose	45-165 µm	for common purification
Superflow	Superflow 6, 6% agarose, crosslinked	60-160 µm	for purification of large protein complexes
MacroPrep	MacroPrep, polymethacrylate	50 µm	when high non-specific protein binding with other two resins

Crude membrane extracts of the strain expressing *PilA1-StrepII* were prepared on ice in one of listed buffers (Table 3.5) as described in chapter 3.9. Membranes were solubilized during one hour of incubation in 1% buffer solution of dodecylmaltoside (DDM), octylglucoside (OG), or decylmaltoside (DM) and centrifuged at 18 000 x g for 20 min.

The supernatant containing solubilized proteins was filtered (0.22 µm) and diluted to obtain a final detergent concentration and loaded on a column filled with 100 µl of resin (Table 3.5). When the sample passed through, the column was washed with 100 x column volume, and the PilA1-StrepII protein was eluted by 500 µl of 2.5 mM desthiobiotin in used buffer supplemented by final detergent concentration. Finally, the eluate was concentrated 4 times using Microcon Centrifugal Filter molecular weight of 10 000 kDa (Millipore).

Table 3.5. List of conditions tested for the PilA1-StrepII purification.

Buffer	Detergent final concentration	pH	Tested resin
25 mM HEPES, 10 mM $MgCl_2$, 0.5 M manitol	0.04% DDM	7.6	Sepharose
25 mM HEPES, 10 mM $MgCl_2$, 0.5 M manitol	0.067% DDM	7.6	Sepharose, Superflow, MacroPrep
25 mM HEPES, 10 mM $MgCl_2$, 0.5 M manitol	1% OG	7.6	Sepharose, Superflow, MacroPrep
25 mM HEPES, 10 mM $MgCl_2$, 0.5 M manitol	0.4% OG	7.6	Sepharose, Superflow, MacroPrep
50 mM K-Pi	0.04% DDM	8.0	MacroPrep
11.8 mM K-Pi, 140 mM NaCl, 2.7 mM KCl	0.33% DM	7.3	MacroPrep
25 mM MES, 10mM $CaCl_2$, 20mM $MgCl_2$, 20% glycerole	0.33% DM	6.5	MacroPrep
25 mM HEPES, 10mM $CaCl_2$, 20mM $MgCl_2$, 20% glycerole	0.33% DM	7.4, 7.6	Sepharose and Superflow (7.4), MacroPrep

3.12 Detection of a disulphide bond by AMS labelling

To identify whether the PilA1-StrepII protein contains a disulphide bond, free –SH groups were visualized using AMS (4-acetamido-4'maleimidyl-stilbene-2,2' disulfonate) labelling method (Motohashi *et al.*, 2001). This approach is based on the fact that binding of AMS to one thiol causes 0.5 kDa shift in a protein migration detectable on SDS-PAGE. The purified PilA1-StrepII protein was first partly reduced by incubation with 5mM selective sulphydryl reductant tris(2-carboxyethyl)phosphine (TCEP) for one hour and then 10 mM AMS was added into samples for three hours. A change in protein mobility was analyzed using SDS-PAGE.

3.13 Analysis of pili structures using electron microscopy

Cells in logarithmic phase of growth were applied on a Formvar-covered grid and allowed to adhere for 5 min. Then the grid was drained and negatively stained for 1 min with 3% uranyl acetate and examined by JEOL JEM-1010 transmission electron microscope.

4. RESULTS

4.1. The presence of the pili on the cell surface

The surface of the motile *Synechocystis* sp. PCC 6803 cells contains both thin and thick pili. The presence of pili on the cell surface of the PCC and GT was analyzed using an electron microscopy. Highly resolving transmission electron microscope micrographs were obtained and no obvious differences in the pili structure between the motile PCC and the GT strain were observed (Figure 4.1). Thus, the motility of the GT strain is not abolished due to an effect on pili formation.

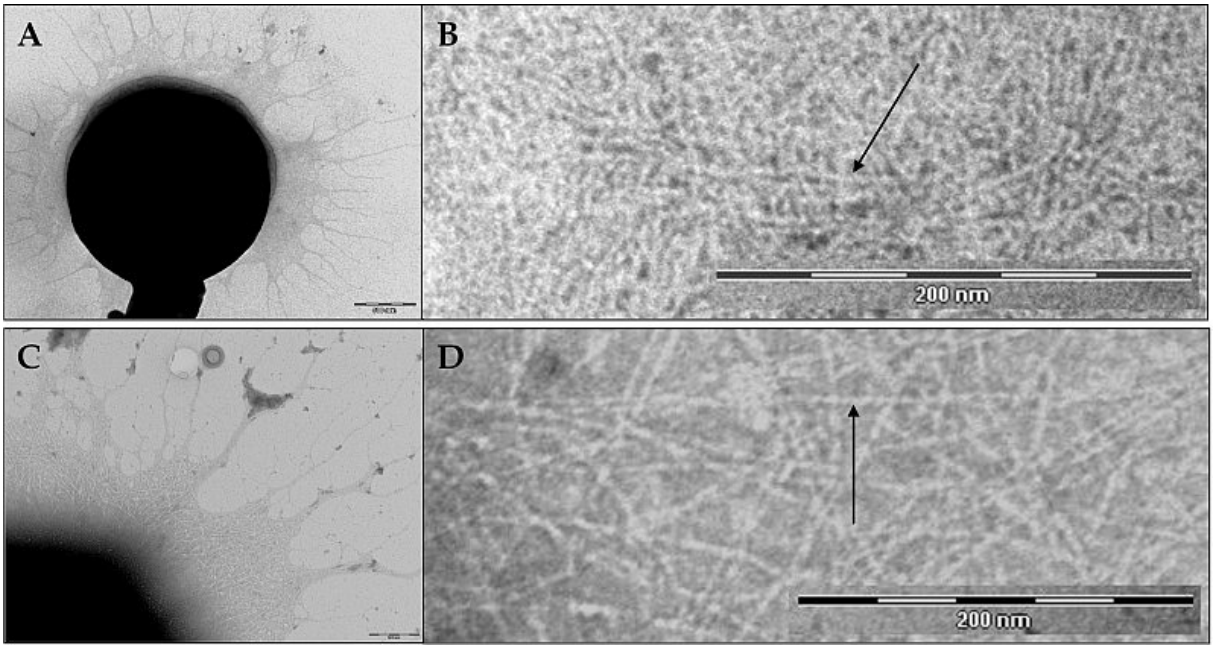

Figure 4.1. Transmission electron microscopy of *Synechocystis* pili. Logarithmically growing cells were stained with 1% uranyl acetate for 1 min. A) A single cell of the PCC motile strain, the bar indicates 500 nm. B) Thick pili of the PCC strain, a pilus filament is highlighted by the arrow, the bar indicates 200 nm. C) A single cell of the GT non-motile strain, the bar indicates 200 nm. D) Thick pili of the GT strain, a pilus is highlighted by the arrow, the bar indicates 200 nm.

4.2. Phenotype of the mutants lacking PilA proteins

As the non-motile GT strain displays similar pili structure to the motile PCC strain, it is a good model to study 'alternative functions´ of pili in cyanobacteria. To study proposed alternative functions of pili structures, several mutants lacking *pilA* genes were prepared in the GT background. Particularly, two mutants with deleted *pilA1/A2* operon (double mutant) and *pilA1/A2/A4* genes (triple mutant) were prepared and their phenotype was analyzed together with the single *pilA4*$^{-}$ mutant obtained as a gift from Professor W. Vermaas (Arizona University). The PilA1, PilA2 and PilA4 proteins were selected on the basis of their

sequence similarity to the PilA proteins of *Pseudomonas aeruginosa* and *Myxococcus xanthus* (Figure 1.5). The double *pilA1/A2* mutant was prepared using the megaprimer mutagenesis method based on PCR (Figure 4.2.A, see chapter 3.4 for details). The GT cells were transformed by a final PCR product and the full segregation of the double mutant was verified by PCR (Figure 4.2.B). To prepare the triple *pilA1/A2/A4* mutant, chromosomal DNA of the double *pilA1/A2* mutant was used for transformation of the *pilA4*$^-$ strain.

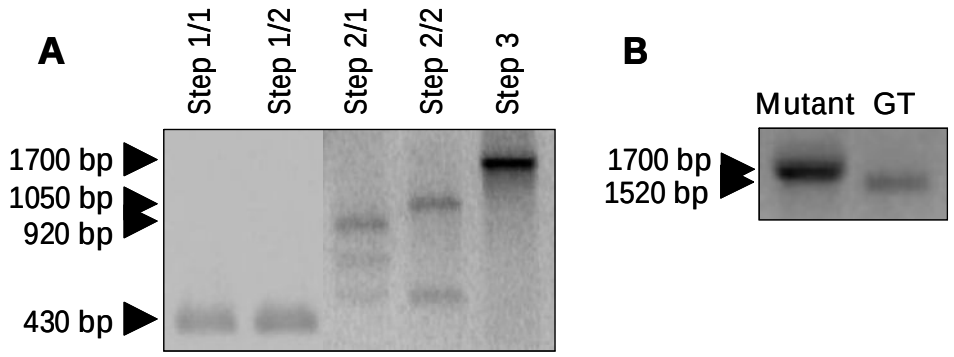

Figure 4.2. A) Preparation of the *pilA1/A2* mutant using PCR mutagenesis. Two megaprimers were prepared in Steps 1/1 and 1/2 and extended in Steps 2/1 and 2/2. The whole construct was finished in Step 3. Mutagenesis is described in chapter 3.4. **B) Full segregation of the *pilA1/A2* deletion.** The presence of the zeocin resistance cassette in the genome of the *pilA1/A2* mutant was verified by the PCR product of 1700 bp whereas original sequence in the GT strain has of 1520 bp.

Phenotype of all *pilA* mutants was analyzed under several growth conditions. First, the *pilA* mutant phenotypes were compared to the control GT phenotype under photoautotrophic conditions. Double and triple mutants appeared to bleach when grown on plates for a week. I have never observed such cell bleaching in the GT strain. There were no significant differences in doubling times among the GT, *pilA4*, *pilA1/A2*, and *pilA1/A2/A4* mutants grown in liquid cultures. Nevertheless, I observed differences in pigment composition between the GT and *pilA* mutants. All three mutant strains contained less carotenoids and more chlorophyll than the GT as is apparent from whole cell absorption spectra (Figure 4.3). Even the *pilA4* mutant (more similar to the GT strain than the other mutants) contained higher level of chlorophyll than the GT strain. The spectra of double and triple mutants were very similar, suggesting that the observed differences in pigment composition were caused specifically by the *pilA1/A2* deletion. Cells of the double and triple mutant strains also partly aggregated when grown to higher optical density in liquid medium (Table 4.1).

Table 4.1. Summary of the cell aggregation and bleaching phenotypes. The presence (+) and absence (-) of the bleaching and aggregation of a given strain, number of pluses indicates the intensity of the cell aggregation (subjectively assessed).

	Cell aggregation		
Studied strain	**Autotrophic growth**	**Photomixotrophic growth**	**Bleaching**
WT	-	-	-
pilA4—	-	-	-
pilA1/A2—	+	+++	+
pilA1/A2/A4—	+	+++	+

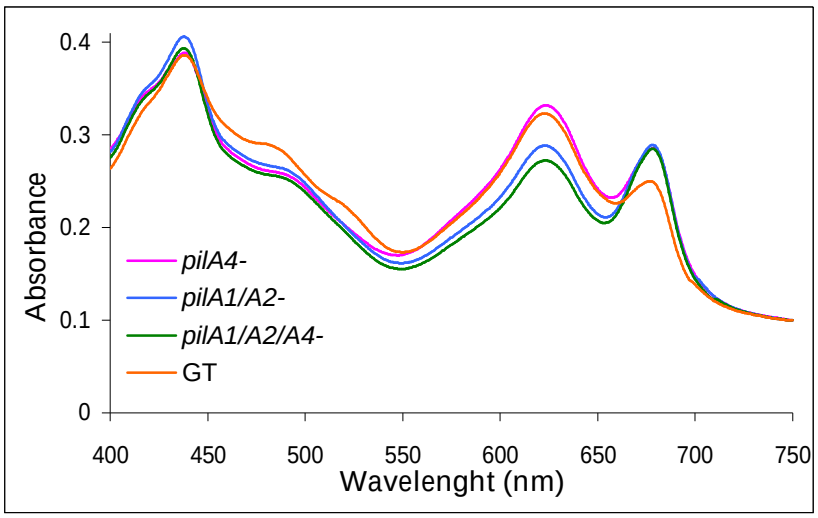

Figure 4.3. The whole cell absorption spectra of the GT and mutant strains grown under photoautotrophic conditions. The chlorophyll *a* is presented by 440 and 682 nm peaks, phycobiliproteins are represented by 625 nm peak and carotenoids by the range of 450 - 500 nm. Spectra were normalized to light scattering at $OD_{750\ nm}$.

To analyze the effects of oxygen on phenotypes, strains were grown under microaerobic conditions with liquid cultures bubbled with the mixture of the 3% CO_2 and 97% N_2. Interestingly, the cells of the double mutant completely aggregated under these conditions. No such effect was observed in the case of the GT strain, or when both strains were bubbled with plain air (Figure 4.4). Moreover, under microaerobic conditions, the medium of the double mutant was highly viscous implying the secretion of compound(s) into the growth medium. The chemical nature of the viscous compounds remains to be determined.

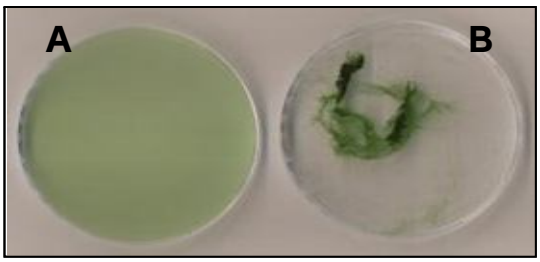

Figure 4.4. The aggregation of the *pilA1/A2* mutant under microaerobic conditions. A) Bubbled with plain air. B) Completely aggregated when bubbled with 3% CO_2 and 97% N_2 mixture.

The growth under photomixotrophic conditions was also analyzed. Surprisingly, cell aggregation, similar to that described under microaerobic conditions, was induced in the double and triple mutants when the growth medium was supplemented by 5 mM glucose (Table 4.1). In contrast, the GT and *pilA4*$^{-}$ strains did not aggregated under any of conditions tested. To estimate the level of aggregation, cultures of the *pilA1/A2*$^{-}$ and *pilA1/A2/A4*$^{-}$ strains grown photomixotrophically were filtered through 4 µm filter and the optical density of the filtered media was measured at 730 nm. The *Synechocystis* cell, having a diameter of approximately 1 - 2 µm, passes through. As the OD_{730} of the double and triple mutant filtered media was zero, the media did not contain single cells but only aggregates larger than 4 µm. Whole cell absorption spectra of the *pilA1/A2*$^{-}$ and *pilA1/A2/A4*$^{-}$ strains were impossible to measure under photomixotrophic conditions due to cell aggregation. For this reason, only the GT and *pilA4*$^{-}$ absorption spectra were measured. The *pilA4*$^{-}$ strain contained less carotenoids and lower amounts of chlorophyll in comparison to the GT strain (Figure 4.5).

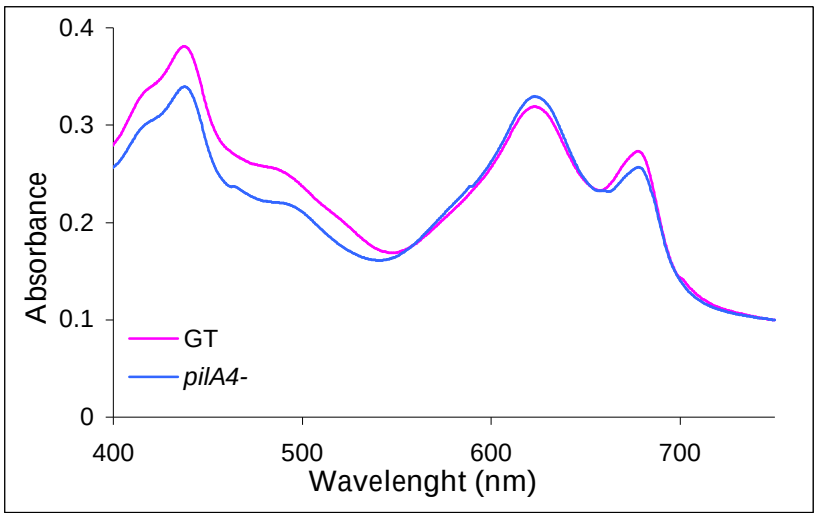

Figure 4.5. The whole cell absorption spectra of the GT and *pilA4*- strains grown under photomixotrophic conditions. It was not possible to measure cell spectra of other strains due to high level of the cell aggregation. See Figure 4.3 for description of the peaks.

4.3 The sensitivity of the PilA mutants to various stress conditions

I have found not only aggregation phenotype but also a difference in the pigment composition between the GT strain and *pilA* mutants. PilA mutants appear to be impaired in carotenoids biosynthesis under all tested conditions. To see whether they are really impaired, I blocked the carotenoids biosynthesis by using specific inhibitor fluridone under autotrophic and photomixotrophic conditions. Strain cultures were diluted to the same OD_{730} and 15 µl of each culture was dropped onto autotrophic and photomixotrophic plates

containing 0, 0.1, and 0.2 µM fluridone and illuminated by 30 µmol m^{-2} s^{-1} of photons (standard light) for five days. All mutant strains were more sensitive to fluridone than the GT strain, especially when grown under photomixotrophic conditions (Figure 4.6). The *pilA1/A2/A4* mutant appears to be the most sensitive strain.

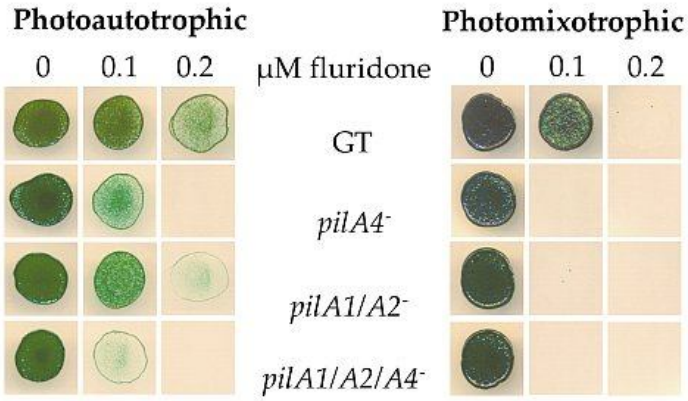

Figure 4.6. The strains viability was influenced when carotenoids biosynthesis was blocked. The *pilA* mutants were more sensitive to fluridone than the GT strain under both the photoautotropic and photomixotrophic growth conditions.

As the *pilA* mutants were found to be sensitive to lower carotenoid content per cell, I anticipated increased sensitivity of these strains to high light. Cultures were diluted to the same OD_{730} and 15 µl of each culture was dropped onto autotrophic plate and placed under illumination of 30, 200, and 300 µmol m^{-2} s^{-1} photons for five days. Surprisingly the control GT strain exhibited the greatest sensitivity to high light and did not grow under an illumination of 300 µmol m^{-2} s^{-1} photons. In contrast, all tested *pilA* mutants were more resistant to high light (Figure 4.7).

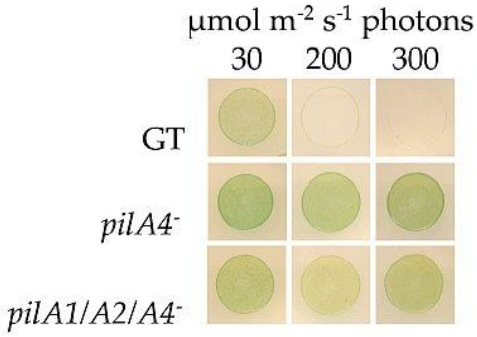

Figure 4.7. Sensitivity of the studied strains to high light. In contrast to the GT strain, the *pilA* mutants grew under 300 µmol m^{-2} s^{-1} of photons.

4.4. The *Synechocystis* strains expressing the PilA1-StrepII protein

Two *Synechocystis* strains were prepared containing the *pilA1* gene fused with the StrepII tag on its C-terminus (Figure 4.8) in order to localize and purify the PilA1 protein. The GT cells were transformed by this construct and the full segregation of the *pilA1-StrepII* was verified by PCR (Figure 4.9). Chromosomal DNA of the double and triple mutants was

used for the *pilA1-StrepII* mutant transformation in order to prepare *pilA1/A2⁻/pilA1-StrepII* and *pilA1/A2/A4⁻/pilA1-StrepII* strains (see chapter 3.3).

> PilA1-StrepII
MASNFKFKLLSQLSKKRAEGGFTLIELLVVVIIIGVLAAIALPNLLGQVGKARESEAKSTIGALNRAQQGYFT
EKGTFATDTETLEVPAPDGNFFSFAVNTADNTEAIQDATALNWEADGTRSMSGGTFYDSGTRAFSTVVCR
AEAGSEDTPPTPGGANDCGGAEVIWSHPQFEK

Figure 4.8. Amino acid sequence of the PilA1-StrepII protein. The StrepII tag sequence on the C-terminus of the protein, the first amino acid after maturation, and two cysteines are highlighted in grey.

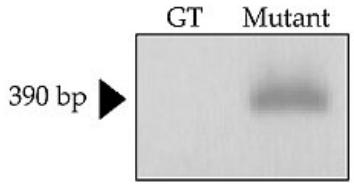

Figure 4.9. A 390 bp PCR product confirming the presence of the *pilA1-StrepII* gene in the mutant genome.

The presence of the StrepII tag or transcription from an artificial promoter could abolish the PilA protein function. Therefore, strains expressing the *pilA1-StrepII* gene were analyzed to determine whether the presence of the PilA1-StrepII protein complemented the mutant phenotype as expected for a functional PilA1 protein. Importantly, *pilA1/A2⁻/pilA1-StrepII* cells did not aggregate when grown under either photoautotrophic or microaerobic conditions and the culture medium did not seem to be viscous in contrast to medium of the *pilA1/A2⁻* strain. Some cell aggregates were observed when mutant strains containing the *pilA1-StrepII* gene were cultivated under photomixotrophic conditions, which suggest that the PilA1-StrepII protein does not fully complemented the *pilA1/A2* deletion. However, the degree of the cell aggregation was much weaker than observed for the *pilA1/A2* mutant (Figure 4.10 and Table 4.2). The most striking phenotype of *pilA* mutants, extensive cell aggregation, was clearly improved demonstrating at least partial functioning of the PilA1-StrepII in the cell.

Table 4.2. Summary of cell aggregation and bleaching phenotypes. The presence (+) or absence (-) of bleaching and aggregation in given strains, the number of pluses indicates the intensity of the cell aggregation (subjectively assessed).

	Cell aggregation		
Studied strain	**Autotrophic growth**	**Photomixotrophic growth**	**Bleaching**
pilA1/A2—	+	+++	+
pilA1/A2—/pilA1-StrepII	-	+	+
pilA1/A2/A4—	+	+++	+
pilA1/A2/A4—/pilA1-StrepII	-	+	+

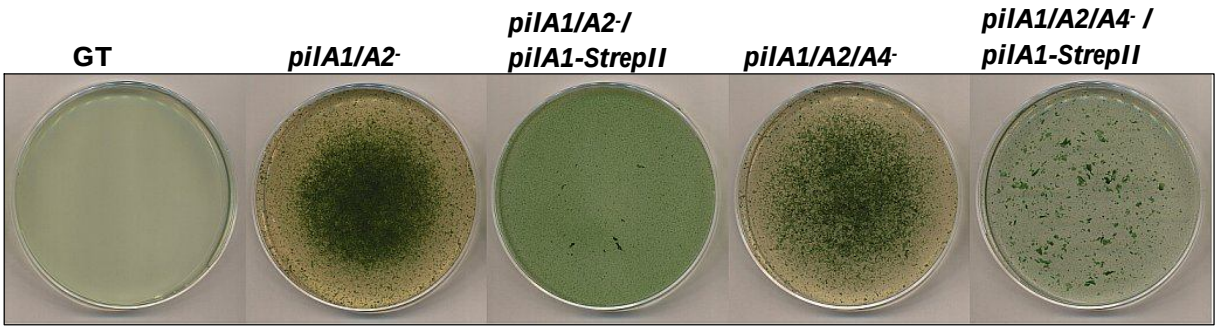

Figure 4.10. Cell aggregation in studied strains. All strains were cultivated in liquid medium supplemented with the 5 mM glucose.

The inability of the PilA1-StrepII protein to fully complement the deletion of the *pilA1/A2* genes is also demonstrated by using high light intensities. The same experiment was repeated as was described in the Figure 4.7. The high light tolerance of the *pilA1/A2/A4- pilA1-StrepII* strain did not differ from the *pilA1/A2/A4-* strain (Figure 4.11).

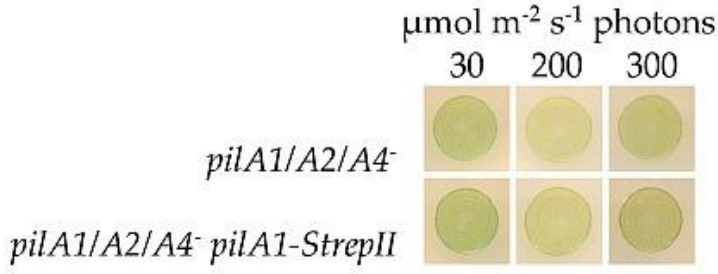

Figure 4.11. Lack of high light sensitivity of the strain expressing the PilA-StrepII protein. Both tested mutant strains grew photoautotrphically under light of 300 µmol m^{-2} s^{-1} photons.

4.5. Localization of the PilA1 protein

The StrepII tag is easily detected by a commercial antibody and the strain expressing the PilA1-StrepII protein was used to localize the PilA1 protein in the cell. The leader peptide of the PilA1 protein is believed to allow its localization into the cytoplasmic membrane. However, experimental localization of the PilA1 protein in the *Synechocystis* cell has not been reported.

The soluble and membrane protein fractions were prepared from GT and *pilA1-StrepII* mutants in order to localize the PilA1 protein. Immunodetection of the PilA1-StrepII protein using antibody against the StrepII tag demonstrated that this protein is present only in the membrane cell fraction (Figure 4.12). To find out whether the PilA1 is an integral or peripheral membrane protein, membranes were washed with high a salt solution (1M $CaCl_2$ or 0.1 M Na_2CO_3 in MES, pH 6.5 buffer) which solubilized and removed peripherally associated proteins. The two resulting fractions containing integral

and peripheral proteins were then analyzed for the presence of the PilA1-StrepII protein. The StrepII tag was detected only in the fraction of integral membrane proteins (Figure 4.13).

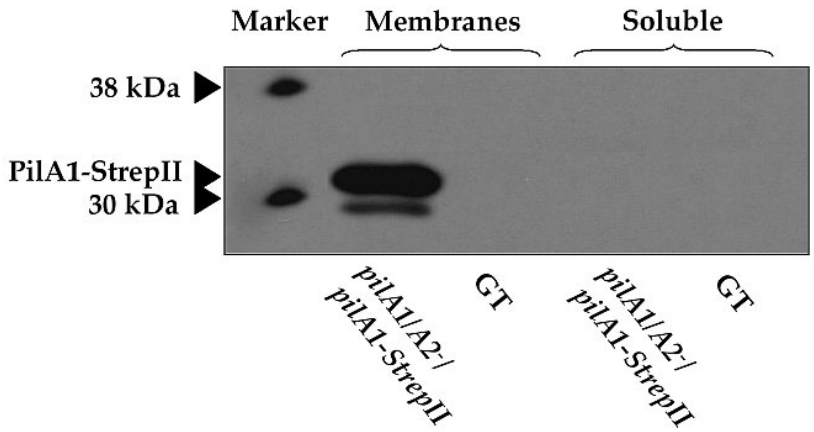

Figure 4.12. PilA1-StrepII is a membrane protein. Immunodetection of the PilA1-StrepII protein in the p*ilA1/A2-/pilA1-StrepII* and GT membrane and soluble protein fractions using the StrepII antibody (gradient 12-20% SDS-PAGE).

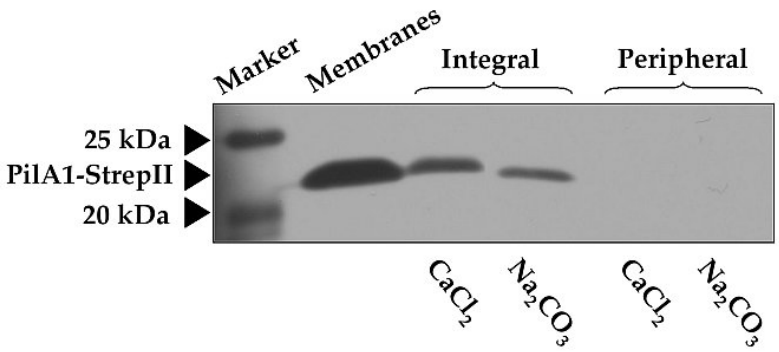

Figure 4.13. The PilA1-StrepII is an integral membrane protein. Isolated membranes of the *pilA1-StrepII* strain were washed with a high salt buffer and both peripheral and integral membrane protein fractions were obatined. The PilA1-StrepII protein was immunodetected using the StrepII antibody.

To determine whether the PilA1-StrepII protein is a part of a high mass protein-complex, the membrane fraction was analyzed using 2D gel electrophoresis system. The solubilized membrane complexes prepared from the *pilA1/A2- pilA1-StrepII* strain were separated first using a blue-native system (according to Dobáková *et al.*, 2007). A gel strip containing separated complexes from the first dimension was cut out and the proteins were separated again in a second dimension using SDS-electrophoresis. Separated proteins were then transferred onto a PVDF membrane and the PilA1-StrepII protein was immunodetected. The majority of the PilA1-StrepII protein was detected on the edge of the gel migrating as a single protein or in a small complex (< 40-50 kDa). Interestingly, a small amount of the PilA1-StrepII protein migrated to a position close to the monomer and dimer forms of the photosystem II (Figure 4.14).

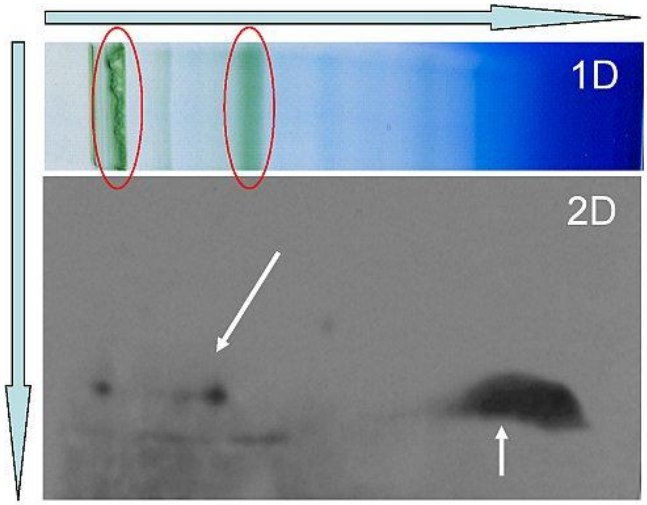

Figure 4.14. Separation of the PilA1-StrepII protein using 2D gel electrophoresis. Membrane protein complexes from the *pilA1/A2-/pilA1-StrepII* strain were separated using two-dimensional electrophoresis and blotted onto PVDF membrane. The PilA1-StrepII protein was detected, using antibodies against the StrepII tag, mainly as a low-mass protein/complex (large spot on the right side of the membrane indicated by an arrow). The left arrow indicates several bands of the PilA1-StrepII protein detected as higher-mass complexes. Dimeric and monomeric forms of photosystem II are indicated by red ovals on the native gel.

4.6. Purification of the PilA1 protein

In order to characterize in detail the PilA1 protein or to isolate and identify a putative protein complex containing the PilA1, this protein was purified from the membrane fraction using the StrepII tag. First, a method for the purification of membrane proteins from *Synechocystis* using the StrepII tag had to be developed. Three Tactin-based resins (IBA, Germany) with an affinity to StrepII-tag were tested in combination with different detergents, various buffers, and pH to obtain the pure PilA1 protein under native conditions.

I have found that dodecylmaltoside and octylglucoside detergents were not suitable for purification using StrepII-tag system as eluate contained a large number of contaminating proteins under all conditions I tested (*e.g.* Figure 4.15). I obtained much better results using decylmaltosite detergent. I prepared almost pure protein using the 0.33% decylmaltoside (final concentration), 11.8 mM K-Pi, 140 mM NaCl, 2.7 mM KCl (pH 7.3) and MacroPrep resin. Purified protein was separated using electrophoresis and then stained with Commassie.

Only a single polypeptide was visible when the eluate containing the purified PilA1-StrepII protein was separated by SDS-PAGE and stained with Commassie (Figure 4.16). As some proteins do not interact well with the Commassie, the more sensitive (than the Commassie) SYPRO system (Sigma, Germany) was also used and another protein was visualized together with the PilA1-StrepII (Figure 4.16). The identity of the PilA1 protein showed in the column 1 on Figure 4.16 was confirmed using the mass spectrometry.

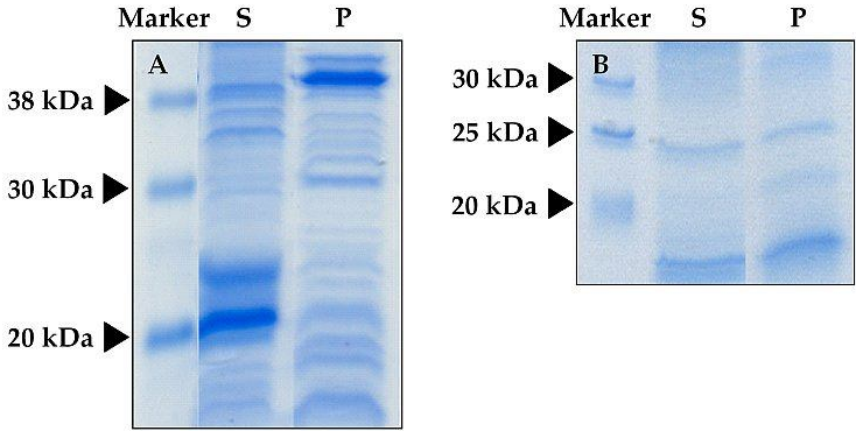

Figure 4.15. Two examples of unsuccessful purification. A) 25 mM HEPES, 10 mM $MgCl_2$, 0.5 M manitol, pH 7.6 buffer was used in combination with Sepharose resin and dodecylmaltoside as a detergent. B) 25 mM HEPES, 10 mM $MgCl_2$, 0.5 M manitol, pH 7.6 buffer was used in combination with MacroPrep resin and dodecylmaltoside as a detergent. S represents membrane samples that were loaded on the column for purification and P represents purified eluate.

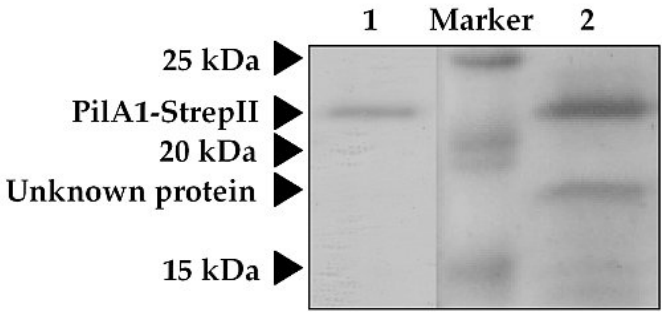

Figure 4.16. The purification of the PilA1-StrepII protein. Only the PilA1-StrepII protein was visualized in the eluate by Commassie staining (column 1), another protein with different molecular-mass was stained with SYPRO (column 2).

4.7. Detection of a disulphide bond in the PilA1 protein

When the purified PilA1-StrepII protein was analyzed on the membrane and I have noticed that after addition of the 1% reductant DTT into the eluate, the fully reduced PilA1-StrepII protein migrated much slowly on the gel (Figure 4.17). Such a change in protein mobility often indicates presence of a disulphide bond.

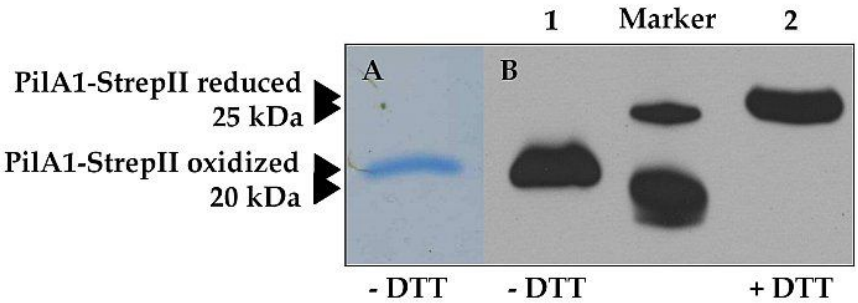

Figure 4.17. The mobility of the PilA1-StrepII protein changes after incubation with DTT. A) The PilA1-StrepII protein was the only protein visible on the Commassie stained SDS-PAGE gel. B) Immunodetection using the anti-StrepII antibody revealed different mobility of the PilA1-StrepII protein in dependence on its redox state. Oxidized form (column 1) and reduced form (column 2).

The sequence of the PilA1-StrepII protein contains only two cysteines (at positions 142 and 161). To demostrate the presence of a redox sensitive disulphide bond between these two cysteines, AMS labeling of this protein was employed for visualization of –SH groups. In principle, binding of AMS to one or both thiols caused 0.5 or 1 KDa shift in protein mobility on the SDS-PAGE. The purified PilA1-StrepII protein appears to be fully oxidized, so I first partly reduced it through incubation with the selective sulphydryl reductant TCEP to obtain a mix of both oxidized and reduced forms of the protein. If the fast migrating form of protein contains a disuphide bridge, the absence of thiol groups should prevent it from reacting with AMS, whereas reduced protein should migrate slower after AMS binding to reactive -SH groups. The result of labeling corresponds to this expectation implying that the purified PilA1-StrepII protein possesses a disulphide bond (Figure 4.18).

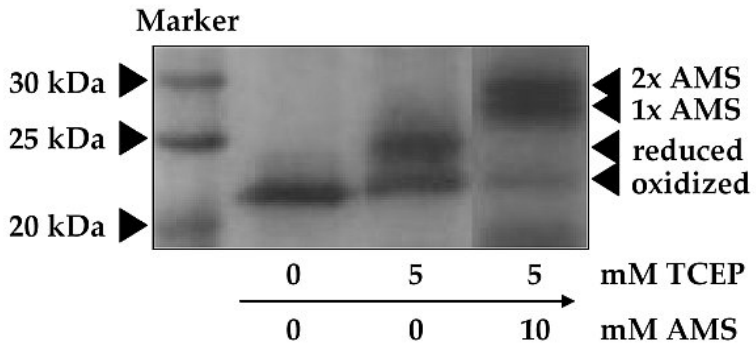

Figure 4.18. The AMS labeling of the PilA1-StrepII protein. The fully oxidized PilA1-StrepII protein (line 1) was partly reduced after the TCEP treatment (line 2). Binding of AMS to one or both thiols caused shift in the mobility of the reduced protein form (line 3). SYPRO stained SDS-PAGE.

5. DISCUSSION

5.1 The GT strain as a tool for studies of the pili functions

The non-motile GT strain of *Synechocystis* was used as the wild type in this project. This GT strain is used as a model organism for studying photosynthesis. In contrast, the original PCC strain is used for examination of pili and motility. As many *pil* mutants are non-motile, I decided to use the GT strain because I was not focused on motility but in other proposed functions of pili or PilA proteins in cyanobacteria. Finally, use of the non-motile strain led to the fortuitous discover of an improved sensitivity to high light of the *pilA* mutants in comparison to the original GT strain. Non-motile strains often lack pili (Bhaya *et al.*, 2000), but I demonstrated that the non-motile GT strain possesses similar pili structures as the motile PCC strain. All experiments should be repeated with the same *pilA* mutants prepared in the motile PCC background in order to strengthen my preliminary conclusions about PilA proteins function in cell aggregation, pigment accumulation or high light acclimatization.

5.2 Cell aggregation

The phenotype of the *pilA1/A2* and *pilA1/A2/A4* mutant strains differed in a number of characteristics from the GT strain. The most distinct changes were the aggregation and altered levels of photosynthetic pigments. Strains formed domed colonies, cells in colonies appeared to be more tightly aggregated than the parent strain and it was difficult to collect those cells from agar plates with a loop. Cells tended to form large clumps when grown in liquid BG11.

I observed the same aggregation phenotype in the *pilA1/A2* and *pilA1/A2/A4* mutants as was already published for the *pilA1* and *pilA1/A2* mutants (PCC strain) by Yoshihara *et al.* (2001). In addition, Yoshihara *et al.* (2001) reported that the *pilA4* mutant did not aggregate or form domed colonies, which is also in agreement with my data. I would speculate that *pilA1/A2* genes are responsible for avoiding aggregation. So it is possible that the *pilA1/A2* operon is mainly responsible for the formation of cell surface structures which prevent cell aggregation. Some pili, that are still present on the surface of *pilA1/A2*$^-$ cells (Bhaya *et al.*, 1999), could be formed by the PilA4 protein. The presence of PilA4 protein on the cell surface

is not responsible for the aggregation in the absence of PilA1 and PilA2 proteins because the *pilA1/A2/A4*- strain also forms aggregates.

Yoshihara *et al.* (2001) described aggregation under photoautotrophic conditions but I found a much stronger phenotype under photomixotrophic and microaerobic conditions. Reasons for cell aggregation under both conditions could be similar. Glucose strongly downregulated the expression levels of the *pilT* and *pilD* genes in pathogenic bacterium *Clostridium perfringens* and thus completely repressed Tfp gliding motility (Mendez *et al.*, 2008). PilA proteins are processed by the PilD peptidase so the glucose could indirectly influence pili formation and consequently their functions. In other words, glucose possibly caused low level of all Pil proteins which could explain stronger phenotype of *pilA1/A2* mutant under photomixotrophic growth. Another possibility could be that highly reducing conditions under both photomixotrophic and microaerobic conditions may be somehow related to the formation of the disulphide bridge needed for the proper Pil proteins function (see chapter 5.7).

I detected that the medium of the *pilA1/A2* double mutant was highly viscous under microaerobic conditions. This finding suggests imply secretion of some compound(s) into the growth medium. Bhaya *et al.* (1999) investigated the *sigF* mutant phenotype, which has no detectable *pilA1/A2* mRNA and does not form pili, and found that this strain released yellowish-brown pigments and a glycoprotein into the growth medium. Thus, it is possible that the *pilA1/A2* mutant produces similar glycoprotein under microaerobic conditions and the nature of the secreted compound will be characterized in the future.

5.3 The accumulation of pigments in *pilA* mutants

The effect of *pilA* gene deletion on pigment levels appears to be quite complex. The *pilA1* and *pilA1/A2* mutants bleached (yellow-green) after prolonged period on plates (Bhaya *et al.*, 2000). I also observed such bleaching (yellow-brown-green) in my *pilA1/A2* and *pilA1/A2/A4* mutants. This bleaching may be connected to pigment composition.

The *pilA* mutants had lower levels of carotenoids and higher levels of chlorophyll under photoautotrophic conditions. This result is in line with accumulating evidences that there is a link between pigment metabolism and PilA proteins (He and Vermaas, 1999; Singh *et al.*, 2005). There is no clue how the PilA proteins could be implicated in the chlorophyll metabolism. For example the deletion of *pilA1/A2* genes had stronger effect on

the chlorophyll biosynthesis rate during the early stages of greening than deletion of all three *pilA1/A2/A4* genes (He and Vermaas, 1999). In spite of PilA1, PilA2, and PilA4 proteins do not have significant similarity to any known chlorophyll-binding proteins, PilA1 may be a part of a chlorophyll-protein complex (He and Vermaas, 1999). Another report indicates that the IsiA protein and the products of the *sll1693*, *pilA1*, *pilA2*, and *sll1696* gene cluster could play an inter-related role in the storage or assembly of chlorophyll intended for pigment-protein complexes as these proteins partially compensate for loss of IsiA in the protection of PSII (Singh *et al.*, 2005).

The discovery of high-light sensitivity and inhibition of the carotenoid biosynthesis appears contradictory. The *pilA* mutants are more tolerant to high light but more sensitive to inhibition of the carotenoid biosynthesis in comparison to the GT. A possible explication is that the *pilA* mutants have very low levels of phototoxic compound(s) *e.g.* intermediate of chlorophyll/heme biosynthesis, which helps them to grow under high light intensities. Such compound could be also a signal for carotenoid accumulation. When carotenoid biosynthesis is blocked by fluridone, carotenoid content drops down below a threshold needed for the cell viability. However, the carotenoid content is sufficient for growth of the *pilA* mutants under high light, as these strains are 'deficient' for a putative highly phototoxic compound.

5.4 Expression of the PilA1-StrepII in the *pilA* mutants

The fact that introduction of the PilA1-StrepII protein at least partly complements the *pilA1* gene deletion as it is apparent from the aggregation phenotype. In contrast to the *pilA1/A2* mutant, the same strain expressing the PilA1-StrepII protein did not aggregate under autotrophic or microaerobic conditions. The absorption spectra of strains expressing the PilA1-StrepII protein were also more similar to spectra of the GT than to *pilA1/A2*$^{-}$ and *pilA1/A2/A4*$^{-}$. In contrast, some data suggest that the PilA1-StrepII protein did not fully complemented *pilA1/A2* mutant phenotype. For example the *pilA1/A2*$^{-}$*/pilA1-StrepII* mutant is more resistant to high light than the GT strain. One possibility could be that the PilA2 protein has a different function than PilA1 which can not be complemented by the PilA1-StrepII protein. The PilA2 protein function is unknown. The only known phenotype of the *pilA2* mutant is lowered transformation competency (Yoshihara *et al.*, 2001). I can not also exclude that the StrepII tag amino acid sequence affects PilA1 protein

functionality. The StrepII tag is localized at the C-terminus of the protein, which is critical for many protein interactions (Craig *et al.*, 2004). However, localization of the StrepII tag at the N-terminus of PilA1 is not possible due to protein modification.

An interesting example of how a peptide tag can influence PilA protein character was described at *Pseudomonas stutzeri* (Graupner *et al.*, 2001). A twitching motility, transformation, and pili deficient *pilAI* mutant was not restored by a plasmid expressing the PilAI protein with a His-tag added to the C-terminus. However, when the six C-terminal amino acids of the PilAI protein were replaced by six histidines, transformability was restored. However, the resulting mutant remained non-motile and did not form pili. The *pilT* mutant was also defect in motility and transformability and when the PilAI with six C-terminal amino acids replaced by six histidines was expressed in the *pilAI/pilT* mutant the transformation of the strain was restored. Thus, introduction of the PilAI protein with modified C-terminus allowed transformation of an 'untransformable' *pilT* mutant suggesting a new, *pilT* independent, DNA uptake (Graupner *et al.*, 2001).

5.5 Localization of the PilA1 protein

The PilA1 protein is predicted to be a part of the cytoplasmic membrane, but experimental evidences are missing. He and Vermaas (1999) suggest the presence of PilA1 protein in a putative chlorophyll-binding complex. Interestingly, the PilA1 protein migrated close to the D1 protein on 2D electrophoresis, which could imply an interaction of the PilA1 protein with subunits of photosystem II (Komenda, unpublished). The PilA1 and PilB1 proteins were copurified with ScpB, a small high light inducible protein with a chlorophyll-binding motif (Kufryk *et al.*, 2007). However, it is possible that this finding is the result of contamination. The localization of proteins encoded by *sll1693*, *pilA1*, *pilA2*, *sll1696*, and *isiA* genes could also implicate the interaction of cytoplasmic and thylakoid membrane during the photosystem II complex assembly (Singh *et al.*, 2005).

Is is not easy to confirm the presence of PilA1 in thylakoid membrane due to difficulties in preparation of a 'pure´ thylakoid membrane. It will be needed to employ new methods for isolation of pure cytoplasmic and thylakoid membrane (*e.g.* Srivastava *et al.*, 2005), that should confirm or refute theories about the occurrence and function of PilA proteins in the thylakoid membrane.

5.6 Purification of the PilA1 protein

Purification of membrane proteins is generally difficult and often requires laborious testing of different buffer conditions and detergents. This is the case of the PilA1-StrepII protein as the resins used during purification are not compatible with commonly used detergents for the solubilization of membrane proteins. I have developed a completely new protocol for the purification of membrane proteins under native conditions using the StrepII tag and decylmaltoside detergent. I received very pure eluate with minimal amount of contaminating proteins using this method. In addition, another protein visible only in the SYPRO stain was copurified with the PilA1-StrepII protein (Figure 4.16). This unknown protein might be another PilA or a protein involved in the general secretory pathway protein because the PilA protein in *Pseudomonas aeruginosa* is isolated as a homodimer or heterodimer mixed with the general secretory pathway proteins (Nunn, 1999).

5.7 Disulphide bond in the PilA1 protein

The disulphide bond in the PilA protein in cyanobacteria has not been analyzed yet. In pathogens, pilins share a carboxy-terminal disulphide bond essential for pilus assembly (Craig *et al.*, 2004). Both cysteines are important for binding to human cells in *Pseudomonas aeruginosa* and can not be replaced by another amino acid. Pilins always include one or two disulphide bonds in their structure. The pilus binding domain of epithelial cells contains a cycteine-cysteine bridge, which is a conserved antigenic determinant (Craig *et al.*, 2004; Strom and Lory, 1993). I found a disulphide bond in the PilA1 protein from *Synechocystis*. The importance of the disulphide bond in the PilA1 protein of *Synechocystis* could be further tested using mutants where cysteine is replaced by glycine or serine. Sequences of all eleven PilA proteins in *Synechocystis* (except for PilA3 that I referred before it is not real PilA protein, see chapter 1.3) contain even number of cysteines, mostly at its C-terminus. PilA1, PilA2, PilA5, and PilA10 proteins contain two cysteines; PilA4, PilA6, pilA7, and PilA11 contain four cysteines; and PilA8 and PilA9 contain six cysteines. The disulphide bonds seem to be highly conserved not only in PilA proteins but also in other Pil proteins. The PilD protein in 11 of 13 different bacteria species including the *Synechocystis* contains a cluster of cysteines arranged in two pair motif with the cysteine pair separated by two amino acids and with each pair separated by 21 amino acids (C-X-X-C-X_{21}-C-X-X-C) (Lory and Strom, 1997).

6. CONCLUSION

My results show that the type IV pili apparatus in cyanobacteria is much more complex than could be generally expected. The PilA proteins were shown to function directly or indirectly also in cell aggregation and photosynthetic pigment metabolism. These functions are consistent with those already described; directed movement towards light is regulated by phototaxis, which cooperates with photosynthesis. Aggregation is a self-protecting process related to photoprotection and thus modulates pigment composition.

The modification of the PilA1 protein by the StrepII tag addition and its successful purification provides practical information useful also for purification of other proteins from *Synechocystis*. The construct of a mutant strain expressing the modified PilA1 protein with a detectable tag will allow further research efforts to determine the relationship between pili and protein secretion machinery, pili visualization using transmission electron microscopy, and a detail localization of the PilA proteins in photosynthetic cells.

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