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Bachelor Thesis

Isolation and characterisation of light harvesting complexes from bacteria of the genus *Gemmatimonas*

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Bachelor thesis

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Annotation

Photosynthetic bacteria are highly abundant. The isolation and characterization of the light-harvesting complex from the *Gemmatimonas phototrophica* AP64^{T} which belongs to the seventh phylum of the evolutionary important (bacterio)chlorophyll-containing bacteria are reported here. The purification of the complex was achieved by gel-filtration and electrophoresis. HPLC was used for identification of the pigments. The protein subunits were further identified by MS analysis.

Affirmation

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1. Introduction

1.1 Photosynthesis

Photosynthesis is a process where light energy is captured and converted into chemical energy which drives the synthesis of organic compounds necessary for life. The whole process appeared early in Earth's history and influenced its whole surrounding system. The early stages of photosynthesis were undeniably anoxygenic (non-oxygen evolving). Approximately 2.4 billion years ago massive amount of atmospheric oxygen was released by Cyanobacteria that lead to a new modification of chain electron transport that slowly developed into advanced photosynthetic cells [1]. The discoveries in biochemical processes in photosynthesis contributed to identification of the products of this process, ATP and NADPH, as common compounds for a huge amount of metabolic reactions within the photosynthetic cells. NADP is reduced by protons and electrons from water in the last reaction of the electron transport chain of photosynthesis, forming NADPH [2]. ATP is formed in a process called photophosphorylation, where energy of the proton gradient established across the membrane thanks to the earlier reactions of photosynthesis is used for addition of the phosphate group to adenosine diphosphate (ADP).

Photosynthesis consists of two phases. The *light phase* covers the absorption of light and conversion to chemical energy in process of oxidation of the electron donor, reduction of NADP to NADPH, and phosphorylation of ADP into ATP. Consequently, the *dark phase* covers the reduction of CO_2 and synthesis of carbohydrates and other organic molecules [3]**.** The general equation of photosynthesis is presented by the following expression (1).

$$CO_2 + 2H_2A \xrightarrow{\text{photons}} [CH_2O] + 2A + H_2O \qquad (1)$$

H_2A represents the electron donor (water is used as an electron donor in oxygenic photosynthesis) and A is the oxidized electron donor (2A is replaced by O_2 in oxygenic photosynthesis). In anoxygenic photosyntheis, the electron donor can be an inorganic hydrogen (H_2S), where A would be the elemental sulphur.

1.2 Photosynthesis in bacteria

Bacterial photosynthesis is rather diverse, differences appearing in the source of electrons, electron transport chain, amount of reaction centers (RCs) used etc.

There are seven groups of chlorophototrophic bacteria, i.e those containing BChl-based photosynthetic reaction centers (RCs) [4]. They are divided into seven groups: Cyanobacteria, Proteobacteria, Chlorobi, Chloroflexi, Firmicutes, Acidobacteria and Gemmatimonadetes. Chlorophototrops that do not require oxygen are: Proteobacteria, Chlorobi, Chloroflexi and Firmicutes [5]. There are species of Acidobacteria that favour semiaerobic as well as aerobic conditions [6,7]. Another aerobic group of chlorophototrophic bacteria, whose members even evolve oxygen, are the Cyanobacteria [8]. However, the knowledge of the newly identified photosyntetic species of Gemmatimonadetes about their genetic and physiological characteristics is sparse, which was the motivation of this study.

According to the terminal acceptors of the electrons the RCs can be divided into two groups: type 1 of RC using Fe-S clusters and type 2 of RC using quinones. The reaction centers for each bacteria phylum is presented in Table 1. The species that use only type 1 RC are Chlorobi, Firmicutes and Acidobacteria; those with type 2 RC are Chloroflexi, Proteobacteria and Gemmatimonadetes [4]. Some species have both type 1 and type 2 reaction centers which are oxygenic cyanobacteria and their descendants (all photosynthetic eukaryotes) [4]. However, despite the analogous properties of both RCs, the question whether these reaction centers originate from a common ancestor is still evasive [9].

RC1	RC2
Chlorobi	Chloroflexi
Firmicutes	Proteobacteria
Acidobacteria	Gemmatimonadetes
Cyanobacteria	

Table 1: Types of reaction centers in each bacterial phyla.

While the variability of RCs is rather low and the structure conserved among various phyla of photosynthetic organisms, the opposite is true about the light harvesting complexes (also called antenna complexes), whose task is to collect enough photons and transfer their energy to the reaction centers. Thus, the aim of this thesis is to explore if the recently described members of the phylum Gemmatimonadetes add to this variability, potentially revealing new light harvesting strategies of Nature.

1.3 Bacterial phylum *Gemmatimonadetes*

The phototrophic bacteria that belong to the phylum Gemmatimonadates have been very little studied. This rod shaped Gram-negative bacterium was discovered in 2003 and the first cultivated strain was named *Gemmatimonas aurantiaca* $T27^T$ [10]. The 16S rRNA showed that Gemmatimonadetes are widely spread among the microbial flora in soils ranging from 0.2% to 6.5% [11]. Other sources revealed that Gemmatimonadetes are found in different environments such as sludge wastewater [10], soils [11] and lake water [12]. However due to the abundance in many different natural environments many of representative species of this phylum have not been cultivated yet, therefore there is an incomplete physiological description.

Until now only two genomes, i.e *Gemmatimonas aurantiaca* $T27^T$ isolated from semi-aerobic wastewater reactor and *Gemmatimonas phototrophica* $AP64^T$ from freshwater Swan Lake in western Gobi Desert [9,10] have been released. The *Gemmatirosa kalamazoonesis* $KBS708^T$ has been isolated from a wheat-corn-soybean agricultural soil (USA) and it is yet to be analyzed [11]. The 16S rRNA gene sequence showed high similarity of 96.1% identity between *G. aurantiaca* $T27^T$ and *G. phototrophica* $AP64^T$. However, 89% of 16S rRNA identity was described between *G. kalamazoonezis* $KBS708^T$ and *G. aurantiaca* $T27^T$, respectively.

1.4 *Gemmatimonas phototrophica* $AP64^T$

In this work we are focused on the Gram Negative bacterium *Gemmatimonas phototrophica* $AP64^T$. Described as a red-pigmented and rod-shaped species, this BChl-a containing bacterium is 1～6 μm long and 0.3-0.5 μm wide (Figure 3). The difference between the $AP64^T$ and the other representative in this phylum (G. *aurantiaca* $T27^T$) is the apparent presence of the fully functional RC of type 2 [4].

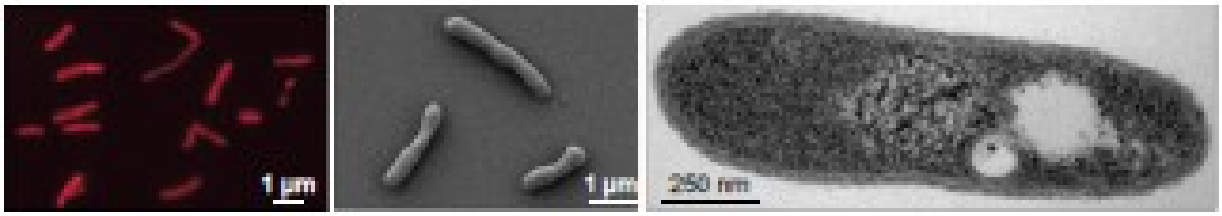

Figure 3: Images of *G. phototrophica* AP64. The left image is under red-infrared fluorescence microscope. In the center, scanning electron microscopy (SEM). The right, transmission electron microscopy (TEM) [4].

1.5 Photosynthetic pigments and pigment-protein complexes

The absorption of light by the photosynthetic organisms can not be achieved without the presence of pigment molecules. There is an immense number of pigments present that supply the light-harvesting process. Generally they can be divided into 2 main groups. The first group is the magnesium containing bacterio-chlorophyll (B)Chl (typical example). The second group is represented by terpenoids which are represented by the most important carotenoids [13].

Carotenoids are highly abundant in nature. Most carotenoids are linear molecules, contatinig 40 carbon atoms, and are divided according to the oxygen content into two groups: xanthophylls (oxygen present) and carotenes (no oxygen). There are two most essential roles of the carotenoids. They serve as light-harvesting pigments mainly in the blue-green spectral range, where chlorophylls (B)Chl have low absorption, and they protect the organism from the damaging photo reactions that takes place in the presence of oxygen, i.e photoprotection [14]. Regarding the light-harvesting role, the direction of the energy flow is described by the so called funnel effect, which is practically explained by the energy conservation law (Figure 4). The energy is transferred from higher-energy (shorter-wavelength) absorbing carotenoids to lower-energy (longer-wavelength) absorbing (B)Chls.

The photophysics of carotenoid excited states can be explained by the energy level diagram (Figure 5). The absorption of a photon promotes the molecule from the S_0 (ground state) to the second excited S_2 state. Steady-state absorption spectrum in the visible spectral range refers to this transition. The S_0-S_1 transition is forbidden due to the carotenoid symmetry. The lifetimes of S_2 and S_1 are incredibly short (hundreds of femtoseconds to tens of picoseconds). Consequently, the energy transfer process must proceed on an even shorter time scale because both excited states serve as energy donors to (B)Chls [15,16].

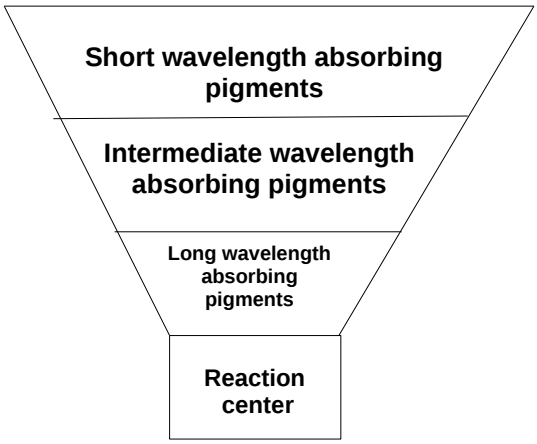

Figure 4: The funnel effect in a photosynthetic antenna complex.

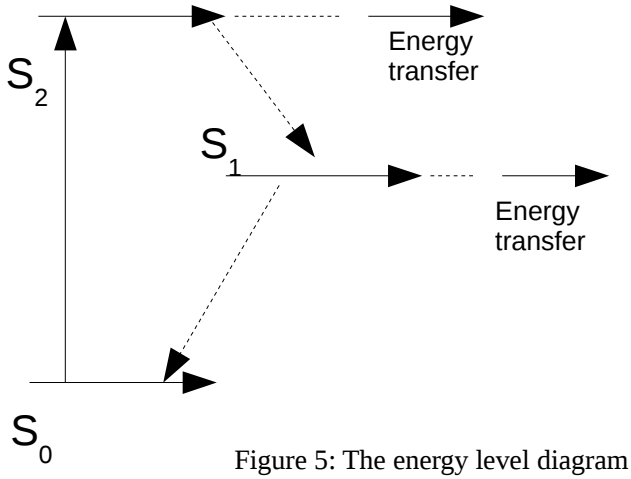

Figure 5: The energy level diagram of a carotenoid.

The major light absorbing pigments of non-cyanobacterial prokaryotic phototrophs, carotenoids and BChls, are non-covalently attached to the pigment-protein complexes (RCs and antenna complexes), which are all placed in the intracytoplasmic membrane (ICM). Altogether this pigment-protein complex is known as the photosynthetic unit (PSU). [17]. The reaction center consists of several proteins and pigments that conduct the energy conversion reactions of photosynthesis. The RC is usually constructed from three protein subunits: L (light), M (medium), and H (heavy). The L and M subunits provide the frame for the chromophors, while subunit H is exposed to the intracellular domain [18]. The reaction center (RC) of the thoroughly-studied Proteobacteria is surrounded by the light-harvesting complex 1 (LH1). Some species contain a second antenna complex (LH2) and the ratio of LH2/LH1 can change [19]. The LH1 and LH2 complexes are both integral membrane antennas.

Generally, the structure of these complexes can be described as oligomers with α and β subunits each containing 2 BChl molecules and 1-2 carotenoid molecules. The LH1 complex surrounds the RC [19]. The structure of LH2 complex is similar to LH1 (ring like structure, oligomer of alpha and beta subunits), but they differ spectroscopically. The upper closely packed molecules in LH2 exhibit absorption of 850 nm (B850) (green subunits in Figure 6, Panel A), while the loosely packed BChls absorb at 800 nm (B800) (blue subunits in Figure 6, Panel A) [20,21]. Due to the closely interacting pigments, LH1 typically has strong near-infrared absorption band around 875 nm (B875). Some other types of bacteria contain a third type of antenna complex (LH3) with absorption 800 and 820 nm [20]. The LH1 complex transfers the energy directly to the RC with the support from LH2 (LH3) rings (Figure 6).

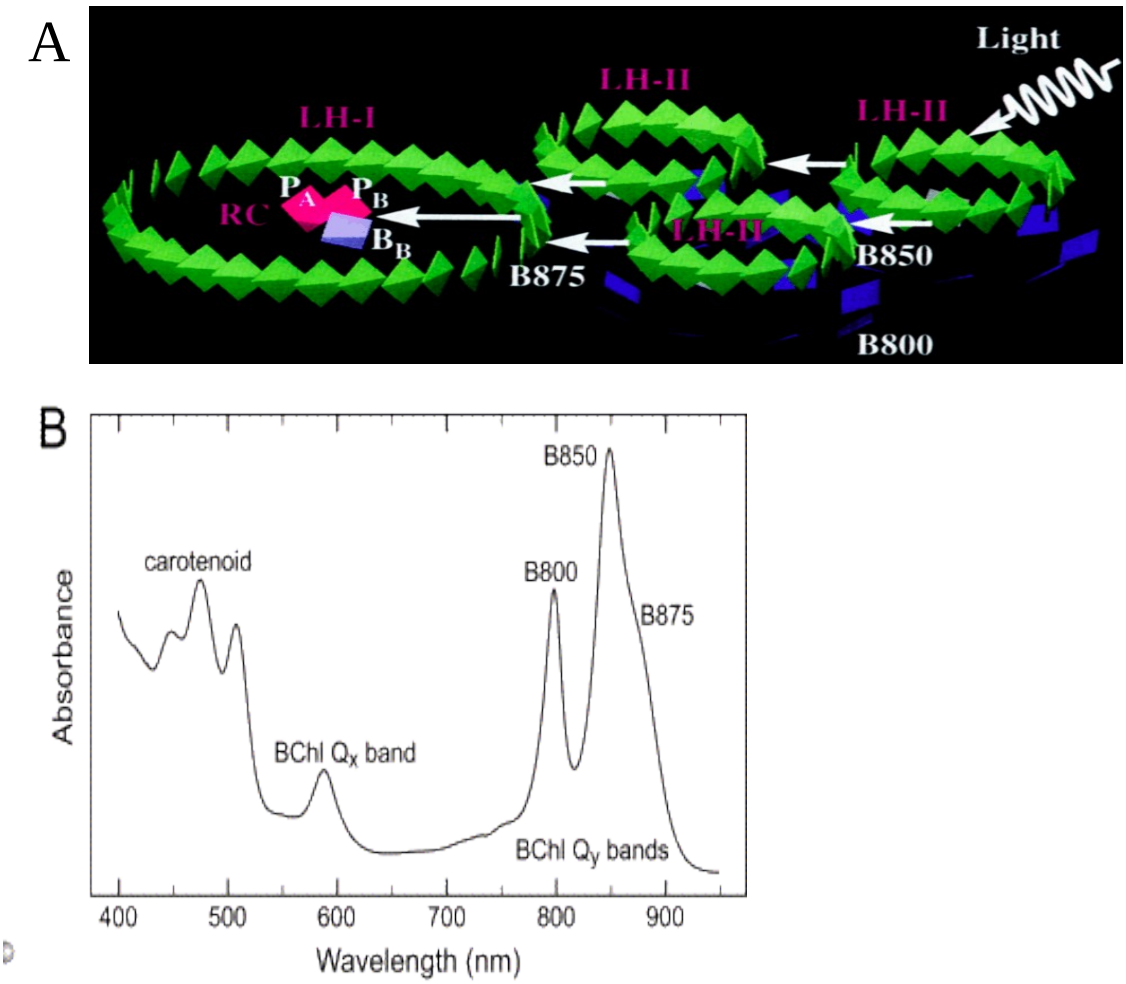

Figure 6. Panel A: Structure and an energy flow in the PSU of *Rba. sphaeroides* [22]. Panel B: Absorption spectrum of photosynthetic membranes from *Rb. Sphaeroides* [23].

1.6 Purpose and objective

This work is focused on the isolation and characterization of the light-harvesting complexes from *Gemmatimonas phototrophica* AP64^T, as well as the optimization of the cultivation process with the aim of growing a larger amount of bacteria necessary for potential future measurements (CD spectroscopy, femtosecond time-resolved spectroscopy).

2. Methods

In this section a general introduction of the methods and a detailed description of the procedure are presented.

2.1 Cell cultivation and growth conditions

The strain AP64^T was grown on R_2A agar plates under optimized semiaerobic conditions at the pressure of 200 mbar under 24-h light regimen. The media pH was adjusted to 8, due to the alkaline conditions in the natural environment [4]. The formation of the red-pigmented colonies occurred after 2-4 weeks at an optimum temperature of 25 °C.

To identify the presence of the positive colonies, the BChl-containing AP64^T was checked by a custom made NIR imaging system as described before [4].

R_2A medium	Amount
Yeast extract	0.5 g
Proteose peptone	0.5 g
Glucose	0.5 g
Soluble starch	0.5 g
Na-pyruvate	0.3 g
K_2HPO_4	0.3 g
$MgSO_4 \cdot 7H_2O$	0.05 g
Distilled water	1 L

Table 2: Composition of the growth medium for strain AP64^T.

2.2 Pigment analysis by liquid chromatography

The pigment composition was analyzed by using reverse phase high-performance liquid chromatography (RP-HPLC) as described before [31]. This HPLC is an efficient and fast way of separating the analytes. The working principle lays in the hydrophobic interactions of the analyte with the stationary phase, which makes it quite efficient in a comparison with classical chromatography methods. The method was used to compare the pigment content of the newly grown culture with the one published in a recent paper [4].

Detailed description of the procedure

The cells were scrapped from the plates and then washed twice with buffer A, containing 50 mM Tris, pH 8, 1 mM EDTA, 50mM NaCl. The collected solution was centrifuged (10 000 × g). Subsequently, extraction with 1ml of Acetone/Methanol (7:2, vol/vol) was performed followed by another centrifugation and the supernatant was analyzed by Shimadzu® Prominence-i LC2030C 3D HPLC (column type, Luna® 3 µm C8(2) 100 Å, LC Column 150 x 4.6 mm, flow rate of 0.8 ml/min, column temperature of 40°C). The sample was automatically injected into the column.

The purpose of this experiment was to analyze the pigment composition of the membranes and compare it with previously reported results [4]. Two forms of BChl-a were identified which contain geranyl-geranyl(1) or phytol(2) ester chain (Figure 7). In addition, AP64^T appeared to contain several main carotenoids. The presence of the putative (2S,2'S)-oscillol 2,2'-di-(alpha-l-rhamnoside) (3,4), spirilloxanthin(9) and some other non-defined carotenoids, which are presumed to be derivatives of spririlloxanthin and oscillol(5-8), was confirmed (Figure 7) [9,24]. The results were not significantly different compared to the previous study [4].

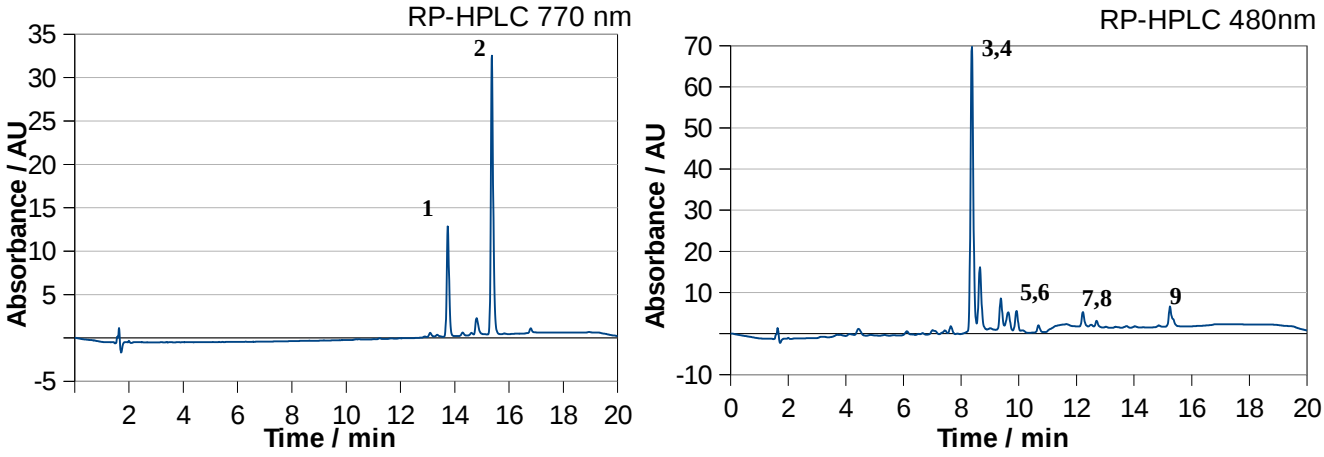

Figure 7: Left: RP-HPLC elution profile for BChl-a at 770 nm. Right: RP-HPLC elution profile for carotenoids at 480 nm from strain *G. phototrophica* AP64^T. 1: BChl with geranyl-geranyl side chain, 2: BChl-a with phytol, 3,4: (2S,2' S)-oscillol 2,2'-di-(alpha-l-rhamnoside), 5-8: unknown carotenoids, 9: spririlloxanthin.

2.3 Spectrophotometry

This method is used to study the absorption of light by molecules in the sample. When light is absorbed by a sample, the intensity of the light is decreased. The basis of the method can be explained by the Beer-Lambert's law by using the following expression (2):

$$A = \ln (I_0 / I) = \epsilon\, l\, c \qquad (2)$$

where A is the absorbance, I_0 and I are the intensity of incident and transmitted light, ϵ is the molar absorptivity (L mol^{-1} cm^{-1}), *l* is the pathlength (cm) and *c* is the concentration (mol/L).
This method was used in this work for many purposes, e.g basic characterization of the cells *in vivo* and in particular, the spectrometer type was Shimadzu® UV-2700 with double monochromator.

2.4 Membrane isolation

The scrapped cells were resuspended in the buffer A as previosly described. Additionally, the cells were broken by vortex with an addition of glass beads (Ballotini, SIGMA®). The cell disruption cycles were interrupted by incubation for 60 s on ice to avoid heating of the sample. The sample was suspended in 0.5 ml of buffer A, PMSF (phenylmethylsulfonyl fluoride) was added to final concentration of 1 mM to inhibit protease activity. Unbroken cells were pelleted by low-speed centrifugation (2 500 × g). The absorption spectrum of the membranes was recorded as described before [4].

2.5 Gel filtration

The gel acts as a molecular sieve which separates molecules according to their size and shape. The suspension moves through porous beads. Small molecules enter the pores, therefore moving through the beads more slowly, while larger molecules cannot enter the pores, so they move faster. The main purpose of this method was to purify the sample by separating the light-harvesting complex for further analysis.

Detailed description of the procedure

The membrane containing sample was mixed with 13.3 µl of n-Dodecyl β-D -maltoside (DDM) (15%) and centrifuged for 10 min (18 000 × g). For a comparison, second attempt was with a similar procedure differing in addition of 2% of Triton. The protein samples were separated by using Agilent® 1200 Series Liquid Chromatography at a flow rate of 0.2 µl and column temperature of 15°C. The column type, Yarra® 3 µm SEC-3000 290 Å, LC Column 300 x 7.8 mm, was used. In addition the system was equipped with diode array detectors.

2.6 Native Gel Electrophoresis

Gel electrophoresis enables separation of charged molecules in an electric field based on their mass, charge, and structure. The native electrophoresis is carried out in a polyacrylamide gel electrophoresis (PAGE). There are two distinguished types of native electrophoresis [25]. "Blue native electrophoresis" (BN-PAGE) involves binding of proteins with Coomasie brilliant blue G-250. This anionic dye provides the charge to the proteins but sometimes can lead to undesirable dissociation [25,26]. The "colorless-native electrophoresis" (CN-PAGE) is used for both water soluble and membrane proteins (pI < 5.4), and is suitable for further analysis, which gives advantage over BN-PAGE [27]. There are other types of native electrophoresis developed for higher resolution separation and analysis of membrane proteins i.e high-resolution clear native electrophoresis (hrCN-PAGE) [28].

Detailed description of the procedure

In this work, CN-PAGE (Biorad® 1 mm, 16 cm) was used for dissociation of the protein complexes. The sample was resuspended in buffer B containing: 25mM MES/NaOH, pH 6.5, 10mM $MgCl_2$, 10mM $CaCl_2$ and 25% glycerol. The buffer B was supplemented with 10% (DDM) in water [w/v] and water was added to get sample volume/DDM = 10. The sample was gently mixed by hand and spined down (18 000 × g, 10 min, 4°C). The supernatant was loaded into the well. The separation was carried out at 4°C. The 1.5 mm thick gel was prepared and included 4% bis-Acrylamide (4% Upper gel) for stacking and 4-14% for gradient gel (Concentration gel with decreasing pore size). The gel buffer(C) contained 3M aminocaproic acid (ACA), 300 mM Bis-Tris/HCl, pH 7. The acrylamide solution was prepared with 50% acrylic acid (AA) and 1.33% bis-Acrylamide (BIS). The electrophoresis was run by using cathode buffer (0.5M Tricine, 150mM Bis-Tris/HCl, pH 7)

and anode buffer (0.5M Bis-Tris/HCl, pH 7) at 11mA, 1000V for 60 min until the front reached cca. 7 cm from the boundary between the stacking and gradient gel. Bands from CN-PAGE were further separated in the second dimension by SDS-PAGE.

Gel type			
Chemicals	**4%**	**14%**	**4% Upper**
Glycerol	0	3.2 g	0
Water	6.4 ml	6.7 ml	3.7ml
Buffer C	1.4 ml	2.8 ml	0.85ml
AB	0.68 ml	4.8 ml	0.4ml
TEMED	3.5 µl	7 µl	5µl
10% APS	19 µl	38 µl	40µl
Volume	8.5 ml	17 ml	5ml

Table 3: Chemical composition of the Gradient gel (4-14%) and the stacking gel (4%).

2.7 2D-PAGE

2D gel electrophoresis is a powerful technique for separating and analyzing protein mixtures. The procedure consists of two electrophoretic steps, conducted in sequence in perpendicular directions. The protein mixture can thus be separated based on two different properties, increasing the separation specificity. A typical 2D-PAGE is conducted under native conditions in one dimension (see above) and under denaturating conditions in the other, which was used also in our study. As a denaturating agent serves the detergent sodium dodecyl sulfate (SDS), which provides a uniform negative charge to the proteins. In the second dimension step, the molecules are separated according to their molecular weight/size.

Detailed description of the procedure

In this work 2D electrophoresis was used for separating the pigment-protein complexes. After the CN-PAGE, single lines were cut and incubated in 3 ml 25mM Tris/HCl, pH 7.5 containing 1% SDS, 120 mg dithiothreitol (DTT) for 20 min at room temperature. Gradient gel (12-20%) was prepared with a thickness of 1.5 mm. The gel buffer (D) contained 2.8M Tris/HCl, pH 8.6. The acrylamide solution was prepared with 50% acrylic acid (AA) and 1.33% (BIS). The electrophoresis was run by using cathode buffer (250 mM Tris, 1.92M glycin, 2% SDS) and anode buffer (2M Tris/HCl, pH 8.3) at 31mA, 350V for 16 hours. The staining was done by Coomasie brilliant blue G-250. The excess dye integrated in the gel was removed by destaining with the gel buffer (D). The further identification of the proteins was done by Mass Spectrometry (MS).

Gel type			
Chemicals	**12%**	**20%**	**5% Upper**
Sucrose	0	1.8 g	0
Urea	7.7 g	7.7 g	0
Water	4.1 ml	0 ml	6 ml
Buffer D	4.2 ml	4.2 ml	2.1 ml
Acrylamide solution	4.3 µl	7.2 µl	0.9 µl
TEMED	9µl	9µl	9µl
10 % SDS	360 µl	360 µl	180 µl
10 % APS	36 µl	36 µl	36 µl

Table 4: Chemical composition of the Gradient gel (12-20%) and the stacking gel (5%).

Staining solution	**Destaining solution**
0.025 % Coomasie brilliant blue	25% Methanol
25 % Methanol	10 % Acetic acid
10 % Acetic acid	

Table 5: Staining and destaining solutions.

2.8 Mass spectrometry

Mass spectrometer is an analytical tool designed for separating ions based on their mass to charge ratio (m/z), *"z"* being the multiple of elementary charge. It ultimately enables to determine the structural properties of the charged ionized fragment. The basic components of each mass spectrometer are the ionization source, mass analyzer, and detector. In the ionization process ions are formed either by a loss or a gain of charge from a neutral species.

Different ion sources provide different ionization mechanisms. Electron Ionization (EI) and Chemical Ionization (CI) are the most common ionization methods where most scientists are familiar with. However some of the most sophisticated methods used in modern mass spectrometry is the electron spray ionization (ESI) which occurs at atmospheric pressure (outside the vacuum chamber). The reason for using this method in this work is because it forms a protonated molecule and shows a great compatibility with liquid chromatography (LC). In this experiment the isolated proteins from the 2D electrophoresis were analyzed by using LC-MS technique with Waters® Q-Tof Premier™ Mass Spectrometer (Proteomics Laboratory, Faculty of Science, České Budějovice).

This instruments uses Quadrupole and Time of Flight (TOF) mass analyzers coupled with Waters Nano Acquity UPLC (Ultra Performance Liquid Chromatography). The particular reason for the application of UPLC is because it delivers a higher resolution separation in a short time then a regular LC method.

3. Results

The absorption spectrum of the AP64^T membranes is shown in Figure 8. The near-infrared absorption is dominated by BChl-a bands at 819 nm and 866 nm, while in visible mostly carotenoids contribute with absorption peaks at 478, 507, 542 nm, in accordance with ref. [4]. The rather unusually positioned BChl-a near-IR bands raise the question of their origin because they have spectral characteristics of neither typical LH1 nor LH2 complexes as reported in the similar photosynthetic Proteobacteria [9]. To resolve this problem, various methods have been applied, whose results are presented below.

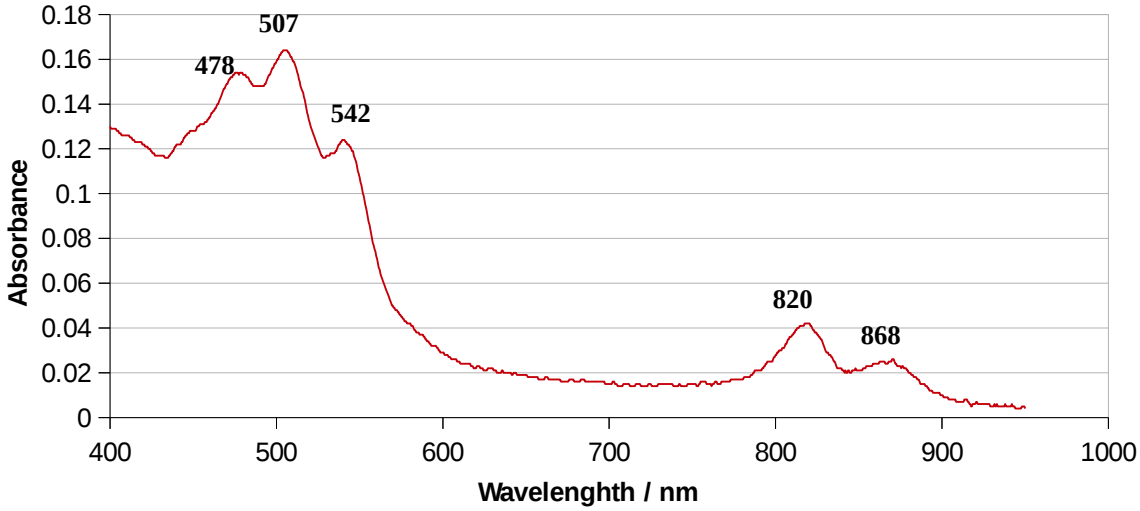

Figure 8: Absorption spectrum of the membranes from *G. phototrophica* AP64^T.

3.1 Gel filtration

The isolation of the antenna complexes continued with solubilization of the membrane with n-dodecyl *β*-D-maltoside (DDM) followed by centrifugation and application to Phenomenex® column. The separation is graphically presented in Figure 9. It shows absorption spectra of the time fractions of the antenna-containing zone. The peak positions are very similar to those obtained from the membranes. The important outcome is that the both BChl (Q_y) bands appear in all fractions, suggesting strong binding of the protein complexes, or association of both spectral forms with a single complex.

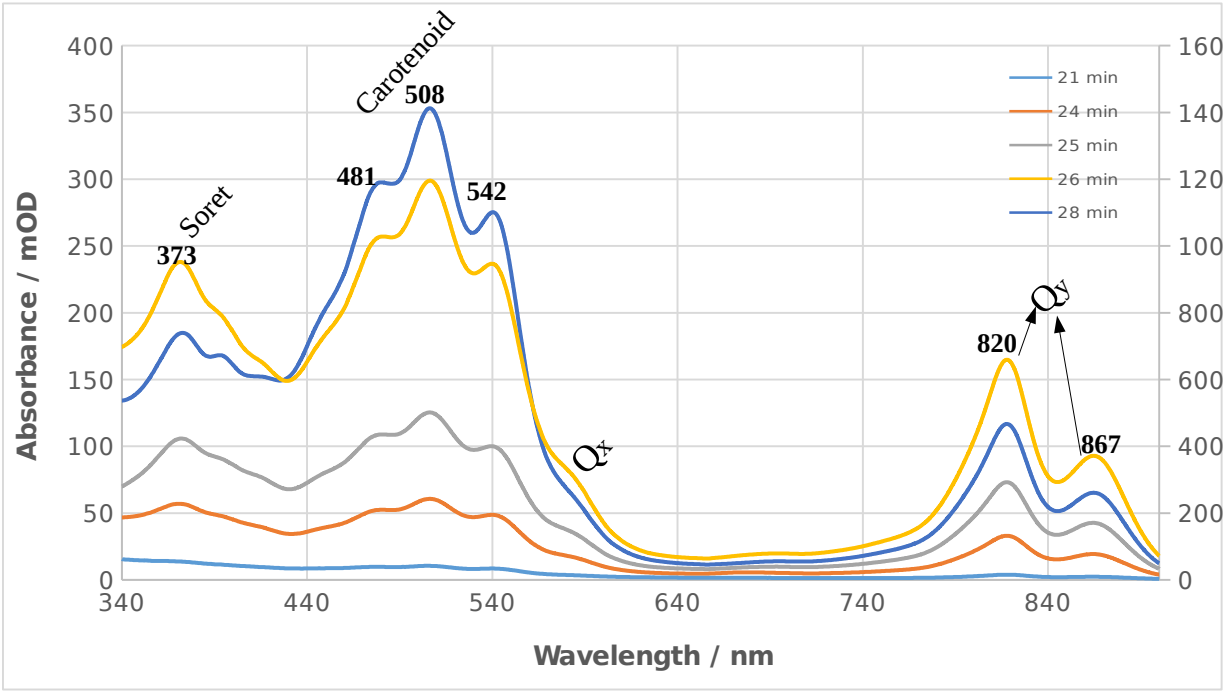

Figure 9: Absorption spectrum of the time fractions after gel filtration from the *G. phototrophica* AP64^T. The "yellow" fraction (26 min) was divided by two. The y-axis (Right) is multiplied by four and is used to scale the last "blue" fraction (28 min).

The strong band at 373 nm appears to be the bacteriochlorophyll Soret peak. The bands between 480 and 540 nm are predominantly from the carotenoids. The small peak around 600 nm is obtained from the BChl (Q_x) and the Q_y peaks appears in the near-infrared region.

The HPLC chromatogram and absorption spectrum from the second attempt of isolation are presented below.

Figure 10 presents the elution profile detected at 480 nm. Subsequently, by using a mixture of stronger detergents, a better separation of the photosynthetc pigments was demonstrated (Figure 11,12). The carotenoid absorption bands between 480 nm and 540 nm appeared (Figure 11). However, in contrast to the previous absorption spectrum (Figure 9, where a weaker detergent was used), two distinct bands were obtained (Figure 10): one protein contained only carotenoids (Figure 11), the other contained the light-harvesting complex (Figure 12).

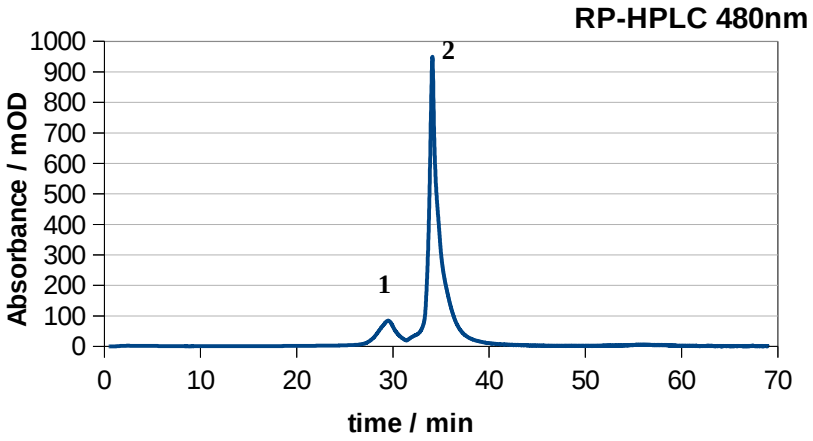

Figure 10: RP-HPLC elution profile from the strain *G. phototrophica* AP64^T after application of DDM (15%) and Triton (2%).

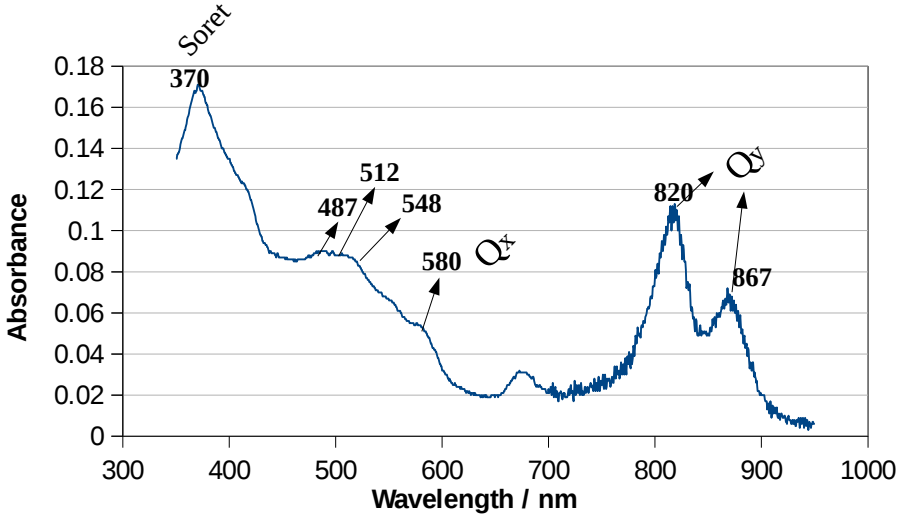

Figure 12: Absorption spectrum of the light-harvesting complex from the strain *G. phototrophica* AP64^T after application of DDM (15%) and Triton (2%) (peak 1, Figure 10).

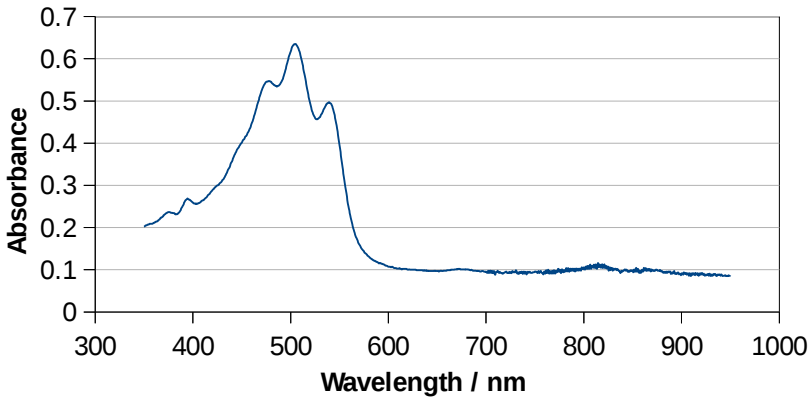

Figure 12: Absorption spectrum of the carotenoid-containing hypothetical protein from the strain *G. phototrophica* AP64^T after application of DDM (15%) and Triton (2%) (peak 2, Figure 10).

3.2 Native electrophoresis

The colorless-native electrophoresis (CN-PAGE) was used to furhter purify the light-harvesting complexes and possibly separate the near-IR Bchl bands, if they arise from different complexes. (Figure 13a). The band contained ~1 MDa antenna-RC complex. The near-IR complex bands were not separated even with this method (Figure 13b).

The lower band appeared to be smaller in size (~0.1 MDa) and was assigned, based on its absorption spectrum, as the carotenoid-only-containing protein (Figure 13c).

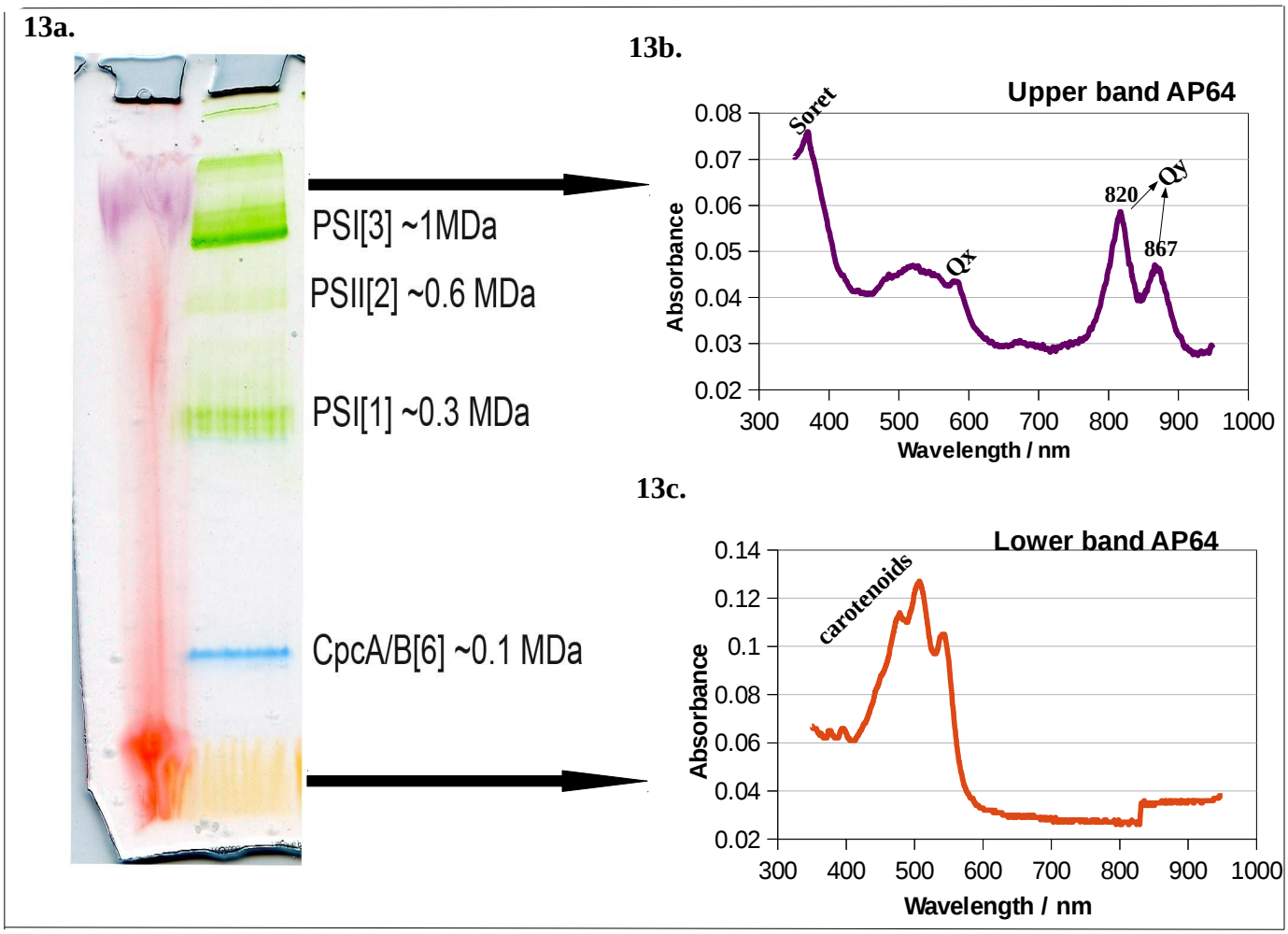

Figure 13: Results of the native gel electrophoresis from the gel filtration from the cells of *G. phototrophica* AP64^{T}. Pigment-protein complexes from cyanobacterium *Synechocystis* sp. PCC6803 are shown in the right line for comparison. 13a: Native electrophoresis gel. 13b: Absorption spectra of the upper band. 13c: Absorption spectra of the lower band with the instrumental artifact above 820 nm.

3.2 2D gel electrophoresis

The protein complexes separated by the first dimension of the native gel electrophoresis were analyzed by 2D SDS-PAGE. After the staining, the Coomassie blue–stained bands were digested with trypsin and identified by mass spectrometry. The antenna band at 7.7 kDa exhibited similarity with α-polypeptides from the LH1 complex [29]. The band at 19 kDa has been identified as a larger subunit of the H subunit protein of the RC, which successively is consisted of two subunits (α and β). The low molecular bands at 5.3 and 5.1 kDa have been identified as polypeptides of novel antenna proteins. Additionally, the large bands with 118, 111, 52.4 kDa were observed. It appears that the complex is large and it can not migrate easily under electrophoresis, thus remains intact. The question remains whether carotenoids from the "orange" band are associated with any of the proteins separated in the second dimension.

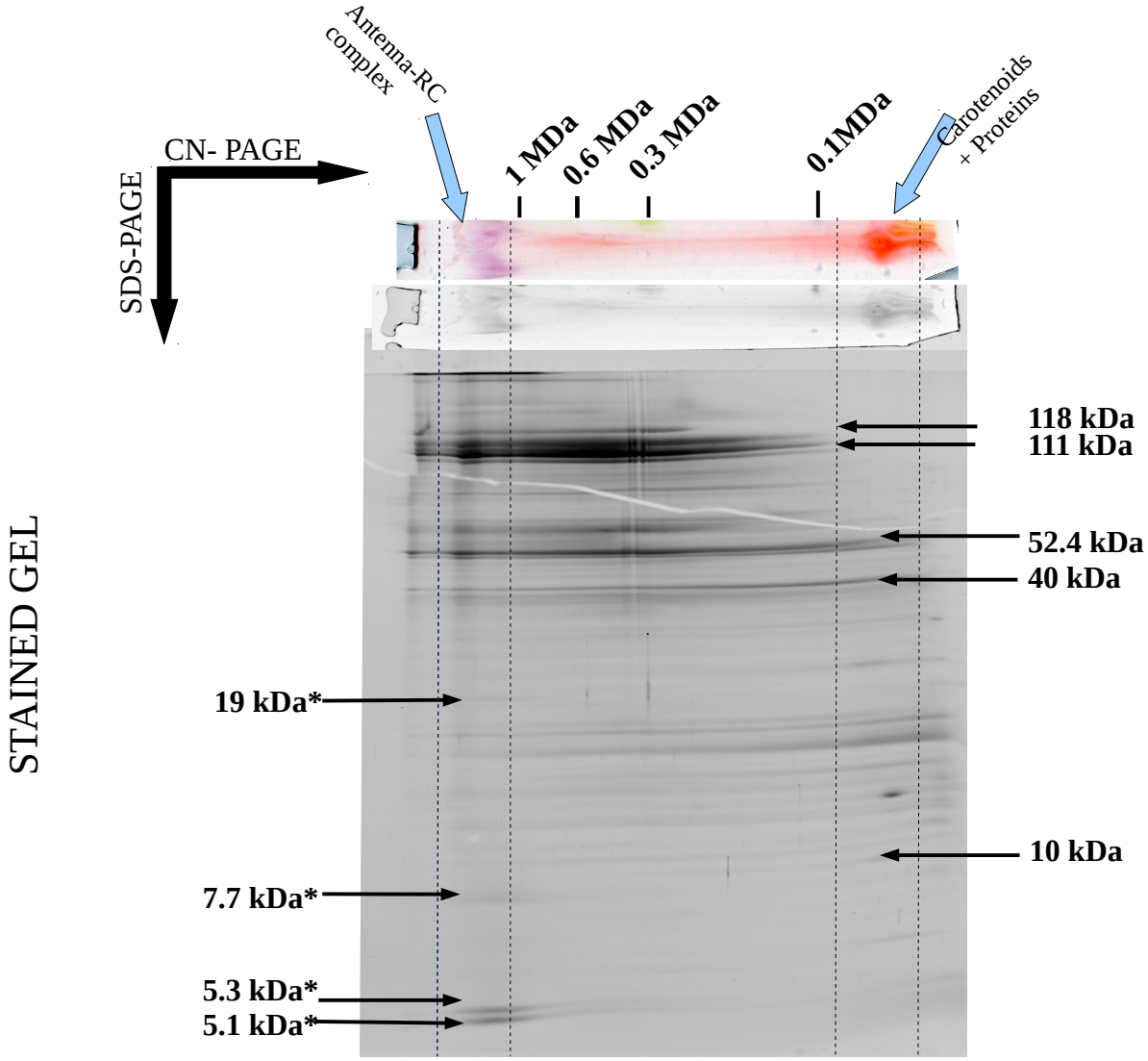

Figure 14: 2D Electrophoresis of the light-harvesting complex of *G. phototrophica* AP64^{T} and molecular size of individual proteins.

3.3 MS-analysis

Individual identification of the protein spots was done by MS. The table 6, presents the precise molecular weight size of the identified proteins, according to their amino acid sequence. A structural homology modeling server for proteins in 3D was used to generate a model based on template search in Blast and HHBlits performed by Swiss Model server.

Gene	Protein	Molecular weight	Model
puh B (1)	Photosynthetic RC, H subunit	7.81 kDa	Figure 15
puf A (α)	Light-harvesting protein B-880, alpha chain	6.85 kDa	Figure 16

Table 6: Description of models built based on the target-template alignment.

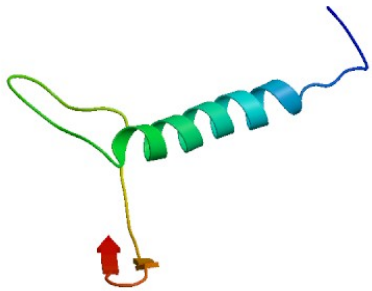

Figure 15:SWISS-MODEL hetero-oligomer display of photosynthetic RC. Puh B, H-subunit (1).

Figure 16: SWISS-MODEL hetero-oligomer display of photosynthetic LH1-like alpha polypeptide.

4. Discussion:

In phototrophic bacteria the light energy necessary for photosynthesis is accumulated by the antenna complex. The proteins and pigments composing the antenna system are organized around photosynthetic reaction center, together forming the fundamental unit of bacterial photosynthesis. In this work, we aim at isolation and characterization of the light-harvesting complex(es) from the bacterium *Gemmatimonas phototrophica* AP64^T.

This round, red-shaped, bacterium was grown under semi-aerobic conditions. All performed experiments were based on the cells grown on agar plates. The slow growing process (2-4 weeks) yielded an optimum amount of culture which was further used for the biochemical and chromatography analysis.

The spectroscopic properties of the isolated membranes as shown (Figure 8) were similar to those of some other species of phototrophic bacteria [30]. However, the position of the near-IR BChl-a Q_y bands is rather unusual, having spectral characteristics typical neither for LH1, nor LH2 complexes. The antenna with the most similar spectral properties is the B808-B866 complex of *Chloroflexus aurantiacus* [31]. The question is thus whether the spectral bands of G. *phototrophica AP64^T* membranes in near-IR come from the same complex (LH1-like), or from more complexes.

Attempts aiming at separation of near-IR bands with both weaker (Figure 9) and stronger detergents (Figure 12) were unsuccessful. From this analysis follows that the two bands arise either from the same complex, or from multiple strongly bound complexes.

To minimize the possibility that more complexes give rise to near-IR bands, gel electrophoresis was applied as another separation method. The result was similar to that when the stronger detergent was used in gel filtration: two main bands were obtained, one with carotenoid-only spectrum, and the other with the spectrum of the light-harvesting complex with both near-IR bands. It thus follows from the conducted analyses that those bands arise from a single complex, having structural-functional characteristics of LH1 (RC is contained, which is supported by the presence of the H subunit of the RC in the same band as the light harvsting complex, see the 2D gel in Figure 14 and spectroscopic characteristics of LH2 complex (two spectral sets of BChls). The structural similarity with LH1 complexes is also supported by the fact that the MS analysis revealed presence of a polypeptide similar to the *α*-chain of LH1 (Figure 16).

Both gel filtration with stronger detergent and gel electrophoresis lead to extraction of another protein, which contains only carotenoids (see Figures 11 and 13c). Although the possibility that the carotenoids are not part of the complex can not be excluded mainly in case of gel electrophoresis, the fact that essentially the same spectra were obtained upon application of two various methods strongly suggests that the carotenoids are associated with the complex. The function of such a

complex is however, unknown. Because no energy acceptor is present in the vicinity of the carotenoid, the light harvesting function of the bound carotenoids can be excluded, leaving the most probable function of photoprotection for this carotenoid-protein complex.

Finally, the question of the carotenoid contained in the light-harvesting complex from *G. phototrophica AP64*^{T} will be opened. The carotenoid spectra in the two main pigment-protein complexes (compare Figure 13b and 13c) are obviously different: the carotenoid spectrum in the light harvesting complex has barely resolved vibrational structure. A group of carotenoids is well-known for this features, i.e. carotenoids with a carbonyl group in conjugation [32]. Although no such carotenoid is among the identified ones (see Figure 7), the fact that many so far unidentified carotenoids are present in the cells gives hope that one of them is the on with a carbonyl group in conjugation.

As follows also from this study, many photosynthetic features of *G. phototrophica* AP64^{T} are quite different from those of the other known phototrophic bacteria. That makes it an interesting object to study, offering to deepen our understanding of Nature’s photosynthetic strategies.

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