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Three C-phycoerythrin-associated linker polypeptides in the phycobilisome of green-light-grown *Calothrix* sp. PCC 7601 (cyanobacteria)*

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Microanalyses by SDS-PAGE and microsequencing demonstrate that, under green-light conditions, 3 C-phycoerythrin associated rod-linker polypeptides with different N-terminal amino acid sequences are present in phycobilisomes (PBS) from *Calothrix* sp. 7601 cells. Two of these polypeptides, corresponding to SDS-PAGE bands at 36 and 37 kDa, could be assigned, respectively, to the *cpe*C and *cpc*D genes found on a separate *cpe*CD-operon in *Calothrix* sp. 7601 (Federspiel, N.A. and Grossman, A.R. (1990) J. Bacteriol. 172, 4072–4081). The third C-PE rod-linker polypeptide, $L_{R,2}^{PE,33}$, requires, therefore, a third gene with the suggested locus designation '*cpe*E'. A C-PE $(\alpha\beta)_6$–$L_{R,2}^{PE,33}$ complex containing this third rod-linker polypeptide could be isolated from phycobilisomes and characterized. PBS from both green- and red-light cells of *Calothrix* contain a single, unique L_{RC}^{28} rod-core linker polypeptide which is not altered during chromatic adaptation.

Cyanobacteria; *Calothrix*; Phycobilisome; Phycoerythrin; Linker polypeptide; Amino acid sequence; Chromatic adaptation

1. INTRODUCTION

As in other cyanobacteria, the light-harvesting antenna in *Calothrix* sp. PCC 7601 is the phycobilisome (PBS), a large pigment–protein complex of about 8.35 × 10^6 Da. The PBS consists of both pigmented phycobiliproteins (78%) and non-pigmented (12%) linker proteins. The colors of the phycobiliproteins originate from open-chain tetrapyrrole chromophores, the red phycoerythrobilin (PEB) and the blue phycocyanobilin (PCB) [1–3]. In electron micrographs of *Calothrix* 7601 PBS [4], 2 PBS-subdomains are visible. The first subdomain is the PBS-core, seen in Fig. 4 in front view as 3 circles arranged in a triangle. This subdomain of 3 cylinders, each containing 4 trimers, is largely composed of allophycocyanin. From this allophycocyanin-core, 6 rods, forming the second subdomain, radiate outwards attached by rod-core linker polypeptides. C-phycocyanin (C-PC) and C-phycoerythrin (C-PE)-hexamers, or C-PC-hexamers alone form the rods, depending on light conditions as well as the size of the rods. The core-proximal end of the rods is a C-PC-hexamer under all light conditions. Two additional C-PC-hexamers are added during red-light growth, while during green- or white-light growth, additional red-pigmented C-PE-hexamers are added. This response to different light-wavelength conditions is referred to as complementary chromatic adaptation [5–7]. In *Calothrix* PCC 7601 3 copies of the genes encoding the α-C-PC subunits (*cpc*A1,2,3) and the β-C-PC subunits (*cpc*B1,2,3) were found and sequenced [8–11]. Under green-light conditions, only the *cpc*1 operon encoding the constitutive C-PC is turned on, while under red-light conditions, the *cpc*2 operon encoding the inducible C-PC is also transcribed. The *cpc*3-operon is expressed

Abbreviations: $L_{X,n}^{Y}$, refers to a linker polypeptide (L) having a mass of Y, located at a position X in the phycobilisome, where X can be R (rod), RC (rod-core junction), C (core) or CM (core-membrane junction) and n is the number of the linker polypeptide where more than one linker have the same mass. When necessary the abbreviation for a linker is appended to that of its associated phycobiliprotein; APC, allophycocyanin; C-PC, C-phycocyanin; C-PE, C-phycoerythrin; *apc*E, gene encoding the core-membrane linker phycobiliprotein; *cpc*A,B,C,D,E,F, genes encoding the C-PC α- and β-subunits of 3 different C-phycocyanins; *cpe*C,G,H,I, genes encoding C-PC-associated rod-core and rod-linker polypeptides; *cpe*A,B, genes encoding the C-PE α- and β-subunits; *cpe*C,D,E, genes encoding C-PE-associated linker polypeptides; CpeC-, CpeD-polypeptide, gene product encoded by *cpe*C, *cpe*D, etc.; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate; PTH, phenylthiohydantoin; PMSF, phenylmethanesulfonyl fluoride.

*Dedicated to Professor W. Wehrmeyer on the occasion of his 60th birthday.

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only under sulfur-limited growth conditions [12]. With each *cpc* operon different C-PC-associated linker polypeptides are expressed [13].

The *cpe*BA-operon, coding for the C-PE α- and β-subunits expressed under white-light and green-light conditions, was found to contain a single set of genes for the α- and β-C-PE subunits [14–17]. Federspiel and Grossman [15] have reported the DNA-sequences of 2 genes (*cpe*C and *cpe*D) encoding phycoerythrin-associated linker polypeptides. The *cpe*C and *cpe*D genes are not a part of the *cpe*BA operon but form a separate and unlinked *cpe*CD-operon.

In this study we demonstrate that under green-light conditions 3 C-PE-hexamers per PBS-rod, containing 3 different C-PE-associated linker polypeptides, are present in the PBS of *Calothrix* sp. PCC 7601. We have analyzed the linker polypeptides of the *Calothrix* PBS, grown under green, or red-light conditions, by SDS-PAGE, protein-microsequencing and by re-examination of the PBS by electron microscopy.

2. EXPERIMENTAL

2.1. *Biological material*

Cells of *Calothrix* sp. PCC 7601 (also known as *Fremyella diplosiphon* 33 or strain UTEX 481) were grown in 10-liter batch cultures in medium BG-11 [18] as described previously [19]. Cinemoid Masterline filters, Red No. 6 and Dark Green No. 24 (Rank-Strand GmbH, Wolfenbüttel, Germany), were placed around the culture vessel to induce complementary chromatic adaptation.

2.2. *Isolation of PBS from* Calothrix *sp. PCC 7601*

PBS were isolated by ultracentrifugation on a discontinuous sucrose gradient consisting of 4 ml of 2.0 M sucrose, 6 ml of 1.0 M sucrose, 8 ml of 0.75 M sucrose and 8.0 ml of 0.5 M sucrose in the isolation buffer as previously described [19,20]. PBS were recovered from the 1.0 M sucrose zone of the step gradients and dialyzed against isolation buffer without 0.5 M sucrose. Dialyzed PBS solutions were centrifuged at 38,700 × *g* for 30 min to pellet out contaminating aggregated membrane fragments, and were then stored at 4°C until used.

2.3. *Isolation and characterization of a C-PE* $(\alpha\beta)_6$ $L_{R,2}^{PE,33}$ *complex*

White-light grown cells (70 g) of *Calothrix* 7601 suspended in 600 ml of 0.9 M potassium phosphate buffer, pH 7.1, containing 1 mM PMSF, 1 mM EDTA and 1 mM NaN_3 (hereafter designated as PBS buffer), were disrupted under cooling by 2 passages through a Manton Gaulin press. The cell debris was removed by centrifugation. PBS were precipitated from the supernatant by addition of 144 g/l ammonium sulfate and harvested by centrifugation for 45 min at 10,500 × *g* (8,000 rpm) at 4°C. The PBS were dissolved in 1.3 liter of 5 mM PBS buffer, containing 5 mM EDTA, and allowed to dissociate for 4 h. The C-PE complexes were precipitated by ammonium sulfate (34% saturation).

After centrifugation, the C-PE pellet was dissolved in 100 ml of 5 mM PBS buffer and dialyzed against three 10 liter portions of 5 mM PBS buffer. C-PE complexes were separated by ion-exchange chromatography using a TSK DEAE-650 (S) Fractogel column (4 × 60 cm) equilibrated in 5 mM PBS buffer and eluted by a linear gradient of 5–150 mM potassium phosphate (4.5 liter total volume). The pooled fractions with different C-PE complexes were re-chromatographed by size-exclusion chromatography on a Biogel A-0.5m column (4 × 90 cm) in 50 mM PBS buffer followed by ultracentrifugation of the eluted complexes on continuous sucrose gradients consisting of 30 ml of 5–18% sucrose in 0.6 M PBS buffer. B-PE-$(\alpha\beta)_{6\gamma}$ from *Porphyridium cruentum* was used as a molecular weight standard. The gradients were centrifuged for 20 h at 25,000 rpm in a Kontron TFT 70.38 rotor, or for 12 h at 25,000 rpm in a Kontron TST 28.38 rotor at 4°C.

2.4. *Spectroscopic analysis*

Absorption spectra were measured with a Perkin Elmer Lambda 5 UV/VIS spectrophotometer and fluorescence emission spectra were recorded on a SPEX Fluorolog spectrofluorometer (SPEX Industries Inc.) in 1 mm × 1 cm cuvettes.

2.5. *Polyacrylamide gel electrophoresis*

Analytical and preparative SDS-PAGE based on the system of Laemmli [21] was performed using 6% isopropanol [22] exactly as described previously [19].

2.6. *Electroelution and amino acid sequence analysis of linker polypeptides*

Electroelution from minced acrylamide gel slices was performed with a Model 422 Electroeluter (Bio-Rad Laboratories, Richmond, CA, USA) using the electrode buffer of Laemmli [21] without SDS at 8–10 mA per tube for 12–15 h at room temperature directly onto 2 superimposed circles (*d* 4 mm) of Immobilon-P (Millipore Corpora-

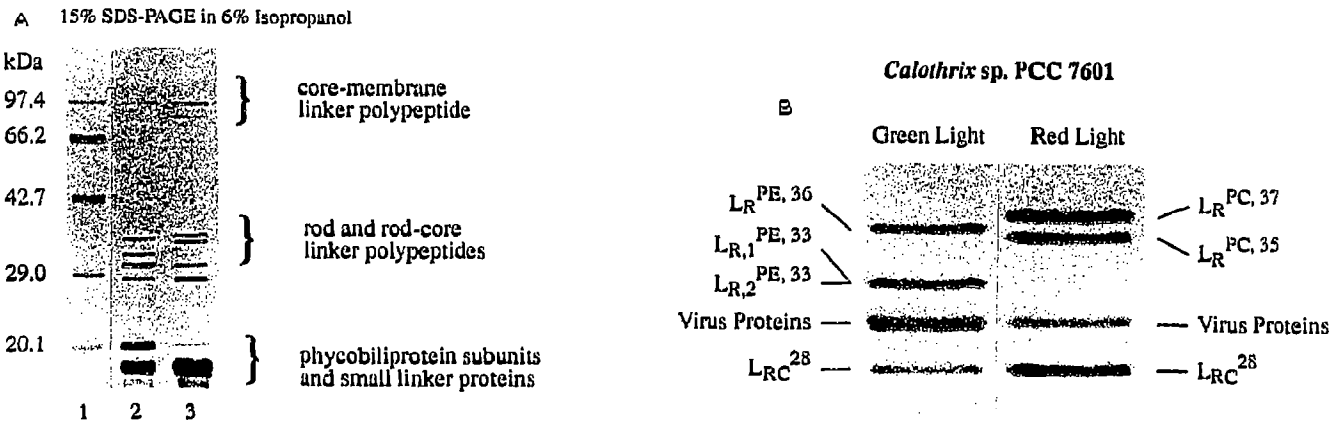


Fig. 1. (A) Analytical SDS-PAGE of isolated PBS from *Calothrix* 7601 grown under red light (lane 2) and green light (lane 3); (lane 1) mol. wt. standards. Conditions for maximal resolution of the rod and rod-core linker polypeptide bands were chosen (system of Laemmli [21]) using an analytical Mini-Protean II dual slab cell system (Bio-Rad Laboratories, Richmond, CA, USA). The stacking gel consisted of 5% acrylamide/0.13% bis-acrylamide (w/v) and the separating gel of 15% acrylamide/0.4% bis-acrylamide (w/v), containing 6% isopropanol [22]. (B) Magnified view of the rod and rod-core linker polypeptide bands from the PBS of *Calothrix* 7601 grown under green or red light.

tion, Bedford, MA, USA). After electroelution, the polyvinylidenedifluoride membranes were washed once in 100% methanol and twice in 20% (v/v) aqueous methanol for 1–3 min, dried, and applied to a protein-microsequencer (Knauer Model 810 protein microsequencer, Dr. Ing. H. Knauer GmbH, Berlin, Germany) equipped with an isocratic HPLC-system as described previously [23].

2.7. *Electron microscopy of green- and red-light* Calothrix *sp. PCC 7601 PBS*

Preparations of freshly isolated PBS were examined with a Philips EM 301 electron microscope as described previously [19,20].

3. RESULTS AND DISCUSSION

Fig. 1 shows the phycobiliprotein and linker polypeptide composition on SDS-PAGE of green- and red-light PBS isolated from *Calothrix* 7601. In order to achieve optimal resolution of the rod and rod-core linker polypeptides in the 27–37.5 kDa region, modified electrophoresis conditions using 15% SDS-PAGE in 6% isopropanol [22] were used. A significantly inferior separation of the phycobiliprotein subunits and small linker polypeptides was, however, obtained in this modified system.

The L_{CM} polypeptide of *Calothrix* 7601 (Fig. 1A, lanes 2 and 3, mol. wt. standard lane 1) had an apparent mass of 94 kDa although the *apc*E gene from this cyanobacterium contains 1,080 amino acid codons and predicts a protein of 120 kDa [24]. In PBS types of both cells grown in green- or red-light, 4 polypeptide bands in the 27–37.5 kDa range were resolved (Fig. 1). One of these bands (apparent mol. wt. 30.5 kDa) is not actually a PBS component but has been attributed to co-migrating proteins originating from a defective virus which is sometimes observed in this organism (N. Tandeau de Marsac, Centre National de la Recherche Scientifique, Département de Biochimie et Génétique Moléculaire, Institut Pasteur, Paris, France; personal communication).

Fig. 2 shows the amino acid sequences as determined from the linker polypeptide bands. SDS-PAGE gels in 6% isopropanol were used and the rod and rod-core polypeptides were excised from preparative gels for subsequent analysis. The N-terminal amino acid sequence of the L_{CM} polypeptide (Fig. 2, sequence I) was identical to that deduced from the nucleotide sequence of the *apc*E gene except for the absence of the initiator methionine residue. This suggests that a C-terminal processing of the ApcE gene product most likely occurs thus reducing its mol. wt. from approximately 120 to 94 kDa [24].

N-terminal sequence analysis of the component originating from a defective virus (apparent mol. wt. 30.5 kDa on SDS-PAGE from *Calothrix* 7601 grown under either green or red light) produced a mixture of 2 polypeptides, neither of which were related to the known linker polypeptides of this organism. The major sequence of this polypeptide mixture was: QAPSELADxLGGLxYA..., where x represents an unidentified amino acid residue (not shown in Fig. 2).

The 28-kDa linker polypeptide of *Calothrix* 7601 (Fig. 1) was clearly identified as an L_{RC} linker due to the high homology of its N-terminal amino acid sequence to the L_{RC} linker polypeptide of *Mastigocladus laminosus* (sequences II and III in Fig. 2). The sequence of this polypeptide was identical regardless of whether PBS were isolated from cells grown in green or in red light. This indicates that this polypeptide is not altered during the chromatic adaptation response in contrast to the peripheral PE- and PC-associated rod linkers (Figs. 1 and 2). No other L_{RC} linker polypeptides were identified in the PBS of *Calothrix* 7601. This contrasts with the PBS of the related filamentous, heterocystous

				10 20 30 40 50 60
I	Ca	L_{CM}^{94}	(ApcE)	SVKASGGSSSV/
II	Ma	$L_{RC}^{29.5}$	(CpcG2)	AIPLLQYKPSSQNQRVPGYEVPNEDTPR
III	Ca	L_{RC}^{28}	(CpcG)	AIPLLEYTPISQNQRVASLEVPGDEQPRTFSTDN/
IV	Ma	$L_R^{PC,36}$	(CpcC)	TAAASRLGTEPFSNAAKIELRSDASREEVEAVINAVYREVLG/
V	Ca	$L_R^{PC,35}$	(CpcH2)	TSSTAAR
VI	Ca	$L_R^{PC,37}$	(CpcI2)	PITSAA-SRLGTTAY-QTNPIEL/
VII	Ca	$L_R^{PE,36}$	(CpeC)	PFGPasrlgvslfdetppvewvpgrsqeeaetiiralyrqvlgnayvm
VIII	Ca	$L_{R,1}^{PE,33}$	(CpeD)	ASQTILELwPSSSLEEVQTIIRAVYKQVLGN
IX	Ca	$L_{R,2}^{PE,33}$	(CpeE)	ALxIETESVELRPNATEEDLQAT/
X	Ca	$L_{R,2}^{PE,33}$	(CpeE)	ALWIETESVELRPNATEEDLQATIRAVYRQVLGNAxxFENQDLTN/

Fig. 2. Comparison of the N-terminal amino acid sequences of core-membrane, rod-core and rod linker polypeptides of the cyanobacteria *Calothrix* 7601 (Ca) with some of those of *Mastigocladus laminosus* (Ma). The apparent molecular masses from SDS-PAGE are indicated. Amino acid sequences deduced from nucleotide sequences are written in lowercase letters, gaps are indicated by hyphens. Unidentified amino acid residues are denoted by x. N-Terminal amino acid sequences: (I) L_{CM} polypeptide of *Calothrix* 7601; (II) L_{RC} polypeptide of *M. laminosus*; (III) L_{RC} polypeptide from PBS of *Calothrix* 7601 cells grown in either green or red light; L_R polypeptides associated with PC hexamers from: (V) *M. laminosus*; (V and VI) *Calothrix* 7601 cells grown in red light; (VII, VIII and IX) L_R polypeptides associated with C-PE hexamers of *Calothrix* 7601, grown in green light; (X) $L_{RC,2}^{33}$, polypeptide from the isolated C-PE $(\alpha\beta)_6$–$L_{R,2}^{PE,33}$ complex.

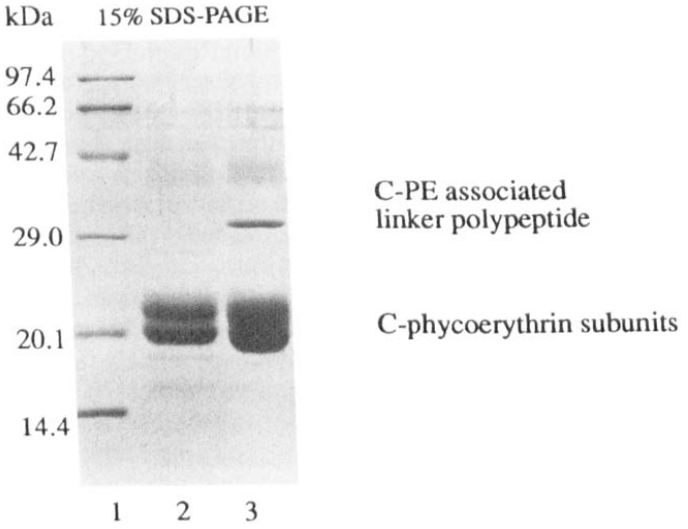
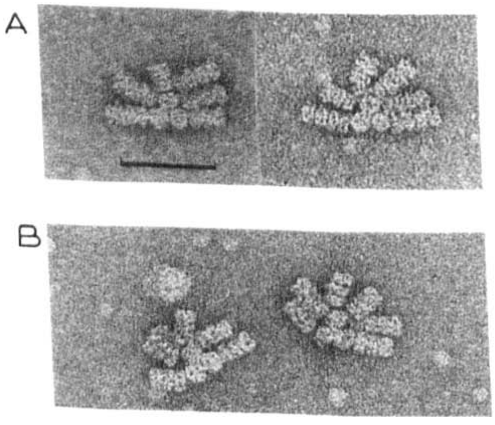


Fig. 3. Analytical SDS-PAGE of the C-PE $(\alpha\beta)_3$ complex (lane 2) and of the C-PE$(\alpha\beta)_6$–$L_{R,2}^{PE,33}$ complex (lane 3). (Lane 1) molecular weight standards based on the system of Laemmli [21] using a separating gel of 15% (without isopropyl alcohol).

cyanobacteria *Anabaenaa* sp. PCC 7120 and *M. laminosus*, which contain 4 rod-core linkers [19]. The gene encoding for the L_{RC} linker polypeptide of *Calothrix* 7601 has not yet been cloned and sequenced at this time.

In *Calothrix* 7601 cells grown in red light, no PE-associated rod linkers were detected, but the PC-associated rod linkers $L_R^{PC,35}$ and $L_R^{PC,37}$ were identified as the gene products of the chromatically induced *cpc*H2 and *cpc*I2 genes, respectively [11] (Fig. 1, lane 2 and Fig. 2, sequences V and VI). The $L_R^{PC,37}$ linker polypeptide has a proline residue at its N-terminus which was poorly cleaved during Edman degradation and therefore only small quantities of PTH-amino acid derivatives could be detected. In *Calothrix* 7601 cells grown in green light, the PE-associated $L_R^{PE,36}$ linker polypeptide on SDS-PAGE (Fig. 1) was identified as the CpeC gene product [15]. Again, due to a poorly cleaved N-terminal proline residue, only traces of the N-terminal 4 amino acid residues Pro-Phe-Gly-Pro (initiator methionine is post-transcriptionally removed) could be detected by Edman degradation (sequence VII in Fig. 2).

Analysis of the 33-kDa polypeptide band yielded 2 sequences, one of which was identical to that predicted by the *cpe*D gene ($L_{R,1}^{PE,33}$ in Figs. 1 and 2, sequence VIII, [15]). The second sequence (sequence IX in Fig. 2), obtained by subtraction of the CpeD sequence was distantly related to those of the identified CpeC and CpeD polypeptides, but showed no similarity to that of the L_{RC}^{28} polypeptide. Hence a third PE-associated peripheral rod linker ($L_{R,2}^{PE,33}$) is indicated, co-migrating on SDS-PAGE with the CpeD gene product (Fig. 1). The N-terminal sequence determined for this third PE-associated linker polypeptide is identical to the deduced N-terminus of an open reading frame (*orf*) downstream from the *cpe*CD operon in *Calothrix* 7601, denoted as *cpe*E, except for the removal of the initiator methionine (N.A. Federspiel and L.A. Scott, University of Idaho, USA; unpublished results). Thus, the $L_{R,2}^{PE,33}$ polypeptide is the CpeE gene product and might belong to a defined C-PE hexamer rod–linker polypeptide complex. This complex, C-PE$(\alpha\beta)_6$–$L_{R,2}^{PE,33}$, was isolated from *Calothrix* 7601 cells by ion-exchange chromatography on DEAE-Fractogel. By ultracentrifugation experiments using B-Phycoerythrin (B-PE$(\alpha\beta)_{6\gamma}$ from *Porphyridium cruentum*) as a standard, it was shown that this C-PE-complex with the $L_{R,2}^{PE,33}$ rod-linker is a hexameric complex. The absorption maximum of this C-PE $(\alpha\beta)_6$–$L_{R,2}^{PE,33}$ complex was at 564.6 nm and the fluorescence emission maximum at 579 nm. Analysis of the excised pure linker polypeptide from SDS-PAGE, (Fig. 3, lane 3) resulted in a single-polypeptide sequence (Fig. 2, sequence X) and confirmed the deduced sequence (sequence IX in Fig. 2). In the first 22 amino acid residues the $L_{R,2}^{PE,33}$ linker polypeptide is more homologous to the $L_R^{PE,36}$ linker with 7 identical amino acid residues and an aromatic residue at position 3 than to the $L_{R,1}^{PE,33}$ linker (5 identical amino acid residues) and



Calothrix sp. PCC 7601
green light

Calothrix sp. PCC 7601
red light

Fig. 4. Electron micrographs of PBS isolated from the cyanobacterium *Calothrix* 7601 grown under (A) green light and (B) red light. The bar represents 50 nm (magnification = × 250,000).

after position 22, it exhibits a high homology to both the $L_{R.1}^{PE,33}$ and $L_R^{PE,36}$ linker polypeptides. The differences in the first residues might be determinative for the location in the rods.

In order to correlate directly the number of C-PE- or induced C-PC-associated linker polypeptides with the number of phycobiliprotein-hexamers per rod, PBS were prepared and inspected by electron microscopy from the same cells used for PBS analysis by preparative SDS-PAGE. Prior to analysis by electron microscopy, PBS from *Calothrix* 7601 cells, grown in either green or red light, were isolated by sucrose gradient centrifugation. These PBS, shown in Fig. 4, were found to have the typical hemidiscoidal structure observed in most cyanobacteria: a tricylindrical core surrounded by 6 peripheral rods composed of phycobiliprotein hexamers. In green-light PBS, most rods contained four 6 × 11 nm hexamers while in red-light PBS rods composed of 3 hexamers were most commonly observed. The results, based on analysis of the linker polypeptides, suggest that each hexamer in the rod possesses a unique linker polypeptide. Consequently, a rod composition of (1 C-PC + 3 C-PE) in green light and (3 C-PC) in red light is proposed. This is largely in agreement with previous studies on the energy transfer pathway [25] and pigment assignment to components of the PBS in the electron micrographs [26], summarized in the PBS model established earlier for *Calothrix* 7601 and related species [4,20].

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