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Short communication

Characterization of the single *psbA* gene of *Prochlorococcus marinus* CCMP 1375 (Prochlorophyta)

Wolfgang R. Hess [1,*], Andreas Weihe [1], Susan Loiseaux-de Goër [2], Frédéric Partensky [2] and Daniel Vaulot [2]

[1] *Humboldt University, Department of Biology, Invalidenstrasse 43, D-10115 Berlin, Germany (*author for correspondence);*

[2] *Observatoire Océanologique de Roscoff CNRS-UPR 4601, BP 74, F-29680 Roscoff Cedex, France*

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Abstract

DNA sequence, copy number, expression and phylogenetic relevance of the *psbA* gene from the abundant marine prokaryote *P. marinus* CCMP 1375 was analyzed. The 7 amino acids near the C-terminus missing in higher plant and in *Prochlorothrix hollandica* D1 proteins are present in the derived amino acid sequence. *P. marinus* contains only a single *psbA* gene. Thus, this organism lacks the ability to adapt its photosystem II by replacement of one type of D1 by another, as several cyanobacteria do. Phylogenetic trees suggested the D1-1 *iso*-form from *Synechococcus* PCC 7942 as the next related D1 protein and place *P. marinus* separately from *Prochlorothrix hollandica* among the cyanobacteria.

P. marinus [8] is the only known free-living marine prokaryote containing chlorophyll *a*, *b* and *c*, but no phycobilisomes. Due to the latter features *P. marinus* was placed into the group termed prochlorophyta [8, 23]. Prochlorophyta-like organisms were originally supposed to represent the endosymbiotic ancestors of higher plant chloroplasts (see [4] for a review). Later, by comparison of DNA sequences from a part of the 16S rRNA gene [46] and a portion of the *rpo*C1 gene [38], it was shown that none of the known prochlorophytes is found on a lineage leading directly to the green chloroplasts. Based on the same data *Prochlorococcus* was suggested to be more closely related to cyanobacteria from the genus *Synechococcus* [46] or to other cyanobacteria [38]. *Prochlorococcus* was found in high cell numbers ubiquitously in the world's tropical and subtropical oceans. It contributes substantially to the photosynthetic biomass and primary production [6, 7, 17]. In the present study the *psbA* gene was analyzed to obtain information relevant for the physiology of the photosynthetic apparatus as well as for the exact phylogenetic position of *P. marinus*.

The *Prochlorococcus* SARG strain was origi-

The nucleotide sequence data reported will appear in the EMBL, DDBJ and GenBank Nucleotide Sequence Databases under the accession numbers Z22779 and Z22780.

nally isolated from a depth of 120 m from the Sargasso Sea in 1988 [8]. It gave rise by serial dilution to the clonal strain SS120, designated as CCMP 1375 at the Center for the Culture of Marine Phytoplankton, West Boothbay Harbor, USA, and is the type strain of *P. marinus* [8]. Unialgal strains of *P. marinus* CCMP 1375 were cultured as described previously [39]. Genomic DNA of *P. marinus* was isolated according to Franche and Damerval [15]. The degenerate primers 5′-ATGATCCCCACCCT(G/C)(C/T)TGAC(T/C)GC(G/C)A(C/T)-3′ and 5′-GGGAAGTTGTGGGCATT(G/A)CG(C/T)TCGTG-3′ were used for amplification of a 994 bp fragment of *Prochlorococcus psbA* by polymerase chain reaction. By hybridization with a heterologous gene probe from *Prochlorothrix hollandica*, we identified one amplified fragment of the expected size that contained part of a *Prochlorococcus psbA* gene, as confirmed by partial sequencing. This fragment was subsequently used to screen a genomic library established in bacteriophage λgt10. Several independent clones were isolated. Two of them, containing the coding region of *psbA*, were used for subcloning the inserts into plasmid pT7T318U and sequence determination.

The DNA encodes a reading frame whose A/T content of 55.5% is biased towards A/T-rich codons. It is followed by a putative terminator region consisting of a pair of 12 nucleotide inverted repeats able to form a stem-loop structure. The reading frame has a potential length of 370 amino acids, from which 360 amino acids beginning with the second possible in frame methionine are highly similar to known D1 polypeptides. This 360 residue reading frame has exactly the size of the D1 proteins in cyanobacteria and non-chlorophytic plastids. Whether the 10 amino acids theoretically preceding this frame (MSVWDFF-HVL) are functional is unknown. The N-terminal part of the 360 residue reading frame is rather different from known D1 proteins and contains one additional amino acid (Fig. 1; see accession number Z22779 and Z22780 for the entire sequence). This deduced D1 protein is 86% identical to both D1 *iso*-forms in *Synechococcus* PCC 7942 and lower for all other known D1 polypeptides. The putative membrane spanning regions and all known amino acids of high functional conservation are present in *P. marinus* D1: Tyr-162 ('Z' = $P680^+$ electron donor [12]); Ser-265 (Q_B-binding [20]), His-216 and -273 (iron binding[43]); Asn-171, Gln-166 and -188 (putative function in manganese ligation [47]); amino acids 226–239 and 242–246 (QEEET motif and PEST-like region [45]). The gene is transcribed as a 1300 nt monocistronic mRNA (Fig. 2). *Prochlorococcus* is photoadaptable [39], including a reorganization of photosynthetic membranes as indicated by the changes in the pigment composition [30, 39]. However, the *psbA* mRNA steady-state level was not or only very little influenced in a light shift experiment from 8 to 57 $\mu E\ m^{-2}\ s^{-1}$ (Fig. 2).

The presence or absence of seven amino acids near the carboxy-terminus of D1 proteins has previously been used as a phylogenetic marker [24, 26, 31, 49]. While all land plant chloroplasts lack these 7 amino acids, they are present in

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P. marinus              MTTIRQQRSSLLKGWPQFCEWVTSTDNR 28
P.hollandica            ***ALR**E*-ANA*E***Q*IA**E** 27
Synechococcus 7942-1    **S*LREQRR-DNV*DR*********** 27
Synechococcus 7942-2    ***AL*R*E*-ASL*Q*********** 27
Synechocystis 6803-2    ***TL***E*-ASL*E***Q*****N** 27
Euglena gracilis        **SPVLKKYARPSL*YR**A**A*KK** 28
Cyanophora paradoxa     **ATLERNA*-VSL*E***GFI***E** 27
Chlamydomonas moew.     **A*LER*E*-TSL*AR****I***E** 27
Marchantia polymorpha   **ATLER*E*-ASI*GR**D*****E** 27
Oryza sativa            **A*LER*E*-TSL*GR**N*I***E** 27
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Fig. 1. Deduced amino-terminal sequence of the D1 protein from *P. marinus* compared with its homologues from several other organisms. Asterisks indicate residues identical to the *P. marinus* sequence, gaps are indicated by a hyphen, the total number of residues is given at the end of each lane. See Fig. 4 for sequence references.

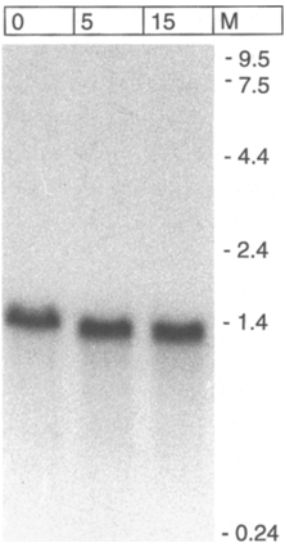

Fig. 2. The *psbA* gene gives rise to a 1.3 kb monocistronic transcript. RNA was isolated at the indicated time points (in minutes) during a light shift from 8 to 57 $\mu E\ m^{-2}\ s^{-1}$. The sizes of molecular weight standards (Gibco-BRL) are indicated in lane M. RNA was isolated in minipreparations from 1 l cultures by a method adapted from Logemann *et al.* [25].

Cyanophora, in all cyanobacteria, and in non-green algal plastids. In this respect, one prochlorophyte, *Prochlorothrix hollandica*, was found to be very similar to higher-plant chloroplasts [31]. In contrast, another prochlorophyte, *Prochloron didemni*, does contain these 7 amino acids [24]. We show here that *P. marinus* also possesses these 7 amino acids (Fig. 3). Bootstrapping analysis was performed to evaluate the degree of relationship of this protein to those of other, especially prokaryotic, organisms. All 'neighbor joining' trees based upon distance values (as well as the consensus tree based upon 100 replicates) show the same topology as that presented in Fig. 4. This topology remained unchanged whether the seven amino acid C-terminal deletion found in green plastids and in *P. hollandica* is counted as one deletion (6 positions deleted in alignments) or as 7 deletions (all positions conserved in alignments), and also with different sets of species included in the analysis. The high bootstrap values shown in Fig. 4 are found at the same places in all the trees. The same topology is also found in the consensus tree obtained from 100 replicates of parsimony trees when the 7 amino acid deletion is counted as one deletion (6 positions omitted in the alignment), although some of the bootstrap values are lower (i.e., 86 between plastid and cyanobacterial branches, 68 between *P. marinus*, *Synechococcus* 7942-1 and the other species). When the seven amino acid deletion is counted as seven deletions, *P. hollandica* moves to the green plastid branch and the red, heterokont and *Cyanophora* plastids group together, separated from the green branch by a cyanobacterial branch (consisting of *Synechococcus vulcanus* and *S. elongatus*). Other branches remain unchanged; however bootstrap values between plastid and cyanobacterial branches are very low in this case. Bootstrap values linking *P. hollandica* to one of

Prochlorococcus marinus	PLDLAAAESTSVALVAPSI-G	360
Prochlorothrix hollandica	******VK-------****I*	353
Prochloron sp.	******G*AAP***T****N*	n.k.
Synechococcus PCC 7942 I	******G*A*P***T****H*	360
Synechococcus PCC 7942 II	******G*A*P***T**A*N*	360
Synechocystis PCC 6803 II	*****SG*QAP***T**AVN*	360
Antithamnion sp.	*****SN**LPL****A*N*	360
Cyanidium caldarium	*****SEV*LP***NKVE*N*	360
Bumilleropsis filiformis	******G*VLP**VS**AVHA	360
Ectocarpus silicolosus	*****SN*ILP**IS***VV*	360
Euglena gracilis	*****----------------	345
Cyanophora paradoxa	*****SG*VMP***T****NA	353
Chlamydomonas moewusii	******F*-------****NA	353
Marchantia polymorpha	******V*--------**AVN*	353
Oryza sativa	******L*-------V**LN*	353

Fig. 3. Comparison of C-terminal amino acid sequences of D1 proteins from all major groups of photosynthetic organisms. The deduced amino acids from residue 341 (according to *P. marinus*) to the termination codon are shown for each sequence; n.k. = not known. Source of the sequence data is as indicated in the legend to Fig. 4, for *Euglena gracilis* [21], for *Prochloron* sp. [24].

the other branch, or separating plastid lineage branches one from the other are always fairly low in all trees whatever method is used. Removing the more rapidly evolving and not expressed *Synechocystis* 6803 D1-1 [29] and *Synechococcus elongatus* D1-2 proteins from the alignment does not change the topology or the higher bootstrapping values in a distance consensus tree. From all the trees constructed during this work, *P. marinus* D1 seems to be more related to *Synechococcus* 7492 D1-1 than to any other known cyanobacterial D1 polypeptide. This position is stable. The D1 of *P. hollandica* is generally found on the same branch, more closely related to *Synechococcus*

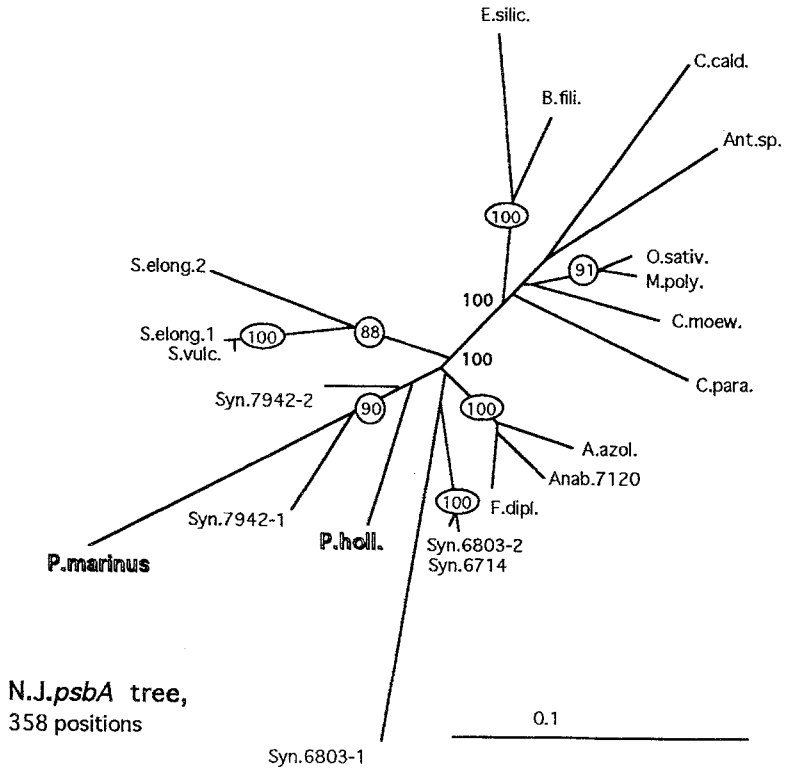

Fig. 4. Neighbor-Joining tree for values of divergence between D1 amino-acid sequences of 13 prokaryotic and some representative plastid proteins as measured with the PROTPARS (Dayhoff option) method (PHYLIP 3.52c release). Bootstrap confidence values [13] are shown at nodes when above 90%. The 358 amino acid positions analyzed include the entire C-terminal region, which means that the 7 amino acids deletion found in green plastids and in *P. hollandica* D1 proteins is counted as 7 separate amino acid mutations. The scale bar indicates divergence of 0.1 amino acid substitutions per site (corrected). Branches are drawn to scale. Abbreviations: Syn.7942-1 and -2: *Synechococcus* PCC 7941 D1-1 and D1-2 [18]; P. holl.: *Prochlorothrix hollandica* [31]; Syn.6803-1: *Synechocystis* PCC 6803 *psbA*I-gene product [36]; Syn.6803-2: *Synechocystis* PCC 6803 *psbA*I and II gene product [28, 40]; Syn.6714: *Synechocystis* PCC 6714 *psbA*I gene product [1]; S.vulc.: *Synechococcus vulcanus* (EMBL X79222); S.elong.1 and 2: *Synechococcus elongatus* 1 and 2 (Swiss-Prot P35877 and P35876); F.dipl.: *Freymyella diplosiphon* [32]; Anab. 7120: *Anabaena* PCC 7120 *psbA*I-gene product [11]; A.azoll.: *Anabaena azollae* (Swiss-Prot P29270); C.para.: *Cyanophora paradoxa* [19]; C.moew.: *Chlamydomonas moewusii* [44]; M.poly.: *Marchantia polymorpha* [34]; O.sativ.: *Oryza sativa* [50]; E.silic.: *Ectocarpus siliculosus* [49]; B.fili.: *Bumilleropsis filiformis* [42]; C.cald.: *Cyanidium caldarum* [26]; Ant.sp.: *Antithamnion* sp. [49]. D1 polypeptide sequences of 13 prokaryotic and 8 eukaryotic organisms were aligned without difficulty using the LINEUP program of the GCG program. The C-terminal deletion of seven amino acids found in green plastid and *Prochlorothrix hollandica psbA* genes was either suppressed, leaving only one deletion, or left entirely. Matrices of distance values were constructed using the PROTDIST program included in the PHYLIP 3.52c release [14] and were analyzed with the 'neighbor-joining' method [41]. Bootstrapping analyses were done with either method using the SECBOOT program (PHYLIP 3.52c), with 100 replicates of distance matrices or 100 replicates of alignments in the case of parsimony, and leading to consensus trees.

7492-2, but its position is not as stable since it shows relationship to green plastids in parsimony as long as an exaggerated weight is put on the 7 amino acid deletion. *P. marinus* D1 is clearly not directly related to plastid D1 proteins. The data presented here extend previous reports on the heterogeneity among prochlorophytes on basis of sequence data [38, 46]. They strongly support the conclusions derived previously from ribosomal RNA sequences of *Synechococcus* as the cyanobacterium most closely related to *P. marinus* [46], whereas conclusions made by analysis of another protein-coding gene seem not to be suitable as long as the exact position of *Prochlorococcus* inside the cyanobacteria was considered [38].

The 7 amino acid difference is part of the processed C-terminus, which is cleaved off post-translationally [27] by a processing protease [2]. Function and importance of this processing step are not yet fully elaborated, but it seems to be important for formation of a functional Mn cluster and establishment of the water oxidation activity [33]. Hence, a certain degree of conservation can be expected for this part of the D1 polypeptide. The cleaved off fragment is mostly either 9 (chlorophytes, *P. hollandica*), or 16 amino acids (cyanobacteria, *P. didemni*). An exception is *Euglena*, where the C-terminal part of the protein is not encoded by the *psbA* gene at all [21]. The D1 of *P. marinus* is also in this respect more similar to cyanobacterial D1 proteins, but the length of the processed sequence part is only 15 amino acids (Fig. 3).

Several lines of evidence suggest that *P. marinus* CCMP 1375 possesses a single *psbA* gene only. Southern hybridization with genomic DNA digested with five different restriction enzymes resulted in four cases in one band only (Fig. 5). With *Hin*d III two bands were obtained due to an internal *Hin*d III site. This result is indicative of an absence of additional physically unlinked *psbA* gene copies in this particular strain of *P. marinus*. The smaller hybridizing fragments obtained by double digests with *Hin*d III/*Eco* RI and *Hin*d III/*Pst* I further show that there also is no second tightly arranged gene copy, for example in a tandem array (Fig. 5). This latter result is further substantiated by sequence determination of 327 nucleotides downstream and 362 nucleotides upstream of the coding region. The two independent clones from the library used for sequence analysis were identical in the overlap containing the coding region. The fact that *P. marinus* contains only one *psbA* gene copy distinguishes it from cyanobacteria, but also from the prochlorophyte *P. hollandica* [31]. In the latter, two different genes were found but encoding proteins of identical amino acid sequence [31]. In cyanobacteria, the *Anabaena* strain PCC 7120 possesses four *psbA* genes [48], whereas the *Synechococcus* strains PCC 7002, 7942 and *Synechocystis* PCC 6714 and 6803 each contain three *psbA* genes [3, 16, 18]. While some of the respective copies are not expressed or encode identical polypeptides, usually two *iso*-forms of the D1 protein can be present. The regulation of different *psbA* gene copies and of the two D1 *iso*-forms is best understood in *Synechococcus* PCC 7942, which are also the most closely related to *P. marinus* [5, 9, 10, 22]. Functional importance with respect to *in vivo* adaptation to varying photon irradiation has recently been demonstrated for these different light-dependent expressed D1 *iso*-forms, from which one dominates at high, the other at moderate illumination [9, 22]. *Prochlorococcus* is present under natural conditions in depths of > 100 m as well as near the water surface [6, 35]. An appropriate adaptability of the photosynthetic apparatus, possibly involving *iso*-forms of the D1 protein, might be expected for such an organism. However, the CCMP 1375 strain of *P. marinus* can produce only one type of D1 protein. Phylogenetic analysis places this protein somewhat closer to the D1-1 type of *Synechococcus* PCC 7942 versus the D1-2 type whatever tree making method is used with fairly high bootstrapping values (Fig. 4). The D1-1 protein is the "lower light" *iso*-form in *Synechococcus* 7942 [9, 22]. Thus, the D1 type present in *P. marinus* CCMP 1375 seems to correlate with the environmental conditions of low light intensity this strain was isolated as described [8]. An adaptation of this strain for growth at low light intensities was also demonstrated under laboratory conditions [30]. Recent

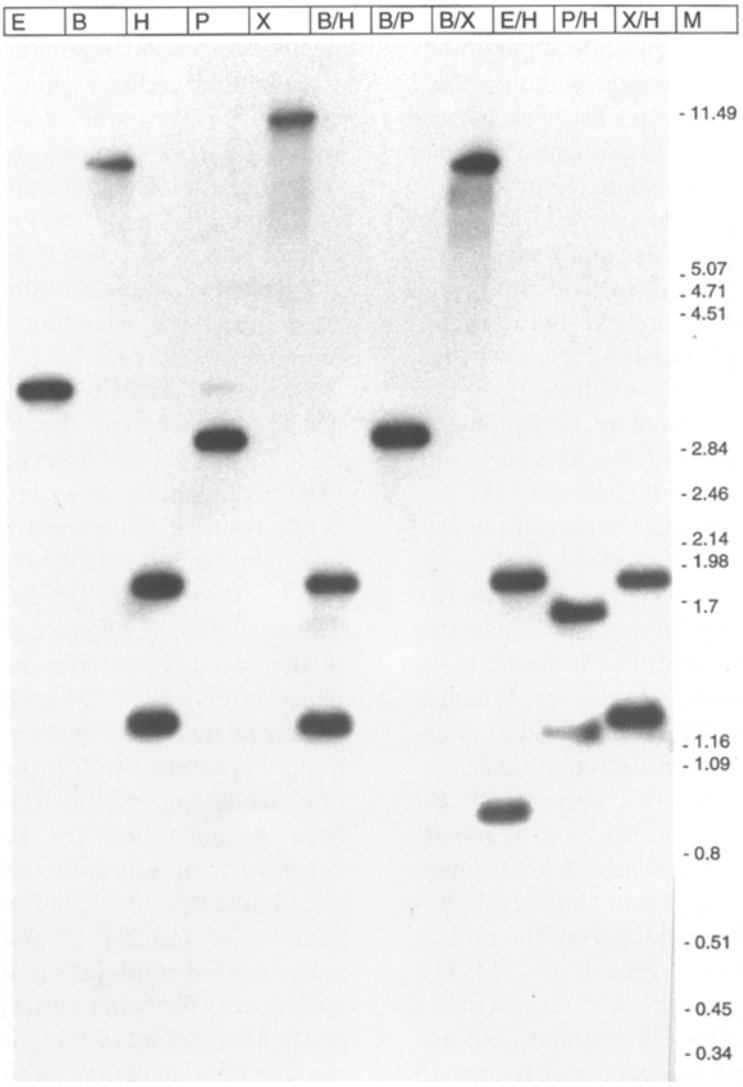

Fig. 5. Genomic DNA of *P. marinus* CCMP 1375 was digested with the restriction endonucleases *Eco* RI (E), *Bam* HI (B), *Hin*d III (H), *Pst* I (P), *Xho* I (X), individually or in pairwise double digests and subjected to Southern hybridization. The probe used was a 1406 nt DNA fragment comprising 1080 nt of the *Prochlorococcus psb*A coding region and 326 nt of downstream sequences. M = molecular weight standard (bacteriophage λ DNA cleaved by the restriction endonuclease *Pst* I).

field studies suggest that natural *Prochlorococcus* communities consist of very different genotypes, perhaps genetically as divergent as different genera of, for example, enteric bacteria [37]. Hence, it can be speculated that such genotype differences might also include the exclusive presence of specialized types of D1 proteins. The D1 *iso*-forms in *Synechococcus* 7492 differ from one another in only 25 of the total 360 amino acids (= 93% identity [18]), obviously including the residues providing the functional difference [9]. From that 25 amino acids 12 occur in the first 16 residues, and 7 within the putative transmembrane helices II and III [9]. It is interesting to note in this context that the somewhat higher similarity of the *P. marinus* D1 to *Synechococcus* D1-1 observed here is based solely on the sequence composition in the transmembrane regions (cf. Fig. 1).

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