Cyanobacteria of the genus *Prochlorothrix*[†]

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Green cyanobacteria differ from the blue–green cyanobacteria by the possession of a chlorophyll-containing light-harvesting antenna. Three genera of the green cyanobacteria namely *Acaryochloris*, *Prochlorococcus,* and *Prochloron* are unicellular and inhabit marine environments. *Prochlorococcus marinus* attracts most attention due to its prominent role in marine primary productivity. The fourth genus *Prochlorothrix* is represented by the filamentous freshwater strains. Unlike the other green cyanobacteria, *Prochlorothrix* strains are remarkably rare: to date, living isolates have been limited to two European locations. Taking into account fluctuating blooms, morphological resemblance to *Planktothrix* and *Pseudanabaena*, and unsuccessful attempts to obtain enrichments of *Prochlorothrix*, the most successful strategy to search for this cyanobacterium involves PCR with environmental DNA and *Prochlorothrix*-specific primers. This approach has revealed a broader distribution of *Prochlorothrix*. Marker genes have been found in at least two additional locations. Despite of the growing evidence for naturally occurring *Prochlorothrix*, there are only a few cultured strains with one of them (PCC 9006) being claimed to be axenic. In multixenic cultures, *Prochlorothrix* is accompanied by heterotrophic bacteria indicating a consortium-type association. The genus *Prochlorothrix* includes two species: *P. hollandica* and *P. scandica* based on distinctions in genomic DNA, cell size, temperature optimum, and fatty acid composition of membrane lipids. In this short review the properties of cyanobacteria of the genus *Prochlorothrix* are described. In addition, the evolutionary scenario for green cyanobacteria is suggested taking into account their possible role in the origin of simple chloroplast.

Keywords: cyanobacteria, *Prochlorothrix*, Prochlorophytes

INTRODUCTION

Oxygenic photosynthetic bacteria are represented by blue–green cyanobacteria and green cyanobacteria. This distinction is analogous to that between green chlorosome-containing and purple non-chlorosome-containing anoxygenic photosynthetic bacteria and is based on the supramolecular organization of the light-harvesting complex rather than cell color (certain blue–green cyanobacteria are blue, green, red, violet, or brownish). Blue–green cyanobacteria are characterized by a constantly expressed phycobiliprotein light-harvesting antenna, or phycobilisome, with the exception of inducible mutants and terminally differentiated wild type cells called heterocysts. In contrast, green cyanobacteria have a constitutive chlorophyll-protein light-harvesting antenna instead of the phycobilisome (the former is expressed in some blue–green cyanobacteria in stress conditions). In terms of pigments, green cyanobacteria possess accessory chlorophyll(s), whereas blue–green cyanobacteria have only *chl a* (Partensky and Garczarek, 2003).

Green cyanobacteria are worth interest primarily for two reasons. First of all, *Prochlorococcus marinus* dominates pelagic environments for large regions of the world's oceans. Secondly, the remaining four species (*Acaryochloris marina*, *Prochloron didemni*, *Prochlorothrix hollandica*, and *Prochlorothrix scandica*) are limited to specific eutrophic habitats, in contrast to ubiquitously distributed *P. marinus* and blue–green cyanobacteria (Partensky et al., 1999).

Cyanobacteria of the genus *Prochlorothrix* are unique among green cyanobacteria being filamentous and freshwater, as opposed to the other three marine unicellular species.

In this review, the properties of cyanobacteria of the genus *Prochlorothrix* are summarized, and the phylogeny of green cyanobacteria is reconsidered in the light of their role in the origin of simple chloroplast.

SCOPE OF OBJECTS
CIRCUMSTANCES OF DISCOVERY
Green cyanobacteria were initially discovered by the chromatographic detection of the accessory *chl b* and/or other accessory chlorophylls (a_2, b_2, *c*, etc) in collected samples. This approach lead to the discovery of two unicellular species *P. didemni* (Lewin, 1981) and *A. marina* (Miyashita et al., 1996), as well as the filamentous species *P. hollandica* (Burger-Wiersma et al., 1989) and *P. scandica* (Pinevich et al., 1999). The third unicellular species,

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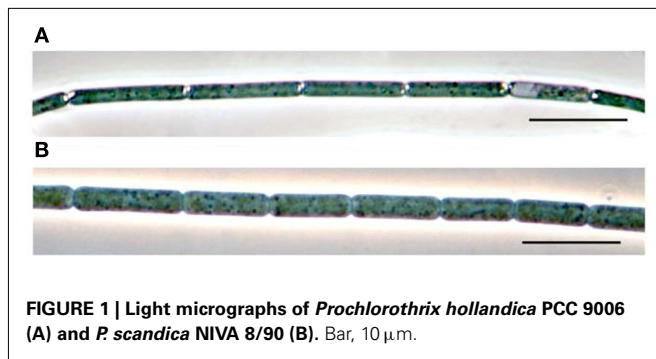
FIGURE 1 | Light micrographs of *Prochlorothrix hollandica* PCC 9006 (A) and *P. scandica* NIVA 8/90 (B). Bar, 10 µm.

Table 1 | Genetic distinction between *P. hollandica* and *P. scandica*.

Criteria of distinction	Measure of assignment to different species (%)	
	Conventional	Actual
Δ Mol% GC	>5	6.6
DNA–DNA hybridization degree	<30	17.9
Positional matches in the complete sequence of 16S rRNA gene	>3	2

Table 2 | Cellular fatty acid composition (%) of *P. scandica* and *P. hollandica* grown under identical conditions.

Fatty acid	*P. scandica* NIVA-8/90	*P. hollandica* CCAP 1490/1
STRAIGHT-CHAIN		
$C_{12:0}$	0.5	0.6
$C_{14:0}$	10.7	8.7
$C_{15:0}$	0.8	1.1
$C_{16:0}$	18.1	17.7
$C_{18:0}$	**1.2**	**0.5**
BRANCHED		
Iso-$C_{16:1}$*	1.4	2.0
Anteiso-$C_{16:1}$*	6.7	6.0
UNSATURATED		
$C_{12:1}$*	1.3	0.8
$C_{14:1}$*	14.3	17.0
$C_{15:1}$*	8.0	9.6
$C_{16:1}\omega9$*	5.5	3.7
$C_{16:1}\omega7$*	**11.9**	**21.4**
$C_{16:1}\omega5$*	**8.7**	**5.0**
$C_{16:2}\omega4$*	**1.8**	**1.0**
$C_{16:3}\omega3$*	**1.5**	**0.7**
$C_{18:1}\omega9$*	**3.4**	**0.5**
$C_{18:1}\omega11$*	0.7	0.5
$C_{18:2}\omega6$*	0.2	0.3
Unknown	4.0	2.2

The position(s) of double-bond(s) is not thoroughly determined; trans-cis isomeric state of unsaturated fatty acids is not determined. The most prominent differences between the two species are in bold.

P. marinus, was revealed by flow cytometry in oceanic picoplankton (Chisholm et al., 1988). Later on, pigment-based methods were supplemented with or gave way to PCR detection of the 16S rRNA gene with specific primers (Castiglioni et al., 2004). In order to increase the sensitivity of detection, as well as for a better separation of gene clusters, internal transcribed spacers of 16S–23S-rRNA were also tested (Laloui et al., 2002; Rocap et al., 2002). Screening for green cyanobacteria is also performed using other primers which help amplify genes which code for photosynthetic proteins, e.g., Pcb proteins of *chl a/b* antenna distinct from CAB proteins in green algae, euglenae, and higher plants (Geiß et al., 2003).

REPRESENTATIVES

The first member of the genus *Prochlorothrix*, the type species *P. hollandica* (**Figure 1A**) was validly described according to the rules of Bacteriological Code (Burger-Wiersma et al., 1989). In the modern taxonomic system, it is ascribed to Subsection III of Phylum X. *Cyanobacteria* (Oxygenic Photosynthetic Bacteria) or the order "*Oscillatoriales*" (Castenholz, 2001).

The second member of the genus *Prochlorothrix* is *Prochlorothrix* sp. NIVA 8/90 (**Figure 1B**). This strain was named *P. scandica* (Pinevich et al., 1999) legitimized under the rules of Botanical Code (Skulberg, 2008).

Prochlorothrix sp. NIVA 8/90 is considered as separate species primarily based on genomic DNA differences with *P. hollandica* (**Table 1**). Noteworthy, the previous demand for 3% dissimilarity of 16S rRNA is now considered to be not obligatory in species delimitation (Wayne et al., 1987). *Prochlorothrix hollandica* and *P. scandica* also differ in 16S–23S-ITS, and have one (1,100 bp) and two (1,100 and 620 bp) 16S–23S-ITS, respectively. The larger ITS in *Prochlorothrix* sp. NIVA 8/90 is 99% identical to the single ITS in *P. hollandica*. At the same time, the two ITS in *Prochlorothrix* sp. NIVA 8/90 demonstrate low mutual similarity (Velichko et al., unpublished). In addition, these two cyanobacteria differ by another genetic criterion: the electrophoretic profile of PCR-amplified highly iterated palindromic sequences (HIP-1; Robinson et al., 1995; Smith et al., 1998; Velichko et al., unpublished).

Phenotypic criteria for the species difference between *P. hollandica* and *P. scandica* are cell size, temperature optimum (see below), and fatty acid composition of the membrane lipids (**Table 2**).

Due to the presence of $C_{16:2}$ and $C_{18:2}$ fatty acids both species should be categorized as group 5 cyanobacteria (Cohen et al., 1995). In both species, $C_{14:0}$, $C_{16:0}$, $C_{14:1}$, and $C_{16:1}$ fatty acids are among the major components. The profile of saturated (straight-chain and branched) fatty acids is comparable in the two species, except for a double amount of $C_{18:0}$ stearic acid in *P. scandica*. At the same time, cellular content of unsaturated fatty acids is species-specific. The most prominent differences of *P. scandica* from *P. hollandica* are a twofold increase (8.7% vs 5.0%) in the amount of the $C_{16:1}\omega5$ isomer palmitoleic acid, sevenfold increase (3.4% vs 0.5%) in the amount of $C_{18:1}\omega9$ oleic acid, and a twofold decrease (11.9% vs 21.4%) in the amount of the $C_{16:1}\omega7$ isomer palmitoleic acid. In addition, the trace amounts of dienoic $C_{16:2}\omega4$ and trienoic $C_{16:3}\omega3$ acids are 1.8% vs 1.0% and 1.5% vs 0.7% in *P. scandica* and *P. hollandica*, respectively.

DISTRIBUTION

Evidence for the presence of the genus *Prochlorothrix* has been restricted to eutrophic freshwater or brackish habitats of North Europe (**Figure 2**). *Prochlorothrix hollandica* was accidentally encountered during a routine study of cyanobacterial blooms in the Loosdrecht lake system near Amsterdam (The Netherlands; Burger-Wiersma et al., 1989). These polymictic and highly eutrophic ($\sim$60 mg m^{-3} *chl a*) water pools of average depth $\sim$2 m originate from a flooded turf pit, with phytoplankton represented mostly by blue–green filamentous cyanobacteria of the group *Limnothrix/Pseudanabaena* (Zwart et al., 2005). In addition, "Dutch" green cyanobacteria were also found in a shallow eutrophic Lake Tjeukemeer (van Liere et al., 1989). Finally, according to personal communication by J. van der Does (van Liere et al., 1989), the presence of filamentous green cyanobacteria was registered in other shallow eutrophic Dutch lakes by fluorescence microscopy.

The second representative of the genus *Prochlorothrix, P. scandica* was isolated from Lake Mälaren (1,140 km^2, medium depth $\sim$13 m) near Stockholm, Sweden (Skulberg, 2008).

Based on these discoveries, the representatives of the genus *Prochlorothrix* are common in eutrophic lakes occupied mainly by cyanobacteria (Lewin et al., 1980; van Liere et al., 1989).

In addition to direct evidence for the presence of these cyanobacteria in eutrophic lakes, they have been indirectly detected by the PCR-amplification of 16S rRNA gene and *pcb* genes (Geiß et al., 2003). Natural DNA was obtained from water samples of estuary Darss-Zingst located near the South-West coast of the Baltic Sea between the cities Rostock and Stralsund (Germany). This polymictic and eutrophic water body is represented by a chain of lagoons fed by the mouth of Prerow River, separated from the Baltic Sea by the Peninsula Frischland/Darss, and is characterized by opposite gradients of dissolved organic substances and salinity (Geiß et al., 2004). It is reasonable to expect that dynamic combination of these (a)biotic factors favors the development of cyanobacteria from the genus *Prochlorothrix*.

In summer 2011 *Prochlorothrix*-like 16S rRNA nucleotide sequences were detected in the brackish waters of the Finnish Bay in the environs of St. Petersburg, Russia (Velichko et al., unpublished data). Also, we came across an environmental *Prochlorothrix*-like 16S RNA gene sequence revealed during the metagenomic screening of New Orleans area water systems after Hurricanes Katrina and Rita (Amaral-Zettler et al., 2008).

Phylogenetic reconstruction of all available *Prochlorothrix* 16S rRNA gene sequences demonstrates close clustering of the environmental sequences from different regions of the world within the *Prochlorothrix* group (**Figure 3**). In fact, within the *Prochlorothrix* group sequences of the 16S rRNA gene are 98–99% similar.

In seems that the strains of *Prochlorothrix* detected in brackish coastal waters are merely temporary invaders from the mainland, and they could even sporadically occur in open waters of the Baltic

FIGURE 2 | Sampling location of *Prochlorothrix* strains and *Prochlorothrix*-type environmental DNA.
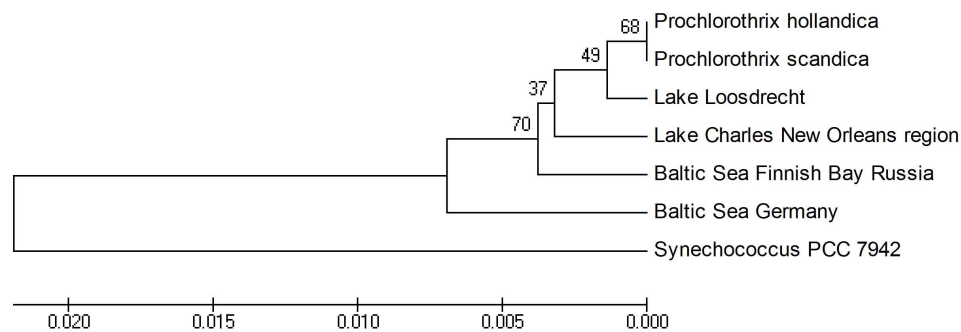
FIGURE 3 | Unrooted dendrogram of a 375-bp fragment of *Prochlorothrix* 16S rDNA sequences obtained by the UPGMA method using MEGA version 4 (Tamura et al., 2007**).** Bootstrap values based on 1,000 replications are shown at the nodes.

Sea (Geiß et al., 2003). At the same time, there is no indication of their presence in any oligotrophic lake.

FUNCTIONAL ECOLOGY

Filamentous green cyanobacteria never dominate the phytoplankton of small polymictic lakes. Growth rate of natural populations of *P. hollandica* is usually 30–40% that of *Oscillatoria*-like blue–green cyanobacteria (0.02–0.14 day^{-1}; see Pel et al., 2004). This is possibly the reason why these blue–green cyanobacteria dominate and even suppress green cyanobacteria in natural environment.

PRIMARY PRODUCTIVITY

The biomass of *Prochlorothrix* which reaches its maximum during the bloom season can be used as a principal nutrient by the zooplankton. In particular, the number of *Euchlanis dilatata* (Rotatoria, Nemathelminthes) which is among the main primary suppliers in the Loosdrecht Lakes (Gulati et al., 1993), is increased in late summer and in early spring which coincides with mass development of *Prochlorothrix*. The intensity of *Prochlorothrix* biomass consumption by this invertebrate is commeasurable with that of the blue–green cyanobacterium *Aphanizomenon flos-aquae* (Gulati et al., 1993).

PHOTOACCLIMATION

Genes *pcbABC* in *P. hollandica* constitute an operon (Nikolaitchik and Bullerjahn, 1998; van der Staay et al., 1998). The antenna proteins pcbAB selectively associate with PSII, while the antenna protein PcbC is attached to PSI (Bumba et al., 2005). Photosystems I and II in *P. hollandica* have the peripheral antennae which produce a 18-meric ring around the PSI trimer and a 14-meric ring around the PSII dimer (Boichenko et al., 2007). *Prochlorothrix hollandica* is the only green cyanobacterium in which a shift of photosynthetic States 1 ↔ 2 (i.e., preferential light channeling) was demonstrated. However, the details of the structural/functional association between the *chl a/b* antenna with photosystems remain obscure. According to one of the two conflicting models, in State 1 the main *chl a/b* antenna (which consists of proteins PcbB and PcbC) transiently associates both PS. In State 2, the minor *chl a/b* antenna (which consists of PcbA protein) dissociates from the super complex of the main *chl a/b* antenna and PSII (Post et al., 1992). In another model (van der Staay and Staehelin, 1994), in State 1 the stacked thylakoids are enriched with the super complex of *chl a/b* antenna and PSII, whereas unstacked thylakoids contain PSI.

Later experimental data suggest a compromise model (Partensky and Garczarek, 2003) based on the spatial redistribution of (de)phosphorylated Pcb proteins.

TEMPERATURE

The culture optimum growth of *P. hollandica* PCC 9006 and *P. scandica* NIVA-8/90 is 20 and 24°C respectively (Pinevich et al., 1999; Skulberg, 2008). However, in nature they were shown to grow at lower temperatures. Compared to other green cyanobacteria, *Prochlorothrix* occupies a narrow geographic range characterized by significant seasonal changes in temperature, as well as by the episodic formation of ice cover in winter time. For instance in July 1984 when *P. hollandica* PCC 9006 was discovered in Lake Loosdrecht the water temperature was 18°C (Burger-Wiersma et al., 1989), whereas in April 1998 it was only 8°C (Zwart et al., 2005). As a rule, mass development of *Prochlorothrix* in this lake occurs in the end of summer when water temperature is 15–18°C (Post and Bullerjahn, 1994).

Water sampling from Lake Mälaren, where *P. scandica* NIVA-8/90 was detected was performed in October 1990. During this period, the temperature is 10–13°C throughout the water column due to the active mixing (Skulberg, 2008).

SALINITY RESPONSE

Cyanobacteria of the genus *Prochlorothrix* are traditionally considered to inhabit freshwater pools, and they are sensitive even to a small increase in salinity. *Prochlorothrix hollandica* PCC 9006 demonstrated poor growth in the presence of 25 mM NaCl, and the growth was fully suppressed at 100 mM or after the addition of sea water equivalent (Burger-Wiersma et al., 1989). Our data on the comparative salt resistance in *P. hollandica* PCC 9006 and *P. scandica* NIVA-8/90 confirm the inhibitory effect of 50–100 mM NaCl (Pinevich et al., unpublished data). Increased salinity provoked morphological changes in both species: cells were elongated, and the filaments became zigzag. At >200 mM NaCl, the filaments of both species degraded after a week growth.

Although high sensitivity to salt can be easily explained by the inability to synthesize osmotic protectors, salt adaptation

may depend on the local ion climate. In particular, *P. hollandica* was resistant to 170 mM NaCl when grown in a semi-synthetic medium supplied with water from the estuary Darss-Zingst. Under these conditions, an increase in the cellular content of sucrose was registered (Bergmann et al., 2008). This data are in agreement with the detection of *Prochlorothrix*-like marker genes in the phytoplankton of the Baltic Sea open waters (Geiß et al., 2003).

INVOLVEMENT IN SYMBIOSES

In contrast to the rest of green cyanobacteria, the strains of *Prochlorothrix* are easily cultured on liquid mineral media (see below). However, most attempts to obtain pure cultures on solid medium proved unsuccessful. Cultures ceased to grow, when attempts to get rid of heterotrophic contaminants were made by the authors. FISH hybridization with taxon-specific probes EUB338II, EUB338III, ALF968, BET42a, GAM42a, and DELTA495a revealed a vast diversity of heterotrophic bacteria associated with *P. hollandica* PCC 9006 and *P. scandica* NIVA-8/90. In case of *P. hollandica* PCC 9006, a positive signal was obtained with probes specific to alpha-, beta-, and gammaproteobacteria. On the other hand, heterotrophic epiphytes of *P. scandica* NIVA-8/90 belong to alpha- and betaproteobacteria (Velichko et al., unpublished data).

These data, along with the fact that bacteria-free filaments of *P. hollandica* PCC 9006 demonstrated negligible growth in liquid culture (Schyns et al., 1997), indicate a mutually favorable consortium-type association between the filamentous green cyanobacteria and their heterotrophic satellites.

CULTIVATION

MEDIA AND STRAINS

Cyanobacteria of the genus *Prochlorothrix* are grown in mineral media FPG, BG-11, and Z8 which contain relatively low amounts of inorganic salts and are routinely used for the cultivation of freshwater blue–green cyanobacteria (**Table 3**). Double-distilled water is necessary. Otherwise, unidentified contaminating chemicals inhibit growth. For slant cultivation, semisolid agar ($\leq 0.5\%$) is recommended, since $\geq 1\%$ inhibits growth. In addition to double-distilled water and mineral salts of high quality, purified agar (e.g., Difco) is needed. Cell growth is suppressed by elevated concentrations of NaCl, and the degree of salt resistance depends on ion content of the medium (Burger-Wiersma et al., 1989; Bergmann et al., 2008). Since *Prochlorothrix* sp. is unable to fix nitrogen even in microaerobic conditions, an addition of combined nitrogen is necessary.

In collections *Prochlorothrix* sp. is represented by several cultured strains. One of them, isolated in the Netherlands and validly described as *P. hollandica* (Burger-Wiersma et al., 1989), is maintained under different strain identifiers (CALU 1027, CCAP 1490/1, NIVA-5/89, PCC 9006, and SAG 10.89). Several multixenic strains isolated in Sweden are stored in the Norwegian Institute for Water Research (Oslo, Norway) collection, namely NIVA 8/90, 9/90, and 16/90 (Skulberg, 2008). Strain *P. hollandica* PCC 9006 (CALU 1027) and strain *P. scandica* NIVA-8/90 (CALU 1205) are stored at the collection of St. Petersburg University (St. Petersburg, Russia). Various methods of axenization were attempted including streaking, repeated washing, and micromanipulation, as well as

Table 3 | Nutrition media for the cultivation of *Prochlorothrix*.

Component	Medium		
	FPG*	BG-11**	Z8***
MACRO NUTRIENTS (mg L^{-1})			
NaHCO$_3$	100.0	–	–
Na$_2$CO$_3$	–	20.0	20.0
NaNO$_3$	500.0	1500.0 (300.0)	467.0
NH$_4$Cl	–	–	31.0
K$_2$HPO$_4$	25.0	40.0	–
MgSO$_4$·7H$_2$O	50.0	75.0	250.0
CaCl$_2$·2H$_2$O	13.0	36.0	–
Ca(NO$_3$)$_2$·4H$_2$O	–	–	59.0
MICRO NUTRIENTS (mg L^{-1})			
FeCl$_3$·2H$_2$O	0.3	–	2.8
H$_3$BO$_3$	2.68	2.86	3.1
MnC1$_2$·4H$_2$O	1.81	1.81	–
MnSO$_4$·4H$_2$O	–	–	2.23
ZnSO$_4$·7H$_2$O	0.22	0.22	0.22
(NH$_4$)$_6$Mo$_7$O$_{24}$·4H$_2$O	0.002	–	0.08
Na$_2$MoO$_4$·2H$_2$O	–	0.39	–
CuSO$_4$·5H$_2$O	–	0.079	–
Co(NO$_3$)$_2$·6H$_2$O	0.08	0.05	0.146
NH$_4$VO$_3$	0.01	–	–
VOSO$_4$·6H$_2$O	–	–	0.054
Al$_2$(SO$_4$)$_3$·K$_2$SO$_4$·2H$_2$O	–	–	0.474
NiSO$_4$·(NH$_4$)$_2$SO$_4$·6H$_2$O	–	–	0.198
Cr(NO$_3$)$_3$·7H$_2$O	–	–	0.037
Cd(NO$_3$)$_2$·4H$_2$O	–	–	0.154
Na$_2$WO$_4$·2H$_2$O	–	–	0.033
KBr	–	–	0.119
KI	–	–	0.083
CHELATORS (mg L^{-1})			
Citric acid	–	6.0	–
EDTA	0.3	1.0	3.7

*Extracted from Burger-Wiersma et al. (1989); **Rippka et al. (1979); ***Kotai (1972). Double-distilled water is generally used.*

differential sensitivity to antibiotics (Burger-Wiersma et al., 1989; Pinevich et al., 1999; Skulberg, 2008). All of them proved unsuccessful because of the loss of viability in both liquid and solid medium. In the latter case, filaments grow not as colonies, but rather as films surrounded by dim layer of colorless heterotrophic bacteria.

GROWTH PARAMETERS

Batch cultures of *P. hollandica* PCC 9006 in FPG medium at $40\,\mu\text{E}\,\text{m}^{-2}\,\text{s}^{-1}$ and 20°C grew at a maximum rate of $0.02\,\text{h}^{-1}$ (Burger-Wiersma et al., 1989). When the BG-11 medium was used, grow rate varied from $0.007\,\text{h}^{-1}$ (Burger-Wiersma et al., 1989) to $0.026\,\text{h}^{-1}$ (Bergmann et al., 2008). Optimum growth temperature for *P. hollandica* PCC 9006 is 20°C (Burger-Wiersma et al., 1989; Pinevich et al., 1999), while that for *P. scandica* is 24°C (Pinevich et al., 1999). Stirring or air bubbling failed to stimulate growth and even resulted in decreased viability due to mechanical damage of the filaments.

MORPHOLOGY

Immotile, non-branching filaments of *Prochlorothrix* sp. grow by equal binary fission of intercalary cells and multiply by fortuitous fragmentation. Hormogonia or other types of differentiated structures are never observed.

Filaments of *P. hollandica* PCC 9006 and *P. scandica* NIVA-8/90 (**Figure 1**) are ≤250 μm long. Mean cell size of *P. hollandica* PCC 9006 is $11.8 \pm 0.9 \times 1.6 \pm 0.1$ μm (length vs width, 7.4:1), while that of *P. scandica* NIVA-8/90 being $7.4 \pm 0.7 \times 2.1 \pm 0.1$ μm (3.5:1). In both cases, the width of cell-to-cell junction is 1/8–1/5 of filament diameter. In this respect, the filaments of *Prochlorothrix* more closely resemble those of *Limnothrix* than those of *Pseudanabaena*. In the latter case, the width of cell-to-cell junction is 1/2 of filament diameter (Castenholz, 2001).

SELECTED FEATURES OF ULTRASTRUCTURE

THYLAKOID STACKING

In addition to unstacked thylakoids which are typical of blue–green cyanobacteria, *P. hollandica* PCC 9006 contains stacked thylakoids vaguely resembling grana thylakoids in chloroplasts (Giddings et al., 1980; Schuster et al., 1984; Miller et al., 1988; Golecki and Jürgens, 1989). In both cases, membrane adhesion is maintained by the PSII proteins and *chl a/b* antenna proteins (van der Staay and Staehelin, 1994).

Thylakoids in freeze-fractured cells of *P. hollandica* PCC 9006 were shown to be laterally heterogeneous (van der Staay and Staehelin, 1994). Similarly to chloroplasts, stacked regions are enriched with globular particles which correspond to the super complexes of PSII with *chl a/b* antenna. By contrast, PSI and ATP synthase are preferentially localized in the unstacked regions (Schuster et al., 1984; van der Staay and Staehelin, 1994).

Membranes of stacked thylakoids in *P. scandica* NIVA-8/90 are covered with particles (viridosomes) which are 10.0 ± 2.3 nm diameter and are located with the periodicity of 20.0 ± 5.6 nm. These are suggested to correspond to the epimembrane domain of *chl a/b* antenna (Pinevich et al., 1999). The analogous particles (xanthosomes) are present in both the chromophyte algae (e.g., *Bacillariophyceae* and *Phaeophyceae*) and *Dinozoa* (Katoh et al., 1993; Green and Durnford, 1996).

Thylakoid stacking in *Prochlorothrix* may help redistribute excitation energy between the adjoining thylakoids (Dekker and Boekema, 2005). Alternatively, it may be a functionless result of segregation of PSII centers in thylakoid membranes (Bullerjahn and Post, 1993; see Functional Ecology and Photoacclimation).

THYLAKOID CENTERS

An intriguing detail of ultrastructure in *P. hollandica* PCC 9006 and *P. scandica* NIVA-8/90, also rarely observed in blue–green cyanobacteria, is the thylakoid center. The latter corresponds to a ~200-nm electron dense zone between cell membrane and two to four radiating thylakoids (Pinevich et al., 1996). These metastable structures possibly channel the molecules of *chl-ide a*$_{GG}$ (the precursor of *chl* a) from the sites of synthesis in cell membrane to the functional sites in thylakoids (Nierzwicki-Bauer et al., 1983; Bryant, 1986; Hinterstoisser et al., 1993).

GAS VESICLES

Similarly to many planktonic filamentous blue–green cyanobacteria, *P. hollandica* PCC 9006 contains honeycomb-like aggregates of gas vesicles. An individual vesicle is a cylinder of 50 nm diameter with conical ends. Its 125–265 nm length mirrors different stages of self-assembly (Golecki and Jürgens, 1989; Pinevich et al., 1999).

CARBOXYSOMES

Similarly to blue–green cyanobacteria, the carboxysomes of *P. hollandica* PCC 9006 are enveloped in a non-unit membrane. However, the carboxysomes of *P. scandica* NIVA-8/90 lack this shell resembling the pyrenoid of green algae and higher plants (McKay and Gibbs, 1990; Morden and Golden, 1991; Pinevich et al., 1996). According to the immune electron microscopy data, all RuBisCO of *P. hollandica* PCC 9006 is associated with carboxysomes. Whereas, the RuBisCO in *P. scandica* NIVA-8/90 exists in both the immobilized and soluble form (Pinevich et al., 1996). Carboxysomes in *Prochlorothrix* were shown to contact thylakoids (Pinevich et al., 1999), which is not the case in blue–green cyanobacteria. This association may promote phosphorylation of ribulose-5-phosphate, as well as the activity of carbonic anhydrase (Pinevich et al., 1999).

GENETICS

Genome size of *Prochlorothrix* is ~5.5 Mb (Post and Bullerjahn, 1994; Schyns et al., 1997) which is within the range of most cyanobacteria. The G + C Mol% of genomic DNA is 51–53 (Herdman, 1981; Coleman and Lewin, 1983; Burger-Wiersma et al., 1989; Dufresne et al., 2003).

GENOME SEQUENCING

Genome sequencing of *P. hollandica* PCC 9006 is currently performed by the authors. Automatic annotation using The RAST Server (Rapid Annotations using Subsystems Technology; Aziz et al., 2008) was performed on the draft sequence, and the manual curation of automatically annotated genes is being done. We are focusing primarily on photosynthetic genes. Phylogenetic affiliation for two of them, namely *psbO* and *psaF*, are given below (see Phylogeny).

ENERGY ASSIMILATION

In contrast to unicellular green cyanobacteria, *Prochlorothrix* has not been tested for dark chemotrophy; the only studied mode of energy assimilation in this case is phototrophy.

PHOTOSYNTHETIC PIGMENTS

Prochlorothrix hollandica PCC 9006 contains accessory *chl b* (Burger-Wiersma et al., 1989). It is suggested to be synthesized from *chl b*$_2$ in one stage using 4-vinyl-reductase (Helfrich et al., 1999). The alternative way involves *chl a* oxygenase which was the enzyme also found in *Prochloron didemni* and higher plants (Tanaka et al., 1998; Tomitani et al., 1999; Nagata et al., 2005).

Among the carotenoids in *P. hollandica* PCC 9006 are β-carotene and zeaxanthin (van der Staay et al., 1992). The content of β-carotene in *P. hollandica* PCC 9006 is four times that of zeaxanthin. The former preferentially associates with PSI, while the latter complexes with 56/58 kDa proteins integrated in the cell membrane (Omata et al., 1985; Engle et al., 1991).

LIGHT-HARVESTING ANTENNA

In contrast to blue–green cyanobacteria, *P. hollandica* PCC 9006 and *P. scandica* NIVA 8/90 possess the light-harvesting antenna

containing both *chl a* and *chl b* (Bryant, 1986; Burger-Wiersma and Post, 1989; Hess et al., 1996; Marquardt et al., 1998). Although it was originally thought that Pcb proteins of this antenna share the origin with CAB proteins in chloroplasts, in fact they have no similarity (La Roche et al., 1996; van der Staay et al., 1998). In particular, Pcb proteins of *P. hollandica* PCC 9006 do not react with antibodies raised against CAB proteins of green algae and higher plants (Hiller and Larkum, 1985; Bullerjahn et al., 1990; van der Staay and Staehelin, 1994). At the same time, Pcb proteins of *P. hollandica* PCC 9006 are similar to PsbB and PsbC (CP43) proteins of PSII core antenna. In addition, they share similarity with *IsiA* (CP43′) protein of PSI stress antenna which is formed in blue–green cyanobacteria under iron starvation (Bullerjahn et al., 1987, 1990; van der Staay and Staehelin, 1994; Nikolaitchik and Bullerjahn, 1998; van der Staay et al., 1998; Partensky et al., 1999). Hexadomain proteins Pcb/Cp43/*IsiA* vary in the length of the loop exposed at the thylakoid exoplasmic surface between helices V and VI (Herbstová et al., 2010).

Genes *pcbABC* of *P. hollandica* PCC 9006 form an operon (Nikolaitchik and Bullerjahn, 1998; van der Staay et al., 1998). The genes *pcbA* and *pcbB* genes belong to the evolutionary lineage of *IsiA* and code for polypeptides of 32 and 33 kDa. The third gene, *pcbC*, belongs to a separate lineage and codes for a polypeptide of 38 kDa (van der Staay and Staehelin, 1994; van der Staay et al., 1998; Garczarek et al., 2000).

Pcb proteins aggregate in multimeric super complexes (Bibby et al., 2001, 2003; Chen et al., 2002, 2005; Partensky and Garczarek, 2003) which surround the oligomers of PSI and PSII (Bibby et al., 2003; Boichenko et al., 2007). PcbA/PcbB proteins build up the mobile antenna of both photosystems, while the PcbC proteins associate with a portion of antenna tightly bound to PSI (Herbstová et al., 2010).

PHOTOSYSTEMS

PSI polypeptides of *P. hollandica* PCC 9006 are the same as in blue–green cyanobacteria (Schuster et al., 1984; Bullerjahn et al., 1987; van der Staay et al., 1998; Garczarek et al., 2000). They include major PsaA/PsaB proteins (66 kDa) and 6 minor proteins PsaC–F, I–M (20 kDa; Burger-Wiersma and Post, 1989; van der Staay et al., 1993). PSII proteins of *P. hollandica* PCC 9006 include CP43/CP47, D1/D2, PsbH, and PsbO, as well as low molecular weight polypeptides PsbJ and PsbK (Greer, 1991; Greer and Golden, 1992; Mor et al., 1993; Post and Bullerjahn, 1994). Interestingly, *psbA* gene has a 3′-terminal 21-nucleotide deletion; the same deletion is present in the chloroplast ortholog (Morden and Golden, 1991).

The oxygen evolving complex (OEC) in *P. hollandica* PCC 9006 lacks proteins PsbP (17 kDa) and PsbQ (23 kDa) typical of chloroplasts (Mor et al., 1993). The interaction of OEC with Cl— and Ca2+ is provided by the hydrophobic Mn-stabilizing protein PsbO (37 kDa) different from the more amphiphilic analog in blue–green cyanobacteria (26.5 kDa; Mor et al., 1993; Barber and Kühlbrandt, 1999).

FACULTATIVE ANOXYGENIC PHOTOSYNTHESIS

Among green cyanobacteria, P. *hollandica* PCC 9006 is uniquely capable of photosynthesis with sulfide as electron donor and,

respectively, without dioxygen production (Post and Arieli, 1997). OEC of this cyanobacterium retains activity at ≤ 1 mM sulfide. High resistance to sulfide stress is provided by the PsbO protein which protects the catalytic site of OEC (Mor et al., 1993; Post and Arieli, 1997). The transition to anoxygenic photosynthesis occurs at >1 mM sulfide when this substance inhibits both the biogenesis and activity of PSII, especially that of OEC.

A relative resistance to sulfide allows *P. hollandica* dominate the phytoplankton of water bodies with sulfide accumulating in warm seasons (Post and Arieli, 1997). Given the trophic state of shallow waters harboring *Prochlorothrix*, sulfide production in the anoxic sediments may mix with surface waters. Thus, sulfide resistance may be an essential adaptation in this habitat.

SUBSTRATE ASSIMILATION AND ENZYMOLOGY
CARBON ASSIMILATION

Data on the ability to use organic substrates (photomixotrophy or photoheterotrophy, in the light; chemoheterotrophy, in darkness) in *Prochlorothrix* is absent. Similarly to most blue–green cyanobacteria, *Prochlorothrix* demonstrates photoautoptrophy based on Calvin cycle with RuBisCO as key enzyme. *Prochlorothrix hollandica* PCC 9006 contains IB-subform of RuBisCO which clusters together with RuBisCO of blue–green β-cyanobacteria from the so-called "green" phylogenetic branch of the enzyme (Morden and Golden, 1991; Shimada et al., 1995; Chen et al., 2004; Badger et al., 2006).

RuBisCO of *P. hollandica* PCC 9006 is encoded by the dicistronic operon *rbcLS* which consists of genes for the large (L, 57 kDa) and small (S, 13 kDa) subunits, respectively. The holoenzyme (560 kDa) belongs to the L_8S_8-type (Hawthornthwaite et al., 1990; Morden and Golden, 1991). Enzymatic activity is maximum at 30°C and pH 8; Mg^{2+} is used as a cofactor (Hawthornthwaite et al., 1990).

NITROGEN ASSIMILATION

There are no data on the ability of *P. hollandica* PCC 9006 for aerobic nitrogen fixation. Initial attempts to induce nitrogenase under the conditions of nitrogen starvation proved unsuccessful (Burger-Wiersma et al., 1989). Furthermore, there are no *nif* genes sequences in the partially reconstructed genome of this cyanobacterium (Pinevich et al., unpublished data).

At the same time, *P. hollandica* PCC 9006 assimilates both ammonium and nitrate. Maximum growth rate in culture conditions is observed at 0.75 mM NH_4^+, whereas growth is inhibited at 3 mM. Growth is also absent if 1.5 mM urea substitutes for nitrate, indicating lack of the urease enzyme (Burger-Wiersma et al., 1989).

PHOSPHORUS ASSIMILATION

According to field experiments, the most extensive growth of *P. hollandica* PCC 9006 takes place in those parts of water bodies where phytoplankton is limited by phosphorus deficiency (Burger-Wiersma et al., 1989). *Prochlorothrix hollandica* PCC 9006 is characterized by higher maximum rate of phosphorus uptake and by lower saturation constant for this substrate as compared to other filamentous cyanobacteria, in particular *Planktothrix* sp. (Ducobu et al., 2002; Dignum et al., 2005).

Special enzymology: RNAse P

This enzyme performs the maturation of 5′-end of tRNA and is ubiquitous in prokaryotes including plastids and mitochondria. *Prochlorothrix hollandica* PCC 9006 contains a rare type of this RNAse characterized by long domain P15/16, as well as by a GGU motif which binds 3′-CCA of pre-tRNA and is absent from the ribozymes of other cyanobacteria (Fingerhut and Schön, 1998).

PHYLOGENY

Tracing phylogeny of green cyanobacteria raises the questions regarding: (i) relations within the green cyanobacteria group; (ii) relations between green and blue–green cyanobacteria; (iii) relations between green cyanobacteria and simple plastids; (iv) the ancestor of green cyanobacteria; and (v) the origin of chlorophyll light-harvesting antenna.

Here, two phylogenies are involved. One of them is the general evolution of cyanobacteria reconstructed by the comparative analysis of the major informational (in particular, 16S rNNA gene and *rpoS*) and photosynthetic genes (in particular, *psbA* and *petE*). Secondly, it is the evolution of green cyanobacteria traced by the analysis of the *pcb* genes coding for the chlorophyll light-harvesting antenna.
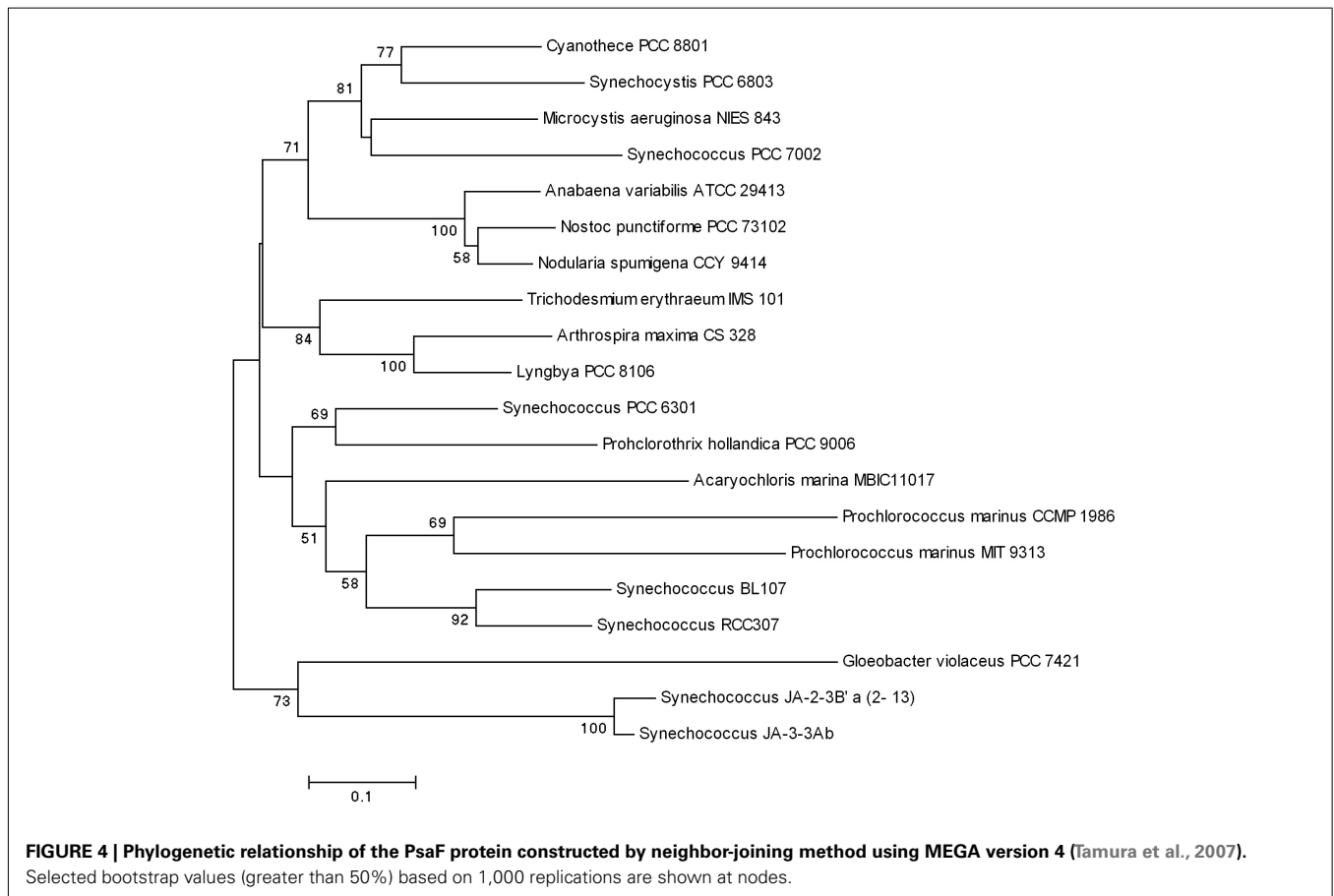
16S rRNA GENE

According to this evolutionary criterion, green cyanobacteria are polyphyletic (Urbach et al., 1992). Paradoxically, *P. hollandica* PCC 9006 clusters close to the unicellular strain *Synechococcus* PCC 6301 (Pinevich et al., 2010). Detailed dendrograms showing the distribution of green cyanobacteria between separate clades of cyanobacterial tree, as well as mutual subordination of strains from the same genus, are presented elsewhere (Turner, 1997; Moore et al., 1998; Partensky et al., 1999; Miyashita et al., 2003; Partensky and Garczarek, 2003; Rocap et al., 2003; de los Rios et al., 2007; Münchhoff et al., 2007; Scanlan et al., 2009).
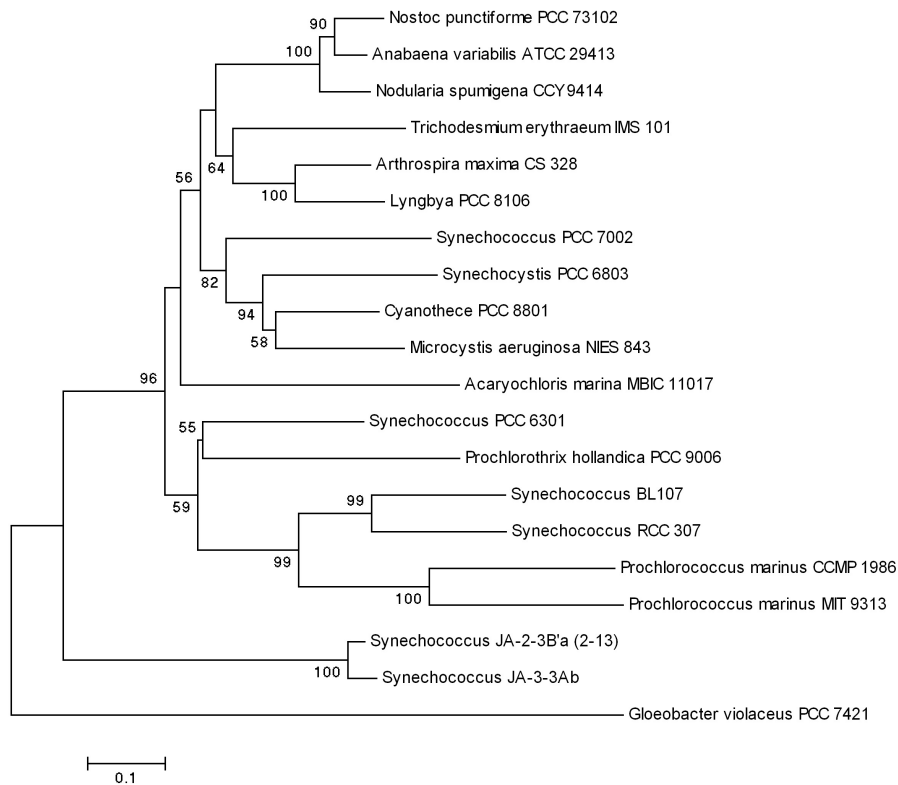
RpoC GENE

Another informational gene which helps reconstruct the evolutionary history of cyanobacteria is *rpoC1* coding for a subunit of the DNA dependent RNA polymerase. Here, again, green cyanobacteria were shown to be polyphyletic, and *P. hollandica* PCC 9006 groups with *Synechococcus* PCC 6301 (Palenik and Haselkorn, 1992).

The phylogenies of the major photosynthesis genes [*psaF*, coding for the conservative PsaF protein, a 21-kDa three-domain component of the reaction center complex; *psbA*, coding for the 32 kDa core PSII protein; *psbO*, coding for the Mn-stabilizing protein PsbO; and *petE*, coding for the copper-containing soluble protein plastocyanin (∼11 kDa), which donates electrons to PSI] repeat that of the 16S rRNA gene with green cyanobacteria being polyphyletic and *P. hollandica* PCC 9006 clustering with *Synechococcus* PCC 6301 (**Figures 4** and **5**; Pinevich et al., 2010).

FIGURE 4 | Phylogenetic relationship of the PsaF protein constructed by neighbor-joining method using MEGA version 4 (Tamura et al., 2007). Selected bootstrap values (greater than 50%) based on 1,000 replications are shown at nodes.

FIGURE 5 | Phylogenetic relationship of PsbO protein constructed by neighbor-joining method using MEGA version 4 (Tamura et al., 2007**).** Selected bootstrap values (greater than 50%) based on 1,000 replications are shown at nodes.

Pcb GENES

The *pcbABC* genes of *P. hollandica* PCC 9006 which code for the antenna proteins of family Pcb/*IsiA*/CP43 cluster together with their counterparts in unicellular green cyanobacteria from the genera *Acaryochloris* and *Prochloron* (Pinevich et al., 2010). The highly conserved proteins of Pcb/*IsiA*/CP43 family are thought to be inherited from a common ancestor of cyanobacteria. They might evolve according to two scenarios.

According to the first scenario, these proteins may have appeared in *Synechococcus*-like ancestor of *P. marinus* which inhabited oceanic systems poor in iron (Ting et al., 2002). In these stress conditions, they constitutively expressed both the *IsiA* (CP43′) protein and the phycobilisome. Among the descendants of this hypothetical cyanobacterium were phycoerythrin-rich strains of *Synechococcus*, as well as phycobiliprotein free or phycobiliprotein-poor (phycobilisome free) strains of *P. marinus*.

By the second scenario, *pcb* and *isiA* genes evolved independently by duplication and subsequent specialization of the copied *psbC*-like gene in a protocyanobacterium (Chen et al., 2005). This scenario is more plausible since it explains the existence of a set of *chl*-binding proteins in blue–green and green cyanobacteria.

Both scenarios assume a transient coexistence of the *chl*-based antenna with the phycobiliprotein-based antenna (Shimada et al., 2003).

GENES CODING FOR ENZYMES OF THE CHROMOPHORE BIOSYNTHESIS

The evolution of these genes is distinct from that of the *pcb* genes. In fact, presence of the conservative His residues in Pcb proteins is only a prerequisite of *chl* binding. In other words, the formation of *chl*-containing antennae, in particular the *chl a/b* antenna, needs biosynthetic enzymes for the respective chromatophores. Since the ancestor of Pcb proteins is thought to be *chl a*-binding protein (see above), the incorporation of *chl b*, a_2, b_2, and *d* in antennae of different green cyanobacteria suggests the formation of these pigments as byproducts in the metabolic pathway of *chl a*. In fact, monovinyl *chls* are synthesized from divinyl precursors by reductases, whereas *chl b* and *chl d* are formed by oxygenases (Beale, 2008).

The *cao* genes coding for *chl a* oxygenase in *P. hollandica* PCC 9006 and *P. didemni* are highly similar (Tomitani et al., 1999). Correspondingly, these green cyanobacteria did not acquire this gene independently, but rather the ability to synthesize *chl b* was a trait of common ancestor of cyanobactetria.

The evolutionary loss of phycobilins resulted in the appearance of green cyanobacteria like *P. hollandica* PCC 9006 and *P. didemni*, while the loss of *chl b* resulted in the lineage of blue–green cyanobacteria, which allows to consider both these green cyanobacteria as a model for the protocyanobacterium (Tomitani et al., 1999).

RELATION OF GREEN CYANOBACTERIA TO THE ORIGIN OF THE SIMPLE CHLOROPLAST

Although cyanobacterial origin of the simple chloroplast is beyond doubt (Morden et al., 1992; McFadden, 1996), the specific nature of ancestry is debatable.

The *Prochloron*-type green cyanobacteria were rejected as a candidate, since Pcb proteins are dissimilar with the members of CAB family (La Roche et al., 1996; van der Staay et al., 1998). This conclusion prematurely deprived green cyanobacteria of any role in the origin of the simple chloroplast.

However, it should be noted that Pcb proteins cluster with PsbC/*IsiA*(CP43′) proteins (Bullerjahn et al., 1987, 1990; van der Staay and Staehelin, 1994; Nikolaitchik and Bullerjahn, 1998; van der Staay et al., 1998; Partensky et al., 1999). This finding, as well as data on the relationships between green cyanobacteria and their blue–green relatives (see above) help restore the role of the former as both the ancestor of the chloroplast, as well as the ancestor of oxygenic phototrophic bacteria.

CHLOROPLAST ORIGINS

The ancestor of chloroplasts is hypothesized to possess both the *pcb* genes, coding for *chl*-binding antenna proteins, and the *pbp* genes coding for phycobilisome (Tomitani et al., 1999). This cyanobacterium, engulfed by a non-photosynthetic protist, became a precursor of three lineages of the simple chloroplasts (**Figure 6**).

One lineage is represented by the chloroplast of *Chlorophyceae*/*Charophyceae*/*Plantae* which lost the ancestral *pcb* and *pbp* genes. The loss of "old" *chl a*/*b*-binding Pcb proteins was compensated by the acquisition of convergent "new" *chl a*/*b*-binding CAB proteins.

Two other lineages are represented by the chloroplasts of *Glaucocystophyceae* and *Rhodophyceae* which lost the ancestral *pcb* genes but retained the ancestral *pbp* genes.

CYANOBACTERIAL ORIGINS

Cyanobacteria are hypothesized to share the ancestor with chloroplasts, i.e., the primordial cyanobacterium had both the *pcb* genes, coding for *chl*-binding antenna proteins, and the *pbp* genes coding for phycobilisome (Tomitani et al., 1999). This cyanobacterium became a precursor of two lineages of cyanobacteria (**Figure 6**).
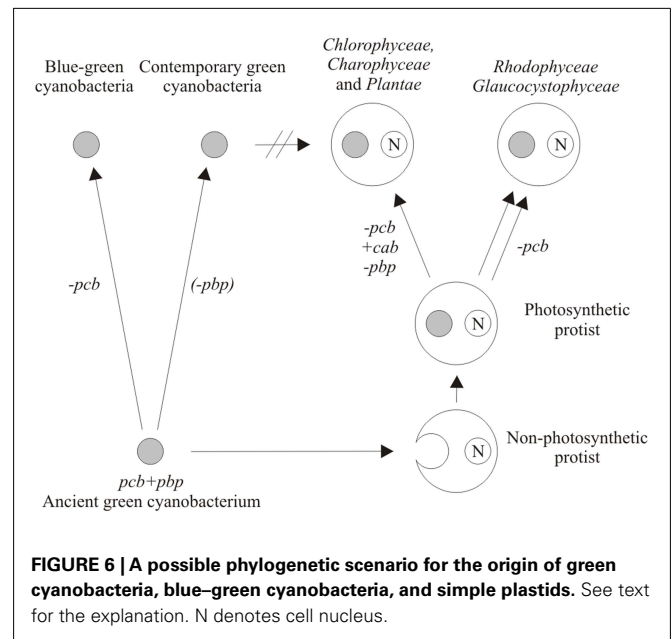
One lineage, resulting from the loss of ancestral *pcb* genes is represented by blue–green cyanobacteria. The other lineage originating from the complete or partial loss of ancestral *pbp* genes is represented by green cyanobacteria. Noteworthy, the latter were not chloroplast ancestors.

FIGURE 6 | A possible phylogenetic scenario for the origin of green cyanobacteria, blue–green cyanobacteria, and simple plastids. See text for the explanation. N denotes cell nucleus.

Interestingly, the genes responsible for *chl b* synthesis were divergently inherited by green cyanobacteria and chloroplasts.

GENERAL CONCLUSION

The primordial cyanobacterium which possessed both the *pcb* and *pbp* genes is by definition a green cyanobacterium Therefore, the ancient green bacteria should be reconsidered as key players of the evolutionary process which resulted in the biodiversity of cyanobacteria and plastids.

CONCLUDING REMARKS

Cyanobacteria of the genus *Prochlorothrix*, in contrast to the other cyanobacteria, are both very rare and largely incapable of axenic growth. They combine trivial features (primarily, oxygenic photosynthesis) with specific, yet obscure aspects of homeostasis and competitive potential. The analysis of their environmental and symbiotic ecology can help better understand this uniqueness. Thereby, the complete annotation of *P. hollandica* PCC 9006 genome is important, as well as search and isolation of new strains of the genus.

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