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Prochlorococcus marinus nov. gen. nov. sp.: an oxyphototrophic marine prokaryote containing divinyl chlorophyll *a* and *b*

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Abstract. Several years ago, prochlorophyte picoplankton were discovered in the N. Atlantic. They have since been found to be abundant within the euphotic zone of the world's tropical and temperate oceans. The cells are extremely small, lack phycobiliproteins, and contain divinyl chlorophyll *a* and *b* as their primary photosynthetic pigments. Phylogenies constructed from DNA sequence data indicate that these cells are more closely related to a cluster of marine cyanobacteria than to their prochlorophyte 'relatives' *Prochlorothrix* and *Prochloron*. Several strains of this organism have recently been brought into culture, and herewith are given the name *Prochlorococcus marinus*.

Key words: Prokaryote – Prochlorophyte – *Prochlorococcus marinus* – Cyanobacteria – Picoplankton – Molecular phylogeny – Divinyl chlorophyll

Over a decade ago, Johnson and Sieburth (1979) published the first electron micrographs documenting the diversity of chroococcoid cyanobacteria in the sea. The members of one group, which they called "Type II" cells, were distinct from the others in that their peripheral thylakoids were closely appressed to one another. The cells were isolated from deep water, were "difficult to culture", were hypothesized to lack phycoerythrin, and were, at one point, suspected to being nitrifying bacteria (Sieburth 1983). Their true identity remained unclear until several years ago, when we associated them with the flow cytometric signature of abundant red-fluorescing picoplankton in seawater samples (Chisholm et al. 1988). The cells were not in culture at that time, but we inferred from fluorescence measurements of field samples that they did indeed lack phycobiliproteins and, more significantly, contained chlorophyll *b*. We were also able to establish from field samples that the cells contained "red-shifted" chlorophyll *a* (Gieskes and Kraay 1983, 1986) as their major chlorophyll, which we hypothesized to be divinyl chlorophyll *a*. We called the cells "prochlorophytes" (sensu Lewin 1981) until they could be cultured and described in more detail.

Here we review briefly the progress that has been made in characterizing and culturing these cells, and establish for this organism a new genus and species: *Prochlorococcus marinus*.

Distribution and abundance

The abundance and ecology of prochlorophyte picoplankton is most well established for the N. Atlantic (Li and Wood 1988; Neveux et al. 1989; Olson et al. 1990), but they are much more cosmopolitan than this. Through flow cytometric analysis, they have been shown to be abundant in the Mediterranean Sea (Vaulot et al. 1990), equatorial Pacific (Chavez et al. 1991), subtropical N. Atlantic (Veldhuis and Kraay 1990), Panama Basin, Gulf of Mexico, Caribbean, and the Southern California Bight (Chisholm et al. 1988). If the presence of "red-shifted" or divinyl chlorophyll *a* in the water column signals the presence of these cells, as has been shown to be the case for the Sargasso Sea (Goericke 1990) and the subtropical N. Atlantic (Veldhuis and Kraay 1990), prochlorophytes are also abundant in the tropical Atlantic (Gieskes and Kraay 1983, 1986) and the Banda Sea (Gieskes et al. 1988). Finally, through inference from pigment ratios, Everitt et al. (1990) have provided evidence for abundance of prochlorophytes in the western tropical Pacific. The cells are usually found at concentrations between 10^4 to 10^5 cells ml^{-1} throughout the euphotic zone, and dominate the subsurface chlorophyll maximum layer in highly stratified waters during the summer at temperate latitudes (Olson et al. 1990).

Isolation and culture

The first successfully cultured isolate of this organism was obtained by B. Palenik from a depth of 120 m in the Sargasso Sea during May 1988. Subsequently, two additional isolates have been cultured from the Mediterranean Sea and the N. Atlantic using the same procedures

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(D. Vaulot and F. Partensky, personal communication). Trace-metal clean techniques (Fitzwater et al. 1982) were used for the sample collection and processing, including the use of GOFLO bottles on a Dacron coated Kevlar line. Once collected, the prochlorophytes were separated from *Synechococcus* by filtering the sample twice successively through 0.6 μm pore size Nuclepore filters by gravity. This removed 99–100% of the *Synechococcus*, and allowed about 5% of the prochlorophytes to pass through the filter. The efficacy of the fractionation procedure in terms of excluding *Synechococcus* was monitored by flow cytometric analysis at sea (Olson et al. 1990).

Following fractionation, five to ten milliliter portions of the samples were transferred into polycarbonate tubes and enriched with single or combined additions of 2 μM phosphate (β-glycerophosphate) and 2 μM nitrogen (as urea). The chelator CPTC (50–100 μM cis,cis,cis,cis-1,2,3,4-cyclopentanetetracarboxylic acid, Aldrich Milwaukee, Wis., USA, neutralized with Na_2CO_3), without metal additions, was added to some tubes, while others were enriched with the EDTA-based "K/10 (-Cu)" trace metal mix of Keller et al. (1987) (Table 1). After isolation, samples were incubated at 18 °C in a shipboard incubator on a 14:10 L:D cycle at light levels between 1 and 15 μE m^{-2} s^{-1} (0.07 to 1.0% surface irradiance) supplied by cool white fluorescent lamps, passed through 1/8″ thick blue plexiglass for the initial isolations. The cultures were monitored using an EPICS V flow cytometer modified for high sensitivity (Olson et al. 1990), or, more recently, using a Becton Dickinson (San Jose, Calif., USA) FACScan. Both the CPTC and EDTA trace metal mixes, with added urea and glycerophosphate, led to growth of the prochlorophytes.

Since the initial isolation of the cells, we have shown that they grow quite well with inorganic nitrogen and phosphorus sources (Table 1), with growth rates of about 0.50 day^{-1} at photon flux densities of 60 μE m^{-2} s^{-1} on a 14:10 L:D photocycle at 24 °C. The cells reach densities of greater than 10^8 cells ml^{-1} in K/10 (-Cu) medium (Table 1), but transfer best during exponential growth at densities of less than 5×10^7 cells ml^{-1}. Although flow cytometry is necessary to enumerate the cells at low densities, in vivo fluorometry (Brand et al. 1983) can be used to monitor them at high densities, when they are approaching stationary phase.

Our isolates are not bacteria free. All attempts at growing the cells on agar plates have been unsuccessful, and since the contaminating bacteria outnumber *Prochlorococcus*, they cannot be eliminated through dilution. We have, however, established clonal cultures of both the Sargasso and Mediterranean Sea strains by serial dilution.

Since these cells require the special treatment necessary for maintaining phytoplankton from oligotrophic oceans, they have been deposited in the Center for Culture of Marine Phytoplankton at the Bigelow Laboratories (see below) rather than at the American Type Culture Collection.

Table 1. Media for the isolation and culture of marine prochlorophytes. For isolation, 2 μM urea and glycerophosphate were added to the seawater, along with CPTC (100 μM) or the "K/10 (-Cu)" trace metal mix. After isolation, either of the full media described below can be used for culturing the cells. Either filtered Sargasso seawater or artificial seawater (Morel et al. 1979) can be used as a basal medium. The basal media can be tyndallized (Brand et al. 1983), microwaved (Keller et al. 1988) or sterilized by autoclaving in teflon bottles. The trace metals with chelator can be made as one stock solution (10^4 X). "K/10 (-Cu)" medium is derived from the K medium described in Keller et al. 1987. Elevated concentrations of nitrogen and phosphorus can be used for higher cell yields

Nutrient	"CPTC-based"	"K/10 (-Cu)"
Urea	20 μM	
NH_4Cl		50 μM
Glycerophosphate	10 μM	
NaH_2PO_4		10 μM
EDTA		10 μM
CPTC	100 μM	
Trace metals		
$FeCl_3$	0.1 μM ($FeSO_4$)	1.2 μM
$MnCl_2$	10 nM	90 nM
$ZnCl_2$		8 nM
$CoCl_2$		5 nM
Na_2MoO_4	10 nM	3 nM
Na_2SeO_3		10 nM

Ultrastructure and pigments

Transmission electron micrographs of the cultured cells confirm that they do indeed appear to be the same organism that was sectioned and described by Johnson and Sieburth (1979) and Chisholm et al. (1988) in samples collected from the deep euphotic zone in the Sargasso Sea (Fig. 1). The pigments of the cultured cells have been analyzed in detail by Goericke and Repeta (1992), and generally confirm our earlier report from the field samples (Table 2). Goericke and Repeta (1992) have demonstrated that the "red-shifted" chlorophyll that we and others have observed in field samples is indeed divinyl chlorophyll *a*, as postulated earlier (Chisholm et al. 1988). In addition, all of the chlorophyll *b* in the Sargasso Sea isolate is of the divinyl type; we were mistaken when we reported this pigment to be "normal" chlorophyll *b* from our analysis of the field samples (Chisholm et al. 1988). The cells also contain zeaxanthin, α-carotene, an unidentified carotenoid, and a chlorophyll *c*-like pigment, possibly Mg 3,8-divinylpheoporphyrin a_5. This suite of pigments is unique among oxygenic phototrophs, and distinctly different from that of *Prochloron* and *Prochlorothrix*, which contain "normal" (monovinyl) chlorophyll *a* and *b*, zeaxanthin, and β-carotene.

Molecular phylogenetic position of *P. marinus*

A portion of the DNA-dependent RNA polymerase gene (Palenik and Haselkorn 1992) and most of the 16S ribosomal RNA gene (Urbach et al. 1992) of this organism

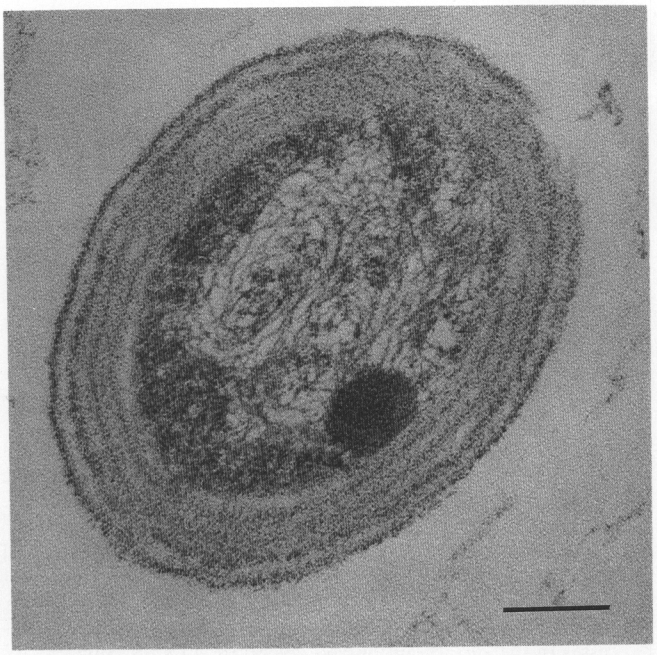

Fig. 1. Electron micrograph of a thin section of *P. marinus* (strain CCMP-1375). The cells were grown at 20 °C in a 14-h light (10 μE m^{-2} s^{-1})/10-h dark cycle in "K/10(-Cu)" medium. The ultrastructural features of the cultured cells are similar to those of cells concentrated and fixed directly from natural seawater samples (Chisholm et al. 1988). The possession of closely appressed photosynthetic thylakoids is the primary ultrastructural feature that distinguishes *P. marinus* from open-ocean strains of *Synechococcus* sp. *Scale bar* = 100 nm

Table 2. Pigment composition of cultured *Prochlorococcus marinus* (Strain CCMP-1375). The cells were grown at 18 °C on a 10:14 L:D cycle at a photon flux density of 25 μE m^{-2} s^{-1} (Goericke and Repeta 1991). This suite of pigments is distinct from that of other known prochlorophytes, which contain monovinyl chlorophyll *a* and *b*, zeaxanthin, and β-carotene

Pigment	Concentration (fg $cell^{-1}$)
Divinyl chlorophyll *a*	3.11
Divinyl chlorophyll *b*	3.56
Zeaxanthin	2.15
α-Carotene	0.90
Chlorophyll *c*-like pigment	0.29
Unknown carotenoid	0.24

have been sequenced and analyzed to assess the relationship of *Prochlorococcus marinus* to other prochlorophytes, cyanobacteria, and plant chloroplasts. The analysis of both gene sequences yields the same general picture: *Prochlorococcus marinus* is phylogenetically affiliated with cyanobacteria of marine cluster A of the *Synechococcus* Group (Waterbury and Rippka 1989) which includes the closely related *Synechococcus* clones WH8103 and WH7805. The phylogenetic tree constructed from the 16S rRNA sequences indicates unequivocally that *Prochlorococcus* is distinct from the *Prochloron* and *Prochlorothrix* lineages, and the shallowness of the cluster containing *Prochlorococcus* and marine cluster A of *Synechococcus* suggests a relatively recent origin for the *Prochlorococcus* pigment phenotype. The demonstration that the prochlorophytes are derived from multiple lineages within the cyanobacterial radiation indicates that *Prochlorococcus marinus* should be included in the cyanobacteria (Palenik and Haselkorn 1992; Urbach et al. 1992).

None of the known prochlorophytes are on the lineage that leads to green plant chloroplasts (Turner et al. 1989; Morden and Golden 1989a, b; Urbach et al. 1992; Palenik and Haselkorn 1992). It appears most likely, therefore, that the three groups of prochlorophytes and the chloroplast ancestor lost phycobiliproteins and gained chlorophyll *b* independently, in convergent evolutionary events.

Formal description of *P. marinus*

Prochlorococcus nov. gen.: *Prochlorococcus* [Pro·chlo·ro·coc′·cus. Gr. pref. *pro*, before (primitive); Gr. adj *chloros*, green; M.L. masc n. *coccus*, berry. Primitive green sphere.]. The prokaryotic cells are spherical to rod shaped just prior to division, occur singly or in pairs and divide by transverse binary fission. They possess a gram-negative cell wall and have peripheral photosynthetic thylakoids that are closely appressed to one another. Phycobilisomes are absent. They are oxygenic photoautotrophs that possess divinyl chlorophylls *a* and *b* as their principal photosynthetic pigments, and lack phycobiliproteins.

Prochlorococcus marinus nov. sp.: *Prochlorococcus marinus* (ma·ri′·nus. *marinus* L. adj., marine). Cells are 0.6 to 0.8 μm in diameter and 1.2 to 1.6 μm in length just prior to division. In addition to divinyl chlorophylls *a* and *b*, *P. marinus* contains zeaxanthin, α-carotene, an unidentified carotenoid, and a chlorophyll *c*-like pigment possibly Mg 3,8-divinylpheoporphyrin a_5.

P. marinus occurs at high cell concentrations in the euphotic zone of the open oceans. In the temperate oceans its distribution and abundance follow seasonal patterns. Two strains of *P. marinus* are in culture; both strains are clonal (derived from a single cell through serial dilution) but neither is bacteria-free. The strains were isolated from the Sargasso Sea in May 1988 (28° 58.9′ N, 64° 21.5′ W, by B. Palenik) and the Mediterranean Sea in Jan. 1989 (43° 12.15′ N, 6° 52′ E, by D. Vaulot and F. Partensky). Both are grown in minimal media with a seawater base at light intensities of 60 μE m^{-2} s^{-1}, which is saturating for growth. The strain from the Sargasso Sea grows optimally at 25 °C and fails to grow above 29 °C.

The Sargasso Sea strain (CCMP-1375) of *P. marinus* has been designated the type strain. It, along with the Mediterranean strain (CCMP-1378), is deposited at the Center for the Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME 04575, USA.

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