***Prochlorococcus marinus* Chisholm et al. 1992 subsp. *pastoris* subsp. nov. strain PCC 9511, the first axenic chlorophyll a_2/b_2 -containing cyanobacterium (*Oxyphotobacteria*)**

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The formal description of *Prochlorococcus marinus* Chisholm et al. 1992, 299 was based on the non-axenic nomenclatural type, strain CCMP 1375^T. The purification and properties of the axenic strain PCC 9511, derived from the same primary culture (SARG) as the type species, are reported here.

***Prochlorococcus* PCC 9511 differs from the latter in possessing horseshoe-shaped thylakoids, exhibiting a low chlorophyll b_2 content and lacking phycoerythrin, but shares these phenotypic properties with *Prochlorococcus* strain CCMP 1378. This relationship was confirmed by 16S rRNA sequence analyses, which clearly demonstrated that the axenic isolate is not co-identical with the nomenclatural type. Strain PCC 9511 has a low mean DNA base composition (32 mol% G+C) and harbours the smallest genome of all known oxyphotobacteria (genome complexity 1.3 GDa = 2 Mbp). Urea and ammonia are the preferred sources of nitrogen for growth, whereas nitrate is not utilized. Several different organic phosphorus compounds efficiently replace phosphate in the culture medium, indicative of ecto-phosphohydrolase activity. In order to distinguish strain PCC 9511 from the nomenclatural type, a new subspecies is proposed, *Prochlorococcus marinus* Chisholm et al. 1992 subsp. *pastoris* subsp. nov.**

Keywords: *Prochlorales*, cyanobacteria, nitrogen assimilation, phosphohydrolases, 16S rRNA

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Abbreviations: chl, chlorophyll; HL, high light; LL, low light; PPFD, photosynthetic photon flux density; PE, phycoerythrin; T_m , temperature mid-point of denaturation.

The GenBank accession numbers for the 16S rRNA sequences of PCC 9511, CCMP 1426 and NATL1 are AF180967, AF133833 and AF133834, respectively.

INTRODUCTION

In the last edition of *Bergey's Manual of Systematic Bacteriology*, the class *Oxyphotobacteria* was subdivided into the *Cyanobacteria*, whose ordinal recognition still awaits validation, and the order *Prochlorales* Lewin 1977 (Castenholz & Waterbury, 1989). In the light, like algae and plants, these photosynthetic prokaryotes use H_2O for the generation of chemical energy and reducing power and liberate molecular O_2 as a by-product. The demand for cellular carbon is met by CO_2 fixation. Cyanobacteria synthesize monovinyl chlorophyll a (chl a_1) and harvest light by aid of water-soluble multimeric complexes, the phycobilisomes, composed of biliproteins and linker polypeptides

(Glazer, 1987, 1989; Sidler, 1994). In contrast, oxyphotobacteria of the order *Prochlorales* Lewin 1977 either lack phycobiliproteins entirely, or synthesize only trace amounts (Lewin, 1977, 1989; Burger-Wiersma *et al.*, 1986; Chisholm *et al.*, 1992; Hess *et al.*, 1996). Their light-harvesting complexes are membrane-associated proteins containing chlorophyll *a* (a_1 or a_2) and chlorophyll *b* (b_1 or b_2) as the major photosynthetic pigments (Burger-Wiersma *et al.*, 1986; Goericke & Repeta, 1992; Partensky *et al.*, 1997). In spite of these unifying traits, the representatives of the *Prochlorales*, *Prochloron didemni* Lewin 1977, *Prochlorothrix hollandica* Burger-Wiersma *et al.* 1986 and *Prochlorococcus marinus* Chisholm *et al.* 1992, do not form a monophyletic lineage but evolved in multiple and independent events within the cyanobacterial radiation (Turner *et al.*, 1989; Kishino *et al.*, 1990; Lockhart *et al.*, 1992; Palenik & Haselkorn, 1992; Urbach *et al.*, 1992; Hess *et al.*, 1995). Consequently, the order *Prochlorales* is no longer justified, its members merely being atypical representatives of the Cyanobacteria (Palenik & Haselkorn, 1992; Urbach *et al.*, 1992, 1998; Pinevich *et al.*, 1997).

The genus *Prochlorococcus* was created for two non-axenic isolates, both of which were assigned to a single species, *P. marinus* Chisholm *et al.* 1992, 299 (Chisholm *et al.*, 1992). The holotype, strain CCMP 1375^T, originated from a depth of 120 m in the Sargasso Sea, near the bottom of the euphotic zone, whereas strain CCMP 1378 was isolated from surface water (5 m) of the northwestern Mediterranean Sea (Chisholm *et al.*, 1992; Partensky *et al.*, 1993). The former strain is also known as SS120, whereas the latter has been cited as MED4 (Moore *et al.*, 1995; Partensky *et al.*, 1999). Both isolates have very small, coccoid to rod-shaped cells (diameter 0.6–0.8 µm) that exhibit red fluorescence when excited by blue light (488 nm) (Chisholm *et al.*, 1992; Goericke & Repeta, 1992). Representatives of this novel group of oxygenic phototrophs, first characterized by aid of flow cytometry, pigment analysis and electron microscopy (Chisholm *et al.*, 1988, 1992; Li & Wood, 1988), are ubiquitous and abundant (10^4 – 5×10^5 cells ml⁻¹) in the water column (0–200 m) of most tropical and temperate oceans (for a review, see Partensky *et al.*, 1999). They generally dominate the bottom of the euphotic zone (100–200 m), whereas in the upper layers (0–100 m) they often co-exist at variable ratios with phycoerythrin-rich unicellular cyanobacteria assigned to *Synechococcus* (see Partensky *et al.*, 1999), a botanical form genus encompassing several distinct genetic clusters (Herdman *et al.*, 1979b; Waterbury & Rippka, 1989; Urbach *et al.*, 1998; Honda *et al.*, 1999).

Members of the genus *Prochlorococcus* synthesize divinyl-chlorophyll *a* and *b* (a_2 and b_2) (Goericke & Repeta, 1993), may contain monovinyl-chlorophyll *b* (b_1) (Partensky *et al.*, 1993; Moore *et al.*, 1995), but lack monovinyl chlorophyll *a* (a_1) (Goericke & Repeta, 1993; Partensky *et al.*, 1993; Moore *et al.*, 1995). Minor quantities of a chlorophyll *c*-like pigment

(Mg, 3-8 divinyl phaeoporphyrin a_3) and α -carotene, rather than β -carotene, are additional characteristic features (Chisholm *et al.*, 1992; Goericke & Repeta, 1992, 1993). Most isolates lack phycobiliproteins, but the holotype *Prochlorococcus marinus* CCMP 1375^T synthesizes small amounts of a novel phycoerythrin (PE), called PE-III (Hess *et al.*, 1996, 1999). Based on sequence analyses of the respective genes (*cpeA* and *cpeB*), PE-III is distinct both from the common cyanobacterial phycoerythrin, C-PE-I and from PE-II, mainly found in marine cyanobacteria (Ong & Glazer, 1991; Swanson *et al.*, 1991). However, none of the *Prochlorococcus* isolates examined, including strain CCMP 1375^T, contain phycobilisomes (Chisholm *et al.*, 1992; Lichtlé *et al.*, 1995; Hess *et al.*, 1999). Therefore, the cellular location and role of PE-III in the latter strain remains unknown, although a function as a light-harvesting pigment has recently been suggested (Lokstein *et al.*, 1999).

On the basis of their pigment composition and genetic affiliations inferred by 16S rRNA sequence analyses, *Prochlorococcus* isolates have been assigned to a 'low-light-adapted clade' (LL) and to a 'high-light-adapted clade' (HL) (Urbach *et al.*, 1998; Partensky *et al.*, 1999; West & Scanlan, 1999). The former is mainly composed of ecotypes originating from deep waters (80 m or more) and exhibiting high chl *b/a*₂ ratios (0.4–2.4) (Moore *et al.*, 1995; Urbach *et al.*, 1998). The members of the HL clade are thought to preferentially inhabit the more illuminated surface layers and are characterized by much lower chl *b/a*₂ ratios (< 0.15) (Goericke & Repeta, 1993; Moore *et al.*, 1995, 1998; Urbach *et al.*, 1998; Partensky *et al.*, 1999). They also differ with respect to light sensitivity, the LL representatives being photoinhibited at irradiances that are optimal for growth of the members of the HL clade (Moore *et al.*, 1995, 1998; Urbach *et al.*, 1998). However, two strains (MIT 9302 from the Sargasso Sea and MIT 9312 from the Gulf Stream) seem to be somewhat exceptional in having a chl *b/a*₂ ratio of about 0.7, considered to be typical of the LL clade (see above), but being genetically assignable to the HL clade (Moore *et al.*, 1998). It therefore seems possible that pigment signatures are not always sufficient to predict phylogenetic affiliations among *Prochlorococcus* isolates or field populations and that the apparent correlation of light penetration in the habitat with pigment phenotype and genotype may be oversimplified. Furthermore, the use of light-harvesting pigments as a discriminatory taxonomic marker requires caution since, as shown for phycobiliprotein-rich cyanobacteria (Tandeau de Marsac & Houmard, 1993; Grossman *et al.*, 1994; Liotenberg *et al.*, 1996), their complement may to be influenced by the nutritional status of the cells. The latter, however, is difficult to assess at present, since all previous studies, including the description of the type species *P. marinus* CCMP 1375^T, were performed on impure cultures that, depending on their original habitat or degree of contamination, harbour a commensal bacterial flora

of rather variable metabolic properties which may affect pigment composition. In order to permit defined physiological studies and sequence analyses of a wider range of genes (i.e. beyond those encoding components of the photosynthetic apparatus or the highly conserved *rrn* genes), we attempted to purify the type strain of the genus *Prochlorococcus*. In this paper, we report some physiological and genetic properties of *P. marinus* strain PCC 9511, the first axenic isolate. A new subspecies, *Prochlorococcus marinus* subsp. *pastoris*, is proposed.

METHODS

Cyanobacterial strains. The axenic strain PCC 9511, deposited in the Pasteur Culture Collection of Cyanobacteria (PCC, Institut Pasteur, Paris, France) was purified from a primary culture of the genus *Prochlorococcus* originally designated 'LG' (Palenik & Haselkorn, 1992; Palenik, 1994) or 'SARG' (Partensky *et al.*, 1993; Morel *et al.*, 1993). It had been kindly provided in 1990 to F. Partensky by L. Moore and S. W. Chisholm (MIT, Cambridge, USA) and was maintained in the Roscoff Culture Collection (France) until 1993, from where it was sent to the principal investigator of the present study. This isolate, rendered axenic in 1995, was first thought to be co-identical with two non-axenic clonal isolates equally derived from 'LG' and designated 'SSW5' (Urbach *et al.*, 1992) and 'SS120' (Moore *et al.*, 1995). The latter was described as the nomenclatural type, *P. marinus*, strain CCMP 1375^T and deposited in the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Chisholm *et al.*, 1992). The characterization of strain PCC 9511, however, demonstrated that it is phenotypically and genotypically distinct from the type strain (see Results). *Synechococcus* PCC 7001, used for comparative photomicroscopy, has been described previously (Rippka *et al.*, 1979).

Growth media. The media employed during this study are shown in Table 1. The slightly modified Gaffron's trace metal solution (Rippka, 1988) used for most of the early

media (PCR-S8 to PCR-S16) contained the following (μM): H₃BO₃ (300); MnSO₄·H₂O (60); Na₂WO₄·2H₂O (0·6); (NH₄)₆Mo₇O₂₄·4H₂O (0·4); KBr (6·0); KI (3·0); ZnSO₄·7H₂O (6·0); Cd(NO₃)₂·4H₂O (3·0); Co(NO₃)₂·6H₂O (3·0); CuSO₄·5H₂O (3·0); NiCl₂·6H₂O (3·0); Cr(NO₃)₃·9H₂O (0·6); VSO₅·5H₂O (0·6); KAl(SO₄)₂·12H₂O (6·0); SeO₂ (3·0). The trace metal stock solution A5 + Co (Rippka, 1988) for media PCR-SA₆ and PCR-Tu₂ was diluted 10-fold prior to use. For both trace element solutions, the compounds were dissolved separately in sterile water, mixed, and adjusted to the final volume. The Na₂-EDTA/FeCl₃ complex was prepared by dissolving FeCl₃·6H₂O (270 mg) and Na₂-EDTA (372 mg) in 10 ml HCl (0·1 M) and 10 ml NaOH (0·1 M), respectively, before mixing and diluting with sterile water to a final volume of 500 ml (Skulberg, 1990). All stock solutions added to the autoclaved seawater were filter-sterilized (0·22 μm, Millex GP; Millipore) and stored at 4 °C. The recipe of Turks Island Salts solution (Merck Index no. 9954) used for the artificial seawater medium PCR-Tu₂ is as follows (mM): NaCl (480); MgSO₄ (28); MgCl₂ (27); KCl (9); CaCl₂ (10). To avoid precipitation, the latter salt was dissolved in 1 vol. H₂O and added only after all other ingredients had each been dissolved separately in aliquots of a total of 9 vols. The vitamin mix 8A (Table 1) was purchased from Sigma (ref. P 9931), its composition being described by McLachlan (1973).

Growth on solid media. Purification by isolation of single colonies was attempted with several different media (PCR-S8, PCR-S11, PCR-S12 and PCR-Tu₂) using different solidifying agents (washed agar A 8678, agarose A6013 and A 9539, Sigma; agarose Litex LSL 4000, FMC) at 0·6 and 0·8 % (w/v). For the former three seawater-based media, the solidifying agents were dissolved and autoclaved separately in H₂O (20 % of the final volume), cooled to 50 °C, and then added to 4 vols pre-warmed medium. For the artificial medium PCR-Tu₂, both the Turks Island Salts mix and the solidifying agents were prepared at double strength, before mixing equal volumes after sterilization and cooling. The appropriate nutrient supplements were added prior to pouring the plates.

Purification. Based on the known correspondence (R.

Table 1. Media employed for *Prochlorococcus* PCC 9511

Components	PCR-S8	PCR-S10	PCR-S11	PCR-S12	PCR-S16	PCR-SA ₆	PCR-Tu ₂
Seawater (autoclaved) (ml)	900	1000	1000	1000	1000	1000	
Seawater (filter-sterilized) (ml)	100						
Turks Island Salt solution* (ml)							1000
HEPES-NaOH (pH 7·5) (mM)	1	1	1	1	1	1	1
NaHCO ₃ (mM)							2
Trace metals 'Gaffron + Se'* (ml l ⁻¹)	0·5	0·5	0·5	0·5	0·05		
Trace metals A5 + Co* (ml l ⁻¹)						0·5	0·5
Na ₂ -β-glycerophosphate (μM)	20	50					
Na-PO ₄ (pH 7·5) (μM)			50	50	5	50	50
Na ₂ -EDTA/FeCl ₃ * (μM)	8	8	8	8	1·6	8	8
(NH ₄) ₂ SO ₄ (μM)	125	400	400	400	40	400	400
Vitamin B ₁₂ (μg l ⁻¹)	10	10	10			10	10
Vitamin mix 8A† (ml l ⁻¹)				1	0·1		

* Prepared or modified as described in Methods.

† For composition, see McLachlan (1973).

Rippka, unpublished) of OD_{750} (1.0), cell size (diameter $2.5\ \mu\text{m}$), cell volume ($8.18\ \mu\text{m}^3$) and viable cell counts ($2\text{--}3 \times 10^7\ \text{ml}^{-1}$) for *Synechocystis* PCC 6803, it was estimated that an $OD_{750} = 0.1$ of *Prochlorococcus marinus* (cell diameter about $0.5\ \mu\text{m}$) would correspond to about $2\text{--}3 \times 10^8$ cells ml^{-1} . Consequently, for the purification of clonal isolates, serial dilutions (10^{-1} – 10^{-9}) were performed in Pyrex test tubes (18 ml) containing 7 ml growth media (see Results and Table 1). Dilutions were 10-fold up to 10^{-8} , but were twofold in the final range. The tubes were incubated at $18\ ^\circ\text{C}$, receiving white light (Osram White Universal White) of a photosynthetic photon flux density (PPFD) of $10\ \mu\text{mol quanta m}^{-2}\ \text{s}^{-1}$ (measured with a LICOR LI-185B quantum/radiometer/photometer equipped with a LI-190SB quantum sensor) over a light/dark cycle of 14 h/10 h. Every 14 d, loss of liquid by evaporation was compensated for by addition of an appropriate volume of sterile water.

Purity tests. A putative axenic culture, as judged by oil immersion phase-contrast microscopy, was tested for purity on plates of medium ASNIII (Rippka *et al.*, 1979), supplemented with glucose and Casamino acids (0.2 and 0.02 %, respectively) and solidified with Difco Bacto agar (1 %). After several days' incubation in the dark (at $18\ ^\circ\text{C}$ and at room temperature), and in contrast to the original impure culture, bacterial growth was not observed. Purity was also confirmed on plates of medium PCR-S11, solidified with Sigma washed agar (0.8 %, w/v) and containing 5 % (v/v) of medium Luria–Bertani (Sambrook *et al.*, 1989), in addition to the aforementioned organic supplements. Finally, the lack of bacterial contaminants was confirmed by experiments in liquid media, testing for the growth-promoting effects of a wide range of different organic compounds (see below).

Growth experiments. The effect of various sources of nitrogen and phosphate or other growth supplements was examined in duplicate or triplicate, and was judged visually by comparison to control cultures lacking the additions. These experiments were performed in test tubes (18 ml) containing 6 ml of the growth medium specified. Incubation was at $20\ ^\circ\text{C}$ and fluorescent tubes (Claude/GTE Blanc Industriel) were used to provide a PPFD of $40\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ over a light/dark cycle of 14 h/10 h.

Large-scale cultivation. Cell material for the extraction of chromosomal DNA was accumulated over the course of 4 months. For this, the cultures (58 in total) were grown in medium PCR-S11 (200 ml), individually prepared from stock solutions in Erlenmeyer flasks (500 ml), and incubated at $18\ ^\circ\text{C}$ under the conditions described for the serial dilutions (see above). The volumes for inoculation (20–30 ml) were adjusted depending on the densities of the pre-cultures, whose growth phase (in the range of OD_{750} 0.10–0.15) was monitored by measurements in plastic cuvettes (3 ml, 1 cm light path) in a Kontron Uvikon 860 spectrophotometer. The cells were harvested after 10–14 d by centrifugation for 30 min at $20\ ^\circ\text{C}$ and 6000 *g*. The resulting pellets, containing a large amount of salt precipitate from the seawater-based medium, were purified by resuspension in 20 ml sterile seawater or NaCl (150 mM) and centrifugation for 2 min at 300 *g* and $20\ ^\circ\text{C}$. The supernatants were recentrifuged for 15 min at 20000 *g*. The pellets were collected in 2 ml screwcap microtubes (Sarstedt), centrifuged again for 15 min and finally stored at $-20\ ^\circ\text{C}$ until use.

Ultrastructure. For transmission electron microscopy, cells

from a culture (200 ml, OD_{750} 0.13) grown under the conditions described for large-scale cultivation (see above) were concentrated by centrifugation and fixed for 90 min at $4\ ^\circ\text{C}$ in glutaraldehyde (2 %, v/v) in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.25 M sucrose. Post-fixation was performed for 120 min with OsO_4 (1 %, v/v). After dehydration, the cells were embedded in Spurr medium resin. Sectioning and viewing were performed as described by Lichtlé *et al.* (1995).

Spectrometry. Absorption spectra were recorded in a Kontron Uvikon 933 spectrophotometer, on cell suspensions (1 ml, OD_{750} 0.08–0.13) and after concentration of 2 ml by centrifugation (15 min at $20\ ^\circ\text{C}$ and 20000 *g*) and extraction for 30 min in 1 ml acetone (90 %, v/v) at room temperature in the dark. In order to reduce light scattering, *in vivo* spectra were recorded by subtracting the absorbance of a depigmented cell sample, obtained by addition of 20 μl household bleach ('Eau de Javel') and incubation for 30 min. Both *in vivo* and acetone spectra were standardized to represent the absorbance values of cultures (1 ml) of an $OD_{750} = 0.1$ and, for the former, the OD_{750} was subtracted. Fluorescence excitation and emission spectra were performed on a 15-fold concentrated cell suspension at room temperature using a Perkin Elmer LS50 spectrofluorometer.

Pigment analyses. For pigment determinations, triplicate samples (25 ml) were filtered on Whatman GF/F at very low vacuum pressure ($< 5\ \text{mm Hg}$) and analysed by reverse-phase HPLC on a Zorbax ODS C18 column ($25 \times 4.6\ \text{cm}$), according to Kraay *et al.* (1992) with the modifications reported by Garczarek *et al.* (1998).

Analysis by SDS-PAGE and Western blotting. A polyclonal antiserum to the α subunit of phycoerythrin (α PE) of *Prochlorococcus marinus* CCMP 1375^T was raised in rabbits by immunization with the recombinant fusion protein expressed in *Escherichia coli* (Hess *et al.*, 1999). Western blots of *Prochlorococcus* PCC 9511 were prepared from total protein extracts separated on 15 % SDS-polyacrylamide gels and blotted on PVDF membranes (NEN Life Sciences) using a semi-dry blotter (Bio-Rad). Total proteins of *Chamaesiphon* PCC 6605, a cyanobacterium that contains high levels of C-phycoerythrin (Bryant, 1982), as well as the α PE recombinant fusion protein of *P. marinus* CCMP 1375^T, were included as positive controls. Incubation with IgG-purified anti- α PE-serum as a primary antibody was performed at a titre of 1:600 for 1 h at room temperature. The secondary antiserum (anti-rabbit IgG; Sigma) was conjugated with alkaline phosphatase and used at a titre of 1:8000. The blots were developed using the chromogenic substrates nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate from Sigma.

Mean DNA base composition and genome size. DNA of *Prochlorococcus* PCC 9511 was extracted from the pooled pellets (1.75 g wet wt) and purified on hydroxyapatite as described by Herdman *et al.* (1979b). The mean DNA base composition (mol% G+C) was calculated from the temperature midpoint of denaturation (T_m) following thermal denaturation of DNA (OD_{260} approx. 0.3 in $0.1 \times \text{SSC}$, pH 7.0; $\text{SSC} = 150\ \text{mM NaCl}$, 15 mM sodium citrate), as described by Herdman *et al.* (1979b), except that a Kontron Uvikon 933 spectrophotometer was employed, in the dual-beam mode. The temperature of the DNA solutions in sealed cuvettes (1.0 ml total volume, 1 cm light path) was increased at a rate of $0.1\ ^\circ\text{C min}^{-1}$. The reference cuvette contained a solution of guanine (OD_{260} 0.02 units less than

that of the DNA) to correct for electronic drift of the instrument and for solvent expansion. The temperature probe of the instrument was calibrated by denaturing cyanobacterial DNA samples of known base composition (Herdman *et al.*, 1979b): *Synechocystis* sp. PCC 6308, 34.7 mol% G+C; *Oscillatoria agardhii* PCC 7821, 40.0 mol% G+C; *Arthrospira platensis* PCC 7345, 44.3 mol% G+C; *Synechocystis* sp. PCC 6803, 47.5 mol% G+C; *Synechococcus elongatus* PCC 6301, 55.1 mol% G+C; *Cyanobium gracile* (previously *Synechococcus* sp.) PCC 6307, 69.7 mol% G+C. The mean T_m of *Prochlorococcus* strain PCC 9511 DNA was determined in duplicates in three independent denaturation experiments, in which DNA of *Synechocystis* sp. PCC 6308 was included to control for daily fluctuations in the sensitivity of the temperature probe.

Genetic complexity (genome size) was determined from the kinetics of renaturation of DNA at the optimal temperature of renaturation. DNA was thermally denatured in $0.1 \times$ SSC and the renaturation reaction was initiated by addition of an appropriate pre-warmed volume of NaCl to bring the final concentration of Na^+ to 1 M (Herdman *et al.*, 1979a; Schyns *et al.*, 1997). The molecular mass of the genome of *Prochlorococcus* strain PCC 9511 was calculated from the second-order renaturation rate constant k_2 , determined as described by Schyns *et al.* (1997), and by reference to that of cyanobacteria of known genetic complexity (Herdman *et al.*, 1979a).

Analysis by PFGE. Inclusions for PFGE analyses were performed by modification of a protocol described by Churin *et al.* (1995). Cells (250 ml of a culture containing approx. 10^8 cells ml^{-1}) were concentrated by centrifugation, transferred to Eppendorf microtubes and washed twice in 2 ml diluted seawater (50%, v/v, and 20%, v/v, for the first and second wash, respectively), supplemented with 50 mM EDTA, pH 8.0. The pellet was resuspended in 1 ml of the second washing solution and embedded by mixing with an equal volume of low-melting-point agarose (1.2%, w/v; BRL), dissolved in 50 mM EDTA, pH 8.0 and equilibrated to 50 °C. After immediate transfer into plug formers, the agarose inclusions were hardened consecutively at room temperature (0.5–1 h) and at 4 °C (0.5–1 h). They were then cut into pieces ($5 \times 8 \text{ mm}^2$) and digested for 16 h at 55 °C with proteinase K (0.1% in a total volume of 5 ml 0.5 M EDTA, pH 8.0, containing 1%, w/v, SDS). Subsequently the agarose plugs were washed three times in TE (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and twice in TE supplemented with fresh PMSF for 30 min each. Prior to use and equilibration in the appropriate restriction enzyme buffer, samples were washed again three times in TE. Restriction was performed overnight at 37 °C (*Mlu*I, *Not*I) or at 25 °C (*Sma*I). Separation of restriction fragments was performed with a CHEF-MAPPER (contour-clamped homogeneous field electrophoresis; Bio-Rad Laboratories) in 1% agarose gels in $0.5 \times$ TBE (Sambrook *et al.*, 1989) at a temperature of 13.8 °C, using the auto algorithm option. The exact conditions for the separation of fragments in the range 5–30 kbp were 9.0 V cm^{-1} for the forward voltage gradient and 6.0 V cm^{-1} for the reverse voltage gradient. The initial swap time was 0.11 s, the final swap time 0.46 s. For the separation of fragments in the range 30–300 kbp, the voltage gradient was 6 V cm^{-1} throughout, and the initial and final swap times were 2.16 and 26.29 s, respectively. After the run was complete, the bands were stained with ethidium bromide ($1 \mu\text{g ml}^{-1}$) and photographed by a UVP Image Store 7500 System.

Determination of 16S rRNA gene sequences. The 16S rRNA gene of *Prochlorococcus* PCC 9511 was amplified by PCR in a Perkin-Elmer Cetus Gene Amp 9600 Thermal Cycler, using chromosomal DNA prepared from cultures (25–50 ml) grown to an OD_{750} of about 0.05–0.1, as described by Cai & Wolk (1990). The reactions were performed in 500 μl microtubes (MoBiTec) containing the following: 10 μl of $10 \times$ Taq polymerase buffer (Appligène); 10 μl dNTP (2.5 mM); 5 μl (100 ng μl^{-1}) each of primers A2 (5'-AGA-GTTTGATCCTGGCTCAG-3') and S17 (5'-GGCTACC-TTGTTACGAC-3') (*E. coli* numbering 8–27 and 1494–1510, respectively, Ruimy *et al.*, 1994); 200 ng of *Prochlorococcus* chromosomal DNA and 5 U Taq polymerase (Appligène). The volumes were adjusted to 100 μl with sterile high-purity H_2O . The thermal cycling profiles and cycle numbers were: one cycle of denaturation (95 °C) for 3 min, annealing at 51 °C for 2 min and extension at 72 °C for 30 s; 25 cycles at 94 °C (30 s), 51 °C (2 min) and 72 °C (1.5 min); final cycle of 94 °C (30 s), 51 °C (1 min) and 72 °C (5 min), followed by storage at 10 °C. The PCR products were analysed for the expected size (about 1450 bp) by gel electrophoresis, purified using the Wizard PCR PREPS DNA purification system (Promega), and cloned into the plasmid pGEM-T (Promega) as suggested by the manufacturer. Transformation into *E. coli* DH5 α was performed by electroporation (Bio-Rad Gene Pulser II, Pulse Controller PLUS module) following the procedures provided in the manual. Extraction of plasmid DNA from 12 pooled clones was performed with the kit Nucleobond (Macheray–Nagel). Sequences were determined manually for both strands using the T7 sequencing kit (Promega) and the primers described by Ruimy *et al.* (1994). They were reconfirmed by automatic sequencing (Genome Express). The complete 3' end of the 16S rRNA was determined after amplification and sequencing of the intergenic transcribed spacer (ITS) of the *rrn* operon using primers 322 (5'-TGACACACCGCCCGTC-3') and 340 (5'-CTCTGTGT-GCCTAGGTATCC-3'), complementary to regions near the 3' end of the 16S rRNA and the 5' end of the 23S rRNA genes, respectively (Willmott *et al.*, 1993; Itean *et al.*, 1999). The 16S rRNA sequences of *Prochlorococcus* strain NATL1 and CCMP 1426 (Partensky *et al.*, 1993; Morel *et al.*, 1993; Scanlan *et al.*, 1996) were determined as described previously (Urbach *et al.*, 1998).

Phylogenetic analyses. Sequences of 16S rRNA were obtained from GenBank under the following accession numbers: *Cyanobium gracile* (previously *Synechococcus* sp.) PCC 6307, AF001477; *Prochlorococcus marinus* CCMP 1375^T, X63140; *Prochlorococcus marinus* CCMP 1378, AF001466; *Prochlorococcus* sp. GP2, AF001472; *Prochlorococcus* sp. MIT 9107, AF001468; *Prochlorococcus* sp. MIT9302, AF053396; *Prochlorococcus* sp. MIT9303, AF053397; *Prochlorococcus* sp. MIT9312, AF053398; *Prochlorococcus* sp. MIT9313, AF053399; *Prochlorococcus* sp. NATL2, AF001470; *Prochlorococcus* sp. NATL2A, AF001467; *Prochlorococcus* sp. PAC1, AF001471; *Prochlorococcus* sp. SB, AF001473; *Prochlorococcus* sp. TATL1A, AF001474; *Prochlorococcus* sp. TATL1B, AF001475; *Prochlorococcus* sp. TATL2, AF001476; *Synechococcus* sp. Biwa-B0, D50614; *Synechococcus* sp. Biwa-G0, D50615; *Synechococcus* sp. Biwa-P0, D50616; *Synechococcus elongatus* PCC 6301, X01296, X01982 and X03538; *Synechococcus* sp. PCC 7001, AB015058; *Synechococcus* sp. WH 7805, AF001478; *Synechococcus* sp. WH 8101, AF001480; *Synechococcus* sp. WH 8103, AF001479; uncultured Clone 100, M63813; uncultured Clone 139,

M63814; uncultured SARG6, X52169; uncultured SARG7, X52171. The sequences of strains PCC 9511, CCMP 1426 and NATL1 have been deposited in GenBank with the accession numbers AF180967, AF133833 and AF133834, respectively.

The sequences were aligned, in the ARB program (<http://www.mikro.biologie.tu-muenchen.de/pub/ARB>), with the PCC database that now contains 315 cyanobacterial 16S rRNA sequences. Phylogenetic trees were inferred with distance matrix methods in the PHYLIP package (Felsenstein, 1993) and by fastDNAm1 (Olsen *et al.*, 1994), excluding regions that either carried gaps in more than 50% of the sequences, or where precise alignment was doubtful. The tree shown was inferred with fastDNAm1 and is consistent with the other methods used; it was extracted from a larger tree based on approximately 110 complete (or almost) cyanobacterial sequences in order to avoid artefacts in branching order that arise when a limited data set is employed (M. Herdman, unpublished results).

RESULTS

Purification and growth properties

The impure culture *Prochlorococcus* strain SARG (see Methods) was initially cultivated in medium K/10-Cu (Chisholm *et al.*, 1992). However, the maximal cell densities obtained even near the stationary phase of growth were too low to be measurable by spectroscopy without concentrating the cultures. Consequently, new growth media were developed and tested. Growth of the impure strain proved superior in media PCR-S8, PCR-S10, PCR-S11 and PCR-S12 (see Table 1), in the latter three giving rise to lemon-green suspensions of optical densities (OD_{750}) of 0.1–0.15. However, purification attempts by classical streaking techniques on plates prepared with these media, using different solidifying agents (see Methods) proved unsuccessful. If growth was achieved, single colonies were only observed after several months in the heavily contaminated parts of the plates. Therefore we attempted purification by serial dilution in PCR-S8, PCR-S10 and PCR-S11 liquid media, choosing a range of dilutions appropriate for the expected initial number of cells ($1\text{--}4 \times 10^8$ cells ml^{-1}) based on cell size and volume. However, growth at higher dilutions ($> 10^{-5}$) proved inconsistent and, if observed, only occurred in tubes still harbouring one or more of the commensal bacterial contaminants. Eventually, after many independent attempts, microscopic inspection of a culture derived from a 2×10^8 -fold dilution in medium PCR-S12 revealed that it contained *Prochlorococcus*, accompanied by a common laboratory contaminant (a yellow sarcina forming mucilaginous aggregates and exhibiting relatively large cells), but lacked the small marine bacteria typical of the original impure culture. This suggested that the contaminant had modified the medium by some unknown means (e.g. detoxification) to permit growth of the initial single cell theoretically expected in the range of $1\text{--}4 \times 10^8$ -fold dilutions. The fortuitously large cell size of the contaminant allowed further purification attempts by selective elimination. For this, a culture was centrifuged ($3 \times$) at low speed

(300 g), and the final supernatant was diluted (10^{-1} – 10^{-3}) in two different media, PCR-S12 and PCR-S16 (Table 1), with and without the addition of either glutamine (50 μM) or methionine (50 μM). Microscopic examination of all cultures resulting from these dilutions suggested the purity of one of them, raised in medium PCR-S16 and supplemented with glutamine. This was confirmed by extensive purity tests (see Methods). The axenic isolate entered the Pasteur Culture Collection of Cyanobacteria under strain number PCC 9511 and is also available from the American Type Culture Collection, Manassas, VA, USA (ATCC 700925).

Experiments comparing growth of the axenic strain PCC 9511 in media PCR-S16, PCR-S11, PCR-S12, with or without glutamine (50 μM), revealed neither a beneficial effect of Provasoli's vitamin 8A supplements nor an obligate requirement for the amino acid. Thus, the successful isolation of the axenic strain in the more nutrient-poor medium PCR-S16 (see Table 1), containing vitamin mix 8A and glutamine, was coincidental. Since growth yields in medium PCR-S11 were superior to those obtained in medium PCR-S16, the former was initially chosen as the maintenance medium for the axenic strain (but see below). In this medium *Prochlorococcus* PCC 9511 reaches an OD_{750} of 0.1–0.15 (corresponding to about $3\text{--}4 \times 10^8$ cells ml^{-1}) within 10–14 d from an initial OD_{750} of about 0.01, if incubated at 20 °C and receiving a PPFD of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ over a light/dark cycle of 14 h/10 h. The mean growth constant (μ) was in the range 0.23–0.3 d^{-1} .

Sources of phosphate

In the absence of phosphate, cultures of strain PCC 9511 ceased to grow upon the third transfer, whereas replacement of Na_2PO_4 (pH 7.5) in medium PCR-S11 by $\text{Na}_2\beta$ -glycerophosphate, Na_4 -pyrophosphate, glucose 6-phosphate and ATP at equimolar concentrations (100 μM) resulted in good growth rates and yields over the same period of subculturing. These observations indicate that *Prochlorococcus* is capable of synthesizing one or more phosphohydrolysing enzymes.

Sources of combined nitrogen

Replacement of ammonia in medium PCR-S11 by urea, nitrate, nitrite, arginine, glutamine and glutamate, at the same concentration (400 μM), resulted in comparable growth only with urea, whereas the other compounds gave rise to culture densities similar to controls that lacked a source of combined nitrogen. Urea has been used as the standard source of nitrogen for impure cultures of *Prochlorococcus* (Chisholm *et al.*, 1992; Partensky *et al.*, 1993), but urease activity of these oxyphototrophs, rather than of the commensal bacterial contaminants, could only be deduced reliably with the axenic strain.

Effect of diverse compounds on growth

In an attempt to increase the growth rates and yields, or to discover stimulatory factors eventually permitting growth of single colonies on solid media, a variety of additions were tested in PCR-S11. The following compounds (100 μM) are tolerated by *Prochlorococcus* PCC 9511, but have no beneficial effect: glycine, proline, glutamine, arginine, tryptophan, methionine, dehydroascorbate, glutathione (oxidized), thiosulphate, uracil, thymine, δ -aminolaevulinic acid, salicylic acid and *p*-OH benzoate. Difco yeast extract (10 $\mu\text{g ml}^{-1}$), Sigma yeast concentrate (100 $\mu\text{g ml}^{-1}$), putrescine, spermine and cAMP (all 50 μM), haemin-chloride and α -lipoic acid (0.1 and 1 $\mu\text{g ml}^{-1}$) and NaHCO_3 (1 mM) were equally without effect. In contrast, ferulic acid (50 and 100 μM), adenine and ascorbate (10 μM) inhibited growth. Na-pyruvate (5 mM), glucose, fructose and sucrose (10 mM) were tolerated and seemed to slightly prolong survival, but had no major beneficial effect on the growth rates or yields.

Chromatic light

Using the coloured filters described by Tandeau de Marsac & Houmard (1988), growth of the axenic strain was observed in red, but not in green, light. These results are in agreement with the observed lack of phycoerythrinoid light-harvesting antennae (see below).

Trace metals and development of a synthetic seawater medium

Given the rather low concentration of the trace metal additions in medium PCR-S11 compared to the elements present in natural seawater (Bruland, 1983), it seemed possible that some of the trace metal supplements may not be necessary. Indeed, medium PCR-SA₆, a modification of medium PCR-S11 in which Gaffron's +Se trace metal solution (see Methods and Table 1) was replaced by a 10-fold dilution of trace metals A5 + Co (composed of only six rather than 16 trace metals, see Methods), gave growth rates and yields comparable to those typically observed in medium PCR-S11 (see above). For refined physiological studies, a synthetic seawater medium of a more defined composition seemed desirable. Consequently, the natural seawater in medium PCR-SA₆ was replaced by a solution of Turks Island Salts (see Methods and Table 1). This resulted in equivalent growth in both media, provided that NaHCO_3 (2 mM) was added to raise the pH of the artificial salt mix from about 5 to 7.8. With the latter medium, designated PCR-Tu₂ (see Table 1), it was shown that vitamin B₁₂, routinely added to all previous media, was not essential, since no growth limitation occurred even after 10 successive transfers in the absence of this supplement. The addition to medium PCR-Tu₂ of boron (10 and 100 μM), manganese (1, 10 and 20 μM), molybdenum

Fig. 1. Light micrographs of *Prochlorococcus* PCC 9511 (a) and *Synechococcus* PCC 7001 (b). Bar, 5 μm .

(1 and 20 μM), zinc (1 μM), nickel (1 μM) and selenium (1 μM) had no effect, whereas zinc (10 μM) and cobalt (1 μM) totally inhibited growth. Copper, toxic to some marine cyanobacteria (Waterbury & Willey, 1988), was only tested and tolerated at 100 nM. Finally, the total omission of copper in medium PCR-Tu₂ resulted in sustained growth comparable to controls ($\text{Cu} = 16 \text{ nM}$, see Methods) for more than eight successive transfers, demonstrating a lack of, or only low, copper requirement, possibly being met by impurities introduced by the major salts of this artificial seawater medium. The latter result confirms previous studies (Chisholm *et al.*, 1992), performed on impure strains. Lack of growth in medium PCR-Tu₂ with nitrate as sole source of nitrogen, both in the presence and absence of additional molybdenum (5 and 50 μM), reconfirmed earlier observations in medium PCR-S11 (see above). This suggests that the axenic strain is either deficient in a nitrate transport system or does not possess nitrate/nitrite reductase activity.

Morphology and ultrastructure

Prochlorococcus PCC 9511 exhibited small spherical post-divisional cells, about 0.5–0.6 μm in diameter and about 0.7–0.8 μm in length, that in contrast to the marine *Synechococcus* sp. PCC 7001 were barely visible by light microscopy (Figs 1a, b). Pre-divisional cells were elongated, reaching about 1.2 μm in length (Fig. 2). The ultrastructural features of strain PCC 9511

Fig. 2. Transmission electron micrograph of a thin section of *Prochlorococcus* PCC 9511. C, carboxysomes; T, thylakoids. Bar, 200 nm.

Fig. 3. *In vivo* absorption spectrum of whole cells after growth in white light at a PPFD of $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ (a), and absorption spectrum of an acetone extract (b) prepared from the same culture.

revealed an organization typical of prokaryotes with a Gram-negative cell wall (Fig. 2). Carboxysomes, mainly composed of ribulose 1,5-bisphosphate carboxylase/oxygenase and carbonic anhydrase (Kaplan *et al.*, 1994), two enzymes involved in CO_2 fixation via

Fig. 4. *In vivo* fluorescence excitation and emission spectra at room temperature of whole cells of *Prochlorococcus* PCC 9511 after growth in white light (PPFD $30 \mu\text{mol m}^{-2} \text{s}^{-1}$). The emission spectrum shown was obtained by excitation at 443.5 nm, and is identical to that with excitation at 478.5 nm.

the reductive pentose phosphate cycle, were also observed. The thylakoid disposition was similar to that of *Prochlorococcus* strains CCMP 1378 (Lichtlé *et al.*, 1995) and MIT 9313 (see Partensky *et al.*, 1999), representing an interruption of the internal photosynthetic membranes at one cell pole ('horseshoe' arrangement) (Fig. 2). This is in contrast to the holotype strain CCMP1375^T which has a concentric thylakoid arrangement (Chisholm *et al.*, 1992; Lichtlé *et al.*, 1995).

Absorption and fluorescence spectra

Cell suspensions of strain PCC 9511, cultivated in white light ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$), exhibited an *in vivo* absorption spectrum with a blue maximum at 447 nm and a red maximum at 674 nm (Fig. 3a). The spectral profile was similar to that reported for *Prochlorococcus* CCMP 1378, an isolate containing chl a_2 and synthesizing only low amounts of chl b_2 (Morel *et al.*, 1993; Partensky *et al.*, 1993; Moore *et al.*, 1995) but differs from the holotype CCMP 1375^T. An absorption spectrum of an acetone extract of the same culture of *Prochlorococcus* PCC 9511 is shown in Fig. 3(b). The three maxima at 441, 620 and 663 nm correspond with minor shifts to the reported Soret bands of chl a_2 , the shoulder around 475 nm being attributable to the combined absorption by chl b_2 and carotenoids (Morel *et al.*, 1993; Partensky *et al.*, 1993; Moore *et al.*, 1995).

Typical room temperature fluorescence excitation and emission spectra of whole cells of *Prochlorococcus* PCC 9511 are shown in Fig. 4. As expected for members of this genus (Moore *et al.*, 1995; Partensky *et al.*, 1997), the *in vivo* fluorescence emission peak was located at 678.5 nm, and the presence of chlorophylls a_2 and b_2 is evidenced by fluorescence excitation peaks at 443.5 nm and 478.5 nm, respectively, both being red

Table 2. Pigment content (fg cell⁻¹) of *Prochlorococcus* PCC 9511 grown at different irradiance values

Irradiance*	Cells ml ⁻¹	chl <i>a</i> ₂	chl <i>b</i> ₂	chl <i>c</i> -like	Zeaxanthin	α-Carotene	chl <i>b</i> ₂ /chl <i>a</i> ₂
8	3.9 × 10 ⁸	1.234	0.061	0.029	0.645	0.235	0.050
30	3.2 × 10 ⁸	1.151	0.062	0.026	0.739	0.204	0.054
80	2.6 × 10 ⁸	0.680	0.012	0.006	0.740	0.116	0.017

* PPFD (μmol quanta m⁻² s⁻¹).

shifted by 9 nm with respect to chlorophylls *a*₁ and *b*₁ (F. Partensky, unpublished). The large difference in the heights of these two fluorescence excitation peaks is in agreement with the low divinyl-chlorophyll *b* to *a* ratio anticipated from the absorption spectra (see above). In contrast, fluorescence excitation due to chl *b*₂ absorption was higher in the holotype CCMP 1375^T and, at low growth irradiances, exceeded that of chl *a*₂ (Moore *et al.*, 1995).

Quantitative determinations of chlorophylls and carotenoids

Prochlorococcus PCC 9511, grown at various irradiances (8, 30 and 80 μE m⁻² s⁻¹), contained chl *a*₂, chl *b*₂ and traces of a chlorophyll *c*-like pigment (Table 2), but no monovinyl-chlorophyll *b*. Zeaxanthin and α-carotene were the only carotenoids detected in the axenic strain. Therefore the traces of unknown carotenoids reported for other non-axenic members of this genus (Chisholm *et al.*, 1988, 1992) are either strain specific or may be attributable to bacterial contaminants. In agreement with the absorption and fluorescence spectra (Figs 3a, b and 4), the chl *b*₂/chl *a*₂ ratios were very low (less than 0.1) under all light conditions. This pigment complement resembled that reported for *Prochlorococcus* CCMP 1378 but differed from that of the holotype CCMP 1375^T which, in addition to the former pigments, contained small amounts of chl *b*₁ (Partensky *et al.*, 1993; Morel *et al.*, 1993; Moore *et al.*, 1995) and exhibited a ratio of total chlorophyll *b* (chlorophylls *b*₁ plus *b*₂) over chl *a*₂ in the range of 0.4–2.4 (Moore *et al.*, 1995; Urbach *et al.*, 1998).

Search for phycoerythrin

Western blots were performed using a polyclonal antiserum obtained by aid of a recombinant fusion protein of the cloned α PE-III of strain CCMP 1375^T after expression in *E. coli* (Hess *et al.*, 1999). A band of an apparent molecular mass of about 15 kDa was revealed (Fig. 5) in extracts of *Prochlorococcus marinus* CCMP 1375^T and for the recombinant α PE-III, released from the fusion protein by cleavage with factor X (Hess *et al.*, 1999). This result was in good agreement with the molecular mass of 17.3 kDa,

Fig. 5. Immunoblot for detection of phycoerythrin. An antiserum against the α-subunit of phycoerythrin of *Prochlorococcus* CCMP 1375^T, was cross used to develop a Western blot containing crude extracts of *Prochlorococcus* PCC 9511, *Prochlorococcus* CCMP 1375^T and *Chamaesiphon* PCC 6605. The recombinant fusion protein (MP-αX), cleaved after purification by factor X and resulting in release of α PE and the maltose-binding protein (MBP) part from *E. coli* used for expression, was included as a positive control. Only the recombinant protein and strains known to synthesize PE (PCC 6605 and CCMP 1375^T) show strong immunodecoration of bands with an appropriate molecular mass.

estimated on the basis of the α PE gene sequence (Hess *et al.*, 1996). Two major polypeptides of about 14 and 18 kDa were recognized in *Chamaesiphon* PCC 6605, a PE-I containing cyanobacterium that adapts chromatically (Bryant, 1982) and assembles typical phycobilisomes (Waterbury & Stanier, 1977). Whereas the 14 kDa band remains to be identified (possibly a degradation product), the subunit of 18 kDa corresponds well to the molecular mass deduced by SDS-PAGE for other cyanobacterial α PEs (Bogorad, 1975; Alberte *et al.*, 1984). In contrast, no PE was detectable in *Prochlorococcus* PCC 9511 (Fig. 5). This was

Fig. 6. PFGE analysis. Cells of *Prochlorococcus* PCC 9511 were included in agarose followed by overnight restriction with the enzyme *MluI* and separation of the resulting fragments by contour-clamped homogeneous field electrophoresis. The left panel shows resolution of 12 large bands (molecular mass 30–250 kb); the right panel shows resolution of bands of less than 30 kb. The run was performed using parameter settings appropriate for the respective size range to be separated (see Methods). Mid Range I (MR) and Low Range (LR) PFG Marker from New England Biolabs served as molecular mass markers.

confirmed by PCR studies and Southern blot hybridizations, neither of which revealed the corresponding gene (data not shown).

Genetic characterization

Prochlorococcus PCC 9511 was shown to have a low mean DNA base composition (32.0 ± 0.1 mol% G + C) and the smallest genome (1.30 ± 0.11 GDa = 2 Mbp, determined from DNA renaturation kinetics) of all known oxyphotobacteria. PFGE analyses (Fig. 6) after digestion of chromosomal DNA by the restriction endonuclease *MluI* resulted in 13 bands larger or equal to 30 kbp that could be easily identified (size of individual bands in kbp: 250, 245, 220, 190, 160, 120, 115, 105, 74, 73, 40, 38, 30; Σ 1660). The number of bands < 30 kbp was about 20. The following sizes (in kbp) may be deduced: 28, 27.5, 26, 25.5, 24, 23, 21, 19, 18, 17 (2 x), 16.5, 16, 15.5, 13, 11, 10.5, 10, 9.5, 8; Σ 356. From these data an estimation of a total genome size of about 2 Mbp ($1660 + 356$) was concluded for *Prochlorococcus* PCC 9511, in perfect agreement with the renaturation studies. Two other enzymes tested for PFGE (*SmaI* and *NotI*) also cut the chromosomal DNA of this strain, but resulted in only a few large bands (data not shown). Given the recognition specificities of the latter enzymes (highly rich in G + C),

these results were consistent with the low mean DNA base content of the genome determined by thermal denaturation (see above).

Phylogeny

Phylogenetic analysis (Fig. 7) of an almost complete 16S rRNA gene sequence of strain PCC 9511 and those of 17 previously determined sequences of cultured and uncultured members of the genus *Prochlorococcus* revealed that the axenic strain was identical to *Prochlorococcus* strain CCMP 1378 and exhibited only one base difference with the comparable region (1271 bases common to all *Prochlorococcus* sequences) of strain CCMP 1426. The two latter strains were both derived from a primary culture, 'MED', isolated from the Mediterranean Sea (Chisholm *et al.*, 1992; Partensky *et al.*, 1993). The same high degree of relatedness (1 base difference, but in a different position) was found for *Prochlorococcus* NATL1, isolated from the North Atlantic (Partensky *et al.*, 1993), whose 16S rRNA sequence was also determined in this study. In contrast, the sequence of strain PCC 9511 differed by 21 bases from that of its supposedly parental culture, *Prochlorococcus* CCMP 1375^T, which excluded the possibility that the latter two isolates are co-identical.

Fig. 7. Phylogenetic tree inferred from 16S rRNA gene sequences by maximum likelihood (fast DNAML, Olsen *et al.*, 1994) as described in Methods. The axenic strain PCC 9511 is underlined.

DISCUSSION

The axenic strain *Prochlorococcus* PCC 9511 will be extremely valuable for research in fundamental microbiology and oceanography. For the first time, physiological, biochemical and genetic studies are possible that can be interpreted without the complexity introduced by potential interactions or interference with commensal contaminants. Although it is unfortunate that growth as single colonies on plates has not yet been achieved, significant progress with respect to the characterization of this axenic strain has been made. It has been shown unambiguously that this isolate is nutritionally relatively versatile, capable of using orthophosphate, polyphosphate and organic phosphates. Since the latter two types of substrates require dephosphorylation prior to crossing the cytoplasmic membrane (Ammerman, 1991), it is clear that strain PCC 9511 must possess one or more ecto-phosphohydrolases. Whether utilization of ATP is mediated by alkaline phosphatase or by a specific 5'-nucleotidase remains to be examined. Both types of ecto-phosphohydrolases have been found in bacteria (Ammerman, 1991; Martinez & Azam, 1993; Björkman & Karl, 1994) and are of great importance for efficient recycling of orthophosphate from organic matter. The presence of such enzyme(s) in *Prochloro-*

coccus is undoubtedly a beneficial adaptation of the organism to its oceanic ecosystem, in which the supply of orthophosphate is thought to be a limiting factor for primary production (Vaulot *et al.*, 1996; Scanlan & Wilson, 1999). Ammonia and urea are the preferred sources of combined nitrogen for strain PCC 9511. In contrast, nitrate, commonly used by other cyanobacteria (Flores *et al.*, 1983; Flores & Herrero, 1994) does not support growth. Since molybdenum is a co-factor of nitrate reductase (Guerrero & Lara, 1987) and uptake of this element may be inhibited at the high concentrations of sulphate typical of seawater (Howarth & Cole, 1985), lack of growth on nitrate was reconfirmed after increasing the molybdenum concentration in the artificial seawater medium PCR-Tu₂ from 80 nM to 5 and 50 µM. Inability to utilize nitrate seems to be an ecological contradiction, since field studies demonstrated a positive correlation of *Prochlorococcus* abundance with nitrate availability (Olson *et al.*, 1990). Thus either the preferential positioning of *Prochlorococcus* near the nitracline is coincidental or the available nitrate is converted to a utilizable source of nitrogen by other constituents of the autochthonous microbial community. Alternatively, it is possible that *Prochlorococcus* ecotypes typical of the LL clade, which are adapted to grow at the bottom of the euphotic zone (i.e. near or below the nitracline), may be able to use nitrate more efficiently than members of the HL clade, which generally inhabit the more nitrate-depleted, surface waters (see Partensky *et al.*, 1999). This is suggested by the ability of strain CCMP 1375^T to grow on nitrate as the sole source of nitrogen (Moore, 1997). However, since the latter isolate is not axenic, this hypothesis can only be confirmed once pure strains of the LL clade are available.

In the phylogenetic tree inferred from 16S rRNA sequences, strain PCC 9511 clusters with members of the HL clade and is identical or closely related to strains CCMP 1378, CCMP 1426, NATL1, TATL2 and NATL2 (Fig. 7). Interestingly, all *Prochlorococcus* isolates sequenced to date group with marine and freshwater *Synechococcus* strains, one of which (PCC 6307) was assigned to the 'Cyanobium cluster' (Rippka & Cohen-Bazire, 1983; Waterbury & Rippka, 1989). These cyanobacteria have a mean DNA base composition of 59–70 mol% G+C (Herdman *et al.*, 1979b; Waterbury & Rippka, 1989) and a genome size of about 4 Mbp (Herdman *et al.*, 1979a). However, strain PCC 9511 has a much lower mean DNA base composition (32 mol% G+C) and its genome is the smallest (2 Mbp) of all photosynthetic prokaryotes. These important genetic differences among members of the same phylogenetic clade raise questions with respect to overall genomic evolutionary events. The low mean DNA base composition of strain PCC 9511 is confirmed by sequence analyses of several genes of cultured and uncultured members of the genus *Prochlorococcus*: in contrast to marine *Synechococcus* genotypes of the same 16S rRNA gene clade (i.e.

strains WH 7803, WH 7805 and WH 8103), the *rpoC*, *psbA*, *psbB* or *petB/D* genes of most *Prochlorococcus* isolates studied are characterized by a very low G+C content (maximum 38 mol%) in their third codon position (Urbach *et al.*, 1998). An exception to this rule is *Prochlorococcus* MIT 9303, an isolate at the root of the *Prochlorococcus* clade (see Fig. 7) that, based on its relatively high G+C content (52–58 mol%) of the third codon position of the *psbB* and *petB/D* genes (Urbach *et al.*, 1998), may represent a species of a much higher mean DNA base composition.

In contrast to strain SARG and its clonal derivative CCMP 1375^T, both of which are members of the LL clade (see Partensky *et al.*, 1999), the axenic strain PCC 9511 is characterized by chl *b/a₂* ratios lower than 0.1 and a pigment signature typical of the HL clade (Urbach *et al.*, 1998; Partensky *et al.*, 1999). The low content of chl *b* in strain PCC 9511 is a stable feature, having been observed during four years of sub-culturing. Furthermore, the 'horseshoe-like' thylakoid arrangement of strain PCC 9511 is more comparable to that described for *P. marinus* CCMP 1378, a member of the HL clade, than to the concentric thylakoids described for the holotype strain CCMP 1375^T (Chisholm *et al.*, 1992; Lichtlé *et al.*, 1995). However, this character may not be clade-specific since strain MIT 9313, a LL representative (Moore *et al.*, 1998), also exhibits in longitudinal sections a 'horseshoe-like' opening of the thylakoids at one pole of the cell (Partensky *et al.*, 1999). As expected for all members of *Prochlorococcus*, phycobilisomes are lacking in strain PCC 9511 (Fig. 2). In contrast to the holotype CCMP 1375^T, the genes encoding the novel phycoerythrin (PE-III) (Hess *et al.*, 1996, 1999) seem to be lacking in PCC 9511, and an antiserum to α PE-III did not reveal the corresponding protein subunit (Fig. 5).

In conclusion, the axenic strain PCC 9511 exhibits an ensemble of properties that strongly excludes its co-identity with *P. marinus* strain CCMP 1375^T, suggesting that the parental culture contained a major and a minor representative of the genus *Prochlorococcus*, of which only the latter has been rendered axenic. Alternatively, human error of mislabelling cultures, or accidental contamination with a HL representative, cannot entirely be excluded. However, we favour the former explanation, particularly since several other *Prochlorococcus* cultures have been shown to contain more than one genotype (such as NATL2 versus NATL2A and TATL1A versus TATL1B, Fig. 7; see also Urbach *et al.*, 1998; West & Scanlan, 1999). Although convincing evidence for the vertical distribution and distinct niche-partitioning of the HL and LL ecotypes, at least in well-stratified water columns, has been obtained for uncultured populations by RNA polymerase gene sequence analyses and 16S rRNA-specific gene probing (Ferris & Palenik, 1998; West & Scanlan, 1999), it is equally clear that both HL and LL genotypes co-exist at the same depth (Ferris & Palenik, 1998; Palenik, 1994; Moore *et al.*, 1998). Consequently, primary non-clonal cultures established from

such habitats may change both phenotypic and genotypic signatures over prolonged periods of sub-culturing as a result of selection by the growth conditions employed. This is exemplified by strain NATL1, which originally exhibited the LL phenotype (chl *b/a₂* ratio in the range of 0.4–0.9) (Partensky *et al.*, 1993; Morel *et al.*, 1993), but changed in the course of maintenance towards a phenotype of a low chl *b/a₂* signature (F. Partensky, unpublished data) and was found to be phylogenetically a member of the HL clade (see Fig. 7). However, the same isolate maintained in another laboratory (S. Chisholm, MIT) as NATL1-MIT retained its initial LL pigment signature and yielded a second 16S rRNA gene sequence clustering with LL genotypes (N. West and D. Scanlan, unpublished results).

In order to distinguish strain PCC 9511 and its close relatives of the HL clade from the nomenclatural type, *Prochlorococcus marinus* strain CCMP 1375^T (Chisholm *et al.*, 1992), which is phenotypically and genetically distinct, we propose strain PCC 9511 as the type strain of *Prochlorococcus marinus* Chisholm *et al.* 1992 subsp. *pastoris* subsp. nov. We chose to propose the separation at the subspecies level because no other axenic isolates are presently available for DNA–DNA hybridization studies, necessary for valid separation at the level of species or genus. If the degree of 16S rRNA gene sequence divergence between the low- and high-light clades of *Prochlorococcus* can be correlated with percentages of DNA–DNA hybridizations and more drastic separations can be justified, the proposed subspecies *Prochlorococcus marinus* subsp. *pastoris* could be elevated to a higher taxonomic rank. The epithet of the new subspecies, *pastoris*, was chosen in honour of Louis Pasteur, since his famous citation (Vallery-Radot, 1939) 'dans le champ d'observation, le hasard ne favorise que les esprits préparés' (in the field of observations, chance only favours the prepared mind) is highly appropriate in the context of the rather fortuitous purification of strain PCC 9511.

Description of *Prochlorococcus marinus* subsp. *pastoris* subsp. nov.

***Prochlorococcus marinus* Chisholm *et al.* 1992, 299.** The diagnoses of the latter genus and type species correspond to those formulated by Chisholm *et al.* (1992). They are here extended, in order to distinguish between two of the different pheno- and genotypes within the type species, leading to the proposal of the following subspecies.

***Prochlorococcus marinus* Chisholm *et al.* 1992 subsp. *marinus* subsp. nov.** Chisholm *et al.* 1992. The type strain of this subspecies is the non-axenic holotype of the genus, strain CCMP 1375^T. The cells are spherical to slightly elongated (0.6–0.8 μ m in diameter; pre-divisional cells are 1.2–1.6 μ m in length); thylakoids are arranged concentrically at the periphery of the cells; the genes encoding the α and β subunits of phycoerythrin (PE-III) are present and translated; chlorophylls *a₂*, *b₁*, *b₂*

and Mg, 3-8 divinyl phaeoporphyrin a_5 are synthesized; the ratios of chlorophyll b (b_1 and b_2) over chl a_2 are in the range of 0.4–2.4. Zeaxanthin and α -carotene are the major carotenoids. The mean DNA base composition and genome size are unknown.

***Prochlorococcus marinus* Chisholm et al. 1992 subsp. *pastoris* subsp. nov.** The type strain of the new subspecies, *pastoris* (pa.sto'ris. Latinized form of Pasteur), is the axenic isolate PCC 9511 (= ATCC 700925). The cells of this strain are 0.5–0.6 μ m in width and 0.7–0.8 μ m in length; thylakoids have a 'horseshoe-like' opening at one pole of the cells; phycoerythrin is not produced and the genes encoding the α and β subunits of phycoerythrin (PE-III) have not been revealed in the genome; chlorophylls a_2 , b_2 and Mg, 3-8 divinyl phaeoporphyrin a_5 are synthesized, but chl b_1 is lacking; the ratios of chlorophyll b_2/a_2 are less than 0.1. As observed for all other non-axenic members of this genus, zeaxanthin and α -carotene are the major carotenoids. Strain PCC 9511 uses ammonia and urea as the preferred sources of nitrogen, but is incapable of using nitrate. Utilizable sources of phosphate are: Na-PO_4 (pH 7.5), Na_2 - β -glycerophosphate, Na_4 -pyrophosphate, glucose 6-phosphate and ATP; the mean DNA base composition is 32 mol% G + C; genome size is 2 Mbp. Based on morphology, pigment content and 16S rRNA sequence analyses, the type strain of this new subspecies is identical to the impure strain CCMP 1378 (MED4), isolated from the Mediterranean Sea. Both are highly related to four other non-axenic isolates, CCMP 1426, NATL1, TATL2 and NATL2, which most likely are also assignable to the subspecies *pastoris*. The former two differ from PCC 9511 by one base at two different positions; the latter exhibit two different base changes in the respective 16S rRNA sequences that cover a total of 1271 comparable bases. In contrast, the type strain CCMP 1375^T of the genus and proposed as *Prochlorococcus marinus* Chisholm et al. 1992 subsp. *marinus* subsp. nov., differs in the 16S rRNA gene sequence by 20–21 bases from all six members of the subspecies *pastoris*.

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