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High degree of genetic variation in *Prochlorococcus* (Prochlorophyta) revealed by RFLP analysis

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A genetic characterization of nine strains of *Prochlorococcus* originating from various depths of the Mediterranean Sea, the Sargasso Sea, the North and the tropical Atlantic Ocean and the Pacific Ocean was performed by restriction fragment length polymorphism (RFLP) mapping using probes for *rbcL*, *rbcS*, *psbA* and *woxA*. This study revealed extensive genetic variation among strains, which were grouped into two distinct clusters. Unexpectedly, with one exception (TATL1 strain), the strains clustered by isolation depth (i.e. near surface versus deep isolates) rather than by geographic origin. These relationships were confirmed by secondary RFLP analysis of *psbA* amplification products using *Prochlorococcus*-specific polymerase chain reaction (PCR) primers.

Key words: PCR amplification, *Prochlorococcus*, *psbA*, RFLP mapping.

Introduction

The discovery of unicellular prochlorophytes as major components of the picophytoplankton (i.e. photosynthetic cells 0·2–2·0 μm in diameter) in wide areas of the world's oceans has had profound implications for our understanding of primary productivity in the marine environment (Chisholm *et al.*, 1988; Olson *et al.*, 1990). Flow cytometric analysis has shown that prochlorophyte picoplankton are present at high cell densities (10^4–10^5 cells per ml) in the North and subtropical Atlantic Ocean (Li & Wood, 1988; Olson *et al.*, 1990), the Mediterranean Sea (Vaulot *et al.*, 1990), the subtropical and equatorial Pacific (Chavez *et al.*, 1991; Campbell & Vaulot, 1993), the Red Sea (Lindell, 1993; Veldhuis & Kraay, 1993), the Indian Ocean (Pollehne *et al.*, 1993) and in areas around the Panama Basin, Gulf of Mexico, Caribbean and the Southern California Bight (Chisholm *et al.*, 1988). These organisms may account for a large proportion of the biomass in oligotrophic environments: for example, 40% of the chlorophyll and 30% of living carbon in the central Pacific off Hawaii (Campbell *et al.*, 1994). All isolates have so far been assigned to the same species, *Prochlorococcus marinus* Chisholm *et al.* (1992). The cells are very small (0·6 μm average diameter), and characteristically lack phycobiliproteins, but contain divinyl chlorophyll *a* and *b* (DV-chl *a* and DV-chl *b*) as their primary photosynthetic pigments. Accessory pigments include zeaxanthin, α-carotene and a chlorophyll *c*-like pigment (Goericke & Repeta, 1992).

Although marine *Prochlorococcus* can be discriminated from other phytoplankton taxa by their small size, their typically dim red fluorescence when excited by blue light (488 nm) and their absence of phycoerythrin fluorescence, investigations of the genetic diversity of the *Prochlorococcus* genus in nature are just beginning (Palenik, 1994). The characterization of the genetic relatedness of isolates from different geographical and ecological situations is of great interest in view of their ubiquitous presence, high abundance and ecological importance.

Recent isolation of several *Prochlorococcus* strains from different environments, e.g. the Mediterranean Sea, the Sargasso Sea and the North Atlantic Ocean, into unialgal or clonal (but not axenic) cultures has greatly facilitated laboratory study of these organisms, and initiated intense study of their photosynthetic apparatus: carbon assimilation capacity (Partensky *et al.*, 1993), optical properties (Morel *et al.*, 1993; Moore *et al.*, 1995), pigment–protein complexes (Partensky & LaRoche, 1994) and photosynthetic genes (Hess *et al.*, 1995).

Studies of the photophysiology of several *Prochlorococcus* isolates have revealed marked differences in pigment content between strains, especially in the ratios of DV-chl *b* to DV-chl *a* (Partensky *et al.*, 1993; Moore *et al.*, 1995). In addition, sequencing of the DNA-dependent RNA polymerase genes from two marine prochlorophytes, *Prochlorococcus marinus* (LG) [≡ *Prochlorococcus* SARG] and *Prochlorococcus* sp. (DV1) [≡ *Prochlorococcus* MED] suggests they are fairly distantly related phylogenetically (Palenik & Haselkorn, 1992). Dendrograms constructed using amino acid or nucleotide sequence comparisons of 16S ribosomal RNA and DNA-dependent RNA polymerase sequences (Palenik & Haselkorn, 1992; Urbach *et al.*, 1992) have shown that *Prochlorococcus*

Table 1. *Prochlorococcus* strains and their origin

Strain	Unialgal/clonal	Origin	Location	Date	Isolated by	Depth (m)
MED	U	Mediterranean Sea	43°12·15′ N, 6° 52′ E	January 1989	D. Vaulot	5 m
CCMP 1378	C		MED strain derivation			
COMP 1426	C		MED strain derivative			
NATL1	U	North Atlantic	37° 39′ N, 40° 01′ W	April 1990	F. Partensky	30 m
NATL2	U	North Atlantic	38° 59′ N, 49° 33′ W	April 1990	F. Partensky	10 m
SARG	U	Sargasso Sea	28° 59′ N, 64° 21·5′ W	May 1988	B. Palenik	120 m
CCMP 1375	C		SARG strain derivative			
TATL1	U	Tropical Atlantic	21° 02′ N, 31° 08′ W	October 1991	F. Partensky	20 m
TATL2	U	Tropical Atlantic	20° 25′ N, 31° 08′ W	October 1991	F. Partensky	30 m
PACI	U	Tropical Pacific	22° 45′ N, 158° W	April 1992	L. Campbell	100 m

forms a shallowly branching cluster with marine A *Synechococcus* strains WH7805 and WH8103, and suggests a closer phylogenetic link between *Prochlorococcus* and the oceanic cyanobacteria than between the former and the other prochlorophyte genera *Prochloron* (Lewin, 1976) and *Prochlorothrix* (Burger-Wiersma *et al.*, 1986). *Prochlorococcus* has also been shown to be closely related to a number of shotgun-cloned 16S rRNA and RNA polymerase gene sequences from the Sargasso Sea and north Pacific Ocean (Urbach *et al.*, 1992; Palenik, 1994).

In this study we investigate the genetic relatedness of nine strains of *Prochlorococcus*, including new isolates from the tropical Atlantic and Pacific Ocean. We have made use of marine and freshwater *Synechococcus* strains as putative outgroups using both published data (Douglas & Carr, 1988; Wood & Townsend, 1990) and data described herein. We have used restriction fragment length polymorphisms (RFLPs), which detect variations in restriction sites using radioactively labelled DNA, specific for a given gene. Previous work has examined the genetic relatedness within the WH7803 serogroup of the marine *Synechococcus* (Douglas & Carr, 1988; Wood & Townsend, 1990), and immunologically investigated the temporal and depth-related distribution of the WH7803 serogroup (Campbell & Iturriaga, 1988). The data presented here extend the genetic studies to the *Prochlorococcus* genus, a potentially more significant component of the photosynthetic picoplankton.

Materials and methods

Strains

The *Prochlorococcus* strains used in this study are described in Table 1. Clonal isolates have been obtained by serial dilution (Chisholm *et al.*, 1992). Cultures were grown in continuous blue light at 21 ± 1°C, in a modification of K/10 medium (Keller *et al.*, 1987) consisting of 50 μM NH_4Cl, 10 μM glycerophosphate and trace metals as in K/10-Cu with 10^{-8} M $NiCl_2$ and 10^{-8} M H_2SeO_3 added. Marine *Synechococcus* strains were grown in artificial seawater medium at 25°C under constant illumination (Wyman *et al.*, 1985). The freshwater cyanobacterial strain *Synechococcus* sp. PCC7942 was grown in BG11 medium as described in Rippka *et al.* (1979).

DNA isolation

DNA was prepared from all *Prochlorococcus* and *Synechococcus* strains using either frozen cell pastes containing between 1×10^{11} and 4×10^{11} cells in total, by a method described previously (Scanlan *et al.*, 1990), or lyophilized cells. In the latter case *Prochlorococcus* cells were resuspended in 0·3 ml lysis buffer [100 mM EDTA, 10 mM Tris-HCl, pH 8·0, 1% (w/v) sodium dodecyl sulphate (SDS), 100 μg ml^{-1} proteinase K] and incubated at 50°C for 2 h. After extraction with phenol/chlorofom/isoamyl alcohol (25 : 24 : 1) and chloroform/isoamyl alcohol (24 : 1) the DNA was precipitated with 0·8 volumes of isopropanol at room temperature. The pellet was washed in 70% ethanol, dissolved in water and once more precipitated with 2·5 volumes of chilled ethanol. This method typically yielded about 100 μg DNA from 10^{10} cells.

RFLP analysis

Prochlorococcus and *Synechococcus* chromosomal DNAs (approx. 1 μg) were digested with various restriction enzymes using conditions recommended by the manufacturer (Gibco BRL). Southern blotting of the DNA onto nylon or nitrocellulose membranes (Amersham) was performed as described in Maniatis *et al.* (1982). DNA fragments used as hybridization probes (see Table 2) were labelled to high specific activity using [α-^{32}P] dCTP by the random priming method (Feinberg & Vogelstein, 1984). For the RFLP analysis, prehybridization and hybridization of nitrocellulose filters was carried out at low stringency in 5 × SSPE [1 × SSPE is 0·18 M NaCl, 10 mM sodium phosphate, 1 mM EDTA (pH 7·4)], 5 × Denhardt's solution (Maniatis *et al.*, 1982), 0·1% (w/v) SDS and 100 μg ml^{-1} herring sperm DNA (Sigma) at 55°C. Blots were washed in 6× SSC, 0·1% SDS at 55°C for 30 min. For homologous probes membranes were washed at high stringency (2 × SSC, 1% SDS; 1 × SSC, 0·5% SDS; 0·1 × SSC; 0·1% SDS, each for 20 min) at 65°C.

Table 2. Photosynthetic gene probes used in the RFLP analysis

Gene	Organism	Gene product	Restriction fragment	Plasmid/source
rbcL	*Synechococcus* PCC 6301	Large subunit of Rubisco	1·47 kb *PstI–EcoRI*	pLS401 (Reichelt & Delaney, 1983)
	Nostoc sp. MAC PCC8009		0·9 kb *HindIII*	pNM51·1 (M.R.K. Alley)
rbcS	*Prochlorothrix hollandica*	Small subunit of Rubisco	1·65 kb *BamHI*	pAM636 (Golden *et al.*, 1992)
woxA (*psbO*)	*Synechococcus* WH7803	33 kDa extrinsic protein of PSII	3·4 kb *HindIII*[a] 0·75 kb *BamHI*[b]	pDO455 (G. Watson)
psbA	*Prochlorothrix hollandica*	32 kDa reaction centre protein of PSII	2·5 kb *BamHI*	pAM396 (Golden *et al.*, 1992)
psbA	*Prochlorococcus marinus* SARG		0·98 kb *PstI–KpnI* fragment	pPC*psbA*1364A (Hess *et al.*, 1995)
psbA	*Prochlorococcus marinus* TATL1		3·8 kb *BglII* fragment	This study
cab	*Dunaliella tertiolecta*	29 kDa LHC II apoprotein	1·1 kb *EcoRI*	pDTcab1 (LaRoche *et al.*, 1990)

PSII, photosystem II.
[a] This fragment contains both *woxA* and an ATP sulphurylase gene with homology to *Met3* from *Saccharomyces cerevisiae* (G. Watson, personal communication).
[b] This is a *woxA*-specific fragment.

Amplification of an intragenic fragment of psbA

The partially degenerate oligonucleotides 5′-CTGTG-AGTGGGTTACATCCACTGATAA-3′ and 5′-GGG-AAGTTGTGGGCATT(G/A)CG(C/T)TCGTG-3′ were used to amplify a fragment of the *psbA* gene encoding the 32 kDa D1 protein of photosystem II from *Prochlorococcus*. The antisense primer was chosen according to conserved regions in the published sequences of *Anabaena, Prochlorothrix and Synechocystis* (Osiewacz & McIntosh, 1987; Morden & Golden, 1990) and the sense primer from the published *psbA* sequence of the SARG CCMP 1375 strain (Hess *et al.*, 1995). The expected length of a fragment amplified using these primers is 968 base pairs (bp). Polymerase chain reactions (PCR) contained 0·5 μg of each primer, 0·2 mM dNTPs final concentration, 1·25 U *Taq*-polymerase and 100 ng template DNA in a total volume of 100 μl. Standard reaction conditions were: 2 min at 94°C, followed by 35 cycles of 60 s at 94°C, 90 s at 50°C, 90 s at 72°C, followed by 5 min at 72°C, and 15°C until analysis. Reactions were performed in a Hybaid Thermocycler.

Data analysis

Cluster analysis was achieved using NTSYS-pc (Exeter Publishing, Setauket, NY, USA), after converting the RFLP data into binary matrix form. Similarity values were calculated for the data matrix using SIMQUAL in conjunction with the Simple Matching coefficient. A variety of clustering algorithms were then used to cluster the strains. Phenograms were created in SAHN using the unweighted pair group method with arithmetic averages (UPGMA) (Sokal & Michener, 1958), single and complete linkage (Lance & Williams, 1967), and by principal component analysis (Sneath & Sokal, 1973).

Results and discussion

RFLPs

For this study we employed probes only for photosynthetic genes in order to avoid the problem of possibly hybridizing non-*Prochlorococcus* fragments from the non-axenic cultures.

Hybridization data obtained using several restriction enzymes and strains of *Prochlorococcus* are presented in Table 3. DNA extracted from each *Prochlorococcus* isolate cut to completion with most restriction enzymes. *BamHI* proved slightly refractory to restriction endonuclease digestion (which improved with the addition of spermidine 4 mM final concentration to the reaction mixture), whereas *SalI* and *XhoI* did not cut (data not shown). The latter two enzymes were not used in the RFLP analysis. A freshwater and a marine *Synechococcus* (PCC7942 and WH7803 respectively) were included as outgroups. Both are well-characterized representatives of these groups of cyanobacteria, and their phylogenetic position has been well documented (Wilmotte, 1994). The RFLP data obtained for these strains agreed well with those reported by Douglas & Carr (1988), though discrepancies were noted for both strains with *Hin*dIII-digested DNA

Table 3. RFLPs from *Prochlorococcus* and *Synechococcus* spp.

Strain	Molecular sizes (kb) of fragments obtained with *rbcL*					
	*Bam*HI	*Bgl*II	*Eco*RI	*Eco*RV	*Hin*dIII	*Pst*I
NATL1	14·0	1·4	4·7	6·0	1·6, 1.0	7·2
NATL2	14·0	1·4	4·7	6·0	1·6, 1·0	7·2
MED original strain	14·0	1·4	4·7	6·0	1·6, 1·0	7·2
MEd clone CCMP1378	14·0	1·4	4·7	6·0	1·6, 1·0	7·2
MED clone CCMP1426	14·0	1·4	4·7	6·0	1·6, 1·0	7·2
TATL1	6·0	3·0	3·15	13·0	6·6	5·9
TATL2	14·0	1·4	4·0	11·5	0·7, 0·5[a]	3·5
SARG original strain	8·0	2·3, 1·7	4·95	2·8,[a] 1·8	1·65, 1·35	1·65
SARG clone CCMP 1375	8·0	2·3	4·95	1·8	1·65	1·65
PAC1	12·0	2·3	4·35	0·9	1·5	9·5
Outgroup						
WH 7803	4·0	4·3	12·0, 3·3	18·0	6·6	1·5
PCC 7942	> 20·0	18·0	9·0	>20·0	5·0	2·0

	Molecular sizes (kb) obtained with: *rbcS*[d]					
	*Bgl*II	*Cla*I	*Eco*RI	*Eco*RV	*Hin*dIII	*Pst*I
NATL1	6·6	5·2	6·4	3·9[a]	4·5[b]	7·4
NATL2	6·6	nd	9·0	9·0	6·2	7·4
MED original strain	6·6	3·7	6·4, 3·1	5·1, 3·9, 2·65	4·5	7·4,[b] 6·0
MED clone CCMP 1378	6·6	nd	9·0[b]	3·9	4·5	ns
TATL1	5·2	nd	6·8	10·6	4·9	6·0
TATL2	ns	ns	ns	ns	ns	ns
SARG original strain	3·55	8·0	11·0	4·7	2·75	1·4
PAC1	nd	nd	nd	ns	nd	nd

	Molecular sizes (kb) obtained with *psbA*				
	*Bam*HI	*Bgl*II	*Eco*RI	*Hin*dIII	*Pst*I
NATL1	20·0	10·0	9·6	5·3, 3·3, 1·8	8·0, 2·2
NATL2	17·51	14·0,[a] 5·2	12·3, 4·8	2·5, 1·8, 1·6	14·0, 2·9
MED original strain	17·5	5·2	4·8	5·3	14·0
MED clone CCMP 1378	17·5	5·2	4·8	5·3, 1·8	14·0
MED clone CCMP1426	nd	5·2	4·8	5·3, 1·8	14·0
TATL1	20·0	3·8	11·0	5·8	8·6
TATL2	7·4	14·0,[a] 3·6	6·8	3·2, 1·8	12·0
SARG original strain	14·0	2·3, 1·8	4·0	2·3,[a] 2·0, 1·4	3·2
SARG clone CCMP 1375	14·0	2·3	4·0	2·3, 2·0, 1·4	3·2
PAC1	16·0, 9·5	4·3, 2·0	9·6, 2·2	2·45, 1·4	12·0
Outgroup					
WH 7803	6·0, 4·5, 3·0, 2·0	10·0, 6·0	14·0, 10·0	11·5, 10·0, 7·0, 4·2	9·5, 3·6, 2·7, 1·0
PCC 7942	12·0, 8·0, 3·5	6·5, 4·8	10·0, 9·0, 7·5	7·2	5·0, 1·8, 1·6, 1·2, 1·0

	Molecular sizes (kb) obtained with *woxA*					
	*Bgl*II	*Eco*RI	*Eco*RV	*Hin*dIII	*Pst*I	*Xba*I
NATL1	4·8, 2·6,[c] 1·1[c]	3·5, 1·0	4·1	5·2,[a] 2·9	1·35	15·0, 6·0
NATL2	4·8	5·4, 3·5, 1·0	4·1	2·9	1·35	15·0
MED original strain	4·8	3·5, 1·0	4·1	3·45, 2·1[a]	3·95	15·0
MED clone CCMP 1378	4·8	3·5, 1·0	4·1	3·45	3·95	15·0, 6·0
MED clone CCMP 1426	3·4[c]	nd	nd	nd	2·3[c]	nd
TATL1	5·4	11·0	11·0	7·4, 5·4	5·0	11·5, 8·0
TATL2	5·4,[a] 1·3	4·0	2·35	7·0, 5·2	2·25	10·5
SARG original strain	2·9	5·4, 1·8	2·8	4·8, 2·7,[a] 2·15	nd	1·1
SARG clone	nd	5·4[c]	nd	nd	2·6[c]	nd

nd, not determined; nc, not cut; ns, no signal.

[a] Indicates a weakly hybridizing fragment.

[b] Very faint signal.

[c] Indicates a *woxA* specific fragment.

[d] The *rbcS* probe hybridizes strongly to SARG and TATL1 strains, poorly to NATL and MED strains and not at all to TATL2.

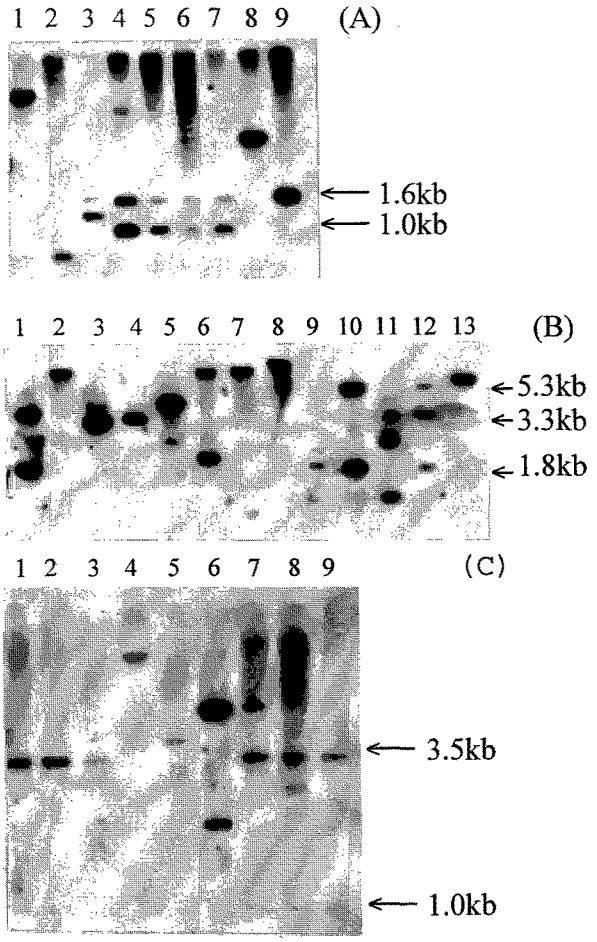

Fig. 1. Southern hybridization of various *Prochlorococcus* strains probed with (*A*) a 1·47 kb *Pst*I–*Eco*RI fragment from *Synechococcus* PCC7942 encoding *rbcL*. Lanes 1–7 were digested with *Hin*dIII, and 8–9 with *Bgl*II. Lanes: 1, TATL1; 2, TATL2; 3, SARG original strain; 4, NATL1; 5, NATL2; 6, MED original strain; 7, MED clone CCMP 1378; 8, TATL1; 9, TATL2. (*B*) A 2·5 kb *Bam*HI fragment from *Prochlorothrix hollandica* encoding *psbA*. Lanes 1–3 were digested with *Bgl*II, 4–8 with *Eco*RI and 9–13 with *Hin*dIII, Lanes: 1, 6 and 11, PAC1; 2, 7 and 12, NATL1; 3, 8 and 13, TATL1; 4 and 9, SARG clone CCMP 1375; 5 and 10, MED clone CCMP 1426. (*C*) A 3·4 kb *Hin*dIII fragment from *Synechococcus* WH7803 encoding *woxA* (*psbO*). All lanes were digested with *Eco*RI. Lanes: 1, NATL1A; 2, NATL1B; 3, NATL1C; 4, TATL1; 5, TATL2; 6, SARG original strain; 7, NATL2; 8, MED original strain; 9, MED cone CCMP 1378. Arrows show the sizes of the major hybridizing bands.

probed with *psbA*. Representative Southern hybridizations are shown in Fig. 1. The *cab* gene probe from *Dunaliella tertiolecta* Butcher failed to hybridize to DNA from all the *Prochlorococcus* strains under study even at low stringency (data not shown). Hence, the *cab* gene of *Prochlorococcus* is probably very different. This is supported by the recent finding in *Prochlorococcus* of a chl *a*/*b*-binding protein complex, whose N-terminal sequence showed 82% homology with a pigment protein complex (CP43′) encoded by the *isiA* gene from *Synechococcus* sp. (Partensky & LaRoche, 1994). The similarities in hybridization patterns among the different isolates are well evident. Cluster analysis using the NTSYS software package and UPGMA, single linkage and principal component algorithms (see Data Analysis) with the more complete *rbcL* and *psbA* RFLP data set in binary matrix form (using the character of shared restriction fragments) gives rise to two distinct clusters of strains (Fig. 2*A*). The Mediterranean Sea and North Atlantic isolates group tightly together and less closely to one of the tropical Atlantic strains (TATL2). The latter strain showed some similarity to another tropical Atlantic strain TATL1. A second, deeply branched cluster was formed by the Sargasso Sea and Pacific Ocean strains. Complete linkage analysis of the same data set clustered the Pacific Ocean strain closer to one of the tropical Atlantic strains (TATL1) than to the Sargasso Sea strain (Fig. 2*B*). With the exception of TATL1, the two clusters gather together strains from very different geographic origins but from similar isolation depths. Thus, the two strains from deep waters (PAC1 and SARG) cluster together and separately from the other strains isolated from surface waters or the upper mixed layer. Firm confirmation of this finding requires the analysis of more strains.

The number of bands hybridizing to the *psbA* gene probe might indicate the presence of more than one gene copy, particularly in strains NATL1 and PAC1 (Fig. 1*B*), as is known from cyanobacteria (Golden *et al.*, 1986; Vrba & Curtis, 1990). In a detailed analysis, however, a single gene was found in the genome of the SARG CCMP1375 strain (Hess *et al.*, 1995). Sequences upstream and downstream of the *psbA* coding region (about 1100 bp of the 2·5 kilobase (kb) probe from *Prochlorothrix hollandica* Burger-Wiersma, Stal *et* Mur used here: cf. Table 2) might contribute to this relatively high number of hybridizing bands.

To clarify the position of TATL1 within the groupings, the *psbA* gene from this strain was cloned as a 3·8 kb *Bgl*II fragment from a size-fractionated library in pUC19. This fragment was used as a probe in Southern hybridization (Fig. 3). The same filter was stripped and reprobed with a 0·98 kb *Pst*I–*Kpn*I fragment from the SARG CCMP 1375 strain (Hess *et al.*, 1995). The relative hybridization intensities again suggested a closer relationship of TATL1 with TATL2, the Mediterranean Sea and North Atlantic strains, than with the SARG strain.

Amplification of psbA

In the section above, describing the results of Southern hybridizations, relatively large polymorphisms were detected for the *psbA* gene encoding the extremely conserved D1 reaction centre protein of photosystem II, one of the most conserved proteins known. The percentage amino acid identity between the D1 proteins of higher land plants and cyanobacteria is in the region of 80%. Due to this extremely high protein conservation it would be surprising to find a high degree of sequence variation at the DNA level if the coding region alone is considered among representatives of one species. From the sequence of the SARG CCMP 1375 strain *psbA* gene (Hess *et al.*, 1995) a specific PCR primer was derived (see Materials and Methods) which routinely amplified *Prochlorococcus* DNA but which amplified cyanobacterial

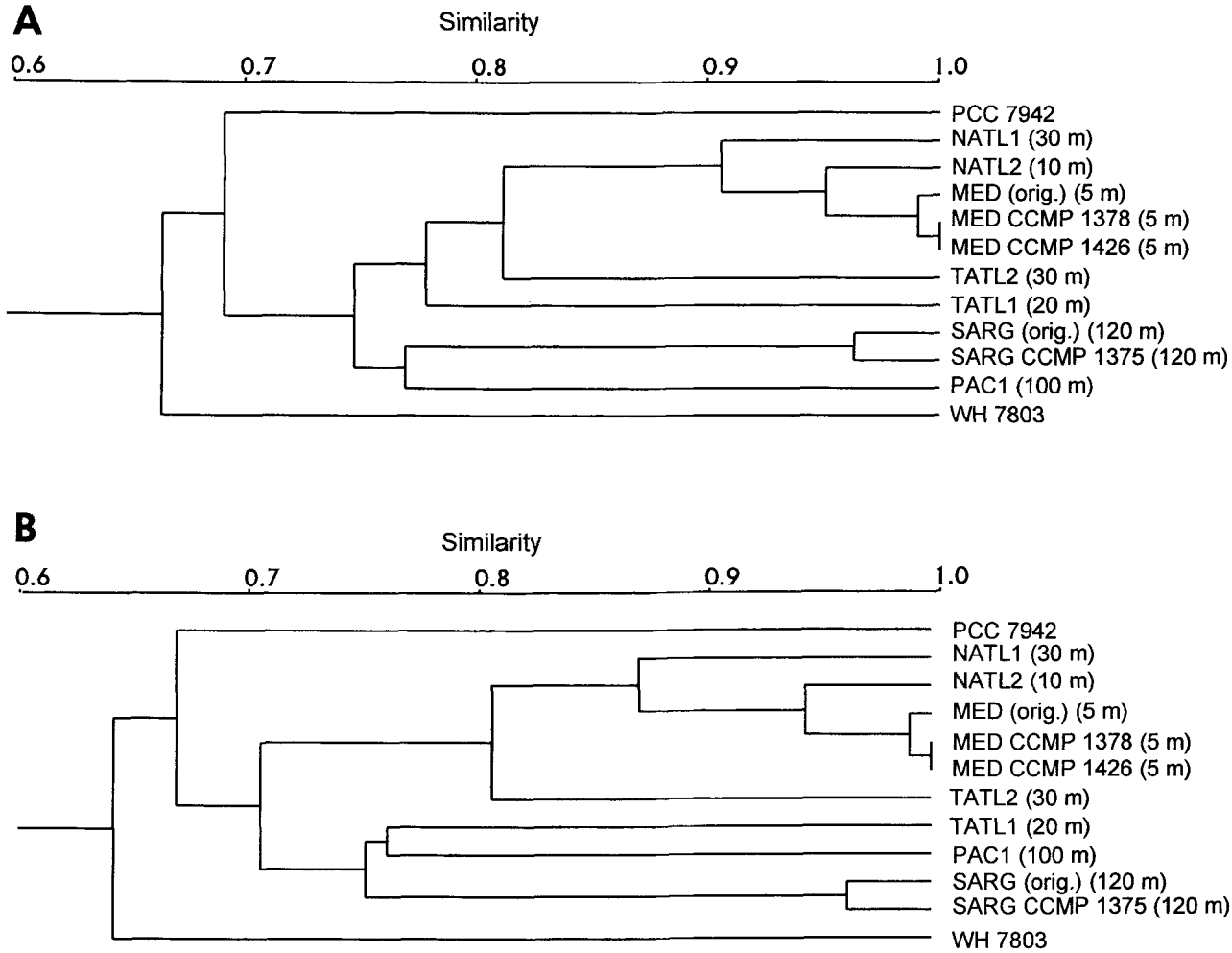

Fig. 2. Cluster analysis for various *Prochlorococcus* strains (showing depth of isolation) using the NTSYS software package and the *rbcL* and *psbA* data set in binary matrix form on the basis of shared restriction fragments. (*A*) UPGMA analysis. (*B*) Complete linkage analysis. *Synechococcus* PCC 7942 and *Synechococcus* WH 7803 are designated as outgroups.

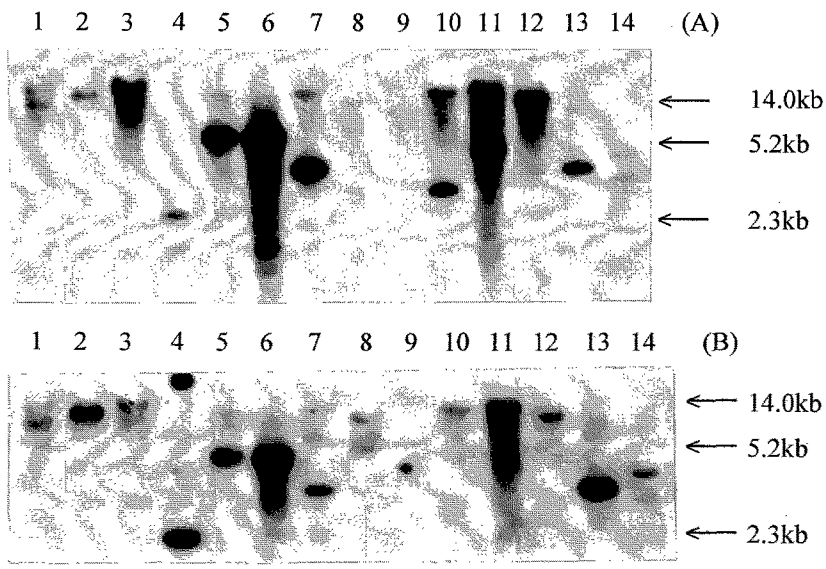

Fig. 3. Southern hybridization of various *Prochlorococcus* and *Synechococcus* strains probed with (*A*) a 3·8 kb *Bgl*II fragment encoding *psbA* from TATL1 and (*B*) a 0·98 kb *Pst*I–*Kpn*I fragment encoding *psbA* from the SARG strain. Lanes 1–3 were digested with *Bam*HI, lanes 4–9 with *Bgl*II and 10–14 with *Pst*I. Lanes: 1, PAC1; 2 and 13, SARG clone CCMP 1375; 3, MED clone CCMP 1426; 4, SARG original strain; 5 and 10, NATL2; 6 and 11, MED clone CCMP 1378; 7 and 12, TATL2; 8 and 14, *Synechococcus* WH7803; 9, *Synechococcus* sp. PCC 7942. Arrows show the sizes of the major hybridizing bands.

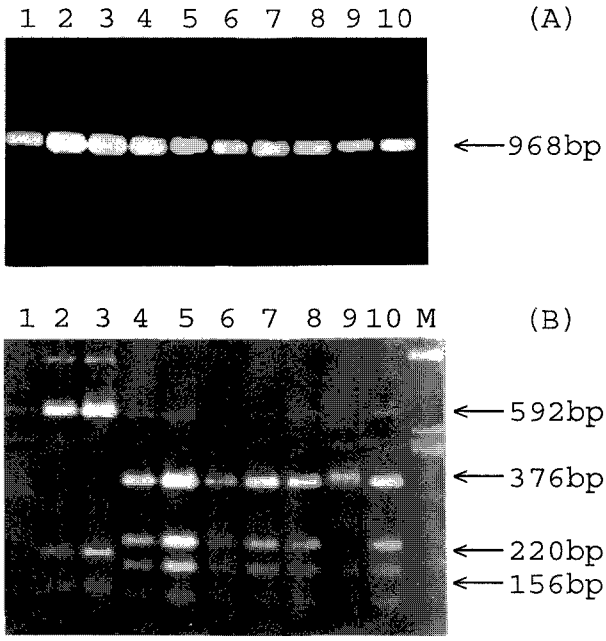

Fig. 4. (*A*) Products of PCR using *psbA*-specific primers. The 968 bp product contains only coding region of *psbA*. Lanes: 1, PAC1; 2, SARG original strain; 3, SARG clone CCMP 1375; 4, MED clone CCMP 1378; 5, MED clone CCMP 1426; 6, MED original strain; 7, NATL1; 8, NATL2; 9, TATL1; 10, TATL2. (*B*) PCR products produced in (*A*) cleaved with the restriction enzyme *Sau*3AI. M, marker lane. Arrows show the sizes of the major fragments.

template DNA very poorly. For amplification of *Synechococcus psbA* a different sense primer has to be used: 5′-ATGATCCCCACCCT(G/C)(C/T)TGAC(T/C)GC(G/C)A(C/T)-3′ (Data not shown).

Amplification of a single putative *psbA* fragment of the expected size of 968 bp is shown in Fig. 4*A*. The identity of the amplified fragment as representing most of the coding region of *psbA* was confirmed by Southern hybridization (data not shown). The products of PCR amplification were cut with various restriction enzymes. Only enzymes having tetranucleotide recognition sequences were found to produce a sufficient number of bands for analysis due to the relatively short length of the amplification product. The enzyme *Sau*3AI recognizing the motif GATC was highly informative in this respect (Fig. 4*B*). As expected, the degree of difference was much lower than that detected by Southern hybridization. Nevertheless, using all the clustering algorithms, the analysed strains clearly grouped into two different clusters (Fig. 5). One cluster, designated cluster 1, is formed by the MED strain and its derivatives, both the North Atlantic isolates and the tropical Atlantic isolate TATL2, all having four identical fragments, and with TATL1 sharing two of these. Comparison with the SARG strain and its clonal derivative CCMP1375 shows that these strains differ most widely from the former cluster, because there are no common fragments. PAC1, however, shows some relationship to both the SARG strains and to cluster 1. Only in the case of the PAC1 strain do the amplification products suggest that there might be more than one *psbA* gene copy present. Here, the total length of all visible fragments equals about twice the total length of the primary product of amplification. Insofar as there is a band shared with the SARG strains and two bands shared with *psbA*s from cluster 1, these might derive from two different types of *psbA* genes present in this strain. Alternatively, this strain may be polyclonal. These data support the grouping recovered from RFLP analysis.

Conclusions

The genetic difference between the two tropical Atlantic strains is noteworthy because they were both isolated from surface waters of geographically close sites and using similar procedures. TATL2 grouped together with the North Atlantic and Mediterranean Sea isolates despite their much more distant geographical origin. Furthermore, the PAC1 isolate showed some relatedness to the other strains, especially to the Sargasso Sea isolate. Although we know nothing of the frequency of exchange of genetic material between strains *in situ*, their relatedness probably reflects adaptation to the comparable environmental conditions around the deep chlorophyll maximum, from which both strains were isolated.

Immunological characterisation of *Prochlorococcus* spp. using monoclonal antibodies (MAbs) raised against whole cells of the SARG CCMP 1375 strain and the MED strain

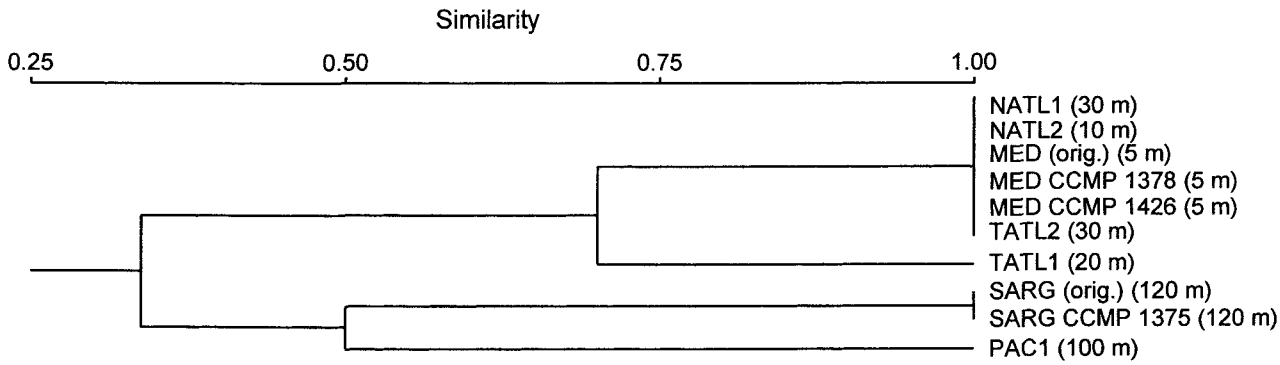

Fig. 5. Cluster analysis for various *Prochlorococcus* strains using the NTSYS software package with the UPGMA algorithm and the *psbA* PCR product *Sau*3AI data set in binary matrix form.

showed the SARG MAb to be specific, whereas the MED MAb cross-reacted with both NATL2 and TATL2 (L. Campbell, personal communication). These data agree well with the groupings resulting from the RFLP data presented here.

An initial physiological characterization of growth and photosynthesis in the MED strain seemed to indicate that this strain was low-light selected (as opposed to acclimated), probably because it was isolated and maintained for a long period at very low light prior to the measurements (Partensky *et al.*, 1993). However, the clonal isolate CCMP 1378 showed unambiguous features of adaptation to high light (Moore *et al.*, 1995). This physiological evidence suggests a polyclonal nature of the original MED isolate.

The degree of genetic variation among several of the *Prochlorococcus* strains analysed suggests that they may belong to different species, and is comparable to previous RFLP data obtained for *Synechococcus* (Douglas & Carr, 1988; Wood & Townsend, 1990). A similar degree of variation was observed in a single seawater sample taken from the Sargasso Sea at a 65 m site during the winter—a time when the water column was relatively well mixed (Palenik, 1994). In the latter study, using RNA polymerase gene sequence analysis, two distinct *Prochlorococcus*-like clusters were identified. The first one (cluster A) was significantly more closely related to the Mediterranean Sea isolate (DV1 = MED) than to either cluster B or the LG1 (= SARG) isolate. This suggests that populations from near the surface might be less genetically variable than populations from deep layers. Physiological approaches should yield an even better understanding of the relatedness and adaptability to varying light and nutrient regimes of these important marine prokaryotes.

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