

Molecular diversity among marine picophytoplankton as revealed by *psbA* analyses

Gil Zeidner,¹ Christina M. Preston,² Edward F. Delong,² Ramon Massana,³ Anton F. Post,⁴ David J. Scanlan⁵ and Oded Béjà^{1*}

¹Department of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel.

²Monterey Bay Aquarium Research Institute, Moss Landing, CA 95039-0628, USA.

³Departament de Biologia Marina i Oceanografia, Institut de Ciències del Mar, CSIC, E-08003 Barcelona, Spain.

⁴H. Steinitz Marine Biology Laboratory, Interuniversity Institute for Marine Sciences, Eilat 88103, and Department of Microbial and Molecular Ecology, Hebrew University of Jerusalem, Jerusalem, Israel.

⁵Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK.

Summary

Photosynthetic microorganisms play a crucial role in the marine environment. In vast areas of the oceans, marine primary productivity is performed by cells smaller than 2–3 µm (picoplankton). Here, we report on molecular analyses of the conserved photosynthetic *psbA* gene (coding for protein D1 of photosystem II reaction centre) as a diversity indicator of naturally occurring marine oxygenic picophytoplankton. The *psbA* genes proved to be good indicators of the presence of a wide variety of photosynthetic marine microbial groups, including new cyanobacterial groups and eukaryotic algae (prasinophytes). Furthermore, using environmental bacterial artificial chromosome (BAC) libraries, we were able to correlate *psbA* genes with small subunit rRNAs and, therefore, to confirm their phylogenetic affiliation.

Introduction

Both phototrophic bacteria and eukaryotes containing chlorophyll use light as their main energy source. Based on satellite measurements of ocean colour and production models, it is estimated that phytoplankton fix about 55 GT of carbon yearly, about 50% of total global photosynthetic

production (Behrenfeld *et al.*, 2001). Oxygenic ('green-plant') photosynthesis has been a key metabolic process on Earth, and a major driver in the evolution of plant and metazoan species. The photosynthetic apparatus is composed of two photosystems, I and II, of which photosystem II (PSII) is a membrane-embedded protein complex that transfers electrons across the photosynthetic membrane from water to the plastoquinone pool. The D1 and D2 proteins form the reaction centre dimer of PSII that bind the primary electron donors and acceptors.

Cultivation-independent surveys of microbial rRNA genes have greatly expanded the known phylogenetic variety of microbial species on earth (Pace, 1997). The accepted distributions of major microbial groups have been dramatically altered by such molecular analyses of naturally occurring microbes (DeLong, 2001). It is now well established that many new microbial groups discovered via cultivation-independent surveys represent major components of natural microbial assemblages in marine systems (Karl, 2002).

Results and discussion

Motivated by recent discoveries of novel planktonic microorganisms in the ocean (DeLong *et al.*, 1994; Béjà *et al.*, 2000a; 2001; 2002; Karner *et al.*, 2001; López-García *et al.*, 2001; Moon-van der Staay *et al.*, 2001; Zehr *et al.*, 2001), we searched for molecular diversity among 'green' photosynthetic planktonic microorganisms. Although prior studies focused on the survey of rRNA operons, we decided to use a functional, photosynthetic protein-coding gene. For that purpose, DNA primers were designed to well-conserved amino acid regions of the photosystem II core protein, protein D1, coded by the *psbA* gene. This gene is found in cyanobacteria and in chloroplast genomes of algae and higher plants and is used widely as a phylogenetic marker (Morden and Golden, 1989; Scherer *et al.*, 1991; Morden *et al.*, 1992; Zhang *et al.*, 2000). General degenerate primers were designed to target all known oxygenic photosynthetic lineages, both prokaryotic (cyanobacteria) and eukaryotic (algae and higher plants).

PsbA genes were polymerase chain reaction (PCR) amplified from DNA extracts of mixed picoplankton assemblages from the Red Sea (RED clones), the Mediterranean Sea (MED clones) and the central North Pacific

Received 14 August, 2002; revised 13 November, 2002; accepted 14 November, 2002. *For correspondence. E-mail beja@tx.technion.ac.il; Tel. (+972) 4829 3961; Fax (+972) 4822 5153.

Ocean (HOT clones). The *PsbA* protein phylogenetic tree encompassed two major clades, one containing eukaryotic plastid *PsbA* sequences and another with cyanobacterial representatives. Although some clones clustered close to known species (e.g. haptophytes, cryptophytes, stramenopiles and the marine cyanobacterium *Prochlorococcus*), some formed distinct clades (MED2-4, RED132-6-2, RED132-6-4 and RED132-6-6 branching close to marine *Synechococcus*; MED2-23, RED122-2-1, RED122-4-9 and RED122-4-3 branching close to green algae). *PsbA* clones from dinoflagellates are missing from our analysis because of size constraints of our cell fractionation procedure, which selected specifically for the picoplankton size fraction (<3 µm).

The topology observed for the marine cyanobacterial *PsbA* clade was similar to that observed by resolution of *Prochlorococcus* and *Synechococcus* ecotypes using cyanobacterial RNA polymerase C1 gene (*rpoC1*) (Palenik and Haselkorn, 1992), 16S rRNA (West *et al.*, 2001) or 16S–23S rDNA internal transcribed spacer (ITS) sequences (Rocap *et al.*, 2002). The *PsbA* tree topology also separated between low light (LL)- and high light (HL)-adapted *Prochlorococcus* strains and placed the LL-adapted *Prochlorococcus* deeper in the tree, in proximity to marine *Synechococcus*, again in agreement with 16S or 16S–23S rDNA ITS tree topologies (West *et al.*, 2001; Rocap *et al.*, 2002). The broad phylogenetic diversity observed for the *Prochlorococcus*-like *PsbA* sequences coming from the Hawaiian HOT station might hint at many different ecological niches occupied by the different *Prochlorococcus* strains.

In order to correlate the different *PsbA* groups identified to known rRNA phylotypes, we screened a Bacterial Artificial Chromosome (BAC) library (Béjà *et al.*, 2000b) from Monterey Bay, California (<http://www.mbari.org/rd/tigr> and <http://www.tigr.org/tdb/MBMO>) for clones containing *psbA* genes. Five *psbA*-containing BAC clones were identified (eBAC). The BACs *PsbA* sequences fell into two groups, three (eBAC64, eBAC23D01 and eBAC23D10) clustering with the newly identified green algal clade and two (eBAC65-3 and eBAC65-10) with the distinctive marine *Synechococcus* clade. Recently, the representation of small subunit (SSU) rRNA-containing BAC clone sequences in the surface Monterey Bay BAC library was determined (Béjà *et al.*, 2000b). All BAC-encoded plastid-like or cyanobacterial rRNAs analysed clustered with either 'uncultured Prasinophyceae' (Rappé *et al.*, 1998; 2000), or the newly described (yet to be cultivated) *Synechococcus* clade IV (Rocap *et al.*, 2002). This new *Synechococcus* clade has been described so far solely in BAC- and PCR-amplified rRNA gene libraries (Béjà *et al.*, 2000b; Suzuki *et al.*, 2001).

We therefore checked for the presence of rRNA genes in Monterey Bay BAC clones that contained a *psbA*

gene. The *psbA*-containing BACs related to green algae did indeed contain rRNAs most highly similar to prasinophyte chloroplast rRNA (eBAC23D01 and eBAC23D10 – prasinophyceae in Fig. 1; identical to AF268223 rRNA observed previously by Béjà *et al.*, 2000b). *Synechococcus*-related *psbA* BAC clones did not contain an rRNA gene, and so we can only speculate that these sequences belong to clade IV of uncultured *Synechococcus* (Rocap *et al.*, 2002), based on unlinked SSU rRNA data from the BAC library (Béjà *et al.*, 2000b).

The Prasinophyceae group contains many cultured representatives beside *Nephroselmis olivacea* (*PsbA* of *N. olivacea* chloroplast shown in Fig. 1). SSU rDNA genes of many cultured prasinophytes have been sequenced and are similar to many PCR-amplified rRNA genes recovered from natural samples (L. Guillou and R. Massana, unpublished results). We therefore searched for more *psbA* sequences found in unpublished Prasinophyceae genome projects. Access to unfinished genomic data (Derelle *et al.*, 2002) from the marine prasinophyte *Ostreococcus tauri* (Courties *et al.*, 1994) became available (H. Moreau, personal communication) and revealed that the *O. tauri* *PsbA* sequence is identical to eBAC64 and eBAC23D01 *PsbA* sequences reported here. Therefore, the 'uncultured Prasinophyceae' reported previously by our group (Béjà *et al.*, 2000b) and others (Rappé *et al.*, 1997; 1998; Frias-Lopez *et al.*, 2002) is now known to be derived from *O. tauri* species, a cultivated group.

Our results, based on a functional photosynthetic gene, document naturally occurring genetic diversity among marine picophytoplankton, and provide a reliable marker for photosynthetic picoplankton groups including picoeukaryotic green algae (prasinophytes) and cyanobacteria. Our results confirm and extend and, in one case link with *psbA* gene sequence data, previous results obtained from SSU rRNA sequences (Rappé *et al.*, 1998; Díez *et al.*, 2001; Moon-van der Staay *et al.*, 2001; Rocap *et al.*, 2002).

Experimental procedures

Environmental DNA collection

Red Sea samples (29.28°N, 34.55°E and 27.17°N, 34.22°E) were collected in February 1999 (5 and 50 m; fraction >0.45 µm) (Lindell and Post, 2001). Mediterranean samples were from the Alborán Sea (36.0°N, 4.25°W) collected in May 1998 (5 and 50 m; fraction 0.2–2 µm) and from the Catalano-Balear Sea (42.7°N, 2.8°E) in January 2000 (5 and 60 m; fraction 0.2–5 µm). HOT station (22.4°N, 158.0°W) waters were collected in March 1998. DNA was extracted from the samples according to the method of Massana *et al.* (1997). The surface BAC library (Béjà *et al.*, 2000b) was prepared from sea water harvested at Monterey Bay, California (36.7°N, 122.4°W; fraction 0.2–1.6 µm).

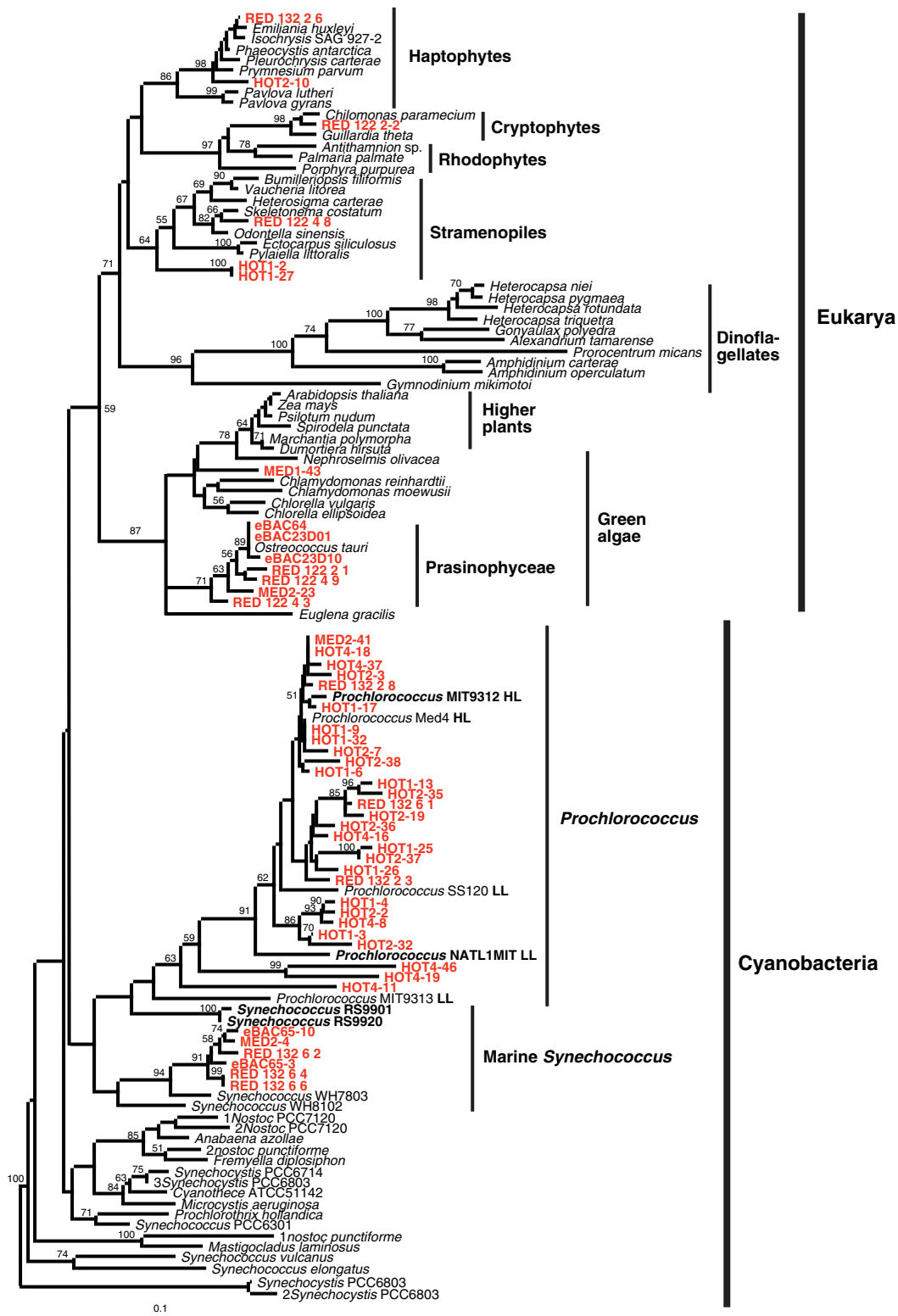


Fig. 1. Phylogenetic relationships of PsbA proteins. Evolutionary distances for the PsbA proteins were determined from an alignment of 320 amino acid positions using neighbour-joining analysis (Thompson *et al.*, 1997). PsbA sequences that were amplified by PCR in this study are indicated by bold letters; environmental sequences are marked in red, with MED indicating Mediterranean Sea, RED indicating Red Sea and HOT indicating Hawaii ocean time series. Low light- and High light-adapted *Prochlorococcus* strains are marked in bold LL and HL respectively. Bootstrap values >50% are indicated above the branches. The scale bar represents the number of substitutions per site.

PsbA amplification and cloning

The general degenerate primers used to amplify the *psbA* gene from environmental samples were 58-VIDIGIREP-66 (5'-GTNGAYATHGAYGGNATHMNGNGARCC-3') and 331-MHERNAHNF-340 (5'-GGRAARTTRTGNGCRTTNCKYT CRTGC-AT-3'). PCR amplification was carried out in a total volume of 20 µl containing 10 ng of template DNA, 200 µM dNTPs, 1.5 mM MgCl₂, 0.2 µM primers and 2.5 U of BIO-X-ACT DNA polymerase (Bioline). The amplification conditions comprised steps at 92°C for 4 min, and 35 cycles at 92°C for 1 min, 55°C for 1 min and 68°C for 1 min. PCR-amplified *psbA* from each depth of the different sampling sites was ligated into the pDrive cloning vector (Qiagen) before transformation into *Escherichia coli* DH10B. DNA was isolated with a Qiagen Miniprep kit, and clones were analysed by amplification with the specific *psbA* primers, followed by *EcoRI* and *AluI* restriction fragment length polymorphism (RFLP) analysis, before sequencing of representative RFLP groups.

Synechococcus isolates

Synechococcus RS9901 and RS9920 were isolated from Station A (29.28°N, 34.55°E) in the Gulf of Aqaba, Red Sea, during March 1999 (1 m depth) and November 1999 (150 m depth), respectively (D. J. Scanlan *et al.*, unpublished) aboard the RV *Sea Surveyor*.

Data deposition

Sequences reported in this paper have been submitted to GenBank under the following accession numbers: AY176592–AY176643.

Acknowledgements

We thank J. Zehr and D. Karl for the HOT samples, and H. Moreau for access to unpublished genomic data from *O. tauri*. Red Sea samples were taken during the 1999 RV *Meteor* cruise in the northern Red Sea and Gulf of Aqaba. The data for the *psbA* sequences from *Nostoc punctiforme*, *Prochlorococcus marinus* MED4, *Prochlorococcus marinus* MIT9313 and *Synechococcus* WH8102 were obtained from the US DOE Joint Genome Institute (JGI) at http://www.jgi.doe.gov/JGI_microbial/html/index.html for use in this publication only. This work was supported in part by the Human Frontiers Science Program (O.B.), Israel Science Foundation grant 525/98 (A.F.P.), the European PICODIV programme EVK3-CT1999-00021 (D.J.S. and R.M.), NSF grants OCE-0001619 and MCB-0084211 (E.F.D.), and by support provided to MBARI by the David and Lucile Packard Foundation.

References

Behrenfeld, M.J., Randerson, J.T., McClain, C.R., Feldman, G.C., Los, S.O., Tucker, C.J., *et al.* (2001) Biospheric pri-

- mary production during an ENSO transition. *Science* **291**: 2594–2597.
- Béjà, O., Aravind, L., Koonin, E.V., Suzuki, M.T., Hadd, A., Nguyen, L.P., *et al.* (2000a) Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. *Science* **289**: 1902–1906.
- Béjà, O., Suzuki, M.T., Koonin, E.V., Aravind, L., Hadd, A., Nguyen, L.P., *et al.* (2000b) Construction and analysis of bacterial artificial chromosome libraries from a marine microbial assemblage. *Environ Microbiol* **2**: 516–529.
- Béjà, O., Spudich, E.N., Spudich, J.L., Leclerc, M., and DeLong, E.F. (2001) Proteorhodopsin phototrophy in the ocean. *Nature* **411**: 786–789.
- Béjà, O., Suzuki, M.T., Heidelberg, J.F., Nelson, W.C., Preston, C.M., Hamada, T., *et al.* (2002) Unsuspected diversity among marine aerobic anoxygenic phototrophs. *Nature* **415**: 630–633.
- Courties, C., Vaquer, A., Troussellier, M., Lautier, J., Chrétiennot-Dinet, M.J., Neveux, J., *et al.* (1994) Smallest eukaryotic organism. *Nature* **370**: 255.
- DeLong, E.F. (2001) Microbial seascapes revisited. *Curr Opin Microbiol* **4**: 290–295.
- DeLong, E.F., Wu, K.Y., Prezelin, B.B., and Jovine, R.V. (1994) High abundance of Archaea in Antarctic marine picoplankton. *Nature* **371**: 695–697.
- Derelle, E., Ferraz, C., Lagoda, P., Eychenié, S., Cooke, R., Regad, F., *et al.* (2002) DNA libraries for sequencing the genome of *Ostreococcus tauri* (Chlorophyta, Prasinophyceae): the smallest free-living eukaryotic cell. *J Phycol* **38**: 1150–1156.
- Díez, B., Pedrós-Alió, C., and Massana, R. (2001) Study of genetic diversity of eukaryotic picoplankton in different oceanic regions by small-subunit rRNA gene cloning and sequencing. *Appl Environ Microbiol* **67**: 2932–2941.
- Frias-Lopez, J., Zerkle, A.L., Bonheyo, G.T., and Fouke, B.W. (2002) Partitioning of bacterial communities between seawater and healthy, black band diseased, and dead coral surfaces. *Appl Environ Microbiol* **68**: 2214–2228.
- Karl, D.M. (2002) Hidden in a sea of microbes. *Nature* **415**: 590–591.
- Karner, M.B., DeLong, E.F., and Karl, D.M. (2001) Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature* **409**: 507–510.
- Lindell, D., and Post, A.F. (2001) Ecological aspects of *ntcA* gene expression and its use as an indicator of the nitrogen status of marine *Synechococcus* spp. *Appl Environ Microbiol* **67**: 3340–3349.
- López-García, P., Rodríguez-Valera, F., Pedrós-Alió, C., and Moreira, D. (2001) Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. *Nature* **409**: 603–607.
- Massana, R., Murray, A.E., Preston, C.M., and DeLong, E.D. (1997) Vertical distribution and phylogenetic characterization of marine planktonic Archaea in the Santa Barbara channel. *Appl Environ Microbiol* **63**: 50–56.
- Moon-van der Staay, S.Y., De Wachter, R., and Vaultot, D. (2001) Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. *Nature* **409**: 607–610.
- Morden, C.W., and Golden, S.S. (1989) *psbA* genes indicate common ancestry of prochlorophytes and chloroplasts. *Nature* **337**: 382–385.

- Morden, C.W., Delwiche, C.F., Kuhsel, M., and Palmer, J.D. (1992) Gene phylogenies and the endosymbiotic origin of plastids. *Biosystems* **28**: 75–90.
- Pace, N.R. (1997) A molecular view of microbial diversity and the biosphere. *Science* **276**: 734–740.
- Palenik, B., and Haselkorn, R. (1992) Multiple evolutionary origins of prochlorophytes, the chlorophyll b-containing prokaryotes. *Nature* **355**: 265–267.
- Rappé, M.S., Kemp, P.F., and Giovannoni, S.J. (1997) Phylogenetic diversity of marine coastal picoplankton 16S rRNA genes cloned from the continental shelf off Cape Hatteras, North Carolina. *Limnol Oceanogr* **42**: 811–826.
- Rappé, M.S., Suzuki, M.T., Vergin, K.L., and Giovannoni, S.J. (1998) Phylogenetic diversity of ultraplankton plastid small-subunit rRNA genes recovered in environmental nucleic acid samples from the Pacific and Atlantic coasts of the United States. *Appl Environ Microbiol* **64**: 294–303.
- Rappé, M.S., Vergin, K., and Giovannoni, S.J. (2000) Phylogenetic comparisons of a coastal bacterioplankton community with its counterparts in open ocean and freshwater systems. *FEMS Microbiol Ecol* **33**: 219–232.
- Rocap, G., Distel, D.L., Waterbury, J.B., and Chisholm, S.W. (2002) Resolution of *Prochlorococcus* and *Synechococcus* ecotypes by using 16S–23S ribosomal DNA internal transcribed spacer sequences. *Appl Environ Microbiol* **68**: 1180–1191.
- Scherer, S., Herrmann, G., Hirschberg, J., and Boger, P. (1991) Evidence for multiple xenogenous origins of plastids: comparison of psbA-genes with a xanthophyte sequence. *Curr Genet* **19**: 503–507.
- Suzuki, M.T., Béjà, O., Taylor, L.T., and DeLong, E.F. (2001) Phylogenetic analysis of ribosomal RNA operons from uncultivated coastal marine bacterioplankton. *Environ Microbiol* **3**: 323–331.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**: 4876–4882.
- West, N.J., Schonhuber, W.A., Fuller, N.J., Amann, R.I., Rippka, R., Post, A.F., and Scanlan, D.J. (2001) Closely related *Prochlorococcus* genotypes show remarkably different depth distributions in two oceanic regions as revealed by in situ hybridization using 16S rRNA-targeted oligonucleotides. *Microbiology* **147**: 1731–1744.
- Zehr, J.P., Waterbury, J.B., Turner, P.J., Montoya, J.P., Omoregie, E., Steward, G.F., et al. (2001) Unicellular cyanobacteria fix N₂ in the subtropical North Pacific Ocean. *Nature* **412**: 635–638.
- Zhang, Z., Green, B.R., and Cavalier-Smith, T. (2000) Phylogeny of ultra-rapidly evolving dinoflagellate chloroplast genes: a possible common origin for sporozoan and dinoflagellate plastids. *J Mol Evol* **51**: 26–40.