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Picoplankton culture assessment using single strand conformation polymorphism and partial 18S sequencing

KLAUS VALENTIN, HELGA MEHL AND LINDA MEDLIN*
ALFRED WEGENER INSTITUT, AM HANDELSHAFEN 12, 27570 BREMERHAVEN, GERMANY
*CORRESPONDING AUTHOR: lkmedlin@awi-bremerhaven.de

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Environmental water samples were taken throughout 2001 from fractioned water samples at the Helgoland time series site. The less than 3-μm fraction was inoculated into various media. Serial dilutions from these inoculations produced a large number of rough cultures from which several hundred well-growing picoplankton cultures were started. We established a combination of crude DNA extraction, single strand conformation polymorphism (SSCP) fingerprinting and subsequent sequence analysis to screen these large numbers of cultures, assess their purity/clonality and determine their identity. Picoplankton species (i.e., cells smaller than 3 μm) are difficult to distinguish because of their small size and the lack of morphological characters. Therefore, molecular techniques provide the most reliable method to achieve their identification. Cultures were enriched for photoautotroph cells, i.e., no carbon source was added, and cultures were grown in the light. From these cultures, crude DNA was extracted, which was used for partial 18S polymerase chain reaction (PCR). On average, 50% of the cultures produced a PCR product. SSCP analysis of PCR products revealed the clonality of a given culture. If clonal, then there was only a single SSCP band; if not clonal, then there were multiple SSCP bands. Single SSCP bands were subsequently sequenced, and sequences were used to identify the culture. For this study, 300 cultures were screened resulting in the identification of 63 potentially clonal cultures. These methods proved to be relatively easy to apply to assess the clonality and purity of the cultures.

INTRODUCTION

Cells smaller than 3 μm (=picoplankton) are an important component of the marine plankton (Caron *et al.*, 1999). In many oceanic regions, namely those with nutrient limitations, the less than 3-μm fraction can contribute more than 50% of the total chlorophyll (Joint and Pomeroy, 1986). Therefore, the molecular characterization of photosynthetic picoplankton has received considerable attention during the last decade (Caron *et al.*, 1999; Díez *et al.*, 2001a,b; Lopez-Garcia *et al.*, 2001; Moon-van der Stay *et al.*, 2001; Massana *et al.*, 2002, 2004a,b; Romari and Vaulot, 2004). The prokaryotic members of the picoplankton mainly belong to two cyanobacterial genera *Synechococcus* and *Prochlorococcus*. For both, a considerable number of geographical and physiological ecotypes have been isolated and are held in culture (Scanlan, 2003). Eukaryotic photoautotrophs are also common in the picoplankton fraction, the smallest of which are less than 1 μm in diameter. In contrast to the cyanobacterial picoplankton, the eukaryotic one is highly diverse in that all major algal groups are contributing (Fuller *et al.*, 2006.). In addition, new algal classes have been detected in the eukaryotic marine picoplankton, e.g. the bolidophytes, the pelagophytes, the prasinophytes and the pinguinophytes (Moestrup, 1991; Andersen *et al.*, 1993; Guillou *et al.*, 1999; Kawachi *et al.*, 2002). Major

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progress on eukaryotic picoplankton diversity has been made by sequencing of environmental clone libraries (Caron *et al.*, 1999; Diez *et al.*, 2001a,b; Lopez-Garcia *et al.*, 2001; Moon-van der Stay *et al.*, 2001; Massana *et al.*, 2002, 2004a,b; Romari and Vaulot, 2004). That approach has revealed that especially among the stramenopiles and alveolates, novel clades exist that contain large numbers of potential new picoplankton species. However, the clone library approach only indicates the presence of new species, because no culturable organism is seen. In order to identify the organisms that are associated with the new sequences, and to study their biology and ecology, it is necessary to establish cultures, to screen and taxonomically identify them. As in the bacterial world, probably many of the new species are currently not accessible, because we are unable to culture them.

Although establishing picoplankton cultures is no more difficult than in other plankton fractions; however, screening for their purity and clonality is. Because of their small size, they are not easy to visualize by light microscopy and, moreover, they mostly lack morphological characters. Cultures may look homogenous in light microscopy but consist of different species (Porter *et al.*, 1996). Electron microscopy can unravel such dilemmas, but it is too time consuming to analyze large numbers of cultures, and it is virtually impossible to judge whether a culture is clonal or axenic with this method. Analysis of pigment or lipid composition of cultures can be done via high performance liquid chromatography (HPLC) for many samples in a short time, but results will only give hints to the possible identity of a culture, and uniformity of a culture cannot be proven. Pigment and lipid composition is also strongly dependent on the physiological state and the growth conditions and may, thus, be misleading. It would be easy to determine the identity of a culture by sequencing a relevant gene, e.g., the 18S rRNA gene, provided this is accessible. Pure cultures will produce clear sequences, and the sequence will easily allow an identification of the culture phylogenetically. With this method, those cultures not unialgal can be culled or subject to reisolation. However, isolating the DNA, amplifying the 18S gene and sequencing the product is still time consuming and costly, at least when every single culture has to be sequenced.

Therefore, there is a need to establish a method that allows a fast, cost-efficient and gene sequence-based analysis of large numbers of cultures. Here we describe such a method that is based on a simple DNA isolation procedure, partial 18S polymerase chain reaction (PCR) amplification, fingerprinting of the product and subsequent sequencing of only that subset of the cultures producing single bands in the fingerprint.

METHOD

Establishment of picoplankton cultures

Surface water samples were taken at Helgoland 'Kabeltonne' (North Sea 54°11.3′ N, 7°54.0′ E), the same location that is used since 1962 for the 'Helgoland Time Series'. Samples were immediately transferred to the laboratory and filtered through a 3-μm polycarbonate filter by gravity flow. From the filtrate, serial dilutions (1:10, 1:100, 1:1000 and 1:10000) were prepared in Drebes (Drebes, 1966) and Institute of Marine Research (Eppley *et al.*, 1967) medium in 24 × 2 mL microtiter plates. Plates were incubated in 10 and 15°C culture rooms at a 14:10 h light: dark cycle and 50 μmole m^{-2} s^{-1}. These culture conditions, except for the daylength, matched approximately the environmental conditions at the times when the samples were taken. Cultures were routinely checked by eye, and those showing visible growth were transferred to 50-mL flasks. The time before visible growth occurred varied considerably and is dependent on each species cultured.

DNA extraction and PCR

Visibly grown cultures were checked by light microscopy, photographed, and those that looked uniform and consisted of small cells were further analyzed. One-and-a-half milliliters of these cultures were spun down (5000 g, 3 min), resuspended in 20 μL of water, shock-frozen in liquid nitrogen and subsequently heated to 94°C for 10 min. After another centrifugation step (5 min, 13 000 g), Ten microliters of the supernatant was used for PCR amplification. We applied primers 528F (eukaryote specific) and 926R (universal) to amplify an ~500 bp large fragment of the 18S rRNA gene (Elwood *et al.*, 1985). The 926R primer was phosphorylated in order to allow subsequent single strand digestion (see next section). PCR was performed in an Eppendorf cycler with Perkin Elmer polymerase (2U/reaction) and the following program: 5′ 94°C, 35x 1′ 94°C/1′ 50°C/2′ 72°C, followed by 7′ 72°C extension. The reaction volume was 50 μL. The success of the PCR reaction was monitored by agarose gel electrophoresis.

Single strand conformation polymorphism and subsequent sequence analysis

For single strand conformation polymorphism (SSCP) analysis, PCR products were purified on Qiagen PCR purification columns and finally eluted from the columns in 50 μL deionized water. Twenty-five microliters of the elutant were kept at −20°C and the remaining 25 μL were treated with 10 U Lambda exonuclease (New England Bioloabs) at 37°C for 1 h. After exonuclease treatment,

products were again purified on Qiagen PCR minielute columns in order to remove nucleotides, the enzyme and salts. Samples were eluted with 10 µL of deionized water, boiled for 4 min and 10 µL of SSCP loading buffer (95% formamide, 4.9% 0.1M NaOH, 0.1% Bromophenol blue) was added. Samples were then immediately loaded to a gel or stored at −20°C. For polyacrylamide gel electrophoresis, we used a macrophor apparatus, which was kept at 20°C by means of a thermostat plate and a recycling buffer in a cryostat. The gel contained 6.25% acrylamide (any premixed solution), 1× Tris Borate EDTA (TBE) buffer and had a size of 35 × 25 × 0.04 cm. Electrophoresis was performed at <400 V, <8 mA and <5 W for 16–20 h, and 4 µL of the samples were loaded. Bands were visualized by silver staining. For samples producing single bands, the remaining 25 µL (see above) were used for sequence analysis using the 528F primer.

RESULTS

Picoplankton cultures

We chose Helgoland Kabeltonne (North Sea) as our sampling site, because this site is routinely used for plankton sampling for the Helgoland time series. On an almost daily basis, samples have been taken since 1962; plankton are counted, and parameters, such as salinity, temperature and nutrient concentrations are measured. Throughout 2001 and 2002, samples were taken on for our purposes on almost monthly basis, and per sampling 96 pre-cultures were started resulting in more than 1000 pre-cultures and several hundred well-growing 50 mL cultures. There was no obvious effect of either growth medium or the temperature on the success of culture establishment. Best growth was obtained with 1:10–1:100-fold dilution of filtered seawater. Higher dilutions rarely produced growing cultures, thus we can assume that cultures from these dilutions represent clonal cultures. Culturing was more successful, and average size of cells in cultures was smaller when filtration was done by gravity flow rather than vacuum filtration (data not shown).

Crude DNA extraction and partial 18S PCR

Extraction of DNA from cultures by phenol, CTAB extraction or by the use of DNA binding columns is time consuming and does not always guarantee good results (Valentin *et al.*, 2005). Therefore, we developed a crude extraction protocol based on centrifugation, shock-freezing and subsequent boiling. Although the DNA resulting from this procedure is not pure in terms of its OD 280/260 ratio, it proved to be a good template for PCR amplification in about 50% of the preparations (see Discussion).

For PCR amplification, we chose primer combinations of 528 forward (F)/926 reverse (R). Primer 528F only binds to eukaryotic rDNA and 926R is universal, ensuring that only eukaryotic rDNA can be amplified. Using 528F was a prerequisite, because this primer was also used for large-scale partial sequencing of environmental clone libraries in our laboratory (Medlin *et al.*, 2006) thus allowing the comparison of sequences from cultures to those from these other libraries. Primer 926R was phosphorylated to allow specific digestion of the anti-sense strand. We also tested the oligo pair 528F/1055R, which was less efficient (data not shown). On average, 50% of the cultures produced a product in the PCR reaction. We assume that those not producing a PCR product were prokaryotes, but we did not test this with prokaryotic PCR primers. Alternatively, the PCR might have failed because of impurities in the DNA extraction; this could be checked by spiking the PCR with known DNA. Products had to be purified prior to subsequent single strand digestion. Additional purification after single strand digestion improved the resolution in gel electrophoresis (data not shown) but is not essential where large numbers of cultures have to be screened in a most cost-efficient way.

Gel electrophoresis of SSCP products

SSCP products were analyzed on 6% polyacrylamide gels at 20°C allowing secondary structure formation. The gels were run on old-style manual sequencing electrophoresis units, which are often found in laboratories. In theory, a pure culture should produce a single band, whereas mixed cultures should result in multiple bands. We tested this by analyzing pure and mixed cultures. Whereas mixed cultures always produced multiple bands (data not shown) of the four pure cultures tested one (*Skeletonema costatum*) gave rise to more than one band (Fig. 1). However, given the recent revision of this species, this culture may not have been clonal (Sarno *et al.*, 2005). Multiple bands resulting from microheterogeneity should only differ by a few bases and thus should be tightly clustered on the gel. Grossly different band sizes are likely different species. On average, about 10% of the cultures produced a single band on the SSCP gel (Fig. 1). From these cultures, the 528F/926R PCR products were subsequently sequenced with the 528F primer. More than 90% of these sequencing reactions produced clean sequences.

Sequences were compared to Genbank, the Ribosomal Data Bank (RDP) or to the ARB databank in order to determine their phylogenetic affiliation. Table I summarizes the results. In total, we could determine homogeneity and identity of 61 cultures from 300 tested. These sequences

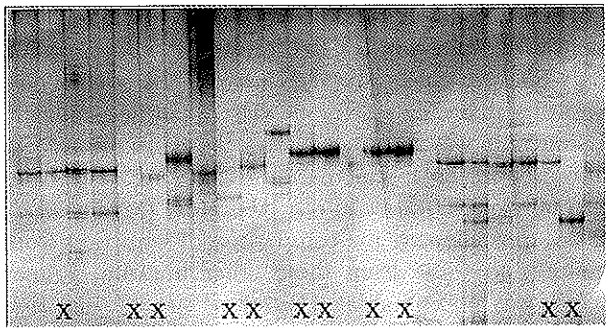

Fig. 1. Single strand conformation polymorphism (SSCP) analysis of Helgoland cultures. Lanes that displayed a single band (labeled X) were chosen for subsequent sequencing and produced clean sequences indicating the presence of a unialgal culture.

have been deposited in Genbank under the accession numbers AM041070-129.

DISCUSSION

Here we present a procedure for fast and exact identification of large numbers of cultures and applied it to picoplankton. We could identify a variety of potentially new species belonging to a wide phylogenetic range (Chlorophyta, heterkont algae). Thus, the method proved to be useful for the analysis of cultures that are otherwise difficult to identify; the method seems not to be biased by the PCR step or the primers used. It should, in principle, also be applicable to other plankton classed, i.e., those larger than 3 μm. The use of PCR-specific

Table I: A list of the Helgoland (He) cultures (date, location in microtiter plate) assessed by sequencing to be unialgal and their closest taxonomic affiliation determined by sequence identity

Strain	Closest taxonomic identification
He010322, I1_C3	*Bathycoccus*
He010710, D1_B6	*Phaeocystis*
He010710, D1_C6	*Ostereococcus*
HE000427, U_D4	*Bathycoccus*
He001206, D1_C1	*Nanochlorum*
He001206, D2_B1	*Phaeocystis*
HE000427, U_D3	*Nanochlorum*
HE000427, U_D6	*Nanochlorum*
He010619, D1_A1	*Bathycoccus*
He010619, D2_A1	*Bathycoccus*
He010619, D2_A3	*Cafeteria*
He010619, D2_B4	*Bathycoccus*
He010619, I1_A1	*Cafeteria*
He010619, I1_A2	*Micromonas*
He010619, I1_A3	Stramenopiles, Group VI
He010619, I1_A4	*Cafeteria*
He010619, I1_C1	*Spumella*
He010619, I1_D1	*Cafeteria*
He010619, I2_A6	*Nanochlorum*
He010619, I2_B2	*Ostereococcus*
He010619, I2_B3	*Cafeteria*
He010418, D1_C2	*Bathycoccus*
He010418, D1_C6	*Bathycoccus*
He010418, D2_A2	*Nanochlorum*
He010418, D2_B1	*Bathycoccus*
He010418, I1_A6	*Chlorella*
He010418, I2_A1	*Nanochlorum*
He010418, I2_A4	*Nanochlorum*
He010418, D1_C4	*Bathycoccus*
He010418, I1_C4	*Bathycoccus*
He010516, D1_B6	*Chlorella*
He010516, D2_A1	*Bathycoccus*
He010516, D2_B5	*Bathycoccus*
He010619, D2_C6	*Micromonas*
He010710, D1_C1	Streptophyta
He010710, D2_A2	Streptophyta
He010322, I2_C6	*Bathycoccus*
He010518, D1_A2	*Bathycoccus*
He001206, D2_C1	Ciliophora
He010322, D2_B6	*Cafeteria*
He010322, D2_C4	*Bathycoccus*
He010322, D2_C5	*Bathycoccus*
He010322, D3_A1	*Micromonas*
He010322, D3_A2	*Nanochlorum*
He010322, I2_C6	*Bathycoccus*
He001206, D2_B1	*Phaeocystis*
He010322, D2_A5	Ciliophora
He010322, I3_A6	*Ochromonas*
He010418, I1_B3	*Bathycoccus*
He010322, I3_A3	*Symbiomonas*
He010322, D1_A2	*Apedinella*
He010322, I1_A3	Alveolate
HE010322, D2_A6	*Crypthecomonas*
He010322, I1_C5	*Cafeteria*
He010218, I2_D1	*Nanochlorum*
He010218, I3_A5	*Bathycoccus*
He010322, D3_B4	*Bathycoccus*
He010117, D1_D5	*Micromonas*
He001206, I1_D1	*Phaeodactylum*
He010117, I1_C1	*Micromonas*
He010117, I1_C4	*Nanochlorum*
He011322, I1_A2	*Bathycoccus*

oligos for other groups (i.e., universal, prokaryote specific) could also allow screening for other taxonomic groups.

The method could also be scaled up to 96 well plate scale. Crude DNA extraction, sufficient to produce amplifiable DNA, can be performed for many cultures within less than 1 h. The PCR cycle takes about 4 h, subsequent purification on Qiagen columns in a 96 scale could be done in about 1 h. Exonuclease digestion needs 1 h, followed by another purification step of 1 h. All these steps can be performed in one day. The most time-consuming step is the gel electrophoresis and subsequent silver staining, which takes altogether an additional 20 h (overnight). Another limitation is the capacity of the gel. Conventional 40 cm gels hold about 45–60 samples when using a shark tooth comb. Thus, two such gels are necessary to analyze 96 samples. This limitation could possibly be overcome by using fluorescently labeled 528F primers and a capillary sequencer. Sequence analysis can then be done on a 96 capillary capacity scale. Therefore, the procedure could allow the routine SSCP analysis of up to 200 cultures per week when using conventional gels. Using a 96 well system for SSCP gels, this number can be increased to 400/week. This means that about 20–40 new isolates could be identified in this time span.

Obviously, there are also some shortcomings of the method. First, the crude DNA extraction applied produced amplifiable DNA only for about 50% of the cultures. Probably, some of these were prokaryotic and could not be amplified with eukaryotic 528F. Nevertheless, in these cases, one could perform a 'traditional' DNA extraction protocol or increase the amount culture used for DNA isolation in order to increase the amount of PCR products. Secondly, it is possible that purportedly pure cultures produce more than one band and thus could be regarded as mixed cultures. The only way to overcome this problem would be to sequence all SSCP products, which in turn would make the SSCP analysis itself dispensable. However, costs would be about 5-fold higher then.

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