Preparation of DNA Suitable for PCR Amplification from Fresh or Fixed Single Dinoflagellate Cells

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ABSTRACT

A method is described to prepare total DNA from single cells of dinoflagellates, which can be used for PCR amplification. As model organisms, we used a stock strain of Alexandrium catenella *and cells of* Dinophysis acuminata *harvested from the Atlantic Ocean. Fresh grown cells or cells maintained in different preservatives were tested as sources for DNA preparation. The method used to prepare DNA combines physicochemical and enzymatic procedures on cells embedded in agarose plugs or beads. The agarose pieces containing the DNA were used to perform PCR amplification of a fragment of DNA containing a 5.8S rRNA gene and the flanking internal transcribed spacers (ITS1 and ITS2).*

INTRODUCTION

Dinoflagellates have been studied mainly from two points of view: (*i*) from the applied one, with the hope that the knowledge acquired could help prevent the economical and health problems caused by blooms of toxin-producing species that contaminate mollusks and fishes (12); and (*ii*) from an academic point of view, since these organisms are at the beginning of eukaryotic evolution and possess unique structural and physiological characteristics (11,16). However, progress has been slow due to the lack of suitable molecular techniques, not only because of the particular characteristics of these microorganisms but also, in some cases, no laboratory culture methods are available, as is the case for *Dinophysis* species (5). The application of common methods of DNA purification to these organisms is not appropriate because they do not remove contaminants that, for example, prevent the DNA from being used for PCR amplification (1). The nature of these contaminants is not known in the case of dinoflagellates, but in other organisms, polysaccharides, hemoglobin, or urea have been reported to contaminate DNA, preventing its use for PCR assays (reviewed in Reference 17). Some methods to prepare DNA from dinoflagellates have been described that are able to yield clean DNA (1,14,15), and the method described by Adachi et al. (1), which uses cetyltrimethylammonium bromide (CTAB) extraction, is widely used. However, the many purification steps necessary with this method to obtain pure DNA require large amounts of cells, which is often a problem with dinoflagellates and prohibitive in species that cannot be cultured in the laboratory. For these reasons, a key to advance dinoflagellates studies, from both academic and applied points of view, is to design a method to obtain pure DNA for molecular approaches from small numbers of cells. Several protocols for PCR amplification of DNA from single cells have been reported for different organisms such as bacteria, cyanobacteria, humans, etc. (4,8,9), but no methods have been described for dinoflagellates. Such procedures are of particular value for organisms with complex life histories but in which the stages cannot be unequivocally assigned to particular species using morphological or cytological criteria. Here, we describe a method that provides DNA suitable for PCR amplification, starting with as few as a single cell.

MATERIALS AND METHODS

Dinoflagellate Strains and Culture Conditions

Two species were used in this work: *Alexandrium catenella* clone ACC01 (isolated from Canal Jacaf in Chile) and *Dinophysis acuminata* (isolated from the Rias Bajas in the NW Spain).

A. catenella ACC01 (obtained from Biotoxin Marine Laboratory, Chile) was grown in batch culture in f/2-enriched seawater medium (6,7) for 10 days at 15°C under a daily 16:8 light/dark cycle at 100 µEinsteins s^{-1} m^{-2}.

D. acuminata cells were collected using vertical phytoplankton net-hauls (10–20 µm mesh size) or water samples taken at specific depths with oceanographic bottles in the Rias Bajas (NW Spain) during different Diarrhoeic Shellfish Poisoning (DSP) outbreaks and seasons between 1994 and 1999. Samples were screened through a 100–150-µm sieve to eliminate large microplanktonic organisms. Aliquots of water samples were placed in sedimentation chambers, and *Dinophysis* cells were identified under inverted microscope with 25× and 100× magnification.

D. acuminata cells, kept for up to five years in frozen methanol (stored at -20°C), neutral formaline (4%) (stored at 4°C), or lugol (6.66 g/L KI; 3.33 g/L I_2) (stored at 4°C), and of *A. catenella*, kept for one year in ethanol (stored at -20°C), methanol (stored at -20°C), neutral formaline (4%) (stored at 4°C), lugol (stored at 4°C), or glutaraldehyde (5%) (stored at 4°C), were also used in addition to fresh cells for DNA preparation.

Preparation of Intact DNA

A. catenella cultures were treated for 30 h with ampicillin and streptomycin at final concentrations of 1.6 and 0.8 mg/mL, respectively (3), before harvesting the cells by centrifugation (2000× *g* for 10 min). This step is included to kill bacteria that usually are present in dinoflagellate cultures. Cells were resuspended with 1× PBS and pelleted again. Finally, the cells were resuspended in buffer A (1/2 volume of 1× PBS, 1/2 volume of 0.5 M EDTA pH 9.5). Intact cells were picked up with a capillary pipet and placed in 10 µL buffer A and mixed with 10 µL 1.6% low melting agarose (InCert® Agarose; BMA, Rockland, ME, USA) at 37°C. The cell-agarose mixture was then deposited as a drop on parafilm and kept at 4°C until solidification. The agarose beads were incubated at 26°C in buffer B [0.4 M D-mannitol (Sigma, St. Louis, MO, USA), pH 5.8, 1% cellulase (Calbiochem-Novabiochem, San Diego, CA, USA), 1% hemicellulase (Sigma)] for 3 h and then with ESP solution [0.5 M EDTA, pH 9.5, 1% lauroyl-sarcosine (Sigma), 1 mg/mL Proteinase K (Sigma)] for 48 h at 50°C.

For *D. acuminata*, cells were indi-

Table 1. Sequence Similarity of the ITS1-5.8S rRNA Gene-ITS2 Region of *A. catenella* ACC01 and Other *Alexandrium* Species

	A. catenella ACC01		
	5.8S rRNA	**ITS1**	**ITS2**
A. tamarense FK-788 A gene	98.8	98.8	99.5
A. tamarense FK-788 B gene	91.9	95.1	95.9
A. tamarense WKS-1	94.4	69.5	81.8
A. tamarense CU-15	90	48.5	67.1
A. catenella MI7	90.6	65.7	80.6

Percentages of identity are shown for each region. *A. tamarense* FK-788 (Funka Bay, Japan) A gene (accession no. AB006993); *A. tamarense* FK-788 B gene (accession no. AB006994); *A. tamarense* WKS-1 (Kushimoto, Japan) (accession no. AB006991); *A. tamarense* CU-15 (Gulf of Thailand, Thailand) (accession no. AB006992); *A. catenella* MI7 (Harima Nada, Japan) (accession no. AB006990).

vidually picked up with a capillary pipet and transferred to 0.22 µm filtered sea water. Cells were further transferred 2–3 times to drops of filtered sea water to eliminate possible DNA contaminants from other broken cells. Finally, the cells were placed in buffer A and embedded in low melting agarose the same as for *A. catenella*.

Agarose-embedded DNA was stored in ESP at 4°C until use. Samples were washed twice for 2 h in 10 volumes TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and once overnight. Two hours before PCR, a final wash was performed.

The fixed cells were pelleted and resuspended in PBS three times before being embedded in agarose, and the DNA was extracted the same as for fresh cells.

PCR Amplification and Analysis of Products

The 5.8S rDNA and flanking internal transcribed spacers (ITS1 and ITS2 regions) were amplified from agarose beads containing 1, 5, 7, 10, 23, or 50 cells using ITSA and ITSB primers (1). The primers were synthesized by Isogen Bioscience BV (Maarssen, The Netherlands). Amplifications were performed in a GeneAmp® PCR System 2400 thermal cycler (Applied Biosystems, Foster City, CA, USA). Reactions contained in a 100-µL mixture: agarose-embedded genomic DNA, 250 µM each dATP, dCTP, dGTP, dTTP, 100 pmol each primer, 2.5 mM $MgCl_2$, 1× reaction buffer, and 2.5 U *Taq* DNA polymerase (both from Applied Biosystems). Thermal cycling was as follows: one cycle of hot start at 94°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 40°C, and 1 min at 72°C, with an additional final elongation step of 7 min at 72°C. The same protocol was applied to agarose beads without cells as a negative control of PCR amplification.

The PCR products were analyzed by electrophoresis of 5 µL reaction mixture on 1% agarose gels. The band containing the 5.8S rRNA gene and the ITS1 and ITS2, from 10 µL reaction mixture separated by electrophoresis, was purified from the agarose gel with QIAquick™ Gel Extraction Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Sequencing of both strands of the purified DNA was performed by the DNA sequencing Service at the Department of Molecular Biology, Universidad Autónoma de Madrid, using the BigDye™ terminator ready reaction kit and a 377 automatic sequencer (both from Applied Biosystems). Nucleotide sequence comparisons were carried out by using the Basic Local Alignment Search Tool (BLAST) and PILEUP programs (GCG software package).

GenBank® accession numbers for the sequences reported here are AJ272-119 for *D. acuminata* and AJ272120 for *A. catenella* (*A. catenella* ACC01).

RESULTS AND DISCUSSION

DNA preparations of dinoflagellate species performed by regular procedures are usually contaminated by unknown substances that prevent the DNA from being used in enzymatic assays such as PCR or restriction enzyme digestions. For some organisms, the whole cells can be used for PCR assays without previous DNA isolation since cell lysis can easily be achieved by heat. However, in cases when contaminants in the cells can prevent the PCR assay from working, this method cannot be used because cell lysis does not eliminate contaminants from the solution. For this reason, a special procedure with many purification steps was developed for the isolation of DNA from dinoflagellates containing such PCR inhibitors (1). This procedure needs large numbers of cells, which can be achieved only for species that can be cultured, though often at considerable cost in time and resources. It is quite difficult or impossible for those species that cannot be cultured and have to be collected from wild populations when seasonal blooms take place. A procedure then is badly needed that (*i*) can be used to prepare clean DNA suitable for enzymatic analysis and (*ii*) can be performed on small amounts of fresh cells and on fixed and preserved cells if possible, thus circumventing the seasonality of the DNA preparation.

The main problem in preparing DNA from a few cells is the loss of cells or DNA during manipulation. We used agarose-trapped cells that, in this way, can easily be manipulated. The DNA extraction can then be performed using physicochemical and enzymatic procedures on the agarose beads. Similar procedures have been described for different species to prepare intact DNA for use in pulsed field gel electrophoresis techniques (10) but have not been described for dinoflagellate species. Ob-

taining DNA from embedded cells also allows the DNA to be kept trapped in the agarose, while other lower molecular weight components can diffuse freely into the surrounding medium. This may be an effective way to remove compounds that usually contaminate dinoflagellate DNA preparations. The purity of the DNA preparations was assessed using a PCR amplification test. In this test, we tried to amplify the DNA region containing the 5.8S rRNA and the flanking ITS regions using previously described primers specific for this region (1). For *Dinophysis* species, no sequence of this region is available. Since the primers have been used successfully for different species of dinoflagellates, we tried them on *Dinophysis* DNA. Preparations of DNA were made using agarose beads containing 1, 5, 7, 10, 23, or 50 cells. All the samples were successfully amplified both for *D. acuminata* (Figure 1) and *A. catenella* cells (data not shown). The amplified bands in both species were of similar size, around 650 bp. To confirm the specificity of the amplification, the bands from the two species were purified from the agarose gel and sequenced.

Analysis of the sequences shows that both correspond to the expected locus. The 5.8S rDNA sequence of *A. catenella* clone ACC01 is similar to those of other Alexandrium species found in GenBank (Table 1). It is particularly related to the reported sequences of *A. tamarense* and *A. catenella* (2). It may seem surprising that this sequence is more like some of the *A. tamarense* sequences than that of *A. catenella*, but similar results have been reported that show higher sequence similarity among different species from the same geographical region than among isolates of the same species from different places (1,14). The ITS regions of the strain ACC01 are also quite similar to those of the other *Alexandrium* species, showing a higher similarity among the ITS2 sequences than among the ITS1 sequences. The highest similarity among the ITS1 and ITS2 sequences is found with the A gene of *A. tamarense* FK-788 (from Funka Bay, Japan), while the lowest is from a different strain of the same species, CU-15 (from the Gulf of Thailand). The latter is intermediate between *A. tamarense* WKS-1 (from Kushimoto, Japan) and *A. catenella* MI7 (from Harima Nada, Japan). Even though more differences are seen by comparison of the ITS sequences than of the 5.8S rDNA ones, the three regions show the same order of similarity: *A. tamarense* FK-788 gene A > *A. tamarense* FK-788 gene B > *A. tamarense* WKS-1 > *A. catenella* MI7 > *A. tamarense* CU-15. Gene A of *A. tamarense* FK-788 has been assigned to the North American large ribosomal subunit RNA (LSU) ribotype, while the *A. tamarense* WKS-1 has been assigned to the Western European LSU ribotype (1,14); *A. catenella* MI7 and *A. tamarense* CU-15 have not been assigned to any regional LSU ribotype. These results indicate than *A. catenella* ACC01 from Chile may be more closely related to the North American ribotype than to the Western European ribotype and less closely related to *A. tamarense* CU-15 from Thailand.

The sequence of *D. acuminata* is the first one of this region described for a *Dinophysis* species. The 5.8S rDNA sequence of this region is quite similar to that of other dinoflagellates species, showing the highest similarity with the sequence of *Prorocentrum micans* (84.9%), followed by those of *Gyrodinium impudicum* (84.3%) and *Gymnodinium sanguineum* (83.6%). The ITS regions show less similarity, the highest similarity being with *P. micans* for both ITS regions. These results indicate that ITS sequences can be used to distinguish between *D. acuminata* and other species, which, based on the 5.8S rDNA sequences, are quite similar.

The positive results of the PCR test indicate that the method used for DNA preparation allows DNA to be obtained from 1 to 50 cells and, with sufficient purity, to be used in an enzymatic molecular biology assay such as PCR amplification. The PCR test for DNA samples prepared from preserved cells is

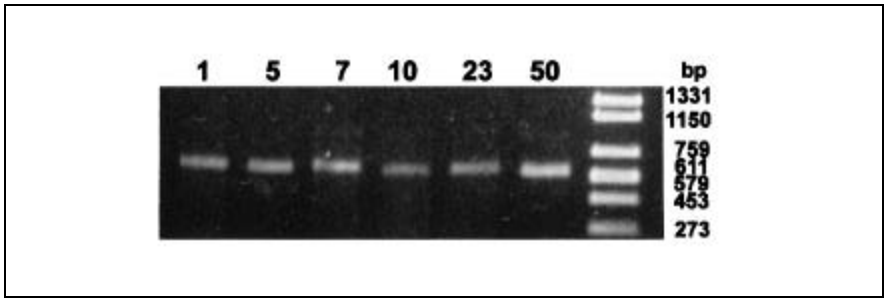

Figure 1. PCR products obtained from 1, 5, 7, 10, 23, or 50 cells of *D. acuminata* with the specific primers for the ITSA-5.8S rRNA gene-ITSB genomic region (1). Size markers are shown on the right.

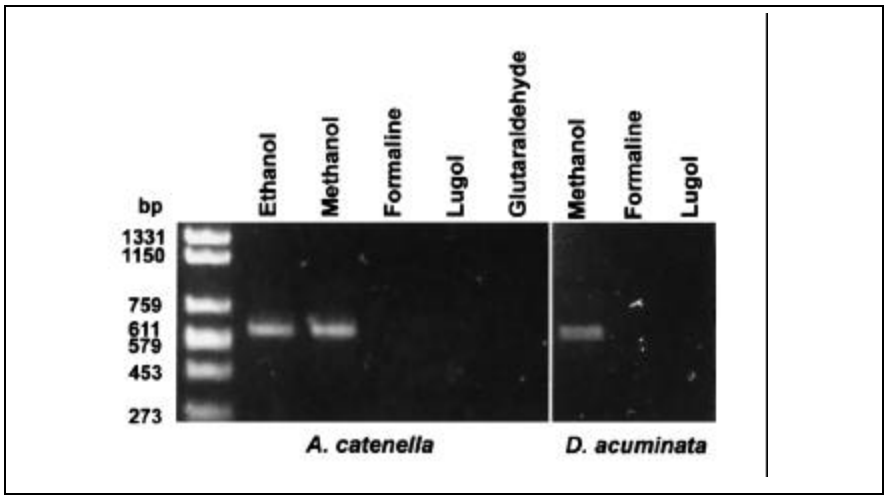

Figure 2. PCR products obtained from *A. catenella* clone ACC01 and *D. acuminata* cells preserved in ethanol, methanol, neutral formaline, lugol, or glutaraldehyde.

shown in Figure 2. As can be seen, only those cells maintained in alcoholic media (methanol or ethanol) were suitable for PCR amplification. These then must be the media used to preserve dinoflagellate cells to be able to prepare DNA samples suitable for PCR analysis.

The results presented here constitute a real breakthrough in the application of molecular biology tools to research the life history and genetic characterization of nonculturable toxic dinoflagellates, such as species of *Dinophysis*, that are usually present in low numbers (< 200 cell/L) have complex polymorphic life cycles that have not yet been fully described and have a highly plastic cell cover that varies in form in response to feeding behavior, environmental conditions, and vegetative and sexual stages (13).

The DNA preparation procedure described here offers several advantages, the main one being the small number of cells needed for DNA extraction. DNA preparation from single cells will be of interest for the recognition of the different morphotypes of the same species or cells in different stages of their life cycle, particularly in nonculturable species that have to be isolated one by one from field samples. The possibility of using alcohol-preserved samples, not reported until now, can circumvent the seasonality of DNA preparation when blooms of these species occur.

The use of PCR on these DNA samples will allow us to obtain genomic DNA sequences that can be useful, for instance, to develop species-specific DNA probes to identify and study the biology, ecology, and genetics of these organisms and speed up the monitoring procedures used up to now.

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