Co-monitoring bacterial and dinoflagellates communities by denaturing gradient gel electrophoresis (DGGE) and SSU rRNA sequencing during a dinoflagellates bloom

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Abstract
Dinoflagellates are unicellular eukaryotic protists that dominate in all coastal waters, and are also present in oceanic waters. Despite the central importance of dinoflagellates in global primary production, the relationship between dinoflagellates and bacteria are still poorly understood. In order to understand the ecological interaction between bacterial and dinoflagellates communities, denaturing gradient gel electrophoresis (DGGE) and SSU rRNA sequencing were applied to monitoring the population dynamics of bacteria and dinoflagellates from the onset to disappearance of a dinoflagellates bloom occurred in Baltimore Inner Harbor, from April 15 to 24, 2002. Although *Prorocentrum minimum* was the major bloom forming species under the light microscopy, DGGE method with dinoflagellate specific primers demonstrated that *Prorocentrum micans, Gymnodinium galatheanum* and *Gyrodinium uncatenum* were also present during the bloom. Population shifts among the minor dinoflagellate groups were observed. DGGE of PCR-amplified 16S rRNA gene fragments indicated that cyanobacteria, α, β, γ-proteobacteria, *Flavobacterium–Bacteroides–Cytophaga* (FBC), and *Planctomycetes* were the major components of bacterial assemblages during the bloom. DGGE analysis showed that Cytophagales and α-proteobacteria played important roles at different stages of dinoflagellates bloom. DGGE can be used as a rapid tool to simultaneously monitor population dynamics of both bacterial and dinoflagellates communities in aquatic environments, which is demonstrated here.

Key words: bacteria, dinoflagellates, DGGE, SSU rDNA sequencing

1 Introduction
Dinoflagellates are important primary producers in both coastal and oceanic waters, and could play remarkable ecological roles on pelagic energy flow and nutrient cycling (Cole et al., 1982; Doucette et al., 1998). Many species of dinoflagellates are also capable of forming massive algal blooms. It is expected that availability of organic matters changed dramatically at different stages of an algal bloom. Bacterial biomass and production are known to be correlated with amount of organic matters released from bloom-forming species (Palumbo et al.,...
1984; Smith et al., 1995; Riemann et al., 2000). However, there is only limited information on the phylogenetic affiliations of bacteria associated with marine algal blooms (Gonzalez et al., 2000). Moreover, little is known about the effect of bacterial succession on population structure of bloom-forming species.

The development of molecular approaches greatly enhanced our ability to study the population diversity of microorganisms in marine environments (Giovannoni et al., 1990; Ward et al., 1990; Lopez-Garcia et al., 2001; Moon-van der Staay et al., 2001). Recently, DGGE technology has been widely used as a rapid method to examine the complexity of microbial communities including prokaryotes and eukaryotes (Muyzer et al., 1993; van Hannen et al., 1999; Bano and Hollibaugh, 2002). Theoretically, DGGE can separate different PCR fragments even with single GC pair difference (Muyzer et al., 1993; Ferris et al., 1996). Therefore, diversity profile from different microbial communities can be compared according to their gel patterns and the sequences of representative bands.

In this study, we intended to monitor bacterial and dinoflagellate population succession during a dinoflagellates bloom using DGGE method. PCR primers used in the study are specific for eubacteria and dinoflagellates, respectively. Bacterial and dinoflagellate communities at different stages of bloom were compared based on their DGGE fingerprints. The major DGGE bands were sequenced and identified based on the phylogenetic relationship with known species from the GenBank database.

2 Materials and methods

2.1 Sample collection

An algal bloom with dark brown color was observed at Baltimore Inner Harbor on April 15, 2002. Water samples were collected daily from Pier 5 of Baltimore Inner Harbor during the bloom period (April 15–24, 2002) using a bucket. Water temperature and salinity were recorded, respectively. When the samples were taken, 250 mL water was filtered through 0.2-mm-pore-size polycarbonate filters (47 μm in diameter; millipore, Bedford, Mass.) immediately. Microbes retained on the filters were stored at −20 °C for further analysis. Meanwhile, additional 50 mL water was fixed by 1% glutaraldehyde for total bacterial and dinoflagellate counting. Microbial cells were stained by SYBR Gold (Molecular Probes, Inc., Eugene, Oreg.) as described by Chen et al. (2001) and enumerated using an epifluorescence microscope, Zeiss Axiplan (Zeiss, Germany). At least 200 cells were counted for bacteria and dinoflagellates, respectively.

2.2 Nucleic acid extraction

Total DNA was extracted according to a protocol developed by Schmidt et al. (1991) with minor modifications. DNA from bacteria and dinoflagellates was extracted by treating with lysozyme and proteinase K concomitant with phenol extraction and isopropanol precipitation. DNA was dissolved in ddH₂O and stored at 4 °C for further analysis.

2.3 PCR amplification of SSU rDNA

PCR amplification was performed in a 50 mL volume containing approximately 100 ng of template DNA, 1×PCR buffer, 1.5 mmol/dm³ MgCl₂, 0.5 mmol/dm³ (each) primer, 200 mmol/dm³ (each) deoxynucleotide, and 2.5 u of Taq DNA polymerase (Promega, WI, USA).

The primers used to amplify eubacterial 16 S ribosomal DNA (rDNA) were 1070F (eubacteria) and 1392R(GC) (universal) which contained a 40 bp GC-rich clamp (Ferris et al., 1996). The oligonucleotide primers for PCR amplification of dinoflagellate 18S rDNA were EUK4618R and DinoF(GC) (Oldach et al.,
2000). The sequences, target sites and specificity of the primers were shown in Table 1.

Table 1. Oligonucleotide sequences used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
<th>Target site</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1070F</td>
<td>ATGGGCTGT</td>
<td>16S(1055-1070)</td>
<td>bacteria</td>
<td>Amann et al. (1995), Ferris et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>CGTCAAGCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1392R (GC)a</td>
<td>ACAGGCGG</td>
<td>16S(1392-1406)</td>
<td>universal</td>
<td>Amann et al. (1995), Ferris et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>TGTGTA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DinoF(GC)a</td>
<td>CGATGGAGTGAGT</td>
<td>Dino18S</td>
<td>dinoflagellates</td>
<td>Oldach et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>GATCCGTTGAAATA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EUK4618R</td>
<td>TGATCTTCTGCT</td>
<td>18S</td>
<td>universal</td>
<td>Oldach et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>AGGTTCACTAC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) The GC clamp sequence is CGCCCGCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCC.

PCR amplification was carried out using a PTC-200 thermal cycler (MJ Research, Waltham, Mass.). For 16S rDNA, PCR program included an initial activation at 94 °C for 5 min followed by 27 cycles using a touchdown PCR program developed by Muyzer (1993) to minimize nonspecific amplification. The 27 cycles were performed at 94 °C for 0.5 min, T1 for 1 min, and 72 °C for 3 min. In the first 20 cycles, T1 decreased by 1 °C, stepwise, each 2 cycles, from 65 °C in the first cycle to 56 °C in the 20th. In the last 5 cycles, T1 was 55 °C. Cycling was followed by 5 min of incubation at 72 °C. The PCR cycle for dinoflagellates was performed as described by Oldach et al. (2000). One activation step at 95 °C for 15 min was followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 40 s and then a final extension step at 72 °C for 5 min. Agarose gel (1%) electrophoresis was used to detect the PCR products.

2.4 DGGE analysis and sequencing

DGGE was performed using Dcode™ universal simulation detection system (Bio-Rad, Hercules, Calif.). PCR products were separated on a 1.5-mm-thick vertical gel containing polyacrylamide (acrylamide–bisacrylamide, 37:5:1) and a linear gradient of the denaturants urea and formamide, increasing from 40% at the top of the gel to 65% at the bottom. Equal amount of PCR products was loaded on the DGGE gel. Electrophoresis was performed at 60 °C in a 0.5×TAE buffer, and 70 V of electricity was applied to the submerged gel for at least 16 h. DNA bands were visualized by staining with SYBR Gold and photographed (Ovreas et al., 1997).

Prominent DNA bands were excised from the gels, re-amplified and electrophoresed again in DGGE gels (at least twice). PCR products were purified by Qiaquick PCR purification kit (Qiagen Inc., Valencia, CA). Prism ready reaction dye deoxy termination kit (Applied Biosystems, Foster City, Calif.) was used for sequencing in conjunction with Taq polymerase in a 373A DNA sequencer (Applied Biosystems, Foster City, Calif.). The PCR primers without GC clamp were used for sequencing. The sequences were submitted to GenBank and blasted against the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/).

2.5 Phylogenetic analysis

Sequence alignment and phylogenetic tree reconstruction were performed using MacVector 7.1 program (Accelrys, San Diego, Calif.). Evolution distance was calculated by the Jukes–Can-
tor method (Jukes and Cantor, 1969) and a distance tree was constructed with the neighbor-joining algorithm (Saitou and Nei, 1987).

3 Results

3.1 Microbial abundance, water temperature and salinity

During the bloom period (April 15 to 24, 2002), microscopic examination indicated that *Prorocenrum minimum* was the dominant bloom-forming species (data not shown). *P. minimum* reached the maximum cell density (2×10^5 cells/mL) on April 17 and decreased to 1 700 cells/mL on April 24. Total bacterial counts were more than 5×10^6 cells/mL on April 19, and declined to 2×10^6 cells/mL on the day when the *P. minimum* cell density started to declined (Fig. 1). During the bloom, bacterial abundance showed a positive correlation with dinoflagellate density (r²=0.985, P<0.05). However, bacterial abundance had a postponed shift in relevance to dinoflagellates.

3.2 Population structure of dinoflagellates

Five different dinoflagellate populations were detected by DGGE throughout the bloom period, and four of them were sequenced and identified. During the bloom period, *P. minimum* (Band D3) was always detected and other minor dinoflagellate populations shifted dramatically according to their DGGE profiles (Fig. 2, Lanes 6–10). For example, species of Bands D1 and D2 were relatively more abundant at the beginning of bloom, and species of Band D4 emerged towards the end of bloom. Species of Band D3 were detected at all stages of bloom. Phylogenetic analysis based on the 18S rDNA sequences indicated that species of Bands D1, D2, D3 and D4 are closely related to *P. micans*, *Gyrodinum galatheanum*, *P. minimum*, and *G. uncatenam*, respectively (see Fig. 3).

![Fig. 1. Bacterial and dinoflagellate counts, water temperature and salinity during the bloom.](image)

![Fig. 2. DGGE fingerprints of bacterial (lanes 1–5) and dinoflagellate populations (lanes 6–10). Sequenced bands from both communities were indicated. Lanes 1–5 represented the bacterial community profiles at day 1, 3, 5, 9 and 10 of the bloom period. Lanes 6–10 represented the dinoflagellate community profiles at day 1, 3, 5, 9 and 10 of the bloom period.](image)
about 20 major bands were visible on the DGGE gel. In general, bacterial communities appeared to be stable during the bloom. A total of 19 bands were excised and sequenced (Fig. 2, Lanes 1–5). Bands B14 and B17 were dual bands that could be separated better after re-amplification. There were multiple bands in B18 but only 1 band was re-amplified and sequenced in the following analysis. Bands B2 and B16 could not be re-amplified and thereof their sequences were not available. Bands from different samples with identical vertical positions in the DGGE gel were assumed to have identical sequences. The 19 bacterial sequences were closely related to cyanobacteria and plastids (29%), α-proteobacteria (19%), β-proteobacteria (14%), and γ-proteobacteria (10%), Flavobacterium–Bacteroides–Clytophaga (FBC) (14%), and Planctomycetes (5%) respectively.

As shown in Fig. 4, 6 bands (B1, B3, B4, B5, B8 and B17a) were related to photosynthetic organisms. B17a shared high similarity with Synechococcus sp. PS723. All other 5 bands were closely related to phytoplankton plastids. Four bands (B11, B14a, B17b and B18) were associated with a-proteobacteria. Bands B12, B13 and B15 were related to β-proteobacteria. Three bands (B7, B9 and B19) were affiliated with Flavobacterium–Bacteroides–Clytophaga (FBC) group, which were composed of 14% of the whole community.

3.4 Bacteria–dinoflagellate interaction

An interesting interaction between bacteria and dinoflagellates was observed with epifluorescence microscopy (see Fig. 5). Figure 5a is a typical view of P. minimum cells during the peak of bloom. When the bloom began to fade off, nucleus of dinoflagellates became brighter and bacterial cells began to increase around the dinoflagellate nucleus (see Figs 5b and c). It is common to see that bacterial cells aggregated around the nucleus of P. minimum when the cells were crashed (see Fig. 5d).

4 Discussion

Dinoflagellate blooms occurred frequently from late spring to summer at the Baltimore In-
Fig. 4. Phylogenetic affiliations of representative 16S rDNA sequences taken from the bloom samples. B1 to B19 represented 19 bands excised from bacterial DGGE gel. An Archaea (Nanoarchaeum equitans) was used as an outgroup. The scale bar indicates substitutions per nucleotide position. Bootstrap values lower than 50% are not shown. B14 and B17 had two bands sequenced and shown as a and b. B18 (?) had multiple bands but only one was re-amplified and sequenced.
ner Harbor. Located in the northern part of the Chesapeake Bay, spring runoff from the Patapsco River provides Baltimore Inner Harbor massive organic matter and nutrients that in turn trigger dynamic responses of both dinoflagellates and bacterial communities. The close relationship between bacterial and phytoplankton biomass has been studied (Pinhassi et al., 1999; Riemann et al., 2000; Fandino et al., 2001). However, very few studies have been conducted to study population composition of bacteria and phytoplankton (Gonzalez et al. 2000; Fandino et al. 2001). Differentiating similar phytoplankton species by microscopy could be problematic due to their similar morphological characteristics. In this study, we demonstrated that multiple dinoflagellate species could be detected using PCR–DGGE method during the *P. minimum* bloom. For example, *P. micans* and *P. minimum* were well separated on the DGGE gel. Our result also indicates that DGGE method is not biased for the dominant species like *P. minimum*. The minor species like *Gymnodinium galatheanum* and *G. uncate-num* that were difficult to be found under the light microscopy were also detectable with dinoflagellate specific PCR primers. With such a detection sensitivity, DGGE method can be a useful tool to monitor population shift of phytoplankton.

During the bloom, phylotypes related to α-proteobacteria (Bands B11, B14a, B17b and B18) were present in all the analyzed samples. Intensity of Bands B11 (*Roseobacter*) and B17b (marine α-proteobacteria) was much higher in bloom samples indicating that these groups of bacteria could be numerically abundant in the bloom samples. The *Roseobacter* lineage made up over 20% of the bacterial rDNA associated with the *Emiliania huxleyi* bloom (Gonzalez et al., 2000). High proportion of α-proteobacteria was also found in a mesocosm diatom bloom (Reimann et al., 2000) and a *Lingulodinium polyedrum* bloom in off the southern California coast (Fandino et al., 2001). It is thought that *Roseobacter* may play...
a role in cycling of organic sulfur compounds produced during the bloom (Gonzalez et al., 2000). 

\( \alpha \)-proteobacteria could accelerate uptake of amino acid in marine waters (Cottrell and Kirchman, 2000).

Three *Cytophagale* phylotypes were identified during the bloom. The *Cytophagele* lineage (Band B7) appeared to increase band intensity during the late stage of bloom. *Cytophaga*-related species are abundant in marine environments (Glöckner et al., 1999) and known to be involved in the degradation of complex macromolecules (Shewan and McMeekin, 1983). *Cytophagale* phylotypes were more abundant during the late stage of the diatom bloom (Riemann et al., 2000). Organic particles retrieved from decay of phytoplankton provide *Cytophaga* an ecological niche for colonization and hydrolysis of organic matters.

\( \gamma \)-proteobacteria (Bands B6 and B10) also emerged at the late stage of bloom. In consistent with our results, Fandino et al. (2001) found \( \gamma \)-proteobacteria showed phylotype richness and predominance of abundance in a dinoflagellates bloom. SAR86, the ubiquitous cluster of \( \gamma \)-proteobacteria was also found prominent in heterotrophic bacterial communities during a North Atlantic algal bloom (Gonzalez et al., 2000).

Bands B13 and B15 sharing high similarity with \( \beta \)-proteobacteria also showed high intensities in bloom samples. Recently, transmission electron microscopy (TEM) and in-situ hybridization showed that \( \beta \)-proteobacteria were the major endonuclear and endocyttoplasmic bacteria in dinoflagellates (Alverca et al., 2002). Intracellular symbiotic bacteria associated with different dinoflagellate species have been studied for a long time (Silva, 1978). For example, FBC groups were found in cytoplasm of the dinoflagellate cells, but absent from the nucleus (Alverca et al., 2002; Biegal et al., 2002). Although a tight interaction between bacterial and dinoflagellate cells was observed in our study, it is not clear whether the bacteria are associated with nucleus or cytoplasm of dinoflagellates (see Fig. 5). In-situ molecular tools (e.g., FISH and in-situ PCR) coupled with laser confocal microscopy can be exploited to further understand the interaction between bacteria and dinoflagellates.

Some potential biases associated with PCR and DGGE methods have been discussed elsewhere (Muyzer, 1999; Diez et al., 2001). For eukaryotes, some bias may be due to rRNA gene copy numbers because eukaryotes usually have very high copy numbers for some genes (Long and Dawid, 1980). It is worth pointing out that DGGE is not a quantitative approach, but its high reproducibility demonstrates that it reflects the major variations of PCR-amplifiable phylotypes in natural communities (Riemann et al., 1999). Changes in band patterns and intensity of same bands should reflect the actual changes in the relative abundance of compositions of microbial communities.

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