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Randomly Amplified Polymorphic DNA PCR as a Tool for Assessment of Marine Viral Richness▽†

Danielle M. Winget[1] and K. Eric Wommack[1,2]*

College of Marine and Earth Studies, University of Delaware, Newark, Delaware 19711,[1] and Department of Plant and Soil Sciences, University of Delaware, Newark, Delaware 19711[2]

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Recent discoveries have uncovered considerable genetic diversity among aquatic viruses and raised questions about the variability of this diversity within and between environments. Studies of the temporal and spatial dynamics of aquatic viral assemblages have been hindered by the lack of a common genetic marker among viruses for rapid diversity assessments. Randomly amplified polymorphic DNA (RAPD) PCR bypasses this obstacle by sampling at the genetic level without requiring viral isolation or previous sequence knowledge. In this study, the utility of RAPD-PCR for assessing DNA viral richness within Chesapeake Bay water samples was evaluated. RAPD-PCR using single 10-mer oligonucleotide primers successfully produced amplicons from a variety of viral samples, and banding patterns were highly reproducible, indicating that each band likely represents a single amplicon originating from viral template DNA. In agreement with observations from other community profiling techniques, resulting RAPD-PCR banding patterns revealed more temporal than spatial variability in Chesapeake Bay virioplankton assemblages. High-quality hybridization probes and sequence information were also easily generated from single RAPD-PCR products or whole reactions. Thus, the RAPD-PCR technique appears to be practical and efficient for routine use in high-resolution viral diversity studies by providing assemblage comparisons through fingerprinting, probing, or sequence information.

The widespread recognition of viruses as the most abundant biological entities in the marine environment (for reviews, see references 32 and 38) spurred more recent metagenome surveys, which indicated that DNA viruses may also represent vast genetic diversity in this ecosystem (3, 5, 8). These studies of marine viral assemblages discovered that between 30 and 90% of viral sequences are entirely novel (3, 5, 8). Comparative studies of phage isolates infecting the same mycobacterial host species revealed low sequence similarity between these viruses (27). High viral diversity not only in aquatic environments but also in soils (17), sediments (6), and humans (7) inspires curiosity about the unique traits and underlying genetic similarities hidden within these most numerous members of the biosphere. However, viral metagenome studies provide only a single snapshot of a viral assemblage at a given place and time and remain too expensive for describing viral diversity on fine temporal and spatial scales. Due to the lack of a common genetic marker among viruses, characterization of the diversity, population genetics, and variations of natural viral assemblages has been elusive, leaving a gap in the understanding of viral communities (29).

To overcome this dilemma, several methods have been developed to quantify viral diversity and examine changes in virioplankton assemblage composition. Viral capsid sizes and morphologies as observed via transmission electron microscopy (TEM) can be compared, between either virioplankton isolates (35) or whole communities (20, 33). Concentrates of whole virioplankton assemblages or individual viral strains can be examined by pulsed-field gel electrophoresis (PFGE), which produces fingerprints from the variety of intact viral genomes in a sample and has become a common tool for marine virologists (28, 31, 40, 41). However, the ability of TEM and PFGE to discriminate between genetically dissimilar viral groups remains well below that of DNA sequence-based methods.

DNA-DNA hybridizations between viral isolates (14, 35) or portions of viral assemblages (41) and radiolabeled, purified phage DNA (26) can determine genetic similarity between viruses and track specific viruses over time and space. Sequence polymorphisms within specific genes (10, 11, 15, 19, 44) have also been used successfully as proxy measures of viral diversity within a range of aquatic environments. Unfortunately, these approaches assess the diversity and dynamics of only those viruses which carry the targeted genes, thus failing to encompass total viral diversity (29, 31).

An alternative approach that overcomes the low resolution of PFGE, the limitation of targeted PCR approaches, and the expense of high-throughput metagenome sequencing is randomly amplified polymorphic DNA (RAPD) PCR. RAPD-PCR employs a 10-mer oligonucleotide primer to amplify viral DNA and produce several amplicons. Subsequent electrophoretic separation of the amplicons has been used to compare the genotypic diversity of viral isolates (4, 12, 13, 22). This technique has also been successfully used for strain-typing bacteria (23–25, 42) and comparing aquatic and soil microbial communities (18, 43). To date, RAPD-PCR has not been applied to the analysis of whole-viral-assemblage diversity. Because RAPD-PCR requires minimal amounts of template DNA (13, 18) and no a priori knowledge of gene sequence data, it is particularly well suited to work with genetically

* Corresponding author. Mailing address: Delaware Biotechnology Institute, 15 Innovation Way, University of Delaware, Newark, DE 19711. Phone: (302) 831-4510. Fax: (302) 831-3447. E-mail: wommack@dbi.udel.edu.

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diverse environmental viral samples. In this study, RAPD-PCR was evaluated as a technique for tracking virioplankton assemblage richness dynamics in the Chesapeake Bay.

MATERIALS AND METHODS

Viral community sampling, concentration, and PFGE. Water samples from the main stem of the Chesapeake Bay were collected in August 1995, May, June, and July 1996, September 2002, and March 2003. Sampling stations were as follows: 724 (37°24′N, 76°24′W), 744 (37°44′N, 76°11′W), 818 (38°18′N, 76°17′W), 834 (38°34′N, 76°24′W), 845 (38°45′N, 76°26′W), 858 (38°58′N, 76°23′W), and 908 (39°08′N, 76°20′W) (41). Sample names followed by a "T" or "B" were collected approximately 1 m from the surface and 2 m from the sediment-water interface, respectively. Samples collected in 1995 and 1996 were concentrated as described elsewhere (39, 41). More recently obtained viral community samples (2002 and 2003) were concentrated by sequential tangential flow filtration (TFF). In general, 50 liters of water was filtered through a 25-mm string-wound polypropylene sediment filter to remove large particles. Samples were then filtered through a 0.5-m^2, 0.22-μm Pellicon TFF cartridge filter (Millipore) followed by concentration to 2 liters using a 30-kDa spiral-wound TFF filter (Amicon S10Y30; Millipore) (11). Two-liter samples were then reduced to 300 ml by TFF through a 30-kDa Prep/Scale TFF filter (Millipore) and stored at −20°C. Final concentration to approximately 2 ml was achieved by centrifugation through 30-kDa spin filters (Centricon Plus-80; Millipore). Completed viral concentrates were stored at 4°C until RAPD-PCR analysis, which occurred within a year of the collection date. Viral concentrates were subjected to PFGE as previously described (41) to isolate DNA from single virioplankton genomic bands.

Amplification of viral DNA by RAPD-PCR. RAPD-PCR mixtures contained the following components: 0.16 mM each of dATP, dCTP, dTTP, and dGTP; 4 μM of primer; a 1× final concentration of *Taq* polymerase reaction buffer; and 2.5 U of TaKaRa ExTaq polymerase, HotStart version (TaKaRa Bio Inc.). Either 1 μl of virioplankton concentrate or 1 to 5 μl of melted single bands cut from a PFGE fingerprint of total virioplankton viral DNA (41) served as the template DNA. The decamer primers OPA-6 (5′-GGT CCC TGA C-3′), OPA-9 (5′-GGG TAA CGC C-3′), OPA-13 (5′-CAG CAC CCA C-3′), CRA-22 (5′-CCG CAG CCA A-3′), and CRA-23 (5′-GCG ATC CCC A-3′) were used to amplify virioplankton template DNA. In each reaction, only one primer was used, acting as both the forward and reverse primer (23, 41, 42). PCR conditions were as follows: (i) 94°C for 10 min; (ii) 35°C for 3 min; (iii) 72°C for 1 min; (iv) 94°C for 30s; (v) repeat steps 2 to 4 for a total of 30 cycles; (vi) 35°C for 3 min; (vii) 72°C for 10 min; (viii) hold at 4°C. All RAPD-PCR products were separated by gel electrophoresis on 2% MetaPhor agarose (Lonza) or 1.8% GenePure HiRes agarose (ISC BioExpress) gels in 0.5× Tris-borate-EDTA run at 4 V/cm and visualized by ethidium bromide (1 mg/ml) or SYBR gold staining (0.5× stock concentration; Molecular Probes).

To investigate whether free, nonviral DNA contributed to the production of RAPD-PCR products, a viral concentrate was subjected to DNase treatment prior to RAPD-PCR amplification. The DNase reaction mixtures contained either 1 U or 5 U of RQ1 RNase-free DNase (Promega), 1× DNase buffer (final concentration), 1 μl of viral concentrate, and diethyl pyrocarbonate-treated water to a final volume of 20 μl. Reaction mixtures were incubated for 30 min at 37°C followed by 15 min at 75°C. In addition, a viral concentrate sample was heated to 98°C for 15 min, cooled to room temperature, and then treated with DNase as described above. One microliter of product from each of these reactions served as the RAPD-PCR template DNA, and results were compared to those from non-DNase-treated but similarly diluted (1 μl in 19 μl diethyl pyrocarbonate-treated water) viral concentrate.

Reproducibility of RAPD-PCR banding patterns was assessed by repetition of the same reaction on three different days. The same thermocycler and template DNA but different aliquots of stock solutions were used for each trial. The similarity of resulting banding patterns was assessed by unweighted pair group method with arithmetic average clustering based on the Jaccard similarity index in GelCompar II software (v4.0; Applied Maths) (13, 18).

RAPD-PCR bands as hybridization probes and DNA sequence analyses. Approaches for production of radiolabeled single RAPD-PCR bands for use as DNA probes are described elsewhere (41). To obtain DNA sequence from individual RAPD-PCR amplicons, single bands were cut from a 0.75% SeaPlaque (Lonza) gel and purified using a QIAquick gel extraction kit (Qiagen). A QIAquick PCR purification kit (Qiagen) was used to purify RAPD-PCR products from a whole reaction.

Purified PCR products were ligated into pCRII-TOPO or pCR4-TOPO TA cloning vectors (Invitrogen) and transformed into OneShot chemically competent TOP10 *Escherichia coli* cells using either the fast or long chemical transformation procedure provided by the manufacturer. Mixtures for cloning reactions (ligation followed by transformation) contained either a single gel-purified RAPD-PCR product or a collection of products from a whole RAPD-PCR, named PFGE band RAPD band (PBRB) and whole-reaction (WR) sequences, respectively. Colonies identified as containing a plasmid with an insert of the same size as the initial RAPD-PCR product were picked and grown overnight at 37°C with shaking in 2 to 4 ml of LB broth with 50 μg ml^{-1} kanamycin.

Plasmid DNA was purified from overnight cultures using a QIAprep spin miniprep kit (Qiagen) and sequenced on an automated ABI Prism 377 DNA sequencer (Applied Biosystems). Sequence trimming and contigs were formed from forward and reverse sequences using Sequencher 4.1 software (Gene Codes Corp.). Consensus RAPD-PCR amplicon sequences were analyzed using BLAST against the GenBank subject databases nt, nr, env-nt, and env-nr and smaller databases containing environmental viral sequences from the Chesapeake Bay, Delaware soil, and coastal California sediments and surface waters (2). BLAST homologs were considered significant at an expectation value (e value) of $\leq 10^{-3}$ (8).

Nucleotide sequence accession numbers. Sequences were deposited in the GenBank database with accession numbers ET165566 to ET165585.

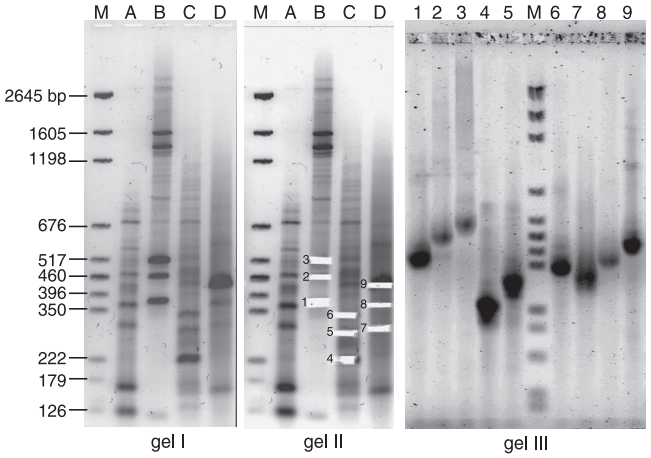

FIG. 1. Generation and purification of RAPD-PCR products from virioplankton concentrates using primer OPA-6. (Gels I and II) Lanes: A, 834B (June 1996); B, 724T (July 1996); C, 845T (August 1995); D, 908T (May 1996); M, pGEM DNA marker. (Gel III) Lane numbers correspond to cut-out bands labeled in gel II.

RESULTS

Amplification of virioplankton DNA using RAPD-PCR. RAPD-PCR of Chesapeake Bay viral concentrates collected from 1995 to 2003 using a variety of primers successfully produced DNA amplicons. Profiles contained an average of seven distinct bands ranging in size from approximately 125 bp to 3,000 bp (Fig. 1 and 2). The number of distinct amplicons produced per sample ranged from 4 to 12 (Fig. 1 and 2). Of the 11 viral assemblages assayed in this study, only 724T from September 2002 did not produce any amplicons. Gel extraction, purification, and reamplification of single bands produced single products in eight of nine trials (Fig. 1, gel III), indicating that the majority of RAPD-PCR products represent single, distinct products not prone to concatemerization. The smearing of the reamplified single bands in Fig. 1, gel III, is likely due to overloading of reaction products on the gel rather than production of multiple bands of similar sizes.

Comparison of RAPD-PCR banding patterns generated from Chesapeake Bay virioplankton concentrates collected

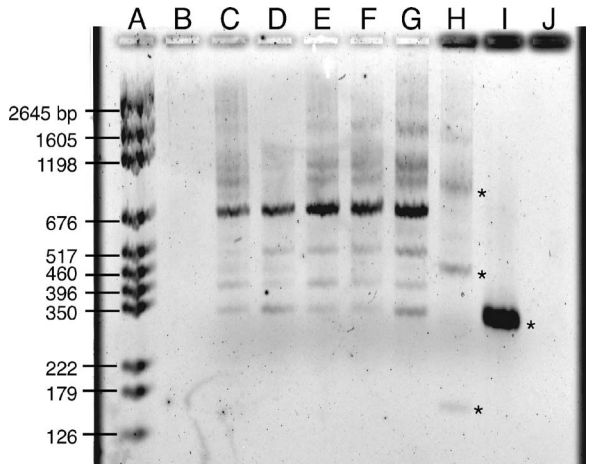

FIG. 2. Agarose gel of primer CRA-23 RAPD-PCR products from Chesapeake Bay viral concentrates. Lanes: A, pGEM DNA marker; B, 724T; C, 744T; D, 818T; E, 858T; F, 858B; G, 908T; H, 818T; I, positive control; J, no-template control. All samples were collected in September 2002, with the exception of 818T, which was collected in March 2003. Asterisks indicate bands that were cloned and sequenced.

during different seasons at different locations revealed high genetic variability in viral assemblages over time (Fig. 1, gel I, and Fig. 2, lanes D and H). In contrast, virioplankton concentrates collected over the course of a few days in September 2002 during a bay transect revealed highly similar RAPD-PCR fingerprints (Fig. 2, lanes C to G). High similarity was also observed between depths, as surface and bottom water viral assemblages at station 858 produced identical RAPD-PCR banding patterns (Fig. 2, lanes E and F).

To confirm that RAPD-PCR products were generated from viral DNA templates, the viral concentrate from sample 818T (March 2003) was subjected to treatment with combinations of DNase and heat (Fig. 3). Heating the viral concentrate for 15 min at 98°C prior to PCR had no impact on the presence of bands, but a lightening of larger products was observed compared to the control reaction (Fig. 3, lanes A and B). Exposure to 1 or 5 U of DNase prior to PCR similarly reduced band intensity but did not remove any bands (Fig. 3, lanes C and D), indicating that template DNA encapsulated within virus particles was DNase resistant. Heat treatment followed by DNase digestion significantly reduced both the number and intensity of bands produced by RAPD-PCR (Fig. 3, lanes E and F). The same result of fewer bands with heat and DNase treatment but not complete removal of all bands was observed in a similar experiment with the viral concentrate from sample 908T (September 2002) (data not shown). In both experiments, some template was always resistant to DNase activity, even with as much as 15 U of DNase in the reaction (data not shown).

To determine the reproducibility of RAPD-PCRs, viral concentrates from samples 818T (September 2002) and 908T and 818T (March 2003) were amplified on three separate days and the resulting banding patterns compared (Fig. 4). The repeated profiles were an average of 88% similar, ranging from 79.2% to 100% similarity, and visually indistinguishable. Slight differences between triplicate reactions resulted from band loss and not band acquisition in all but one occurrence (sample 818T, September 2002).

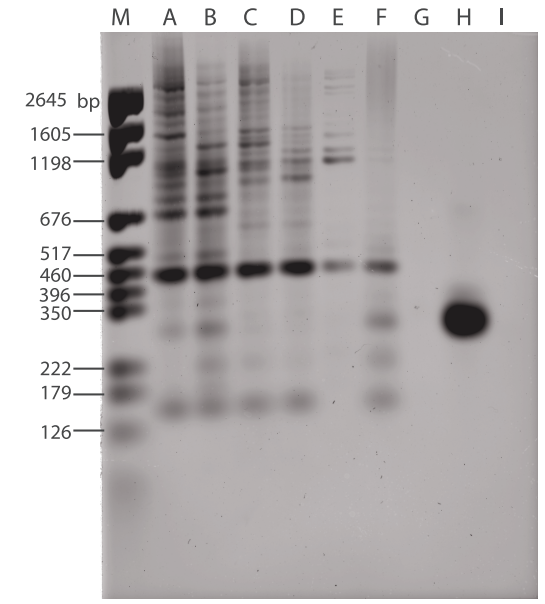

FIG. 3. RAPD-PCR amplification after heat and DNase treatment of viral concentrates. All products are from sample 818T (March 2003) viral concentrate amplified with primer CRA-23. Heat treatment exposed viral concentrates to 98°C for 15 min prior to DNase treatment or PCR. One μl viral concentrate was used in each treatment, diluted in a total volume of 20 μl. Five microliters of reaction product was loaded in each lane, except for the positive control, where 3 μl was loaded. Lanes: M, pGEM DNA marker; A, 818T alone; B, 818T plus heat; C, 818T plus 1 U DNase; D, 818T plus 5 U DNase; E, 818T plus heat plus 1 U DNase; F, 818T plus heat plus 5 U DNase; G, 5 U DNase plus water; H, PCR-positive control; I, PCR-negative control (no template DNA).

Use of RAPD-PCR amplicons as hybridization probes. As demonstrated in Fig. 5, virioplankton genomic DNA collected from a single PFGE band can serve as a template in RAPD-PCRs to generate single radiolabeled probes suitable for use in

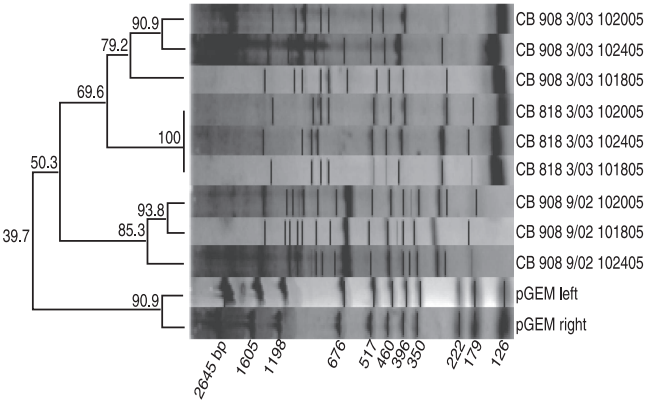

FIG. 4. Reproducibility of RAPD-PCR banding patterns. RAPD-PCRs were repeated on three days (18, 20, and 24 October 2005) using viral community concentrates from samples 908T (March 2003), 818T (March 2003), and 908T (September 2002) as template DNA. The banding patterns produced were run on the same gel and are shown with bands detected by GelCompar II analysis depicted as an overlay of black lines. Percent similarity between banding patterns and stations, as determined by the Jaccard similarity index with optimization and tolerances set to 2%, is shown in the tree (left). DNA markers from the gel were used as an outgroup for the banding pattern comparison. CB, Chesapeake Bay.

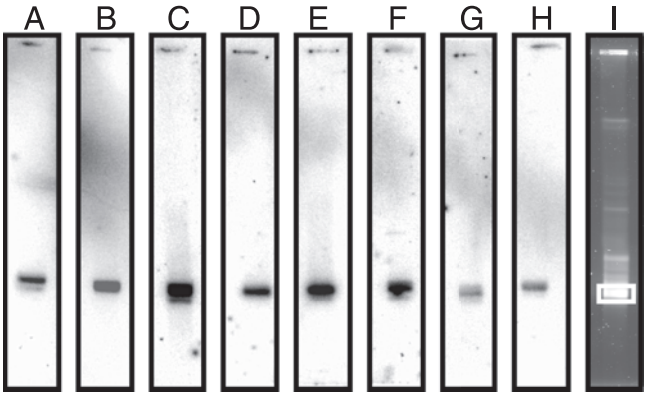

FIG. 5. Use of RAPD-PCR products as DNA probes in Southern hybridization. Lane I, PFGE of virioplankton concentrate from sample 744T (June 1996). DNA within the boxed area was purified and used as the template in RAPD-PCR. Lanes A to H, hybridizations of RAPD-PCR products to Southern blots of 744T virioplankton concentrate. Blots were probed with RAPD-PCR products from the primers OPA-9 (A to C), CRA-23 (D), OPA-13 (E and F), and CRA-22 (G and H).

Southern hybridizations. Virioplankton DNA recovered from a small region of a virioplankton PFGE fingerprint (Fig. 5, lane I) served as a DNA template in RAPD-PCR. Subsequently, RAPD-PCR products were electrophoretically separated, and single bands were excised from the gel and subjected to a second RAPD-PCR to give high yields of single RAPD-PCR amplicons (see Fig. 1 for an example). Finally, single-band RAPD-PCR amplicons were radiolabeled by random priming and hybridized against virioplankton genomic DNA separated by PFGE (Fig. 5). In all cases, RAPD-PCR probes bound only to the original DNA template PFGE band.

Sequencing of RAPD-PCR products. To independently confirm the viral origin of RAPD-PCR amplicons, seven distinct RAPD-PCR bands were cut from gels, purified, cloned, and sequenced. In addition, RAPD-PCR products from a whole reaction of the virioplankton concentrate from sample 818T (March 2003) were purified, cloned, and sequenced without prior band selection and gel extraction. Seventeen unique clones were picked and bidirectionally sequenced from these two procedures. PBRB band D (PBRB_D) produced three clones with various insert lengths, which were nonhomologous after sequencing (see the supplemental material). Similarly, PBRB_F produced nonhomologous clones with inserts of two sizes, PBRB_F1 and PBRB_F2 (see the supplemental material). Three sequences from cloning of the whole PCR from sample 818T had lengths matching PCR products previously identified by gel electrophoresis (Fig. 2). Sequencing from this experiment also revealed that several clones contained the same sequence, and the resulting consensus sequences were used for BLAST analysis. Eleven of the seventeen sequences had a significant BLAST homolog (e value $\leq 10^{-3}$) within one of the databases (see the supplemental material). Five of the seventeen (29%) had a significant homolog within GenBank database nr. However, only one sequence, WR4, had its best homolog within the nr subject database. Most of the significant homologs were environmental database sequences, five of which could be tentatively identified as viruses or prophages from their known sequence source (WR12) or TBLASTX results of their homologous environmental sequences (PBRB_D1, PBRB_D2, PBRB_E, and PBRB_F1). Three other tentative identifications of environmental database sequences to which RAPD-PCR amplicons showed significant homology were of bacterial and fungal origin (see the supplemental material).

DISCUSSION

Evaluation of RAPD-PCR. The goal of this study was to appraise the usefulness of RAPD-PCR as an approach to routine assessment of the genetic richness of aquatic viral assemblages while circumventing the need for a common or single genetic marker. Using this technique, PCR products within the expected RAPD product size class (13, 16, 25) were amplified from Chesapeake Bay viral concentrates and produced banding patterns for community comparison, probe generation, DNA sequencing, and bioinformatic analysis. To further establish the utility of RAPD-PCR, several assumptions inherent to the method must be addressed.

First, it is essential to assume that RAPD-PCR products in this study were amplified from viral templates. Treating viral concentrates with DNase prior to PCR amplification confirmed the viral origin of RAPD-PCR products in this study. Exposure to DNase prior to PCR removed few or no products from the final reaction (Fig. 3), indicating that amplicons were from encapsulated viral DNA and not from free, dissolved DNA in the sample. Heating followed by DNase treatment was expected to remove all DNA templates, as heat treatment compromises viral protein capsids, releasing viral genomic DNA, which is subsequently digested by DNase (37). Most but not all bands were lost from RAPD-PCRs of viral concentrates that were heated and then exposed to DNase prior to PCR. A few bands always remained after heat and DNase treatment. This population of DNase-resistant templates could be viral DNA that was associated with heat-resistant proteins (21) or bound in an unknown form (9) and still suitable as template DNA. The same pattern of largely unknown sequences, a small proportion with homologs in GenBank nr, and a large proportion of high-quality viral gene homologs has been observed in other viral sequencing projects (3, 5–8, 17) and is at present a general characteristic of viral sequences. Finally, the methods used for viral concentration prior to RAPD-PCR resulted in little or no bacterial contamination, as demonstrated by the lack of bacteria as assessed by epifluorescence microscopy and 16S rRNA gene amplification in freshly collected viral concentrates (data not shown). RAPD-PCR results have previously been shown to be resistant to large amounts of host DNA contamination (13).

Second, the use of RAPD-PCR as a method for comparing communities relies heavily on the assumption that the banding patterns are reproducible between reactions. In previous reports, RAPD-PCR patterns from a variety of DNA templates (1, 18, 25), including *Vibrio parahaemolyticus* phage isolates (12), were highly reproducible. Overall, the lowest similarity between replicate banding patterns in this study was 79% (Fig. 4). This is similar to the 89% similarity between RAPD fingerprints of creek microbial communities (18) and the 80% similarity between replicate sediment viral RAPD-PCR fingerprints (R. R. Helton, personal communication).

Thirdly, RAPD-PCR may underestimate viral richness if genetically different DNA template molecules produce PCR amplicons of the same size. Probe generation and band purification from RAPD products (Fig. 1 and 5) indicate that most bands are likely derived from single viral strains within the original assemblage. Sequencing of RAPD-PCR insert DNA from RAPD gel bands PBRB_D and PBRB_F did reveal multiple nonhomologous products from a single purified PCR band. For some of the sequences, the observed differences may be due to variations in sequencing read length. However, PBRB_D1 and PBRB_D2 are not homologous despite similar read lengths and generation from the same cloning reaction, indicating that underestimation of total community diversity may occur when this technique is used. RAPD-PCR banding patterns are only a proxy for actual diversity, as only viral strains that contain priming sites are detected and viruses larger than 0.2 μm are excluded from samples by filtration. RAPD-PCR can also only assess changes in viral richness, or the number of different viral strains present, and not changes in viral diversity, which would require measurements of richness and evenness. The RAPD-PCR fingerprints generated in this study may also underestimate viral richness, since the sample storage conditions used do not prevent viral particle decay (34). However, subsequent research has produced stable, reproducible RAPD-PCR banding patterns in viral concentrates stored at 4°C for over a year between repeated PCR analyses. For example, banding patterns produced from the 818T (March 2003) viral concentrate using the same primer over the course of 2 years (Fig. 2, lane H [2003], and Fig. 4 [2005]) amplified the same dominant bands with little variation, proving that significant viral richness can still be detected even in degraded samples. Unlike targeted gene approaches, RAPD-PCR amplifies sequences from a random collection of viruses without assuming shared gene content and by extension, similar biology. Thus, RAPD-PCR potentially garners data from viruses with a broader range of lifestyles and host ranges.

Finally, RAPD-PCR may overestimate viral genetic diversity if a single viral genome contains more than one priming site, resulting in multiple bands from the same virus in the final banding pattern. Sequence results from this study found no homology between bands from the same profile. Through hybridization experiments, Akopyanz et al. (1) also found that bands of different sizes were not homologous. Thus, RAPD-PCR patterns are likely a reliable representation for the scale of true genetic diversity within a given virioplankton assemblage. PCR may also produce biased results due to differences in the efficiency of priming sites. However, the large numbers of bands produced suggests unbiased production, and previously reported experiments applying random amplification yielded unbiased amplification of the entire bacteriophage λ genome (30). Chimeric DNA can occur in any PCR, but sequencing results in this study and that of Rohwer et al. (30) found no chimeric products. Additionally, the number of cycles used in RAPD-PCR was intentionally kept low (30 cycles) to prevent chimera formation (41). From these results, RAPD-PCR reliably produces a range of amplicons from virioplankton concentrates, and the capacity for this technique to under- or overestimate richness appears to be low, although these assumptions must always be acknowledged when this technique is used to compare viral assemblages.

Applications of RAPD-PCR to viral samples. Comparisons of Chesapeake Bay virioplankton genetic richness using RAPD-PCR indicate that these viral assemblages display more temporal than spatial variability (Fig. 2). Banding patterns from virioplankton collected at six stations spanning the Chesapeake Bay over a 2-day period generated similar fingerprints, while the pattern generated by a sample from one of the same stations (818T) sampled 6 months later was distinct from those of the other samples (Fig. 2). These results mirror previous PFGE profiling methods that showed greater temporal than geographic divergence between Chesapeake Bay viral assemblages (40). A high degree of genetic similarity between vibriophages isolated from similar environments, but separated by broad geographic distances was also reported in a RAPD-PCR-based study using a degenerate primer (12). Together these results support the argument that viral assemblages may be genetically homogenous within a given environment, such as the length of the Chesapeake Bay.

Recently, Comeau et al. (13) advocated against using RAPD-PCR results alone for comparing viral communities because altering the proportions of viral strains within the assemblage affected the resulting fingerprints. However, these proportional changes in viral species do represent shifts in viral assemblage composition, which RAPD-PCR clearly detected. Differences in RAPD-PCR banding patterns can result from either absolute change in assemblage composition (loss or gain of viral strains) or shifts in the relative proportion of viral strains within the assemblage. Since these two factors cannot be separated, it is critical to standardize template concentration (amount of DNA or viral particles) across RAPD-PCRs to ensure that banding patterns relate only to changes in the viral assemblage, not to differences in template concentration. Because template concentrations were not standardized in this study, more in-depth comparisons of viral assemblages were not attempted.

Comeau et al. (13) also argued against using sequence-specific 10-mer primers and instead favored use of degenerate 10-mers, as the smaller genomes of viruses may not contain enough priming sites for one primer to produce an adequate number of bands for analysis. Results from this study and a study of *Baculovirus* isolates indicate that nondegenerate 10-mer primers can produce robust banding patterns for RAPD-PCR fingerprint analysis (Fig. 1 and 2) (4). In addition, pooling RAPD-PCR banding patterns resulting from at least two different primer sequences may allow greater sensitivity to differences between samples and strains (4, 18, 23, 25, 36).

Use of RAPD-PCR for ecological investigations of changes in viral genetic richness was the principal consideration of this study. Alternately, previous studies have demonstrated that RAPD-PCR products, serving as hybridization probes, can be useful in ecological surveys of viral (41), bacterial (16), and eukaryotic (36) samples. In this work, the most successful strategy for probe construction was the use of a single virioplankton PFGE band as template DNA in the RAPD-PCR. The use of RAPD-PCR bands obtained from whole virioplankton concentrates, as opposed to PFGE genomic DNA bands, was less successful, with only ca. 20% of these probes showing successful hybridization (data not shown). Presumably this poor suc-

cess rate was due to the contribution of rare viral genotypes to amplicons within RAPD-PCR of whole viral assemblages. Finally, RAPD-PCR can also serve as a means of investigating gene composition within a viral assemblage through cloning and sequencing of amplicon DNA. Thus, RAPD-PCR enables qualitative comparisons of viral assemblage diversity not only through banding patterns but also through DNA sequence information.

In conclusion, RAPD-PCR is particularly applicable to ecological investigations of natural aquatic viral assemblages by eliminating the need for cultivation, prior sequence knowledge, or a common genetic marker. RAPD-PCR assays offer a robust and economical approach for routine observations of viral richness and can provide ancillary data on the genetic complement within a given viral assemblage. This technique enables sampling of a larger and less selective cross-section of the virioplankton assemblage without the costs associated with other methods. The application of RAPD-PCR to viral diversity studies can perhaps lead to a better understanding and constraint of the dynamics of viral assemblages in aquatic environments and the genetic diversity they harbor.

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