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Viral Contribution to Dissolved DNA in the Marine Environment as Determined by Differential Centrifugation and Kingdom Probing

SUNNY C. JIANG AND JOHN H. PAUL*

University of South Florida, St. Petersburg, Florida 33701

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Dissolved or filterable (<0.2-μm-pore-size filter) DNA is a ubiquitous component of the dissolved organic matter in the surface waters of this planet. In an effort to understand the composition and possible sources, we subjected dissolved DNA concentrated by vortex flow filtration from offshore and coastal environments to differential centrifugation and probing with 16S rRNA-targeted kingdom oligonucleotide probes. Initial studies with calf thymus soluble DNA and T2 phage particles indicated that high-speed ultracentrifugation (201,000 × *g* for 90 min), a method to separate viral particles from soluble DNA used by other investigators, resulted in pelleting of nearly all the DNA and virus particles. Lower-speed centrifugation (11,200 to 25,800 × *g* for 90 min) resulted in >99% of the virus particles being collected in the pellet and ~65% of the calf thymus DNA remaining in the supernatant. Employing this approach, we estimate that approximately 50% of the filterable DNA from marine environments is truly soluble or free DNA and that the other half is composed of bound forms (viral particles and, potentially, colloids). Of the bound form, 17 to 30% could be accounted for by viral particles, by calculating the amount of viral DNA on the basis of viral abundance, leaving a portion of the bound form uncharacterized. Kingdom probing with universal, eubacterial, and eucaryotic probes indicated that dissolved DNA hybridized with all of these probes, while purified standard viral DNAs did not, or hybridized only slightly with the universal probe (tailed oligonucleotide only). Collectively, these data indicate that DNA in viral particles is a small component of the dissolved DNA, the majority being of eubacterial and eucaryotic origin.

Dissolved DNA (that which passes through a 0.2-μm-pore-size filter) has been found in nearly all the freshwater and marine environments that have been examined for its presence (3, 7, 8, 17, 22, 27, 31, 37). This material is generally found at concentrations of 5 to 44 μg/liter for estuarine environments, 2 to 15 μg/liter for coastal oceanic environments, 1 to 5 μg/liter for oligotrophic ocean surface waters, and <1 μg/liter for deep-sea environments (3, 8, 27). The molecular size range is broad, from <500 bp to >23 kbp, with DNA in offshore environments at the lower end of this range. By using radioactive precursors, heterotrophic bacterioplankton (26, 27) and exogenously added genetically altered bacteria (25) were shown to be a source of dissolved DNA and RNA, while actively photosynthesizing phytoplankton was shown to be a source of dissolved RNA and protein, but not DNA (26). Subsequent studies indicated the presence of the ribulose bisphosphate large subunit gene (*rbcL*), a phytoplankton gene, in dissolved DNA from freshwater and marine environments (24).

The discovery of the abundance of virus particles (2, 33), also predominantly in the <0.2-μm fraction, led us to investigate the proportion of the dissolved DNA contained in viral particles. We had previously considered DNA encapsulated in viral particles to be unimportant (8), because all dissolved DNAs we had measured were DNase sensitive, and we assumed DNA in viral particles would be resistant to DNase. However, all our previous dissolved-DNA measurements were made by using samples that had been ethanol precipitated, and this procedure liberates DNA from viral particles, or at least makes the capsids permeable to DNase (28). In 1991, we published a report on the use of vortex flow filtration (VFF) to concentrate both viral particles and dissolved DNA without ethanol precipitation (28). That report indicated that a portion of the dissolved DNA behaved like DNA in virus particles in that it was resistant to digestion by DNase and that ethanol precipitation resulted in higher Hoechst fluorescence DNA measurements and increased DNase sensitivity. However, the amount of dissolved DNA was much larger than that which could be contained in viral particles, on the basis of direct counts and calculation of viral DNA (vDNA) content. Subsequent to that report, Maruyama et al. (21), using a direct method for the measurement of dissolved DNA which did not require sample concentration, found most of the DNA to be DNase insensitive (termed "coated"), which they attributed to vDNA. Beebee (1) reported the separation of vDNA from soluble DNA (sDNA) by ultracentrifugation at 140,000 × *g* for 4 h followed by chromatographic purification of the two DNA fractions. By this procedure, the majority (>80%) of the filterable DNA was in the vDNA fraction, with only a small proportion in the sDNA fraction. The vDNA fraction was macromolecular (>20 kb), while the sDNA fraction was ≤500 bp. In an attempt to reconcile the discrepancies between the reports of the relative importance of viral particles in dissolved DNA measurements, we have employed differential ultracentrifugation (1) to try to separate viral particles from sDNA. In an attempt to further characterize dissolved DNA, we have used 16S rRNA-targeted oligonucleotides to probe dissolved DNA and various microbial and vDNA standards (15, 36). The results of these studies, presented in this paper, suggest that viral particles are a small component of the dissolved DNA, at least for the environments we have sampled.

MATERIALS AND METHODS

Sampling locations. For vDNA and dissolved DNA studies based upon ethanol precipitation and/or ultracentrifugation, samples were taken from Bayboro

* Corresponding author. Mailing address: University of South Florida, 140 Seventh Ave. South, St. Petersburg, FL 33701. Phone: (813) 893-9168. Fax: (813) 893-9189.

Harbor, St. Petersburg, Fla., in Tampa Bay, on various dates from 1990 to 1993; from the St. Petersburg Pier, Tampa Bay; and from the Fort Desoto fishing pier in Tampa Bay on 28 May, 25 July, and 2 October 1991. Samples were also taken during cruises aboard the R/V Pelican in June 1992, at the mouth of Tampa Bay (27°35′ N, 82°43′W) and in the Gulf of Mexico (station 3, 27°15′N, 83°17′W; and station 7, 26°06′N, 84°53′W). For comparison of ultracentrifugation with concentration of samples by VFF for phage enumeration, samples were taken during cruises aboard the R/V Pelican in June of 1993 at the mouth of Charlotte Harbor, Fla. (26°42′N, 82°19′W), and in the Gulf of Mexico at station 2A (26°58.3′N, 83°25′W) and stations 5A and D (24°24′N, 83°42′W). For kingdom probing of dissolved DNA, samples were taken from Fort Desoto; from the mouth of Tampa Bay (June 1992 and June 1993); from the dock off the National Underseas Research Center (NURC) in the Port Largo Canal, Key Largo, Fla.; from Garden Key, Dry Tortugas (24°37.35′N, 82°52.20′W) (March 1992); from Key West Harbor, Charlotte Harbor (26°42′N, 82°19′W); and from station 6 in the Gulf of Mexico (24°39′N, 82°56′W).

Purification of dissolved DNA. DNA in filtered seawater samples (0.2-μm-pore-size filter) was concentrated either by VFF (20 to 40 liters) (28) or by ethanol precipitation (8 to 10 liters) (7, 8) as previously described. Samples were further purified by Elutip-d chromatography (Schleicher and Schuell, Keene, N.H.) as previously described (8).

Standard DNAs and virus stocks. Calf thymus DNA and salmon sperm DNA were obtained from Sigma Chemical Co. (St. Louis, Mo.). Viral particles used in ultracentrifugation studies included T2 phage, which was grown on *Escherichia coli* B on LB media (19), and the marine phages ϕ16 and ϕ13. These phages were isolated from Tampa Bay and were maintained on marine hosts 16 and 13, respectively, also isolated from Tampa Bay. All marine phages were grown on ASWJP media (23) at 28°C. For preparation of phage stocks, soft agar overlay of the host (12 plates) was performed at a high phage titer and the phages were eluted with 5.0 ml of 0.5 M Tris-HCl (pH 8.0) per plate. The lysates were subjected to a low-speed centrifugation to remove debris and filtered through a 0.2-μm-pore-size filter.

For purified phage preparations, the phages were purified from cell lysates by following standard phage lambda purification protocols (34). Viral lysates were clarified at 8,000 rpm (SS34 rotor; DuPont-Sorvall, Inc.) for 5 min. Supernatants were treated with DNase I (50 μg/ml) and RNase I (5 μg/ml) at room temperature for 1 h, followed by NaCl-polyethylene glycol 8000 (1 M and 10%, respectively) precipitation. The pellets were resuspended in SM buffer (0.1 M NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl, 0.01% gelatin) and extracted with an equal volume of chloroform-isoamyl alcohol (24:1). To the aqueous phase, 0.5 g of $CsCl_2$ was added, and this mixture was layered carefully onto a $CsCl_2$ step gradient (steps of 1.7, 1.5, and 1.45 g/ml). After centrifugation at 22,000 rpm for 2 h (Beckman SW41 rotor), the viral band was collected by puncturing the side of the centrifuge tube. The collected band was dialyzed against a low-salt buffer (10 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4).

Preparation of purified phage DNA. For kingdom probing, crude viral lysates were DNase and RNase digested as described above, phenol-chloroform extracted, and ethanol precipitated (19). For comparison of DNA quantitation methods, $CsCl_2$-banded phage preparations were further purified by phenol-chloroform fractionation and ethanol precipitation.

DNA quantitation. For DNA quantitation of most samples, the Hoechst 33258 method (29) was employed. Purified phage DNA was also quantitated by determining A_{260} and A_{280} by using a Cary 17 D spectrophotometer (Varian Instruments Group, Sugarland, Tex.). For comparison of DNA quantitation methods, purified T2 phage DNA was also quantitated by the ethidium bromide fluorometric method (34) and the diphenylamine colorimetric method (4).

Purification of microbial DNA for kingdom probing. Eubacterial DNAs from *E. coli* B and marine bacterial host 16 (tentatively identified as *Vibrio parahaemolyticus*) were purified by the Marmur method (20). Archaebacterial DNA was purified from *Haloferax volcanii* ATCC 29605 (formerly *Halobacterium volcanii*) and *Sulfolobus sulfotaricus* ATCC 35091. *H. volcanii* was maintained on medium 974 (13) at 30°C, and *S. sulfotaricus* was maintained on medium 1304 (13) at 75°C. Chromosomal DNA was purified by the method of Marmur (20). Eucaryotic DNA was purified from *Saccharomyces cerevisiae* ATCC 834-161591 by the method of Cryer et al. (6).

Kingdom probing. The following oligonucleotide probes were synthesized by the University of Florida DNA Synthesis Laboratory: archaebacterial probe 915 (5′-GTGCTCCCCCGCCAATTCCT-3′ [35]), archaebacterial probe 361 (5′-GCGCCTGG/CTGCG/CCCCCGTAGGGCC-3′ [14]), eubacterial probe 338 (5′-GCTGCCTCCCGTAGGAGT-3′ [35]), eucaryotic probe 1209 (5′-GGGCA TCACAGACCTG-3′ [15]), and universal probe 1407 (5′-ACGGGCGGTGTGT G/AC-3′ [36]). Nonradioactive probe labelling was accomplished by using the Genius oligonucleotide Labelling Kit and the Genius Oligonucleotide Tailing Kit (Boehringer Mannheim). Probe labelling was verified by dotting probes in a dilution series and detecting them by chemiluminescence as recommended by the manufacturer of the kits. Initially, Zeta-probe charged nylon filters (Bio-Rad) were used, but better results were obtained subsequently with MSY Magnagraph charged nylon membranes. Probe stringency was evaluated by adjusting hybridization and washing temperatures by using DNA standard blots only. Chemiluminescence was detected by autoradiography with X-ray film (Kodak X-Omat AR film).

RESULTS AND DISCUSSION

Table 1 shows the effect of ethanol precipitation on the fluorometric quantitation of various DNA preparations. For VFF-concentrated natural water samples and viral lysates of laboratory cultures, ethanol precipitation resulted in a 1.4- to 7.8-fold increase in apparent DNA concentration. The average increases were 3.4-fold for environmental samples and 2.4-fold for viral lysates. However, no increase was found upon ethanol precipitation of calf thymus DNA. In fact, a slight decrease was observed, consistent with losses expected in ethanol precipitation, centrifugation, and resuspension of small-volume samples. These results suggested to us that DNA in VFF concentrates of natural water samples behaved similarly to crude phage lysates and purified phage preparations. We interpret these data to reflect the inability of the Hoechst stain to fully penetrate viral capsids, or to indicate that the conformation of such encapsulated DNA prevented efficient binding by the stain. Ethanol precipitation resulted in partial release of the DNA from the viral particle, or increased conformational freedom which allowed interaction with the dye.

Table 1 also shows the estimated vDNA contents of these samples, calculated on the basis of viral direct counts and assuming an average viral genome weight of 9×10^{-17} g per phage (3, 12, 28). For naturally occurring dissolved DNA samples, the estimated amount of vDNA is significantly below the amount of total DNA, ranging from <1% to up to 22% and averaging 7.7%. For cultivated marine phages (ϕ16 and ϕ13) which had been purified by $CsCl_2$, there is good agreement between the estimated vDNA and the measured DNA. In fact, vDNA estimates based on direct counts exceeded measured DNA for these two viruses by 30 to 60%. For ϕ13, we have assumed an average genome size of 9×10^{-17} g of DNA per phage. If we employ the size of ϕ16, which we estimated by restriction analysis to be 5.58×10^{-17} g (18), the estimated vDNA becomes 15.6 μg, or 99% of the measured DNA. Unlike the marine phages, T2 vDNA estimates based upon viral counts were far below measured values, both for lysates and $CsCl_2$-purified phage preparations. Therefore, the T2 vDNA preparations behaved more like environmental dissolved DNA samples than cultivated marine phage. The reason for this is not known. We speculate that some form of bound DNA may be present in T2 lysates as well as in natural retentates.

Concerned that we may have overestimated the DNA content by our Hoechst 33258 method or underestimated viral direct counts by our VFF-transmission electron microscopy (TEM) technique, we compared our DNA quantitation results with those obtained by three other methods and we also enumerated viral populations by the technique of ultracentrifugation onto grids (2, 32) (Table 2). Because the diphenylamine and A_{260} techniques for DNA quantitation require relatively pure DNA, we used T2 phage DNA from cesium-banded preparations. The results obtained for the various methods of DNA quantitation were all relatively close to each other (no significant difference; $P < 0.2$). We expected some difference because each method detects DNA on a different principle: UV absorbance is based on purine and pyrimidine absorbance, diphenylamine reacts with deoxyribose, ethidium bromide intercalates itself between base pairs, and Hoechst 33258 is believed to interact with the major groove of AT-rich sequences of DNA. Thus, differences between standards and target DNA in any one of these properties would result in differences in apparent concentration, but this was not found. Therefore, we do not feel that the Hoechst method was overestimating DNA concentration.

We have previously shown that the VFF technique for con-

TABLE 1. Effect of ethanol precipitation on DNA measurements of VFF-concentrated dissolved DNA and viral lysates

Sample and/or location and date (mo/day/yr)	Amt of DNA (μg/liter)		After/before ratio	Viral direct count (10^9/liter)	Estimated vDNA (μg/liter)[c]	vDNA/total (%)	Source or reference
	Before[a]	After[b]					
Dissolved DNA							
Medard reservoir	4.2 ± 0.4	12.1 ± 1.2	2.9	4.9 ± 2.5	0.44	3.6	28
Tampa Bay							
Bayboro Harbor	3.1 ± 0.17	5.5 ± 1.1	1.8	4.2 ± 0.7	0.378	6.9	28
St. Petersburg pier	ND[d]	4.4	ND	6.3 ± 2.01	0.567	12.9	This study
Fort Desoto, 5/28/91	2.1 ± 0	11.7 ± 0.14	5.6	9.8 ± 1.4	0.882	6.6	This study
Fort Desoto, 7/25/91	1.25 ± 0.08	9.64 ± 0	7.7	23.8 ± 8.0	2.1	21.8	This study
Fort Desoto, 10/2/91	0.94 ± 0.04	7.39 ± 0.83	7.8	13.8 ± 2.1	1.2	16.3	This study
Mouth of Tampa Bay	5.0 ± 1.5	11.1 ± 1.0	2.2	1.8 ± 0.34	0.162	1.46	28
Miami sea buoy	2.6 ± 1.3	7.5 ± 1.1	2.9	0.74 ± 0.2	0.066	0.88	28
Bahamas, Chub Cay	0.9 ± 0.67	1.82 ± 0.93	2.0	0.4	0.036	2.0	28
Gulf of Mexico							
Station 3a	2.0	4.4	2.2	1.61 ± 0.4	0.144	3.3	This study
Station 7	0.83	2.0	2.4	0.49 ± 0.2	0.044	2.2	This study
Viruses and DNA							
ϕ13 lysate	410	670	1.6	ND	ND	ND	This study
ϕ13 lysate, $CsCl_2$ band	11,500	15,800	1.4	280,000	25,200	159	This study
ϕ16 lysate, $CsCl_2$ band	32,700	134,000	4.1	3,250,000	175,000	130	This study
T2 lysate							
1	5,600 ± 60	12,500 ± 600	2.2	7,170	1,150	9.2	This study
2	5,210 ± 120	12,200 ± 300	2.3	8,000	1,280	10.5	This study
3	3,150 ± 20	9,540 ± 190	3.0	3,350	5,650	5.92	This study
$CsCl_2$ band	47,100	94,200	2.0	100,000	16,000	17	This study
Calf thymus DNA	24,000	18,900	0.78	ND	ND	ND	This study

[a] Before denotes before ethanol precipitation.
[b] After denotes after ethanol precipitation.
[c] vDNA content was estimated by using an average phage genome weight of 9×10^{-17} g per phage particle (12), except for ϕ16, whose genome weight was estimated to be 5.58×10^{-17} g by restriction analysis, and T2 phage, whose genome weight was 1.6×10^{-16} g per phage particle (12).
[d] ND, not determined.

centrating viral particles was efficient and that TEM enumeration of T2 viral lysates gave values similar to those obtained with plaque assays (28). However, T2 is a large phage and the smaller indigenous phage might not be efficiently concentrated and enumerated. Table 2 shows the results of viral counts performed by ultracentrifugation onto grids and VFF concentration followed by TEM. There was no significant difference between viral direct counts obtained by the two methods. At low viral concentrations, as found in oligotrophic waters of the Gulf of Mexico, we could not enumerate viral particles using the ultracentrifugation technique because the viral concentration was below this method's limit of detection, at least in our hands. These samples resulted in far too many TEM fields with no viral particles present. Thus, an advantage of the VFF technique is the ability to concentrate large volumes of water when low viral abundance is expected, resulting in accurate enumeration in dilute samples. These studies indicate that we were not underestimating viral abundance.

Confident in both our DNA determination and viral count technologies, we attempted to separate sDNA from vDNA in dissolved-DNA samples by ultracentrifugation. Beebee (1) reported successful separation of sDNA from vDNA by ultracentrifugation at 140,000 × *g* for 4 h, with the viral fraction being collected in the pellet and the sDNA remaining in the supernatant. He concluded that most of the dissolved DNA was viral, with only a small portion, ≤500 bp in size, being soluble. Table 3 shows the results of ultracentrifugation of calf thymus DNA and T2 phage. The 201,000 × *g* treatment, similar to that used by Beebee (1), resulted in only 6% of the calf thymus DNA, our model sDNA, remaining in the supernatant, with 60% being collected in the pellet and 33% being unaccounted for. We believe that the high-speed *g* force sheared or denatured this missing one-third of the DNA. The lowest speed used, 25,800 × *g*, resulted in 65% of the DNA remaining in the supernatant and 23.5% still being collected in the pellet. The assumption that Beebee made about separation of DNA by ultracentrifugation at high speeds did not hold true for sDNA even at low speeds, which resulted in appreciable levels of sDNA being collected in the pellet. Centrifugation at forces as low as 25,800 × *g* resulted in essentially all the phages being pelleted, with no appreciable levels of phage in the supernatant.

On the basis of these observations, it seemed reasonable that all the virus particles could be efficiently collected in the pellet fraction from a natural water sample at a relatively low speed, with a majority of the sDNA remaining in the supernatant. Table 3 shows our application of these principles to VFF-concentrated retentates of seawater samples. For the Fort Desoto sample, 30.7% of the dissolved DNA was in the supernatant of the lowest-speed fraction and all the viruses were in the pellet. There was no significant difference in the viral direct counts of any of the pellet fractions, irrespective of the *g* force employed. Increasing the *g* force resulted in increasing amounts of dissolved DNA being collected in the pellet

TABLE 2. Comparison of methods to measure vDNA and viral direct counts

Measurement and expt no. or sampling location	Method	DNA content (μg/ml)	Viral counts (10^6/ml)	% of value obtained by Hoechst 33258 method	Confidence interval
DNA measurements with T2 phage DNA					
Expt 1	H33258[a]	522 ± 125	ND[b]		
	A_{260}[c]	422	ND	81	
	EtBr[d]	535 ± 86	ND	102	$P < 0.2$
	Diphenyl[e]	348 ± 23	ND	67	
Expt 2	H33258	282 ± 58	ND		$P < 0.2$
	A_{260}	223 ± 7	ND	79	
Viral direct counts					
Tampa Bay, Bayboro Harbor	VFF	ND	19.7 ± 4.4		
	UC[f]	ND	24.3 ± 12.5	123	$P < 0.5$
Mouth of Tampa Bay	VFF	ND	13.7 ± 2.17		
	UC	ND	10.7 ± 3.7	87.1	$0.2 < P < 0.5$
Charlotte Harbor	VFF	ND	18.1 ± 6.9		
	UC	ND	9.7 ± 3.6	53.6	$0.1 < P < 0.2$
Gulf of Mexico					
Station 2A	VFF	ND	0.099 ± 0.05		
	UC	ND	BDL[g]	ND	
Station 5A	VFF	ND	0.047 ± 0.013		
	UC	ND	BDL	ND	
Station 5D	VFF	ND	0.36 ± 0.21		
	UC	ND	BDL		

[a] H33258, Hoechst 33258 fluorometric method (29).
[b] ND, not determined.
[c] A_{260}, A_{260} spectrophotometric method.
[d] EtBr, ethidium bromide fluorometric method (34).
[e] Diphenyl, diphenylamine method of Burton (4).
[f] UC, ultracentrifugation method for collecting viruses (2).
[g] BDL, below detection limit.

fraction, which we interpret as the higher-molecular-weight sDNA. If the fractionation observed in the calf thymus DNA experiments can be assumed also to occur with the VFF retentates (that is, 65% of the sDNA remaining in the supernatant), then another 35% of the true sDNA might be expected to be in the low-speed pellet fraction. Thus, expressed as a percentage of the total dissolved DNA, the amount of sDNA is 30.7/0.65, or 47.2%. Subtracting the amount in the supernatant (47.2 − 30.7) leaves an additional 16.5% of the dissolved DNA that should be soluble in the pellet. Fifty-two percent of the total dissolved DNA would be bound and/or viral in the pellet, 16.5% of the total would be sDNA in the pellet fraction, and 30.7% would be soluble in the supernatant fraction. Thus, a total of 47.2% would be soluble and

TABLE 3. Effect of differential centrifugation on calf thymus DNA, T2 phage, and dissolved DNA samples

Sample and treatment (*g* force employed)	% in:		% Unaccounted for	Viral counts in pellet (10^6/ml)
	Pellet	Supernatant		
Calf thymus DNA				
25,800 × *g*	23.5	65.0	11.5	
37,000 × *g*	26.9	45.2	27.9	
103,000 × *g*	62.4	24.9	12.7	
201,000 × *g*	60.6	6.1	33.7	
T2 phage lysate[a]				
25,800 × *g*	>99	<0.1	0	1.7×10^4
201,000 × *g*				
Run 1	>99	0.77	0	2.04×10^5
Run 2	82.1	1.3	16.6	4.3×10^3
VFF-concentrated dissolved DNA				
Fort DeSoto				
11,200 × *g*	69.2	30.7	0	19.4 ± 8.7
16,500 × *g*	89.7	10.3	0	16.9 ± 6.6
201,000 × *g*	94.8	5.2	0	23.8 ± 3.9
Gulf of Mexico, Station 3				
11,200 × *g*	42.4	57.6	0	1.05 ± 0.2
201,000 × *g*	50.8	49.2	0	1.61 ± 0.44

[a] Results in each row come from a different experiment.

TABLE 4. Effect of ethanol precipitation on samples that were fractionated by differential centrifugation

Sample and treatment	Total DNA (μg/sample)	Amt DNase sensitive (μg/sample)	% DNase sensitive	Viral direct counts (10^6/sample)	Estimated vDNA (μg/sample)	% of vDNA DNase sensitive
Calf thymus DNA						
201,000 × *g* pellet	24	23.4	97.5			
201,000 × *g* pellet, EtOH ppt.[a]	18.9	18.4	97.3			
T2 phage lysate						
201,000 × *g* pellet	37.4	12.4	33	4,300	0.68	5.4
201,000 × *g* pellet, EtOH ppt.	139.0	121.6	87.4	4,300	0.68	0.56
VFF-concentrated dissolved DNA						
Gulf of Mexico, station 7						
11,200 × *g* supernatant	0.27	0.17	63	ND[b]	ND	ND
11,200 × *g* supernatant, EtOH ppt.	0.17	0.14	82.3	ND	ND	ND
11,200 × *g* pellet	0.27	0.13	48.0	0.97	0.087	66.9
11,200 × *g* pellet, EtOH ppt.	0.45	0.42	93.0	0.97	0.087	20.7
201,000 × *g* pellet	0.21	0.045	21.0	0.49	0.044	97.0
201,000 × *g* pellet, EtOH ppt.	0.625	0.57	91.2	0.49	0.044	7.7
Gulf of Mexico, station 3						
11,200 × *g* supernatant	0.56	0.38	67.9	ND	ND	ND
11,200 × *g* supernatant, EtOH ppt.	0.42	0.38	90.5	ND	ND	ND
11,200 × *g* pellet	0.6	0.28	46.6	1.05	0.095	33.9
11,200 × *g* pellet, EtOH ppt.	0.89	0.85	95.5	1.05	0.095	11.1
201,000 × *g* pellet	0.49	0.32	65.3	1.61	0.145	45.3
201,000 × *g* pellet, EtOH ppt.	0.58	0.54	93.1	1.61	0.145	26.9

[a] EtOH ppt., ethanol precipitated.
[b] ND, not determined.

52.7% would be bound. The pellet fraction contained 1.17 μg of DNA and 1.94×10^7 phages. In terms of vDNA, this corresponds to 1 ng of DNA, or well below the total amount of bound DNA.

For samples from the offshore Gulf of Mexico, about one-half of the DNA remained in the supernatant at low- or high-*g*-force centrifugation. The viral counts in the pellet increased from 1.05×10^6 to 1.61×10^6 (a 35% increase), with only an 8% increase in the amount of DNA in the pellet. This further argues for the insignificance of vDNA in dissolved-DNA measurements.

Because ethanol precipitation increased the amount of Hoechst DNA fluorescence in both viral lysates and VFF-concentrated dissolved-DNA samples, we examined the effect of ethanol precipitation on samples that had been treated by differential centrifugation (Table 4). As a model sDNA, we again used calf thymus DNA, and as a model phage particle, we used T2 phage. We examined and compared the effects of ethanol precipitation on the DNase digestibility of the DNA (an indication of encapsulation or binding) by using materials that had been pelleted by ultracentrifugation. For calf thymus DNA, ethanol precipitation had little effect, except that some DNA was lost, probably during collection by centrifugation in the ethanol precipitation step. The DNA was completely digestible with DNase prior to or after ethanol precipitation. This was not the case with the T2 phage lysate, which was only 33% digestible prior to ethanol precipitation and 87% digestible afterwards. Ethanol precipitation caused an increase in measurable DNA signal, from 37.4 to 139 μg (a 3.7-fold increase). Although it is tempting to conclude that this was DNA released from viral particles, such a hypothesis was not supported by estimation of vDNA by viral direct counts. Less than 1% of the ethanol-precipitable DNA could be due to the amount of viruses present. This suggests (as indicated in Table 1) that there is a form of encapsulated, membrane-enclosed, or simply bound DNA in these preparations.

The results of similar treatments of VFF-concentrated retentates of natural waters also appear in Table 4. The low-speed supernatant fraction behaved very much like calf thymus DNA, yielding no increase in fluorescence upon ethanol precipitation and possessing a high degree of DNase digestibility prior to and after precipitation. The low- and high-speed pellet fractions behaved like the T2 viral lysate, with a low degree of DNase digestibility prior to ethanol precipitation, an increase in Hoechst DNA fluorescence after ethanol precipitation, and near-complete DNase sensitivity after precipitation. The calculated amount of vDNA in these pellets approached the measured amount of DNA, particularly prior to ethanol precipitation (averaging 61%). One interpretation of these data is that the increase in Hoechst fluorescence of DNA has nothing to do with viral particles, but rather reflects the presence of membrane-bound DNA. Although this seems to be the case with the T2 phage lysates, we are reluctant to accept this hypothesis because of the apparent agreement between amounts of measured and calculated DNA for the marine phages, ϕ16 and ϕ13 (Table 1). The ethanol-precipitated pellet fraction DNA of the marine samples listed in Table 4 averaged 16.6% of the calculated vDNA, which is significantly greater than the value for those samples not fractionated by ultracentrifugation (7.1%) (Table 1). Thus, it seems that the low-speed ultracentrifugation effectively removed some of the sDNA from the viral fraction but that other components (behaving like particulates) still

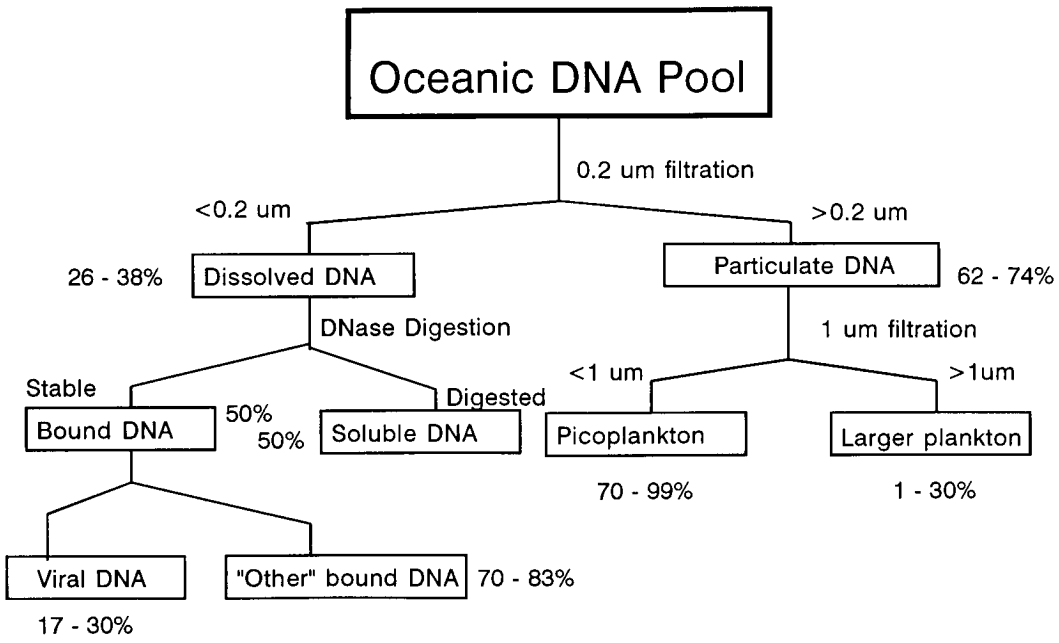

FIG. 1. Conceptual model of the properties of the oceanic DNA pool. Percentages are additive at each horizontal level.

contained greater amounts of DNA than did the viral particles in the pellet.

For Gulf of Mexico station 7, if we assume that 65% of the dissolved DNA is in the supernatant and another 35% is in the pellet (as for calf thymus DNA [Table 3]), then the total amount of sDNA is 0.17/0.65 μg, 0.262 μg. Subtracting the DNase-sensitive amount in the supernatant (0.262 − 0.17) gives 0.092 μg, or the amount of sDNA in the 11,200 × *g* pellet (DNase sensitive). A direct measure may be the amount of DNase-digestible DNA in the pellet prior to ethanol precipitation, which is 0.13 μg. Thus, the total amount of sDNA, calculated on the basis of the DNase digestibility of the sample, is 0.17 (in the supernatant) + 0.13 μg, or 0.3 μg. The amount of nonsoluble or bound DNA in the 11,200 × *g* pellet is the amount of ethanol-precipitable, DNase-sensitive DNA, 0.42 − 0.13 μg or 0.29 μg. The entire amount of dissolved DNA is then the 0.3 μg of sDNA (50.8%) plus the 0.29 μg of bound DNA (49.2%), a total 0.59 μg. Note that this value is close to that for the 201,000 × *g* pellet after ethanol precipitation (0.57 μg), which we interpret as the maximum of all forms of dissolved DNA (viral, soluble, and bound). Of the 0.29 μg of bound DNA, the calculated amount of vDNA (0.087 μg) represents 30%.

Similar calculations can be made for station 3 by using the low-speed centrifugation data. The true sDNA was the 0.38 μg in the 11,200 × *g* supernatant plus the DNase-digestible 0.28 μg in the low-speed pellet, a total of 0.66 μg. The total amount of dissolved DNA is the sum of the amounts of sDNA in the supernatant (0.38 μg) and the ethanol-precipitable, DNase-sensitive DNA in the pellet (0.85 μg), or 1.23 μg. From this, the amount of sDNA is 0.66/1.23, or 53.6% of the total, and the amount of bound DNA is 0.57/1.23, or 46.4%. Note that in this instance, the total is much larger than for the 201,000 × *g* pellet after ethanol precipitation. This may be because the high speeds could have resulted in significant destruction of DNA in these samples, or at least denaturation, such that detection by Hoechst 33258 fluorescence becomes impossible. Of the 0.57 μg of bound DNA, the calculated amount of vDNA (0.095 μg) is 16.7%.

These calculations are put in the context of the total DNA pool (particulate and dissolved) in Fig. 1. This figure synthesizes data presented by DeFlaun et al. (8), Boehme et al. (3), and Paul et al. (27) and is based on data for oceanic environments. The total DNA pool is composed of the dissolved and particulate fractions, with the amount of particulate DNA being approximately twice that of the dissolved DNA. A combination of DNase digestion and low-speed ultracentrifugation indicates that roughly one-half of the dissolved DNA is soluble (or free DNA), while the other half is bound (viruses plus an as-yet-uncharacterized bound form). The bound form can be further divided into vDNA (DNA in viral particles) on the basis of viral direct counts and estimation of vDNA content. vDNA therefore constitutes 17 to 30% of this bound form.

Wells and Goldberg (38–40) have reported the presence of large amounts of marine colloids ($>10^7$/ml) in seawater. On the basis of morphology, many of these are distinct from viruses. We do not know if these colloids contain DNA. We have observed amorphous, bleb-like materials by TEM in both T2 phage lysates and VFF retentates of natural populations (16). Dorwood et al. (9–11) have described DNA-containing membrane blebs produced by bacteria. Similar structures are believed to participate in gene transfer by natural transformation ("transformasomes" [5, 30]). If such DNA-containing particles are abundant in the marine environment, they could explain the discrepancies between the large amount of dissolved-DNA signals and the small amount of estimated vDNA. These colloids could be the third component of dissolved DNA, in addition to sDNA and viral particles. We have not yet devised a method to separate these particles from viral particles; however, their existence could explain the overabundance of bound forms of dissolved DNA in the oceans.

Kingdom probing of dissolved DNA. The rationale for kingdom probing is that viruses lack ribosomes and therefore should lack genes encoding the rRNAs, whereas all other forms of life contain ribosomes. Thus, the level of hybridization per quantity of dissolved DNA may be a means of estimating viral contribution to dissolved DNA. That is, no hybridization should indicate a potential large viral contribution,

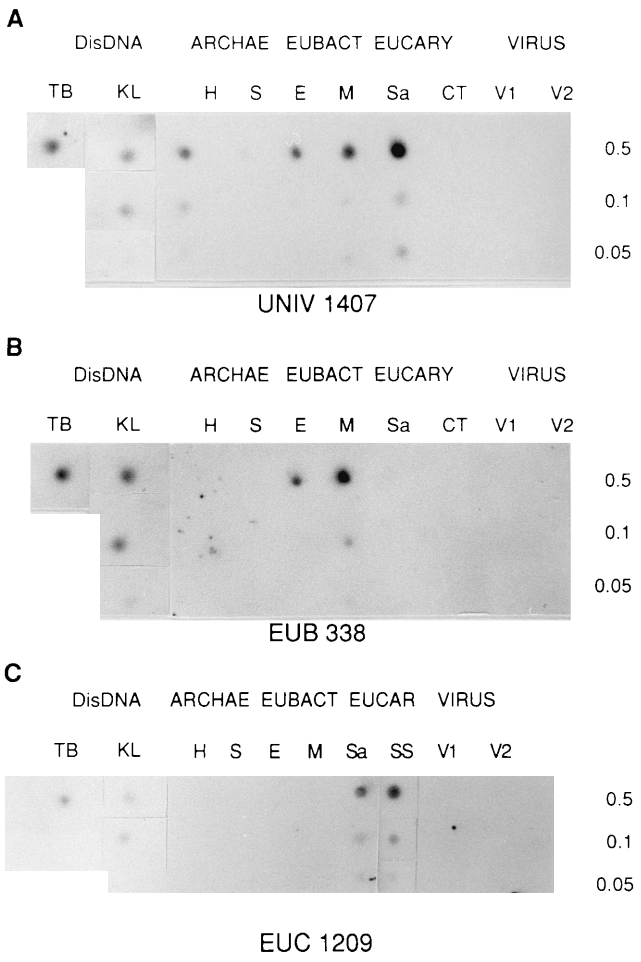

FIG. 2. Kingdom probing of dissolved-DNA (DisDNA) samples and DNA standards by using the universal (UNIV) 1407 (A), eubacterial (EUB) 338 (B), and eucaryotic (EUC) 1209 (C) oligonucleotide probes. Equal amounts of DNA standard or DisDNA were dotted in each row (amounts in micrograms are given to the right of the rows). DisDNA samples were from Fort Desoto, Tampa Bay (lane labelled TB; 3.68 μg obtained from 10 liters of seawater), and the NURC facility in Key Largo (lane labelled KL; 9.8 μg from 10.5 liters of seawater). Standards include the archaebacteria (ARCHAE) *H. volcanii* (lane H) and *S. sulfotaricus* (lane S), the eubacteria (EUBACT) *E. coli* (lane E) and marine bacterium host 16 (lane M), and the eucaryotes (EUCARY) *S. cerevisiae* (lane Sa) and calf thymus DNA (lane CT). Viral standards (VIRUS) include T2 phage (lane V1) and ϕ16 (lane V2).

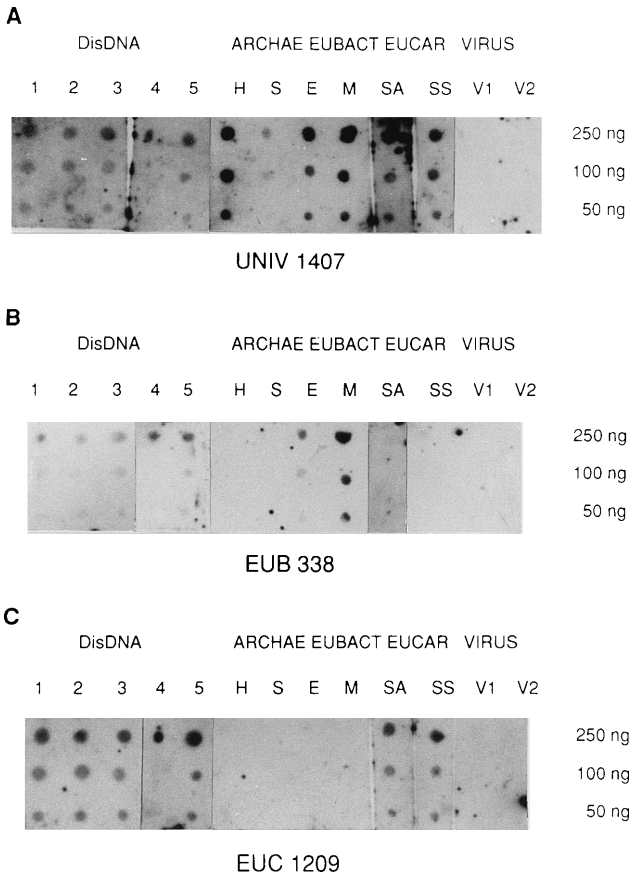

FIG. 3. Kingdom probing of dissolved-DNA (DisDNA) samples and standard DNA. Panels and abbreviations are as described for Fig. 1. DisDNA samples are from the Dry Tortugas (lane 1, 3.8 μg of DNA from 8.8 liters of seawater, ethanol precipitated; lane 2, 4.4 μg of concentrated DNA from 80 liters of seawater, obtained by Amicon ultrafiltration), from Key West Harbor (lane 3, 7.2 μg from 9 liters, ethanol precipitated), and from the NURC dock in Key Largo (lane 4, top row only, ethanol precipitated; lane 5, 20 liters, concentrated by VFF). Standards are as described for Fig. 1, except that salmon sperm DNA (lane SS) replaces the calf thymus DNA and lane V2 contains ϕ13 DNA.

whereas a high level of hybridization should indicate DNA from higher forms of life, as opposed to viruses. Figures 2, 3, and 4 show the results of kingdom probing of standard DNAs and dissolved DNA samples by using untailed (Fig. 2 and 3) and tailed (Fig. 4) digoxigenin-labelled probes and chemiluminescent detection. Preliminary studies were performed with DNA standards only to determine the stringency of hybridization and washing. Preliminary studies using archaebacterial probes 316 and 915 indicated a significant hybridization with vDNAs. Therefore, we chose not to use these probes for probing dissolved-DNA samples.

In our initial experiments, universal probe 1407 hybridized with all standards except the two vDNAs (T2 and ϕ16) and calf thymus DNA and only weakly hybridized with the archaebacterial DNA from *S. sulfotaricus*. The reason for the lack of binding with calf thymus DNA is not known, and in later blots (Fig. 3 and 4), we replaced calf thymus DNA with salmon sperm DNA. The dissolved-DNA samples from Fort Desoto and the NURC dock in Key Largo also hybridized with the universal probe. In a second series of blots using universal probe 1407, the weakest hybridization was obtained with *S. sulfotaricus*. The vDNAs did not hybridize, whereas all dissolved-DNA samples did; although they hybridized more weakly than the eubacterial standards, they hybridized more strongly than *S. sulfotaricus*. The dissolved-DNA samples were from Fort Jefferson in the Dry Tortugas, Key West Harbor, and the NURC dock in Key Largo; the last two are eutrophic environments, and the former is an oligotrophic to mesotrophic environment.

In probing with the tailed oligonucleotide probes, a slight hybridization was obtained for the dissolved-DNA samples with the universal probe (Fig. 4). However, the vDNA standards also hybridized slightly with the universal probe. Additionally, the eubacterial probe also hybridized slightly with the eucaryotic standards. We hypothesize that the tailing process may have resulted in a decrease in the specificity of these probes, as alluded to by the manufacturer of the kits. The dissolved-DNA samples also hybridized with the eubacterial probe and the eucaryotic probes.

To summarize, in two of three experiments, the kingdom

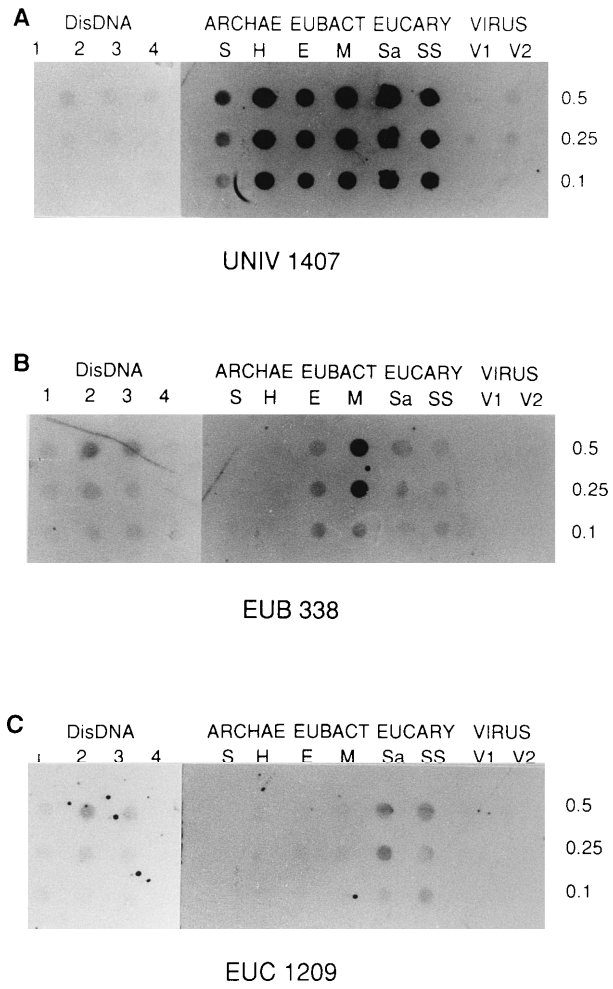

FIG. 4. Kingdom probing of dissolved-DNA (DisDNA) samples and standards by using tailed oligonucleotide probes. DisDNA samples include those collected from the mouth of Tampa Bay in September 1993 (lane 1, 15.2 μg of DNA from 20 liters of seawater, concentrated by VFF) and June 1993 (lane 2, 8.4 μg from 20 liters, concentrated by VFF), from station 6 in the Gulf of Mexico (lane 3, 9.7 μg from 20 liters, concentrated by VFF), and from outside the mouth of Charlotte Harbor (lane 4, 17.3 μg from 9.4 liters, ethanol precipitated). Abbreviations and standard DNAs are as described for Fig. 2, except that lane V2 contains the vibriophage KL6 (18).

probing results indicated significant hybridization of dissolved DNA with the universal, eubacterial, and eucaryotic probes, while the vDNA standards did not hybridize. In only one set of experiments did the standard vDNAs hybridize with any kingdom probes. We interpret these results to mean that the majority of the dissolved DNA in the diversity of samples examined was more like eubacterial and eucaryotic DNA than phage DNA. This is in agreement with the circumstantial evidence provided by differential centrifugation and calculated vDNA values, which both indicate that DNA in viral particles is a small portion of the dissolved DNA.

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