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Isolation of viruses infecting photosynthetic and nonphotosynthetic protists

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Abstract

Viruses are the most abundant biological entities in aquatic environments and our understanding of their ecological significance has increased tremendously since the first discovery of their high abundance in natural waters. About 40 viruses infecting eukaryotic algae and 4 viruses infecting nonphotosynthetic protists have so far been isolated and characterized to different extents. The isolated viruses infecting phytoplankton (Chlorophyceae, Prasinophyceae, Haptophyceae, Dinophyceae, Pelagophyceae, Raphidophyceae, and Bacillariophyceae) and heterotrophic protists (Bicosoecophyceae, Acanthamoebidae, and Thraustochytriaceae) are all lytic. Some of the brown algal phaeoviruses, which infect host spores or gametes, have also been found in a latent form (lysogeny) in vegetative cells. Viruses infecting eukaryotic photosynthetic and nonphotosynthetic protists are highly diverse both in size (ca. 20–220 nm in diameter), genome type (double-strand deoxyribonucleic acid [dsDNA], single-strand [ss]DNA, ds–ribonucleic acid [dsRNA], ssRNA), and genome size [4.4–560 kb]). Availability of host–virus laboratory cultures is a necessary prerequisite for characterization of the viruses and for investigation of host–virus interactions. In this report we summarize and comment on the techniques used for preparation of host cultures and for screening, cloning, culturing, and maintaining viruses in the laboratory.

Introduction

Table 1 shows a list of viruses infecting eukaryotic algae and nonphotosynthetic protists that have been characterized using typical clonal virus isolates. So far, viruses infectious to Chlorophyceae, Chrysophyceae, Pelagophyceae, Prasinophyceae, Haptophyceae, Dinophyceae, Raphidophyceae, Bacillariophyceae, Bicosoecophyceae (Mastigophorea), Acanthamoebidae, and Thraustochytriaceae have been isolated.

Research on algal and protistan viruses has now passed a first stage of development and each year a number of published reports disclosing their roles in natural environments, their host relationships, their molecular and biological characteristics, and other information, including genome informatics and nanostructures, are published. Many of these studies, which are often advanced, have been possible only thanks to successful isolation of novel viruses (Table 1). Virus isolation itself is not at all a very advanced activity, but its success is often critically dependent on the researchers' skill and experience. A number of different approaches and strategies have been used in various laboratories. All of these techniques are useful (i.e., they have led to successful isolation of a virus), but because no comparative studies are available it is impossible to state that one method is better or more efficient than the other. Nevertheless, summarizing the methods described, as well as additional information, notes, and observations that are scattered in the literature but relevant for a rational and successful virus isolation protocol, may be instructive for all workers in the field. A typical isolation procedure is shown in Fig. 1. Appropriate modifications must be made to meet

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Table 1. Viruses infecting eukaryotic algae and the methods used for their isolation.

Virus	Host (class)	Size, nm	Genome	Source
Viruses infecting unicellular algae				
AaV (BtV)	*Aureococcus anophagefferens* (Pelagophyceae)	140	dsDNA	Water sample Water sample
CbV	*Chrysochromulina brevifilum* (Haptophyceae)	145–170	dsDNA	Water sample
CdebDNAV	*Chaetoceros debilis* (Bacillariophyceae)	30	ssDNA, fragmented?	Water sample
CeV	*Chrysochromlina ericina* (Haptophyceae)	160	dsDNA, 510 kbp	
Chlorella virus (e.g., ATCV-1, ATCV-2)	*Chlorella* SAG 3.83 (Chlorophyceae) (symbiont of *Acanthocystis turfacea*)	140–190	dsDNA, 288 kbp	Water sample
Chlorella virus (e.g., PBCV-1, NY-2A, AR158)	*Chlorella* NC64A (Chlorophyceae) (symbiont of *Paramecium bursaria*)	150–190	dsDNA, 331–369 kbp	Water sample
Chlorella virus (e.g., MT325, FR483)	*Chlorella* Pbi (Chlorophyceae) (symbiont of *Paramecium bursaria*)	140–150	dsDNA, 314–321 kbp	
CsNIV	*Chaetoceros salsugineum* (Bacillariophyceae)	38	(ss+ds)DNA, 6.0 kb	Sediment sample
CspNIV	*Chaetoceros* cf. *gracilis* (Bacillariophyceae)	25	—	Water sample
CsfrRNAV	*Chaetoceros socialis* f. *radians* (Bacillariophyceae)	22	ssRNA, 9.5 kb	Water sample
CtenRNAV	*Chaetoceros tenuissimus* (Bacillariophyceae)	31	ssRNA, 8.9 and 4.3 kb	Water sample
CwNIV	*Chaetoceros* cf. *wighamii* (Bacillariophyceae)	22–28	—	Water sample
EhV	*Emiliania huxleyi* (Haptophyceae)	170–200	dsDNA, 410–415 kbp	Water sample,† mesocosm with host bloom
HaNIV	*Heterosigma akashiwo* (Raphidophyceae)	30	—	Water sample, integrated from 0–75-m depth
HaV	*Heterosigma akashiwo* (Raphidophyceae)	202	dsDNA, 294 kbp	Water sample, host bloom†
HaRNAV	*Heterosigma akashiwo* (Raphidophyceae)	25	ssRNA, 9.1 kb	Water sample, Chl maximum
HcRNAV	*Heterocapsa circularisquama* (Dinophyceae)	30	ssRNA, 4.4 kb	Water sample
HcV	*Heterocapsa circularisquama* (Dinophyceae)	197	dsDNA, 356 kbp	Water sample
MpRV	*Micromonas pusilla* (Prasinophyceae)	50–60	dsRNA, 24.6 kbp in total (segmented)	Lysed host culture
MpV	*Micromonas pusilla* (Prasinophyceae)	115	dsDNA, 200 kbp	Water sample
MpVN1	*Micromonas pusilla* (Prasinophyceae)	110–130	(ds?)DNA	Water sample
MpVN2	*Micromonas pusilla* (Prasinophyceae)	110–130	(ds?)DNA	Water sample
OIs1	*Heterosigma akashiwo* (Raphidophyceae)	30 and 80	dsDNA, 20 and 130 kbp, respectively for 30 and 80 nm	
PgV-102P	*Phaeocystis globosa* (Haptophyceae)	98	dsDNA, 176 kbp	Water sample
PgV Group I	*Phaeocystis globosa* (Haptophyceae)	150	dsDNA, 466 kbp	Water sample
PgV Group II	*Phaeocystis globosa* (Haptophyceae)	100	dsDNA, 177 kbp	Water sample
PoV	*Pyramimonas orientalis* (Prasinophyceae)	180–220	dsDNA, 560 kbp	
PpV	*Phaeocystis pouchetii* (Haptophyceae)	130–160	dsDNA, 485 kbp	Water sample, host bloom†
RsRNAV	*Rhizosolenia setigera* (Bacillariophyceae)	32	ssRNA, 11.2 kb	Water sample, nonhost bloom
TampV	*Teleaulax amphioxeia* (Cryptophyceae)	203	—	Water sample, nonhost bloom
Viruses infecting multicellular algae				
EsV	*Ectocarpus siliculosus*(Phaeophyceae)	130–150	dsDNA, 336 kbp	Plants showing symptoms of infection
EfasV	*Ectocarpus fasciculatus* (Phaeophyceae)	135–140	dsDNA, 340 kbp	Plants showing symptoms of infection
FlexV	*Feldmannia simplex* (Phaeophyceae)	120–150	dsDNA, 170 kbp	Plants showing symptoms of infection
FirrV	*Feldmannia irresguralis* (Phaeophyceae)	140–170	dsDNA, 180 kbp	Plants showing symptoms of infection
FsV	*Feldmannia* species (Phaeophyceae)	150	dsDNA, 158 and 178 kbp	Plants showing symptoms of infection
HincV	*Hincksia hinckiae* (Phaeophyceae)	140–170	dsDNA, 220 kbp	Plants showing symptoms of infection
MclaV	*Myriotrichia clavaeformis* (Phaeophyceae)	170–180	dsDNA, 340 kbp	Plants showing symptoms of infection
PlitV	*Pilayella littoralis* (Phaeophyceae)	161	dsDNA, 280 kbp	Plants showing symptoms of infection
Viruses infecting marine heterotrophic protists				
CroV (BV-PW1)	*Cafeteria roenbergensis* (Bicosoecophyceae/ Mastigophorea) (reported as *Bodo* sp.)	230–300	dsDNA, 730 kbp	Water sample
Mimivirus	*Acanthamoeba polyphaga* (Acanthamoebidae)	750	dsDNA, 1.2 Mb	Viral lysate
SssRNAV	*Aurantiochytrium* sp. NIBH N1-27 (Thraustochytriaceae) (reported as *Schizochytrium* sp. NIBH N1-27)	25	ssRNA, 10.2 kb	Water sample
SmDNAV	*Sicyoidochytrium minutum* (Thraustochytriaceae)	140	dsDNA, 250 kbp	Water sample

*PC, polycarbonate membrane filters; GF, Glassfiber filter; PVD, polyvinylidene difluoride filters (Durapore, Millipore); kbp, kilobase pair; kb, kilobase; NC, nitrocellulose membrane; CA, cellulose acetate filters (Schleicher & Schuell); HT, HT Tuffryn low-protein-binding polysulphonate membrane.
†UV treatment applied but not essential.

Virus sample filtration, pore size and type*	Virus concentration and inoculation	Lysate filtration	Principal references, unpublished data, or personal communication
0.2-µm PC	Ultracentrifugation 105,000*g*, 3 h	0.2-µm filter	Garry et al. 1998; Gastrich et al. 2004
0.2-µm polypropylene filter capsules (MSI)	Ultrafiltration 30,000 MW cutoff	0.2-µm; GV low-protein binding filter from Millipore	Gobler et al. 2004, 2007; Rowe et al. 2008
1.2-µm GF and 0.22 or 0.45 µm PVD	Ultrafiltration 30,000 MW cutoff	0.8-µm GF and 0.22-µm PVD	Suttle and Chan 1995
0.2-µm PC	Non, 20% v/v	0.1-µm PC	Tomaru et al. 2008
0.45-µm Supor filters (Gelman)	Plankton concentrated by continuous flow centrifugation	0.2-µm Meditron syringe filters (Schleicher & Schuell)	Sandaa et al. 2001; Thyrhaug et al. 2003; Monier et al. 2008
0.45-µm NC	Plaque assay after 2-week incubation in liquid culture		Bubeck and Pfitzner 2005; Fitzgerald et al. 2007c
0.4-µm PC		0.4-µm PC	Van Etten et al. 1983, 1991, 2002; Van Etten and Meints 1999; Yamada et al. 1999, 2006; Fitzgerald et al. 2007b Reisser et al. 1988a,b; Van Etten et al. 1991; Yamada et al. 2006; Fitzgerald et al. 2007a
0.7-µm GF and 0.2-µm Dismic-25cs filters (Advantec)	Non, 20% v/v	0.1-µm PC	Nagasaki et al. 2005c
0.22-µm Tangential-flow membrane	Ultrafiltration 30,000 MW cutoff		Bettarel et al. 2005
0.2-µm (DISMIC-25, Advantec)	Non, 33% v/v	0.1-µm PC	Tomaru et al. 2009
0.2-µm PC	Non, 20% v/v	0.1-µm PC	Shirai et al. 2008
	Ultrafiltration 30 000 MW cutoff	0.22-µm	Eissler et al. 2009
	Non, 10% v/v	0.2-µm Syringe filter (FP30/0.2CA-S [Schleicher & Schuell])	Castberg et al. 2002; Wilson et al. 2002, 2005; Schroeder et al. 2003; Thyrhaug et al. 2003; Allen et al. 2006
1.2-µm GF and 0.4-µm PC cartridge filter	Ultrafiltration 30,000 MW cutoff	0.22-µm filter	Lawrence et al. 2001
0.2-µm PC	Non, 4% v/v	0.2-µm (DISMIC-25, Advantec)	Nagasaki and Yamaguchi 1997; Nagasaki et al. 1999, 2005b; Tarutani et al. 2000; Tomaru et al. 2004b
1.2-µm GF and 0.2-µm PC cartridge filter	Ultrafiltration 30,000 MW cutoff	0.22-µm GV Durapore filter (Millipore)	Tai et al. 2003; Lang et al. 2004
0.8-µm PC	Non, 40% v/v	0.1-µm PC	Tomaru et al. 2004a; Nagasaki et al. 2004a, 2005a, 2006; Mizumoto et al. 2007, 2008
0.2-µm PC	Non, 50% v/v	0.2-µm PC	Tarutani et al. 2001; Nagasaki et al. 2003, 2005b, 2006
	Non, 10% v/v	0.45- and 0.2-µm CA, and 0.1-µm filter	Brussaard et al. 2004b; Attoui et al. 2006
0.2-µm PC or 0.45-µm PVD			Cottrell and Suttle 1991; 1995; Mayer and Taylor 1979; Waters and Chan 1982
0.22-µm HT	Non, 10–0.01%		Zingone et al. 2006
0.22-µm HT	Non, 10–0.01%		Zingone et al. 2006
			Lawrence et al. 2006; Lawrence unpubl. data
0.2-µm	Non, 2% v/v	0.2-µm	Wilson et al. 2006
0.7-µm GF	Non, 10–20% v/v	0.2-µm CA	Brussaard et al. 2004a, 2007; Baudoux and Brussaard 2005
0.7-µm GF	Non, 10–20% v/v	0.2-µm CA	Brussaard et al. 2004a, 2007; Baudoux and Brussaard 2005
0.45-µm Supor filters (Gelman)	Plankton concentrated by continuous flow centrifugation	0.2-µm Meditron syringe filters (Schleicher & Schuell)	Sandaa et al. 2001; Thyrhaug 2003; Monier et al. 2008
	Plankton concentrated by continuous flow centrifugation, 1% v/v		Jacobsen et al. 1996; Bratbak et al. 1998; Yan et al. 2005; Monier et al. 2008
0.2-µm PC	Non, 25% v/v	0.1-µm PC	Nagasaki et al. 2004b; Shirai et al. 2006
0.2-µm PC	Non, 50% v/v	0.2-µm PC	Nagasaki et al. 2009
			Müller 1991; Lanka et al. 1993; Müller et al. 1996, 1998; Van Etten et al. 2002
			Müller et al. 1996, 1998
			Friess-Klebl et al. 1994; Müller et al. 1998
			Kapp et al. 1997; Müller et al. 1998
			Henry and Meints 1992; Müller et al. 1998; Meints et al. 2008
			Kapp et al. 1997; Müller et al. 1998
			Kapp et al. 1997; Müller et al. 1998
			Maier et al. 1998; Müller et al. 1998
1.2-µm GF and 0.2 or 0.45µm PVD	Ultrafiltration 30 000 MW cutoff	0.2-µm PC	Garza and Suttle 1995; Suttle pers. comm.
No filtration; sequential centrifugation		Sucrose cushion centrifugation	La Scora et al. 2003; Raoult et al. 2004
0.2-µm PC	Non, 40% v/v	0.2-µm PC	Takao et al. 2005, 2006
0.2-µm PC	Non, 40% v/v	0.2-µm PC	Takao et al. 2007

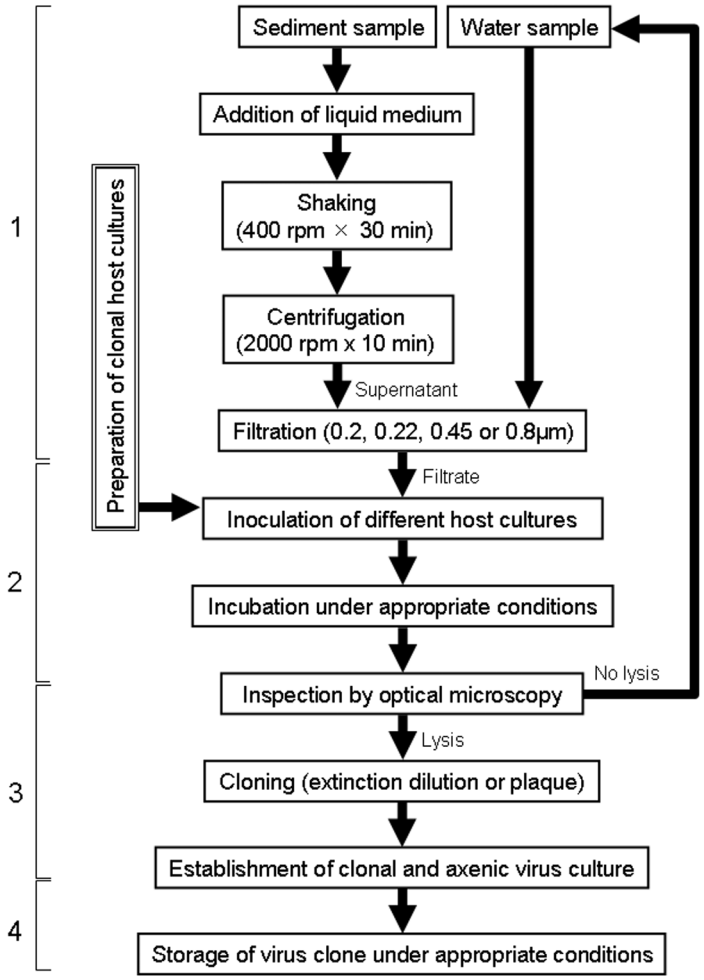

Fig. 1. Summarized scheme of virus isolation procedure. A typical protocol for isolation of virus includes the following steps: (1) Preparation of inoculum: a natural water sample, or the supernatant left after low-speed centrifugation of a sediment suspension, is filtered through a 0.2-μm (0.22-, 0.45-, or 0.8-μm) nuclepore filter to obtain a virus sized fraction. (2) Propagation: the filtrate with the virus sized fraction is inoculated into vigorously growing monoclonal algal host cultures and incubated under appropriate conditions. (3) Purification: when algal lysis is observed by optical microscopy, the lytic agent (virus) is purified using an extinction dilution procedure or plaque assay method (if possible). (4) Storage: the established monoclonal virus culture is stored under appropriate conditions. See main text for details.

requirements and characteristics of the host organisms. Here we discuss some of the practical issues involved in each step.

Preparation of host cultures

Preparation of host cultures is essential for isolation of viruses. Use of unialgal and axenic host cultures is preferable because bacterial contaminants can cause sudden and unexpected cell lysis in the cultures. Contaminating bacteria may possibly also introduce their associated phages into downstream applications and analysis, creating havoc with the results. To establish axenic microalgal cultures, micropipetting or other washing methods are commonly employed, often in combination with use of various antibiotics (Paasche 1971; Stein 1973; Lee 1993; Imai and Yamaguchi 1994; Connell and Cattolico 1996).

For nonphotosynthetic protists only a few examples of successful virus isolation have been reported in the literature. A large double-stranded deoxyribonucleic acid (dsDNA) virus (CroV) infecting *Cafeteria roenbergensis* was isolated by Garza and Suttle (1995) (reported as BV-PW1 infecting *Bodo* sp., C. Suttle pers. comm.). Cultures of *C. roenbergensis* and other bacterivors will inevitably be difficult or impossible to make axenic. Takao et al. (2005, 2007) succeeded in isolating a single-stranded ribonucleic acid (ssRNA) virus infecting *Schizochytrium* sp. and a dsDNA virus infecting *Sicyoidchytrium minutum*. To make the host cultures axenic these investigators used liquid and solid media containing chloramphenicol (0.2% w/v), and because these protists are able to form colonies on agar plates, they can easily be isolated and purified by picking single colonies.

Generally, viruses of eukaryotic algae and nonphotosynthetic protists are strain specific as well as species specific; i.e., virus sensitivity spectra can differ among host clones (e.g., Sahlsten 1998; Tarutani et al. 2000; Tomaru et al. 2004a,b; Zingone et al 2006). Thus, the use of several different clones of the same host species for isolating viruses is often advantageous. In most cases, exponentially growing cultures tend to be more sensitive to viral infection than stationary phase cultures (e.g., Nagasaki et al. 2003). Considering that viruses use the biosynthetic apparatus of the host cell for DNA and protein synthesis and that the host cells in exponential growth have a higher biosynthetic activity, use of vigorously growing host cultures is also important.

Screening for viruses

Information on the screening methods used for isolation of various algal and protist viruses (including considerations such as origin and sample preparation) is included in Table 1.

Microalgal viruses are found not only in seawater but also in marine sediments (Lawrence et al. 2002; Nagasaki et al. 2004a), and samples from both have been used as inoculums for virus isolation (Table 1). The most straightforward method for isolating viruses infecting algae or protists from natural seawater is to add (10% to 50% by volume) a filtered (0.2–0.8-μm pore size) or unfiltered water sample potentially containing viruses to a prospective host culture (see Table 1 for references). Two more elaborate methods have also been used: the virus concentration method and the virus induction by ultraviolet (UV)-treatment method. In the former method, the viruses in a large water sample (typically 10–100 L) are separated from larger particles by filtration (0.2- or 0.45-μm pore size) and concentrated by ultrafiltration (typically 30,000 MW cutoff); the concentrated virus-size fraction is then used as inoculum (Suttle et al. 1991). Many viruses have been successfully isolated by this technique, including viruses infecting

Micromonas pusilla (MpV) (Cottrell and Suttle 1991), *Chrysochromulina brevifilum* (CbV) (Suttle and Chan 1995), *Heterosigma akashiwo* (HaV, HaNIV, HaRNAV) (Nagasaki and Yamaguchi 1997; Lawrence et al. 2001; Tai et al. 2003), and *C. roenbergensis* (CroV, reported as BV-PW1 infecting *Bodo* sp.) (Garza and Suttle 1995; C. Suttle pers. comm.). A detailed protocol of the ultrafiltration technique is given by Suttle (1993).

The intention of the UV-treatment technique is to cause induction and production of latent viruses in natural phytoplankton populations. Briefly, natural seawater containing the target phytoplankton species is collected and concentrated by centrifugation (Jacobsen et al. 1996). The resulting cell concentrate is then poured into a petri dish and exposed to UV irradiation for an appropriate time. Next, the treated cell suspension is incubated overnight in the dark, centrifuged to remove cell debris, and then inoculated into a growing host culture. By using this method viruses infectious to *Emiliania huxleyi* (EhV) (Bratbak et al. 1996) and *Phaeocystis pouchetii* (PpV) (Jacobsen et al. 1996) have been isolated. Assuming latent viruses to be present, use of other inducing agents, e.g., mitomycin C, may also be possible for boosting virus production. However, the efficiency of the UV-treatment technique has not been verified, and no lysogenic viruses infecting unicellular algae or protists have so far been reported.

Preincubation of natural water samples has in some cases also been observed to increase the abundance of free viruses and facilitate virus isolation. Brussaard et al. (2004a) reported that nutrient addition and incubation of natural seawater for a week at in situ temperatures promoted the isolation of viruses infecting *Phaeocystis globosa*. Nagasaki et al. (1994) found an increased fraction of virus containing *H. akashiwo* cells after incubation of the collected samples for 26 h. The mechanisms involved may include increased abundance of host cells supporting an increased production of associated viruses and maturation and lysis of already infected cells. Both of these mechanisms bring about an increase in free viral particles and thus an increase in the probability for successful virus isolation after filtration of the samples.

Mussel mantel fluid and material from gills of fish and mussels have successfully been used as inoculum for isolating novel viruses infecting *Tetraselmis viridis* and *Phaeodactylum tricornutum* (O. A. Stepanova pers. comm., Institute of Biology of the Southern Seas, National Academy of Sciences of Ukraine, Sevastopol). The concept behind this approach is that particles, including viruses and infected cells, are retained and concentrated on the gills of fish and filter feeders and that the probability for isolating a virus will be greater using this material as inoculum rather than just seawater. Further detailed information concerning this new method is of much interest.

Virus in sediments can be extracted by shaking with a Voltex Shaker (VR-36, Taitec) (e.g., 400 rpm × 30 min) or vortexing sediment samples with a suitable medium such as SWM3 (Chen et al. 1969), phosphate-buffered saline, or sodium pyrophosphate (Lawrence et al. 2002), followed by low-speed centrifugation (e.g., 600*g* × 10 min) and filtration (0.2–0.45 µm) to separate viruses from bacteria, sediment particles, and debris (Maranger and Bird 1996; Danovaro and Serresi 2000; Lawrence et al. 2002).

Prior to inoculation of the virus suspension to host cultures, bacteria and larger organisms (including zooplankton, phytoplankton, nanoflagellates, and fungi) should be removed by filtration. Considering the size of microalgal viruses (20–220 nm in diameter), 0.2-, 0.22-, or 0.45-µm pore-size polycarbonate membrane filters (Nuclepore) are suitable. The resulting filtrate (viral fraction) is then added to vigorously growing potential host cultures. Growth of hosts is monitored and compared to that of control cultures without virus inoculation.

When the host algae are able to grow and form a lawn on solid media, viruses may also be isolated by means of plaque assay. Cultivation of algal flagellates on solid media may be difficult, especially in the case of marine forms (Nagasaki and Imai 1994), but the approach has nevertheless been applied with success on a number of occasions, e.g., *Chlorella* viruses (van Etten et al 1991), *M. pusilla* (Cottrell and Suttle 1991), *E. huxleyi* (Bratbak et al. 1996), and *Chaetoceros ceratosporum* (Sakata et al. 1991). As mentioned above, fungoid protists such as *Schizochytrium* sp. (*Aurantiochytrium* sp.) can grow well on agar plate (Y. Takao pers. comm., Fukui Prefectural University, Japan); thus, plaque assay is a plain option. For viruses that replicate very slowly and form small plaques that may be hard to observe, it may help to reduce the initial cell density in the soft agar overlay or, in case of algal hosts, put the plates in the dark for a couple of days. This may allow the plaques to develop and not become overgrown by cells that grow faster than the virus. Moreover, as long as it is possible, plaque purification and cloning of viruses is also much faster and easier than end-point dilution.

One possible method conceivable on the basis of a recent report by Mizumoto et al. (2007) is the use of a gene gun. In the case of HcRNAV (Table 1), purified genome RNA extracted from virions caused a regular infection when transfected into its host cell by using a gene gun. Based on these data, isolation of unknown viruses might be possible if environmental viral RNA is extracted from sediments or seawater and physically injected into potential host cells. This method would be a kind of "enrichment culturing" of viruses, in which only viruses that can replicate inside the cells and produce descendant virions that infect the same cells are selectively propagated. No successful results have been obtained and reported with this method so far, however.

Cloning and maintenance of microalgal viruses

When decay (i.e., bleaching, decrease in chlorophyll *a* fluorescence, clearing, etc.) of the tested host algal culture is detected in the screening procedure, the lytic factor should be cloned as soon as possible. In many cases clones have been obtained by using an extinction dilution procedure (e.g., Suttle 1993; Tomaru et al. 2004a,b). Briefly, the culture lysate is

diluted with an adequate liquid medium in a series of 10-fold dilution steps. Aliquots (100 µL) of each dilution are added to 8 wells in cell-culture plates with 96 round-bottom wells, mixed with 150 µL of exponentially growing host culture, and incubated under the conditions suitable for the host's growth. Lysed cultures are removed from the most diluted wells in which lysis occurred, and the entire procedure is repeated. The lysate in the most diluted wells of the second assay is sterilized by filtration through 0.1-µm (for ssRNA, ssDNA, or dsRNA viruses) or 0.2-µm pore size polycarbonate membrane filters (phycodnaviruses or large dsDNA viruses) and transferred into an exponentially growing host culture; certification of the lytic activity of the lysate is essential. After cell debris is removed by low-speed centrifugation, the supernatant is used as the clonal pathogen suspension.

Microalgal viruses are diverse in terms of stability, and a suitable protocol for maintaining infective viruses must be set up in each case. *Chlorella* viruses (PBCV-1) are so durable that significant decreases in titer are rarely seen as long as the viruses are kept refrigerated. In contrast, the titers of HaV, HcV, and PpV gradually decrease even when stored at 4˚C in the dark. Isolated viruses may also be maintained in culture by routine transfer of the viral lysate to fresh host cultures. Loss of infectivity, however, caused for example by defective interfering particles (Bratbak et al. 1996), is a possible risk that should be taken seriously. Cryopreservation may, at least in some cases, be an alternative, and HaV and PpV have for example been stored at –196˚C in 10–20% dimethyl sulfoxide and at –70˚C in 10–20 % sucrose, respectively (Nagasaki and Yamaguchi 1999; Nagasaki 2001).

Comments

Filtration of the inoculum used for isolating viruses through 0.2–0.45-µm filters and later of the obtained lysates through 0.1–0.2-µm filters is a common procedure (see Table 1). Several workers have noted that some viruses do not pass or lose infectivity when filtered through certain types of filters and through filers with small pore size (i.e., 0.2 µm) (Van Etten et al. 1981; 1983; Suttle et al. 1991; Bratbak unpubl. data). Exceptionally large viruses such as the Mimivirus (~750 nm; La Scora et al. 2003; Xiao et al. 2005) will also be lost during filtration.

With use of the extinction dilution method for cloning, it should be noted that only the most abundant virus showing lytic activity to the host culture will be isolated. In other words, the less dominant viruses (if there are any) will be lost.

In most cases, lysis of the host culture will be a key criterion for detection of virus infection. Recovery and regrowth of the host in lysed cultures appears to be a common occurrence, however, and if this phenomenon occurs lysis may pass unnoticed if cultures are infected with a too high virus dose that results in weak lysis and rapid regrowth, or if cultures are left for too long before being inspected for lysis (Thyrhaug et al. 2003). Moreover, Mizumoto et al. (2008) have recently demonstrated that microscopic observation alone may be inadequate for detecting viral lysis, and infected cultures may be erroneously disposed of if the symptoms of lysis are weak.

Another issue that should be considered when preparing host cultures for isolation of virus is that viral susceptibility may change between life cycle stages with different ploidy levels in unicellular eukaryotes. Frada et al. (2008) has recently demonstrated that the haploid phase of *E. huxleyi* is resistant to EhVs that kill the diploid phase and that exposure of diploid *E. huxleyi* to EhVs induces transition to the haploid phase. The ensuing hypothesis, that the ploidy level of the host cultures may explain earlier unsuccessful attempts to isolate viruses and that viral induced transition between host ploidy levels may result in an apparent loss of infectivity during isolation, should be tested.

The lytic activity differs between various host–virus systems on the species level and also between various clonal combinations of host and virus within the same species (Nagasaki and Yamaguchi 1998; Mizumoto et al. 2008). The isolation procedures used so far may have selected for viruses having strong lytic activity because researchers inadvertently may prefer host–virus systems showing massive cell lysis. Viruses that are slow, latent, cause chronic infections, or are produced and released without killing or lysing the host may be hard to isolate but have an important ecological impact. The conception of viruses in natural ecosystems may thus be miscalculated if based only on the properties of the possibly "extreme" host–virus systems available in culture.

Brown algal phaeoviruses infect only the host spores or gametes and are reproduced in the sporangia. Virus particles are not present in vegetative cells, but the genome of the FsV virus infecting *Feldmannia* sp. has recently been found integrated into the host genome in vegetative cells (Meints et al. 2008). Protocols for isolation and culturing of viruses infecting these macroalgae are described by Lanka et al. (1992) and Müller (1996).

List of materials and reagents:

- Bucket or water sampler (e.g., Van Dorn water sampler)
- Sediment sampler (e.g., Ekman bottom grab sampler)
- Centrifuge and centrifuge tubes (e.g., 15- or 50-mL Falcon tubes)
- Sterilized filter holder and membrane filters (0.2, 0.22, 0.45 or 0.8µm)
- Vacuum pump
- Sterile medium for algal culture (e.g., SWM-III, f/2)
- Sterile test tubes, pipette with tips and vortexer (for serial dilution)
- 24-well and 96-well cell culture plates (e.g., Falcon)
- 8-channel pipette and sterile reservoir tray for filling (for extinction dilution procedure)
- Plastic tape (for sealing the culture plates to avoid drying)
- Incubator (with light and temperature control)
- Inverted microscope
- Refrigerator, freezer, deep freezer, or liquid nitrogen container

Tips and hints

Detailed outline of the extinction dilution method:

All work should be done in a clean bench.

Preparation of dilution series:

(1) Prepare 9 tubes (numbered #1–#9) with 4.5 mL medium.
(2) Add 500 μL of the virus filtrate (virus size fraction) to tube #1 and vortex.
(3) Change the pipette tip and transfer 500 μL of suspension #1 to tube #2 and vortex.
(4) Repeat the procedure to tube #8.

Preparation of cell culture plates:

Use an 8-channel pipetter filled from a reservoir tray (an ordinary pipette may be used but makes the work more tedious). When starting with the most diluted samples it is not necessary to change tips or reservoir tray while working with the same virus.

(5) Pour vigorously growing algal host culture into the reservoir tray, fill the pipetter and add 150 μL culture to each well in lines 1–9 of a 96-well cell culture plate. Empty the tray.
(6) Pour dilution tube #9 (control medium, no virus) into the reservoir tray, fill the pipetter and add 100 μL of to each well in line 9. Empty the tray.
(7) Repeat procedure (6) for dilution tube #8–#1 and fill the respective well lines in the culture plate.
(8) Put on the lid and seal tightly with plastic tape to avoid drying.
(9) Incubate under appropriate conditions.

Inspection:

(10) Use an inverted microscope and inspect the culture plates for signs of cell lysis at regular intervals.
(11) Mark wells where lysis is observed and continue the incubation with daily inspections until no more lysis occurs.
(12) Prepare a second extinction dilution with virus from the most-diluted well.
(13) Propagate virus clone from the most diluted well in a larger volume and store appropriately.

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