MANUAL
of
AQUATIC VIRAL ECOLOGY

MAVE Chapter 4, 2010, 30–33

Detection of lysogeny in marine environments

John H. Paul[*1] *and Markus Weinbauer*[2]
[1]College of Marine Science, University of South Florida, 140 Seventh Ave. S, St. Petersburg, FL 33701
[2]CNRS and Université Pierre et Marie Curie-Paris 6, Laboratoire d'Océanographie de Villefranche, 06230 Villefranche-sur-Mer, France

Abstract

Silent viral infections occur in all forms of life, from bacteria to humans, as indicated from genomic sequencing. Temperate phages can infect bacteria and establish a symbiotic relationship termed *lysogeny,* enabling the phage genome to be propagated in host daughter cells. The expression of prophage genes often results in an altered bacterial phenotype, often turning benign bacteria into virulent pathogens. The most widely used method to detect lysogens is to chemically induce their prophages and detect these via microscopy or flow cytometry. Although chemical induction is the gold standard in prophage detection, not all prophages can be detected by it. This review gives two methods for prophage induction in heterotrophic bacterioplankton, a method for induction of *Synechococcus* populations, and a method for isolating temperate phage, as well as a simple method to recognize prophage-like elements in bacterial genomes.

Introduction

Lysogeny occurs when a temperate phage establishes a stable symbiosis with its bacterial host. This is accomplished most often by integration of the host genome into one of the host's replicons, although prophages that exist as autonomous plasmids have also been described in marine bacteria (Mobberley et al. 2008). The integrated temperate phage genome is termed a *prophage*. The prophage usually confers an altered phenotype to the host. When this is the result of expression of phage genes, it is termed *conversion*. A phage infection that results in both the production of high phage titers and host cells is termed *pseudolysogeny* (Ackermann and DuBow 1987). Historically, lysogeny has been regarded to impart increased fitness to the lysogenized host compared with the uninfected host (Edlin et al. 1975), yet there are few demonstrations of this in the literature, and none in marine bacteria (Paul 2008). A similar process of viral integration occurs in eukaryotes, termed *latency*, is outside the scope of this review.

The operational definition of a lysogen is a bacterium that contains an inducible prophage particle, most often detected through the use of an inducing agent (Ackermann and DuBow 1987). "The most sensitive method is thus induction by mitomycin C or UV light (or a combination of both) followed by the spot test in combination with electron microscopic examination" (Ackermann and DuBow 1987). However, not all prophages are inducible with mitomycin C (Ackermann and DuBow 1987). A more stringent definition of a lysogen is a bacterium that contains a prophage that is capable of infecting other hosts and establishing a lysogenic relationship. For this to occur, one needs an uninfected yet sensitive host, and such complete systems (temperate phage, lysogen, and uninfected host) have seldom been described for marine bacteria.

The value of lysogeny can only be inferred from ecological studies in natural populations. Lysogeny seems to occur during conditions that are unfavorable for rapid vegetative host growth. Such conditions may be manifested in deep sea environments (Weinbauer et al. 2003), oligotrophic surface waters (Long et al. 2008), Antarctic lakes (Lisle and Priscu 2004), Arctic lakes (Laybourne-Perry et al. 2007), or during winter months (McDaniel et al. 2002, Williamson et al. 2002). These conditions are characterized by low host abundance and growth rates, which would result in a low probability of a successful host encounter and lytic infection. Survival as a prophage also ensures protection from some of the viral inactivating factors that free phage particles encounter (i.e., UV inactivation and grazing; Wommack and Colwell 2000).

*Corresponding author: E-mail: jpaul@marine.usf.edu

Acknowledgments

Publication costs for the Manual of Aquatic Viral Ecology were provided by the Gordon and Betty Moore Foundation. This document is based on work partially supported by the U.S. National Science Foundation (NSF) to the Scientific Committee for Oceanographic Research under Grant OCE-0608600. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the NSF.

This work was supported by NSF grants OCE-0221763 and EF-0801593 to J. H. Paul.

ISBN 978-0-9845591-0-7, DOI 10.4319/mave.2010.978-0-9845591-0-7.30

Suggested citation format: Paul, J. H., and M. Weinbauer. 2010. Detection of lysogeny in marine environments, p. 30–33. *In* S. W. Wilhelm, M. G. Weinbauer, and C. A. Suttle [eds.], Manual of Aquatic Viral Ecology. ASLO.

In this review, we provide methods for detection of lysogeny in natural populations of marine bacteria and cyanobacteria, a method for isolating temperate phages from marine viral concentrates, and a simple method for detecting prophage-like elements in marine bacterial genomes bioinformatically.

Natural populations of heterotrophic bacteria, no viral reduction

Materials

- Freshly filtered (0.02 µm; Whatman Anodisc) formaldehyde solution (formalin; 37%);
- Mitomycin C (Sigma; 1 mg/mL stock solution, dissolved in deionized water [DI]);
- Materials for epifluorescence or flow cytometry enumeration of viral particles;
- Electron microscopy–grade glutaraldehyde (Sigma-Aldrich).

Prophage induction—For unconcentrated seawater samples, add 25 mL each to a control or treatment, 50-mL sterile, conical centrifuge tubes. If many inducing agents are to be investigated, increase the number of treatment tubes accordingly. Take an additional sample (25 mL) and fix with 1% 0.02-µm filtered formalin. For the treatment samples, add 1 µg/mL mitomycin C (or 0.5 µg/mL in oligotrophic environments). If other mutagens are to be used, it is a good idea to include a mitomycin C treatment as a positive control. Mutagens can be added at any concentration desired, but this can be limited by the solubility of the mutagen (e.g., polynuclear aromatic hydrocarbons; Jiang and Paul 1996). The samples are incubated for 16–24 h at room temperature and fixed with either 2% glutaraldehyde (for TEM), 1% formalin (epifluorescence microscopy), or 1% formalin/0.5% glutaraldehyde (flow cytometry [FCM]).

Samples for enumeration by epifluorescence microscopy should be counted within 24 h of collection or stored as frozen slides stained with SYBR Gold (Chen et al. 2001; *see* Danovaro and Middelboe 2010, this volume). Count both bacteria and viruses in control and treated samples. For induction to have occurred, viral counts in the treatment must exceed those in the control (i.e., be statistically different).

Calculate the % lysogenic bacteria as follows:

$$\% \text{ lysogens} = [(VDC_T - VDC_C)/B_Z]/BDC_{T=0},$$

where VDC_T is the viral direct counts (in viruses/mL) in the treatment, VDC_C is the viral direct counts in the control, B_Z is the average burst size, and $BDC_{T=0}$ is the bacterial counts at the set up of the experiment ($T = 0$). The average burst size can be derived by TEM observation of bacterial bursts (i.e., when viruses become visible in the cell at the end of the latent period; Ackermann/Heldal, this volume). We have found an average for our samples from the Gulf of Mexico of 30, whereas taking an average of the literature from a recent review (Wommack and Colwell 2000) indicates a value of 53.5 ± 48.

Natural populations of heterotrophic bacteria, viral reduction

Materials

- Freshly filtered (0.02 µm) formaldehyde;
- Mitomycin C (Sigma);
- Materials for epifluorescence or flow cytometry enumeration of viral particles;
- Cartridge (30- or 100-kDa cutoff) to make virus-free water;
- Filtration (0.2 µm pore size) to reduce viral abundance (see also Weinbauer et al., this volume).

Prophage induction—The rationale of the virus reduction approach is to avoid new infection by reducing the number of viruses and, thus, the encounter rates with hosts (Weinbauer and Suttle 1996). This can be accomplished by several methods (see Weinbauer et al., this volume). Prokaryotic cells with reduced viral abundance (25–50 mL) are incubated at in situ temperature in triplicates with or without inducing agent C (see also above). Samples for enumeration of prokaryotes and viruses are taken periodically and fixed as described above. Calculation of induced viral production and the percentage of cells containing a prophage (% lysogens) is calculated as described above.

Prophage induction in marine Synechococcus

Materials

- Sterile 96 well microtiter plates
- Indicator host culture (i.e., *Synechococcus* WH7803)

Prophage induction—The samples for prophage induction are pretreated by the technique of viral reduction (Weinbauer and Suttle 1996). Each sample is filtered through a 0.2-µm filter to a volume of approximately 5 mL to remove most of the ambient viruses. Virus-free (0.02-µm filtered) water prepared from the same sample is added and the volume reduced a second time. The retentate is then returned to its original volume by addition of virus-free seawater, divided into aliquots, and incubated with and without inducing agent. Treated samples are amended with the inducing agent mitomycin C at a concentration of 1 µg/mL or with the inducing agent of choice.

To enumerate the cyanophage population, the most probable number (MPN) method is employed (Suttle and Chan 1994). By this method, a one- to five-dilution series of the environmental or prophage induction treatment sample is prepared using 96-well microtiter plates (Costar, Corning Inc.). A susceptible *Synechococcus* host is then freshly diluted 1:10 and placed in each well (either *Synechococcus* isolate WH7803, our own isolate GM9901, or both). Control plates are prepared similarly using sterile SN media in the first column of wells. Three replicate treatment and control plates are prepared from each site. The plates are incubated until good growth of the host organism is evident (10–14 days). Wells are scored as positive for virus if lysis of the host organism is evident as a well clearing. Viral abundance is calculated for each plate using an MPN program (Hurley and Roscoe 1983).

Data analysis—Treatment and control cyanophage and *Synechococcus* counts are evaluated by paired *t* test between samples using Minitab statistical software. Comparison of induction results and environmental parameters are also performed using linear regression and χ^2 analysis, also using Minitab.

Isolation of temperate phages by plaque agar overlay

The isolation of temperate phages requires a cultivatable host and a source of concentrated viruses. For example, we isolated a pseudotemperate phage (ϕHSIC) and its host from the same bacterial/viral concentrate (Jiang et al. 1998) that we obtained in the Sand Island Channel, Oahu, HI, USA. The host was first isolated by standard isolation streaking on marine agar using inoculum from a microbial population concentrate. This protocol uses the conventional plaque agar overlay and looking for turbid or haloed plaques, a hallmark of temperate phages.

Materials

- Standard marine agar (1.5%) plates (i.e., Zobell 2216 or ASWJP);
- Sterile marine broth;
- Sterile marine soft agar (1%), 3 mL per 15-mL tube;
- Water bath;
- Marine host bacterial culture in exponential growth, 20–50 mL;
- Viral concentrate.

Procedures—Soft agar overlay tubes are melted in boiling water and placed in the 47°C water bath. The host bacterium should be growing exponentially (this can be verified by A_{600} measurements of about 0.4–0.6). One tube of soft agar is removed from the water bath (the agar should have cooled to 47°C), and 1.0 mL host culture and either 1.0 or 0.1 mL viral concentrate is added. The contents of the tube is mixed well by rolling back and forth between two hands, and the tube contents are immediately emptied onto an agar plate. The top agar is gently spread over the agar surface by sliding the plate on the bench surface using a circular motion. The top agar is allowed to harden by not disturbing the plates for 30 min. The plates are incubated (top agar side down) overnight to 48 h.

Temperate phage plaques will appear as turbid or cloudy plaques, whereas purely lytic phage will appear as sharply defined, clear plaques. Plaques may appear haloed (clear area with a larger turbid halo) and are often the result of pseudotemperate phages. Turbid plaques can be picked and replaqued to purify the temperate phage (three replaquings are recommended). It may also be possible to isolate the lysogenized host by carefully picking the turbid plaque and using isolation streaking on marine agar plates. The putative lysogen can be checked for harboring a prophage by mitomycin C induction.

Identification of prophages in marine bacterial genomes

Several initiatives have as their goal sequencing of bacterial and archaeal genomes (www.moore.org/microgenome; genome.jgi-psf.org/mic_home.html). The easily available marine microbial genomes are ideal for the bioinformatic discovery of putative prophage genomes. A computational approach to this task has been published (Phagefinder; Fouts 2005). However, for this approach to be successful, the genome must be reduced to one or very few contigs. Many genomes are now deposited in GenBank in 10's to more than 100 contigs, precluding the Phagefinder approach. J. H. Paul has adopted a simplified approach to prophage finding in marine bacterial genomes that requires no sophisticated bioinformatic software (Paul 2008). Using the NCBI website (www.ncbi.nlm.nih.gov), the genome of the organism in question is found using the genome search engine. Once the genome of the microbe of interest is found, clicking on the accession number brings up the *Genome Results* page, a table of links to various pages of information. In this table, under the *Features* column, find *Protein coding* and click on the link (number) of protein coding features. This opens a page of all open reading frames (ORFs) in order in the genome. Using your browser's "find in this page command" or similar search function, look for phage genes, searching for the term "phage," "terminase," "capsid," "portal," or other phage term. This will locate a phage-like ORF or at least one whose putative identity matches your search term. Once a phage-like ORF is found, scan 10–15 ORFs on either side of the found ORF for additional phage-like ORFs. A typical prophage genomic signature is a stretch of "hypothetical proteins" interspersed with phage proteins that extend for 30–50 kb and lack host metabolic genes. Many prophages begin with an integrase gene, but assigning a start and endpoint of the prophage is often difficult and can be verified only by experimental procedures like PCR and cloning/sequencing of induced lysates. Once a putative prophage is found, it is recommended to export the sequence to a general bioinformatics software program such as Lasergene (DNAStar) or Kodon (Applied Maths). These programs assist in visualizing the prophage gene arrangement and can assist in determining the termini of the prophage.

Assessment

Estimating the occurrence of lysogeny by prophage induction has been used worldwide, from the Arctic to the Antarctic and marine environments in between, with results ranging from 0 to >100% of the ambient population being lysogenized (Williamson et al. 2002). A controversial extension of the assay is to treat cultures with mitomycin C for only 30 min followed by cell collection by centrifugation and resuspension in fresh growth media (Chen et al. 2006). This procedure minimizes the general toxicity of mitomycin C and reportedly has resulted in greater yields of temperate phages.

The virus-reduction approach has the advantage that the control is likely more reliable, since new infection is largely stopped, and thus, the approach is not affected by interference of new infection with the mitomycin C treatment. However, there are also cons with this approach. For example, it

involves manipulation of samples. Another problem is that the virus reduction cannot be applied to all environments. For example, testing mitomycin C in anoxic or suboxic environments without changing the oxygen concentration is feasible (within reasonable constraints) only with the nonreduction approach.

Discussion

Clearly our understanding of lysogeny is only as good as our methods. Much of the detection of prophages in isolates or natural populations rests on induction by mitomycin C. Clearly not all lysogens are inducible by mitomycin C. Some prophage-like particles don't seem to be inducible by any common agents, but rather increase in concentration in the growth media as the culture reaches late stationary phase (similar to that observed for gene transfer agents [GTAs]). Unfortunately, there are no alternate methods to induction to detect prophages (save for testing other inducing agents such as UV). There are few conserved lysogeny genes that could be detected by amplification.

Comments and recommendations

The approach suggested here to assess prophage induction heterotrophic prokaryotes can be expanded to specific prokaryotic groups. Examples discussed here are *Synechococcus* and cyanophages. Other groups can be targeted with the development of primers for qPCR. Prophage induction assays have also been applied to marine sediments (Mei and Danovaro 2004). For the modification of the protocols, see the cited literature.

References

Ackermann, H. W., and M. S. DuBow. 1987. Viruses of prokaryotes. V. 2, General properties of bacteriophages. CRC Press.

Chen, F., J. R. Lu, B. J. Binder, Y. C. Liu, and R. E. Hodson. 2001. Application of digital image analysis and flow cytometry to enumerate marine viruses stained with SYBR Gold. Appl. Environ. Microbiol. 67:539-545.

———, K. Wang, J. Stewart, and B. Belas. 2006. Induction of multiple prophages from a marine bacterium: A genomic approach. Appl. Environ. Microbiol. 72:4995-5001

Danovaro, R., and M. Middelboe. 2010. Separation of free virus particles from sediments in aquatic systems, p. 74-81. *In* S. W. Wilhelm, M. G. Weinbauer, and C. A. Suttle [eds.], Manual of Aquatic Viral Ecology. ASLO.

Edlin G., L. Lin, and R. Bitner. 1975. Lambda lysogens of *Escherichia coli* reproduce more rapidly than non-lysogens. Nature 255:735-737.

Fouts, D. E. 2005. Phage_Finder: Automated identification and classification of prophage regions in complete bacterial genome sequences. Nucleic Acids Res. 34:5839-5851.

Hurley, M. A., and M. E. Roscoe. 1983. Automated statistical analysis of microbial enumeration by dilution series. J. Appl. Bacteriol. 55:159-164.

Jiang, S. C., and J. H. Paul. 1996. The abundance of lysogenic bacteria in marine microbial communities as determined by prophage induction. Mar. Ecol. Prog. Ser. 142:27-38.

———, C. A. Kellogg, and J. H. Paul. 1998. Characterization of marine temperate phage-host systems isolated from Mamala Bay, Oahu, Hawaii. Appl. Environ. Microbiol. 64:535-542.

Laybourn-Parry, J., W. A. Marshall, and N. J. Madan. 2007. Viral dynamics and patterns of lysogeny in saline Antarctic lakes. Polar Biol. 30:351–358.

Lisle, J. T., and J. C. Priscu. 2004. The occurrence of lysogenic bacteria and microbial aggregates in the lakes of the McMurdo Dry Valleys, Antarctica. Microb. Ecol. 47:427-439.

Long, A., L. D. McDaniel, J. Mobberly, and J. H. Paul. 2008. Differences in prophage induction between heterotrophic and autotrophic microbial populations in the Gulf of Mexico. ISME J 2:132-144.

McDaniel, L., L. Houchin, S. Williamson, and J. H. Paul. 2002. Lysogeny in natural populations of marine *Synechococcus*. Nature 415:496.

Mei, M. L., and R. Danovaro. 2004. Virus production and life strategies in aquatic sediments. Limnol. Oceanogr. 49:459–470.

Mobberley, J. M, N. Authement, J. H. Paul, J. Koomen, and A. M. Segall. 2008. Complete genome sequence of ΦHAP-1, a linear plasmid-like temperate marine phage of *Halomonas aquamarina*. J. Virol. 82:6618-6630.

Paul, J. H. 2008. Prophages in marine bacteria: Dangerous molecular time bombs or the key to survival in the seas? ISME J. 2:579-589.

Suttle, C. A., and A. M. Chan. 1994. Dynamics and distribution of cyanophages and their effect on marine *Synechoccus* spp. Appl. Environ. Microbiol. 60:3167-3174.

Weinbauer, M. G., and C. A. Suttle. 1996. Potential significance of lysogeny to bacteriophage production and bacterial mortality in coastal waters of the Gulf of Mexico. Appl. Environ. Microbiol. 62:4374-4380.

———, I. Brettar, and M. Hofl. 2003. Lysogeny and virus-induced mortality of bacterioplankton in surface, deep, and anoxic marine waters. Limnol. Oceanogr. 48:1457-1465.

Williamson, S., L. McDaniel, L. Houchin, and J. H. Paul. 2002. Seasonal variation in lysogeny as depicted by prophage induction in Tampa Bay, Florida. Appl. Environ. Microbiol. 68:4307-4314.

Wommack, K. E., and R. R. Colwell. 2000. Virioplankton: Viruses in aquatic ecosystems. Microbiol. Mol. Biol. Rev. 64:69-114.