Applied and Environmental Microbiology

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Appl. Environ. Microbiol. **2012, 78(3):892. DOI: 10.1128/AEM.06580-11.**
Published Ahead of Print 2 December 2011.

AEM
Journals.ASM.org

Cultivated Single-Stranded DNA Phages That Infect Marine *Bacteroidetes* Prove Difficult To Detect with DNA-Binding Stains

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This is the first description of cultivated icosahedral single-stranded DNA (ssDNA) phages isolated on heterotrophic marine bacterioplankton and with *Bacteroidetes* hosts. None of the 8 phages stained well with DNA-binding stains, suggesting that *in situ* abundances of ssDNA phages are drastically underestimated using conventional methods for enumeration.

While the majority of cultivated viruses infecting heterotrophic bacteria (phages) have double-stranded DNA (dsDNA) genomes, recent metagenomic studies suggest that single-stranded DNA (ssDNA) phages similar to the *Microviridae* family are widespread in aquatic viral communities (1, 6, 22, 23). However, the only cultivated representatives of aquatic ssDNA viruses infect cyanobacteria (12) or diatoms (13, 20, 21). So far, phages belonging to *Microviridae* have been cultivated only on a limited set of hosts (*Escherichia coli*, *Chlamydia*, *Bdellovibrio*, *Spiroplasma*) (7), but recently, temperate *Microviridae*-like genomes were reported as integrated provirus in several *Bacteroidetes* genomes (10). Here we report the first description of cultivated ssDNA phages infecting heterotrophic marine bacterioplankton, a member of *Bacteroidetes* (*Cellulophaga baltica*) (9).

Confirmation of the phages' ssDNA nature. The 8 phages were obtained from concentrated surface water (Öresund Strait between Denmark and Sweden, 2005) and were isolated on 6 different *C. baltica* strains using the top-agar plating technique (9, 15). The phages represent 20% of a culture collection of 40 phages that was established by purifying plaques with distinct morphologies from each host (9). Phage stocks (obtained by top-agar plating technique and recovered from the top layer) (15) were ultracentrifuged (222,000 × *g* for 2.5 h at 4°C; Beckman) to obtain high-titer (10^{10} to 10^{12} phage ml^{-1}) phage concentrates for further analysis. DNA extracted from the 8 phages could be detected only through gel-based analysis using SYBR gold (stains ssDNA and dsDNA; Molecular Probes) when the phages were embedded and lysed (EDTA-SDS-proteinase K) in agarose plugs (16). All attempts to extract phage DNA from liquid phage stocks using a λ phage protocol (2) or releasing DNA from phage particles by heating (16) did not yield detectable amounts of DNA as measured by PicoGreen (stains dsDNA; Molecular Probes) or when run on gels stained with SYBR gold or ethidium bromide (EtBr) (stains dsDNA; Sigma). The DNA of the phages was determined to be ssDNA, as the DNA in the agarose plugs was degraded by nucleases DNase I (degrades ssDNA and dsDNA; Roche) and S1 (degrades ssDNA and ssRNA; Promega) (Fig. 1). Restriction enzymes NdeII and HindIII (digest dsDNA; Boehringer Mannheim) did cleave DNA from positive-control dsDNA phages infecting *C. baltica* (9) but not DNA from the 8 ssDNA phages (Fig. 1). While we cannot disregard the possibility that a lack of restriction sites for the two enzymes or genomic DNA modification (e.g., glycosylation) prevented digestion, the data support the view that all 8 phages have ssDNA genomes. CsCl gradients (1 ml of 1.5 g cm^{-3}, 1 ml of 1.4 g cm^{-3}, 3 ml of 1.3 g cm^{-3}, and 4 ml of 1.2 g cm^{-3} from bottom to top; 102,000 × *g* for 4 h at 4°C) (SW40; Beckman) showed that all 8 phages had lower buoyant densities (1.20 to 1.24 g cm^{-3}) (Table 1) than dsDNA phages infecting the same host (1.39 to 1.44 g cm^{-3}) (data not shown), which is consistent with previous comparisons between ssDNA and dsDNA phages (18). This difference in buoyancy is important to keep in mind if preparing viral samples for metagenomic sequencing using CsCl gradients, since ssDNA phages are found in density fractions not normally collected for viral metagenomes (4, 18). Morphological characterization through electron microscopy (2% uranyl acetate, negatively stained; Philips CM12 microscope, accelerating voltage of 80 kV) showed that the 8 phages had tailless icosahedral capsids falling into two size classes (Fig. 2). The smaller phages (capsid diameters, 30 to 32 nm) (Table 1 and Fig. 2A to C) resembled phages of the ssDNA phage family *Microviridae* (7), while the larger phages (capsid diameters, 72 to 73 nm) (Table 1 and Fig. 2D to E) resembled a temperate ssDNA phage induced from a cultivated cyanobacterium (12). Consistent with the different capsid sizes, the phages could be grouped into two groups based on genome size, as estimated by agarose and pulsed-field gel electrophoresis. However, there are no commercially available large ssDNA ladders and thus we compared the 8 phages to dsDNA ladders, which provided notably different size estimations depending on if the DNA was run on regular agarose gels (Fig. 1) or pulsed-field gels (9) and should therefore be interpreted cautiously. While genome sequences would provide the correct genome size and valuable information about the ecology and evolution of the phages, we have thus far not been able to obtain sufficient amounts of DNA for genome sequencing.

Visualization and enumeration of ssDNA phages. Accurate enumeration of marine viruses is essential for analyzing their distribution (8, 14), production (24), and role in marine biogeochemistry. While the effectiveness of today's enumeration meth-

Received 18 August 2011 **Accepted** 17 November 2011
Published ahead of print 2 December 2011
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Supplemental material for this article may be found at http://aem.asm.org/.

doi:10.1128/AEM.06580-11

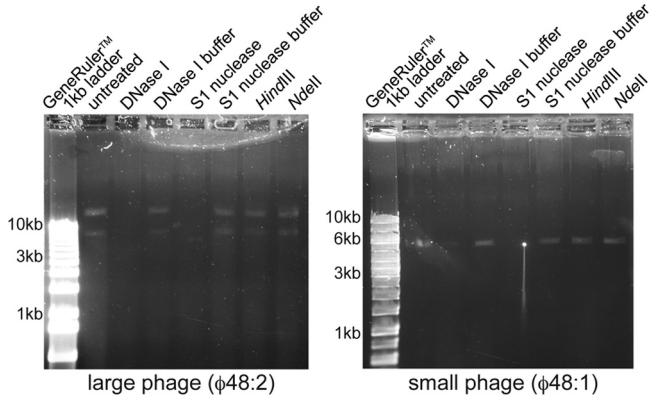

FIG 1 Representative agarose gels showing enzyme digestion of ssDNA from a large phage (left) and small phage (right). The DNA was digested by nucleases DNase I and S1 but was not affected by treatment without nuclease (marked buffer) or by restriction enzymes HindIII and NdeII. A GeneRuler 1-kb DNA Ladder (Fermenta) was used as molecular weight marker. The double bands seen on the gels could be due to double chromosomes (11) or be an artifact produced by supercoiling of circular genomes, which *Microviridae* commonly possess (7).

ods for ssDNA phages has been questioned (17), no major attempt has been made to confirm this. Here, the 8 ssDNA phages and a positive-control dsDNA phage were diluted in various media and stained with 8 different DNA-binding stains (see Table S1 in the supplemental material) either before or after filtration using 0.02-μm Anodisc 25 membrane filters (Whatman) and examined with epifluorescence microscopy (EFM) (Zeiss Axioplan, ×1,250 magnification) as previously described (14). The ssDNA phages could be visualized with EFM only if stained with SYBR gold. If the phages were stained after filtration, all the media used to dilute the phages resulted in positive staining (Tris-EDTA [TE], MSM [450 mM NaCl, 50 mM $MgSO_4$, 50 mM Tris, pH 8] [storage] buffer, or seawater), whereas TE had to be used if the phages were stained prior to filtration. When enumerating SYBR gold-stained virus-like particles, their abundance was always equal to or higher than the number of infectious phage particles measured as PFU, pointing toward a validity of the enumeration (Fig. 3). However, the ssDNA phage particles were very faint and hard to enumerate and would most likely be impossible to enumerate by EFM in environmental samples. Using a flow cytometer (FCM) (FACScan; Becton Dickinson) where phages were stained with either SYBR green or

TABLE 1 Characteristics of the ssDNA phages examined in this study[a]

Phage	Host bacterium	Capsid size ± SD (nm)	Size group	Buoyant density (g cm^{-3})
ϕ3:2	MM#3	73 ± 0.5	Large	1.24
ϕ46:2	NN016046	72 ± 1.9	Large	1.23
ϕ48:2	NN016048	72 ± 1.1	Large	1.23
ϕ12:2	#12	31 ± 2.1	Small	1.20
ϕ12a:1	OL12a	30 ± 1.8	Small	1.20
ϕ14:1	#14	ND	Small	1.21
ϕ18:4	#18	32 ± 2.6	Small	1.24
ϕ48:1	NN016048	ND	Small	1.21

[a] Characteristics are as follows: the host bacterium they were isolated on (the *C. baltica* strain), the size group they are affiliated with based on capsid (diameter, $n = 10$) and genome size, and their buoyant density in CsCl. ND, no data.

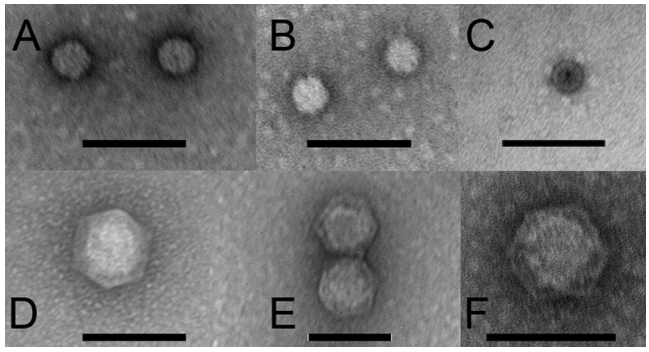

FIG 2 Transmission electron micrographs of negatively stained phages, showing their icosahedral tailless nature: ϕ12:2 (A), ϕ18:4 (B), ϕ12a:1(C), ϕ3:2 (D), ϕ46:2 (E), ϕ48:2 (F). Scale bars equal 100 nm.

SYBR gold (according to references 3 and 5), the dsDNA phages were easily detected (see Fig. S1 in the supplemental material). Among the ssDNA phages, only the large phages were detected above the electronic noise (Fig. S1). However, the calculated abundances were orders of magnitude lower than EFM counts (Fig. 3), pointing toward severe underestimation of the ssDNA phage abundance when enumerating using FCM. This has also been reported for ssDNA phytoplankton viruses (19). In our perception, indigenous ssDNA phages will be difficult if not impossible to enumerate in mixed natural samples by using conventional techniques, as they are barely visible even in pure cultures.

Conclusions. In this study, we present the first characterization of icosahedral ssDNA phages infecting heterotrophic aquatic bacteria and bacteria belonging to the phylum *Bacteroidetes*. The 8 phages, which accounted for as much as 20% of a culture collection, present an excellent opportunity for further investigations where the phylogeny and evolutionary characteristics of these phages can be combined with ecological interrogations to evaluate the role and importance of ssDNA phages in marine waters. In future work, accurate enumeration of ssDNA phages is highly desirable but represents a difficult challenge due to their minuscule size and the single-stranded nature of their genomes.

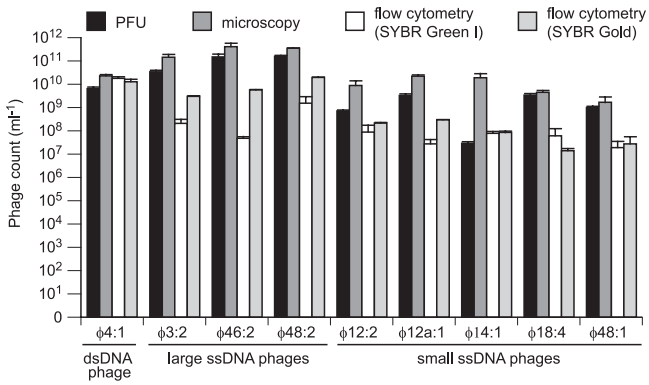

FIG 3 Phage abundance of phage culture stocks estimated by PFU, microscopy (SYBR gold), and flow cytometry (SYBR green I and SYBR gold) counts. Error bars represent standard deviations (duplicate samples). The ssDNA phages are separated into groups with larger and smaller genome sizes (Table 1). Microscopy counts exceeded PFU counts, whereas FCM counts were in most cases lower than PFU counts, indicating severe underestimation.

ACKNOWLEDGMENTS

We thank Natalie Solonenko for doing CsCl and transmission electron microscopy analyses. We also thank three anonymous reviewers for thoughtful comments on the manuscript.

This study was supported by the Linnaeus University and postdoc fellowships from the Sweden-America Foundation and the Swedish Research Council (VR) to K.H., grants from The Royal Swedish Academy of Sciences to L.R., a grant from the Danish Council for Independent Research to M.M., and a Gordon and Betty Moore Foundation grant to M.B.S.

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